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Ovarian tissue cryopreservation andtransplantation in patients withcentral nervous system tumours

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STUDY QUESTION: Is there a possibility of reseeding cancer cells potentially present in frozen ovarian tissue from patients with centralnervous system (CNS) tumours?

SUMMARY ANSWER: Malignancy reseeding in cryopreserved ovarian tissue from 20 patients with CNS tumours was not detected byhistology, immunohistochemistry (IHC), molecular biology or xenotransplantation.

WHAT IS KNOWN ALREADY: Ovarian metastasis potential has been documented in patients with leukaemia, borderline ovariantumours, advanced breast cancer and Ewing sarcoma. However, data on the safety of transplanting frozen-thawed ovarian tissue from can-cer patients with CNS tumours are still lacking.

STUDY DESIGN, SIZE, DURATION: This prospective experimental study was conducted in an academic gynaecology research labora-tory using cryopreserved ovarian cortex from 20 patients suffering from CNS tumours. Long-term (5 months) xenografting was performedin immunodeficient mice.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Subjects enrolled in the study were suffering from one of six types of CNStumours including medulloblastoma, ependymoma, primitive neuroectodermal tumours, astrocytoma, glioblastoma and germinoma. Thepresence of malignant cells was investigated with disease-specific markers for each patient in cryopreserved and xenografted ovarian tissueby histology, IHC via expression of neuron-specific enolase (NSE) and glial fibrillary acidic protein (GFAP), and reverse transcription dropletdigital polymerase chain reaction (RT-ddPCR) for quantification of GFAP and ENO2 gene amplification.

MAIN RESULTS AND THE ROLE OF CHANCE: Serial sections of cryopreserved and xenografted ovarian tissue from 20 patientsshowed no malignant cells by histology. All samples were

negative for NSE and GFAP, although these neural markers were expressed ex-tensively in the patients' primary tumours. Analysis by RT-ddPCR revealed no cancer cells detected in cryopreserved and xenograftedovarian fragments from subjects with astrocytoma, ependymoma, glioblastoma or medulloblastoma. Taken together, the study found noevidence of malignancy seeding in frozen-thawed and xenotransplanted ovarian tissue from patients affected by CNS cancers.

LIMITATIONS, REASONS FOR CAUTION: This analysis cannot guarantee complete elimination of disseminated disease from all cry-opreserved ovarian cortex, since we are unable to examine the fragments used for transplantation.

WIDER IMPLICATIONS OF THE FINDINGS: This is the first study to be conducted in patients with CNS cancers undergoing ovariantissue cryopreservation and transplantation, and clearly demonstrates no tumour seeding in their frozen-thawed and xenografted tissue.This information is vital for doctors to provide patients with meaningful and accurate advice on the possibilities and risks of ovarian tissuereimplantation.

STUDY FUNDING/COMPETING INTEREST(S): This study was supported by grants from the Fonds National de la RechercheScientifique de Belgique–the Excellence of Science (FNRS–EOS), number 30443682 awarded to M.-M.D. and T.Y.T.N., FNRS grant num-ber 5/4/150/5 and FNRS-PDR Convention grant number T.0077.14 awarded to M.-M.D., grant 2018-042 from the Foundation AgainstCancer awarded to A.C., and private donations (Ferrero, de Spoelberch). The authors declare no competing financial interests.

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Introduction

According to Surveillance Epidemiology and End Results (SEER) program statistics from 2005 to 2015, central nervous system (CNS) tumours have the second highest cancer incidence (26%) after leukaemia (29%) in patients aged 0–14 years. By age 15–19, however, CNS cancers emerge as the most prevalent, followed by lymphoma and leukaemia, respectively (Siegel et al., 2018). CNS tumours also occur in adults. Therapeutic modalities for these cancers include surgery and radio-chemotherapy, which have become significantly more effective over recent decades, resulting in considerable improvements in patient survival rates up to 75% (Steliarova-Foucher et al., 2017; Siegel et al., 2018). Nevertheless, these treatments, especially high-dose chemotherapy, also pose a threat to patients' reproductive organs, leading to premature ovarian insufficiency and subsequent infertility (Anderson and Wallace, 2013; Donnez and Dolmans, 2013, 2017). For this reason, patients should be offered an appropriate approach to fertility preservation prior to their toxic treatment. For prepubertal girls, ovarian tissue cryopreservation offers a unique option (Donnez et al., 2006; Donnez and Dolmans, 2013; Wallace et al., 2016). Once patients are in full disease remission, frozen-thawed ovarian tissue can be transplanted back to the pelvic cavity to restore ovarian function (Donnez et al., 2011; Donnez and Dolmans, 2013; Wallace et al., 2014; Meirow et al., 2016; Anderson et al., 2017; Gellert et al., 2018). Thousands of ovarian tissue cryopreservation procedures have already been performed to safeguard fertility and at least 360 cases of frozenthawed ovarian tissue autotransplantation have been reported, resulting in more than 130 live births worldwide (Donnez and Dolmans, 2017; Gellert et al., 2018), although that figure has probably exceeded 200 by now (Dolmans, 2019). One overriding concern when contemplating ovarian tissue cryopreservation and transplantation in cancer patients is their safety. A number of studies have analysed the risk of reintroducing

malignant cells potentially present in the frozen-thawed ovarian tissue, which could induce recurrence of the primary tumour. Different patient categories most frequently undergoing ovarian tissue cryopreservation and transplantation have indeed been the focus of scientific research and malignant cells have been detected in case of leukaemia and borderline ovarian cancer. However, minimal disseminated disease (MDD) has not been documented in ovarian tissues from patients with bone and soft tissue sarcoma or low-grade breast cancer (Meirow et al., 2008; Abir et al., 2010; Dolmans et al., 2010, 2016; Rosendahl et al., 2010; Fabbri et al., 2012; Luyckx et al., 2013; Abir et al., 2014; Masciangelo et al., 2018). Data on the safety of transplanting frozen-thawed ovarian tissue from cancer patients with CNS tumours are still insufficient, even though this pathology is the third most common indication for ovarian tissue cryopreservation among subjects under 15 years of age (Poirotet al., 2019). Frequently encountered CNS cancers in childhood in-clude astrocytoma, medulloblastoma, ependymoma, glioblastoma andgerminoma. These CNS tumours have the capacity for extraneuralmetastases in 0.5–18% of cases (Rickert, 2003; Xu et al., 2018). In theliterature, we found just two publications reporting metastases topatients' ovaries from medulloblastoma (Paterson, 1961; Lamovec andPogae`nik, 2001).By assessing and determining the safety of ovarian tissue cryopreser-vation and transplantation protocols, the present study aims to exam-ine the possible presence of cancer cells in cryopreserved andxenografted ovarian tissue from patients with CNS tumours.

Materials and methods

Patients

There were 31 patients with CNS cancers who underwent ovarian tis-sue cryopreservation in our institution from 2001 to 2018 prior tochemotherapy. In 20 subjects, 12 of whom are still

alive and 8 de-ceased, we had access to primary tumour samples and cryopreservedovarian tissue. The participants were diagnosed with six types of CNStumours, including medulloblastoma (eight patients), ependymoma(three patients), primitive neuroectodermal tumours (PNETs, threepatients), astrocytoma (two patients), glioblastoma (two patients) andCNS germinoma (two patients). One patient underwent ovarian trans-plantation, while the others had their tissue stored in our cryobank.The clinical characteristics of patients are shown in Table I. One cryo-vial per patient, accounting for 9.1–16.7% of their collected ovarian tis-sue, was thawed for MDD testing and xenografting.

Marker selection

To elicit the potential for seeding primary CNS cancers within ovariantissue by immunohistochemistry (IHC) and reverse transcription drop-let digital polymerase chain reaction (RT-ddPCR), a list of possibleneuronal markers was initially identified via a thorough literature re-view. Several markers were then analysed by IHC based on their spe-cificity and sensitivity to different histological features of six types ofCNS cancers. Two markers were selected, namely glial fibrillary acidicprotein (GFAP) and neuron-specific enolase (NSE) (Goyal et al., 2015;Isgro` et al., 2015; Jaiswal, 2016; Tuffaha et al., 2018). Both showedspecific positive immunoexpression in patients' primary tumours, butnot in normal ovarian tissue from healthy women. This made GFAPand NSE good candidates for detection of MDD in ovarian tissue. Accordingly, expressed GFAP and ENO2 genes, which respectively encode these proteins, were the targets of qualification by RT-ddPCR.

Thawing procedure

Ovarian cortex samples were cryopreserved using the slow-freezingprotocol, as previously described (Baird et al., 1994; Donnez et al.,2004). One cryovial of ovarian cortex from each patient was thawedby leaving it at room temperature for 2 min and then plunging it into awater bath at 37C for a further 2 min. The ovarian fragments weresubsequently transferred to culture dishes filled with Leibovitz's L-15medium, followed by three consecutive baths in minimal essentialmedium-GlutaMAX at room temperature for 5 min each. Thawedfragments were cut into several pieces for testing or grafting.

Histological analysis

Small strips of frozen-thawed or xenografted ovarian tissue were fixed in 4% formaldehyde, before embedding in paraffin wax. After trimming the molds, samples were serially sectioned every 5 mm and placed on microscopy slides. Haematoxylin and eosin (H&E)-stained slides were analysed for the presence of metastatic cells in ovarian fragments and compared with the histological morphology of the patients' primary tumours.

Immunohistochemical evaluation

Slides with biopsies from primary tumours were obtained from the pa-thology department and used as positive controls. Negative controlsconsisted of ovarian tissue biopsies from patients with benign uterinepathologies. IHC markers were selected based on anatomical pathol-ogy analysis of the primary tumour. The same neuronal markers,namely GFAP and NSE, were examined in CNS tumours, frozen-thawed ovarian tissues and xenografted ovarian tissues.Immunostaining was automatically achieved by using Ventana'sultraView Universal DAB detection kit on the BenchMark Ultra IHC/ISH module (catalogue number 760-500, Ventana, Roche, Basel,Switzerland). The slides were deparaffinized, rehydrated and

166 treatedwith Epitope retrieval solution 1 (ethylenediaminetetraacetic acidbuffer, pH 8.0) at
167 95C for 8 min, followed by two more baths atroom temperature. Antibodies added were
168 rabbit polyclonal GFAP an-tibody (10 mg/ml, dilution 1:500, catalogue number CP040A, B, C,
169 lot121806, Biocare Medical, CA, USA) or rabbit primary NSE antibody(60 mg/ml, dilution
170 1:1000, catalogue number A598, lot 106, DakoCorporation, CA, USA) for 1 h at room
171 temperature. Tissue sectionswere subsequently incubated in ultraView HRP-conjugated
172 multimerantibody reagent (Igs, Ventana Medical Systems, AZ, USA) and coun-terstained with
173 haematoxylin.
174
175 RT-PCR
176 CNS tumours were kept in frozen section blocks at Saint-Luc's biobank. Primary tumours
177 were retrieved from study participants and patients of similar ages. Patient age was taken
178 into account when selecting samples because CNS cancers have certain characteristics that
179 differ between paediatric and adult subjects. Samples were collected from individuals of
180 similar age to patients in our cohort in an attempt to keep them as representative as
181 possible. Ten sections measuring 20 mm in thickness were used for RNA extraction. After
182 thawing or degrafting, ovarian cortex from patients was cut into smallpieces, immediately
183 submerged in 700 ml RNAlater RNA stabilizationreagent (Qiagen, Ambion, TX, USA), and
184 stored at #20

C until use.Ovarian tissues were removed from RNA-stabilized buffer andtransferred to 2 ml tubes containing 2 mm yttria-zirconium beads(ZrO_2 95%, Y_2O_3 5%) and 350 ml RLT Plus lysis reagent (þbeta-mer-captoethanol). Similarly, frozen sections of CNS tumour samples werealso placed in tubes with beads and 350 ml RLT buffer. The tubeswere then deposited in the FastPrep-24 homogenizer (catalogue num-ber SKU116004500, MP Biomedicals, USA) to disrupt ovarian cortexat a speed of 6.5 m per second for 30 s twice. RNA extraction wasperformed using the RNeasy Plus micro kit (catalogue number 74004,lot 163052364, Qiagen, Germany) following the manufacturer'sinstructions. Genomic DNA was eliminated by adding 80 ml DNase Imixture directly to the spin column membrane and incubating for15 min at room temperature. All extracted RNA was qualified withthe NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific,ND-2000, Wilmington, USA) and purity was checked by assessingA260/280 ratios over 1.90, followed by immediate storage at #80

C.Complementary DNA (cDNA) synthesis was achieved by using theAdvantage RT for PCR kit (catalogue number 639506, lot 1804757 A,Takara Bio Inc, CA, USA) and 0.5 mg total RNA, following the manu-facturer’s protocol. Specifically, the RNA was mixed with 0.5 ml ran-dom hexamer primer (500 ng/ml) and 0.5 ml oligo (dT) 18 primer andDEPC-treated water to obtain a final volume of 13.5 ml. This solutionwas heated to 67

C for 2 min, then cooled rapidly on ice. Reversetranscription was performed by adding 4 ml of 5$ reaction buffer, 1 mlof dNTP mix (10 mM each), 0.5 ml of recombinant RNAse inhibitorand 1 ml of MMLV reverse transcriptase in a final volume of 20 ml. Thesolution was then placed in the thermal cycler (GeneAmp 9700, serialnumber 096S9030939, Applied Biosystems, MA, USA) for 1 h at42

207 C, 5 min at 94

208 C and 3 min at 4

C. All cDNA was then kept fro-zen at #20C.

Primers and probes

Fluorescein amidite (FAM)-labelled assays for genes of interest wereGFAP (Hs00909233_m1, NM_002055.3, RTPrimerDB ID 7735;Applied Biosystems, Thermo Fisher Sc, MA, USA) and ENO2(Hs00157360_m1, NM_001975.2, RTPrimerDB ID 7733; AppliedBiosystems, Thermo Fisher Sc, MA, USA). Abelson murine leukaemiaviral oncogene homolog 1 (ABL1, Hs01104728_m1, NM_005157.4,RTPrimerDB ID 2188), beta 2 microglobulin (B2M, Hs00187842_m1,NM_004048.2, RTPrimerDB ID 1535) and glyceraldehyde 3-phos-phate dehydrogenase (GAPDH, Hs00266705_g1, NM_002046.3,RTPrimerDB ID 1233) were used as housekeeping genes, as in previ-ous studies (Soares et al., 2015; Dolmans et al., 2016; Soares et al.,2017; Manavella et al., 2018; Masciangelo et al., 2018). The reason forusing these housekeeping genes, which belong to different molecularpathways, was to minimize the risk of co-regulation (Vandesompeleet al., 2002; Koppelkamm et al., 2010). Sequences of these primersand probes are shown in Table II.

Determining the limit of blank and limit of detection

The limit of blank (LOB) and limit of detection (LOD) for ddPCRwere measured following Clinical and Laboratory Standards InstituteEP17 guidelines (National Committee for Clinical LaboratoryStandards, 2004). The LOB refers to the highest apparent value expected from measurement of replicates of a sample containing noanalytes (National Committee for Clinical Laboratory Standards,2004). The blank sample in this study consisted of the pool of RNAfrom 10 healthy women’s normal ovarian tissue. To define the LOB,60 replicates of this pooled sample were tested and calculated usingthe following formula: LOB ¼ meanblank þ

1.645 (SDblank)(Armbruster and Pry, 2008).The LOD refers to the lowest analyte value (percentage of gene in-terest) capable of being reliably differentiated from the LOB and atwhich the detection is feasible. To determine the LOD for ENO2 andGFAP expression in human ovarian tissue, the RNA of CNS tumoursamples and pooled samples of 10 normal ovarian tissues were dilutedprior to reverse transcription. Tenfold serial dilution was carriedout with following concentrations: 10, 10-1, 10-2, 10-3, 10-4 and 10-5. A total of eight replicates were obtained using 80 ng cDNA tem-plates at each stage of serial dilution, except at the lowest dilution(10-5 or 0.001%), which required 20 replicates. The LOD wascalculated according to the following formula: LOD ¼ LOB þ 1.645(SDlow concentration sample) (Armbruster and Pry, 2008). Each ddPCR runincluded duplicates of no-template controls and the diluent pool ofnormal ovarian tissue as negative controls.

ddPCR experiments and data analysis

The QX200TM Droplet Digital PCR System (Bio-Rad Laboratories Inc.,CA, USA) was used. The reaction mixture contained 11 ml ddPCRTMSupermix for probes, no dUTP (Bio-Rad), 1 ml assays for the gene ofinterest or the reference gene, 10 ng cDNA and TE buffer. A volumeof 20 ml of this mixture were loaded into a disposable plastic cartridge(catalogue number 1864008) with 70 ml of droplet generation oil forprobes (catalogue number 1863005), and covered with a special rub-ber gasket (Bio-Rad Laboratories). In no-template control wells, thevolume of cDNA was replaced with TE buffer. Droplets generatedfrom each sample were transferred to 96-well PCR plates (Eppendorf,Hamburg, Germany) and covered with pierceable PX1 PCR plate-sealing foil (Bio-Rad Laboratories). PCR amplification was achievedwith the C1000 TouchTM thermal cycler (Bio-Rad Laboratories) star-ring at 95

C for 10 min, then 40 cycles of denaturation at 94C for30 s and annealing at 60C for 1 min, followed by enzyme deactivationat 98C for 10 min, and finally cooling at 4C. The plates were subse-quently loaded into the QX200TM Droplet Reader (Bio-RadLaboratories), where droplets in each well were automatically read ata speed of 85 s per well. Data were analysed with QuantaSoft software, version 1.7 (Bio-Rad Laboratories). Each experiment in-cluded a negative (RNA pool of normal ovarian tissue samples from10 healthy women) and positive (patients' primary tumour) controlsamples, as well as no-template controls. For each sample, the ddPCRtest was carried out with duplex probes using GFAP-FAM and ABL1-VIC dyes and a singleplex primer with GFAP, ABL1, B2M and GAPDHin the same run. Droplets were deemed positive or negative bythresholding based on their fluorescence amplitudes. Results were ana-lysed when the number of tested droplets per well ranged from10 000 to 20 000 and the housekeeping genes provided reliable ampli-fication. The target concentration in each sample was expressed as thenumber of copies per microliter. Outcomes were considered to have'detected' the presence of cancer cells when concentrations of thegene of interest were equal or above the LOD of this gene for eachtype of CNS tumour with a false positive rate of less than 5% for thistarget (type I or a error). Conversely, values below the LOD wereidentified as 'not detected' with respect to cancer cells. GFAP andENO2 ddPCR linear regression curves were calculated from standarddilutions for each type of CNS tumour, as mentioned above.Detailed information is shown in Supplementary Table S1 accordingto Minimum Information for the Publication of Quantitative DigitalPCR Experiments guidelines (Huggett et al., 2013).

Xenotransplantation to immunodeficientmice

To assess the potential of reseeding malignancy through the graft,ovarian tissue from all study subjects was xenotransplanted to femalesevere combined immunodeficient (SCID) mice (Charles RiverLaboratories, France) for 22 weeks. Appropriate housing and breedingconditions were strictly applied, as previously described (Nisolle et al.,2000). Surgical procedures have already been reported (Dolmanset al., 2010). The mice were kept in sterile conditions and regularly fol-lowed during 5 months prior to euthanasia by cervical dislocation.Grafted ovarian tissues were retrieved and the MDD testing processincluded histology, IHC and RT-ddPCR, as described above.

Statistical analysis

Statistical parameters were calculated using GraphPad Prism software,version 8 (GraphPad Software Inc, CA, USA). Statistical significancewas set at P 0.05.

Ethical approval

Use of human ovarian tissue was approved by the Ethics Committeeof the Cliniques Universitaires Saint-Luc and the Institutional ReviewBoard of the Universite´ Catholique de Louvain (IRB reference 2012/23MAR/125, registration number B403201213872). Among the sur-viving participants, one cryovial of ovarian cortex per patient wasthawed after obtaining their written informed consent. Animal welfarewas fully respected and complied with all guidelines endorsed by theCommittee on Animal Research of the institution (reference 2014/UCL/MD/007).

Results

Patients

Of the 20 patients included in the study, the mean age at which ovarian tissue was frozen was 10.5 (SD 7.2) years, and 70% of them were under 13 years of age. Only two patients were aged over 20 years. One patient suffered metastases to the lungs one year after her initial diagnosis. Malignancies recurred in five patients (two cases of ependymoma, one glioblastoma, one medulloblastoma and one PNET) in the period from 2 to 14 years post-diagnosis. Two patients who did notrelapse had ventriculoperitoneal (VP) shunts fitted 1 and 2 monthsprior to ovarian tissue collection for cryopreservation. IHC analysiswas performed in all 20 patients, while molecular biology was investi-gated in 15 subjects. Detailed characteristics of patients are listed inTable I.

Histology and immunohistochemistry

H&E sections of frozen-thawed ovarian cortex from 20 patients withCNS tumours were analysed by light microscopy. All samples showedovarian follicles at different developmental stages. No malignant cellswere detected in the ovarian tissue of any of subjects. The morphol-ogy of patients' primary tumours was also included in the analysis(Fig. 1).Results of GFAP and NSE immunostaining of patients' primarytumours and frozen-thawed ovarian tissue are shown in Table I.Fourteen patients exhibited positive expression of both markers in pri-mary tumours, while the remaining subjects only showed positive ex-pression in one marker. However, all 20 frozen-thawed ovariansamples from these patients were negative for both GFAP and NSE byIHC analysis (Fig. 2) (Table I).

Droplet digital PCR

Limit of blank and limit of detection of ENO2 and GFAP transcriptsThe LOB of ENO2 in normal human ovarian tissue was calculated asthe 95th percentile of the distribution of

blank values, resulting in 28.5copies/ml. The LOD, calculated on sequential medulloblastoma biop-sies in pooled samples of 10 fragments of normal ovarian tissue, was0.068 and more accurately resulted in 30.7 copies/ml for ENO2(Supplementary Fig. S1). In this case, the pool of 10 normal ovarian tis-sue samples, namely blank samples, produced a moderate signal forENO2. This marker was not therefore used for detecting seeding ofmedulloblastoma cells in ovarian tissue.Conversely, the LOB of GFAP in normal ovarian tissue was signifi-cantly lower, yielding 0.1 copies/ml. This value was used to assess theresults from patients suffering from CNS cancers, who showed posi-tive expression of GFAP in primary tumours, considered to have‘detected’ or ‘not detected’ malignant cells in their cryopreservedovarian tissue. LOD values of GFAP for detecting the presence of me-dulloblastoma, astrocytoma, ependymoma and glioblastoma were0.0013 (0.18 copies/ml), 0.00014 (0.27 copies/ml), 0.00017 (0.16 cop-ies/ml) and 0.00013 (0.22 copies/ml) of standard dilutions, respec-tively. Detection of the GFAP gene by ddPCR was achieved from thestandard 0.01% dilution, suggesting the high sensitivity of this tech-nique. In these cases, the R2 value of 10-fold-diluted curves rangedfrom 0.95 to 0.99, indicating remarkable correlation and noconcentration-dependent bias (Fig. 3).

Detection of GFAP gene amplification in cryopreserved ovariantissue

Levels GFAP gene expression determined by RT-ddPCR were quanti-fied absolutely in 15 patients in the present study. No GFAP tran-scripts were detected in frozen-thawed ovarian tissue from sevenpatients (Patients 1–7) with astrocytoma, ependymoma and glioblastoma. Concentrations of GFAP gene amplification in negativecontrols (pool of normal ovarian tissue from 10 healthy women) andpositive controls were quantified at 0.07 copies/ml, 11300 copies/ml(astrocytoma), 2850 copies/ml (ependymoma) and 4170 copies/ml(glioblastoma),

respectively. The appearance of ABL1 transcripts in du-plex ddPCR runs highlighted the reliability of tested samples (Fig. 4).Regarding cryopreserved ovarian tissue from eight patients with me-dulloblastoma, concentrations of GFAP transcripts were calculated between zero and 0.07 copies/ml. All of these results were below theLOD of GFAP in human ovarian tissue from patients with medulloblas-toma. Positive controls yielded 446 copies/ml, while no positive drop-lets appeared in negative control samples (Fig. 5). Sample 15 wasretested in duplicate due to its low concentration of ABL1 and no ma-lignant cells were detected (Supplementary Fig. S2). In these ddPCRtests, the number of analysed droplets ranged from 14 036 to 17 782,which met the requirement for ddPCR. In addition, no positive drop-lets were found in any no-template controls, indicating high testspecificity.In Patient 18, the primary tumour showed low concentrations ofENO2 gene transcripts (57.6 copies/ml), which was not statisticallystrong enough to distinguish the LOB for ENO2 (28.5 copies/ml).Hence, ENO2 could not be used as a marker to detect the presenceof PNET cells in ovarian tissue. In the four remaining subjects (Patients6 and 7 with germinoma; Patients 19 and 20 with PNET), primarytumours were not found, so RT-ddPCR did not apply.

Xenotransplantation

Histology and IHC

After 5.5 months of xenografting, human ovarian tissue was harvestedand investigated. All grafted tissues had decreased in size and no suspi-cious masses were detected in transplanted sites. Ovarian follicles atdifferent stages of development were identified in all samples. None of the 20 ovarian tissue samples showed any evidence of cancer cells from histology analysis (Fig. 1h and i). Similarly, all samples were negative for GFAP and NSE immunostaining (Fig. 2c and g).

RT-ddPCR evaluation

Molecular biology tests were conducted in 15 ovarian samples degrafted after long-term xenotransplantation. In the group of subjects with astrocytoma, ependymoma and glioblastoma, xenografted ovarian tissue from Patient 1 (ependymoma) and Patient 4 (glioblastoma) each expressed 0.07 copies/ml of GFAP transcripts. Both results were below the GFAP LOD in ovarian tissue affected by ependymoma and glioblastoma (which yielded 0.16 copies/ml and 0.22 copies/ml, respectively) (Fig. 6a). No GFAP gene amplifications were detected in the remaining samples, while concentrations of positive and negative controls were 7120 copies/ml (astrocytoma), 2184 copies/ml (ependymoma), 4390 copies/ml (glioblastoma) and 0.15 copies/ml, respectively (Table I and Fig. 6a). GFAP transcripts were also undetected in eight samples from medulloblastoma patients, with concentrations showing 437 copies/ml in positive controls and 0.09 copies/ml in negative controls (Fig. 6b). At the first attempt, amplification from xenografted sample 10 was lost due to problems in droplet generation. This sample was subsequently retested in duplicate and displayed no GFAP transcripts (Supplementary Fig. S2). In summary, no malignant cells were detected in any of the xenotransplanted samples. No-template control wells also found no positive droplets in any ddPCR runs. Concentrations of the ABL1 housekeeping gene appeared stable in duplex reactions and expression of B2M and GAPDH was clearly identified in all simplex wells. These figures emphasize the reliability of tested samples and procedures.

Discussion

The present study is the largest series to date to assess the safety of ovarian tissue cryopreservation and transplantation in patients suffering from CNS cancers (n ¼ 20). CNS tumours are relatively common in children and young adults, showing the second highest

399 incidence after leukaemia (Siegel et al., 2018). Neurological malignancies account for 5% of
400 indications for ovarian tissue cryopreservation in our department (Dolmans et al., 2013;
401 Jadoul et al., 2017). In a study by Poirot et al. (2019), these diseases ranked third in terms of
402 prevalence, making up 11% of patients undergoing ovarian tissue cryopreservation
403 procedures, but we found medulloblastoma to be the most common type of CNS cancer,
404 affecting 40% of subjects having their ovarian tissue frozen. Indeed, this cancer is the most
405 frequently encountered malignant brain tumour in childhood, representing 16.7–20% of all
406 paediatric brain cancers (Rickert and Paulus, 2001; Ostrom et al., 2015). It is assumed that
407 the incidence and subtypes of CNS tumours differ between paediatric and adult populations
408 (Ostrom et al., 2015; Siegel et al., 2018). We used the patients' primary tumours as positive
409 controls to analyse and compare the presence of cancer cells in their ovarian tissue by
410 histology, IHC and RT-ddPCR. None of the tests detected any seeding of cancer cells in
411 cryopreserved and xenotransplanted ovarian tissue, so we did not identify any differences in
412 our findings according to age. Recurrence of primary tumours was observed in 25% of our
413 cases, while metastasis was found in one patient with PNET, accounting for 5% of subjects. In
414 our series with CNS tumours, 40% of patients died, which was a higher rate than that
415 reported by Poirot's team (28.3%) and also higher than general mortality in patients
416 undergoing ovarian tissue cryopreservation published by our team (13.8%) (Jadoul et al.,
417 2017; Poirot et al., 2019). One out of 20 subjects in this study underwent ovarian tissue
418 transplantation. This uptake rate was consistent with the literature, ranging from 2.2% to
419 8.7% and mainly dependent on subject age at the time of ovarian tissue cryopreservation
420 (Rosendahl et al., 2011; Dolmans et al., 2013; Jadoul et al., 2017; Poirot et al., 2019;
421 Hoekman et al., 2020). Indeed, in our study, 70% of patients were under the median age of
422 menarche (13 years) in Belgium (Roelants et al., 2009). As ovarian tissue cryopreservation is

the only way of preserving fertility in prepubertal girls (Donnez and Dolmans, 2017), assessing the presence of MDD in cryopreserved ovarian tissue is of paramount importance.

Extraneural metastasis of CNS tumours

Primary CNS cancers can spread beyond the brain to form extraneural metastases, although the incidence is low, in the range of 0.96–15% (Morrish, 1994; Houston et al., 2000; Rickert, 2003; Varan et al., 2006; Smoll and Villanueva, 2010). Among paediatric patients, medulloblastoma is by far the most common entity, responsible for 21– 56.3% of extraneural metastasis cases (Rickert, 2003; Xu et al., 2018). Common sites of distant metastasis of CNS tumours vary depending on cancer types and mainly occur in regional lymph nodes, lungs and vertebral bones (Schweitzer et al., 2001; Varan et al., 2006; Smoll and Villanueva, 2010). One of our 20 subjects suffered pulmonary metastasis one year after complete resection of her primary PNET. Peritoneal seeding of malignancy has also been found in distant metastases from CNS tumours, but principally in subjects who underwent prior VP shunt placement (Rickert, 2003; Muoio et al., 2011; Xu et al., 2018). In our study, two of the 20 patients received a VP shunt 1–2 months before ovarian tissue collection and cryopreservation, so testing for MDD was crucial. Fortunately, our investigations revealed that the cryopreserved and xenografted ovarian tissue from these patients was not contaminated by malignant cells, as confirmed by histology, IHC and ddPCR. Ovarian metastases from CNS tumours are rarely reported in the literature, despite having been observed in most malignancies (de Waal et al., 2009; Kyono et al., 2010). There have been two docu-mented cases of ovarian metastasis from medulloblastoma. The firstwas published by Paterson in 1961, involving a 4-year-old girl affectedby the disease. She was diagnosed with metastases to the bones,lymph nodes, kidney, uterus and ovary after surgical

intervention, in-cluding an operation and shunt insertion (Paterson, 1961). In 2001,Lamovec and Pogae`nik (2001) described a 33-year-old woman withmedulloblastoma, whose malignancy had spread beyond the brain toher breast and then to her right ovary and peritoneum. Histologicaland immunohistochemical findings showed features identical to her pri-mary cerebellar cancer, medulloblastoma (Lamovec and Pogae`nik,2001). Despite limited data on ovarian metastases from CNS tumours,these two cases obviously highlight the potential risks and call for ut-most caution when cryopreserving and transplanting tissue from thispopulation.

Detection of MDD in cryopreservedovarian tissue from patients with CNStumours

Reports in the literature on MDD incidence in ovarian tissue fromwomen with CNS tumours undergoing ovarian tissue cryopreservationare lacking. Azem et al. (2010) evaluated ovarian biopsies from one patient with medulloblastoma who underwent ovarian tissue cryopreservation. No malignant cells were detected by histology (Azem et al., 2010). Regarding methods to search for malignant cells in ovarian tissue, investigators have used histology, IHC and PCR, each with its pros and cons. Histology is a technique that can identify clusters of cancerous cells, but individual cells may also provoke recurrence of primary tumours when tissue is transplanted. IHC is a sensitive approach requiring specific markers for tumour cells (not usually expressed in normal ovarian tissue). In the present study, we used two IHC markers, namely NSE and GFAP. Both these markers were found to show immunoexpression in over 90% of astrocytomas, glioblastomas and ependymomas. Regarding medulloblastoma and PNET, NSE is expressed in 50–90% of cases, whereas GFAP shows lower positive immunostaining levels (10–50% of cases) (Tuffaha et al., 2018).

RT-PCR, especially RT-ddPCR, is a highly sensitive technique able to detect small quantities of genetic materials. However, it requires a known sequence specific to primary tumours that is not expressed in ovarian tissue from healthy women. In our study, normal ovaries used as controls showed ENO2 expression at moderate background levels assessed by ddPCR. Indeed, ovarian tissue contains nerve structures consisting of neuronal-origin cells that give rise to ENO2 and can be quantified by ddPCR at a molecular level, but cannot be detected by IHC. Consequently, this molecular marker was not applied to diagnose residual malignant cells in our patients' ovarian tissue. Instead, the GFAP gene sequence was used as a target for MDD detection in ovarian tissue from 15 patients with medulloblastoma, astrocytoma, ependymoma and glioblastoma in our series. Unlike its counterpart, GFAP amplification provides high specificity to detect CNS cell infiltration in ovarian tissue, with a low LOB and LOD. In patients with germinoma and PNET, we attempted to identify GFAP gene transcripts but were unsuccessful dueto the unavailability of primary tumours to serve as positive controls.Xenotransplantation to immunodeficient mice may be deemedtime-consuming and costly. However, this method is currently consid-ered the best available strategy to examine ovarian tissue for the pos-sible presence of disseminated malignancy (Rosendahl et al., 2013;Rauff et al., 2016). Representative ovarian fragments are transplantedto immunodeficient host animals like SCID mice, which assume therole of bioincubators, potentially giving rise to viable neoplastic cellsover the course of 20–24 weeks. This approach was applied in ourstudy to investigate MDD in ovarian tissue from all 20 subjects withCNS tumours. Since (i) evidence of the safety of ovarian tissue cryo-preservation and transplantation in patients with CNS tumours is stilllacking, (ii) two publications have reported metastases to patients' ova-ries from medulloblastoma (Paterson, 1961; Lamovec and Pogae`nik,2001) and (iii) some of our patients underwent ovarian tissue collec-tion after

494 insertion of a VP shunt, which may increase the threat ofcancer cell spread to the peritoneal
495 cavity (Xu et al., 2018), we se-lected the xenotransplantation model to determine the risk of
496 reseed-ing cancer cells. We believe that this strategy can overcome some ofthe limitations of
497 other methods.It is, however, undeniable that analyses like pathology and molecularbiology
498 cannot proceed in ovarian fragments destined for transplantationdue to their destructive
499 manipulation. Examined ovarian fragments repre-sent only a small fraction of the total
500 ovary, so do not exclude the possi-bility of malignant cells being present in other pieces of
501 ovarian cortex.The exact number of cancer cells required to cause a relapse is unknownand
502 variable between individuals and malignancies. Therefore, case bycase evaluation is required
503 according to tumour type. We recommendanalysing a sample of cryopreserved ovarian
504 tissue before transplantation.In order to increase the sensitivity of malignant cell detection,
505 a combina-tion of available approaches including histology, IHC, molecular biologyand
506 xenotransplantation should be used. Nevertheless, the risk is verylow in case of CNS
507 tumours, as demonstrated by our results. All in all, our findings revealed no malignancy in
508 cryopreserved ovarian tissue from 20 patients by histology, IHC for NSE and GFAP, RTddPCR
509 for detection of GFAP gene transcripts, or xenotransplantation to SCID mice. The risk of
510 reseeding cancer cells when transplanting ovarian tissue in these patients can therefore be
511 considered low. Indeed, our results indicate that the risk of MDD in CNS patients is minimal.
512 However, although the current literature shows no evidence of ovarian tissue
513 transplantation resulting in recurrence of malignancy (Rosendahl et al., 2013; Meirow et al.,
514 2016; Wallace et al., 2016; Donnez and Dolmans, 2017; Gellert et al., 2018; Lotz et al., 2019),
515 we must remain cautious. The risk of ovarian infiltration cannot be entirely ruled out for any
516 type of cancer due to the lack of highly specific detection methods at the time of
517 transplantation and possible bias in sampling (Bastings et al., 2013). It is vital that we give

patients adequate information and conduct investigations on in cryopreserved ovarian tissue using individualized methods specific to primary cancers, while counselling women looking to undergo ovarian tissue transplantation.

Conclusions

In conclusion, we did not detect the presence of cancer cells in any of our ovarian samples from 20 subjects with CNS tumours by histological analysis, IHC, RT-ddPCR or xenotransplantation. Nevertheless, comprehensive and multidisciplinary investigations are an absolute prerequisite prior to transplantation, using diseasespecific and highly sensitive methods. Further research needs to be conducted on larger series to refine our knowledge of MDD in cryopreserved ovarian tissue in order to ensure the safety of the procedures.

Supplementary data

Supplementary data are available at Human Reproduction online.

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Authors' roles

T.Y.T.N. was responsible for the experimental design, experimental procedures, analyses, statistical analysis, interpretation of results and manuscript preparation; L.C. contributed to the experimental procedures; A.C. was involved in the analyses and interpretation of results; J.R. participated in the experimental procedures; M.D.V. helped interpret results; I.D. helped interpret results; J.D. participated in interpreting results and manuscript revision. M.-M.D. was the principal investigator and contributed to the experimental design, interpretation of results and manuscript revision. All authors revised and approved the final version of the manuscript.

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Conflict of interest

The authors have no competing interests to declare in connection with this study.

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