Cancers dans l'enfance et fertilité à l'âge adulte

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CANCERS DANS L'ENFANCE ET FERTILITÉ À L'ÂGE ADULTE

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Résumé

Les progrès dans le traitement des cancers de l'enfant et de l'adolescent ont permis de réelles améliorations puisque la survie à long terme est maintenant de plus de 80%. De ce fait, la qualité de vie de ces futurs adultes est une préoccupation majeure des professionnels impliqués dans la prise en charge de ces patients. Notre travail se focalise en particulier sur le développement pubertaire et la fertilité, au travers de la recherche épidémiologique et de la recherche de transfert, chez les enfants et adolescents guéris d'un cancer.

Sur le versant « recherche épidémiologique », **le développement pubertaire et la fertilité chez la fille** ont été étudiés à partir de la cohorte L.E.A. (Leucémies de l'Enfant et Adolescent), qui recueille des données concernant le devenir des patients traités pour une leucémie au cours de leur enfance. Bien qu'elle soit plus importante chez les femmes ayant bénéficié d'une greffe de cellules souches hématopoïétiques et/ou ayant rechuté, la gonadotoxicité atteint également celles n'ayant reçu que de la chimiothérapie en première ligne.

En parallèle, **l'accès à la préservation de la fertilité des adolescents et jeunes adultes** pris en charge pour des pathologies malignes en Auvergne a été analysé. Les garçons bénéficient davantage de consultations au CECOS et d'une préservation de leur fertilité. Des progrès devraient se faire grâce à la création de plateformes clinico-biologiques telles que PREFERA (PREservation FERtilité Auvergne) qui assurent une meilleure coordination entre les différentes équipes prenant en charge ces patients.

Sur le versant « recherche de transfert », nous nous sommes intéressés à la cryoconservation des tissus gonadiques, seule option envisageable chez les enfants prépubères notamment, mais comportant un risque potentiel de réintroduction de cellules néoplasiques en cas d'utilisation ultérieure. Nous avons mis au point **des techniques sensibles et spécifiques de détection de la maladie résiduelle** pour deux tumeurs solides pédiatriques dont les traitements actuels sont potentiellement stérilisants : le neuroblastome et la tumeur d'Ewing. L'intérêt étant de disposer d'un test diagnostic utilisable pour les adultes guéris de ces cancers, dont la fertilité a été compromise par les traitements et qui ont bénéficié d'une cryoconservation de tissu ovarien ou testiculaire.

Mots-clés : cancer de l'enfant, gonadotoxicité, préservation de la fertilité, maladie résiduelle

Abstract

Advances in the treatment of childhood and adolescent cancers have led to real improvements as long-term survival is now over 80%. As a result, quality of life of these future adults is a major concern for the professionals involved in the care of these patients. Our work focuses on pubertal development and fertility, through epidemiological research and transfer, research in childhood cancer survivor.

Concerning the "epidemiological research", **female pubertal development and fertility** were studied using the L.E.A cohort (Childhood and Adolescent Leukemias), which collects data about the outcome of patients treated for childhood leukemia. Although it is more important in women who received hematopoietic stem cell transplantation and/or relapsed, gonadotoxicity also affects those who received only first-line chemotherapy.

In parallel, **access to fertility preservation for adolescents and young adults** treated for malignant diseases in Auvergne was analyzed. Boys benefit more from fertility consultation and preservation of their fertility. Progress should be made thanks the creation of clinico-biological platforms such as PREFERA (PREservation FERtilité Auvergne), which ensure better coordination between the different teams that support these patients.

Concerning the "transfer research", we addressed the cryopreservation of gonadal tissues, only option for prepubertal children in particular, but with a potential risk of neoplastic cells reintroduction in case of future use. We developed **sensitive and specific techniques of residual disease detection** for two solid pediatric tumors whose current treatments are potentially sterilizing: neuroblastoma and Ewing's tumor. The interest is to have a diagnostic test usable for adults cured of these cancers, whose fertility has been compromised by the treatments and who have benefited from ovarian or testicular tissue cryopreservation.

Key words: childhood cancer, gonadotoxicity, fertility preservation, residual disease

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I. INTRODUCTION GÉNÉRALE

1. Cancers chez les enfants et adolescents

Avec plus de 310 000 nouveaux cas par an dans le monde, les cancers des enfants (0-14 ans) et adolescents (15-19 ans) ne représentent qu'un faible pourcentage de l'ensemble des cancers. Les données concernant ces jeunes patients sont relativement rares mais la collecte des données et la tenue de registres épidémiologiques sont indispensables pour optimiser la prise en charge de cette population spécifique (International Incidence of Childhood Cancer, volume 3, Steliarova-Foucher et al. 2017).

	Paediatric dataset (age 0–14 years)			General dataset (age 0–19 years)		
	Cases	Person-years (millions)	Incidence per million	Cases	Person-years (millions)	Incidence per million
Sex (WSR)						
Boys	156 721	1072	151·4	169 531	1039	163·2
Girls	127 917	1023	129·4	141 695	987	143·6
Age group, all cases (ASR)						
0–4 years	127 096	676	187·9	93 559	475	197·1
5–9 years	74 175	690	107·6	54 370	487	111·6
10–14 years	83 378	729	114·4	62 438	519	120·3
15–19 years	..	..	..	100 860	544	185·3
Age group, all cases (WSR)						
0–14 years*	284 649	2095	140·6	210 367	1481	147·2
0–19 years†	..	..	..	311 227	2025	155·8
Age group, malignant cases only (WSR)						
0–14 years	274 096	2095	135·6	206 027	1481	144·3
0–19 years	..	..	..	305 233	2025	152·8

ASR=age-specific rate. WSR=age-standardised rate (world standard). *Includes cases with unknown sex (11 in the paediatric dataset and one in the general dataset). †Includes one case with unknown sex.

Table 2: **Numbers of cancer cases, person-years, and overall cancer incidence, by sex, age group, and malignant status**

D'après Steliarova-Foucher et al. 2017.

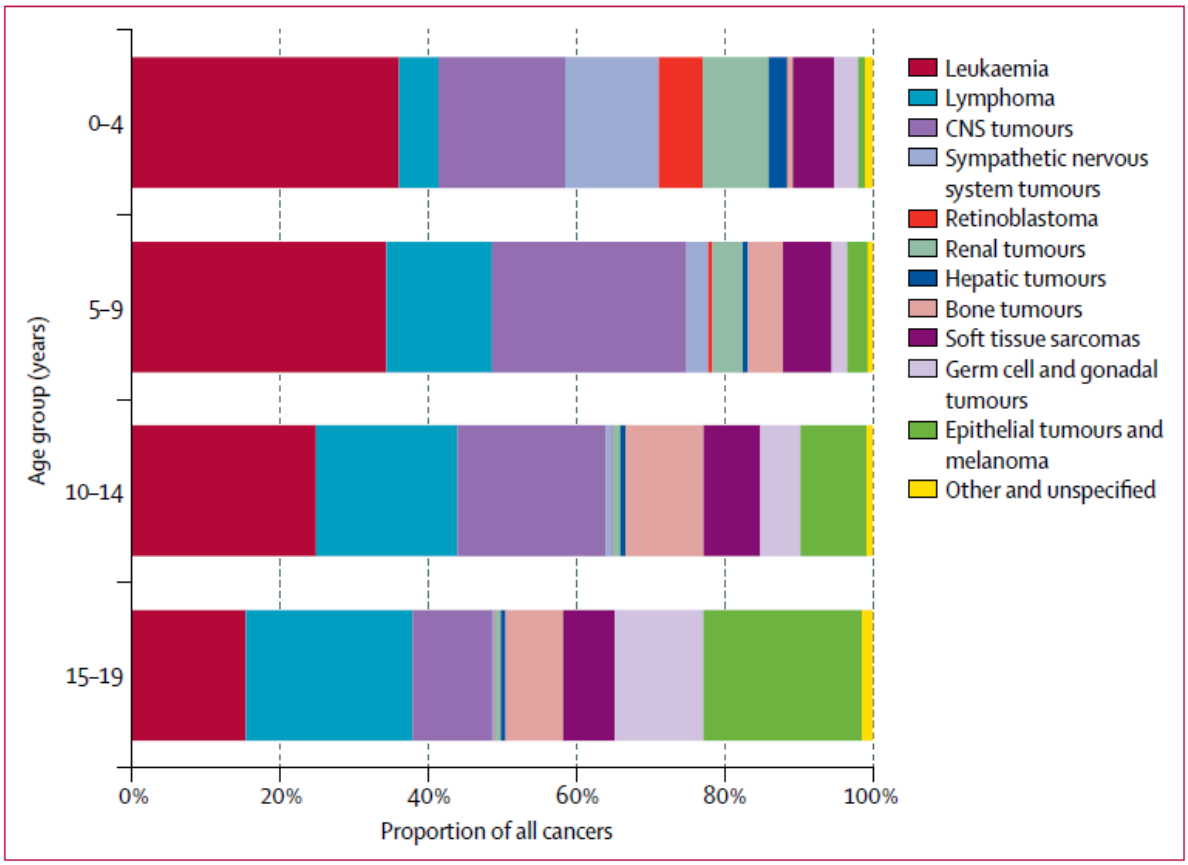

Figure 2: **Proportional distribution of cancer type by age group, 2001–10, all regions combined**
Tumours classified by *International Classification of Childhood Cancer, volume 3*.[6] Statistics for children younger than 15 years are based on the paediatric dataset and the statistics for those aged 15–19 years are based on the general dataset.

D'après Steliarova-Foucher et al. 2017.

Chaque année en Europe, 35 000 nouveaux cas de cancers sont diagnostiqués chez les enfants et adolescents. Un nouveau-né sur 300 va développer un cancer avant l'âge de 20 ans (Vassal et al. 2016). En France, le cancer touche chaque année en moyenne 2 200 nouveaux enfants et adolescents (1 750 chez les moins de 15 ans et 450 chez les adolescents de 15 à 19 ans en 2015) (Institut national du cancer 2015).

Les progrès thérapeutiques ont permis de réelles améliorations puisque la survie à 5 ans des enfants est maintenant d'environ 80% (Gatta et al. 2014 ; Lacour et al. 2014). De ce fait, les effets secondaires des thérapeutiques ne sont pas à négliger et la qualité de vie de ces futurs adultes constitue une préoccupation majeure des professionnels impliqués dans la prise en charge de ces patients.

Les cibles des traitements par chimio- et radiothérapie sont les cellules ayant un pouvoir prolifératif important. Ainsi, ces traitements agissent non seulement sur les cellules

néoplasiques, mais également sur les cellules germinales. Ils sont donc potentiellement toxiques sur la fonction de reproduction, exposant ces patients à un risque d'insuffisance gonadique prématurée. L'évaluation du risque de toxicité gonadique et le recours à une méthode appropriée de préservation de la fertilité sont indispensables pour limiter au maximum l'impact sur la qualité de vie (Lambertini et al. 2016 ; Rousset-Jablonski et al. 2015 ; Hudson et al. 2010 ; Wallace et al. 2005).

2. Toxicité gonadique

a. Chez la fille

Les filles naissent avec un nombre définitif de follicules primordiaux qui décroît avec l'âge, par atrésie ou par maturation (Wallace et al. 2011 ; Wallace et al. 2010). Durant le développement ovarien *in utero*, le pic d'ovocytes est atteint autour de 16-20 semaines d'aménorrhée avec environ 6-7 millions d'ovocytes dans les ovaires fœtaux. Le stock diminue ensuite progressivement jusqu'à la ménopause. Le nombre d'ovocytes n'est déjà plus que de 0.5 à 2 millions à la naissance, pour atteindre 0.3 million lors de la survenue des premières menstruations. Approximativement 400-500 ovocytes vont être sélectionnés pour l'ovulation jusqu'à la ménopause, où il ne reste alors qu'environ 1 000 ovocytes (Algarroba et al. 2018).

La chimiothérapie et la radiothérapie ont une toxicité directe sur les follicules expliquant le risque de survenue d'une aménorrhée secondaire chez les jeunes filles déjà réglées au diagnostic et d'un retard de développement pubertaire avec aménorrhée primaire chez les filles pré-pubères au moment du traitement (Rousset-Jablonski et al. 2015 ; Vatanen et al. 2013). En outre, les données de la littérature rapportent une probabilité moindre de grossesse chez les femmes ayant été traitées pour un cancer dans l'enfance (Rousset-Jablonski et al. 2015 ; Barton et al. 2013). La toxicité ovarienne de la chimiothérapie dépend, d'une part du type d'agent cytotoxique et de la dose cumulée, d'autre part de la réserve ovarienne de la patiente lors du traitement, avec une toxicité qui serait d'autant plus élevée que la patiente est âgée au diagnostic. La toxicité ovarienne de la radiothérapie dépend de la dose et des champs d'irradiation et, à dose égale d'irradiation, la fonction ovarienne sera d'autant plus préservée que la patiente est jeune (Bernier et al. 2010 ; Levine et al. 2010 ; Chemaitilly et al. 2006 ; Wallace et al. 2003). Il existe également une toxicité utérine de la radiothérapie

dépendant de la dose et des champs d'irradiation mais, contrairement à la toxicité ovarienne de la radiothérapie, l'utérus serait plus vulnérable en pré-pubère. Une irradiation vaginale, souvent par curiethérapie, peut également entraîner des synéchies vaginales et une hypofertilité (Sudour-Bonnange et al. 2013).

Tableau 1. Risques pour la fertilité à long terme des filles selon les traitements reçus.

Non évalué	Risque faible	Risque modéré d'infertilité et/ou d'insuffisance ovarienne prématurée	Risque élevé d'insuffisance ovarienne dès l'administration du traitement Discussion d'une conservation de cortex ovarien avant ces traitements
Taxane Oxaliplatine Irinotécan Thérapies ciblées Carboplatine Cisplatine	**Antimétabolites** - Azathioprine - Fludarabine - Méthotrexate - 6-mercaptopurine - Cytarabine **Vinca-alcaloïdes** - Vincristine - Vinblastine **Antibiotiques** - Bléomycine - Actinomycine D **Anthracyclines** - Doxorubicine **Épipodophylotoxines** - Étoposide	**Alkylants** - Cyclophosphamide (> 6 g/m²) - Ifosfamide (> 60 g/m²) - Lomustine (360 mg/m²) - Procarbazine (> 6 g/m²) - Melphalan (140 mg/m²)	**Alkylants** - Busulfan (doses myéloablatives) - Thiotépa (> 600 mg/m²) **Radiothérapie** - Irradiation corporelle totale (12 Gy) - Pelvienne (≥ 4 Gy sur 2 ovaires, sans transposition possible)

D'après Sudour-Bonnange et al. 2013.

b. Chez le garçon

Chez les garçons, la toxicité touche les cellules somatiques assurant support et nutrition (cellules de Sertoli et Leydig) ou la lignée germinale. Les cellules germinales sont plus rapidement affectées par les différents traitements, de manière plus ou moins irréversible.

L'atteinte gonadotoxique de la chimiothérapie et de la radiothérapie existe même si le traitement est administré avant la puberté, car il existe déjà une activité cellulaire importante et essentielle pour la fonction testiculaire future (Rousset-Jablonski et al. 2015 ; Chemes et al. 2001). La chimiothérapie est responsable de troubles de la spermatogenèse, l'effet gonadotoxique étant dépendant de la molécule et de la dose cumulée. Les gonades sont sensibles à l'irradiation, les séquelles étant fonction de la dose délivrée et du schéma de fractionnement (Levine et al. 2010).

Tableau 2. Risques pour la fertilité à long terme des garçons selon les traitements reçus.

Non évalué	Risque faible	Risque modéré	Risque élevé	Risque très élevé
	Conservation de spermatozoïdes chez le garçon pubère			**- Conservation de spermatozoïdes chez garçon pubère** **- Conservation de tissu testiculaire chez le garçon non pubère**
Taxanes Irinotécan Oxaliplatine Thérapies ciblées Cisplatine Gemcitabine Carboplatine	**Alkylants** - Cyclophosphamide ($< 3,5$ g/m^2) - Ifosfamide (< 36 g/m^2) **Nitrosourés** - Lomustine (1 g/m^2) (toujours donné en association avec alkylant) - Carmustine (500 mg/m^2) (toujours donné en association avec alkylant) **Antimétabolites** - Azathioprine - Fludarabine - Méthotrexate - 6-mercaptopurine - Cytarabine **Vinca-alcaloïdes** - Vincristine - Vinblastine **Antibiotiques** - Bléomycine - Actinomycine D **Anthracyclines** - Doxorubicine - Daunorubicine - Mitoxantrone **Épipodophylotoxines** - Étoposide **Antimétabolites** - Dacarbazine	**Alkylants** - Cyclophosphamide (3,5-9 g/m^2) - Procarbazine (< 4 g/m^2) - Ifosfamide (> 36 g/m^2)	**Alkylants** - Cyclophosphamide (> 9 g/m^2) - Procarbazine (> 4 g/m^2) - Thiotépa (400 mg/m^2)	**Alkylants** - Busulfan (doses myéloablatives) - Melphalan (si association) (140 mg/m^2) - Thiotépa (900 mg/m^2) **Radiothérapie** - Irradiation corporelle totale (12 Gy) - Irradiation testiculaire bilatérale (> 3 Gy)

D'après Sudour-Bonnange et al. 2013.

3. Réglementation

Le droit à la préservation de la fertilité est inscrit dans la loi Bioéthique de 2011. L'accès à la préservation de la fertilité est garanti par les lois de bioéthique qui prévoient que « toute personne dont la prise en charge médicale est susceptible d'altérer la fertilité (…) peut bénéficier du recueil et de la conservation (…) en vue de la préservation et de la restauration de sa fertilité ».

Ce droit est également encadré par le dispositif d'autorisation des établissements de santé pour le traitement des cancers et par les critères d'agrément spécifiques pour la prise

en charge des patients de moins de 18 ans. Cet accès à la préservation de la fertilité constitue de ce fait l'un des enjeux du Plan Cancer 2014-2019 (action 8.1), préconisant de systématiser l'information dès la consultation d'annonce et d'assurer un égal accès des patients sur le territoire aux plateformes clinico-biologiques de préservation de la fertilité.

4. Préservation de la fertilité

a. Chez la fille

Différentes stratégies existent pour préserver la fertilité des filles (Algarroba et al. 2018 ; Oktay et al. 2018 ; Sudour-Bonnange et al. 2013 ; De Vos et al. 2014).

Cryoconservation d'ovocytes : elle n'est envisageable que chez la jeune fille pubère, mais requiert que le traitement anti-cancéreux soit retardé de 2 à 3 semaines, permettant la stimulation hormonale par gonadotrophines nécessaire avant le recueil d'un nombre suffisant d'ovocytes (Pereira et al. 2017).

Transposition d'ovaires : elle est possible en cas de nécessité d'irradiation pelvienne (Mossa et al. 2015 ; Gubbala et al. 2014).

Cryoconservation de tissu ovarien : elle représente la seule option chez la jeune fille prépubère (Jadoul et al. 2017). Elle est aussi proposée aux adolescentes chez lesquelles le délai nécessaire à une stimulation ovarienne n'est pas envisageable. Elle ne nécessite pas de stimulation préalable et, de ce fait, ne retarde pas de façon significative la mise en route des traitements anti-cancéreux. En outre, l'intérêt est également de pouvoir restaurer voire instaurer chez les jeunes filles une fonction endocrine permettant le développement d'une puberté si elle n'avait pas encore eu lieu et d'éviter les risques liés à une insuffisance ovarienne précoce (Poirot et al. 2012). Néanmoins, d'une part elle impose un geste chirurgical sous anesthésie générale, d'autre part elle comporte un risque potentiel de réintroduction de cellules tumorales lors de la réimplantation ortho- ou hétérotopique ultérieure des fragments ovariens. Dans le monde, 86 grossesses ont été rapportées après transplantation de tissu ovarien cryopréservé (Jensen et al. 2017). En 2014, la première grossesse obtenue chez une patiente dont le tissu ovarien avait été cryoconservé avant la puberté a été décrite (Demeestere et al. 2015).

b. Chez le garçon

Différentes stratégies existent pour préserver la fertilité des garçons (Oktay et al. 2018 ; Sudour-Bonnange et al. 2013).

Cryopréservation de spermatozoïdes : elle n'est envisageable que chez le garçon pubère et le prélèvement doit, si possible, être réalisé avant toute chimiothérapie pour limiter le risque d'altération de l'ADN. Un ou plusieurs prélèvements recueils, réalisés par masturbation, peuvent être nécessaires pour constituer un stock suffisant.

Cryoconservation de tissu testiculaire : technique réalisé chez des hommes atteints d'azoospermie non obstructive, elle représente la seule option chez le garçon prépubère. Elle implique un prélèvement de pulpe testiculaire sous anesthésie générale avec extraction de gamètes, secondairement cryoconservés. Il faut prélever un quart à un tiers du volume testiculaire, compte tenu de l'asymétrie de répartition des cellules germinales (Wyns et al. 2007). Aucune méthode de restauration de la fertilité à partir de ce tissu n'est actuellement validée. Les options envisagées sont : autotransplantation avec, comme chez la fille, le risque potentiel de réintroduire la maladie, xénotransplantation et, enfin, maturation *in vitro* des spermatogonies. À ce jour, aucune spermatogenèse complète *in vitro* ni grossesse après transplantation de tissu testiculaire n'ont été décrites.

5. Envahissement tumoral du tissu gonadique

Le risque de présence de cellules tumorales dans le tissu gonadique prélevé et conservé dépend du type de cancer considéré. Toutes les tumeurs n'ont pas le même potentiel d'extension aux gonades. Ainsi, le risque de contamination est important pour les leucémies et lymphomes du fait de la présence de cellules malignes dans la circulation sanguine. La recherche de la maladie résiduelle dans les tissus gonadiques a été décrite pour ces cancers (Sadri-Ardekani et al. 2014 ; Rosendahl et al. 2013 ; Meirow et al. 2008).

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Préservation de la fertilité en cancérologie

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Résumé

Avec les progrès diagnostiques et thérapeutiques, la survie des patients atteints de cancers s'est améliorée ces dernières années. La toxicité gonadique reste une conséquence relativement fréquente des traitements utilisés avec différents niveaux d'altération de la fertilité. Dans ce contexte, la préservation de la fertilité doit être proposée aux patient(e)s exposé(e)s à un traitement potentiellement gonadotoxique. Différentes options sont proposées selon l'âge du patient, son sexe, l'urgence thérapeutique et l'évaluation des retentissements potentiels sur la fertilité. Chez les femmes prépubères, la congélation de tissu ovarien est la seule option possible. Après la puberté, la vitrification ovocytaire est de plus en plus utilisée mais nécessite une stimulation hormonale ou une maturation *in vitro* des ovocytes. Lorsque l'urgence thérapeutique ne permet pas le délai d'une stimulation, la

congélation de cortex ovarien est l'option privilégiée. Chez les garçons prépubères, la congélation de tissu testiculaire est proposée. Pour l'utilisation ultérieure du tissu cryoconservé, différentes techniques sont en cours d'évaluation telles que l'injection de cellules souches ou la maturation *in vitro*. Concernant l'homme adulte, la congélation de spermatozoïdes est pratiquée depuis des années et son efficacité en assistance médicale à la procréation est aujourd'hui clairement démontrée. La loi de bioéthique précise clairement que la préservation de la fertilité doit être proposée aux patients exposés à un traitement potentiellement gonadotoxique. De nombreuses méthodes sont aujourd'hui possibles. Les indications de préservation dans le cadre du cancer relèvent d'une concertation multidisciplinaire au sein des plateformes clinicobiologiques de préservation de la fertilité afin d'optimiser la prise en charge des patients.

Mots clés : Préservation fertilité ; Cancer ; Traitements gonadotoxiques ; Cryoconservation gamètes ; Cryoconservation tissus germinaux

Summary

Fertility preservation in oncology

Since the improvement of cancer diagnosis and treatment, survival rates of these patients increase. Gonadal damages are frequent consequences of cancer treatments with different evidence of impaired fertility. In this context, fertility preservation should be proposed to patients exposed to potentially gonadotoxic treatments. Different preservation approaches may be proposed depending on patient age, sex, cancer type and type of treatment. The indications of fertility preservation depend on sexual maturity. In young girls, ovarian cortex cryopreservation is the only technique feasible in order to preserve their reproductive potential. Vitrification of oocytes which needs ovarian stimulation or oocytes *in vitro* maturation is becoming more commonly performed for pubertal women to preserve their fertility. Ovarian cortex freezing could be offered to emergency fertility preservation of adult female cancer patients. In prepubertal boys, testicular tissue cryopreservation is the only line treatment for fertility preservation. For future use, various approaches are being evaluated such as spermatogonial stem cell injection or *in vitro* maturation. Cryopreservation of spermatozoa is today an established and successful technique for male adults. When there are no spermatozoa in ejaculate, sperm can be retrieved after treatment of testicular biopsy.

The French bioethics law clearly indicates that fertility preservation should be proposed to patients exposed to potentially gonadotoxic treatment. Today, many approaches are possible. Fertility preservation indications are based on multidisciplinary consultations within platforms for the fertility preservation in order to optimize the patient care.

Keywords: Fertility preservation; Cancer; Gonadotoxic treatments; Gametes cryopreservation; Germinal tissues cryopreservation

Introduction

Le nombre de cas de cancers diagnostiqués en France a augmenté au cours des dernières décennies à la fois chez l'adulte et l'enfant [1]. Les cancers de l'enfant diffèrent de ceux de l'adulte par leur type histologique. Les hémopathies malignes ainsi que les tumeurs cérébrales sont les cancers les plus fréquemment retrouvés [2]. Leur pronostic est également meilleur. En effet, les progrès thérapeutiques et la précision diagnostique permettent à 80% des enfants qui vont développer un cancer avant l'âge de la puberté de survivre [3]. Chez les enfants âgés de 0 à 15 ans, environ 1750 nouveaux cas sont diagnostiqués chaque année en France [4]. Et on estime qu'un enfant sur 440 est atteint d'un cancer avant sa 16^{e} année [1]. Ainsi, le nombre de personnes de 15 ans et plus en vie en 2008 et ayant eu un cancer est de l'ordre de 3 millions, avec 1 570 000 hommes et 1 412 000 femmes [1], expliquant l'enjeu majeur en santé publique de la prise en charge de cette population.

La toxicité gonadique est une conséquence relativement fréquente des traitements utilisés [5]. De plus, le cancer lui-même peut altérer la gamétogenèse indépendamment du traitement anticancéreux potentiellement stérilisant [6]. Il est donc difficile de prédire avec certitude le risque d'infertilité individuel. En effet, le niveau de toxicité d'un traitement reste difficile à évaluer précisément car il dépend du type de molécules utilisées, de leurs doses et de la fonction ovarienne ou testiculaire au moment de la mise en œuvre du traitement [7]. Il est déjà établi que certaines molécules utilisées en chimiothérapie comme les agents alkylants sont les molécules les plus agressives pour les tissus gonadiques [8,9]. Cependant, il existe des variations individuelles de sensibilité aux agents chimiothérapiques. Ces différences rendent très difficile la prédiction avec certitude de l'état des fonctions reproductrices à l'issue du traitement.

Les mécanismes d'altération de la fonction de la reproduction sont multiples. Toutes les structures anatomiques et physiologiques peuvent être altérées par les traitements. Les principaux effets observés portent sur la gamétogenèse avec des conséquences qui peuvent être définitives ou transitoires. La sévérité des lésions dépend également des associations potentielles des différents traitements. Les différents types cellulaires présents dans les gonades n'ont pas tous la même sensibilité. Les cellules germinales qui sont très sensibles aux effets de la radiothérapie et chimiothérapie peuvent présenter une altération de leur maturation, une mort cellulaire augmentée, voire une disparition totale.

La qualité de vie future de ces patients après traitement est aujourd'hui une préoccupation majeure des équipes médicales. Cette position est encouragée par de nombreuses sociétés savantes qui ont énoncé dans leurs recommandations de bonnes pratiques la préservation de la fertilité pour ces patients, comme l'American Society of Clinical Oncology (ASCO) en 2006. La préservation de la fertilité doit être proposée aux patients et aux parents des enfants atteints d'un cancer, avant la mise en route d'un traitement gonadotoxique. Cette mesure est inscrite dans la loi de Bioéthique de juillet 2011, qui reconnaît que « toute personne peut bénéficier du recueil et de la conservation de ses gamètes ou du tissu germinal... lorsqu'une prise en charge médicale est susceptible d'altérer sa fertilité, ou lorsque sa fertilité risque d'être prématurément altérée ». Par ailleurs, le Plan Cancer 2014-2019 impose aux oncologues de systématiser l'information dès la consultation d'annonce et d'adresser le patient vers un spécialiste compétant en préservation de la fertilité. De plus, ce plan souligne la nécessité cruciale d'assurer un égal accès des patients sur le territoire aux plateformes clinicobiologiques de préservation et développer la recherche sur la prévention et la réduction des risques d'altération de la fertilité. De façon optimale, la préservation de la fertilité doit avoir lieu avant la mise en œuvre du traitement anticancéreux potentiellement gonadotoxique [10]. Wallace et al. (2005) ont classé les cancers pédiatriques et ceux des jeunes adultes les plus fréquents en fonction de leur risque d'atteinte de la fertilité liée à la nature des traitements gonadotoxiques utilisés (tableau I) [8]. Le groupe à haut risque (> 80% d'infertilité) comprend les patients qui reçoivent une irradiation totale corporelle ou pelvienne, une polychimiothérapie avant greffe de cellules souches hématopoïétiques, ou les patient(e)s traité(e)s par agents alkylants pour des sarcomes métastatiques et les lymphomes hodgkiniens, par exemple.

Impact des traitements

Prédire la toxicité gonadique et donc la prise en charge du patient en fonction de son protocole de traitement anticancéreux peut s'avérer difficile, compte tenu des différentes molécules utilisées, de leur dosage, de la sensibilité individuelle des patients. Pour cela, la préservation de la fertilité doit être discutée en amont mais peut être différée ou réalisée après changement ou adaptation d'un nouveau traitement, notamment en cas d'urgence thérapeutique. Le traitement de la maladie reste le premier objectif. Après traitement anticancéreux, une altération totale de la gamétogenèse peut avoir lieu. Mais les conséquences sont moins évidentes lorsque cette atteinte de la gamétogenèse est partielle ou lorsqu'il existe une altération du gamète, à proprement parlé, avec un risque mutagène pour le futur zygote ou produit de conception. Pour cette raison, les indications de préservation de fertilité doivent être discutées et validées au sein d'une plateforme clinicobiologique multidisciplinaire comme le préconise le Plan Cancer et les différentes recommandations professionnelles permettent d'optimiser cette prise en charge.

Radiothérapie

La radiothérapie est utilisée pour le traitement de nombreuses tumeurs pédiatriques et a une place particulière pour le traitement des cancers gynécologiques de l'adulte. Les effets dépendent du site irradié, de la dose totale délivrée et de l'âge au moment du traitement.

Chez la femme

L'irradiation ovarienne entraîne une déplétion du stock de follicules primordiaux générant une insuffisance ovarienne prématurée (IOP). La gravité des lésions dépend de la dose, du schéma de fractionnement et de l'âge au moment de l'irradiation. La radiothérapie abdominopelvienne et l'irradiation corporelle totale exposent également les ovaires à ces effets toxiques. L'importance de la déplétion est proportionnelle à la quantité de follicules primordiaux présents au moment de l'initiation du traitement et donc liée également à l'âge de la patiente. L'atteinte est d'autant plus sévère que l'âge est élevé au moment du traitement.

La dose nécessaire pour détruire 50% des ovocytes existants est < 2 Gy. La dose stérilisante (97,5% d'IOP) est de 20,3 Gy à la naissance ; 18,4 Gy à 10 ans ; 15,5 Gy à 16 ans et

14,3 Gy à 30 ans [11]. La radiothérapie peut également entraîner des lésions utérines de type fibrose ou atteinte de la vascularisation de l'utérus avec comme conséquences des altérations de l'implantation et du développement ultérieur placentaire favorisant les fausses couches, un retard de croissance intra-utérin, des accouchements prématurés ou des ruptures utérines. Contrairement à l'effet toxique constaté sur la gamétogenèse, les lésions utérines sont d'autant plus importantes que la patiente est jeune au moment de l'irradiation. Les grossesses post-radiothérapie sont à considérer comme à risque avec préconisation de césariennes prophylactiques [12]. D'autres conséquences de la radiothérapie sur la vie sexuelle et donc sur la fonction de reproduction existent, notamment en cas de curiethérapie, telles que les atteintes vulvovaginales à type de synéchies ou de fistules.

Après irradiation cérébrale, une atteinte de l'axe hypothalamo-hypophysaire peut entraîner un déficit gonadotrope (dose supérieure à 30 Gy) responsable d'une aménorrhée avec anovulation.

Enfin, les irradiations ont des effets mutagènes sur les cellules germinales et peuvent induire des anomalies chromosomiques [13] avec des altérations de l'ADN transmissibles à la descendance [14].

Chez l'homme

L'irradiation pelvienne ou testiculaire peut entraîner des effets toxiques directs sur le testicule qui est extrêmement sensible, d'autant plus durant la période prépubertaire particulièrement vulnérable. Des différences de sensibilité cellulaire sont observées au niveau du testicule. Les cellules germinales à l'origine des gamètes sont très sensibles avec des altérations de l'épithélium germinal observées dès la dose de 0,15 Gy délivrée. La baisse du nombre de spermatozoïdes dans l'éjaculat (oligospermie) transitoire s'observe entre 3 et 12 Gy et devient définitive avec une azoospermie irréversible au-delà d'une dose délivrée de 12 Gy. La fonction endocrine testiculaire est altérée au-delà de 20 Gy chez l'enfant irradié en période prépubère [15]. Les cellules de Leydig sont, quant à elles, plus radio-résistantes. Une dose de plus de 20 Gy est nécessaire pour observer des lésions permanentes avec un retentissement endocrinien sur la synthèse de testostérone. Le taux de testostérone est alors moins impacté que la production spermatique, même chez les patients recevant des doses relativement fortes de radiothérapie. Des dommages de la chromatine et cassures de l'ADN du spermatozoïde sont rapportés dans l'année suivant la fin du traitement [6,16]. Le risque

principal d'une tentative de procréation trop précoce (délai inférieur à un an post-arrêt du traitement) serait le risque de transmission d'anomalies chromosomiques ou géniques à la future descendance.

Enfin, l'érection et l'éjaculation peuvent être altérées chez les hommes recevant une irradiation pelvienne, expliquées par la réduction du flux vasculaire [17].

Chimiothérapie et hormonothérapie

Les effets observés dépendent du type pharmacologique des molécules utilisées, des doses administrées et de l'effet antimitotique des traitements. La classe des alkylants est celle dont les effets gonadotoxiques sont les mieux connus. L'impact des traitements dépend des doses cumulées avec des effets d'autant plus marqués et irréversibles que les doses cumulées sont élevées. Les chimiothérapies atteignent de préférence les cellules en division et donc, en particulier, en période active de gamétogenèse. Néanmoins, la chimiothérapie a peu d'impact sur la mise en place de la puberté [18].

Chez la femme

La chimiothérapie entraîne une déplétion prématurée de la réserve folliculaire avec un risque d'infertilité ou d'IOP [8]. Les effets gonadotoxiques sont particulièrement dépendants de l'âge de la patiente traitée. Plus la personne est âgée, moins la fonction de récupération gonadique est possible. En effet, le nombre d'ovocytes diminue avec l'âge (1-2 millions à la naissance, 400 000 à la puberté et moins de 1000 à la ménopause) et la cytotoxicité réduit le nombre de follicules ovariens. La réserve ovarienne est donc affectée de façon définitive avec une entrée en croissance importante des follicules. Chez la jeune fille pubère, une aménorrhée peut se produire mais la restauration des cycles à l'arrêt du traitement reste possible. Cependant, l'âge ovarien (évalué par la réserve ovarienne) est affecté avec un vieillissement prématuré d'une dizaine d'années par rapport à l'âge biologique [19]. D'un point de vue histologique, des lésions des cellules de la granulosa et des cellules germinales ont pu être mises en évidence avec possible fibrose, remaniement du stroma et des vaisseaux. Par exemple, le busulfan (agent alkylant, utilisé comme anticancéreux dans le conditionnement préalable à une greffe de cellules souches hématopoïétiques) est très toxique, quel que soit l'âge de la patiente. D'autres traitements tels que l'association ABVD (adriamycine,

bléomycine, vincristine et dacarbazine) administrée dans le lymphome hodgkinien ont peu d'impact cytotoxique sur la fonction ovarienne mais ont une atteinte génotoxique potentielle importante. Cette atteinte peut concerner les ovocytes mais aussi les cellules de la granulosa avec des lésions double-brin de l'ADN, une apoptose par action mitochondriale ou encore des adduits à l'ADN [20].

Chez l'homme

Sur le tissu testiculaire prépubère ou pubère, les chimiothérapies ont des effets cytotoxiques [6,16]. Les lésions concernent principalement la lignée germinale avec une atteinte de la spermatogenèse. Les spermatogonies sont les plus sensibles. La production de testostérone serait quant à elle peu touchée. En effet, la sensibilité des cellules germinales est bien supérieure à celle des cellules de Leydig [17]. Cette conséquence est liée à leurs fortes activités mitotique et méiotique. Par ailleurs, la maturation sexuelle des testicules influence le degré d'atteinte gonadique avec un testicule prépubère moins sensible aux effets cytotoxiques des chimiothérapies qu'un testicule pubère. Un hypogonadisme peut être retrouvé chez les hommes traités pour cancer [21].

Une atteinte génotoxique est possible et consiste en une altération de l'intégrité nucléaire du gamète avec un retentissement potentiel sur la future descendance. Les effets génotoxiques sont différents en fonction du stade de maturation du spermatozoïde avec plusieurs conséquences possibles : fragmentation/cassure de l'ADN au niveau des noyaux des spermatozoïdes ou encore défaut de condensation de la chromatine. À ce risque mutagène s'ajoute une altération du système de réparation de l'ADN. Selon le stade de maturation du gamète mâle, les capacités de réparation de l'ADN peuvent être diminuées, voire inexistantes. À partir du stade spermatocyte, la réparation de l'ADN est quasi impossible avec une incapacité de réparation de l'ADN chez le spermatozoïde mature. Des dommages de l'ADN peuvent être observés plusieurs mois après l'arrêt des traitements anticancéreux [6]. Ces effets incitent fortement à réaliser une cryoconservation des spermatozoïdes avant tout traitement anticancéreux. Ces effets génotoxiques peuvent entraîner une aneuploïdie du spermatozoïde avec par exemple des fausses couches à répétition chez la partenaire dont la grossesse résulte de l'utilisation de ces spermatozoïdes ou des malformations chez le conceptus.

Impact de la chirurgie

Elle peut compromettre la fertilité en particulier lorsqu'elle touche les organes génitaux : ovariectomie ou hystérectomie chez la femme, orchidectomie ou prostatectomie chez l'homme. Cependant, au vu de l'amélioration des thérapies anticancéreuses (chimiothérapie, radiothérapie, immunothérapie), la chirurgie a subi une désescalade permettant de plus en plus aux patient(e)s de conserver leurs organes reproducteurs.

Chez la femme

En cas de cancer du col, le traitement a longtemps été radical à type d'hystérectomie élargie. Aujourd'hui, des progrès majeurs ont été réalisés. En cas de tumeurs cervicales, deux situations se présentent : les lésions dysplasiques précancéreuses et les cancers avérés. En cas de lésion dysplasique (néoplasie cervicale intra épithéliale [CIN] de grades 2 et 3 et les carcinomes in situ [CIS]), un traitement conservateur par conisation est la règle, ceci permet la conservation utérine et ovarienne. Le but est de réaliser une conisation minime mais suffisante afin d'éradiquer la lésion mais de permettre le fonctionnement cervical. Les risques de cette technique étant principalement obstétricaux à type d'incompétence cervicale et d'accouchement prématuré [22]. En cas de cancer de col avéré aux stades précoces IA1 ou pour certaines équipes IA2-IB, une trachélectomie élargie avec conservation utérine est possible. Les critères de sélection sont stricts, tumeurs de moins de deux centimètres et patiente en âge de procréer. Les conséquences obstétricales (fausses couches tardives et accouchements prématurés) sont à évoquer avec les femmes. Enfin, pour les cancers de plus haut stade, les traitements sont souvent castrateurs et actuellement aucune stratégie de préservation n'a fait ses preuves [23].

Concernant les tumeurs de l'endomètre, le traitement a également longtemps été radical à type d'hystérectomie. Récemment, les travaux de Gonthier et al. (2017) ont montré que chez les patientes sélectionnées, en âge de procréer, avec hyperplasie atypique de l'endomètre ou adénocarcinome endométrioïde de stade 1 grade 1, un traitement conservateur est possible [24]. Ce traitement implique une hormonothérapie à type de progestatifs oraux associés à un monitoring par hystéroscopie et biopsies endométriales. Les probabilités de rémission après traitement conservateur sont estimées à 78,0% à 12 mois ; les probabilités de récidive à 29,2% à 24 mois et le risque de progression à 15% (stade 1A avec

invasion myométriale ou plus sur la pièce d'hystérectomie). En termes de fertilité, 32% des femmes obtiennent au moins une grossesse. Il ne s'agit en aucun cas d'un traitement curatif, une fois le projet de grossesse des femmes réalisé, une hystérectomie devient alors nécessaire. En cas de cancer du col ou de l'endomètre de plus haut stade, l'hystérectomie devient indispensable. Si les ovaires ont été conservés, avec ou sans transposition, la gestation pour autrui (GPA) dans les pays où celle-ci est autorisée peut être un recours. En effet en Grande Bretagne où la GPA est permise, les antécédents de cancer en sont la cause la plus fréquente.

Les tumeurs borderline de l'ovaire ont également subi une désescalade chirurgicale, leur prise en charge est maintenant bien codifiée [25]. Actuellement, chez les patientes jeunes présentant un stade précoce et unilatéral, un traitement conservateur à type de kystectomie ou annexectomie unilatérale et « staging » (cytologie et biopsies péritonéales et omentales) est possible, tout en informant la patiente du risque de récidive. Ainsi, dans la méta-analyse de Swanton et al. (2007), regroupant neuf études et 213 patientes ayant eu une chirurgie conservatrice, le taux de grossesses observé est de 48% en cumulant les grossesses spontanées et celles après AMP [26]. Concernant les tumeurs épithéliales de l'ovaire, les dernières données semblent autoriser pour les stades 1, grade 1 ou 2, un traitement conservateur. Dans la série de Park en 2016, 22,2% des patientes étaient enceintes avec un taux de récurrence de 5,6% [27].

Chez l'homme

Chez l'adulte, les traitements castrateurs à type d'orchidectomie ou les chirurgies altérant la vie sexuelle (prostatectomie) peuvent être prévenus par une cryopréservation de spermatozoïdes. Suite aux résultats très favorables et à la faisabilité de cette technique de préservation, les chirurgies radicales demeurent le gold standard. Le problème est plus délicat chez l'enfant prépubère où les solutions en termes de préservation de la fertilité demeurent, à l'heure actuelle, encore du domaine expérimental.

Lambertini et al. (2016) ont classé les principaux traitements anticancéreux (chimiothérapie et/ou radiothérapie) en fonction de leur risque potentiel sur la fertilité (tableau II) [28].

Préservation de la fertilité : techniques, limites et enjeux

Diverses techniques sont aujourd'hui disponibles pour préserver la possibilité d'avoir un enfant à l'issue d'un traitement potentiellement toxique pour l'appareil reproducteur. La préservation de la fertilité féminine est une problématique encore novatrice avec des résultats très prometteurs mais récents du fait de l'efficacité de la méthode de congélation des ovocytes par vitrification et de la greffe de tissu ovarien (TO). Chez l'homme, la cryoconservation de sperme fait preuve de son efficacité depuis de nombreuses années.

Chez la femme

Congélation du cortex ovarien

Chez les jeunes filles prépubères, la cryoconservation de tissu ovarien (CTO) est la seule option envisageable pour préserver leur fertilité [29]. La CTO est également la seule technique applicable chez la femme pubère chez qui le début de la chimiothérapie doit avoir lieu le plus rapidement possible et sans délai compatible avec une stimulation ovarienne pour congélation des ovocytes [30]. En France, depuis 2005, environ 155 prélèvements de TO ont lieu chaque année. Cette CTO est proposée en cas de traitement à fort pouvoir stérilisant [31]. L'idéal est de pouvoir réaliser ce prélèvement avant le traitement gonadotoxique, cependant, environ 80% des patientes ont reçu une chimiothérapie avant la CTO, en particulier lors de pathologies hématologiques (traitement pré-greffe de cellules souches hématopoïétiques). Se pose alors la question des effets de la chimiothérapie sur les follicules primordiaux.

La première greffe de TO cryoconservé a eu lieu en 2000 par K. Oktay et O. Oktem [32]. Il s'agissait d'une greffe orthotopique qui a permis, après stimulation de l'ovulation, un développement folliculaire et une sécrétion d'estradiol. L'autogreffe de TO consiste à replacer les fragments de cortex ovarien au niveau de l'ovaire restant ou dans la fossette péritonéale en cas d'ovariectomie (greffe orthotopique) ou dans un site corporel éloigné de l'ovaire, comme l'avant-bras, au niveau sous-cutané (greffe hétérotopique). La première grossesse après greffe orthotopique de cortex ovarien cryoconservé a été rapportée chez une femme, trois ans après traitement d'un lymphome hodgkinien de stade IV [33]. Depuis, 86 naissances ont été répertoriées dans le monde après CTO dont sept femmes qui avaient eu une préservation de la fertilité avant chimiothérapie [34]. En 2014, a été rapportée la première grossesse évolutive chez une patiente dont le TO cryoconservé puis autogreffé avait été prélevé avant la puberté [35]. En plus de la préservation de la fertilité, l'intérêt de ce greffon

est également de restaurer, voire d'instaurer chez l'adolescente une fonction endocrine permettant le développement d'une puberté si elle n'avait pas encore eu lieu et d'éviter les risques liés à une IOP. La première greffe à cette visée a été publiée par l'équipe de Poirot en 2012 [36].

Le prélèvement de cortex ovarien en vue d'une congélation est en général effectué par laparoscopie, toutefois il peut se programmer simultanément à un autre geste chirurgical (par laparoscopie ou laparotomie) afin d'éviter une chirurgie et une anesthésie supplémentaires. En fonction de l'âge de la patiente et de la nature des traitements envisagés, le prélèvement d'un ovaire ou une résection cunéiforme d'un ou des deux ovaires est discuté. De nombreux follicules primordiaux et primaires sont ainsi conservés dans leur environnement tissulaire. En pratique, les fragments de cortex ovarien prélevés sont placés dans des cryotubes en présence d'un milieu de congélation afin de préserver au mieux la structure du TO et la vitalité des follicules tout en limitant leur apoptose.

L'utilisation de ce TO cryoconservé par greffe présente le risque majeur de réintroduction de la maladie cancéreuse. Il a été démontré que l'autogreffe de TO après décongélation chez des patientes guéries d'une leucémie présente un risque non négli-geable de réintroduction de la pathologie maligne. En effet, les cellules cancéreuses leucémiques circulent dans le sang au moment de la cryopréservation du cortex ovarien [37]. Cette possibilité doit alors être abordée avec la patiente et/ou sa famille avant le prélèvement. La détection de la maladie résiduelle par reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) a été décrite pour les leucémies et les lymphomes [5]. Dans l'espoir de diagnostiquer la maladie résiduelle avant utilisation du TO cryoconservé, des travaux de mise au point de détection de la maladie résiduelle dans les TO ont été récemment réalisés. Grèze et al. (2017) ont validé une méthode de détection de la maladie résiduelle dans le cas de tumeurs solides avec fort potentiel métastatique chez des patientes prépubères [38]. Afin d'éviter le risque de réintroduction des cellules cancéreuses, des techniques encore expérimentales sont en cours de développement avec la greffe de follicules isolés [39] ou avec la culture *in vitro* de follicules [40].

Congélation des ovocytes

Après la puberté, la congélation des ovocytes peut être proposée afin de préserver la fertilité de ces patientes. Une stimulation hormonale est nécessaire afin d'obtenir un nombre

important d'ovocytes matures (au moins huit à dix ovocytes) [41]. Compte tenu du temps nécessaire pour la stimulation (environ deux semaines), elle peut être incompatible avec l'urgence du traitement. Les risques d'hyperœstrogènie liés à l'hyperstimulation ovarienne dans certaines tumeurs hormonodépendantes comme les cancers du sein peuvent être maîtrisés par l'administration à la patiente d'anti-estrogènes ou d'inhibiteur de l'aromatase. La vitrification d'ovocytes devient alors possible chez ces patientes [42]. La méta-analyse de Rodgers et al. (2017) ne retrouve pas de chute des taux de survie chez les patientes ayant reçu une stimulation ovarienne avec du létrozole en comparaison à celles n'ayant pas bénéficié de préservation de la fertilité [42]. Il ne peut conclure sur le tamoxifène en raison d'un nombre de données insuffisantes. Lorsque le traitement doit être mis en place dans un délai très court, le prélèvement d'ovocytes immatures suivi d'une maturation in vitro peut être envisagé. Les ovocytes doivent être prélevés dans des follicules antraux pendant la phase lutéale ou au début de la phase folliculaire. Une maturation *in vitro* (MIV) de 24 à 48h est nécessaire avant vitrification de ces ovocytes devenus matures. L'efficacité de la vitrification ovocytaire est aujourd'hui démontrée pour les patientes prises en charge en assistance médicale à la procréation avec des résultats similaires à ceux obtenus avec des ovocytes frais, non vitrifiés. Les chances d'obtenir un enfant augmentent avec le nombre d'ovocytes obtenus [43]. La probabilité d'obtenir au moins dix ovocytes matures à la ponction diminue avec l'âge de la patiente [41]. La série présentée en 2014 par l'équipe de Martinez est très prometteuse avec un taux de survie ovocytaire à 92,3%, un taux de fécondation à 76,6% et un taux de naissances vivantes à 36,4% [44]. La vitrification ovocytaire semble donc une bonne technique de préservation de la fertilité pour les femmes pubères atteintes de cancers.

Enfin, la maturation *ex vivo* d'ovocytes obtenus sur des fragments d'ovaires prélevés chirurgicalement en vue d'une CTO est une option récente et prometteuse. Cette maturation peut dans certains cas apporter une méthode complémentaire pour optimiser la préservation de fertilité chez les patientes atteintes d'un cancer, en particulier chez les jeunes femmes chez qui la greffe de TO peut présenter un risque de rechute de la pathologie cancéreuse initiale. Le couplage des deux techniques, maturation *ex vivo* et CTO, est une option qui permet de potentialiser les chances de grossesses chez les femmes pubères où la greffe de TO serait contre-indiquée [45]. La première naissance suite à la maturation *in vitro* d'ovocytes obtenus sur des fragments d'ovaires prélevés chirurgicalement en vue d'une CTO a été rapportée par Prasath et al. en 2014 [46].

Congélation d'embryons

Pour les patientes qui sont en couple avec un projet parental, la congélation d'embryons peut être une option à proposer. Cette option soulève alors un problème éthique majeur du devenir des embryons dans le cas d'une séparation du couple ou du décès de l'un des deux partenaires. Les chances de grossesses après décongélation et transfert des embryons congelés dépendent de l'âge de la patiente au moment de la réalisation de la fécondation *in vitro*, du nombre et de la qualité des embryons après décongélation. Il existe peu de données dans la littérature sur les résultats de cette technique dans le cadre de la préservation de la fertilité, en raison d'un nombre restreint de patientes ayant recours à un transfert embryonnaire après guérison d'un cancer. Les résultats en termes de taux de grossesses moyens seraient comparables à ceux obtenus après transfert d'embryons congelés lors de prise en charge en AMP standard [47]. La réponse ovarienne à la stimulation hormonale est inférieure chez ces patientes atteintes d'un cancer, comparée aux résultats obtenus en AMP chez les couples pris en charge pour infertilité [48]. De la même façon que pour la conservation des ovocytes, cette technique n'est pas indiquée chez les patientes pour lesquelles le traitement doit être instauré en urgence. En effet, un délai est nécessaire pour obtenir des ovocytes matures et peut être incompatible avec la mise en route le plus rapidement possible du traitement. La limite principale de cette technique est la nécessité d'un compagnon. La loi française prévoit les conditions d'accès au transfert ultérieur des embryons. Ainsi, il ne sera pas possible de procéder au transfert des embryons en cas de séparation ou de décès de l'un des deux conjoints.

Autres techniques

La transposition des ovaires hors du champ d'irradiation réduit la dose d'irradiation reçue par les ovaires. Elle est proposée avant radiothérapie pelvienne et est facilement réalisée par cœlioscopie. Dans 88% des cas, elle protège efficacement la fonction ovarienne ultérieure [49]. Une protection hormonale ou chimique peut être proposée afin d'inhiber l'axe hypothalamo-hypophysaire en administrant des estroprogestatifs ou agonistes de la GnRH (gonadotropin-releasing hormon). Cette inhibition permettrait en théorie, de bloquer la gamétogenèse et donc de protéger des follicules déjà hormono-sensibles et donc de réduire

la sensibilité des cellules germinales face aux antimitotiques. Cependant, elle serait inefficace sur le pool de follicules primordiaux. Néanmoins, son efficacité est controversée [50].

Chez l'homme

Conservation des spermatozoïdes

Pour préserver la fertilité des jeunes patients ayant déjà réalisé leur puberté ou celle des hommes adultes, les spermatozoïdes provenant d'un ou plusieurs éjaculats de sperme peuvent être congelés pour utilisation ultérieure en AMP. L'AMP peut être réalisée par insémination intra-utérine ou par fécondation *in vitro* (FIV) avec ou sans micro-injection (ICSI). Le choix de la technique sera réalisé en fonction du bilan des deux partenaires et de la qualité des paillettes de sperme autoconservées. Cette conservation de spermatozoïdes est une méthode efficace, pratiquée sans aucun délai nécessaire au laboratoire de biologie de la reproduction depuis environ 30 ans. Un ou plusieurs recueils, réalisés par masturbation, sont nécessaires afin de constituer un stock suffisant. Le nombre de recueils dépend de la qualité de l'éjaculat, qui peut être altérée notamment par l'altération générale du patient suite à sa pathologie cancéreuse. Il est fortement recommandé de réaliser tout prélèvement de sperme avant le début de la chimiothérapie et/ou de la radiothérapie afin d'espérer préserver la qualité et l'intégrité de l'ADN spermatique.

Lorsqu'il n'y a aucun spermatozoïde dans l'éjaculat (azoospermie), la réalisation d'une biopsie testiculaire (BT) peut être discutée afin d'effectuer une extraction tissulaire et une congélation des spermatozoïdes. La BT est réalisée sous anesthésie générale et nécessite un délai supplémentaire.

Le garçon prépubère ne peut pas produire un éjaculat. En effet, même si le testicule prépubère contient des spermatogonies, il n'y a aucun spermatozoïde mature, la spermatogenèse débutant à la puberté. En dépit d'un faible taux de prolifération cellulaire observé au niveau des spermatogonies, le testicule prépubère n'est pas protégé des effets toxiques de la chimiothérapie ou de la radiothérapie. Ainsi, une azoospermie a été décrite comme apparaissant chez près de 18% des hommes guéris d'un cancer traité durant l'enfance [51]. La préservation de la fertilité chez le garçon prépubère est donc un enjeu majeur de leur prise en charge en cas de cancer.

Conservation du tissu testiculaire

Chez le garçon prépubère, une seule méthode peut être conseillée : la cryoconservation de tissu testiculaire (TT). La proposition de congélation du TT doit être discutée de manière collégiale entre l'oncopédiatre, le biologiste de la reproduction et le chirurgien. La congélation de TT peut être envisagée chez l'adulte jeune en cas d'impossibilité de recueil de sperme. Le prélèvement de TT peut être alors effectué sous anesthésie générale simultanément à un autre geste chirurgical (exérèse de la tumeur, pose de voie veineuse centrale...). Un quart à un tiers du volume testiculaire prépubère est prélevé car le testicule est de petite taille. Une analyse histologique est indispensable afin d'évaluer la quantité de cellules souches spermatogoniales et de rechercher la présence éventuelle de cellules tumorales. Aujourd'hui, la production de spermatozoïdes fonctionnels à partir de TT de garçons prépubères n'a pu être obtenue. Trois voies sont développées dans le domaine de la recherche avec des données qui sont prometteuses :

- l'injection directe de cellules souches spermatogoniales en suspension dans les tubes séminifères ou dans le *rete testis* ;
- la greffe de fragments de TT [52] ;
- la maturation *in vitro* des spermatogonies jusqu'à l'obtention de spermatozoïdes pouvant être utilisés en injection intracytoplasmique de spermatozoïdes (ICSI).

Cependant, l'utilisation du TT en vue de restauration de la fertilité n'est pas sans risque. En effet, le TT de patients atteints de cancer pourrait être contaminé par des cellules cancéreuses, d'autant plus que le prélèvement de TT est réalisé de préférence avant le début des traitements. La maturation *in vitro* présente l'avantage d'éviter tout risque de réintroduction de cellules cancéreuses, mais reste expérimentale pour le moment.

Réglementations et recommandations

La loi bioéthique n°2004-800 du 6 août 2004 précise clairement que « toute personne dont la prise en charge médicale est susceptible d'altérer la fertilité (...) peut bénéficier du recueil et de la conservation (...) en vue de la préservation et de la restauration de sa fertilité ». En France, de nombreuses sociétés savantes ont énoncé dans leurs recommandations de bonnes pratiques la préservation de la fertilité pour ces patients : l'Agence francophone des soins oncologiques de support (AFSOS), l'Institut national du cancer (INCa) et les Centres d'étude et de conservation des œufs et du sperme humains (CECOS). Le troisième Plan Cancer

(2014-2019) précise également que la préservation de la fertilité est un enjeu majeur en préconisant de systématiser l'information dès la consultation d'annonce et d'assurer un égal accès des patients sur le territoire aux plateformes clinicobiologiques de préservation de la fertilité. En effet, en France, la conservation des gamètes et des tissus germinaux est assurée essentiellement par les CECOS autorisés pour cette activité par les Agences régionales de santé (ARS). Ces dernières années se sont mises en place des plateformes de préservation de fertilité pour une prise en charge multidisciplinaire optimale. Avant la conservation, les sérologies pour les virus de l'immunodéficience humaine VIH1 et VIH2, de l'hépatite C (VHC) (anticorps anti-HCV), de l'hépatite B (antigène HBs, anticorps anti-HBs et anticorps anti-HBc) et la sérologie pour l'agent de la syphilis doivent être effectuées chez le ou la patient(e) concerné(e) dans les trois mois précédant la conservation [53]. En cas de positivité de l'un des marqueurs, des examens supplémentaires et l'avis d'un expert du domaine concerné peuvent être nécessaires. Le centre qui conserve les gamètes ou les tissus germinaux interroge chaque année la personne concernée sur son souhait de poursuivre la conservation. La personne peut renoncer à l'autoconservation en consentant soit : au don de gamète, au don pour la recherche ou à l'arrêt de la conservation.

L'indication de la préservation de la fertilité relève d'une discussion multidisciplinaire et doit être réalisée par une équipe compétente et autorisée par l'ARS. Les recommandations vont encore évoluer dans les années à venir avec l'amélioration des connaissances des effets sur la fertilité des différents traitements utilisés en cancérologie et compte tenu des nouvelles thérapeutiques en cours de développement (figures 1 et 2). Une collaboration multidisciplinaire est indispensable afin de prendre en charge de façon optimale le patient. Il est important d'intégrer la problématique de la fertilité dans le parcours personnalisé de soins et d'orienter le ou la patient(e) vers une consultation d'oncofertilité. La surveillance du patient et le dépistage des effets secondaires des traitements du cancer sont en général mis en place dans le programme personnalisé de la vie après cancer. L'évaluation de la réserve ovarienne chez la femme ou de la spermatogenèse (spermogramme) chez l'homme et le dépistage de déficits endocriniens en cas de traitements à forte toxicité doivent être intégrés dans le suivi du ou de la patient(e). Le rythme de la surveillance doit être envisagé avec le ou la patient(e) en fonction du traitement, de son âge et du projet personnel. Une préservation de la fertilité complémentaire à distance de l'arrêt des traitements (deux ans après l'arrêt) pourra être envisagée en cas d'atteinte de la réserve ovarienne ou d'altération de la spermatogénèse.

Conclusion

La préservation de la fertilité doit être proposée en cas de traitement gonadotoxique après une concertation multidisciplinaire. L'amélioration de la connaissance des effets des traitements dans les années à venir permettra l'élaboration de nouveaux référentiels qui compléteront ceux déjà existants.

Déclaration de liens d'intérêts

Les auteurs déclarent ne pas avoir de liens d'intérêts.

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TABLEAU I

Évaluation du risque d'infertilité après traitement des cancers fréquents de l'enfant et de l'adolescence selon Wallace et al. (2005) [8]

Risque faible (< 20 %)
Leucémie aiguë lymphoblastique
Tumeur de Wilms
Sarcome des tissus mous : stade I
Tumeurs des cellules germinales (avec préservation gonadique sans radiothérapie)
Rétinoblastome
Tumeur cérébrale : chirurgie seule, irradiation cérébrale < 24 Gy
Risque modéré
Leucémie aiguë myéloïde (difficile à évaluer)
Hépatoblastome
Sarcome osseux
Sarcome d'Ewing non-métastatique
Sarcome des tissus mous : stade II ou III
Neuroblastome
Lymphome non-Hodgkinien
Maladie d'Hodgkin
Tumeur cérébrale : radiothérapie crâniospinale, irradiation cérébrale > 24 Gy
Risque élevé (> 80 %)
Irradiation corporelle totale
Radiothérapie localisée pelvienne ou testiculaire
Chimiothérapie conditionnant greffe de moelle osseuse
Maladie d'Hodgkin : traitement avec agents alkylants
Sarcome des tissus mous : stage IV (métastatique)
Sarcome d'Ewing : métastatique

TABLEAU II

Risques d'infertilité liés aux principaux traitements anticancéreux selon Lambertini et al. (2016) [28]

Niveau de risque	Type de traitement anticancéreux	
	Femme	Homme
Risque élevé (> 80 % d'aménorrhée définitive chez la femme et d'azoospermie chez l'homme)	Greffe de CSH avec cyclophosphamide/TBI ou cyclophosphamide/busulfan Radiothérapie (champs d'irradiation ne comprenant pas les ovaires) CMF, CEF, CAF, TAC × 6 cycles chez les femmes > 40 ans	Radiothérapie > 2,5 Gy des testicules Chlorambucil (1,4 g/m^2) Cyclophosphamide (19 g/m^2) Procarbazine (4 g/m^2) Melphalan (140 mg/m^2) Cisplatine (500 mg/m^2) BCNU (1 g/m^2) et CCNU (500 mg/m^2)
Risque modéré (40–60 % d'aménorrhée définitive chez la femme, probable azoospermie chez l'homme si association avec d'autres agents stérilisants)	BEACOPP CMF, CEF, CAF, TAC × 6 cycles chez les femmes âgées de 30–39 ans AC × 4 cycles chez les femmes ≥ 40 ans AC ou EC × 4 taxanes	Busulfan (600 mg/kg) Ifosfamide (42 g/m^2) BCNU (300 mg/m^2) Moutardes azotées Actinomycine D
Risque faible (< 20 % d'aménorrhée définitive chez la femme, diminutions réversibles des paramètres spermatiques chez l'homme avec d'autres effets possibles)	ABVD chez les femmes > 32 ans CHOP × 4–6 cycles CVP Thérapie pour LAM (anthracycline/cytarabine) Protocole pour LAL CMF, CEF, CAF, TAC × 6 cycles chez les femmes ≤ 40 ans	Carboplatine (2 g/m^2) Doxorubicine (770 mg/m^2) Thiotépa (400 mg/m^2) Cytosine arabinoside Vinblastine (50 g/m^2) Vincristine (8 g/m^2)
Risque très faible ou absence de risque (Risque d'aménorrhée définitive chez la femme; diminutions réversibles des paramètres spermatiques chez l'homme avec d'autres effets possibles)	ABVD chez les femmes < 32 ans Méthotrexate Fluorouracile Vincristine Tamoxifène	Amsacrine Bléomycine Dacarbazine Daunorubicine Epirubicine Etoposide Fludarabine Fluorouracile 6-mercaptopurine Méthotrexate Mitoxantrone Thioguanine Prednisone Interféron-alpha
Risque inconnu (Risque d'aménorrhée définitive chez la femme; effets sur la production spermatique chez l'homme)	Anticorps monoclonaux (trastuzumab, bevacizumab, cetuximab) Inhibiteurs de tyrosine kinase (erlotinib, imatinib)	Oxaliplatine Irinotecan Anticorps monoclonaux (trastuzumab, bevacizumab, cetuximab) Inhibiteurs de tyrosine kinase (erlotinib, imatinib) Taxanes

CSH : cellules souches hématopoïétiques ; TBI : irradiation corporelle totale ; CMF : cyclophosphamide, méthotrexate, fluorouracile ; CEF : cyclophosphamide, épirubicine, fluorouracile ; CAF : cyclophosphamide, doxorubicine, fluorouracile ; TAC : docétaxel, doxorubicine, cyclophosphamide ; BEACOPP : bléomycine, étoposide, doxorubicine, cyclophosphamide, vincristine, procarbazine, prednisone ; BCNU : carmustine ; CCNU lomustine, AC : doxorubicine, cyclophosphamide ; ABVD : doxorubicine, bléomycine, vinblastine, dacarbazine ; CHOP : cyclophosphamide, doxorubucine, vincristine, prednisone, CVP : cyclophosphamide, vincristine, prednisone ; LAM : leucémie aiguë myéloblastique ; LAL : leucémie aiguë lymphoblastique.

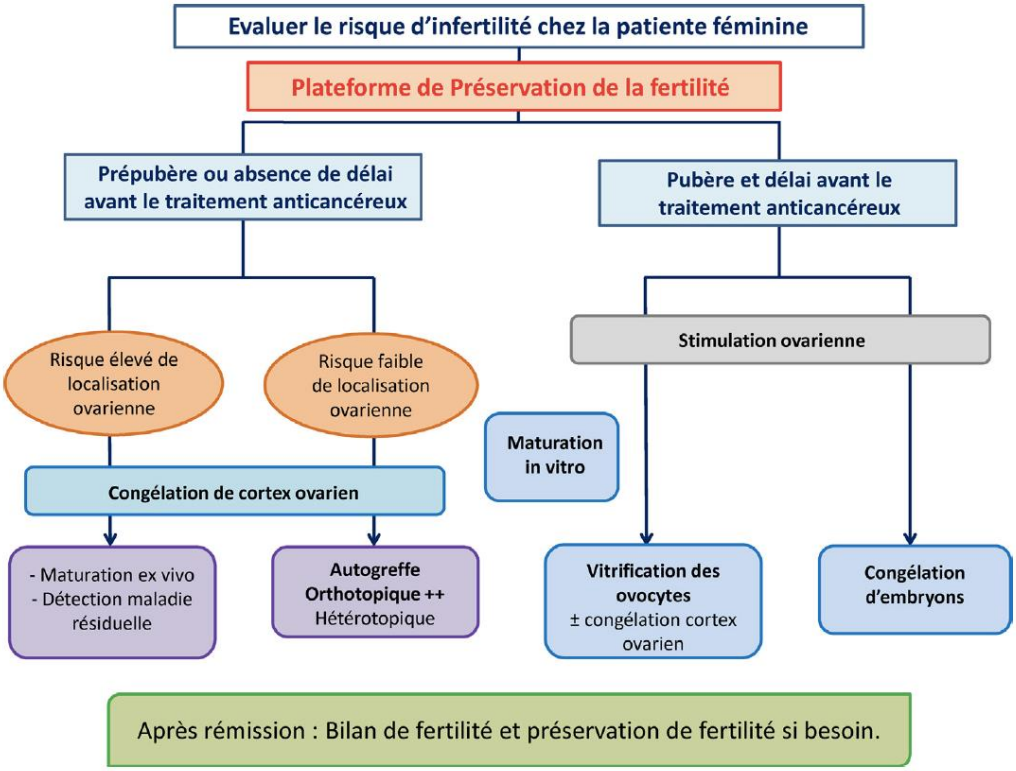

FIGURE 1

Prise en charge de la patiente féminine atteinte d'un cancer au sein par une plateforme de préservation de la fertilité

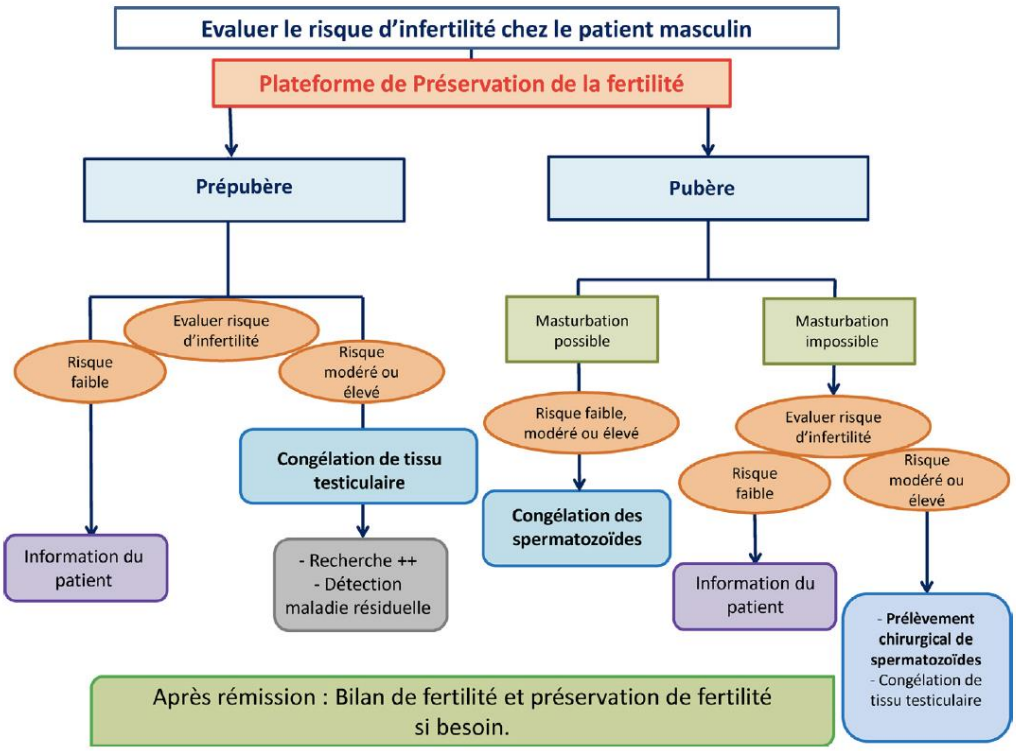

FIGURE 2

Prise en charge du patient masculin atteint d'un cancer au sein par une plateforme de préservation de la fertilité

II. FERTILITÉ APRÈS UNE PATHOLOGIE CANCÉREUSE DANS L'ENFANCE : MODÈLE DE LA LEUCÉMIE CHEZ LA FILLE

1. Rationnel de l'étude

Avec l'amélioration des thérapeutiques au cours des dernières décennies, la survie des patients pris en charge pour une leucémie dans l'enfance est actuellement d'environ 80% pour la leucémie aiguë lymphoblastique et 60% pour la leucémie aiguë myéloblastique (Gatta et al. 2014 ; Pui et al. 2011). De tels progrès encouragent à prendre davantage en considération les effets secondaires des traitements pour assurer à ces futurs adultes la meilleure qualité de vie possible.

L'ovaire est particulièrement sensible aux thérapeutiques anticancéreuses, dont l'impact sur le développement pubertaire et sur la fertilité est connu (Green at al. 2009a ; Green et al. 2009b).

Nous avons la chance de disposer en France depuis 2004 d'une importante base de données sur le suivi à long terme des survivants de leucémies dans l'enfance. La cohorte multicentrique L.E.A. (Leucémie de l'Enfant et l'Adolescent) recense différentes données cliniques, socio-économiques et de qualité de vie chez les patients qui ont été pris en charge pour une leucémie à l'âge pédiatrique depuis 1980. En 2014, un questionnaire plus spécifiquement consacré aux données sur la fertilité a été ajouté au questionnaire général (Berbis et al. 2015 ; Michel et al. 2007).

L'objectif de notre étude était d'évaluer le développement pubertaire et la fertilité des femmes de plus de 18 ans ayant été traitées pour une leucémie durant l'enfance ou l'adolescence, quel que soit le traitement reçu.

2. Article n°2 : *Soumis à Journal of Clinical Oncology*

Pubertal and fertility impairment in females from the French childhood leukemia survivors' cohort

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Running head: Pubertal and fertility impairment in childhood leukemia survivors.
Conflict of interest: none to declare**.**

ABSTRACT

Purpose: The aim of our study was to evaluate pubertal development and fertility outcome in female survivors of childhood leukemia from the French LEA – childhood and adolescent leukemia cohort.

Patients and Methods: Data provided from the medical book completed during specific medical visits at preset dates and from a self-reported specific fertility questionnaire. Comparisons were made with the French general population.

Results: 992 women were included for pubertal progression analysis. Of these, 491 were included for fertility evaluation. Higher prevalence of pubertal abnormalities was found in patients who relapsed and who received hematopoietic stem cell transplantation (HSCT). Relapse, HSCT and thyroid dysfunction were associated with more use of assisted reproduction technology (ART) ($p = 0.013$; $p < 0.001$ and $p = 0.01$ respectively). In survivors who received only first-line chemotherapy treatment, 9% of live births were after ART, which is higher than in the French general population (OR = 3.1 [1.5–6.4]; $p = 0.001$). More children were born after ART in women who received HSCT compared to those who did not (OR = 4.6 [2.4–6.8]). Risk of preterm delivery was significantly greater than expected (OR = 3.0 [1.8–4.9]; $p < 0.001$)], also in women who received only first line treatment (OR = 2.6 [1.4–4.6], $p = 0.001$). The overall incidence of babies with body weight less than 2500 g was significantly higher than expected ($p = 0.025$). The standardized fertility ratio was significantly impacted by HSCT (0.3 [0.1–0.5]; $p = 0.02$) but not by pubertal status at diagnosis.

Conclusion: These data emphasize the importance of long-term follow-up of childhood cancer survivors focusing on fertility and ovarian function.

INTRODUCTION

Continuing advances in childhood leukemia treatment mean that 80% of girls with acute lymphoblastic leukemia (ALL), and 60% of those with acute myeloblastic leukemia (AML), can now expect long-term survival following diagnosis.[1,2] This progress raises important questions concerning quality of life (QoL) and the risk of late effects after successful treatment. One of the greatest concerns associated with late effects is that of reproductive health, especially for young female survivors.

Ovaries are particularly sensitive to the adverse effects of cancer treatment because of the finite number of germ cells present in the postnatal ovary.[3] Ovarian damage resulting in impaired pubertal development and fertility have been described in childhood leukemia survivors.[4,5] Premature ovarian insufficiency (POI) and amenorrhea have often been used as the key outcome in studies of reproductive function in women.[6-8] However, this outcome may not fully reflect the experience of failure to conceive in these patients, who are additionally impacted by the social, psychological and sexual effects of cancer and its treatment.[9-11]

Fertility impairment and pregnancy complications in childhood cancer survivors have already been reported using the large cohort studies such as the British Childhood Cancer Survivor Study in the United Kingdom and the Childhood Cancer Survivor Study in North America.[12-16] However, these studies concern different malignancies and often focus on "at-risk treatment" i.e. radiotherapy and hematopoietic stem cell transplantation (HSCT). Concerning girls with leukemia most reports focus on heavily treated patients[17,18] and it remains unclear whether those who have received standard first-line leukemia treatment are also at risk of fertility deficits.[7,19,20]

The aim of our study was to evaluate pubertal development and fertility outcome in female survivors of childhood leukemia from the French Leucémie de l'Enfant et l'Adolescent (LEA – childhood and adolescent leukemia) cohort, focusing on risks in relation to different therapies.

MATERIALS AND METHODS

The LEA program was implemented in 2004 to prospectively evaluate the long-term health status, QoL and socio-economic status of childhood acute leukemia (AL) survivors

enrolled in treatment programs from 1980 to the present, in 16 cancer centers in France. The details of the program have already been described.[21,22] Data on different long-term complications were logged on medical book during specific medical visits at preset dates (*Online Supplementary Data*).

Since 2014, assessment of fertility impairment has been systematically offered to females aged > 18 years in the LEA program. Data on fertility impairment were collected via a self-reported specific fertility questionnaire. This questionnaire evaluates menstrual cycle characteristics, menopausal status, use of oral contraceptives and hormones, reproductive history, and marital status.

The study was approved by the French National Program for Clinical Research, the National Cancer Institute, and by the review boards of the institutions involved. All the patients gave their written informed consent to take part in the study.

Statistical analyses were performed using Stata software version 15 (StataCorp, College Station, US). The continuous data were expressed as mean ± standard deviation or as median [interquartile range] according to the statistical distribution. Assumption of normality was assessed with the Shapiro-Wilk test. The comparisons between groups (notably self-reported fertility questionnaire completed or not, relapse yes/no, HSCT yes/no, HSCT with TBI yes/no, Chronic GvHD yes/no) concerning continuous parameters (as maternal age at delivery (years), gestational age (weeks) and birth weight (grams)) were performed using the Student t-test or the Mann-Whitney test when assumptions required for the t-test were not met. The homoscedasticity was analyzed using the Fisher-Snedecor test. Categorical parameters were compared between groups using Chi-squared or Fisher's exact tests. The results were expressed as crude odds-ratios and 95% confidence intervals estimated using logistic regression. Sensitivity analyses were then conducted to take into account diagnosis age, as appropriate, applying a multivariable logistic regression with age as adjustment covariate. The results were expressed as adjusted odds-ratios (data not shown). All statistical tests were two-sided with the type-I error set at 5%. As discussed by several authors as Feise,[23] the adjustment of Type I error was not applied. An age-specific fertility rate was calculated as the number of births per 100 women of a given age in a given year. The standardized fertility ratio (SFR) was calculated, as the number of actual births observed to the number of that would be expected in women of same age in the general population. A polynomial regression was realized to estimate the results for LEA cohort women.

For comparison with the metropolitan French general population for the year of the study (2016), data of the national institute of statistics and economic studies, the national institute of demographic studies, the national perinatal registry and the national biomedicine agency were used.[24-27]

RESULTS

At the time of evaluation (2016) the total base LEA cohort comprised 1949 female leukemia survivors, of whom 992 were eligible (aged ≥ 18 years) and included for pubertal progression analysis. Of these, 612 were asked to answer a self-reported specific fertility questionnaire, and 491 were included for fertility impairment evaluation (Figure 1).

Of the 992 included patients, 839 (85%) had had ALL and 142 (14%) AML. The mean follow-up duration from leukemia diagnosis to the last evaluation was 17 ± 6.6 years. Patients were treated according to the protocols in use at the time of AL diagnosis, depending on the leukemia subtype (AML or ALL) (i.e. FRALLE, EORTC, LAME or ELAM). Most of the included patients received chemotherapy only (*n* = 782, i.e. 79%) while 208 (21%) patients received HSCT with (*n* = 140, i.e. 14%) or without (*n* = 68, i.e. 7%) total body irradiation (TBI).

Patients' characteristics are summarized in Table 1. No significant difference was found between patients eligible and included for fertility impairment evaluation (*n* = 491) and eligible but not included (*n* = 501) in terms of age and pubertal status at diagnosis, AL subtype, relapse ratio and treatment modalities ($p > 0.05$).

Puberty

Data on puberty progression are shown in Table 2.

Abnormal progression of puberty occurred in 12% patients who were prepubertal at diagnosis (*n* = 807) with significantly more delayed puberty (89%) than early puberty (11%). No spontaneous onset of menstruation was observed in 10% of prepubertal patients. In 49% of patients who were pubertal at diagnosis (*n* = 178) secondary amenorrhea, transient (63%) or permanent (37%), was observed. The cumulative incidence of early menopause was 5% and there was a significant difference in early menopause incidence between prepubertal groups (4%) and pubertal ones (8%) ($p = 0.03$).

In 755 survivors (613 prepubertal at diagnosis, 136 pubertal at diagnosis) who received only first-line chemotherapy treatment (no relapse and no HSCT), no spontaneous onset of menstruation occurred in 1%, secondary amenorrhea occurred in 36% and early menopause occurred 0.4%. Early menopause was significantly less frequent in these patients than in patients who relapsed and/or received HSCT (OR = 0.02 [0.01–0.05], $p < 0.001$).

Higher prevalence of abnormalities was found in patients who relapsed than in patients who did not: no spontaneous onset of menstruation (37% *vs.* 6%; OR = 9.3 [5.6–15.4]), secondary amenorrhea (92% *vs.* 46%; OR = 13.1 [1.6–104.0]) and early menopause (17% *vs.* 3%; OR = 6.1 [3.2–11.4]), $p < 0.001$.

Higher prevalence of abnormalities was also found in patients who received HSCT than in patients who did not: no spontaneous onset of menstruation (47% *vs.* 0.8%; OR = 107.1 [42.2–272]), secondary amenorrhea (95% *vs.* 37%; OR = 30.1 [6.9–130.5]) and early menopause (23% *vs.* 0.4%; OR = 71.6 [21.9–234.1]), $p < 0.001$. By contrast, women who received HSCT after TBI were not more at risk of developing abnormalities than females from the HSCT without TBI group.

Occurrence of post-transplantation cGvHD is a potentially devastating complication, which can adversely affect pubertal development and post-pubertal ovarian function. This might in part explain the differences observed between the transplanted and non-transplanted groups. We explored this hypothesis in secondary analyses by dividing the transplanted group according to whether post-transplantation cGvHD occurred. Among transplanted survivors with cGvHD ($n = 30$) no significantly higher prevalence of abnormalities was observed compared to those without cGvHD ($n = 70$). There was only a slightly higher incidence of abnormal progression of puberty in the cGvHD group ($p = 0.049$).

In prepubertal patients with thyroid dysfunction, higher prevalence of abnormal progression of puberty (41% *vs.* 17%; OR = 3.4 [2.0–5.6], $p < 0.001$), of no spontaneous onset of menstruation (35% *vs.* 15%; OR = 3.0 [1.8–5.1], $p < 0.001$) and of early menopause (14% *vs.* 7%; OR = 2.3 [1.1–4.9], $p = 0.04$) was observed compared to those without thyroid dysfunction.

Pregnancy intention

Among the 491 women who answered the fertility questionnaire, 82% declared that they had already had sex (72% among women aged < 25 years, 94% among women aged ≥ 25 years) and 46% that they had already been married or had live-in relationship. Median age at

initiation of sexual activity was 17 years. No significant difference in time of sexual activity initiation was observed between women who relapsed and those who did not, between women who received HSCT and those who did not, or between survivors with cGvHD and those without it (Table 3).

Fifty-two women (13%) had been trying to conceive for more than 1 year and 8% declared that they had already used assisted reproduction technology (ART). Relapse, HSCT and thyroid dysfunction were associated with more use of ART (17% *vs.* 7%, $p = 0.013$; 23% *vs.* 4%, $p < 0.001$ and 22% *vs.* 9%, $p = 0.01$ respectively).

Concerning adoption, 31 women (10%) had already thought about adoption. Both in the relapse group and in the HSCT group, the idea of recourse to adoption was significantly more often declared (32% *vs.* 7%, $p < 0.001$ and 33% *vs.* 4%, $p < 0.001$ respectively).

Three hundred sixty four women had never been pregnant (76%). They were significantly younger than those who had already been pregnant (median age 22.1 years (18.0–46.5) *vs.* 29.9 (19.8–43.8), $p < 0.001$). Of these, 10% declared that they wanted a child, 3% had already tried to conceive and 7% had already thought about adoption. Among these women, desire for a child and pregnancy attempts were significantly more often stated by those who relapsed (29% *vs.* 7% and 14% *vs.* 2%, respectively $p = 0.001$). The same was observed in the HSCT group: more desire for a child (20% *vs.* 6%, $p = 0.001$) and more pregnancy attempts (8% *vs.* 2%, $p = 0.03$) than in the no-HSCT group. Fourteen women declared unsuccessful ART, and 9 of them received HSCT (all with TBI).

Pregnancies

Among the 491 female survivors responding to fertility questionnaire, 113 reported that they had already been pregnant. Of these 113 women, 82 (73%) were prepubertal at the time of diagnosis. These 113 women had had a total of 176 pregnancies (Table 4).

The relative probability of being pregnant was not significantly different between those who relapsed and those who did not (prevalence: 19% *vs.* 24%, OR = 1.3 [0.7–2.6], $p = 0.43$), those who received HSCT and those who did not (prevalence: 18% *vs.* 25%, OR = 1.5 [0.9–2.7], $p = 0.139$) and those who received TBI and those who did not (prevalence: 21% *vs.* 12 %, OR = 0.5 [0.2–1.6], $p = 0.24$).

Out of 176 pregnancies, 67 resulted in no live birth (38%) because of miscarriage, either spontaneous (*n* = 33) or elective (*n* = 25), stillbirths (*n* = 2), early termination of tubal pregnancy (*n* = 1), and unknown events (*n* = 6).

The incidence of spontaneous abortions among all the female patients was 19%, not different from the reported incidence of 20% for the French general population *(p* = 0.74). Women who relapsed declared the incidence of 40% of spontaneous abortions, not significantly higher than expected in the French general population (*p* = 0.05). When HSCT recipients were considered, the incidence of 31% of spontaneous abortions was still not substantially higher than expected (*p* = 0.2) and not substantially higher than observed among women treated by chemotherapy only (15%; OR 4.8 [0.8–26.9]; *p* = 0.08). However, all the spontaneous abortions occurred in the TBI group.

There were 110 live births (1 set of twins) and 17 (16%) after ART, which is significantly higher than in the French general population (3.1%) (*p* < 0.001).[27] Also in survivors who received only first-line chemotherapy treatment (no relapse and no HSCT), 8/89 (9%) of live births were after ART, which is significantly higher than in the French general population (OR = 3.1 [1.5–6.4]; *p* = 0.001). There were significantly more children born after ART in women who had received HSCT compared to those who did not (90% *vs.* 8%, OR = 4.6 [2.4–6.8]). On the other hand, 1/10 (10%) live births in HSCT survivors were spontaneously conceived.

Median maternal age at delivery was 26 years (17–37), younger than in the French general population (30.4 years) (*p* < 0.001).[26]

Median gestational age at delivery was 39 weeks (26–41). The risk of preterm delivery among female leukemia survivors (21%) compared with the French general population (8%) was significantly greater than expected (OR = 3.0 [1.8–4.9]; *p* < 0.001)]. It was also significantly more frequent in women who received only first line treatment (19%) (OR = 2.6 [1.4–4.6], *p* = 0.001). Although the risk of preterm delivery was not more pronounced in the relapse, HSCT or cGvHD groups, we note that in HSCT recipients all preterm deliveries occurred in the TBI group.

Infants

A total of 110 infants were born. Of these, 71 (65%) were born after gestational age 37 weeks, and 97 (89%) had birth weights > 2500 g.

Median birth weight at delivery was 3300 g (1000–5200). The overall incidence of babies with body weight less than 2500 g was 11%, significantly higher than expected in the French general population (8.2%) (p = 0.025).[26] However, there were significantly more babies weighing < 2500 g in women who had received HSCT than in those who had not (33% *vs* 9%; OR = 5.4, [1.3–22.1]).

Of 12 babies weighing < 2500 g, 10 were low birth weight (LBW) (between 1800 and 2400 g) and 2 were very low birth weight (VLBW) (between 800 g and 1360 g), these last being born from mothers who received HSCT with TBI.

Ten of the infants were small for their gestational age (SGA): 9 of the 96 live births from women treated with chemotherapy alone and 1 of the 12 live births from women who received HSCT (with TBI). A set of twins born at 34 weeks had birth weights of 2000 g and 3000 g.

Age-specific fertility rate

As our cohort was relatively young, with 98% of survivors younger than age 40 years i.e. still of childbearing age, fertility rate can only be given as age-specific. These age-specific fertility rate estimations for 992 female leukemia survivors from the LEA cohort at the time of evaluation (2016) compared to age-specific fertility rates in France in the same year are presented at Figure 2.[24] The standardized fertility ratio for all patients was 0.5 [0.3–0.6]. It was significantly impacted by HSCT (0.3 [0.1–0.5]; p = 0.02) but not by pubertal status at diagnosis.

DISCUSSION

Our study evaluated pubertal development and fertility outcome in female survivors of childhood leukemia from the French Leucémie de l'Enfant et l'Adolescent (LEA-childhood and adolescent leukemia) cohort.

Reassuringly, our results show no increased risk of pubertal or post-pubertal abnormalities in girls who received only first-line treatment (no relapse and no HSCT), with 2% of abnormal progression of puberty, which is not different from the French general population.[28] This confirms previous findings.[7]

Like others, we found a clear impact of HSCT on pubertal progression and post-pubertal abnormalities (delayed puberty: OR = 109 [43–277], non-spontaneous onset of

menstruation: OR = 107 [42–272] and early menopause: OR = 72 [22–234]).[4,29-31] As previously reported by Bakker et al., almost 50% of prepubertal girls treated with TBI had abnormal progression of puberty.[32] However, we showed no marked differences between TBI and not-TBI groups. This may be due to equivalent impact of high cumulative doses of alkylating agents on ovarian function in patients transplanted without TBI.[8,30]

We found a strong association between relapse and pubertal and post-pubertal abnormalities (delayed puberty: OR = 9 [6–16], non-spontaneous onset of menstruation: OR = 9 [6–15] and early menopause: OR = 6 [3–11]), which was to be expected, because of the higher cumulative doses of treatments received by these patients, who also underwent HSCT more often than patients who did not relapse (80% *vs.* 13%).

Unsurprisingly, our prepubertal patients with thyroid dysfunction showed more anomalies of pubertal development.[33,34]

Age at initiation of sexual activity was not different in our cohort from the French general female population or as reported by other authors.[9,25]

Pui et al. evaluated long-term survivors of childhood acute lymphoblastic leukemia and showed that rates of marriage in a non-irradiated group were similar to the age- and sex-adjusted national averages. However, women in an irradiated group were less likely to be married.[35] In our study, we did not find any impact of different types of treatment on live-in relationship or marriage.

Van Dijk et al. reported no difference in desire to have children between childhood cancer survivors and controls.[36] We found that the desire for a child and pregnancy attempt was more often stated in women who relapsed and those who had HSCT.

It was previously reported that diagnosis of leukemia was associated with consulting a fertility specialist (12%, OR = 2 [1–3]).[36] In our cohort, 8% of patients declared that they had used ART irrespective of the result. This is significantly more than in the French general population (1%) ($p < 0.001$). This is consistent with previous reports of an increase in the use of *in vitro* fertilization in female cancer patients compared with the general population,[37] but contrasts with Barton et al.'s finding that despite being equally likely to seek treatment for infertility, survivors were less likely to be prescribed medication for treatment of infertility than their siblings.[38] HSCT and relapses in our cohort were associated with significantly more recourse to ART and more unsuccessful ART.

Freycon et al. reported that TBI and alkylating agents were negatively correlated with fecundity, with a standardized fertility ratio of 0.62 in patients treated with chemotherapy only *vs*. 0.17 in patients who received TBI conditioning allografts.[19] We found significant decrease in age-specific fertility rate in our patients impacted by HSCT but not by pubertal status at diagnosis. We can only speculate on the impact of ART, which was more often used in these survivors. We can speculate on this difference in the light of ART, which is more and more used in survivors with cancers.

Maternal age in our cohort was younger than in the French general population as already found by Freycon et al.,[19] but this may be merely artefactual owing to the young age of our cohort.

The incidence of no live births in our survivors treated by chemotherapy only (34%) was consistent with that previously reported in female AML survivors.[7] However, in patients who had received HSCT, the incidence in our cohort (54%) was higher than that of Sanders (28%).[17] Concerning spontaneous abortions, overall incidence in our cohort was 19%, reassuringly no higher than in the French general population.[24] The incidence in patients treated with chemotherapy only was 15%, less than previously reported.[7] Concerning our female survivors who had received HSCT with TBI, the incidence of spontaneous abortions (44%) was consistent with the figures of Sanders et al.[17]

Sixteen percent of the born babies were conceived after ART, significantly more than in the French general population (3.1%),[26] with significantly higher incidence in women who had received HSCT (90%, OR = 4.6 [2.4–6.8]).

Although risks of premature delivery and delivering LBW offspring in survivors of childhood cancer were previously reported,[12-16] only few information are focused particularly on childhood leukemia survivors and on impact of each phase of treatment.[7,13,14,17]

We found that in our leukemia survivors the incidence of preterm delivery was significantly higher (21%) than in the French general population (8%). It was higher than expected even in women treated only with first-line treatment (19%), but it was not significantly impacted by relapse, HSCT or cGvHD. Our findings are consistent with those of Mueller et al., with OR = 2.6 [1.8–3.6] for preterm delivery,[14] and Signorello et al. with 19% of preterm births.[13] They differ, however, from Molgaard-Hansen et al.'s findings, with all babies born at term in leukemia survivors treated by chemotherapy only.[7] In our cohort we failed to

find any substantial difference in the incidence of preterm delivery in patients who received HSCT with or without TBI.[17]

In our leukemia survivors, the incidence of LBW babies was significantly higher (11%) than in the French general population (8%),[26] consistent with previous reports,[14] except for women treated only with first-line treatment (9%). The increased incidence of LBW babies born from HSCT survivors in our cohort was similar to that reported by Sanders and coworkers.[17]

CONCLUSION

The main strength of our study is the size of this relatively homogenous cohort. One weakness is the young age of our survivors: 81% were younger than the median maternal age (30.4 yrs) in the French general population.[26] In all treatment groups, standardized fertility ratio was lower than expected. Reassuringly, in girls and women who received only first-line treatment we showed no increased risk of pubertal or post-pubertal abnormality and no increased risk of babies with LBW. We confirm that female survivors of childhood leukemia who have relapsed or received HSCT have more frequently impaired pubertal development and fertility, but we failed to show any impact of TBI. These data emphasize the importance of long-term follow-up of childhood cancer survivors focusing on fertility and ovarian function.

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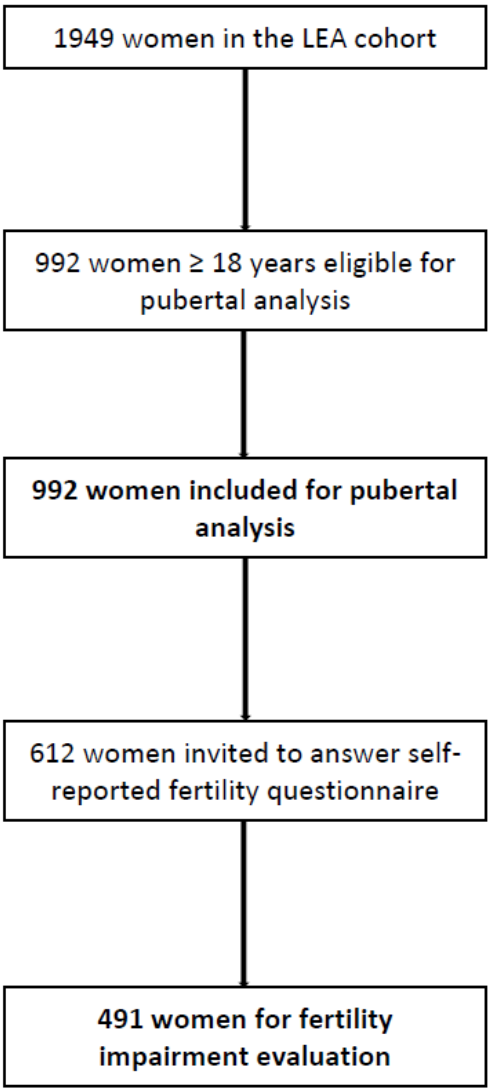

Figure 1: Flow chart of the LEA cohort in 2016

Table 1. Characteristics of 992 Female Leukemia Survivors Aged ≥ 18 Years

	Medical book completed			*P-value*
		Self-Reported Fertility Questionnaire		
		completed	not completed	
Number of patients	992	491 (49.5%)	501 (50.5%)	
Age at evaluation (years)				
Median	25.0	25.0	25.0	
Range	18.0-46.5	18.0-46.5	18.0-43.2	
Initial diagnosis				
ALL	839/992 (84.6%)	403/491 (82.1%)	436/501 (87.0%)	
AML	142/992 (14.3%)	82/491 (16.7%)	60/501 (12.0%)	
Others or unknown	11/992 (1.1%)	6/491 (1.2%)	5/501 (1.0%)	
Pubertal at diagnosis	178/985 (18.1%)	107/484 (22.1%)	71/501 (14.2%)	*NS*
First line treatment only*	755/988 (76.4%)	371/488 (76.0%)	384/500 (69.6%)	
Relapse	124/988 (12.6%)	60/488 (12.3%)	64/500 (12.8%)	
HSCT	208/990 (21.0%)	105/489 (21.5%)	103/501 (20.6%)	
with TBI	140/990 (14.1%)	70/489 (14.3%)	70/501 (14.0%)	
without TBI	68/990 (6.9%)	35/489 (7.2%)	33/501 (6.6%)	
cGvHD	101/216 (46.8%)	49/110 (44.5%)	52/106 (49.1%)	
Thyroid dysfunction	138/426 (32.4%)	67/218 (30.7%)	71/208 (34.1%)	
≥ 1 pregnancy	227/867 (26.2%)	113/433 (26.1%)	114/434 (26.3%)	

ALL: acute lymphoblastic leukemia; AML: acute myeloblastic leukemia; cGvHD: chronic graft versus host disease; HSCT: hematopoietic stem cell transplantation; TBI: total body irradiation.

* No-relapse and no-HSCT

Table 2. Pubertal abnormalities and ovarian insufficiency in 992 female leukemia survivors

	All patients	No Relapse & No HSCT				Relapse				HSCT				HSCT with TBI			
		No	Yes	OR	CI95%	No	Yes	OR	CI95%	No	Yes	OR	CI95%	No	Yes	OR	CI95%
	n/N (%)	n/N (%)	n/N (%)			n/N (%)	n/N (%)			n/N (%)	n/N (%)			n/N (%)	n/N (%)		
Prepubertal at diagnosis	807/985(81.9)	191/232(82.3)	613/749(81.8)			694/857(81.0)	110/124(88.7)			636/776(82.0)	169/207(81.6)			56/67(83.6)	113/140(80.7)		
Abnormal progression of puberty	94/797(11.8)	82/188(43.6)	12/607(2.0)	**0.03**	[0.01-0.05]	51/687(7.4)	43/108(39.8)	**8.3**	[5.1-13.3]	12/630(1.9)	82/166(49.4)	**50.3**	[26.3-96.0]	28/56(50.0)	54/110(49.1)	1.0	[0.5- 1.8]
Delayed puberty	81/797(10.2)	76/185(41.1)	5/607(0.8)	**0.01**	[0.01-0.03]	41/685(6.0)	40/107(37.4)	**9.4**	[5.7-15.5]	5/630(0.8)	76/163(46.6)	**109.2**	[43.0-277.4]	25/55(45.5)	51/108(47.2)	1.1	[0.6- 2.1]
Early puberty	10/797(1.3)	3/185(1.6)	7/607(1.2)	0.7	[0.2-2.8]	8/685(1.2)	2/107(1.9)	1.6	[0.3-7.7]	7/630(1.1)	3/163(1.8)	1.7	[0.4-6.5]	2/55(3.6)	1/108(0.9)	0.2	[0.02- 2.8]
No spontaneous onset of menstruation	81/790(10.3)	76/186(40.9)	5/602(0.8)	**0.01**	[0.01-0.03]	41/681(6.0)	40/107(37.4)	**9.3**	[5.6-15.4]	5/625(0.8)	76/164(46.3)	**107.1**	[42.2-272.0]	26/55(47.3)	50/109(45.9)	1.0	[0.5- 1.8]
Early menopause	31/747(4.1)	28/170(16.5)	3/575(0.5)	**0.03**	[0.01-0.09]	15/644(2.3)	16/101(15.8)	**7.9**	[3.8-16.5]	3/596(0.5)	28/150(18.7)	**45.4**	[13.6-151.6]	9/51(17.6)	19/99(19.2)	1.1	[0.5- 2.7]
Pubertal at diagnosis	178/985(18.1)	41/232(17.7)	136/749(18.2)			163/857(19.0)	14/124(11.3)			140/776(18.0)	38/207(18.4)			11/67(16.4)	27/140(19.3)		
Secondary amenorrhea	85/173(49.1)	36/39(92.3)	48/133(36.1)	**0.05**	[0.01-0.2]	73/160(45.6)	11/12(91.7)	**13.1**	[1.6-104.0]	50/136(36.8)	35/37(94.6)	**30.1**	[6.9-130.5]	11/11(100)	24/26(92.3)	NE	NE
Transient	53/173(30.6)	6/39(15.4)	46/133(34.6)	**2.9**	[1.1-7.5]	49/160(30.6)	3/12(25.0)	0.8	[0.2-2.9]	48/136(35.3)	5/37(13.5)	0.3	[0.1-0.8]	1/11(9.1)	4/26(15.4)	2.8	[0.9- 8.4]
Permanent	31/173(17.9)	29/39(74.5)	2/133(1.5)	**0.01**	[0.01-0.03]	24/160(15.0)	7/12(58.3)	**7.9**	[2.3-27.1]	2/136(1.5)	29/37(78.4)	**242.9**	[49.0-1203.7]	10/11(90.9)	19/26(73.1)	0.03	[0.01- 0.09]
Early menopause	14/173(8.1)	14/38(36.8)	0/135(0)	NE	NE	11/161(6.8)	3/12(25.0)	**4.5**	[1.1-19.2]	0/138(0)	14/35(40.0)	NE	NE	5/10(50.0)	9/25(36.0)	0.6	[0.1- 2.5]

HSCT: hematopoietic stem cell transplantation; NE: non evaluable; TBI: total body irradiation; cGvHD: chronic graft versus host disease
N = number of patients with valid information; n=number of patients who experienced the type of abnormality
OR = Odds Ratio
CI = confidence interval

Table 3. Live-in relationship, pregnancy intention and child desire in 491 female leukemia survivors

	All patients	No Relapse & No HSCT				Relapse				HSCT				HSCT with TBI			
		No	Yes	OR	CI95%	No	Yes	OR	CI95%	No	Yes	OR	CI95%	No	Yes	OR	CI95%
	n/N (%)	n/N (%)	n/N (%)			n/N (%)	n/N (%)			n/N (%)	n/N (%)			n/N (%)	n/N (%)		
Ever had sex	389/477(81.6)	86/113(76.1)	300/361(83.1)	1.5	[0.9-2.6]	342/416(82.2)	44/58(75.9)	0.7	[0.4-1.3]	311/374(83.2)	76/101(75.2)	0.6	[0.4-1.0]	23/34(67.6)	53/67(79.1)	1.8	[0.7-4.6]
Ever married or had live-in relationship	215/468(45.9)	52/112(46.4)	161/353(45.6)	1.0	[0.6-1.5]	187/408(45.8)	26/57(45.6)	1.0	[0.6-1.7]	169/366(46.2)	45/100(45.0)	1.0	[0.6-1.5]	12/34(35.3)	33/66(50.0)	1.8	[0.8-4.3]
Tried to be pregnant for ≥ 1 year	52/388(13.4)	22/91(24.2)	29/294(9.9)	**0.3**	[0.2-0.6]	41/339(12.1)	10/46(21.7)	2.0	[0.9-4.4]	29/305(9.5)	22/81(27.2)	**3.6**	[1.9-6.6]	5/27(18.5)	17/54(31.5)	2.0	[0.7-6.3]
Ever used ART	34/432(7.9)	21/104(20.2)	13/326(4.0)	**0.2**	[0.1-0.3]	25/378(6.6)	9/52(17.3)	**3.0**	[1.3-6.7]	13/339(3.8)	21/92(22.8)	**7.4**	[3.6-15.5]	1/31(3.2)	20/61(32.8)	**14.6**	[1.9-115.1]
Ever thought about adoption	31/320(9.7)	23/76(30.3)	8/243(3.3)	**0.1**	[0.03-0.2]	20/285(7.0)	11/34(32.4)	**6.3**	[2.7-14.8]	9/253(3.6)	22/67(32.8)	**13.3**	[5.7-30.7]	4/21(19.0)	18/46(39.1)	2.7	[0.8-9.4]
Never been pregnant (n= 364)																	
Ever had desire for a child	26/274(9.5)	14/69(20.3)	12/205(5.9)	**0.2**	[0.1-0.6]	17/243(7.0)	9/31(29.0)	**5.4**	[2.2-13.6]	13/210(6.2)	13/64(20.3)	**3.9**	[1.7-8.8]	3/24(12.5)	10/40(25.0)	2.3	[0.6-9.5]
Ever tried to be pregnant	9/274(3.3)	6/66(9.1)	3/208(1.4)	**0.2**	[0.04-0.6]	5/245(2.0)	4/29(13.8)	**7.7**	[1.9-30.5]	4/212(1.9)	5/62(8.1)	**4. 6**	[1.2-17.5]	0/23(0)	5/39(12.8)	NE	
Ever used ART	14/325(4.3)	9/82(11.0)	5/243(2.1)	**0.2**	[0.1-0.5]	9/283(3.2)	5/42(11.9)	**4.1**	[1.3-12.9]	5/250(2.0)	9/75(12.0)	**6.7**	[2.2-20.6]	0/27(0)	9/48(18.8)	NE	
Ever thought about adoption	17/229(7.4)	12/56(21.4)	5/173(2.9)	**0.1**	[0.04-0.3]	10/205(4.9)	7/24(29.2)	**8.0**	[2.7-23.8]	6/177(3.4)	11/52(26.9)	**7.7**	[2.7-21.9]	4/18(22.2)	7/34(20.6)	0.9	[0.2-3.6]

ART: assisted reproductive technology (including: in vitro fertilization, intra-cytoplasmic sperm injection, frozen embryo transfer, intrauterine insemination and ovulation induction); HSCT: hematopoietic stem cell transplantation; TBI: total body irradiation; NE: non evaluable; cGvHD: chronic graft versus host disease

N = number of patients with valid information; n = number of patients who experienced the type of abnormality

OR = Odds Ratio

CI = confidence interval

Table 4. Pregnancy outcome and infant characteristics in 113 female leukemia survivors

	All patients	No Relapse & no HSCT			Relapse			HSCT			HSCT with TBI		
		No	Yes	OR CI95%	No	Yes	OR CI95%	No	Yes	OR CI95%	No	Yes	OR CI95%
	n/N (%)	n/N (%)	n/N (%)		n/N (%)	n/N (%)		n/N (%)	n/N (%)		n/N (%)	n/N (%)	
Pregnancies	176	34	135		154	15		144	26		8	18	
No live births	67/176(38.1)	16/34(47.1)	46/135(34.1)	0.3[0.1-1.7]	55/154(35.7)	7/15(46.7)	2.3[0.2-22.3]	49/144(34.0)	14/26(53.8)	6.4[0.8-49.0]	3/8(37.5)	11/18(61.1)	1.5[0.03-581.3]
Spontaneous miscarriage	33/176(18.8)	10/34(29.4)	20/135(14.8)	0.2[0.1-1.1]	24/154(15.6)	6/15(40.0)	6.5[0.9-46.4]	22/144(15.3)	8/26(30.8)	4.8[0.8-26.9]	0/8(0)	8/18(44.4)	NA
Medical interruption	2/176(1.1)	1/34(2.9)	1/135(0.7)	0.3[0.02-4.0]	2/154(1.3)	0/15(0)	NA	1/144(0.7)	1/26(3.8)	5.7[0.4-94.4]	1/8(12.5)	0/18(0)	NA
Others*	32/176(18.2)	5/34(14.7)	25/135(18.5)	1.3[0.4-3.9]	29/154(18.8)	1/15(6.7)	0.3[0.03-2.5]	26/144(18.1)	5/26(19.2)	1.1[0.4-3.6]	2/8(25.0)	3/18(37.5)	0.1[0.0005- 37.1]
Live births**	110/176(62.5)	18/34(52.9)	90/135(65.9)	3.5[0.6-20.0]	100/154(64.9)	8/15(53.3)	0.4[0.04-4.2]	96/144(66.0)	12/26(46.2)	0.2[0.02-1.2]	5/8(62.5)	7/18(38.9)	0.7[0.002-256.4]
ART	17/110(15.5)	9/16(56.3)	8/89(9.0)	**0.1**[0.02-0.3]	17/99(17.2)	0/6(0)	NA	8/95(8.4)	9/10(90.0)	**97.9**[11.0-873.8]	4/5(80.0)	5/5(100)	NE
Maternal age at delivery (years)													
Median (range)	26(17-37)	28(19-34)	26(17-37)		26(17-37)	29(22-30)		26(17-37)	28(19-34)		26(19-33)	29(22-34)	
< 30	84/104(80.8)	13/18(72.2)	70/85(82.4)	1.8[0.6-5.8]	75/94(79.8)	7/8(87.5)	1.8[0.2-15.1]	74/90(82.2)	8/12(66.7)	0.4[0.1-1.6]	4/5(80.0)	4/7(57.1)	0.3[0.02-4.7]
Gestational age (weeks)													
Median (range)	39(26-41)	38(26-41)	39(31-41)		39(26-41)	37(29-40)		39(31-41)	38(26-41)		41(39-41)	36(26-39)	
< 37	19/90(21.1)	6/15(40.0)	14/74(18.9)	0.4[0.1-1.1]	16/82(19.5)	3/6(50.0)	3.9[0.7-21.0]	14/77(18.2)	5/11(45.5)	3.5[0.9-13.0]	0/4(0)	5/7(71.4)	NA
Birth weight (g)													
Median (range)	3300(1000-5200)	3260(1000-3900)	3280(1970-5200)		3280(1000-5200)	3080(1400-3500)		3300(1970-5200)	3160(1000-3900)		3600(3220-3900)	2400(1000-3300)	
< 2500	12/109(11.0)	4/18(22.2)	8/89(9.0)	0.4[0.1-1.3]	10/99(10.1)	2/8(25.0)	3.0[0.5-16.7]	8/94(8.5)	4/12(33.3)	**5.4**[1.3-22.1]	0/5(0)	4/7(57.1)	NA
LBW	10/12(83.3)	2/4(50.0)	8/8(100)		9/10(90.0)	1/2(50.0)		8/10(80.0)	2/10(20.0)		0/2(0)	2/2(100)	
VLBW	2/12(16.7)	2/4(50.0)	0/8(0)		1/10(10.0)	1/2(50.0)		0/2(0)	2/2(100)		0/2(0)	2/2(100)	

ART: assisted reproductive technology (including: in vitro fertilization, intra-cytoplasmic sperm injection, frozen embryo transfer, intrauterine insemination and ovulation induction); CI: confidence interval; GA: gestational age; HSCT: hematopoietic stem cell transplantation; LBW: low birth weight (1500 g to < 2500 g); NA: non applicable; NE: non evaluable; OR: odds ratio; SGA: small-for-gestational-age; TBI: total body irradiation; VLBW: very low birth weight (< 1500 g)

*Stillborn, ectopic pregnancy, on-going pregnancy, abortion

**One set of twins

N = number of patients with valid information; n = number of patients who experienced the type of abnormality

OR = Odds Ratio

CI = confidence interval

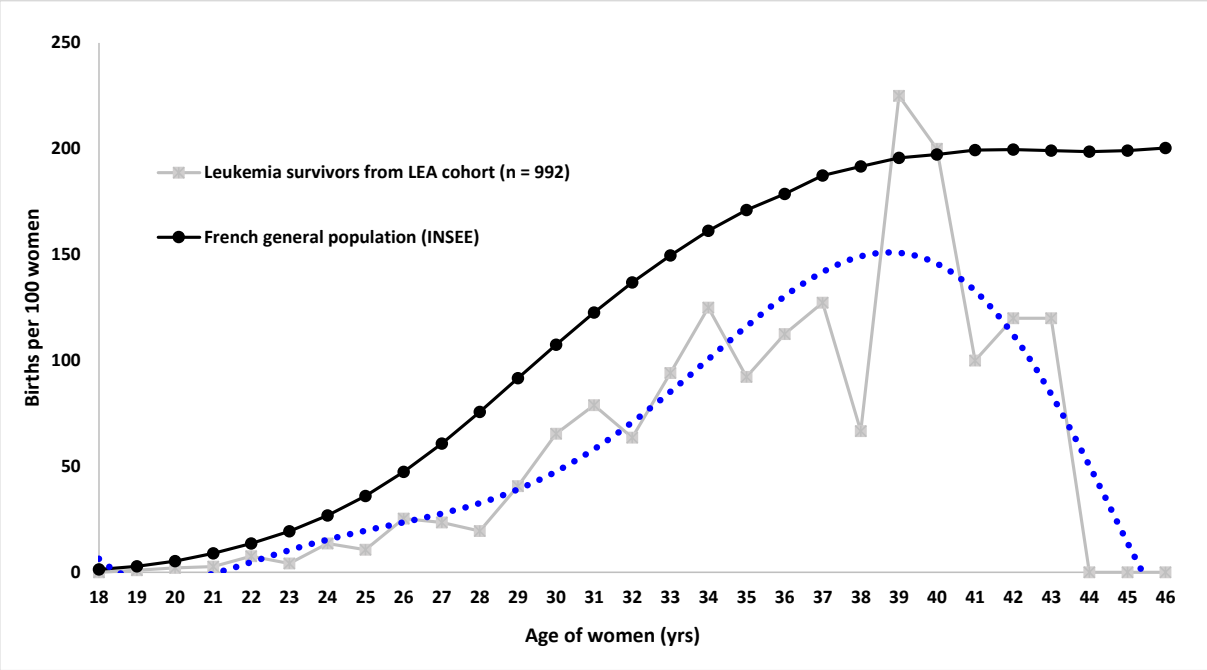

Figure 2. Age-specific fertility rates (calculated as the number of births per 100 women of a given age in a given year) estimated for 992 female leukemia survivors from the LEA cohort at the time of evaluation (2016) with a polynomial regression curb compared to age-specific fertility rates in France in the same year (data from the French National Institute of Statistics and Economic Studies (INSEE)).

Supplementary data: Fertility questionnaire

Merci de bien vouloir répondre à l'ensemble des questions suivantes.

Cette annexe concerne les femmes exclusivement.

Q1. Concernant vos cycles menstruels.

1a. Avez-vous déjà eu vos règles ? ❑ Oui ❑ Non

Si oui, avez-vous actuellement des cycles menstruels ? ❑ Oui ❑ Non

Si non, à quel âge avez-vous eu votre dernier cycle ? .....

1b. Avez-vous déjà pris un traitement hormonal, y compris un contraceptif oral pour avoir vos règles ? ❑ Oui ❑ Non

Q2. Concernant votre statut marital.

2a. Avez-vous déjà été mariée ou vécu en union libre depuis la fin de votre traitement

❑ Oui ❑ Non

2b. Au total, c'est-à-dire tous partenaires confondus, pendant combien de temps avez-vous vécu en couple ?

.....

Q3. Concernant votre contraception et votre descendance

3a. Avez-vous déjà eu des rapports sexuels ? ❑ Oui ❑ Non

Si oui, à quel âge avez-vous eu vos premiers rapports ? ……

3b. Avez-vous actuellement des rapports sexuels ? ❑ Oui ❑ Non

3c. Quel mode de contraception avez-vous déjà utilisé ?

	Déjà utilisé mais pas actuellement	Utilisé actuellement	Jamais
Aucun	□	□	□
Pilule contraceptive	□	□	□
Préservatif masculin	□	□	□
Préservatif féminin	□	□	□
Stérilet	□	□	□
Vasectomie	□	□	□
Ligature des trompes	□	□	□
Autres (Précisez)	□	□	□

3d. Avez-vous déjà été enceinte ? ❑ Oui ❑ Non

3e. Etes-vous actuellement enceinte ? ❑ Oui ❑ Non

Concernant chaque grossesse :

	Issue de la grossesse	Votre âge au début de la grossesse	Terme de la naissance	Poids de naissance
1ère grossesse	……	……	……	……
2ème grossesse	……	……	……	……
3ème grossesse	……	……	……	……
4ème grossesse	……	……	……	……
5ème grossesse	……	……	……	……
6ème grossesse	……	……	……	……

NB : <u>Issue de la grossesse</u> = enfant vivant, mort-né, fausse couche, interruption volontaire de grossesse, interruption médicale de grossesse, grossesse extra utérine
<u>Terme de la naissance</u> : en semaines d'aménorrhées

3f. Pendant les périodes où vous avez vécu en couple, avez-vous déjà cherché à être enceinte <u>pendant 1 an ou plus</u>

❑ Oui ❑ Non

Si oui, Avez-vous, vous et/ou votre partenaire déjà consulté pour infertilité ?.....

❑ Oui ❑ Non

Si oui, une cause a-t-elle été retrouvée ? ❑ Oui ❑ Non

Si oui, précisez ……

Avez-vous fait appel à une technique de Procréation Médicalement Assistée (PMA)

❑ Oui ❑ Non

Si oui, laquelle ?

- ✓ Traitement hormonal ❑ Oui ❑ Non
- ✓ Insémination ❑ Oui ❑ Non
- ✓ Don d'ovocytes ❑ Oui ❑ Non
- ✓ Don de sperme ❑ Oui ❑ Non
- ✓ FIV / ICSI ❑ Oui ❑ Non

La PMA vous a-t-elle permis d'avoir un enfant ? ❑ Oui ❑ Non

Avez-vous envisagé une adoption ? ❑ Oui ❑ Non

Si oui, Avez-vous fait les démarches ? ❑ Oui ❑ Non

Avez-vous adopté un ou plusieurs enfants ? ❑ Oui ❑ Non

Si vous n'avez jamais souhaité être enceinte, pourquoi ?

.....

.....

.....

.....

Q4. <u>Concernant votre fratrie.</u>

Avez-vous une ou plusieurs sœurs ? ❑ Oui ❑ Non

Si oui, quel âge ont-elles ? ……

Ont-elles eu des enfants ? ❑ Oui ❑ Non

Si oui, à quel âge les ont-elles eus ?

……

……..

3. Résultats

Contrairement aux femmes n'ayant reçu que de la chimiothérapie en première ligne, les taux d'anomalies de développement pubertaire, d'aménorrhées secondaires et de ménopauses précoces étaient significativement supérieurs chez les femmes ayant rechuté et/ou ayant bénéficié d'une greffe de cellules souches hématopoïétiques.

Concernant les données sur la fertilité, le nombre de femmes ayant déjà eu recours aux techniques de procréation médicalement assistée (PMA) ou ayant déjà envisagé l'adoption était significativement supérieur parmi celles ayant rechuté ou ayant bénéficié d'une greffe de cellules souches hématopoïétiques. Chez ces mêmes femmes, le désir d'enfant, le taux de tentatives de grossesse et l'idée d'adopter étaient significativement supérieurs.

En analysant les grossesses et naissances, les seules différences mises en évidence concernaient le recours à la PMA et le poids de naissance < 2500 g. En effet, le nombre de nouveau-nés issus de la PMA ainsi que le nombre de nouveau-nés pesant moins de 2500 g étaient significativement plus important chez les femmes ayant bénéficié d'une greffe de cellules souches hématopoïétiques.

Dans cette étude décrivant l'ensemble des patientes, quels que soient les traitements reçus, il est intéressant de constater que la fertilité des femmes n'ayant reçu que de la chimiothérapie en première ligne était tout de même altérée. Le taux de bébés nés après PMA et le taux de prématurité étaient supérieurs aux taux dans la population générale.

4. Conclusion

Après une leucémie durant l'enfance ou l'adolescence, les femmes ayant bénéficié d'une greffe de cellules souches hématopoïétiques et/ou ayant rechuté sont davantage concernées par les anomalies de développement pubertaire et les difficultés de fertilité. Néanmoins, quel que soit le traitement reçu, y compris lorsque les doses cumulées sembent les plus faibles, le risque de conséquences sur la fonction gonadique féminine ne peut être exclu.

Ces séquelles doivent être anticipées. L'information et l'accès aux techniques de préservation de la fertilité doivent faire partie de la prise en charge de ces jeunes patientes.

III. ÉTAT DES LIEUX DE LA PRÉSERVATION DE LA FERTILITÉ AU CHU DE CLERMONT-FERRAND

1. La cryopréservation de tissu ovarien

a. Rationnel de l'étude

Les progrès thérapeutiques ont permis d'améliorer la survie des patients atteints de cancers mais la toxicité gonadique reste une conséquence relativement fréquente. La préservation de la fertilité doit donc être proposée aux patients après évaluation du risque. Différentes options existent selon le sexe, l'âge et le degré d'urgence thérapeutique.

La cryoconservation de tissu ovarien est la seule méthode envisageable chez la fille prépubère. L'accès à ce type de préservation de la fertilité est possible dans notre centre depuis 2000. Nous avons réalisé une étude descriptive rétrospective sur les patientes ayant bénéficié de cette stratégie en Auvergne entre 2000 et 2013 afin de décrire notre expérience.

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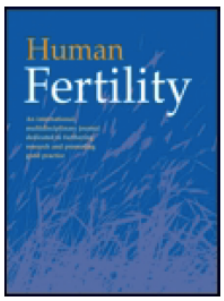

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Cryopreservation of ovarian tissue in pediatric patients undergoing sterilizing chemotherapy

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ABSTRACT

Significantly improved survival rates in children and adolescents with cancer have put fertility preservation high on the pediatric oncology agenda. Here we report a retrospective single-center study of 13 years experience of ovarian tissue cryopreservation (OTC) before sterilizing treatment in order to define the safety/benefits of OTC and study clinical/hormonal outcomes in girls. From 2000 to 2013, OTC was performed in 36 girls: eight had non-malignant disease and 28 had malignant disease. Laparoscopy was used to collect a third of each ovary that was frozen by a slow cooling protocol. Indications for OTC were 13 auto-, 19 allo-stem-cell-transplantation and 4 sterilizing chemotherapy. Ovarian tissue harvested by intraumbilical laparoscopy led to no major postoperative complications and did not delay chemotherapy. Histological analysis of ovarian tissue showed an average of 9 primordial follicles/mm2 [0–83] and no malignant cells were identified. Median post-harvest follow-up was 36 months [1–112]: 26 girls were alive in complete remission and 10 had died. Hormonal results were evaluable for 27 patients (median age 17 yrs [5–26]): 16 patients were in premature ovarian

insufficiency. OTC sampling one third of each ovary appears to be an appropriate approach to preserve fertility in children without consequences on subsequent therapeutic program.

KEYWORDS

Childhood cancer; fertility; ovarian tissue cryopreservation

Introduction

Almost 80% of children and adolescents currently treated for cancer or leukaemia will become long-term survivors (Armenian, Landier, Hudson, Robison, & Bhatia, 2013). This progress makes quality-of-life issues an essential concern for paediatric oncologists. Low fertility, infertility and early menopause rank high on the list of treatment side-effects that reduce quality-of-life in cancer survivors. Indeed, in females, cancer treatments can accelerate the natural decreases in ovarian follicles number and lead to premature ovarian insufficiency (POI) with subsequent absence of puberty, amenorrhea and infertility (Wallace, Anderson, & Irvine, 2005). The extent of ovarian damage depends on age at treatment, cumulative dose, and type of chemotherapeutic agents and radiation doses (Green et al., 2009; Lee et al., 2006).

In France, a child's right to fertility preservation is acknowledged in the national bioethics law (Law n°2004-800 of 6 August 2004, on Bioethics) which states that "everyone should benefit from the collection and preservation of their gametes or germ-tissue, with their consent and – when necessary – the consent of a holder of parental authority (…), when medical care may impair fertility (...)." From a medical standpoint, the question of which medical treatment may impair fertility and therefore which patients should benefit from gamete or germ-tissue preservation has prompted many recommendations, from the American Society of Clinical Oncology (ASCO) recommendations and update (Lee et al., 2006; Loren et al., 2013) to the Société Française des Cancers de l'Enfant (SFCE) long-term monitoring committee guidelines (Sudour-Bonnange et al., 2013).

Ovarian tissue cryopreservation (OTC) is the main option available to preserve fertility in prepubertal girls (von Wolff et al., 2009). However, only a few teams performing OTC in children have reported on their practice (Donnez et al., 2013; Jadoul, Dolmans, & Donnez, 2010; Poirot et al., 2007; Revel et al., 2009). Here we report our experience of OTC in girls treated in the Clermont-Ferrand City Paediatric Oncology Department and assess the efficacy, feasibility and risks of the approach. Subsequent clinical (oncologic and hormonal) outcomes are also reported to provide data about hormonal status after treatment.

Material and methods

Patients

We retrospectively reviewed the data on all females aged less than 20 years old treated in the Pediatric Haematology-Oncology Department and undergoing OTC between September 2000 and September 2013. OTC was offered to all patients who required sterilizing high-dose chemo/radiotherapy followed by auto- or allogeneic hematopoietic stem cell transplantation (HSCT) and to patients who were at high risk of POI. For those latter patients, the decision was based on pathology and treatment on a case-by-case basis and in a multidisciplinary setting (paediatric oncologists, reproductive biologists, gynaecologists and anaesthetists). Written consent was required from both the parent and (if old enough) the patient.

Timing

OTC was performed as soon as possible after diagnosis (i.e. before any treatment in patients with non-malignant disease and at the latest immediately before sterilizing chemotherapy in patients with malignant disease). In patients with malignant disease, we started with chemotherapy in order to obtain an in vivo purging effect of any ovarian infiltration before OTC, taking care not to overstep the theoretical sterilizing drug doses that could compromise follicular reserve before OTC. Surgery was performed after HIV, Hepatitis B & C and syphilis serology was determined.

Ovarian tissue harvest

Ovarian tissue was collected under general anaesthesia via a dedicated procedure or during another surgical abdominal procedure when possible. OTC was performed by intraumbilical laparoscopy in the gynaecology department. Briefly, the optical trocar (5–10 mm depending on patient template) was inserted at the umbilicus after performing safety tests and creating a pneumoperitoneum using a Palmer needle to a pressure of up to 9–18 mmHg. Two surgical trocars (5 mm [3–10]) were then placed at the right and left sides. After exploration of the abdominal cavity and checking the status of both ovaries, bilateral partial oophorectomy (1/3 of each ovary) was performed with scissors and gentle haemostasis of the ovarian slice using bipolar forceps. Once placed in a bag, the ovarian tissue was extracted through the abdominal wall. As previously described, the fragments were then placed in the transport medium, cooled to 4°C and swiftly taken to the reproductive biology lab (Schubert et al., 2005). The

trocars were then removed, the pneumoperitoneum was exsufflated, and the skin carefully closed with inverting sutures.

Freezing protocol

All ovarian tissue fragments were processed at the CECOS (Centre d'Etude et de Conservation des Œufs et Sperme humains) reproductive biology lab in Clermont-Ferrand under fully sterile conditions as previously described (Sanfilippo et al., 2011). The cooling rate was 2°C/min from 4°C down to -11°C. Temperature was then lowered to -40°C at a rate of 2°C/min and down to -150°C at a rate of 10°C/min. Finally, the cryovials were plunged into liquid nitrogen for storage.

Histological analysis

Histological analysis of one random sample of each harvested ovarian tissue fragment was routinely performed before freezing. Each sample was individually embedded in paraffin and 4 µm-thin slice sections were cut perpendicularly to the surface of the ovary and stained with haematoxylin-eosin-saffron. Each section was measured in two dimensions, and primordial/primary follicle counts per mm^2 were determined within the ovarian cortex. Four to eight section slices containing cortex and medulla were examined for follicle count by the same experienced pathologist, who also visually checked for malignant tumour cells.

Post-treatment hormonal status

A clinical evaluation and measures of plasma levels of luteinizing hormone (LH), follicle stimulating hormone (FSH), anti-Müllerian hormone (AMH) and inhibin-B were first scheduled for 6 months post-sterilizing treatment then repeated when requested by the clinician about once a year after completion treatment. Hormonal assays were performed at any point in the cycle, sometimes under hormone replacement therapy (HRT).

Results

Patients

Thirty-six girls underwent OTC, with no parents or patients refusing the procedure. The median age at tissue harvest was 13 years [range: 2–19] and the patient characteristics are shown in Table 1. Eight girls had non-malignant disease requiring HSCT (aplastic anaemia,

myelodysplastic syndrome, sickle cell disease, severe congenital neutropenia and Hurler syndrome) whereas 28 girls received chemo/radiotherapy for malignant disease before OTC. In 32 girls (89%), indication for OTC was high-dose chemo/radiotherapy followed by auto-HSCT (n=13) or allo-HSCT (n=19). Five patients received two successive high-dose regimens: 3 had reduced-intensity conditioning post-auto-HSCT and two had a double auto-HSCT program. Four patients were given standard chemotherapy including alkylating drugs. Details of pre-OTC treatments are shown in Table 2. When classified according to the Brougham and Wallace (2005) list of risk factors for fertility impairment, all our patients were found to be at high risk of premature menopause.

Ovarian tissue harvest

The median duration of surgery was 40 minutes (range: 20–60 min) and the harvesting protocol was followed in all but 2 patients: one presenting ovarian germ cell tumour who had a unilateral healthy ovary fragment harvest and complete oncologic removal of the other ovary during initial surgery; the other, aged 3.6 years, had very small ovaries and the surgeon considered that there was more risk of harming ovarian tissue by sampling each ovary and therefore removed the right ovary and left the left one in place. No healing or infectious problems occurred post-surgery. All patients were discharged the same evening or the next day, except for one girl with sickle cell disease (SCD) and previously unknown protein S deficiency who presented severe left-ovary haemorrhage the day after surgery causing significant blood loss and requiring transfusion and a second surgery to achieve haemostasis. The planned courses of chemotherapy were never delayed and started at a median of 10 [1–38] days post-OTC. An outlier was the SCD patient with post-OTC complications who started pre-allo-HSCT conditioning at 81 days post-OTC.

Histological analysis

Ovarian cortex fragment analysis on slice sections found primary/primordial follicles (median 9/mm² [range: 0–83]) in all except one girl aged 4 years, previously treated for a high-risk neuroblastoma according to HR-NBL-1/SIOPEN protocol. The median number of primordial follicles differed according to patient group age-at-OTC-stratified (age ≤ 10, 11–16 and ≥ 16 yrs) and was significantly correlated to patient age (p=0.029; Table 3). No malignant cells were observed in any ovarian tissue samples.

Follow-up

The median follow-up period was 36 months [range: 1–112] post-harvest and 30 months [0–111] post-sterilizing-treatment. Twenty-six patients were still alive in complete remission and 10 had died, 6 due to disease progression and 4 due to transplantation-related mortality (Table 1). Ovarian tissue of the deceased patients was retained for subsequent analysis of residual disease.

Post-treatment hormonal status

Hormonal status was available for 27 patients at an average of 17 yrs [range: 5–26]. Nine girls had no clinical or hormonal evaluation: one was less than 6 months post-sterilizing treatment, two were lost to follow-up, and six died before the first evaluation. At the time of evaluation, 4 patients had not begun puberty, 1 had normal menstrual cycles, and 22 were primary or secondary amenorrhea, of which 9 took HRT for clinical manifestations of POI. We also found high serum LH and low serum AMH and inhibin-B levels in the patients tested (Tables 1 and 3). In terms of age at OTC, there were no significant differences in hormonal level between age-stratified patient groups. To date, no patient had achieved a pregnancy and none required the use of fragments of cryopreserved ovarian tissue.

Discussion

Indications for OTC depend upon disease and treatment. Brougham and Wallace (2005) classified the most common pediatric and young-adult cancers according to risk of subfertility due to treatment-related gonadotoxicity. The high-risk group (>80% subfertility) included patients after total body irradiation, chemotherapy conditioning for HSCT, irradiated pelvic tumours, metastatic soft tissue sarcomas and Hodgkin lymphomas treated with alkylating agents. However, it is difficult to accurately predict risk to fertility since disease evolution is never completely predictable and patients can change risk level from low/medium to high if they require HSCT or gonadotoxic drugs in response to disease recurrence. Here, the indications (e.g. alkylating agents and chemoradiotherapy graft conditioning) retrospectively corresponded to a high risk of POI.

Ideally, prior to OTC, a patient should not have had any previous chemotherapy that may have reduced follicle numbers. But childhood cancer treatment is often instigated in emergency settings and cannot be delayed. In an effort to save follicular reserve, we – like

others – performed OTC immediately before sterilizing treatment but after induction chemotherapy for malignant disease in order to obtain a theoretic in vivo purging effect of any ovarian infiltration before OTC, which is a theoretical benefit that requires more rigorous investigation (Meirow et al., 2007; Poirot et al., 2007).

In pediatrics, OTC remains the most promising option available to preserve the fertility of prepubertal girls treated with gonadotoxic chemotherapy. It allows a frozen reserve of primordial follicles, but will subsequently require maturation after thawing by autograft or in vitro maturation (IVM) of these immature gametes to achieve a pregnancy. To date, no pregnancy has yet been obtained by reimplanting ovarian tissue harvested before puberty. The question of the minimum age at which it is reasonable to offer OTC is still unresolved and we showed that the follicular reserve of the ovary is age-dependent (Poirot et al., 2007). Indeed, the greater density of primordial follicles in children than in adults might increase the chances of obtaining pregnancy by either ovarian cortex or follicle reimplantation or IVM. Here, we had no technical problem in harvesting the youngest females (<10 years) and all but one of them had follicles in the analyzed sample. Therefore, we did not set a lower limit age for harvesting.

In most cases, the OTC procedure proceeds without complications and the postoperative recovery is straightforward (Jadoul et al., 2010). In our series, there was one bleeding complication likely related to a lack of ovary suture in a patient with previously unknown coagulopathy which was unrelated to the laparoscopic technique itself. Indeed, laparoscopy seems the best way to get ovarian fragments with minimal alteration of the genital tract and minimal postoperative complications (Jadoul et al., 2010). Moreover, as in other studies (Feigin et al., 2007; Poirot et al., 2007), we found that OTC did not delay post-surgery chemotherapy. Indeed, healing is faster than with laparotomy, and the technique quite suitable for children or infants. However, fragment collection still adds a surgery to an already heavy treatment program.

Another important issue is the quantity of ovarian cortex to be harvested for cryopreservation. The decision on how much tissue to remove is dictated by the ovarian volume, especially in very young girls with small-sized ovaries, and by the estimated risk of ovarian failure tied to the planned treatment. There is no consensus on whether to remove an entire ovary or a part of each. In the series published by Poirot et al. (2007), a whole ovary was removed in all cases whereas in the series published by Anderson et al. (2008) the

procedure involved biopsies of cortical tissue. In the study by Jadoul et al. (2010), younger girls were given unilateral oophorectomy while older girls had cortical biopsies without oophorectomy. Donnez et al. (2013) recommend taking at least a 1–1.5 mm-thick biopsy of ovarian cortex. Our choice to perform a bilateral biopsy of 1/3 of each ovary was based on several arguments: (i) it leaves a 2/3 ovarian piece to preserve follicular capital in the event of spontaneous recovery of ovarian and hormonal function (von Wolff et al., 2009); (ii) transplantation of cortical tissue is easier to perform than whole ovary microvascular transplantation (Kagawa, Silber, & Kuwayama, 2009); and (iii) it prevents any subsequent pathology that could alter the remaining ovarian structure (torsion, salpingitis, etc.).

Regarding the harvested tissue, it is legitimate to assume that a younger girl has better chances of restored fertility since she has a greater follicular reserve at the time of harvest. Indeed, total number of primordial and primary follicles per square millimeter showed a strong inverse correlation that was dependent on patient age (Schmidt, Byskov, Nyboe Andersen, Müller, & Yding Andersen, 2003; Poirot et al., 2007). In this study we found a median of 9 follicles/mm² of analyzed tissue (except in one patient), which is consistent with the figures shown in Poirot et al. (2007), but we have to keep in mind that the follicular density in the human ovarian cortex is very uneven (Schmidt et al., 2003).

As IVM of isolated follicles from cryopreserved ovarian cortex has not yet resulted in pregnancy, the autograft technique is for now the only valid option to obtain pregnancies and livebirths. However, the risk of reintroducing cancer cells via an ovarian graft because of residual malignant cells in the frozen–thawed ovarian tissue remains a concern. In our series, no tumour cells were found in any histological analyses whatever the type of cancer, but micrometastases remains possible. All tumours do not have the same potential for extension to the ovaries. To overcome this issue, the ovaries can be taken in remission status. Indeed, some courses of chemotherapy can reduce risk of graft-induced relapse of any initial disease (Shaw, Bowles, Koopman, Wood, & Trounson, 1996). Our data are consistent with Greve et al. (2012) who reported that ovaries from leukaemia patients in complete remission do not appear to contain viable malignant cells (histology, immunohistochemistry and molecular analysis), in contrast to ovarian tissue retrieved before chemotherapy. However, Dolmans et al. (2010) and Rosendahl et al. (2010) showed that graft contamination is frequently detected by reverse-transcriptase-polymerase-chain-reaction (RT-PCR) in acute lymphoblastic leukaemia despite prior chemotherapy. Nevertheless, while it is difficult to ignore the risk of

tumour contamination in ovarian tissue, there are persistent doubts over the pathogenicity of a few residual malignant cells and their ability to effectively induce a relapse of the primary disease. The risk of grafting cancer cells leads scientists to work on detecting minimal residual disease in ovarian tissue before grafting which is an on-going technique. The sensitivity and specificity of detection methods are essential to ensure the safety of autologous ovarian grafts.

Hormonal assessment of gonadal damage is difficult to perform and still not standardized. Interpreting assay results gets more difficult and controversial when patients no longer have spontaneous cycles. Serum FSH is a marker of follicular reserve with good specificity (98%) but low sensitivity (8%) (Barnhart & Osheroff, 1998). Plasma inhibin-B, which is secreted mainly by preantral follicles, is FSH rate-dependent and is only consistently detectable after puberty as it varies during menstrual cycle. AMH, however, emerges as the most useful marker of ovarian reserve (Larsen, Müller, Schmiegelow, Rechnitzer, & Andersen, 2003; van Disseldorp et al., 2010) as levels are relatively constant from mid-childhood to early-adulthood (Hagen et al., 2010) and detectable in girls of all ages, which suggests AMH measurement may be of value for assessing ovarian function in prepubertal girls (Kelsey, Wright, Nelson, Anderson, & Wallace, 2011). Moreover, it can be used regardless of menstrual cycle status (Hehenkamp et al., 2006). In a recent prospective analysis of young females with different cancers at different ages, AMH level fell steadily over the course of repeated chemotherapy treatments, with variable posttreatment recovery (Brougham et al., 2012). Similar results were reported by Miyoshi et al. (2013), who found low AMH levels in 53% of childhood cancer survivors.

Here, taking into account an FSH dosage of over 40 IU/L (Chand, Harrison, & Shelling, 2010) and primary or secondary amenorrhea, we had POI criteria in 22 of the 27 girls tested, all with low AMH values. Although our series is very heterogeneous, with assays performed at a range of times after treatment, AMH still reflected post-chemotherapy ovarian damage. To date, POI is in most cases associated with infertility in adulthood, but since our patients currently have no desire to have children, their fecundity has not been tested.

In conclusion, OTC looks to be an appropriate complication-free strategy to preserve potential fertility in children without affecting the subsequent therapeutic program. It is justifiable that children who require sterilizing treatments should benefit from this technique. The remaining risk of grafting cancer cells has led the medical community to look at detecting minimal residual disease in ovarian tissue before grafting.

Disclosure statement

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Table 1. Characteristics, diagnosis, indications for harvesting and hormonal results of 36 girls who had OTC in the Department of Pediatric Haematology-Oncology in Clermont-Ferrand, France.

		At Cryopreservation		At hormonal evaluation					Disease Status & Follow-up (months)
Pt	Diagnosis	Age (yrs)	Indication	Age (yrs)/months post treatment)	Clinical Status	FSH (IU/L)	LH (IU/L)	AMH (pmol/L)	
1	EW	14	Thio/Mel AutoSCT	17/24	POF	83	–	<0.7	CR (71)
2	OS	16	Thio/Mel AutoSCT[d]	16/8	POF	75	27	–	Dead (52)
3	AML	17	Bu/Cy AlloSCT	17/4	POF	74	34	–	Dead (26)
4	TGM	14	Thio/VP16 AutoSCT	19/60	POF	59.5	78.8	–	CR (60)
5	MDS	18	Bu/Cy AlloSCT	26/95	POF (HRT)	6.9	–	5.6	CR (95)
6	ALL	16	TAM AlloSCT	18/10	POF	88	92	–	Dead (29)
7	AA	19	Bu/Cy AlloSCT	–	–	–	–	–	Dead (2)
8	AA	10	Bu/Cy AlloSCT	20/111	POF (HRT)	113.5	44.5	<0.7	CR (111)
9	RMS	16	Thio/Mel AutoSCT[a]	–	–	–	–	–	Dead (20)
10	HD	16	BEAM AutoSCT	17/8	POF	22	10	–	CR (22)
11	HD	15	BEAM AutoSCT[b,c]	17/12	POF	167	60	<0.7	Dead (35)
12	ALL	9	TAM AlloSCT	16/87	POF (HRT)	0.5	<0.2	1.8	CR (94)
13	SCD	10	Bu/Cy AlloSCT	15/55	POF	71.4	15.4	–	CR (84)
14	PNET	7	Thio/Mel/Cis AutoSCT[d]	12/60	POF (HRT)	79.4	23.8	2.5	CR (61)
15	MPAL	19	RIC AlloSCT	23/51	POF (HRT)	10.2	8.3	–	CR (75)
16	EW	7	Bu/Mel AutoSCT	12/57	Prepub	137.2	47.6	–	CR (75)
17	EW	14	Bu/Mel AutoSCT[c]	19/49	POF (HRT)	76.7	42.6	3.9	CR (52)
18	Neph	6	MEC AutoSCT[d]	–	–	–	–	–	Dead (1)
19	MDS	9	Bu/Cy/Mel AlloSCT	–	–	–	–	–	Dead (29)
20	ALL	9	TBI/VP16 AlloSCT	14/57	POF (HRT)	85	54	–	CR (59)
21	OS	13	OS 2006	–	–	–	–	–	CR (49)
22	OS	11	OS 2006	16/47	NMC	4.6	2.5	11.6	CR (47)
23	RMS	17	Bu/Mel AutoSCT[c]	20/35	POF (HRT)	72.9	36.6	1.2	CR (44)
24	AML	17	Bu/Cy AlloSCT	–	–	–	–	–	Dead (36)
25	HS	2	Bu/Cy AlloSCT	–	–	–	–	–	Dead (0)
26	HD	17	EURONET PHL C1	20/28	POF (HRT)	12	7.4	0.5	CR (31)
27	AML	4	Bu/Cy AlloSCT	–	–	–	–	–	CR (8)
28	SCN	4	RIC AlloSCT	5/13	Prepub	0.8	<0.2	1.5	CR (22)
29	MDS	13	Bu/Cy AlloSCT	14/10	POF	125.9	32.4	3.5	CR (16)
30	AML	19	Bu/Cy AlloSCT	20/12	POF	58.2	30.9	3	CR (15)
31	AML	14	RIC HaploSCT	14/8	POF	21.3	4	2.5	CR (11)
32	ALL	8	TBI/VP16 AlloSCT	9/6	Prepub	0.3	<0.2	–	CR (9)
33	HD	12	EURONET PHL C1	12/7	POF	5	2.2	15	CR (10)
34	MD	10	Thio AutoSCT	10/6	Prepub	7.2	<0.2	2.3	CR (9)
35	AML	16	Bu/Cy AlloSCT	17/6	POF	109.9	71.4	2.3	CR (6)
36	NB	4	Bu/Mel AutoSCT	4/5	Prepub	–	–	–	CR (5)

AA, aplastic anemia; ALL, acute lymphoblastic leukemia; AMH, anti-Müllerian hormone; AML, acute myeloblastic leukemia; EW, Ewing sarcoma; FSH, follicle stimulating hormone; HD, Hodgkin disease; HRT, hormone replacement therapy; HS, hurler syndrome; LH, luteinizing hormone; MD, medulloblastoma; MDS, myelodysplastic syndrom; MPAL, mixed phenotype acute leukemia; NB, neuroblastoma; Neph, nephroblastoma; NMC, normal menstrual cycle; OS, osteosarcoma; PNET, primitive neuroectodermal tumor; POF, premature ovarian failure; Prepub, prepubertal; RMS, rhabdomyosarcoma; SCD, sikle cell disease; SCN, severe congenital neutropenia; TBI, total body irradiation; TGM, germinal tumor.
EURONET PHL C1 = Hodgkin lymphoma treatment protocol; OS 2006 = osteosarcoma treatment protocol.
BEAM = carmustine 300mg/m^2 + VP-16 800mg/m^2 + aracytine 800mg/m^2 + melphalan 140mg/m^2; Bu/Cy = busulfan (480-600mg/m^2) + cyclophosphamide (120-240mg/kg); Bu/Mel = busulfan (480-800mg/m^2) + melphalan (140mg/m^2); MEC = melphalan 350mg/m^2 + etoposide 1350mg/m^2 + carboplatin 400mg/m$^{2;}$ RIC-Allo = fludarabine (180mg/m^2) + busulfan (120-240mg/m^2); RIC-haplo = fludarabine 160mg/m^2 + thiotepa 6mg/m^2 + melphalan 140mg/m$^{2;}$ TAM = TBI 12Gy + aracytine (8-12g/m^2) + melphalan (140mg/m^2); TBI/VP16 = TBI 12 Gy + etoposide 60mg/kg; Thio = thiotepa 1200mg/m^2; Thio/Mel = thiotepa (900-1500mg/m^2) + melphalan (100mg/m^2); Thio/Mel/Cis = thiotepa 240mg/m^2 + melphalan 200mg/m^2 + cisplatine 200mg/m^2.
[a] + pelvic irradiation: 50 Gy; [b] + procarbazine 1500mg/m^2; [c] RIC AlloSCT after AutoSCT; [d] double AutoSCT programme.

Table 2. Chemotherapy before ovarian tissue harvesting in 36 girls who had OTC in the Department of Pediatric Haematology-Oncology in Clermont-Ferrand, France.

Pt	Diagnosis	Age at Cryopreservation (years)	Chemotherapy before harvesting				
			Alkylating agents (g/m^2)	Anthracyclline (mg/m^2)	Platinum (mg/m^2)	VP-16 (mg/m^2)	Others
1	EW	14	IFM (18)	ADR (120)	–	900	VCR
2	OS	16	IFM (24)	ADR (350)	CDDP (600) CBDCA (1600)	1800	MTX
3	AML	17	–	Mitoxantrone (60)	–	–	Amsacrine, Cytarabine
4	TGM	14	IFM (36)	ADR (120)	CDDP (600) CBDCA (900)	2200	Bleomycin, Vinblastine
5	MDS	18	–	–	–	–	–
6	ALL	16	CPM (2)	ADR (120) DNR (270) Mitoxantrone (16)	–	1500	Aspa, Cytarabine, MTX, VCR
7	AA	19	–	–	–	–	–
8	AA	10	–	–	–	–	–
9	RMS	16	IFM (42)	ADR (180) Epirubicin (150)	CBDCA (500)	450	Actinomycin, VCR
10	HD	16	Procarbazine (3)	ADR (160)	–	1800	Bleomycin, Vinblastine, VCR
11	HD	15	Procarbazine (3)	ADR (160)	–	–	VCR
12	ALL	9	IFM (6)	ADR (75) DNR (105)	–	300	Aspa, Cytarabine MTX, Thioguanine, VCR
13	SCD	10	–	–	–	–	–
14	PNET	7	–	–	CBDCA (800)	500	
15	MPAL	19	–	DNR (120) Mitoxantrone (60)	–	200	Amsacrine, Aspa, Cytarabine, VCR
16	EW	7	IFM (60)	ADR (360)	–	2700	VCR
17	EW	14	IFM (27)	ADR (180)	–	1350	VCR
18	Neph	6	–	ADR (100)	–	–	Actinomycin, VCR
19	MDS	9	–	–	–	–	–
20	ALL	9	CPM (2.2)	ADR (115) DNR (160)	–	900	Aspa, Cytarabine, MTX VCR, Vindésine
21	OS	13	–	–	–	–	MTX
22	OS	11	–	–	–	–	MTX
23	RMS	17	IFM (18)	ADR (180)	–	–	Actinomycin, VCR
24	AML	17	–	Mitoxantrone (60)	–	–	Amsacrine, Cytarabine
25	HS	2	–	–	–	–	–
26	HD	17	–	ADR (147)	–	1250	VCR
27	AML	4	–	Mitoxantrone (60)	–	–	Amsacrine, Aspa, Cytarabine
28	SCN	4	–	–	–	–	–
29	MDS	13	–	–	–	–	–
30	AML	19	–	IDR (30) Mitoxantrone (60) DNR (160)	–	400	Amsacrine, Aspa, Cytarabine, Fludarabine,
31	AML	14	–	Daunoxome (180) Mitoxantrone (60)	–	–	Amsacrine, Cytarabine, Fludarabine
32	ALL	8	CPM (6.2) IFM (5.2)	ADR (200) DNR (225)	–	500	Aspa, Cytarabine, Mercaptopurine, MTX, Peg-aspargase, Thioguanine, VCR, Vindésine
33	HD	12	CPM (1.5) Dacarbazine (1.5)	ADR (160)	–	1250	VCR
34	MD	10	–	–	CBDCA (800)	500	–
35	AML	16	–	Mitoxantrone (60)	–	–	Amsacrine, Cytarabine
36	NB	4	CPM (4.2)	–	CDDP (320) CBDCA (1500)	1400	VCR

AA, aplastic anemia; ADR, adriamycine; ALL, acute lymphoblastic leukemia; AML, acute myeloblastic leukemia; Aspa, asparaginase; CBDCA, carboplatin; CDDP, cisplatin; CPM, cyclophosphamide; DNR, daunorubicin; EW, ewing sarcoma; HD, hodgkin disease; HS, hurler syndrome; IDR, idarubicine; IFM, ifosfamide; MDS, myelodysplastic syndrom; MPAL, mixed phenotype acute leukemia; MTX, Methotrexate; NB, neuroblastoma; Neph, nephroblastoma; OS, osteosarcoma; PNET, primitive neuroectodermal tumor; RMS, rhabdomyosarcoma; SCD, sikle cell disease; SCN, severe congenital neutropenia; TGM, germinal tumor; VCR, vincristine.

Table 3. Primordial follicles at harvesting and future hormonal evaluation of 36 girls according to age at OTC (median, range).

	At cryopreservation				At hormonal evaluation							
	Nb of girls	Age (yrs)	Primordial follicles/mm^2		Nb of girls	Age (yrs)	FSH (IU/L)	LH (IU/L)	Oestradiol (pmol/L)	AMH (pmol/L)	Inhibine B (pg/mL)	
≤10 yrs	14	8 (2–10)	14 (0–83)	$p = 0.029$	9	12 (5–20)	71.4 (0.3–137.2)	15.4 (<0.2–54)	<40 (<40–340)	1.8 (<0.7–2.5)	<15	*NS*
11–16 yrs	14	14 (11–16)	9 (3–45)		12	17 (12–19)	75.9 (4.6–167)	32.4 (2.2–92)	40 (<18–139)	3.0 (<0.7–15)	<15	
>16 yrs	8	17 (17–19)	4 (1–20)		6	20 (17–26)	35.1 (6.9–74)	30.9 (7.4–36.6)	62 (<18–201)	2.1 (0.5–5.6)	<15	
All	36	13 (2–19)	9 (0–83)		27	17 (5–26)	71.4 (0.3–167)	27 (<0.2–92)	40 (<18–340)	2.3 (0.5–15)	<15	

FSH, follicle stimulating hormone; LH, luteinizing hormone; AMH, anti-Müllerian hormone.

c. Résultats

Entre 2000 et 2013, 31 filles ont bénéficié de prélèvements de tissus ovariens au CHU de Clermont-Ferrand, après discussion pluridisciplinaire sur les indications et selon des protocoles opératoire et de cryoconservation définis. Une seule patiente, chez laquelle a été mis en évidence *a posteriori* un trouble de coagulation, a présenté une hémorragie ovarienne post-opératoire nécessitant une reprise chirurgicale et une transfusion. Aucune autre complication, y compris infectieuse, n'a été observée.

L'examen anatomo-pathologique réalisé sur un fragment de chaque ovaire prélevé n'a pas mis en évidence de cellule maligne.

d. Conclusion

Compte tenu du risque très faible de complication lié à cette stratégie en l'absence de comorbidité, la cryopréservation de tissu ovarien semble être une méthode de préservation de la fertilité utilisable chez les jeunes filles prises en charge en onco-hématologie pédiatrique après évaluation du risque gonadotoxique.

Si l'absence de mise en évidence de cellule tumorale en anatomopathologie est rassurante, il paraît indispensable de mettre au point des méthodes plus sensibles et spécifiques de détection des cellules malignes dans le tissu ovarien afin de pouvoir, dans le futur, l'utiliser en toute sécurité pour restaurer la fertilité de ces patientes à l'âge adulte.

2. Accès à la préservation de la fertilité chez les adolescents et jeunes adultes pris en charge pour un cancer en Auvergne

a. Rationnel de l'étude

Le parcours de soins des adolescents et jeunes adultes (AJA) de 15 à 24 ans pris en charge pour des pathologies cancéreuses est plus hétérogène que celui des enfants, certains étant traités dans des services pédiatriques, d'autres dans des services adultes.

La préservation de la fertilité est une préoccupation majeure de la prise en charge de ces patients. Toutefois, l'information et l'accès à cette préservation sont probablement insuffisamment ou tardivement proposés.

À la suite du 3ème Plan Cancer, la plateforme PREFERA (PREservation FERtilité Auvergne) a été mise en place en mars 2016, soutenue principalement par la Ligue contre le Cancer et le CHU de Clermont-Ferrand, pour faire face à un besoin important. Elle permet la centralisation des demandes depuis 37 structures régionales qu'elles soient publiques ou privées. Elle est composée de médecins biologistes, de gynécologues, d'une infirmière de coordination, de psychologues, d'une sexologue et de secrétaires. Viennent s'ajouter des médecins référents en cancérologie adulte et pédiatrique mais également en biologie moléculaire et en anatomopathologie. En ce sens, PREFERA assure une collaboration multidisciplinaire entre « prestataires de soins médicaux » (hématologues, oncologues médicaux, gynécologues, ...) et spécialistes de la reproduction humaine, permet de faciliter l'accès à l'information des patients et des professionnels, et d'augmenter le nombre de patients pris en charge en préservation.

Nous avons décrit l'accès à la préservation de la fertilité des AJA pris en charge pour un cancer en Auvergne entre début 2014 et début 2017.

b. Article n°4 : *Soumis au Bulletin du Cancer*

Accès à la préservation de la fertilité des adolescents et jeunes adultes de 15 à 24 ans atteints de cancers en Auvergne, France

Access to fertility preservation for adolescents and young adults aged 15 to 24 years with cancers in Auvergne, France

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Résumé

Introduction : Les cancers des adolescents et jeunes adultes présentent des spécificités épidémiologiques particulières. L'amélioration de leur survie doit s'accompagner d'une prise en considération accrue des effets indésirables des traitements, parmi lesquels la possible diminution de la fertilité. L'objectif de l'étude était de décrire l'accès à la préservation de la fertilité de ces patients au CHU de Clermont-Ferrand sur une période de 3 ans.

Méthodes : Au cours de cette étude descriptive rétrospective, différentes données socio-démographiques et cliniques ont été collectées.

Résultats : Cent-cinquante nouveaux cas de cancers ont été diagnostiqués chez les patients de 15 à 24 ans. Quarante-quatre pourcents ont bénéficié d'au moins une consultation au Centre d'Etude et de Conservation des Œufs et du Sperme humains, 29% chez les filles et 58% chez les garçons ($p < 0.001$). Le nombre de cas n'ayant pas abouti à une préservation de la fertilité

était significativement plus élevé chez les filles que chez les garçons (p = 0.005). La préservation de la fertilité a été réalisée majoritairement par cryoconservation de tissu ovarien chez les adolescentes, d'ovocytes chez les jeunes femmes et de sperme chez les garçons.

Discussion : Nous avons pu observer des disparités dans l'accès à la préservation de la fertilité selon le sexe. Malgré l'existence de recommandations, des progrès restent à faire. La mise en place de plateformes clinico-biologiques devraient permettre une meilleure sensibilisation des patients et professionnels de santé, et ainsi de promouvoir l'accès aux techniques de préservation de la fertilité des jeunes patients atteints de cancers.

Mots-clés

Préservation fertilité ; cancer ; adolescents et jeunes adultes

Abstract

Introduction: Cancers of adolescents and young adults have particular epidemiological specificities. The improvement in their survival should be accompanied by an increased consideration of the treatments' side effects, among which the potential decrease in fertility. The objective of the study was to describe the access to fertility preservation of these patients at the University Hospital of Clermont-Ferrand over a period of 3 years.

Methods: During this retrospective descriptive study, various socio-demographic and clinical data were collected.

Results: One hundred and fifty new cases of cancers were diagnosed in patients aged 15 to 24 years. Forty-four percent received at least one fertility consultation, 29% for girls and 58% for boys (p < 0.001). The number of cases that did not result in fertility preservation was significantly higher for girls than boys (p = 0.005). Fertility preservation was mainly achieved by cryopreservation of ovarian tissue in female adolescents, ovocytes in young women and sperm in boys.

Discussion: We observed sex disparities in access to fertility preservation. Despite the existence of recommendations, progress remains to be made. The establishment of clinico-biological platforms should allow a better awareness of patients and professionals, and thus promote access to fertility preservation techniques for young patients with cancer.

Keywords

Fertility preservation; cancer; adolescents and young adults

Introduction

Avec une incidence annuelle de 219.4 par million chez les 15-19 ans et 293.1 par million chez les 20-24 ans, les cancers des adolescents et jeunes adultes (AJA) sont rares mais constituent la troisième cause de décès en France après les accidents de la voie publique et les suicides [1, 2]. Ces cancers présentent des spécificités épidémiologiques particulières : les plus fréquents sont les leucémies aiguës, lymphomes malins hodgkiniens et non hodgkiniens chez les adolescents de 15 à 19 ans et les tumeurs épithéliales (essentiellement carcinomes thyroïdiens et mélanomes malins) chez les jeunes adultes de 20 à 24 ans [1]. La survie globale à 5 ans s'est améliorée au cours des dernières années : 79% chez les adolescents et 84% chez les jeunes adultes [2], et devrait s'accompagner d'une prise en considération accrue des effets indésirables des traitements, parmi lesquels la diminution de la fertilité tient une place importante pour ces futurs adultes.

Dans un contexte de parcours de soins des AJA qui reste hétérogène (certains étant pris en charge en hématologie-oncologie pédiatrique, d'autres dans des services de médecine adulte) [1, 3], nous avons étudié l'accès à la préservation de la fertilité des AJA atteints de pathologies cancéreuses au CHU de Clermont-Ferrand sur une période de 3 ans.

Méthodes

Les AJA de 15 à 24 ans au moment du diagnostic, atteints de cancers diagnostiqués en première intention (à l'exclusion des rechutes) en Auvergne entre le 1er janvier 2014 et le 1er

janvier 2017, ont été inclus. Dans un souci d'exhaustivité, les données provenaient du Registre des cancers de l'enfant d'Auvergne-Limousin, des Départements d'Information Médicale des centres hospitaliers d'Auvergne et du Centre d'Etude et de Conservation des Œufs et du Sperme humains (CECOS) du CHU de Clermont-Ferrand. Pour chaque patient, les données socio-démographiques et cliniques suivantes ont été collectées : sexe, âge au diagnostic, code postal du domicile, praticien ayant adressé le patient, catégorie socio-professionnelle du patient, structure et service de prise en charge, type de cancer, greffe ou non de cellules souches hématopoïétiques (CSH), réalisation ou non d'une consultation au CECOS, réalisation ou non d'une préservation de fertilité et ses modalités, statut vivant ou décédé au 1er janvier 2018.

Il s'agit donc d'une étude descriptive rétrospective. Les statistiques d'incidence sont présentées selon les groupes et sous-groupes diagnostiques de la 3ème version de l'International Classification of Childhood Cancer (ICCC-3), la plus adaptée aux types de cancers rencontrés dans cette tranche d'âges [4].

Résultats

Cent cinquante nouveaux cas de cancers ont été diagnostiqués chez les AJA de 15 à 24 ans, dont 52% chez les garçons (sex-ratio 1.1) et 31% chez les adolescents de 15 à 18 ans. L'âge médian au diagnostic était de 20 ans. Les cancers les plus fréquemment retrouvés étaient les lymphomes (hodgkiniens et non-hodgkiniens, 31%), les tumeurs de la thyroïde (13%) et les tumeurs germinales (11%). Six patients (4%) ont bénéficié d'une chimiothérapie intensive avant greffe de CSH. Parmi les 46 patients de moins de 19 ans, 22 seulement (48%) étaient pris en charge en hématologie-oncologie pédiatrique.

Concernant les consultations au CECOS, 66 patients (44%) en ont bénéficié. Parmi eux, 58% étaient domiciliés dans le Puy-de-Dôme (n=38) et 73% ont été adressés par le CHU de Clermont-Ferrand (n=48). Ces taux étaient similaires dans les 2 catégories d'âges (Tableau 1). Près de la moitié des moins de 19 ans étaient pris en charge en hématologie-oncologie pédiatrique (48%). Les lymphomes (hodgkiniens et non hodgkiniens) représentaient le cancer le plus fréquent (48%). Parmi ces derniers, seulement 44% des 15-18 ans contre 81% des 19-24 ans (p = 0.02) ont été adressés au CECOS. Quatre-vingt-huit pourcents des patients atteints

de tumeurs germinales et 69% de ceux atteints de sarcomes ont consulté au CECOS (Tableau 1). En regroupant les cancers à haut risque gonadotoxique (leucémies, lymphomes, sarcomes, tumeurs germinales), on ne retrouve pas de différence significative dans les taux de consultations au CECOS entre les 2 catégories d'âges (p = 0.06). En ce qui concerne le sex-ratio et l'âge des patients qui ont consulté au CECOS : 29% étaient des filles (n=21) et 58% des garçons (n=45) (p < 0.001), 46% avaient entre 15 et 18 ans (n=21) et 43% entre 19 et 24 ans (n=45). Nous n'avons pas pu retrouver leur situation professionnelle de 27 patients. Pour les autres patients, sur tous ceux ayant eu une consultation CECOS, 53% étaient étudiants et 39% non-étudiants (travailleurs ou chômeurs). Parmi les 45 patients de 19 à 24 ans ayant consulté au CECOS, 16 étaient étudiants (36%) et 25 non-étudiants (56%).

Parmi les patients ayant consulté au CECOS, le nombre de cas n'ayant pas abouti à une préservation de la fertilité était significativement plus élevé chez les filles que chez les garçons (33% versus 7% ; p = 0.005). Ce taux était plus important chez les patientes de 19-24 ans (43% versus 14%) sans que cette différence ne soit significative. Respectivement 90% des 15-18 ans (n=19) et 82% des 19-24 ans (n=37) adressés au CECOS ont bénéficié d'une préservation de leur fertilité (Tableau 2). La préservation de la fertilité des 14 jeunes femmes a été réalisée par cryoconservation d'ovocytes (n=9) ou par cryoconservation de tissu ovarien (n=5). Il s'agissait majoritairement d'une cryoconservation de tissu ovarien chez les adolescentes (67% des cas) et d'une cryoconservation d'ovocytes chez les jeunes adultes (88% des cas). En-dehors de 2 adolescents ayant bénéficié d'une cryoconservation de tissu testiculaire, la préservation de la fertilité des 42 jeunes hommes a consisté majoritairement en une cryoconservation de sperme (n=40) tant chez les adolescents que chez les jeunes adultes (respectivement 85% et 100% des cas) (Tableau 2).

Discussion

Nous avons recensé 150 patients, âgés de 15 à 24 ans, atteints de pathologies cancéreuses au cours de la période étudiée (3 ans), soit une incidence de 340 nouveaux cas par an et par million d'AJA de 15 à 24 ans en Auvergne. Cette incidence est plus élevée de 34% par rapport à l'incidence nationale de 254 nouveaux cas par an et par million d'AJA rapportée par Desandes et al., ce qui s'explique en partie par quelques patients pris en charge en

Auvergne mais domiciliés dans un département limitrophe. Le sex-ratio et un diagnostic majoritaire de lymphomes sont concordants avec les données de Desandes et al. [2]. Contrairement aux recommandations de la circulaire DHOS/O n°2004/161 du 29 mars 2004, relative à l'organisation des soins en cancérologie pédiatrique, précisant que « la cancérologie pédiatrique concerne les enfants jusqu'à 18 ans atteints de tumeurs solides ou d'hémopathies malignes », 22 seulement (48%) des patients de moins de 19 ans ont été pris en charge en hématologie-oncologie pédiatrique [5].

Les cancers les plus adressés au CECOS étaient les lymphomes, les sarcomes et les tumeurs germinales. Ceci est lié à la localisation des tumeurs germinales et à l'importante gonadotoxicité des traitements des lymphomes et sarcomes, voire des tumeurs germinales. Comme attendu, les cryopréservations d'ovocytes et de sperme constituaient les principales stratégies de préservation [6].

La comparaison des 2 catégories d'âge n'a pas retrouvé de différence significative en termes de proportion de patients ayant consulté au CECOS d'une part, et ayant bénéficié d'une préservation de leur fertilité d'autre part. En revanche, nous avons mis en évidence des disparités entre les 2 sexes : les garçons avaient significativement plus souvent consulté au CECOS que les filles et ont significativement davantage bénéficié d'une préservation de leur fertilité. La raison principale est probablement la plus grande simplicité de préservation de la fertilité chez les patients de sexe masculin, l'option envisagée dans la majorité des cas étant la cryopréservation de sperme.

Certaines études décrivent également ces disparités. Anderson et al. a décrit que, parmi ceux à risque modéré ou élevé d'infertilité, 83% des garçons pubères, 39% des garçons prépubères et 1% des filles étaient adressés en consultation de préservation de la fertilité [7]. Dans une étude de 2011, il a été rapporté que les oncologues prenant en charge des adolescents atteints de cancers déclaraient avoir adressé plus fréquemment les patients de sexe masculin à un spécialiste de la fertilité (respectivement 46% versus 12% seraient adressés dans plus de 50% des cas) [8]. Dans une étude Suédoise, les hommes avaient plus souvent reçu des informations sur l'impact potentiel des traitements sur leur fertilité que les femmes (80% versus 48% ; odd ratio 3.2) et sur les options de préservation (68% versus 14% ; odd ratio 14.4). Alors que 54% des hommes de cette étude ont eu une préservation de leur fertilité, seulement 2% des femmes en ont bénéficié [9]. Une étude américaine a mis en évidence que

80% des hommes et 74% des femmes rapportaient avoir eu une information sur l'impact des traitements sur la fertilité, 29% des hommes et 56% des femmes rapportaient ne pas avoir discuté des modalités de préservation de la fertilité avant le traitement anti-cancéreux, 69% des hommes et 93% des femmes rapportaient ne pas avoir eu de préservation de leur fertilité [10].

Malgré l'existence de recommandations sur la préservation de la fertilité chez les jeunes patients pris en charge en hématologie-oncologie [11, 12] des progrès restent à faire, d'une part en termes d'information sur le risque gonadotoxique, les stratégies de préservation de la fertilité qui existent et leur planification dans les meilleurs délais, d'autre part en termes de communication entre les différents acteurs de la prise en charge de ces patients [13].

A l'annonce d'une pathologie cancéreuse, les patients et leurs familles sont naturellement en priorité focalisés sur le traitement et le pronostic à venir. Néanmoins, les soignants ne doivent pas négliger les effets indésirables des thérapeutiques, qui peuvent avoir des répercussions importantes sur la qualité de vie ultérieure de ces jeunes patients. L'étude de Nieman et al. en 2007 s'est intéressé au vécu des adultes guéris de cancers survenus entre 13 et 21 ans, ainsi qu'à celui de leurs familles. Peu de parents se souvenaient avoir discuté des questions de fertilité au moment du diagnostic [14].

La mise en place de plateformes clinico-biologiques de préservation de la fertilité (PREFERA en Auvergne depuis 2016), ayant pour mission une collaboration pluridisciplinaire et la structuration du parcours de soins des AJA [15] devraient permettre une meilleure sensibilisation des patients et professionnels de santé sur les problématiques de fertilité, et ainsi d'optimiser l'accès aux techniques de préservation de la fertilité des jeunes patients.

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Tableau 1. Caractéristiques des patients ayant consulté au CECOS.

	AJA	15-18 ans	19-24 ans
Nombre de patients	66/150	21/46	45/104
Département			
63	38/66 (58%)	12/21 (57%)	26/45 (58%)
Autres	28/66 (42%)	9/21 (43%)	19/45 (42%)
Service initial de prise en charge			
CHU	48/66 (73%)	15/21 (71%)	33/45 (73%)
Autre	18/66 (27%)	6/21 (29%)	12/45 (27%)
Diagnostic			
Lymphomes* (n=47)	32/47 (68%)	7/16 (44%)	25/31 (81%)
Leucémie (n=11)	6/11 (55%)	2/5 (40%)	4/6 (67%)
Sarcomes (n=13)	9/13 (69%)	6/8(75%)	3/5 (60%)
Tumeur germinale (n=17)	15/17 (88%)	5/5 (100%)	10/12 (83%)
Tumeur cérébrale (n=14)	1/14 (7%)	1/3 (33%)	0/11 (0%)
Tumeur de la thyroïde et autres (n=48)	3/48 (6%)	0/9 (0%)	3/39 (8%)

* Hodgkinien ou non-hodgkinien
AJA : adolescents et jeunes adultes (15-24 ans) ; CECOS : Centre d'Etude et de Conservation des Œufs et du Sperme humains ; CHU : Centre Hospitalier Universitaire

Tableau 2. Préservation de la fertilité chez les AJA en fonction du sexe et de l'âge.

	All patients	15-18 ans	19-24 ans
Filles	n = 72	n = 25	n = 47
CECOS consultation	21/72 (29%)	7/25 (28%)	14/47 (30%)
Préservation d'ovocytes	9/21 (43%)	2/7 (29%)	7/14 (50%)
Préservation de tissu ovarien	5/21 (24%)	4/7 (57%)	1/14 (7%)
Pas de préservation	7/21 (33%)	1/7 (14%)	6/14 (43%)
Garçons	n = 78	n = 21	n = 57
CECOS consultation	45/78 (58%)	14/21 (67%)	31/57 (54%)
Préservation de sperme	40/45 (89%)	11/14 (79%)	29/31 (94%)
Préservation de tissu testiculaire	2/45 (4%)	2/14 (14%)	0/31 (0%)
Pas de préservation	3/45 (7%)	1/14 (7%)	2/31 (6%)

AJA : adolescents et jeunes adultes (15-24 ans) ; CECOS : Centre d'Etude et de Conservation des Œufs et du Sperme humains

c. Résultats

Moins de la moitié des AJA a été mis en contact avec l'équipe de préservation de la fertilité. Les garçons ont davantage été adressés au CECOS et, lorsqu'une consultation a eu lieu, plus de 90% des garçons et moins de 70% des filles ont bénéficié d'une préservation de la fertilité.

La préservation de la fertilité s'est faite essentiellement par cryoconservation de sperme chez les garçons, par congélation de tissu ovarien chez les filles de 15 à 18 ans et par cryoconservation d'ovocytes chez celles de 19 à 24 ans.

d. Conclusion

La mise en œuvre plus simple de la préservation de la fertilité chez le garçon pubère explique en partie les taux différents entre les deux sexes. Dans tous les cas, les AJA doivent être informés du risque potentiel d'atteinte gonadotoxique lié à leur pathologie et ses traitements. Il semble indispensable qu'ils puissent avoir accès à une consultation spécialisée afin de connaître les modalités de préservation de la fertilité possibles pour eux.

La détresse induite par le diagnostic de la maladie et l'urgence éventuelle de mise en place des traitements ne doivent pas faire négliger la prise en compte des effets secondaires à long terme pouvant altérer la qualité de vie après cancer. Si la préservation de la fertilité n'est pas possible au diagnostic, elle peut éventuellement être réalisée après quelques cures de chimiothérapie, le plus tôt possible pour assurer de meilleures chances de succès.

La création de plateformes clinico-biologiques pluridisciplinaires spécialisées permettent une meilleure coordination entre les services prenant en charge les patients atteints de cancers afin d'optimiser l'accès à la préservation de la fertilité, en particulier pour cette tranche d'âges spécifique que représentent les AJA.

IV. DÉTECTION DE CELLULES TUMORALES DANS LE TISSU GONADIQUE : MODÈLES DU NEUROBLASTOME ET DE LA TUMEUR D'EWING

1. Rationnel de l'étude

En France, le neuroblastome est la tumeur pédiatrique extracrânienne la plus fréquente, représentant environ 8% des cancers de l'enfant de moins de 15 ans, soit environ 140 nouveaux diagnostics par an. La tumeur d'Ewing représente environ 2% des cancers de l'enfant de moins de 15 ans, soit environ 33 nouveaux diagnostics par an (Lacour et al. 2010). Ces deux cancers sont à fort potentiel métastatique et impliquent des traitements lourds et potentiellement stérilisants. Néanmoins, ces traitements intensifs ont permis d'améliorer considérablement la survie des patients, et la prise en compte des effets secondaires des traitements ne doit pas être négligée.

Chez l'enfant prépubère, la seule option envisageable pour préserver la fertilité est la cryoconservation de tissu ovarien ou testiculaire. La littérature rapporte plusieurs cas de métastases ovariennes (Steytler et al. 2019 ; Somjee et al. 1999 ; Young et al. 1994 ; Meyer et al. 1979) et testiculaires de neuroblastome (Simon et al. 2000 ; Tourniaire et al. 1997 ; Kumari et al. 1994 ; Kushner et al. 1985 ; Casola et al. 1984) et plusieurs cas de métastases ovariennes (Schifflers et al. 2018 ; Sullivan et al. 2012 ; Abir et al. 2010 ; Sonmezer et al. 2005 ; Young et al. 1994) de tumeur d'Ewing. A notre connaissance, il n'a pas été rapporté de métastase testiculaire de tumeur d'Ewing. Néanmoins, des cellules tumorales circulantes peuvent être mises en évidence chez 20-45% des patients atteints de tumeurs d'Ewing (Scheiermacher et al. 2003), pouvant faire craindre la présence, même infra clinique de cellules malignes dans le tissu gonadique. Afin de pouvoir envisager une éventuelle greffe ultérieure de ces tissus gonadiques cryoconservés, il est donc indispensable de disposer de méthodes sensibles et spécifiques de détection de la maladie résiduelle de la pathologie initiale. Des transcrits détectables en biologie moléculaire existent pour ces deux pathologies.

Pour le neuroblastome, il existe des marqueurs spécifiques de la fonction neuronale, habituellement utilisés lors de la recherche de la maladie résiduelle du neuroblastome dans la moelle ou le sang. Leur présence dans un prélèvement humain constitue un facteur de mauvais pronostic (Viprey et al. 2014) : TH (tyrosine hydroxylase), impliquée dans la synthèse des catécholamines ; PHOX2B (paired-like homeobox 2B), codant pour un facteur de

transcription impliqué dans le développement du système nerveux autonome ; DCX (doublecortin), impliqué dans la migration neuronale. Depuis 1994, nous étudions à Clermont-Ferrand la maladie résiduelle du neuroblastome dans la moelle et le sang. Nous avons ainsi pu mettre au point une technique de quantification des différents transcrits du neuroblastome par RT-qPCR. Nous avons obtenu une sensibilité de détection qui permet de déceler en routine une cellule tumorale parmi 10^6 cellules sanguines ou médullaires (Kanold et al. 2003 ; Tchirkov et al. 2003 ; Kanold et al. 2000 ; Tchirkov et al. 1998).

Pour la tumeur d'Ewing : le transcrit de fusion EWS-FLI1 de type 2 constitue le transcrit de fusion le plus fréquent, résultant de la fusion du gène EWS (22q12) avec le gène FLI1 (11q24), observé dans 85-90% des cas (Ludwig et al. 2008 ; Vermeulen et al. 2006). Il constitue un marqueur moléculaire extrêmement spécifique.

L'objectif de nos études était de mettre au point une technique sensible et spécifique de détection de la maladie résiduelle dans les tissus ovarien et testiculaire par RT-qPCR, applicable aux enfants prépubères atteints de deux types de tumeurs solides : le neuroblastome et la tumeur d'Ewing. L'intérêt étant de disposer d'un test diagnostic utilisable pour les adultes guéris de cancers, dont la fertilité a été compromise par les traitements et qui ont bénéficié d'une cryoconservation de tissu ovarien ou testiculaire. La mise au point a été réalisée sur du tissu gonadique non cancéreux de patients adultes.

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Pediatric Blood & Cancer

RESEARCH ARTICLE

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Highly sensitive assessment of neuroblastoma minimal residual disease in ovarian tissue using RT-qPCR—A strategy for improving the safety of fertility restoration

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Abstract

Background: Ovarian tissue cryopreservation (OTC) is the only option available to preserve fertility in prepubertal girls with neuroblastoma (NB), a childhood solid tumor that can spread to the ovaries, with a risk of reintroducing malignant cells after an ovarian graft. Procedure: We set out to determine whether the analysis of *TH* (*Tyrosine hydroxylase*), *PHOX2B* (*Paired-like homeobox 2b*) and *DCX* (*Doublecortin*) transcripts using quantitative reverse transcriptase polymerase chain reaction (RT-qPCR) could be used to detect NB contamination in ovarian tissue. Analyses were performed on benign ovarian tissue from 20 healthy women between November 2014 and September 2015 at the University Hospital of Clermont-Ferrand. Peri-cystic benign ovarian tissues were collected and contaminated with increasing numbers of human NB cells (cell lines IMR-32 and SK-N-SH) before detection using RT-qPCR. Results: *TH* and *DCX* transcripts were detected in uncontaminated ovarian tissue from all the donors, hampering the detection of small numbers of tumor cells. By contrast, *PHOX2B* was not detected in any uncontaminated ovarian fragment. *PHOX2B* levels were significantly increased from 10 NB cells. Our study is the first to evaluate MRD detection using NB mRNAs in human ovarian tissue. Only *PHOX2B* was a reliable marker of NB cells contaminating ovarian tissue. Conclusions: These results are encouraging and offer hope in the near future for grafting ovarian tissue in women who survive cancer, whose fertility has been jeopardized by treatment, and who have benefited from OTC without oncological risk.

Key words

Neuroblastoma, minimal residual disease, fertility preservation, ovarian tissue, RT-qPCR

Introduction

Neuroblastoma (NB) is the most common extracranial solid tumor in children, with a high metastatic potential. It is also the childhood solid tumor that spreads to the ovaries most frequently.[1-6] In Europe, every year about 250 girls with NB are given high-dose chemotherapy followed by hematopoietic stem cell transplantation (sterilizing treatment), while others are exposed to intensive potentially gonadotoxic treatment. These girls represent the largest NB subgroup, justifying the inclusion of NB in a high-risk subfertility group of pediatric cancers (>80% subfertility) owing to treatment-related gonadotoxicity.[7] Chemo- and radiotherapy are both directly toxic to current growth follicles, causing follicular loss and subsequent primary ovarian failure (POF),[8] and reducing patients' chances of having children later on.

As these high-dose and intensive treatments have led to a remarkable improvement in the long-term survival rate, currently over 40%, also for patients with high-risk diseases,[9] quality of life is an essential concern for pediatric oncologists and families. Infertility ranks high on the list of treatment side-effects that reduce quality of life in cancer survivors.

Given that the typical patient with NB is an infant or young child, ovarian tissue cryopreservation (OTC) is the only option available to preserve fertility in young prepubertal girls at risk of treatment-induced POF.[10,11] It can offer the possibility of restoring gonadal function and fertility after cancer remission.[12,13]

However, because ovarian tissue must be collected before high-dose chemotherapy, it may theoretically still contain malignant cells, implying a potential risk of primary disease recurrence after transplantation.[14,15] Sensitive and specific minimal residual disease (MRD) detection methods are therefore essential to ensure the safety of autologous ovarian grafts.[16-20]

Sensitive detection of MRD using quantitative reverse transcriptase polymerase chain reaction (RT-qPCR) for NB mRNAs in blood and bone marrow is well-established and has a prognostic impact.[21-24] *TH* (*Tyrosine hydroxylase*), *PHOX2B* (*Paired-like homeobox 2b*) and *DCX* (*Doublecortin*) genes, which are strongly expressed in NB tumors, are commonly used as markers in this approach.[24-26] However, no studies on the molecular detection of MDR in ovarian tissue from NB patients have yet been reported. Here we investigated whether the analysis of NB mRNAs using RT-qPCR could be used for the detection of minimal tumor contamination in ovarian tissues.

Materials and Methods

Twenty peri-cystic ovarian tissues were obtained during planned endoscopic surgery for benign ovarian cysts between November 2014 and September 2015 at the University Hospital of Clermont-Ferrand. The written informed consent of patients was obtained at the Biological Resource Centre "GERMETHEQUE" (certified NF 96900, Committee for Personal Protection DC-2008-558) and the study was declared on the clinicaltrial.gov website (No. NCT 02400827). These ovarian tissue fragments were used only when free of any malignancies (histological analysis).

Each sample of ovarian tissue was cut into 6 thin fragments weighing no less than 20mg and frozen in liquid nitrogen (−196°C), either dry (dry freezing) or using a validated slow-freezing procedure as described previously and used in clinical practice for ovarian tissue freezing (slow freezing).[27] The purpose of using these two freezing procedures was to compare them in order to determine the impact of the cryoprotectant.

After thawing, the fragments of ovarian tissue were contaminated with 10, 100 and 1000 tumor cells from human NB cell lines IMR-32 (n=10) or SK-N-SH (n=10) (CCL-127™ and HTB-11™ ATCC LGC Standards, Molsheim, France). In each contamination series, one fragment was not contaminated (negative control). Contaminations with 10 and 100 cells were performed in duplicate for each donor. We tested the cell lines beforehand and found that they strongly expressed *TH*, *PHOX2B* and *DCX* mRNAs (positive controls).

The RT-qPCR procedure was the same for all the series (n=20). RNA was extracted using TRIzol Reagent (Invitrogen, Cergy-Pontoise, France), following the manufacturer's instructions. RNA was treated with DNAse I (Roche Diagnostics, Meylan, France) to remove any contaminating DNA. One microgram of RNA was reverse-transcribed using SuperScript II (Invitrogen). *TH*, *DCX* and *PHOX2B* transcripts and the reference *ß2M* (*Beta-2-microglobulin*) transcript were quantified in the LightCycler 480 real-time PCR system (Roche-Diagnostics, Meylan, France) using previously described primers and probes.[25] Absolute numbers of transcripts was calculated with the LightCycler, using the calibration data obtained with serial dilutions of plasmid standards containing known numbers of cDNA molecules of each gene. Copy numbers of target mRNA transcripts were reported per 10^6 copies of *ß2M* transcript. PCRs were performed in triplicate.

We also studied cryopreserved ovarian tissues from two prepubertal girls with NB in order to validate our molecular results obtained on ovarian tissues from adult patients. On

the same sample, after RNA extraction, RT-qPCR was performed to quantify *TH*, *DCX* and *PHOX2B* transcripts.

Statistical analysis was performed using the Stata software package, version 13 (StataCorp, College Station, TX, US). The tests were two-sided, with a type I error set at α=0.05. The results for quantitative parameters were presented as means ± standard deviations (SD) or medians and interquartile range, according to statistical distribution (assumption of normality checked by Shapiro-Wilk test). ANOVAs for repeated measures or Friedman tests (due to repeated correlated data) were applied according to ANOVA assumptions, i.e. (i) normality and (ii) homoscedasticity studied by Bartlett test. When appropriate, a *post hoc* test for pairwise multiple comparisons was performed (Tukey-Kramer post-ANOVA or Games and Howell post-Friedman). These analyses were supplemented using random-effects models to compare the cell number effect, taking into account within and between subject variability. The assumption of the normality of residuals was studied using the Shapiro-Wilk test. When appropriate (data not exhibiting normal distributions), a log-transformation was proposed to ensure the correct use of these analyses.

Results

We quantitated the expression of *TH*, *DCX*, and *PHOX2B* transcripts in IRM-32 and SK-N-SH cells obtained at five different subcultures and six primary NB tumors (Figure 1). Both cell lines and primary tumors highly expressed these NB-specific mRNAs with mean normalized transcript numbers of $4.5x10^6$ for *TH*, $1.4x10^7$ for *PHOX2B* and $2.7x10^7$ for *DCX*. Overall, average transcript number variations between cell line subcultures were minimal (<1.3 fold). In primary tumors, the variations were higher, but still moderate: 1.8 fold for *TH*, 1.5 for *PHOX2B* and 3.0 for *DCX*. There was no statistical difference between the cell lines and tumors in the *TH* and *PHOX2B* level. Of note, IMR-32 cells showed about 2-fold higher expression of *PHOX2B* than SK-N-SH cells. Finally, *DCX* levels were about 3-fold lower in tumors than in cell lines, but this marker was the most variable. Given that the three mRNAs were expressed in cell lines and primary tumor tissues at high and similar levels, the IRM-32 and SK-N-SH cell lines are appropriate models for NB contamination experiments with ovarian tissue.

Ovarian tissue was collected from 20 women (median age 31 years, range 20–49 years). The median weight of the ovarian tissue samples collected was 248.3mg (168.2–444.0mg). An experienced pathologist found no sign of malignancy in the ovarian tissue after histological

analysis. The median RNA yield from these fragments was 21.8µg (5.4–55.2µg), which corresponds roughly to 727,000–2,180,000 of total cells.

TH and *DCX* transcripts were detected in uncontaminated ovarian tissue from all patients (n=20) frozen with (n=10) or without (n=10) cryoprotectant, which hampered the detection of the 10 contaminating tumor cells (Figure 2a and b). Compared with uncontaminated fragments, the *TH* mRNA level was significantly increased in the case of contamination with 1000 and 100 NB cells ($p < 0.001$ for IMR-32 and SK-N-SH). Similarly, the expression of *DCX* was significantly different between the uncontaminated fragments and those contaminated with 1000 ($p < 0.001$ for IMR-32 and SK-N-SH) and 100 NB cells ($p < 0.001$ for IMR-32 and $p = 0.008$ for SK-N-SH). However, fragments contaminated with 10 NB cells were not significantly different from uncontaminated fragments in *TH* ($p = 0.59$ for IMR-32 and $p = 0.31$ for SK-N-SH) and *DCX* ($p = 0.08$ for IMR-32 and $p = 0.77$ for SK-N-SH) expression levels. We analyzed six additional control samples and confirmed the presence of background expression of *DCX* in all of them, whereas *TH* was undetectable in some controls. We also investigated whether the introduction of cut-off points in *TH* and *DCX* levels could enable the detection of 10 tumor cells. The level of specificity was variable, according to the marker and cell line used: 50–70% for IMR-32 and 60–80% for SK-N-SH.

Importantly, *PHOX2B* was not detected in any uncontaminated ovarian fragment (n=20) (Figure 2c) frozen with (n=10) or without (n=10) cryoprotectant. *PHOX2B* levels were significantly increased from 10 NB cells ($p < 0.001$ for both IMR-32 and SK-N-SH), which provided a specificity and sensitivity of 100% for MRD detection. Moreover, there was a close linear correlation between the number of tumor cells used for contamination and *PHOX2B* transcript numbers ($r^2 = 0.96$ for both IMR-32 and SK-N-SH). The analysis of cryopreserved ovarian tissues from two prepubertal girls with NB revealed the presence of *DCX* expression at a level similar to that of our control samples, and no expression of *TH* and *PHOX2B*.

Discussion

In the light of current progress in NB treatment and the resulting increased survival rates, it is now necessary to consider the long-term side effects of cytotoxic therapies, such as fertility loss. As almost all girls with neuroblastoma are prepubertal, OTC is the only available means of preserving fertility. Since the *in vitro* maturation of isolated follicles from the cryopreserved ovarian cortex has not yet resulted in pregnancy, the autograft of frozen/thawed ovarian

tissue to restore fertility in young patients is today the only valid method for obtaining pregnancies and live births. The first live birth after an autograft of ovarian tissue cryopreserved during childhood before menarche was reported in 2015.[13] It is also proven that endocrine function can be restored.[12,28,29]

However, this reimplantation method raises concerns about the possible presence of residual malignant cells in the frozen-thawed ovarian tissue, which is often collected when the disease is still active, with a risk of reintroducing cancer cells after an ovarian graft of unknown pathogenic potential. As not all tumors have the same potential for extension to the ovaries, the risk depends on the neoplasia considered.[6]

Ovarian spread of tumors has received less attention in young girls than in women of childbearing age. This is because the ovary of a child has minimal endocrine function and lacks rich vascularity. Although several studies have described the presence of metastases in NB, none have systematically sought ovarian metastases or explored the involvement of the ovaries as the study aim. Very few case reports of metastatic ovarian involvement in NB patients have been described in the literature, yet NB is the childhood tumor that most frequently metastases to the ovary.[3-5] Moreover, more than 60% of childhood metastatic neuroblastomas have circulating tumor cells at the time of diagnosis.[23,30]

Ovarian metastasis of NB must be differentiated from rare primary ovarian NB (only a few cases of which have been reported worldwide).[31] The most important feature is the frequent bilaterality of metastatic NB, and the fact that ovarian involvement occurs in the setting of a known primary tumor.

The main study on ovarian metastasis of NB is that of Meyer *et al.*, who found ovarian metastases of NB in 6 out of 21 females in reviewed autopsy files at John Hopkins Hospital.[3] In Young *et al.,* in a series of 14 cases of neoplasms metastatic to the ovaries in children, 8 tumors were neuroblastomas.[4] Somjee *et al.* also reported a case of metastatic ovarian neuroblastoma.[5] Bodian *et al.* found 8 cases in a series of 68 autopsy files, but the number of females in this series was not specified.[1] Himelstein-Braw *et al.* studied the ovaries of 12 children with malignancies at autopsy. Of the 8 cases of neuroblastoma in this series, 2 had ovarian metastatic disease.[2]

As regards OTC, Poirot *et al.* found no sign of ovarian metastases in their histological studies of 20 ovarian tissues from prepubertal girls with NB.[32] The possible presence of tumor cells in the ovarian tissue collected from girls with NB should be borne in mind for several

reasons. In all the previously published studies, only histological analyses were performed, on only one to three randomly selected ovarian fragment sections, and this is not a highly sensitive technique for MRD detection.[1-5,32,33]

Clearly, more extensive studies would not allow the analysis of whole ovaries because all the currently available methods used to evaluate the presence of malignant cell contamination in ovarian tissue fragments are destructive, meaning that the evaluated tissue cannot be used for subsequent transplantation. However, the use of more sensitive and specific MRD detection methods able to detect micrometastases is essential to ensure the safety of ovarian tissue grafts, even if the tissue to be grafted is not checked for MRD. Such techniques have been described for MRD detection in the ovaries for leukemias and more recently for Ewing sarcoma.[16-20]

In children with NB, RT-qPCR is a sensitive and widespread technique used to detect tumor-specific mRNAs as markers of circulating tumor cells in bone marrow and peripheral blood. However, some of these markers are also expressed in normal tissues, and several groups have studied the specificity and predictive value of a large number of mRNAs. *TH*, the first enzyme in the catecholamine synthesis pathway, is one of the most commonly used targets for MRD detection in NB.[21-23] More recently, other gene transcripts have been validated, including *DCX*, involved in neuronal migration[34] and *PHOX2B* encoding a transcription factor involved in the development of the autonomic nervous system.[26,35,36] Given the variability in expression of different molecular markers in the cell lines, and the heterogeneity of neuroblasts, it has been recommended that several markers should be used simultaneously.[37] High levels of *TH*, *PHOX2B* and *DCX* transcripts in bone marrow and blood at diagnosis and at the end of induction were recently shown to be poor prognostic factors in NB patients.[24]

To our knowledge, our study is the first to evaluate MRD detection using NB mRNAs in human ovarian tissue. Human NB cell lines enabled us to perform *in vitro* tumor contamination of ovarian tissue and test the sensitivity and specificity of MRD detection with RT-qPCR for *TH*, *PHOX2B* and *DCX* mRNAs. Our results clearly demonstrate that *PHOX2B* is a reliable marker of NB cells contaminating ovarian tissue, and can be used for molecular MRD diagnosis in prepubertal girls with NB. By contrast, *TH* and *DCX* can be expressed in uncontaminated ovarian tissue, which can lead to false-positive results. They are clearly unreliable for detection in ovarian tissue and so unlikely to be useful for performing diagnosis in this setting.

We also highlight the high sensitivity of RT-qPCR for *PHOX2B* mRNA, which can detect low-level contamination with only 10 neoplastic cells present in small ovarian fragments. The lower level of MRD obtained using the contamination with 10 tumor cells in our experiments may be estimated at 10^{-5}. Thus small ovarian tissue samples may suffice to assess the presence of tumor cells, allowing the preservation of tissue for *in vitro* maturation or transplantation, both of which require optimal amounts of tissue. This is particularly important given the young age of the patients concerned. It may also offer the possibility to evaluate MRD in several very small ovarian tissue fragments taken from different parts of ovaries, thus increasing the probability of tumor cell detection in cases of heterogeneous infiltration. The results of the analysis of cryopreserved ovarian tissues from both prepubertal girls with NB were suggestive of the absence NB cells in the samples, given the absence of expression of *PHOX2B*.

Analyses were satisfactory regardless of freezing mode. The presence of a cryoprotectant does not therefore seem to interfere with our extraction method. Our method of MRD detection can thus be applied to patients' tissues that have already been cryopreserved and stored. In the future, MRD may also be considered on the day of surgical removal for the analysis of small fresh fragments. The diagnosis of MRD could thus be performed at the same time as OTC.

The spread of cancer may be heterogeneous, and so the diagnosis of MRD by RT-qPCR must still be combined with an immunohistochemical analysis of the cryopreserved tissue. This complementary technique will provide both a complementary analysis of residual disease and an evaluation of the functional quality of the ovarian tissue. PCR positivity confirms the presence of malignant cells, but cannot predict their vitality or invasive potential in the case of grafts.[15] Even so, it would seem ethically difficult to envisage grafting ovarian tissue in which tumor cells have been identified.

In conclusion, this approach using sensitive and specific MRD detection can offer a useful pre-conservation (fresh tissues) and/or pre-implantation (thawed tissues) diagnostic test for NB tumor contamination in ovarian tissue. This sensitivity is similar to that in peripheral blood or bone marrow. We will seek to confirm our results on more pediatric ovarian tissues. Meanwhile, these results are encouraging and offer hope in the near future for grafting ovarian tissue in women who survive cancer, whose fertility has been jeopardized by treatment, and who have benefited from OTC without oncological risk.

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Conflicts of interest

The authors declared they have no conflict of interest.

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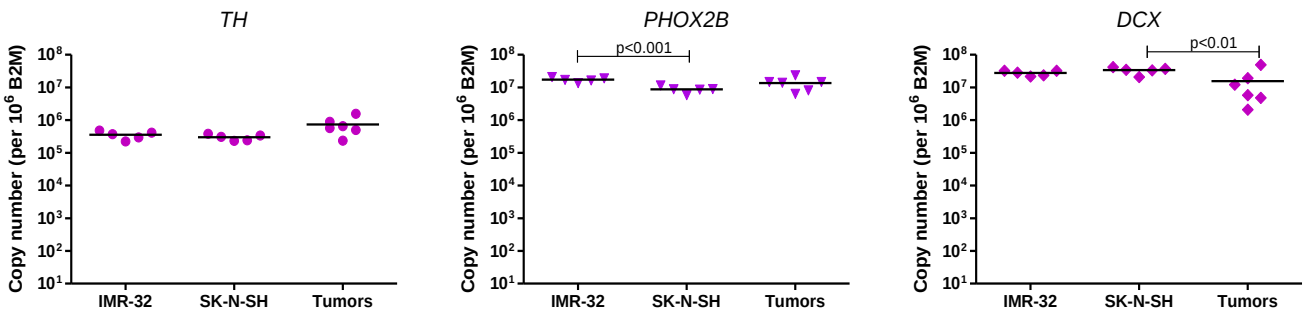

Figure 1. *TH*, *PHOX2B* and *DCX* transcript levels in five subcultures of neuroblastoma cell lines IMR-32 and SK-N-SH and six primary neuroblastoma tumors. There was no statistical difference between the cell lines and tumors in the *TH* and *PHOX2B* levels. IMR-32 cells showed about 2-fold higher expression of *PHOX2B* than SK-N-SH cells (p<0.001). *DCX* levels were about 3-fold lower in tumors than in cell lines (p<0.01), but this marker was the most variable in primary neuroblastoma.

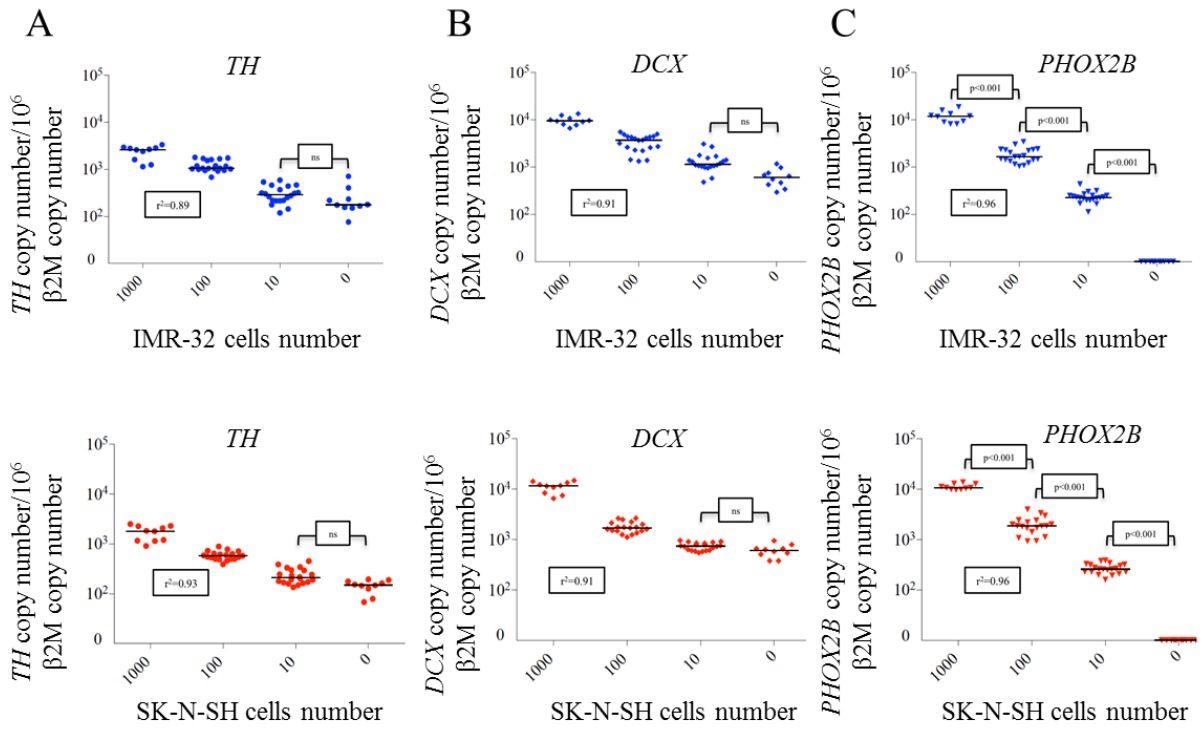

Figure 2. Relative quantifications of *TH* (a), *DCX* (b) and *PHOX2B* (c) transcripts for neuroblastoma cell lines IMR-32 (blue) and SK-N-SH (red). Each symbol represents one ovarian fragment (for each tumor cell line, 20 fragments contaminated with 10 and 100 cells, 10 fragments contaminated with 1000 cells and 10 fragments uncontaminated).
ns: non-significant; r^2: correlation coefficient.

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RT-qPCR for *PHOX2B* mRNA is a highly specific and sensitive method to assess neuroblastoma minimal residual disease in testicular tissue

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Abstract

Neuroblastoma (NB) is the most common type of extracranial solid tumor in children with a high prevalence in toddlers. For childhood cancer survivors, preservation of reproductive potential is an important factor for quality of life. The optimization of NB minimal residual disease (MRD) detection in testicular tissue is crucial to evaluate the risk of malignant cell reintroduction. The first step in the present study was to assess the accuracy of reverse transcription-quantitative polymerase chain reaction (RT-qPCR) to detect tyrosine hydroxylase (*TH*), paired-like homeobox 2b *(PHOX2B)* and doublecortin (*DCX)* mRNA expression in frozen/thawed testicular tissues of patients with non-obstructive azoospermia (NOA) contaminated (*in vitro* model) with an increasing number of IMR-32 and SK-N-SH NB cells. Testicular tissues were frozen by slow or snap freezing. The second step was to determine the expression levels of these markers in testicular samples from 4 pre-pubertal males (2 with stage IV NB and 2 with non-NB malignancy). The yield of extracted RNA was

similar in testicular samples frozen by slow or snap freezing. In the *in vitro* model, *TH* and *DCX* transcripts were detected in uncontaminated testicular tissues, whereas *PHOX2B* mRNA was not detected. There was a strong positive association between the number of NB cells used for contamination and *PHOX2B* transcript levels. For IMR-32 and SK-N-SH NB cell lines, specificity and sensitivity rates of detection were 100% for *PHOX2B* following *in vitro* contamination with 10 tumor cells. In testicular samples from pre-pubertal males with and without NB, *PHOX2B* mRNA expression was not observed, but high expression levels of *TH* and *DCX* mRNA were detected, which were similar to expression detected in the *in vitro* model. Among the markers used in blood and bone marrow for NB MRD studies, the detection of *PHOX2B* transcripts by RT-qPCR may provide an accurate assessment of NB cells in testicular tissues from males who require fertility preservation.

Key words

Neuroblastoma, minimal residual disease, reverse transcription quantitative polymerase chain reaction, testicular tissue, fertility preservation

Introduction

Neuroblastoma (NB) is the most common type of extracranial solid tumor in children, with a high prevalence in toddlers (1). At diagnosis, a high proportion of patients have stage IV metastatic disease (2). Intensive treatments for this type of cancer improved the long-term survival rate, including in children with high risk NB (3). However, since dividing cells are the target of chemo- and radiotherapy, these treatments act not only on cancer cells but also on germ cells. Therefore, they may affect reproductive function, exposing patients to a high risk of infertility (4). Therefore, fertility preservation for boys with NB is currently recommended (5,6). For childhood cancer survivors, preservation of reproductive potential is an important issue for quality of life (7).

When spermatogenesis is effective in pubertal males, sperm cryopreservation should be proposed (8). In pre-pubertal males, since spermatogenesis has not yet started, the preferred strategy is cryopreservation of testicular tissue (9-11). To date, no restoration of human fertility has been reported by the use of frozen/thawed testicular tissue. However, the animal data are promising, and births have been reported following spermatogonial stem cell

transplantation or testicular tissue grafting in rodents or pigs (12‑14). Furthermore, *in vitro* culture of testicular stem cells has been studied in mice for its potential to generate post‑meiotic male gametes (15,16). These preliminary results may offer the potential for fertility restoration in young males in the future. However, fertility restoration using cryopreserved testicular samples needs to be safe, without any risk of reintroducing cancer cells.

As a number of cases of metastatic testicular NB have previously been reported (17‑19), the possible presence of malignant cells in cryopreserved testicular tissue involves a risk of recurrence of the primary disease following fertility restoration by germ cell transplantation. The detection of NB minimal residual disease (MRD) in blood and bone marrow of patients with metastatic NB was developed using reverse transcription‑quantitative polymerase chain reaction (RT-qPCR) for tyrosine hydroxylase (*TH*), paired‑like homeobox 2b *(PHOX2B)* and doublecortin (*DCX)* transcripts (20‑22). These transcripts represent useful and clinically significant biomarkers of MRD in the blood and bone marrow of metastatic NB cells. However, these biomarkers have not been assessed for NB MRD detection in testicular tissue. The optimization of NB MRD detection in testicular tissue is crucial to evaluate the risk of malignant cell reintroduction. This detection method needs to be applicable for testicular samples frozen by slow freezing and for testicular tissues immediately following surgical collection, which could subsequently be frozen by snap freezing until RNA extraction is performed. This would allow detection of MRD for previously cryopreserved testicular tissues and for future testicular sample collections.

Therefore, the first objective of the present study was to assess the accuracy of NB MRD detection. This was performed by quantification of *TH*, *DCX* and *PHOX2B* transcripts in human testicular tissues, cryopreserved by slow or snap freezing and contaminated *in vitro* with increasing number of tumor cells from two NB cell lines. Once accuracy was assessed in this *in vitro* model, the expression levels of these three biomarkers were evaluated in frozen testicular samples from pre-pubertal males with and without NB.

Materials and methods

Experimental design. The specificity and sensitivity of NB MRD detection were assessed by RT-qPCR quantification of *TH*, *PHOX2B* and *DCX* mRNA expression in thawed testicular tissues that were contaminated with IMR-32 and SK-N-SH human NB cell lines (CCL-127 and HTB-11;

American Type Culture Collection, Manassas, VA, USA). Prior to these experiments, IMR-32 and SK-N-SH cells were investigated for positivity and stability of *TH*, *PHOX2B* and *DCX* mRNA expression in the subclones.

Subsequently, thawed testicular tissue from patients with non-obstructive azoospermia (NOA) were contaminated with 0 (negative control), 10, 100 and 1,000 IMR-32 and SK-N-SH tumor cells from human NB cell lines. The contamination procedure was performed at room temperature and took ~20 min. RNA extraction was performed immediately following contamination, without any additional incubation of the samples. Experiments involving contamination with 10 and 100 cells were performed in duplicate for each sample (Fig. 1). Once contamination was achieved, RNA extraction and RT-qPCR were performed. Testicular tissues frozen either by snap or slow freezing methods were used to investigate whether the freezing method may interfere with RT-qPCR analysis.

Following assessment of MRD detection accuracy in the *in vitro* model, thawed testicular samples obtained from pre-pubertal males with stage IV NB and with non-NB malignancy (Ewing tumor) were analyzed in order to expand the method to include pre-pubertal testicular tissues.

Patients and samples. Testicular tissues were obtained from 20 males (mean age, 32.4 years; range, 25-38 years) with NOA between November 2014 and September 2015 at the University Hospital of Clermont-Ferrand (Clermont-Ferrand, France). The patients underwent testicular sperm extraction for intra-cytoplasmic sperm injection. Following sperm retrieval and freezing, the remaining testicular tissue sample would usually be destroyed, but in this instance it was used for the present study.

In addition, testicular tissues obtained from 2 pre-pubertal patients with stage IV NB in January 2012 (patient A, aged 3 years) and in April 2014 (patient B, aged 2 years) and 2 pre-pubertal patients with Ewing sarcoma in January 2016 (patients C and D, aged 6 years) were analyzed. Sample A was obtained at the University Hospital of Rouen (Rouen, France). Samples B, C and D were obtained at the University Hospital of Clermont-Ferrand (Clermont-Ferrand, France). Bilateral testis samples from patients A, B and C were evaluated. For patient D, only one sample of the right testis was available and evaluated. The median weight of the testicular samples for these 4 patients was 12 mg.

The present study was approved by the Committee for Personal Protection (DC 2008 558). Written informed consent was obtained from all patients prior to enrollment in the present study and for inclusion of the testicular samples in the GERMETHEQUE biobank (NFS 96900, www.chu-toulouse. fr/germetheque-centre-de-ressources-biologiques). In the case of two males with NB who succumbed to disease prior to the study, informed and written consent was obtained from their parents. The study was declared on the clinicaltrial.gov website (no. NCT02400970).

Freezing and thawing. For the testicular tissues obtained from adult patients with NOA, the samples were first cut into 6 thin fragments of equal size (~1 mm3) following weighing. Subsequently, the testicular fragments (equally sized samples) were frozen by using the slow (n=10 patients) or snap (n=10 patients) freezing method. The testicular tissues obtained from pre-pubertal males had been previously frozen by slow freezing for the two males with NB and by snap freezing for the males with Ewing sarcoma.

The slow freezing method was performed as previously described and validated by Rives *et al* (10). For the snap freezing method, testicular samples were put in cryogenic vials and immediately frozen by immersion in liquid nitrogen. For both freezing procedures, the samples were stored in cryogenic vials (CRYO BIO SYSTEM, L'Aigle, France). The same thawing procedure was used for testicular tissues frozen by slow or snap freezing. The cryogenic vials were thawed at 37˚C for 3 min and placed into three baths of Leibovitz's L-15 medium (Eurobio, Courtaboeuf, France) for 5 min for each step at room temperature.

Culture of IMR-32 and SK-N-SH subclones. IMR-32 and SK-N-SH are well-established subclones of human cell NB. The IMR-32 cell line was maintained in Dulbecco's modified Eagle's medium (PAN-Biotech, Aidenbach, Germany), supplemented with 10% fetal calf serum (PAN-Biotech), 4 mmol/l L-glutamine, 10 UI/ml penicillin and 10 µg/ml streptomycin (PAN-Biotech) (23). The SK-N-SH cell line was maintained in Iscove's modified Dulbecco's Medium (PAN-Biotech), supplemented with 10% fetal calf serum, 4 mmol/l L-glutamine, 10 UI/ml penicillin and 10 µg/ml streptomycin. Cell counting analysis was performed twice prior to each contamination using the Malassez cell counting chamber. Blue trypan staining (diluted 1/20 with phosphate buffered saline) for 10 min at room temperature was used to assess cell viability. For IMR-32

and SK-N-SH cell lines, the viability was always ≥85%. The cell counting analysis and cell viability test were performed with a light microscope by an experienced observer prior to each contamination test.

Detection of TH, DCX and PHOX2B mRNA expression by RT-qPCR. RNA was extracted using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), according to the manufacturer's instructions. RNA was treated with DNAse I (Roche Diagnostics, Meylan, France) to remove any contaminating DNA. A total of 1 µg RNA was reverse‑transcribed using SuperScript II (Invitrogen; Thermo Fisher Scientific, Inc.). The target *TH*, *DCX* and *PHOX2B* gene transcripts and the reference *β‑2‑microglobulin (β2M)* housekeeping gene transcript were measured using the LightCycler 480 RT‑ PCR system (Roche Diagnostics), using previously reported primers and probes (24). The sequences are provided in supplementary methods of the publication (24). The cycling conditions were as follows: 10 min at 95˚C, followed by 40 cycles of 95˚C for 15 sec and 60˚C for 1 min. Absolute quantification analysis with the LightCycler software was performed to determine transcripts numbers, as described previously (20). Briefly, standard curves were generated using serial dilutions of plasmid containing a known number of molecules of each transcript and used to calculate the number of transcripts in the samples. The number of the reference *β2M* gene transcripts was used for the normalization of data. Normalized data were expressed as the copy numbers of target transcripts per 106 copies of *β 2M* transcripts. The PCR reactions were performed in triplicate.

Statistical analysis. Statistical analysis was performed using Stata software (version 13; StataCorp LP, College Station, TX, US). The results for quantitative parameters were presented as the mean ± standard deviation, according to statistical distribution (assumption of normality assessed by Shapiro‑Wilk's test). The investigation of associations between quantitative parameters was based on the estimation of correlation coefficients (Pearson's or Spearman's rho according to statistical distribution, noted r). Subsequently, in order to take into account differences between and within subject variability (due to several measurements for a given patient), random‑effects for correlated measures were performed instead of the usual statistical tests, which would not be appropriate as the hypothesis of independence of data was not possible. The assumption of normality of residuals was studied using the

Shapiro‑Wilk test. When appropriate (data not exhibiting normal distributions), a logarithmic transformation was performed in order to achieve normality assumption and to ensure the correct use of previous analyses. Finally, receiver operating characteristic curve analysis was performed to evaluate sensitivity and specificity of RT-qPCR to detect the expression of *TH*, *PHOX2B* and *DCX* mRNAs in testicular tissue. All tests described in this section are two‑sided, with a type I error set at $\alpha=0.05$: $P<0.05$ was considered to indicate a statistically significant difference.

Results

Yield of RNA extraction from testicular tissue frozen using snap and slow freezing methods. The median weight of testicular samples from 20 men with NOA was 96 mg (33‑210 mg). Each testicular sample was sectioned into six equal size samples with a median weight of 14 mg (6‑46 mg). The median RNA yield from these samples was 15 µg (0.6‑36.6 µg). The amount of RNA obtained per mg of tissue did not differ between the testicular samples frozen by snap (0.87 µg/mg) or slow freezing (0.80 µg/mg) methods ($P>0.05$).

Expression of TH, PHOX2B and DCX transcripts in IMR-32 and SK-N-SH cell lines. The expression levels of *TH*, *PHOX2B* and *DCX* transcripts in IMR-32 and SK-N-SH cell lines were evaluated for each artificial tumor contamination experiment (Fig. 2). Overall, the cell lines exhibited high levels of expression of the three markers, with the mean normalized values of 3.3x105 for *TH*, 1.9x107 for *PHOX2B* and 3.1x107 for *DCX* in IMR-32 cells and 3.0x105 for *TH*, 9.5x106 for *PHOX2B* and 3.6x107 for *DCX* in SK-N-SH cells. The levels of transcripts indicated minimal variations between different experiments, which were <1.3 fold. The average fold changes in transcript levels in IMR-32 and SK-N-SH cells were 1.26 and 1.22 for *TH*, 1.26 and 1.25 for *PHOX2B* and 1.28 and 1.27 for *DCX*, respectively. These data indicated the high reproducibility of the cell line models in terms of marker mRNA expression.

Detection of TH, PHOX2B and DCX transcripts in frozen testicular samples contaminated with IMR-32 and SK-N-SH NB cells. In the present study, expression of *TH* transcripts was observed in negative control testicular samples (n=10). Expression of *TH* transcripts was detected in samples frozen by snap freezing (IMR-32, 537 copy number/106 *β 2M* copy number; SK-N-SH,

475 copy number/106 *β2M* copy number) and by slow freezing (IMR-32, 363 copy number/106 *β 2M* copy number; SK-N-SH, 490 copy number/106 *β2M* copy number; Fig. 3A). Compared with the negative control (uncontaminated testicular samples), the level of *TH* mRNA was significantly higher in testicular samples contaminated with 100 and 1,000 NB cells from IMR-32 and SK-N-SH cell lines, respectively (P<0.001). There was no significant difference between the levels of *TH* mRNA detected in testicular samples contaminated with 10 NB cells and uncontaminated samples (IMR-32, P=0.18; SK-N-SH, P=0.24). For determination of *TH* expression, the specificity and sensitivity rates were 100% when 100 NB cells were used for contamination.

PHOX2B mRNA was not detected in the uncontaminated testicular samples (Fig. 3B) frozen either by the snap or slow freezing method (n=10). *PHOX2B* transcripts were detected at significantly different levels following contamination of testicular samples with 10, 100 and 1,000 NB cells (P<0.001for IMR-32 and SK-N-SH). Furthermore, there was a strong linear correlation between the number of NB cells used for contamination (10, 100 and 1,000 NB cells) and the levels of *PHOX2B* mRNA (r=0.96 for IMR-32 and SK-N-SH). The specificity and sensitivity rates for *PHOX2B* mRNA detection were 100% following contamination by 10 NB IMR-32 or SK-N-SH cells.

High expression levels of *DCX* mRNA were detected in negative controls (n=10) frozen by snap freezing (IMR-32, 73,231 copy number/106 *β2M* copy number; SK-N-SH, 68,386 copy number/106 *β2M* copy number) and by slow freezing (IMR-32, 64,811 copy number/106 *β2M* copy number; SK-N-SH, 78,810 copy number/106 *β2M* copy number) methods (Fig. 3C). Furthermore, there was no significant difference in *DCX* mRNA levels between the negative controls and samples contaminated with 10, 100 and 1,000 NB cells.

Analysis of TH, DCX and PHOX2B mRNA expression levels in testicular tissues of pre-pubertal males. In the testicular samples obtained from two pre-pubertal NB stage IV males, the levels of *TH* mRNA were similar in the right (patient A, 431 copy number/106 *β2M* copy number; patient B, 469 copy number/106 *β2M* copy number) and left (patient A, 420 copy number/106 *β2M*; patient B, 371 copy number/106 *β2M* copy number) testis tissue samples. *PHOX2B* mRNA was undetectable in the testis samples of patient A and B. Similar high levels of *DCX* mRNA were observed in the right (patient A, 58,700 copy number/106 *β2M*; patient B: 48,297

copy number/106 *β2M*) and left (patient A: 51,280 copy number/106 *β2M*; patient B: 44,738 copy number/106 *β2M*) testis samples.

In the samples obtained from two pre-pubertal males with no ‑NB malignancy (Ewing sarcoma; patients C and D), mRNA *TH* levels were similar in the right (patient C, 684 copy number/106 *β2M*; patient D, 500 copy number/106 *β2M*) and left (patient C, 736 copy number/106 *β 2M* copy number) testis. *PHOX2B* mRNA was not detected in testis samples obtained from either male. High levels of *DCX* mRNA were observed in the right (patient C, 53,690 copy number/106 *β2M*; patient D, 59,380 copy number/106 *β2M* copy number) and left (patient C, 59,380 copy number/106 *β2M* copy number) testis samples.

High expression levels of *TH* and *DCX* observed in the testicular tissues of the four pre-pubertal males were similar to the high expression levels detected in uncontaminated testicular samples (negative controls) in the *in vitro* model described previously in the results section.

Discussion

Given the increased survival rate of patients with NB and the cytotoxic effects of therapies used for treatment of this cancer (25), fertility preservation is highly recommended for these children. Therefore, for a pre-pubertal male facing sterilizing chemotherapy, testicular biopsy may be performed as a preventive strategy. Freezing testicular tissue allows subsequent transplantation either by infusion of a testicular cell suspension into the seminiferous tubules (26) or intra‑testicular grafting of tissue (27). However, >60% of children with metastatic NB have circulating tumor cells at the time of diagnosis (28). Although testis is not the most frequent NB invasion site, numerous cases of testicular NB metastasis have been described previously. Simon *et al* (19) reported testicular metastasis in 10/1,076 male patients with NB using the data of the Cooperative German Neuroblastoma trials. Kushner *et al* (29) demonstrated 11 positive cases in a series of 289 male NB autopsies. Nistal *et al* (30) also reported two cases of testicular metastases confirmed by histological examination in a total of 216 NB patients. Therefore, the possible presence of NB cells in testicular tissue collected from NB males should be taken into account to avoid reintroducing cancer cells when the thawed testicular sample is used to restore fertility. However, in all of the previous studies mentioned, only histological analyses were performed. As the sensitivity of MRD detection by

histological analysis is lower compared with RT-qPCR, the actual frequency of NB infiltration in testis may be higher.

In the present study, sufficient amounts of RNA were extracted from small testicular samples to perform RT-qPCR in optimal conditions and to analyze the level of three transcripts associated with NB. The use of a small quantity of testicular tissue to detect NB MRD ensures that a sufficient amount of sample remains for fertility preservation in pre-pubertal males, in whom large surgical retrieval is not always possible due to age and small testis size. The present study did not observe any effect of the freezing method on the yield of RNA extracted. Therefore, the RT-qPCR method may be performed on testis samples that have already been cryopreserved using slow freezing and on samples, which are cryopreserved by snap freezing at the time of surgical retrieval for subsequent analysis.

In the present study, an *in vitro* model of NB dissemination in human testicular tissue was established to evaluate the sensitivity and specificity for the detection of NB mRNAs. The SK-N-SH and IMR-32 tumor cell lines used for contaminations are well known and were established from human NB. SK-N-SH cells were established from the bone marrow metastasis from a 4 year‑old Caucasian child (31). IMR-32 cells were obtained from a metastatic site in an abdominal mass in a 13 month‑old Caucasian male with NB. The tumor was diagnosed as NB with rare areas of organoid differentiation (32). Therefore, SK-N-SH and IMR-32 human cell lines are highly representative of metastatic NB. In the present study, *TH*, *DCX* and *PHOX2B* mRNAs were expressed at high levels in SK-N-SH and IMR-32 cell lines. The testicular samples that were used to validate the *in vitro* contamination model were obtained from adult males with NOA, as it was unethical to perform these preclinical tests with healthy pre-pubertal testicular samples. Similar to pre-pubertal patients, testicular biopsies from patients with NOA contain low numbers or absence of mature germ cells.

In children with NB, metastatic tumor cells were previously detected in the bone marrow and peripheral blood by RT-qPCR for NB‑specific target genes, including *TH*, *DCX* and *PHOX2B* (24,33,34). To the best of our knowledge, this is the first study to quantify the expression level of these transcripts in human testicular tissues to detect MRD. *TH* encodes for the key enzyme involved in the synthesis of catecholamines, which serves a functional role in the testis. A previous study provided evidence that Leydig cells exhibited close similarity to cathecholaminergic nerve cells with high expression of *TH* (35). This may be explained by a developmental arrest of Leydig cells at the immature state (36). In addition, Leydig cells are

characterized as non-dividing cells with characteristics of early stem cell-like progenitors with endocrine, neuronal and glial cell features (37). Although *TH* is one of the most commonly used targets for MRD detection in peripheral blood and bone marrow of patients with NB (20,21), the use of this transcript as an MRD marker in testicular samples is hindered by its background expression detected in uncontaminated samples of patients with NOA and pre-pubertal males with and without NB. Therefore, it is hypothesized that the background expression of *TH* may be due to the presence of Leydig cells (35,37). Furthermore, 100% sensitivity and specificity rates of *TH* detection were only observed when 100 NB cells were used for contamination, which may suggest that the accuracy of this marker for NB MRD detection is insufficient for detection in human testicular samples.

Previously, the expression level of other transcripts, including *DCX* and *PHOX2B,* were demonstrated to be useful for NB MRD detection in bone marrow and peripheral blood from patients with metastasis (24). *DCX* is involved in the signaling pathway that regulates microtubule in migrating neurons (38). A previous study reported a high expression of a *DCX* homolog, *DCD1*, in human testis (39). This may explain the lack of sensitivity and specificity that was observed for analysis of *DCX* expression in the *in vitro* model in the present study, and the high expression of *DCX* in pre-pubertal males with or without NB. *PHOX2B* encodes a transcription factor involved in the development of the autonomous nervous system (34,40). It was previously revealed that *PHOX2B* is a highly specific marker for sensitive MRD detection of NB in the bone marrow, peripheral blood and harvested hematopoietic stem cells (40). To the best of our knowledge, the present study demonstrated for the first time a high sensitivity and specificity of RT-qPCR analysis for *PHOX2B* mRNA, enabling the detection of as few as 10 NB cells in a testis sample. The absence of background *PHOX2B* expression in uncontaminated testicular samples underlines the high specificity of this marker. Furthermore, *PHOX2B* expression was not detected in testicular samples of pre-pubertal males without NB in the present study. The present study demonstrated that NB MRD assessment in human testicular tissue by the detection of *PHOX2B* by RT-qPCR is highly accurate. It is unknown whether MRD marker genes of NB are up- or downregulated during treatment (23,24), so a panel of PCR targets may be required to overcome tumor heterogeneity (41). Since *TH* and *DCX* transcripts are not sensitive and specific NB markers in testicular tissues, it will be necessary to continue the evaluation of other known NB-specific transcripts. Analysis of NB mRNA is able to detect the presence of malignant cells but cannot predict their vitality or invasive potential.

Therefore, further studies are required to investigate the risk of tumor dissemination on MRD-positive pre-pubertal testicular samples by xenotransplantation in rete testis of nude or severe combined immunodeficiency (SCID) mice.

In conclusion, the present study provides evidence that among the established NB markers for MRD analysis of blood and bone marrow using RT-qPCR, *PHOX2B* mRNA may provide an accurate assessment of MRD in testicular tissues for males that require fertility preservation. These preliminary results require confirmation by a further study of testicular tissues from a large cohort of pre-pubertal males with NB and additional studies are warranted to evaluate the safety threshold by xenotransplantation in nude or SCID mice.

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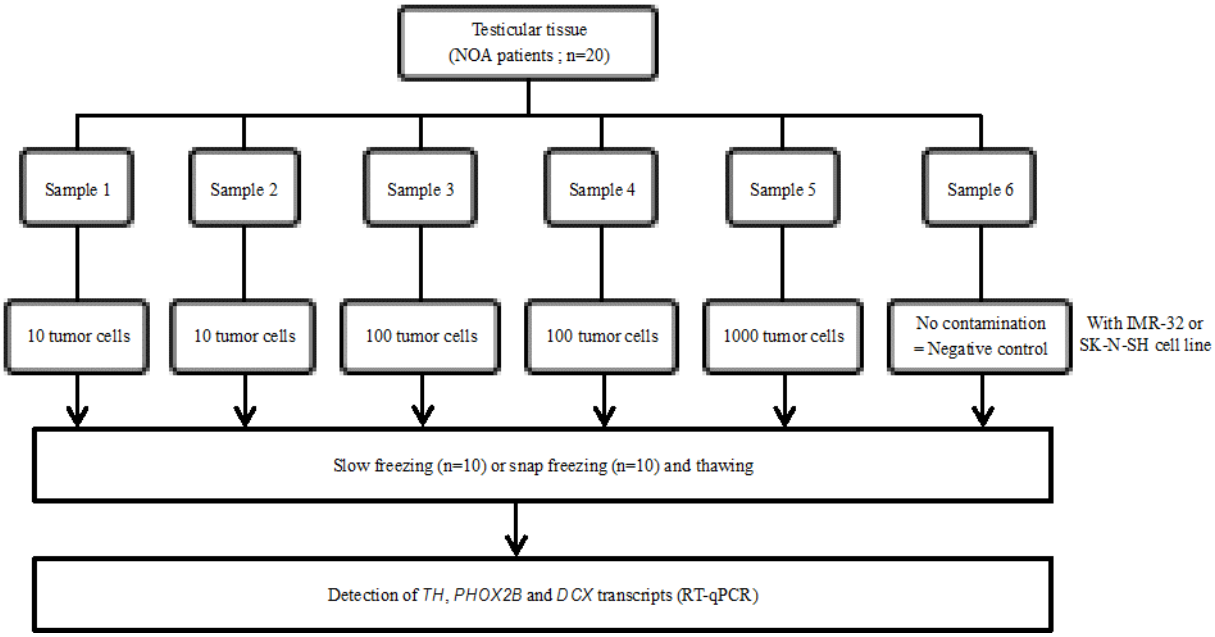

Figure 1. Experimental design for detection of *TH*, *PHOX2B* and *DCX* transcripts in thawed testicular tissues following NB cell contamination by IMR-32 and SK-N-SH cell lines. Each testicular sample from patients with NOA was sectioned into 6 thin samples of equal size (~1 mm^3). Each testicular sample was contaminated with an increasing number of IMR-32 and SK-N-SH NB cells prior to slow or snap freezing and detection of TH, Phox2B and DCX transcripts by RT-qPCR. NOA, non-obstructive azoospermia; NB, neuroblastoma; TH, tyrosine hydroxylase; PHOX2B, paired-like homeobox 2b; DCX, doublecortin; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

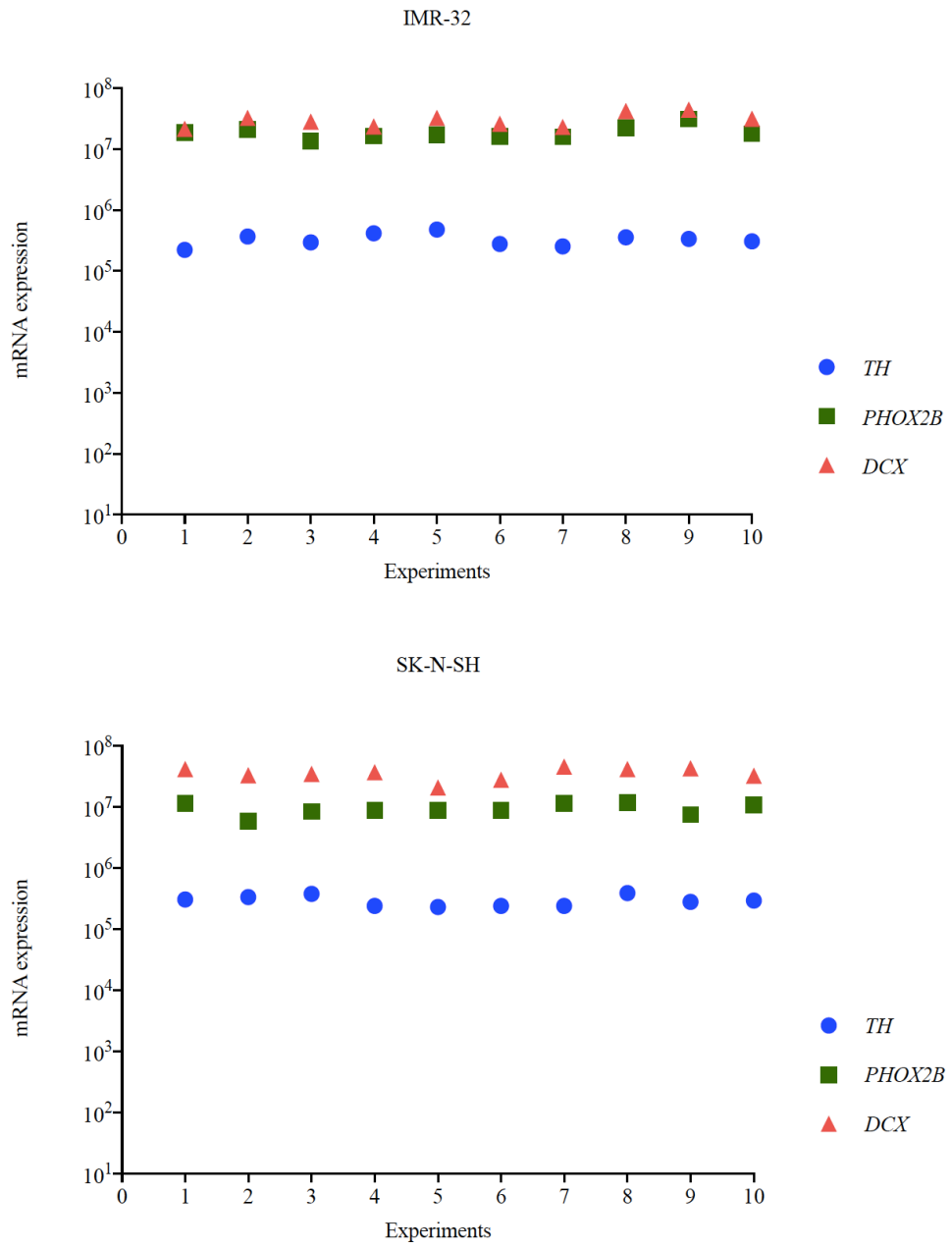

Figure 2. Normalized expression of *TH*, *PHOX2B* and *DCX* in IMR-32 and SK-N-SH cell lines (per 10^6 *β2M* transcripts). For each cell line, 10 different subcultures were used for tumor contamination experiments. These subcultures exhibited minimal variations in the levels of the three mRNAs (<1.3 fold), indicating a good reproducibility of contamination experiments. TH, tyrosine hydroxylase; PHOX2B, paired-like homeobox 2b; DCX, doublecortin; β2M, β-2-microglobulin.

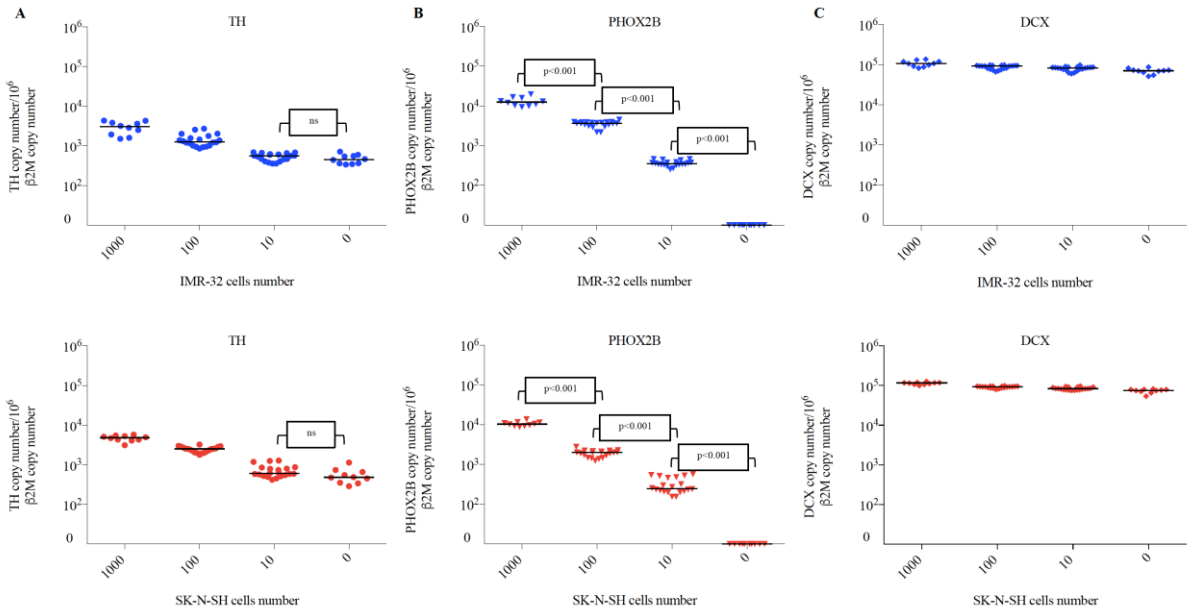

Figure 3. Relative expression of (A) *TH*, (B) *PHOX2B* and (C) *DCX* in thawed testicular tissues following contamination by neuroblastoma cell lines (0, 10, 100 and 1,000 cells). Each symbol represents one testicular tissue fragment. Blue, IMR-32 cells; red, SK-N-SH cells. ns, no significant; TH, tyrosine hydroxylase; PHOX2B, paired-like homeobox 2b; DCX, doublecortin.

4. Article n°7 : *Soumis à Cancers*

Assessment of a sensitive and specific method to detect Ewing sarcoma minimal residual disease in germinal tissues by RT-qPCR

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Abstract

Ewing sarcoma (EWS) is a common pediatric solid tumor with high metastatic potential. Due to toxic effects of treatments on reproductive function, the cryopreservation of ovarian tissue (OT) or testicular tissue (TT) is recommended to preserve fertility. However, the risk to reintroduce residual metastatic tumor cells should be evaluated before fertility restoration. Our goal was to validate a sensitive and specific approach for EWS minimal residual disease (MRD) detection in frozen germinal tissues. Thawed OT (n=12) and TT (n=14) were contaminated with tumor RD-ES cells (10, 100 and 1000 cells) and *EWS-FLI1* tumor-specific transcript was quantified with RT-qPCR. All contaminated samples were found to be positive, with the strong correlation between RD-ES cell numbers and *EWS-FLI1* levels in OT (r=0.93) and TT (r=0.96) ($p<0.001$). No transcript was detected in uncontaminated control samples. The invasive potential of Ewing cells was evaluated using co-culture techniques. After co-culturing, tumor cells were detected in OT/TT with histology, FISH and RT-qPCR. In addition, 5 OT and 3 TT samples from children with EWS were tested, and no MRD was found using RT-qPCR and histology. We demonstrated the high sensitivity and specificity of RT-qPCR to detect EWS MRD in OT/TT samples.

Key words

Fertility preservation - Ewing sarcoma - Ovarian tissue - Testicular tissue - Minimal residual disease detection – RT-qPCR

Introduction

Ewing sarcoma (EWS) is one of the most common solid tumors in children and adolescents, and is characterized by a high metastatic dissemination potential [1–3]. EWS is the second most common bone cancer in children [4]. The study of Schleiermacher et al. [5] reported that 27% of patients had evidence of metastasis at diagnosis. EWS metastases spread hematogenously to lungs, bones, bone marrow and in some cases ovaries [6]. The treatment of EWS most often requires a combination of multi-agent chemotherapy, sometimes followed by hematopoietic stem cell transplantation, which are known to be sterilizing treatments.

Fertility preservation by cryoconservation of germinal tissues and gametes of EWS patients is therefore recommended. Ideally, fertility preservation should take place before the first cycle of chemotherapy by cryoconservation of germinal tissues [7]. For prepubertal children, the option chosen for fertility preservation is the freezing of ovarian and testicular tissues (OT and TT). Today more than 86 births have been reported after grafts of frozen OT [8]. For males, testicular stem cell transplantation into seminiferous tubules to enable spermatogenesis has recently been reported in a macaque model [9] or *in vitro* maturation [10]. Restoration for fertility is thus possible for females and foreseeable for males. However, there may be malignant cells in germinal tissue in metastatic EWS, with a consequent risk of transplanting tumor cells with cryopreserved tissues. It is therefore essential to be able to detect and assess minimal residual disease (MRD) in germinal tissue from such patients.

In 85–90% of cases, EWS tumors are characterized by a specific chromosomal translocation, t(11;22)(q24;q12), resulting in the fusion of the EWSR1 (22q12) gene and a member of the ETS family or transcription factor FLI1 (11q24) gene. This fusion transcript can be detected with a high sensitivity and specificity by RT-qPCR (reverse transcriptase-polymerase chain reaction) and used for MRD evaluation [1,5,11]. EWS MRD detection by RT-PCR has been performed in OT. Schifflers et al. [12] studied MRD in OT from patients with non-metastatic EWS. Abir et al. described EWS MRD detection of cryopreserved ovarian samples by snap freezing [13]. The immunochemistry detection for CD99 and histology were negative and the RT-qPCR (EWS-FLI1 transcript) was positive in one patient out of five tested. Another study evaluated the presence of malignant cells in one piece of OT cryopreserved by slow freezing [14]. Histology and quantitative RT-PCR were performed before and after xenotransplantation to immunodeficient mice and showed negative results. Of note, no MRD evaluation in TT has been reported.

To assess the accuracy of EWS MRD detection, we quantified EWS-FLI1 transcript in human OT and TT cryopreserved by slow or snap freezing and contaminated *in vitro* with increasing numbers of EWS tumor cells using RD-ES cell line. We evaluated the dissemination potential of RD-ES cells within germinal tissue to validate our *in vitro* model. MRD detection was then performed in frozen OT and TT samples from EWS patients. To our knowledge, this is the first MRD study comparing two freezing protocols in OT and TT samples and investigating EWS contamination in testicles.

Materials and Methods

1. Patients and samples

Written informed consent was obtained from all patients for sample inclusion in the Germetheque biobank with the approval of the Committee for Personal Protection (AC 2009-886). The study was declared on the clinicaltrial.gov website (No. NCT 02400970).

OT was collected from ten patients (mean age 31.4 ± 7.6 years, range 23–42 years) during endoscopic surgery for benign ovarian cysts at the University Hospital of Clermont-Ferrand, France between January 2017 and July 2017. For each patient, a piece of ovarian cortex overlying the cyst was excised using scissors and without electrocoagulation.

TT was obtained from eight patients (mean age 36.5 ± 4.7 years, range 30–46 years) with non-obstructive azoospermia between January 2017 and July 2017 at the University Hospital of Clermont-Ferrand, France. These men underwent testicular sperm extraction for intra-cytoplasmic sperm injection (ICSI).

In addition, OT and TT obtained from respectively five girls (mean age 15.8 ± 1.8 years, range 13–16 years) and three boys (mean age 11.6 ± 4.0 years, range 7–14 years) with EWS were analyzed to measure the expression of potential MRD. Each patient had positive detection of EWS-FLI1 type II in the primitive tumor.

2. Freezing and thawing of germinal tissue samples

The samples were first cut into six thin fragments of equal size (1–2 mm^3) after weighing. OT and TT were frozen by slow freezing or snap freezing to compare the two methods. The slow freezing method was performed on OT and TT as previously described respectively by Sanfilippo et al. [15] and Rives et al. [16]. For the snap freezing method, OT and TT samples were placed in cryogenic vials (Cryo Bio System, France) and immediately frozen by immersion in liquid nitrogen. The cryovials were immersed in liquid nitrogen for storage.

The same thawing procedure was used for OT frozen by slow or snap freezing. Cryovials were immersed in a water bath (37 °C, 2 min) and each sample was washed twice in medium A for 5 min at 37 °C. The same thawing procedure was used for TT frozen by slow or snap freezing. Cryogenic vials were thawed in a water bath at 37 °C for 3 min and then incubated in two baths of Leibowitz L15 medium (Eurobio, France) for 5 min at 37 °C.

3. Culture of RD-ES subclones and contamination

We used well-established RD-ES human Ewing sarcoma cell lines (RD-ES, ATCC LGC Standards, Molsheim, France). These cells were maintained in RPMI (Roswell Park Memorial Institute Medium, Fisher Scientific) supplemented with 15% fetal calf serum (Fisher Scientific, France), 1% L-glutamine (Sigma Aldrich, France) and 1% antibiotics (penicillin-streptomycin, Sigma Aldrich, France) at 37 °C. Standard cell counts and blue trypan staining for viability were performed prior to each tissue contamination. The viability of RD-ES cell lines was greater than 80%.

After thawing, fragments of OT ($n = 12$ contamination series) and TT ($n = 14$ contamination series) with respective median weights 37.4 mg [15.2–62.0] and 30.0 mg [16.6–48.0] were contaminated with 10, 100 and 1000 RD-ES cells. One fragment was not contaminated (negative control). Contaminations with 10 and 100 RD-ES cells were assessed in duplicate. The positive control was validated for each contamination series by RT-qPCR analysis of 10^6 RD-ES cells.

4. Detection of EWS-FLI1 of type II mRNA expression by RT-qPCR

The detection of the EWS transcript (EWS-FLI1 of type II) expression was performed by RT-qPCR in thawed tissues using previously described primers and probes [17].

The PCR tests (LightCycler 480 Real Time PCR system, Roche Diagnostics, France) with TaqMan probes were performed in triplicate. Copy numbers of EWS-FLI1 transcript were reported per 10^6 copies of β-2-microglobulin (β2M).

5. Detection of potential dissemination of RD-ES cells in OT and TT after co-cultures

a) OT co-culture

After thawing, pieces of OT (1 mm^3) were individually transferred to a 96-well plate coated with Corning Ultra-Low Attachment surface (Corning, Escolab, Belgium) in a medium consisting of 100 µl/well of pre-equilibrated α-Minimum Essential Medium with GlutaMAX (Invitrogen, Belgium) supplemented with 10% HSA (Vitrolife, Sweden), 100 µg/ml of ascorbic acid, 5 ng/ml of insulin, 5 µg/ml-5 ng/ml of transferrin-selenium and 25 mIU/ml of recombinant FSH (GONAL-f, Merck). Culture lasted 14 days (37 °C, humidified incubator, 5% CO_2). 100 000 RD-ES cells were then added to each culture well.

After co-cultures (on days 7 and 14), we performed RT-qPCR (as previously described), histologic analysis and FISH analysis.

- Histologic analysis

The co-cultured OT was fixed in 4% formalin and embedded in paraffin (FFPE). The 3 µm-thick sections were cut out of the FFPE blocks, stained with hematoxylin and eosin (H&E) and examined under a bright-field microscope.

- Fluorescence *in situ* hybridization (FISH) after co-cultures of ovarian tissue and RD-ES cells

FISH was performed to detect EWSR gene rearrangement using Vysis LSI EWSR1 Break Apart FISH probe kit (Abbott Molecular) in accordance with the manufacturer's recommended procedures as described previously [18]. With pepsin for 25 min at 37 °C, slides were deparaffinized and immersed in ethanol baths. After air drying, hybridization was performed. After counterstaining with 4'-6-diamidino-2-phenylindole dihydrochloride (DAPI). Results were recorded using an Axioplan2 imaging fluorescence microscope (ZEISS, Göttingen, Germany) fitted with appropriate filters, a CCD camera, and the digital-imaging software Isis v3.8.8 (MetaSystems, Altlussheim, Germany).

In normal cells with intact 22q12 regions, each of the EWSR1 gene alleles gives a dual contiguous orange and green signal pattern. A split signal pattern was considered as positive for the EWSR1 rearrangement if the gap between the green and orange signals exceeded the diameter of either one. In the presence of translocations related to the 22q12 region of the gene allele, gapping of one orange signal (denoting the 5' site of the gene) and one green signal (denoting the 3' site of the gene) is expected.

b) TT co-culture

TT was obtained from bilateral orchiectomy performed for gender reassignment of one male-to-female transsexual subject aged from 24 years old (Prof. H. Lejeune) at the University Hospital of Lyon, France. The protocol was approved by the local research ethics committee. The co-culture was carried out after adaptation in humans of published conditions in rats [10]. Isolated seminiferous tubules (20–50 mm^3) after enzymatic digestion and mechanical

dissociation were inoculated into a bicameral chamber with Bio-Alter® technology. Thirty thousand RD-ES cells were then added to each culture well.

After co-cultures (on days 7 and 14), we performed RT-qPCR (as previously described), histologic analysis and immunochemistry (ERG immunoreactivity) in TT (anti-ERG monoclonal mouse antibody, clone 9FY, Zytomed).

- Immunohistochemistry (IHC)

Inserts containing co-culture testicular tissue and the RD-ES cells were fixed in buffered neutral 10% formalin, and glued on glass slides for IHC (Dako FLEX IHC Microscope Slides). After antigen unmasking for 45 min at 98 °C in CC1 buffer (Cell Conditioning 1, Ventana / Roche), incubation with anti-ERG monoclonal antibody (anti-ERG monoclonal mouse antibody, clone 9FY, Zytomed) was performed overnight at 4 °C in a humid chamber. The antigen-antibody reaction was revealed with Dako REAL kit DAB, according to the manufacturer's instructions. The presence of granular brown deposits in the nuclei of Ewing sarcoma cells was considered positive staining. Interpretation of the IHC staining was performed by two researchers independently (LC NR). If they diverged, consensus was reached by conferral with a multi-head microscope.

6. Statistical analysis

Statistical analyses were performed using Stata software, version 13 (StataCorp, College Station, TX, US). The tests were two-sided, with a type I error at 5%. Continuous parameters were expressed as mean ± standard deviation, or median [interquartile range], according to statistical distribution. The assumption of normality was checked using the Shapiro-Wilk test. The study of relationships between quantitative parameters was based on estimation of correlation coefficients (Pearson or Spearman according to statistical distribution, noted *r*). To take into account between- and within-subject variability (due to several measurements for the same patient), random-effects models for correlated data were run rather than the usual statistical tests (inappropriate because the hypothesis of independence of data was not supported). The assumption of residuals normality was checked using the Shapiro-Wilk test. When appropriate, a logarithmic transformation was used to fit the normality assumption and ensure the correct use of previous analyses. A Šidák type I error correction was applied to allow for multiple comparisons. Finally, a ROC (receiver operating

characteristic) curve analysis was carried out to evaluate the sensitivity and specificity of EWS-FLI1 transcript detection (RT-qPCR) in OT and TT samples. The confidence intervals at 95% were presented for area under the curve (AUC), sensitivity and specificity.

Results

1. Experimental model of EWS contamination of germinal tissues

Thawed OT (*n* = 12 contamination series) enclosing ovarian cysts from women with benign cysts, and thawed TT (*n* = 14 contamination series) from patients with azoospermia were contaminated with 0 (negative control), 10, 100 and 1000 human EWS cell line (RD-ES). Quantification of EWS-FL1 transcript of type II expression was performed in thawed OT and TT. Once contamination was achieved, RNA extraction and RT-qPCR were performed. OT and TT frozen by either slow or snap freezing methods were used to determine whether the freezing method could interfere with RT-qPCR analysis. To ensure that RD-ES cell lines had a similar dissemination potential compared to *in vivo* conditions, we co-cultured OT and TT with RD-ES cells. After 7 and 14 days of co-culture we performed RT-qPCR, histological analysis and FISH analysis in germinal tissues to look for dissemination of RD-ES cells. We analyzed TT by immunochemistry with ERG staining.

2. Yield of RNA extraction from germinal tissues frozen using slow or snap freezing

The median weight of OT fragments was 37.4 mg [15.2–62.0 mg]. OT was frozen by slow freezing (*n* = 6) or snap freezing (*n* = 6). The median weight of TT fragments was 28.8 mg [16.6–48.0]. TT was frozen by slow freezing (*n* = 7) or snap freezing (*n* = 7). After freezing by slow and snap freezing, the RNA yield extracted from OT (30.4 µg *vs*. 19.8 µg) and TT (19.8 µg *vs*. 22.8 µg) were not significantly different according to the freezing method ($p > 0.05$).

3. Detection of EWS-FLI1 transcript in frozen OT and TT samples contaminated with RD-ES cells

No expression of EWS-FLI1 transcript was observed in uncontaminated OT (*n* = 12) and TT (*n* = 14) frozen by slow or snap freezing. EWS-FLI1 transcript was detected in all contaminated OT (**Figure 1**) and TT (**Figure 2**). A close correlation between the number of RD-ES cells (10, 100 and 1000 cells) and EWS-FLI1 transcript was observed in OT ($r = 0.93$, $p < 0.001$) and in TT ($r = 0.96$, $p < 0.001$). We studied the sensitivity and specificity of the Ewing

MRD detection by RT-qPCR. For OT, the AUCs (area under the curve, ROC curve) were 0.94 to distinguish 10 and 100 EWS cells CI 95% [0.86–1.00] (**Figure 3a**) and 0.97 CI 95% [0.92–1.00] between 100 and 1000 EWS cells (**Figure 3b**). For TT, the AUCs were respectively 0.98 to characterize 10 and 100 EWS cells CI 95% [0.94–1.00] (**Figure 4a**) and 0.99 CI 95% [0.98–1.00] between 100 and 1000 EWS cells (**Figure 4b**).

4. Measurements of dissemination potential of RD-ES cell lines after co-culture with ovarian and testicular samples

EWS-FLI1 transcript was detected at days 7 and 14 of co-culture in OT and TT. At days 7 and 14, we observed RD-ES cells in the ovarian sample next to follicles (**Figure 5**) and in the testicular sample with typically small, round, blue cell morphology after hematoxylin and eosin (H&E) staining (**Figure 6a**) on tissue biopsy sections. Using FISH, we detected Ewing cells by the presence of a split signal pattern (EWSR1 rearrangement) in OT (**Figure 7**). FISH could not be performed on TT co-culture due to technical limits (specific design of culture chambers). After immunohistochemistry, the ERG intra-nuclear staining of Ewing cells was positive after days 7 and 14 of TT co-culture with RD-ES cells (**Figure 6b**). This demonstrates the dissemination potential of EWS cells in our *in vitro* model of tumor contamination of germinal tissue.

5. MRD analysis in ovarian and testicular tissues of patients with EWS

We analyzed cryopreserved OT (*n* = 5) and TT (*n* = 3) from EWS patients using RT-qPCR (**Table 1**). Most of the patients had received chemotherapy before cryopreservation. Only one boy was not treated prior to TT preservation. The analysis did not detect the presence of EWS-FLI1 transcript. B2M expression was detected at similar levels in OT and TT in the *in vitro* model described previously, indicating good quality of RNA samples. No EWS cells were detected by standard histological analysis and FISH in OT.

Discussion

Because of the risk of infertility after cancer treatment for EWS [19], fertility preservation by OT and TT cryoconservation is recommended for prepubertal patients. Driven by an oncogenic chromosomal translocation resulting in the expression of an aberrant transcription factor, EWS-FLI, the disease is typically aggressive and micrometastatic [4].

Development of sensitive and accurate methods for detecting metastatic EWS cells in germinal tissue is essential to assess the risk of reintroducing cancer when thawed tissue is used to restore fertility and/or induce puberty in patients [20–22]. In the present study, an *in vitro* model of EWS dissemination in human frozen OT and TT was established and its sensitivity and specificity for the detection of EWS-FLI1 transcript evaluated.

The detection of EWS-FLI1 fusion transcript expression by RT-qPCR is a rapid, specific, and sensitive ($1/10^6$ cells) diagnostic test which has mainly been applied for MRD detection in peripheral blood and bone marrow [23]. The RT-PCR assay for detecting sarcoma translocations in tumor tissue is also of clinical utility in differentiating small round blue cell tumors [17]. EWS-FLI1 translocation is detectable with both RT-PCR and FISH in formalin-fixed paraffin-embedded tissue, but FISH seems more reliable than RT-PCR for the diagnosis of EWS in this type of sample [24]. Immunohistochemical markers can assist in differential diagnosis, but the current panels have limited specificity. For this reason, the use of FISH is preferable [17]. FISH and RT-PCR are complementary, and the use of both techniques is needed to clarify the most diagnostically challenging cases [24].

In our *in vitro* contamination model, we demonstrate the high sensitivity and specificity of the EWS-FLI1 transcript detection in frozen OT and TT. Previous studies reported MRD detection in OT but did not evaluate the sensitivity and specificity of the detection. To our knowledge, our study is the first validation of EWS MRD detection in TT. It demonstrates that EWS assessment in OT and TT by RT-qPCR is highly specific, sensitive and accurate. We also show that RD-ES cells used in our *in vitro* model had high dissemination potential in co-cultures with germinal cells. EWS infiltration was detected by RT-qPCR in OT and TT, by FISH in OT and Ewing round blue cells were highlighted by histology and immunochemistry (ERG staining) in TT. FISH could not be performed on TT co-culture owing to technical limits set by the specific design of culture chambers. This co-culture of human seminiferous tubules and RD-ES cells was run with previously defined and validated conditions [10].

Sufficient RNA amounts were extracted from small pieces of ovarian and testicular samples independently of the freezing method applied to germinal tissues. To our knowledge, this is the first study to compare EWS MRD detection in germinal tissues frozen by two protocols. Our results show that the freezing method had no impact on RNA yields. RT-qPCR can therefore be performed on OT and TT samples that have been already cryopreserved using slow freezing and on samples that will be frozen by snap freezing at the time of surgical

retrieval for subsequent analysis. The use of small OT and TT samples for MRD analysis ensures that sufficient amounts of tissue remain for fertility preservation.

The incidence of ovarian metastases in EWS is unknown. Circulating tumor cells can be detected in 20–45% of EWS patients [5]. This suggests that small numbers of malignant cells may be present in ovarian cortex [14] and testicles. Cases of metastatic ovarian involvement in EWS patients have been described in the literature [2,25]. Microscopic ovarian infiltration could be present in non-metastatic EWS [12]. No metastasis in TT has been described [26]. In our study, MRD was not detected in germinal tissues of patients with metastatic or localized EWS. However, the germinal tissues we analyzed were obtained after chemotherapy.

Previously, EWS MRD was studied in ovarian samples with different approaches. In the study of Abir et al., one ovarian sample cryopreserved by snap freezing was evaluated from each EWS patient [13], and no malignant cell was detected by histology, but RT-PCR showed a positive result for the transcript *EWS–FLI1* in one case. Greve et al. investigated the presence of malignant cells in one piece of OT intended for transplantation [14]. All the samples underwent histology and RT-qPCR and none of the pieces revealed Ewing cells or EWS–FLI1 transcript. These results suggest that MRD needs to be evaluated by different techniques before ovarian transplantation. In our study, we performed RT-qPCR, FISH and histology to combine more detection methods. If the MRD detection is positive, OT grafting will be associated with a high risk of reintroducing cancer. In such cases, it may be feasible to graft isolated ovarian follicles or to perform *in vitro* maturation of oocytes to ensure safer transplantation. This would eliminate the risk of transplanting malignant cells hidden in ovarian stromal tissue. Such methods have already produced live offspring in mice [27] and mature oocytes in primates [28].

Conclusion

In conclusion, we show with our *in vitro* model that EWS-FLI1 mRNA detection by qPCR provides an accurate assessment of EWS MRD in ovarian and testicular tissues cryopreserved by slow or snap freezing. Since EWS is highly metastatic, MRD detection of EWS always needs to be confirmed by additional methods (histology, FISH and/or immunostaining) to ensure there is no risk of reintroducing cancer cells. These results now require confirmation by a further study of ovarian and testicular tissues from a large cohort of prepubertal patients with EWS.

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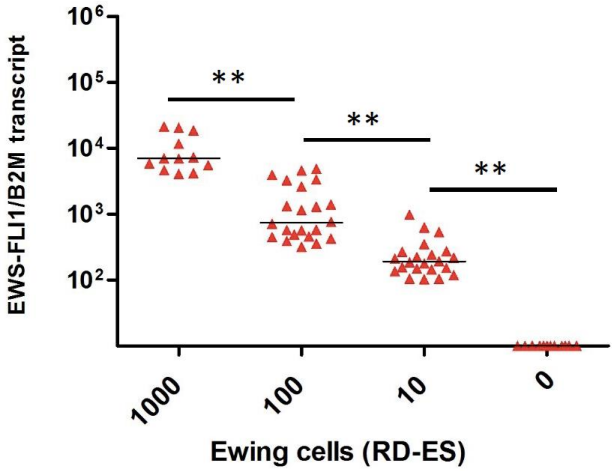


Figure 1. EWS-FLI1 transcript detection in ovarian tissue (*n* = 12). Relative quantification of *EWS-FLI1* transcript (β2M reference gene) for the contamination with 0, 10, 100 and 1000 cells. Each symbol represents one ovarian fragment (the average of the duplicates for 1000 cells or triplicates for 10 and 100 cells). The symbol ** means there was a significant difference and $p < 0.001$.

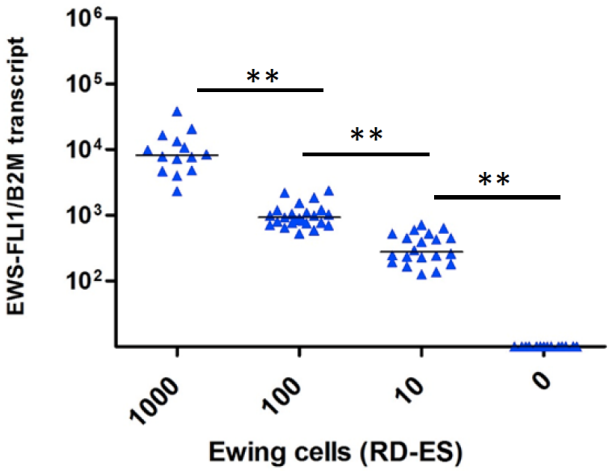
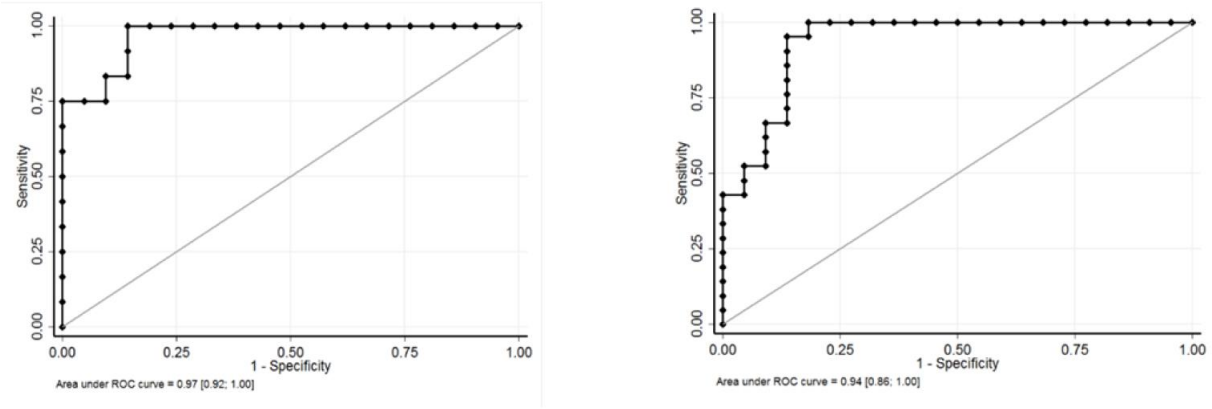
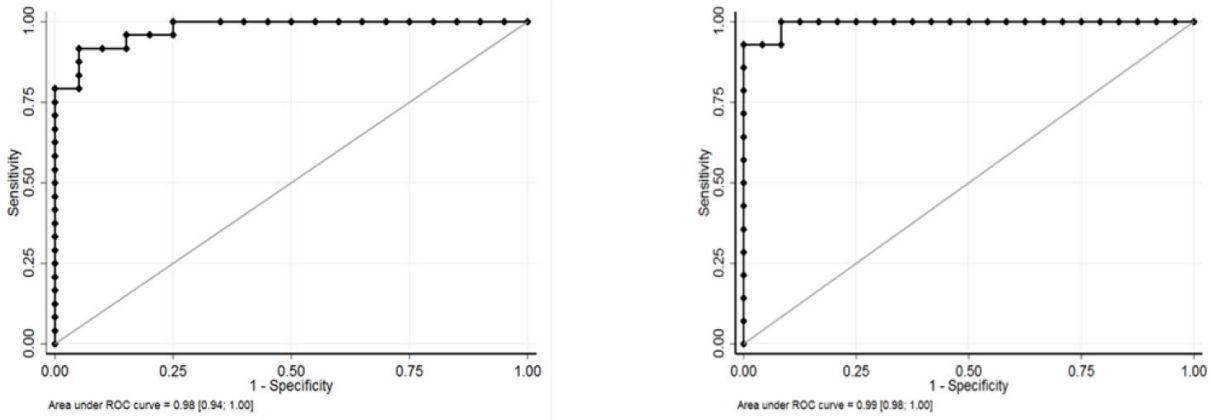


Figure 2. EWS-FLI1 transcript detection in testicular tissue (*n* = 14). Relative quantification of *EWS-FLI1* transcript (β2M reference gene) for the contamination with 0, 10, 100 and 1000 cells. Each symbol represents one testicular fragment (the average of the duplicates for 1000 cells or triplicates for 10 and 100 cells). The symbol ** means there was a significant difference and $p < 0.001$.



(a) (b)

Figure 3. Sensitivity and specificity of detection to distinguish 10 and 100 Ewing cells, and 100 and 1000 Ewing cells in ovarian tissue: (a) The AUC (area under the curve, ROC curve) was 0.94 CI 95% [0.86–1.00] to distinguish 10 and 100 Ewing cells; (b) The AUC was 0.97 CI 95% [0.92–1.00] between 100 and 1000 Ewing cells.



(a) (b)

Figure 4. Sensitivity and specificity of detection to distinguish 10 and 100 EWS cells, and 100 and 1000 EWS cells in testicular tissue: (a) The AUC was 0.98 CI 95% [0.94–1.00] to characterize 10 and 100 EWS cells; (b) The AUC was 0.99 CI 95% [0.98–1.00] between 100 and 1000 EWS cells.

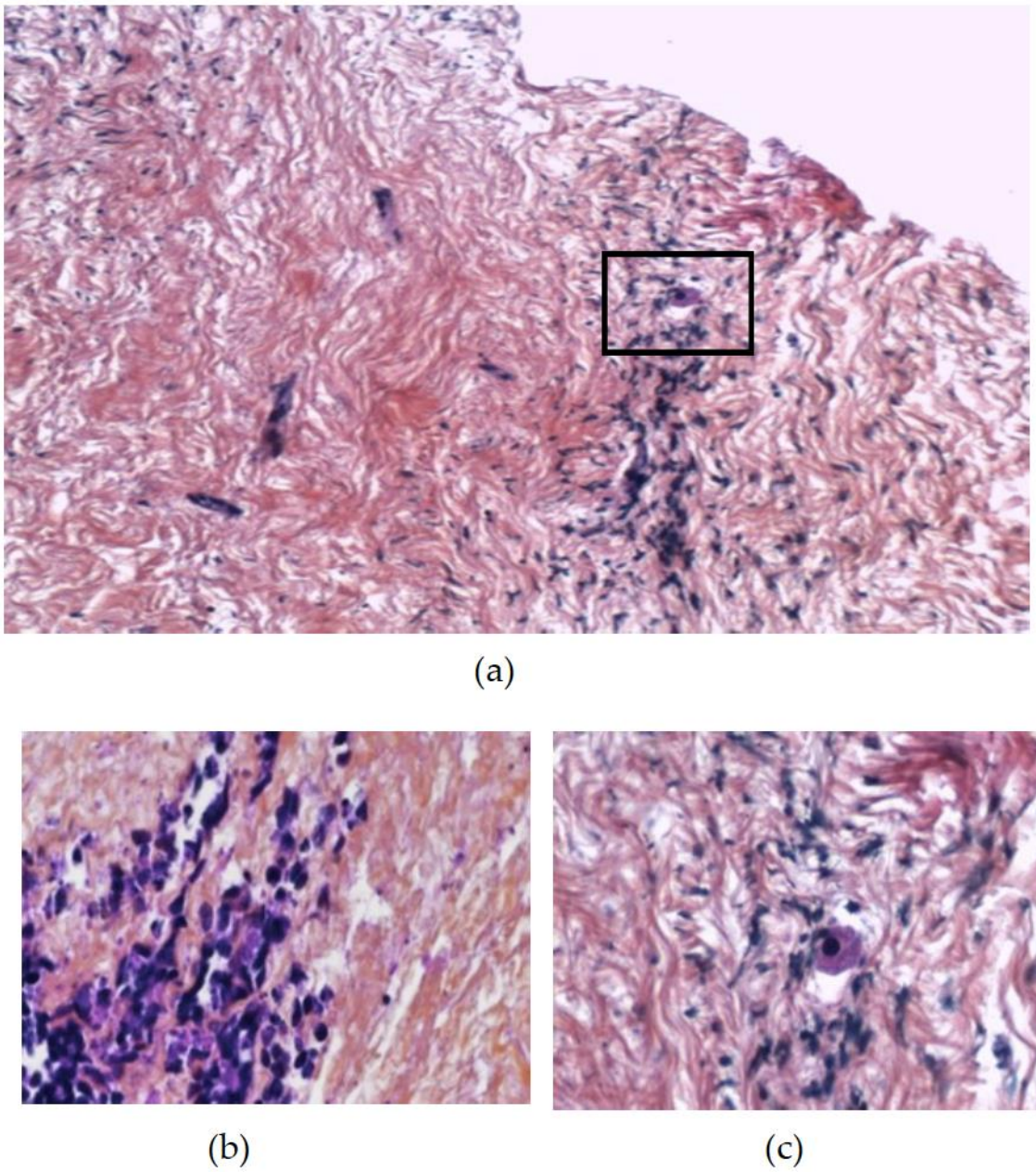


Figure 5. Illustrations of histology: OT after coculture with RD-ES cells (at day 14): (a) Ovarian biopsy stained with hematoxylin and eosin: RD-ES cells (arrow) localized OT after co-culture (at day 7); (b) Area with RD-ES cells infiltrating OT after co-culture; (c) Area with ovarian follicle.

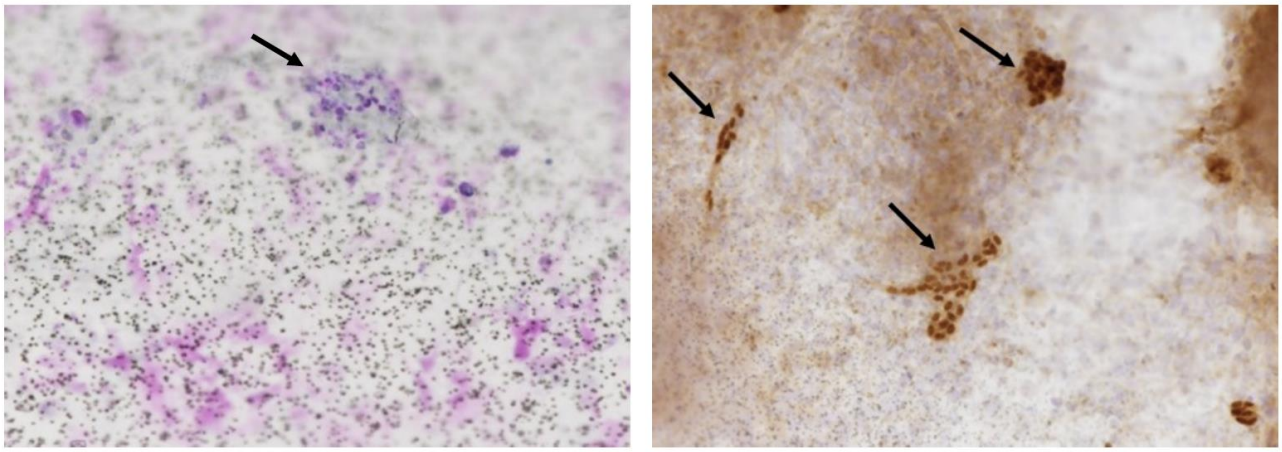


Figure 6. Illustrations of histology and immunohistochemistry: RD-ES cells (black arrow) localized in TT after coculture (at day 14) on insert: (a) Testicular biopsy in insert stained with hematoxylin and eosin; (b) ERG-positive staining of RD-ES cells.

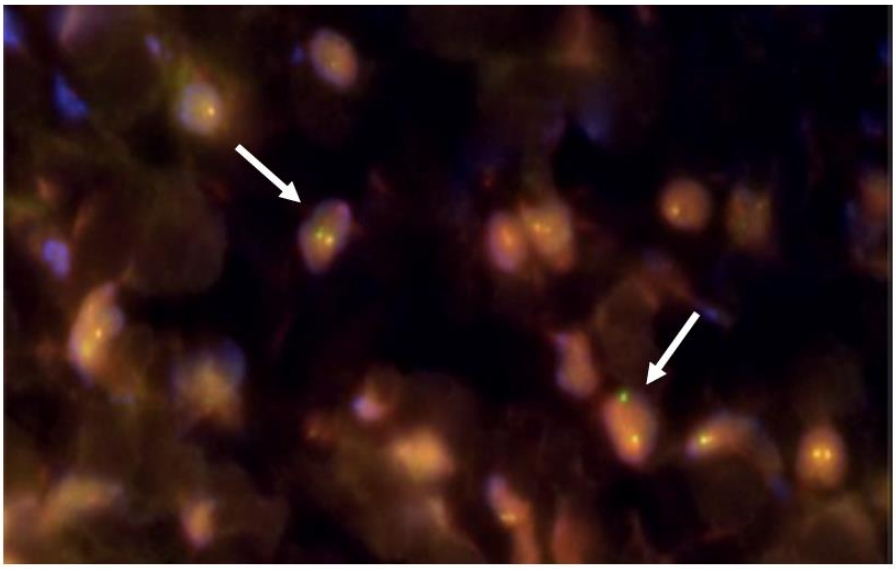


Figure 7. The representative fluorescence *in situ* hybridization (FISH) result using EWSR1 (22q12) Dual Color Break Apart Rearrangement Probe (Vysis): Positive result after OT co-culture with RD-ES cells (at day 14). RD-ES cells displayed one fusion (yellow signal), and the simultaneous split pattern of one orange and one green signal (arrows), indicative of a rearrangement of one copy of the EWSR1 gene. The fusion gene is detected by a yellow signal, corresponding to colocalization of the red and green probes. In this figure, two cells have a split pattern.

Table 1. EWS patient characteristics (OT: Ovarian Tissue; TT: Testicular Tissue).

EWS patients	**Age at cryopreservation (years)**	**Chemotherapy**	**Metastasis**	**Outcome**
OT A	16	Before preservation	Lung	Dead
OT B	14	Before preservation	Lung	Alive
OT C	14	Before preservation	No	Alive
OT D	15	Before preservation	Lung, bone and medullary	Dead
OT E	13	Before preservation	Lung	Alive
TT A	7	Before preservation	Mediastinum	Alive
TT B	14	Before preservation	None	Alive
TT C	14	After preservation	None	Alive

5. Résultats

Nous avons contaminé des tissus gonadiques avec deux lignées tumorales de neuroblastome et une lignée tumorale de tumeur d'Ewing afin de simuler des métastases ovariennes et testiculaires de ces tumeurs. Ensuite, nous avons mesuré l'expression des marqueurs d'intérêt par RT-qPCR.

Concernant le neuroblastome, l'expression des marqueurs TH et DCX était positive dans les tissus ovariens (n=20) et testiculaires (n=20) non contaminés, révélant leur manque de spécificité. En revanche, l'expression de PHOX2B était négative dans les tissus ovariens et testiculaires non contaminés. En outre, une détection de PHOX2B était possible dès la contamination minimale par 10 cellules tumorales dans toutes les gammes réalisées avec les lignées neuroblastiques, avec une très bonne corrélation entre le nombre de cellules tumorales utilisées pour la contamination et le niveau d'expression de PHOX2B, révélant l'excellente sensibilité et spécificité de ce marqueur.

Concernant la tumeur d'Ewing, le transcrit EWS-FLI1 n'a été détecté dans aucun des tissus ovariens (n=12) et testiculaires (n=14) non contaminés. L'expression de ce transcrit était détectée à partir d'une contamination par 10 cellules tumorales, avec une très bonne corrélation entre le nombre de cellules tumorales utilisées pour la contamination et le niveau d'expression du transcrit.

6. Conclusion

PHOX2B et le transcrit EWS-FLI1 constituent des marqueurs fiables, sensibles et spécifiques, pouvant être utilisés pour évaluer la maladie résiduelle, respectivement du neuroblastome et de la tumeur d'Ewing, dans les tissus gonadiques. Cette technique de détection de la maladie résiduelle est réalisable sur de tous petits fragments de tissu gonadique, permettant de conserver un maximum de tissu pour une éventuelle greffe ultérieure. Ces résultats sont très encourageants et permettent d'envisager l'utilisation future de ces tissus en toute sécurité, en limitant au maximum le risque de réintroduire des cellules tumorales.

V. CONCLUSION ET PERSPECTIVES

Avec l'amélioration de la survie des jeunes patients atteints de cancers, l'anticipation et la prise en compte des effets indésirables des traitements font partie intégrante de leur prise en charge. L'altération de la fonction gonadique est bien réelle et dépend du type de cancer, des traitements, mais également de la susceptibilité individuelle du patient, rendant difficile la prédiction avec certitude de la fonction de reproduction à l'issue des traitements (Rousset-Jablonski et al. 2015 ; Levine et al. 2010), comme nous l'avons démontré dans l'article sur la cohorte L.E.A. avec l'atteinte de la fonction gonadique y compris chez les femmes ayant été traitées exclusivement par chimiothérapie de première ligne.

La toxicité gonadique constitue l'une des séquelles les plus invalidantes avec un retentissement majeur sur la qualité de vie ultérieure de ces futurs adultes (Chaume et al. 2007). La question de la préservation de la fertilité des patients atteints de cancer est par conséquent inévitable, sous tendue par la loi bioéthique de 2011 et le troisième Plan Cancer (2014-2019). Le rôle des hématologues et oncologues pédiatres est primordial dans cette démarche.

L'amélioration de la préservation de la fertilité passe par :

- La poursuite du suivi après traitement des jeunes patients guéris d'un cancer pour **améliorer nos connaissances sur les complications** susceptibles de survenir à moyen et long terme, afin de les anticiper et de les prendre en charge. Concernant la toxicité gonadique, les cohortes de grande envergure, comme la cohorte L.E.A. pour les leucémies, permettent d'optimiser le suivi de ces patients. L'analyse de ces données dans quelques années, lorsque l'âge médian des femmes aura augmenté, reflétera davantage les séquelles sur leur fertilité. L'étude FERTILEA a été mise en place plus récemment pour évaluer plus précisément l'atteinte utérine, sur la fonction ovarienne et sur la fonction reproductrice en fonction du type de conditionnement et du statut pubertaire avant greffe de cellules souches hématopoïétiques chez les femmes de la cohorte L.E.A. Afin de mieux appréhender la gonadotoxicité des thérapeutiques et la prise en charge des patients, l'évaluation de la fertilité devrait faire partie intégrante du suivi à long terme de tous les types de cancers.

- **L'information des patients** sur les éventuels risques pour leur fertilité et sur les possibilités de préservation, afin de **faciliter l'accès aux plateformes clinico-biologiques** de préservation de la fertilité. En effet, ce risque gonadotoxique n'est pas une fatalité et des recommandations existent pour optimiser la préservation de la fertilité de ces patients et homogénéiser les pratiques (Oktay et al. 2018 ; Sudour-Bonnange et al. 2013). Au niveau de la région Auvergne, la plateforme PREFERA a vu le jour en mars 2016, assurant une collaboration multidisciplinaire dans la prise en charge des patients. Même si des progrès restent encore à faire, notre plateforme a permis de faciliter l'accès à l'information des patients et des professionnels, d'augmenter le nombre de prises en charge en préservation et de mettre en place des campagnes de sensibilisation auprès du grand public.
- La mise au point de techniques sensibles et spécifiques permettant de détecter la présence de cellules tumorales dans les tissus gonadiques, techniques indispensables pour **sécuriser au maximum la préservation de la fertilité** des jeunes patients ne pouvant bénéficier que de la cryoconservation de tissus ovariens ou testiculaires. Cette mise au point, maintenant possible pour le neuroblastome et la tumeur d'Ewing, est encourageante et potentiellement extrapolable à d'autres cancers pour lesquels des marqueurs biologiques existent (rhabdomyosarcome, lymphome de Hodgkin). L'objectif est ainsi de pouvoir envisager une restauration de la fertilité sans risque carcinologique pour les patients ayant survécu à un cancer dans l'enfance, dont la fertilité a été compromise et ayant bénéficié d'une cryoconservation de tissu gonadique. Dans cette démarche, l'évaluation des bénéfices et risques ainsi que la collaboration entre hémato-oncologues, gynécologues et biologistes sont indispensables, à la fois au moment de la préservation et au moment de l'utilisation des tissus gonadiques.

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