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ORIGINAL ARTICLE *Infertility*

# Evaluation of minimal disseminated disease in cryopreserved ovarian tissue from bone and soft tissue sarcoma patients

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**STUDY QUESTION:** What is the risk of finding malignant cells in cryopreserved ovarian tissue from sarcoma patients?

**SUMMARY ANSWER:** Minimal disseminated disease (MDD) was not detected in frozen-thawed ovarian tissue from 26 patients by any of the sensitive methods applied.

**WHAT IS KNOWN ALREADY:** In case of leukemia, the risk of malignant cell transmission through the graft is well known and widely documented. However, for bone cancer, like Ewing sarcoma or osteosarcoma, only a small number of case reports, have been published. These cancers often affect prepubertal girls, in whom ovarian tissue cryopreservation and transplantation is the only option to preserve fertility.

**STUDY DESIGN, SIZE, DURATION:** The presence of malignant cells in cryopreserved ovarian tissue from patients with bone/soft tissue sarcoma was investigated with disease-specific markers for each patient, using immunohistochemistry (IHC), FISH and real-time quantitative RT-PCR (qPCR), with the original tumor serving as a positive control.

**PARTICIPANTS/MATERIALS, SETTING, METHODS:** Forty-eight sarcoma patients were enrolled in the study, 12 of whom subsequently died. In each case, tissue from the primary tumor was investigated in order to identify markers (immunohistochemical and/or molecular) to analyze the ovarian tissue case by case. Ovarian tissue from osteosarcoma ($n = 15$), liposarcoma ($n = 1$) and undifferentiated sarcoma ($n = 5$) patients could not be evaluated, as no specific markers were detected by FISH or sensitive IHC in any of their primary tumoral tissue. One patient with Li-Fraumeni syndrome was also excluded from the study. IHC analyses were therefore performed on ovarian tissue from 26 patients and qPCR on 19. The primary tumors involved were Ewing sarcoma family of tumors ($n = 14$), rhabdomyosarcoma ($n = 7$), synovial sarcoma ($n = 2$), clear cell sarcoma ($n = 2$) and a malignant peripheral nerve sheath tumor ($n = 1$).

**MAIN RESULTS AND THE ROLE OF CHANCE:** MDD was not detected in any of the 26 analyzed samples using sensitive techniques in this largest reported series, even from patients who subsequently died and/or those who presented with metastasis (11/26), hence the most aggressive forms of bone cancer. Indeed, anti-CD99 IHC and PCR performed on patients presenting with Ewing sarcoma family of tumors ($n = 14$) was negative in all cases. In patients with soft tissue sarcoma ($n = 12$) primitive tumor markers were detected by IHC and were negative in ovarian tissue. PCR could only be performed in 6/12 of these patients, again proving negative.

**LIMITATIONS, REASONS FOR CAUTION:** Cryopreserved ovarian fragments to be transplanted cannot be tested, so this analysis of malignant cells cannot guarantee that all cryopreserved fragments will not contain any disseminated disease. Moreover, molecular markers are not readily available for all types of tumors.

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**WIDER IMPLICATIONS OF THE FINDINGS:** These results are reassuring regarding the risk of malignant cells in the ovary for transplantation, as the study involves a large series including different types of sarcomas. We believe this will help clinicians in their patient counseling for fertility preservation and restoration.

**STUDY FUNDING/COMPETING INTEREST(S):** This work was supported by the Fonds National de la Recherche Scientifique de Belgique-FNRS under Grants Nos 7.4578.14 (Télévie to MS) and 5/4/150/5 to MMD. The authors declare no competing financial interests.

**Key words:** Ewing sarcoma / rhabdomyosarcoma / ovarian tissue cryopreservation / ovarian metastasis / molecular markers / minimal disseminated disease / malignant cell detection / fertility preservation

# Introduction

Sarcomas are one of the most commonly encountered cancers in children, adolescents and young adults. According to Surveillance Epidemiology and End Results (SEER) program statistics from 2005 to 2009, bone and soft tissue tumors account for an estimated 11% of all cancers in patients aged 0 to 14 years (*National cancer institute web site:* http://www.cancer.gov/types/childhood-cancers/hp/unusual-cancers-childhood-pdq#section/_105). Bone and soft tissue tumors have the third highest incidence rate after leukemia (31.3%) and central nervous system tumors (25.4%) in this age group. In patients aged 15 to 19 years, the incidence rate is 13%, making it the fourth highest *(National cancer institute web site:* http://www.cancer.gov/types/childhood-cancers/hp/unusual-cancers-childhood-pdq#section/_105). Recent studies indicate that there were around 114 000 new cases of soft tissue sarcoma (STS) and 3000 cases of bone sarcoma diagnosed in the USA in 2013 (National cancer institute website http://www.cancer.gov/researchandfunding/snapshots/sarcoma). Osteosarcoma develops in bone. Although Ewing sarcoma (EWS) is usually found in bone too, it can be located in soft tissue, especially in older patients. The Ewing sarcoma family of tumors (ESFT) includes bone and soft tissue EWS, as well as peripheral primary neuroectodermal tumors (PNET). Up to 40% of patients with localized EWS have disseminated disease in their bone marrow, associated with an increased risk of systemic relapse (Avigad *et al.*, 2004).

STS forms in fat, muscle, fibrous tissue, blood vessels or other supporting tissue of the body. Among STS cases, rhabdomyosarcoma (RMS) is the most common pediatric manifestation. Around 20% of patients show disseminated disease at diagnosis, and the lungs, lymph nodes and bone marrow are frequently involved (Wexler *et al.*, 2001). Two major histological subtypes of RMS can be identified: embryonal RMS (ERMS), the most common form, and alveolar RMS (ARMS), with a bleak prognosis and a pronounced tendency to disseminate to distant sites. Most of these sarcomas (especially EWS, PNET and ARMS) are characterized by specific genomic aberrations detectable by molecular tools (Davicioni *et al.*, 2009).

Standard treatments for sarcoma include surgery, chemotherapy and radiotherapy. Aggressive chemotherapy and radiotherapy are able to cure increasing numbers of young patients with sarcoma, but these treatments often result in loss of ovarian function. Ovarian tissue cryopreservation is an option for fertility preservation in sarcoma patients of reproductive age, and the only fertility preservation option for prepubertal patients (Donnez and Dolmans, 2013).

Of the malignant diseases that constitute indications for ovarian tissue cryopreservation in our department, STS and bone sarcoma account for a figure as high as 11.1% of all procedures (Dolmans *et al.*, 2013a). However, while transplantation of frozen-thawed ovarian tissue is increasingly performed all over the world, so far resulting in the birth of 60 healthy children (Donnez *et al.*, 2013, 2015), there is a risk that this tissue might harbor malignant cells that could induce disease recurrence after transplantation (Dolmans *et al.*, 2013b). A number of case reports have been published on ovarian involvement in certain types of bone sarcoma, such as EWS (Young and Scully, 1990; Young *et al.*, 1993; Ateser *et al.*, 2007; Sullivan *et al.*, 2012) or osteosarcoma (Eltabbakh *et al.*, 1997; Barbancho *et al.*, 2007). On the other hand, ovarian involvement is rarely reported in RMS (Young and Scully, 1989, 1990), but often diagnosed at autopsy in the presence of advanced-stage disease.

Because sarcomas are a frequent indication for ovarian tissue cryopreservation but concerns have been raised regarding the presence of malignant cells, it is vital to evaluate the occurrence of minimal disseminated disease (MDD) in large series of cryopreserved ovarian tissue from sarcoma patients. Two studies from the literature have already investigated the involvement of frozen-thawed ovarian tissue in cases of EWS in series including, respectively, 8 and 9 patients with the disease (Abir *et al.*, 2010; Greve *et al.*, 2013). One patient proved positive for the *EWSR1* gene in her frozen-thawed ovarian tissue (Abir *et al.*, 2010).

In the present series, the possible presence of malignant cells in cryopreserved ovarian tissue from patients with bone sarcoma and STS was investigated with disease-specific markers for each patient, using immunohistochemistry (IHC), fluorescent in situ hybridization (FISH) and real-time quantitative RT-PCR (qPCR).

# Materials and Methods

## Patients

Forty-eight bone sarcoma and STS patients (Fig. 1) had ovarian tissue stored in our cryobank for fertility preservation purposes at the time of the study, 36 of whom are still alive and 12 deceased. Mean age at ovarian tissue cryopreservation was 16.3 years (16.3 ± SD 7.27). Our objective was to identify immunohistochemical and/or molecular markers in tissue from their primary tumors in order to have disease-specific markers for analysis in ovarian tissue.

Primary tumoral tissue from osteosarcoma ($n$ = 15), liposarcoma ($n$ = 1) and undifferentiated sarcoma ($n$ = 5) patients showed no specific molecular markers by FISH or IHC, so could not be analyzed (Table I). Two patients diagnosed with Li-Fraumeni syndrome, a cancer predisposition disorder, were excluded from this study due to the risk of multifocal cancers (one of whom had an undifferentiated sarcoma with no markers, and the other, a RMS) (see Table I).

Twenty-six samples of frozen-thawed human ovarian tissue were therefore subjected to IHC and 19 to qPCR. The amount of tissue thawed represented less than 10% of the total quantity of cryobanked ovarian cortical tissue. For sarcoma patients with too little tissue cryopreserved to use, RNA was extracted from paraffin blocks ($n = 6$).

Use of human tissue for this study was approved by the ethics committee of the Université Catholique de Louvain (n° B403201213872). For surviving patients, one cryovial of ovarian tissue per patient was thawed after obtaining written informed consent.

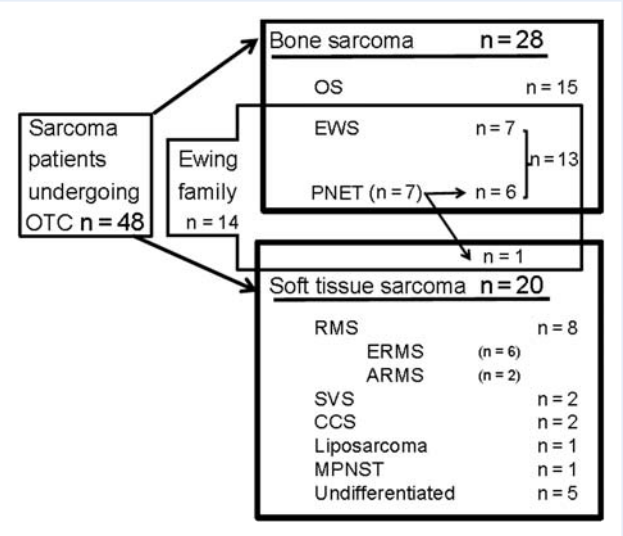


**Figure 1** Diagnosis of patients with sarcoma who have ovarian tissue cryobanked. OTC: ovarian tissue cryopreservation. OS: osteosarcoma. EWS: Ewing sarcoma. PNET: peripheral primary neuroectodermal tumor. RMS: rhabdomyosarcoma. ERMS: embryonal RMS. ARMS: alveolar RMS. SVS: synovial sarcoma. CCS: clear cell sarcoma. MPNST: malignant peripheral nerve sheath tumor.

## Freezing and thawing procedures

Freezing and thawing of ovarian tissue was undertaken according to the protocol described by Gosden *et al.* (1994), as detailed in a previous paper (Dolmans *et al.*, 2010). Briefly, biopsy samples taken at the time of ovarian tissue cryopreservation were cut into small strips (+/– 4 × 1–2 mm), slow-frozen with 10% dimethyl sulfoxide as a cryoprotectant, and placed in cryovials. For quality control and assessment of the absence of malignant cells, one cryovial was thawed. For thawing, the cryogenic vials were kept at room temperature (between 21°C and 23°C) for 2 minutes and then immersed in a water bath at 37°C for another 2 minutes. The tissue was immediately transferred from the vials to tissue culture dishes in Leibovitz (L-15) medium (GIBCO, Paisley, Scotland, UK), and subsequently washed three times (5 minutes each) at room temperature with fresh medium to remove the cryoprotectant before further processing. Tissue from one vial (one large fragment or several small strips) was divided up for IHC and qPCR.

## Histological evaluation and IHC

After thawing, a piece of ovarian tissue was fixed in 4% formaldehyde and embedded in paraffin. Serial 5 µm-thick sections were taken and stained with hematoxylin and eosin (HE) (Merck, Germany) for histological evaluation by expert pathologists to detect the presence of malignant cells and assess follicle density. For each patient, at least four sections of ovarian tissue were stained by IHC. IHC markers were selected based on anatomical pathology analysis of the primary tumor. Paraffin-embedded (5 µm-thick) sections of the samples were deparaffinized (Histosafe, Yvsolab SA, Belgium) and rehydrated in 2-propanol (Merck, Germany).

After blocking endogenous peroxidase activity with $H_2O_2$ 0.3%, a demasking step was performed for 75 minutes at 98°C with citrate buffer and Triton X100. Sections were incubated for 30 minutes with 10% normal goat serum (NGS, GIBCO, New Zealand) and 1% bovine serum albumin (BSA, Sigma-Aldrich, St Louis, MO, USA) to block nonspecific binding sites. They were then incubated overnight at 4°C with primary antibodies (listed in Supplementary Table I).Antibodies against CD99 (for EWS), myogenin and MyoD1 (for RMS), and Bcl-2 (for *synovial* sarcoma) (all from DAKO, Glostrup, Danemark) were used at dilutions of 1:75, 1:200, 1:100

**Table I Number of sarcoma patients with different pathologies from whom primary tumor tissue and ovarian tissue could be analyzed by different techniques.**

| | | Number of patients | Number of deceased patients | Number of Li-Fraumeni syndrome patients | Primary tumors available for analysis (*n*) | | | Ovarian tissue available for analysis (*n*) | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | FISH | qPCR | IHC | qPCR | IHC |
| OS | | 15 | 2 | 0 | 13 | 0 | 0 | 0 | 0 |
| ESFT | | 14 | 5 | 0 | 11 | 12 | 14 | 13 | 14 |
| RMS | ERMS | 6 | 1 | 1 | 4 | 4 | 4 | 0 | 5 |
| | ARMS | 2 | 1 | 0 | 2 | 2 | 2 | 2 | 2 |
| SVS | | 2 | 0 | 0 | 1 | 1 | 1 | 2 | 2 |
| CCS | | 2 | 1 | 0 | 1 | 1 | 1 | 2 | 2 |
| Liposarcoma | | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| MPNST | | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| Undifferentiated | | 5 | 1 | 1 | 4 | 0 | 5 | 0 | 0 |
| **Total** | | **48** | 12 | 2 | 37 | 20 | 28 | **19** | **26** |

OS, osteosarcoma; ESFT, Ewing sarcoma family of tumors; RMS, rhabdomyosarcoma; ERMS, embryonal rhabdomyosarcoma; ARMS, alveolar rhabdomyosarcoma; SVS, synovial sarcoma; CCS, clear cell sarcoma; MPNST, malignant peripheral nerve sheath tumor; IHC, immunohistochemistry; qPCR, quantitative PCR.

and 1:100, respectively (Supplementary Table I). The slides were subsequently incubated with goat anti-mouse IgG (Envision, DAKO, Glostrup, Danemark) for 60 minutes at room temperature. Diaminobenzidine was used as a chromogen (SK 4100, Vector Laboratories, Burlingame, CA). Counterstaining was performed with hematoxylin before mounting the slides with DPX neutral mounting medium (Sigma-Aldrich, St Louis, MO, USA).

For detection of PNET and clear cell sarcoma, staining of NSE (neuron-specific enolase), S100, MDM2 (murine double minute 2), and melanoma cocktail (DAKO, DAKO, Calbiochem and Biocare respectively) were performed automatically using the Ventana HX System BenchMark™ (Ventana Medical Systems, Tueson, USA). Immunoperoxidase staining was carried out according to the manufacturer's instructions (Ventana Medical Systems). Negative controls consisted of the dilution solution without any primary antibody. Paraffin-embedded sections of the original tumor were used as positive controls in each case.

## Karyotyping and FISH

Karyotyping of tumor samples was performed according to standard procedures. Tissues were mechanically and enzymatically disaggregated and cultured in Dulbecco's modified Eagle's medium, Life Technologies, Paisley, UKsupplemented with 20% fetal bovine serum, Grant Island, NY, USA for 2–18 days. Cells were treated overnight with colcemid (0.08 µg/ml). Following hypotonic treatment (0.8% sodium citrate for 20 minutes), the preparations were fixed three times in methanol/glacial acetic acid (at a 3:1 ratio). Metaphase cells were GTW-banded. Karyotypes are expressed according to the 2013 edition of the International System for Human Cytogenetic Nomenclature.

## FISH

Additional dual-color FISH experiments were conducted on fixed nuclei or formalin-fixed paraffin-embedded tissue using the following commercial probes: FLI1/EWSR1, EWSR1/ERG, PAX3, PAX7 (Cytocell, Cambridge, UK) and LSI EWSR1, LSI FKHR/FOXO1, LSI SYT/SS18 (Abbot SA, Wavre, Belgium). Bacterial artificial chromosome (BAC) probes RP11-831J22 and RP11-165G10, used to study the *ATF1* locus by dual-color FISH, were selected from the University of California, Santa Cruz (http://www.genome.ucsc.edu) database. BAC clones were obtained from the BACPAC Resources Center at the Children's Hospital Oakland Research Institute (Oakland, CA, USA). Extraction, labeling and hybridization were performed as previously reported (Duhoux *et al.*, 2011). A minimum of 100 nuclei were examined in each sample, and all hybridized metaphases were captured.

## RNA extraction from frozen-thawed ovarian tissue

Frozen-thawed ovarian tissue was immediately placed in TRIzol reagent (Invitrogen, Paisley, Scotland, UK) after thawing according to the manufacturer's recommendations, then ground before RNA extraction. RNA concentrations were measured using a Quant-iT RNA assay kit with the Qubit fluorometer (Invitrogen) and immediately frozen at –80°C until use.

## RNA extraction from formalin-fixed paraffin-embedded tissue blocks

For RNA extraction and reverse transcription, full-face 7 µm sections were cut from each formalin-fixed paraffin-embedded tissue block with a microtome, and stored in a microfuge tube at –80°C. A single unstained section from each block was deparaffinized and total cellular RNA was extracted using the RecoverAll™ Total Nucleic Acid Isolation Kit (Life Technologies, Carlsbad, CA, USA) according to the supplier's instructions. RNA was quantified on the NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Scientific, Waltham, MA, USA). RNA concentrations were finally adjusted to 1.0 µg of total RNA using the Savant SpeedVac SVC 100 H Centrifugal Evaporator (Thermo Scientific, USA).

## Reverse transcription (RT) and real-time quantitative PCR (qPCR)

cDNA synthesis followed by qPCR reactions were performed with the Ipsogen RT kit (Qiagen, Hilde, Germany) according to the manufacturer's instructions. Briefly, for cDNA synthesis, 1.0 µg of total RNA was denatured at 65°C for 5 minutes, chilled on ice and then 15 µl of RT premix (Qiagen) was added to the RNA in a final volume of 25 µL following the recommended protocol. After a first step at 25°C for 10 minutes, then 50°C for 60 minutes, RT was completed by inactivation at 85°C for 5 minutes. -qPCR amplification reactions were performed on the LightCycler 480 (Roche) using primers and probes listed in Table II. Amplification reactions were performed in simplicate with the LightCycler 480 SYBR Green I Master Mix (Roche,, Mannheim, Germany) in a final volume of 20 µL, with 2 µM of each primer and 5 µL cDNA. Thermal cycling was started with an initial heat activation step at 95°C for 10 minutes, followed by a denaturation step at 95°C for 10 seconds, and 45 cycles of annealing/extension (60°C for 30 seconds/72°C for 1 minute). Original tumor samples of each sarcoma were used as positive controls. For negative controls, we used 2 types of tissue issuing from 16 healthy human ovaries and fibrous scar tissues. Relative quantification was performed by Cq analysis and the results were expressed as the ratio of the target gene versus the housekeeping gene (Abelson: ABL and β2-microglobulin).

# Results

## Patients

Among the 48 sarcoma patients, 37 samples of primary tumoral tissue were analyzed by FISH in an attempt to identify a sensitive molecular marker that could be subjected to qPCR. Molecular markers were detected in 20 samples (Table I). Immunohistochemical markers were found in 28 tumors (Table I). These disease-specific markers were then applied to investigate ovarian tissue from 19 patients by qPCR and 26 by IHC (Table II).

In total, 26 patients (Tables III and IV) had their ovarian tissue analyzed; 14 cases of ESFT and 12 STS were examined for the possible presence of malignant cells using IHC and qPCR. The youngest patient was just 12 months old (no. 20 of STS). Two RMS patients had undergone chemotherapy prior to ovarian tissue cryopreservation. The characteristics of all sarcoma patients are detailed in Tables III and IV.

## Histology and IHC

By light microscopy, no malignant cells were detected in the ovarian tissue of any of the patients. Ovarian follicles were present in all samples.

Details of patients with ESFT are presented in Table III. Of the 14 patients with ESFT and frozen ovarian tissue, all primary tumors were positive for CD99 staining (Table III) and one tumor was also positive for NSE. Details of patients with STS are presented in Table IV. For the RMS subgroup ($n = 7$), both myogenin and MyoD1 were selected as markers. Immunohistochemical staining for myogenin was positive in 6 out of 7 primary RMS tumors. For MyoD1, one patient

**Table II Primer and probe sequences.**

| Assay | Translocation | Forward primer 5′ to 3′ | Reverse primer 5′ to 3′ | Probe 5′ to 3′ |
|---|---|---|---|---|
| Housekeeping gene | β2 -microglobulin | TGACTTTGTCACAGCCCAAGATA | AATCCAAATGCGGCATCTTC | TGATGCTGCTTACATGTCTCGATCCCA |
| | ABL | TGGAGATAACACTCTAAGCATAACTAAAGG | GATGTAGTTGCTTGGGACCCA | CCATTTTTGGTTTGGGCTTCACACCATT |
| Ewing sarcoma | EWS-FLI1 | GTCAACCTCAATCTAGCACAGGG | CTGTCGGAGAGCAGCTCCAG | CTCCTACCAGCTATTCCTCTACACAGCCGACT |
| | EWS-ETV1 | GTCAACCTCAATCTAGCACAGGG | TAAATTCCATGCCTCGACCAG | CTCCTACCAGCTATTCCTCTACACAGCCGACT |
| | EWS-ERG | TCAACCTCAATCTAGCACAGGG | CTGTCCGACAGGAGCTCCAG | CTCCTACCAGCTATTCCTCTACACAGCCGACT |
| Rhabdomyosarcoma | PAX3-FOXO1 | TCCAACCCCATGAACCCC | GCCTTGGAAAACTGTGATCC | ATGCTCAATCCAGAGGGTGGCAAGAG |
| | PAX7-FOXO1 | CAACCACATGAACCCGGTC | GCCATTTGGAAAACTGTGATCC | ATGCTCAATCCAGAGGGTGGCAAGAG |
| | MyoD1 | AGGCGCCTACTACAACGAGG | CAGGCAGTCTAGGCTCGACAC | GCCCAGCGAACCCAGGCCCGGGAA |
| Synovial sarcoma | SYT-SSX | TACCCAGGGCAGCAAGGT | ATCGTTTTGTGGGCCAGATG | TCATGCCCAAGAAGC |
| Clear cell sarcoma | EWS-ATF1 | GTCAA CT AATCTAGCACAGGG | TCCATCAGTGGTCTGTGCATACTG | CTCCTACCAGCTATTCCTCTACACAGCCGACT |

ABL: Abelson.

(no. 17), who had already received chemotherapy prior to surgery, tested negative in her primary tumor (Table IV). For all other STS types (synovial sarcoma (SVS), clear cell sarcoma (CCS), and malignant peripheral nerve sheath tumors (MPNSTs), the selected markers are listed in Table IV. Paraffin-embedded sections from all primary tumor blocks were prepared and confirmed by positive immunohistochemical staining once again. None of the 26 ovarian tissue samples showed any evidence of cancer cells from IHC studies (Fig. 2) (Tables III and IV).

## Cytogenetics

Karyotyping and FISH were performed on available primary tumoral tissue. This allowed us to identify specific genomic aberrations, which were subsequently evaluated in frozen ovarian tissue.

Seven of the 14 ESFT patients presented with tumors harboring fusion genes: five patients with EWS-FLI1 (nos. 4, 6, 8, 12 and 13), one with EWS-ETV1 (no. 5), and one with EWS-ERG (no. 14) (Fig. 3 illustrates FISH in patient no. 4).

## qPCR analysis

RNA yield was satisfactory in all groups (quantification with the NanoDrop and Qubit fluorometer showed good results for RNA extracted, respectively, from formalin-fixed paraffin-embedded (FFPE) tissue and frozen-thawed ovarian tissue). Positive controls and control transcripts showed low cycle thresholds (Cp < 27).

### *Series of ESFT (Table III)*

In the seven samples from ESFT patients with primary tumors harboring fusion genes, qPCR was performed using each identified fusion gene as a molecular marker. None of the fragments of frozen-thawed ovarian tissue were found to express the relevant marker (Table III). The other seven ESFT patients displayed no molecular markers in their primary tumors, so we could not ascertain any information about fusion genes in their ovarian tissue. In these seven patients, qPCR of frozen-thawed ovarian tissue was done using the same set of primers that detects EWS-FLI1, EWS-ERG and EWS-ETV1 fusion genes. However, there was no evidence of this tissue expressing these three fusion genes (Table III). Although five ESFT patients were deceased (nos. 2, 4, 5, 6 and 7), there were no signs of sarcoma in their analyzed frozen-thawed ovarian tissue samples.

### *Series of RMS (Table IV)*

Eight patients had RMS (ARMS: $n = 2$; ERMS: $n = 6$). Primary tumors from both ARMS patients showed fusion genes. (no.15: PAX7-FOXO1; no.19: ARMS PAX3-FOXO1), which are specific for ARMS. qPCR was performed on ovarian tissue using each fusion gene as a marker, but neither of the frozen-thawed ovarian samples from these two ARMS patients expressed these markers (Table IV).

On the other hand, we could not identify any fusion genes in the primary tumors of the 6 ERMS patients. Indeed, MyoD1 was expected to be a good candidate, as previously published (Sartori *et al.*, 2006), but after testing on normal ovarian tissue, proved to be non-specific for sarcoma cells, as it was also found to be expressed by normal ovarian cells.

**Table III Details of patients with Ewing sarcoma family of tumors (ESFT) who underwent ovarian tissue cryopreservation for fertility preservation.**

| Patient no. | Age (y) | Diagnosis | Localized or metastasis | Tumor site | Tumor volume | Primitive tumor IHC markers | Original tumor genetic features | IHC (CD99) on ovarian tissue | Ovarian tissue PCR |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 12 | PNET | Localized | Left fibula | 97.2 ml | CD99(+) | NA | Negative | NA |
| 2# | 19 | PNET | Localized | Left ilium (coxal bone) | 720 ml | CD99(+) | NA | Negative | Negative[a] |
| 3 | 16 | EWS | Localized | Foot (metatarsal bone) | 18 ml | CD99(+) | No fusion gene | Negative | Negative[a] |
| 4# | 19 | PNET (STS) | Localized | Left adductor magnus muscle | 443 ml | CD99(+) | EWS-FLI1 | Negative | Negative |
| 5# | 21 | PNET | Metastasis (lung) | Right femoral bone (proximal) | 227 ml | CD99(+) | EWS-ETV1 | Negative | Negative |
| 6# | 11 | PNET | Localized | Right femoral bone (distal) | 158 ml | CD99(+)/NSE(+) | EWS-FLI1 | Negative/negative | Negative |
| 7# | 9 | PNET | Localized | Left fibula | 65 ml | CD99(+) | No fusion gene | Negative | Negative[a] |
| 8 | 19 | EWS | Localized | Right elbow bone | 63 ml | CD99(+) | EWS-FLI1 | Negative | Negative |
| 9 | 29 | PNET | Localized | NA | NA | CD99(+) | No fusion gene | Negative | Negative[a] |
| 10 | 25 | EWS | Localized | NA | NA | CD99(+) | No fusion gene | Negative | Negative[a] |
| 11 | 17 | EWS | Metastasis (lung) | 9th rib (pleural effusion) | 166 ml | CD99(+) | No fusion gene | Negative | Negative[a] |
| 12 | 15 | EWS | Localized | Right forearm | 3.6 ml | CD99(+) | EWS-FLI1 | Negative | Negative |
| 13 | 13 | EWS | Metastasis (lung + other) | Left ilium (sacrum) | 168 ml | CD99(+) | EWS-FLI1 | Negative | Negative |
| 14 | 14 | EWS | Metastasis (lung + other) | Left ilium | 1914 ml | CD99(+) | EWS-ERG | Negative | Negative |

#, Deceased patients; *PNET, peripheral primitive neuroectodermal tumor; STS, soft tissue sarcoma; NA, no tissue available for analysis.
[a]EWS-FLI1, EWS-ERG, and EWS- ETV1 were examined.

*Series of non-rhabdomyosarcomatous soft tissue sarcoma*

STSs other than RMS are known as non-rhabdomyosarcomatous STSs, whose most frequent presentations include SVS and CCS. Two patients with SVS, two with CCS, and one with MPNST underwent ovarian tissue cryopreservation.

Around 90% of SVS and CCS cases express SYT-SSX and EWS-ATF1 fusion genes (Wexler *et al.*, 2001). We detected these fusion genes in 2 primary tumors (nos. 22 and 24) and used them as molecular markers for each disease (Fig. 4 illustrates FISH in patient no. 22). These two patients (no. 22: SVS; no. 24: CCS) were diagnosed with stage IV disease (according to the Intergroup Rhabdomyosarcoma Study Group) and had lung metastasis. In spite of the high tumor stage, ovarian tissue samples from these patients showed no sign of the relevant markers. (Table IV). We could not evaluate whether the primary tumors of one SVS patient (no. 23) and one CCS patient (no. 25) expressed fusion genes or not, because these patients received cancer treatment in another hospital and primary tumor tissues were not available for analysis.

# Discussion

Sarcoma incidence is relatively high in children, adolescents and young adults, showing the highest rates after leukemia and central nervous system tumors. As ovarian tissue cryopreservation is one of the available options for fertility preservation (and the only one for prepubertal patients), evaluation of MDD in cryopreserved ovarian tissue is crucial.

In our department, indications for ovarian tissue cryopreservation in case of sarcoma (either STS or bone sarcoma) account for 11.1% of all procedures (Dolmans *et al.*, 2013a). The present study is the largest series to date to investigate the incidence of MDD in ovarian tissue from sarcoma patients ($n$ = 26 with disease-specific markers). Nevertheless, it still represents quite a limited number of patients, especially with advanced-stage disease, in whom it may be preferable to perform multicenter analyses.

Two previous studies (Abir *et al.*, 2010; Greve *et al.*, 2013) evaluated ovarian tissue from patients with EWS. In Abir's study, 8 patients were assessed for MDD by IHC (CD99), and 5 patients by identification of the EWS-FLI 1 fusion transcript in ovarian tissue using qPCR. Histology failed to reveal any EWS cells, but one of the five patients was positive for EWS-FLI 1. In Greve's study (Greve *et al.*, 2013), cryopreserved ovarian tissue from nine surviving EWS patients was not found to show any MDD by qPCR. Ovarian tissue from deceased patients could not be utilized, except in one case (Andersen *et al.*, 2014), which proved to be negative for specific molecular markers (Andersen *et al.*, 2014). Moreover, transplantation of ovarian tissue from EWS patients to immunodeficient mice for a period of time failed to induce the disease (Greve *et al.*, 2013). There are no reports in the literature on MDD incidence in the ovarian tissue of women suffering from STS.

**Table IV** **Details of patients with soft tissue sarcoma (STS) who underwent ovarian tissue cryopreservation for fertility preservation.**

| Patient no. | Age (y) | Diagnosis | Recurrence or metastasis | Chemotherapy before OTC | Tumor site | IRS stage or UICC system | IRS group (post-operation) | Primitive tumor IHC markers | Original tumor genetic features | Ovarian pathology and IHC | Ovarian tissue PCR |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 15# | 7 | ARMS | Recurrence | Yes | Right shoulder | Stage II | Group II | Myogenin/MyoD1 | PAX7-FOXO1 | Negative/negative | Negative |
| 16 | 12 | ERMS | No | No | Right eye | Stage I | Group III | NA | NA | Negative/negative | — |
| 17 | 6 | ERMS | No | Yes | Urinary bladder | Stage III | Group III | Myogenin | No fusion gene | Negative | — |
| 18# | 29 | ERMS | No | No | Left sternocleidomastoid | Stage I | Group II | Myogenin/MyoD1 | No fusion gene | Negative/negative | — |
| 19 | 11 | ARMS | No | No | Left nasal cavity | Stage I | Group III | Myogenin/MyoD1 | PAX3-FOXO1 | Negative/negative | Negative |
| 20 | 1 | ERMS | No | No | Vagina | Stage I | Group III | Myogenin/MyoD1 | No fusion gene | Negative/negative | — |
| 21 | 7 | ERMS | Lung meta | No | Nasal cavity | Stage IV | Stage IV | Myogenin/MyoD1 | No fusion gene | Negative/negative | — |
| 22 | 28 | SVS | Lung meta | No | Right femur | Stage IV | - | Bcl-2 | SYT-SSX | Negative | Negative |
| 23 | 13 | SVS | No | No | Right ankle | NA | - | NA | NA | Negative (Bcl-2) | Negative[a] |
| 24# | 10 | CCS | Lung meta | No | Right lower extremity | Stage IV | - | Melanoma cocktail/ S100 | EWS-ATF1 | Negative/negative | Negative |
| 25 | 27 | CCS | No | No | Colon | NA | - | NA | NA | Negative (Melanoma cocktail) | Negative[b] |
| 26# | 18 | MPNST | Lung meta | No | Right femur | Stage IV | - | S100 | No molecular marker | Negative | — |

#: Deceased patients; OTC, ovarian tissue cryopreservation; IRS, Intergroup Rhabdomyosarcoma Study (21, 22); UICC: Union for International Cancer Control; NA, no tissue available for analysis.
[a]SYT-SSX was examined.
[b]EWS-ATF1 was examined.

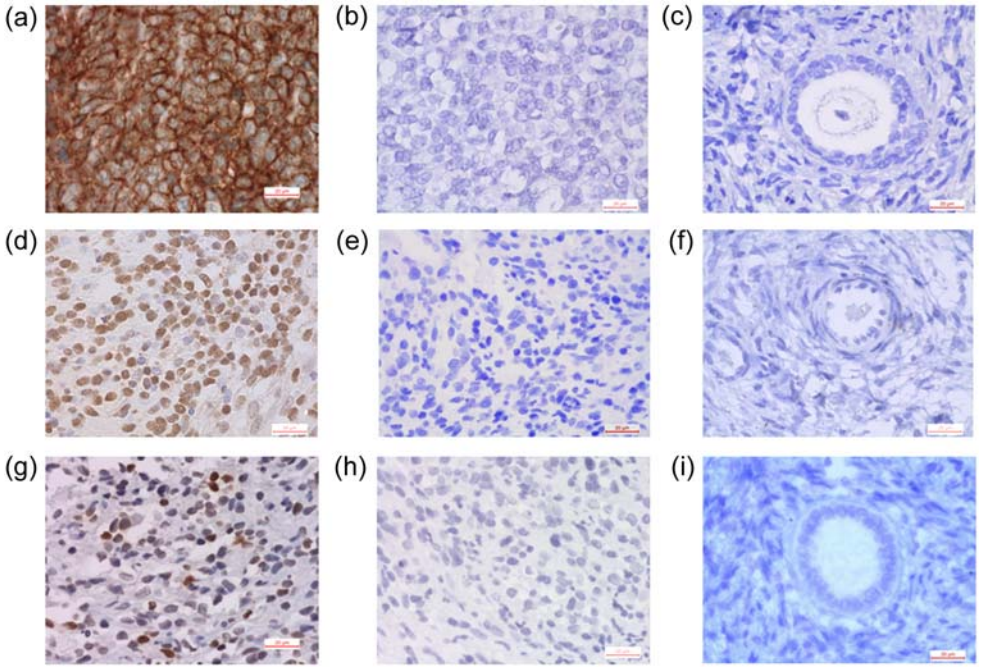


**Figure 2** Illustrations of histology and immunohistochemistry (CD99 and Myo-D1). CD99 immunohistochemistry: Positive (**a**) and negative (**b**) controls of EWS tumors. CD99 immunohistochemistry of frozen-thawed ovarian tissue from EWS patients (**c**). MyoD1 immunohistochemistry: Positive (**d**) and negative (**e**) controls of RMS tumor. MyoD1 immunohistochemistry of frozen-thawed ovarian tissue from RMS patients (**f**). Myogenin immunohistochemistry Positive (**g**) and negative (**h**) controls of RMS tumors. Myogenin immunohistochemistry of frozen-thawed ovarian tissue from RMS patients (**i**).

In our series, 48 sarcoma (7 of whom had EWS) patients had their ovarian tissue cryobanked. Of this number, 28 had bone sarcoma and 20 STS.

## Bone sarcoma

### *ESFT*

Ten of the 14 ESFT patients did not exhibit any metastasis, 2 showed only lung metastasis, and 2 showed lung and other metastasis. The incidence of metastasis in EWS is not low, showing rates as high as 18.4% to 27% (Cotterill *et al.*, 2000; Schleiermacher *et al.*, 2003). Patients with lung metastasis only have a better chance of survival than those with bone metastasis (Cotterill *et al.*, 2000). The Euro-EWING 99 study applied somewhat complex classification scheme that used metastatic status (none, lung, or other) and initial tumor size (with a 200 mL cut-off) (Balamuth *et al.*, 2010; Ladenstein *et al.*, 2010), while in our series, patients were classified according to the risk factors of this study.

### *Immunohistochemistry and IHC*

Eighty-five to 90% of EWS cells express the CD99 adhesion receptor (a 32-kDa cell surface glycoprotein encoded by the MIC2 gene), but CD99 is not specific for EWS, as it is often expressed in red blood cells and lymphoid cells of many normal tissues (Abir *et al.*, 2010).

Sorensen (Sorensen *et al.*, 2014) reported a case of a 12-year-old girl suffering from EWS localized in the thoracic vertebra. Ovarian tissue was biopsied for cryopreservation and revealed the presence of CD99-positive cells. In our study, all primary ESFT cases showed CD99-positive cells, but histology and IHC failed to demonstrate any MDD in ovarian tissue.

Our results corroborate those of Abir (Abir *et al.*, 2010), whose IHC studies also proved negative for eight patients. It should, however, be pointed out that IHC may not be sensitive or specific enough to identify microinvasion, and that other techniques such as qPCR or xenografting should possibly be applied.

Indeed, fusion transcripts are very sensitive and efficient markers of micrometastases. ESFT are characterized by chromosomal translocations involving the *EWSR1*/22q12 locus, mostly through the (11;22)(q24;q12) translocation that generates the EWSR1-FLI1 fusion protein in 90–95% of cases (Lessnick and Ladanyi, 2012).

However, variant translocations have also been reported, leading to fusion with other partner genes (around 10 already reported). In 5–10% of cases, the (21;22)(q22;q12) translocation generates a variant EWSR1-ERG fusion protein, while very rarely (<1% of cases), a t(7;22)(q22;q12) translocation generates the EWSR1-ETV1 fusion protein (Lessnick and Ladanyi, 2012).

In our study, these three types of fusion proteins were used as markers, but our analysis did not reveal any MDD in any of the samples of ovarian tissue analyzed. In Abir's study (Abir *et al.*, 2010), however, one ovarian tissue sample out of 5 was positive for EWSR1-FLI1.

Andersen *et al.* very recently reported a case of an adolescent EWS patient, who relapsed four years after being grafted with frozen-thawed ovarian tissue in order to induce puberty. After her death, all

eight remaining pieces of ovarian tissue were tested by qPCR using the EWSR1-FLI1 fusion protein (Andersen et al., 2014). EWSR1-FLI1 was not detected in any of the fragments, proving that the examined ovarian tissue did not contain any malignant cells. Nevertheless, as stressed by the authors themselves, this could not be ascertained for the two fragments of ovarian cortex that had been transplanted, as they could not be analyzed.

In conclusion, there have been three reports of MDD in the ovary (Sullivan et al., 2012; Sorensen et al., 2014) and ovarian tissue (Abir et al., 2010) from patients with EWS in recent years. We should therefore acknowledge that while the risk of ovarian involvement in case of ESFT is low, it is not zero. Histology and IHC should be carried out with immunohistochemical markers expressed by primary tumor cells. qPCR (fusion transcripts) should also be performed in order to inform patients accordingly.

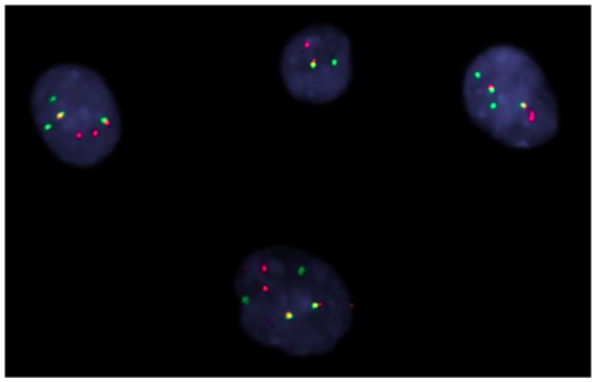

**Figure 3** Detection of the EWSR1-FLI1 fusion gene in primary tumor cells by FISH. FISH hybridization with the FLI1/EWSR1 probe (Cytocell) on a tumor imprint in case of Ewing sarcoma (patient 4): FLI1 is labeled in red (Texas Red® fluorochrome) and EWSR1 in green (FITC fluorochrome). The fusion gene is detected by a yellow signal, corresponding to colocalization of the red and green probes. In this figure, the diploid cell has one fusion gene in addition to its two normal loci (EWSR1 and FLI1), and the three larger cells have two fusion genes, probably corresponding to tetraploid status.

### *Osteosarcoma*

Osteosarcoma is the most common type of bone cancer to occur in children and adolescents. Our cryobank contains ovarian tissue from 15 patients suffering from this disease, 2 of whom have died. Histology of their ovarian tissue did not reveal MDD. We attempted to identify molecular markers in the primary tumor using FISH and qPCR, but were not able to do so. Additional research is now ongoing to determine specific molecular markers. Current efforts (Wang et al, 2016) are directed at detecting circulating microRNA in plasma to use as noninvasive biomarkers for the diagnosis of osteosarcoma.

In a study by Greve et al. (Greve et al., 2013), frozen-thawed ovarian tissue from five osteosarcoma patients was xenotransplanted to SCID (severe combined immunodeficient) mice for 20 weeks. After this period, the removed grafts did not show any MDD and the mice were found to be free of disease. These data on tissue from 20 patients with osteosarcoma are totally reassuring.

## Soft tissue sarcoma

### *Rhabdomyosarcoma*

RMS is the most common form of STS in children and adolescents, accounting for approximately 5% of all pediatric cancers and about 50% of all tissue sarcomas (Wexler et al., 2001; Yang et al., 2014).

As previously mentioned, there are two major histological subtypes of RMS, namely the embryonal form (ERMS), which is the most frequent (six out of eight cases in our series), and the alveolar form (ARMS), characterized by a more pronounced tendency to disseminate to distant sites. Five-year survival is approximately 64% (Yang et al., 2014).

According to clinical stage as defined by the Intergroup Rhabdomyosarcoma Study Group, taking into account size, local invasion, nodal involvement and the presence of distant metastases

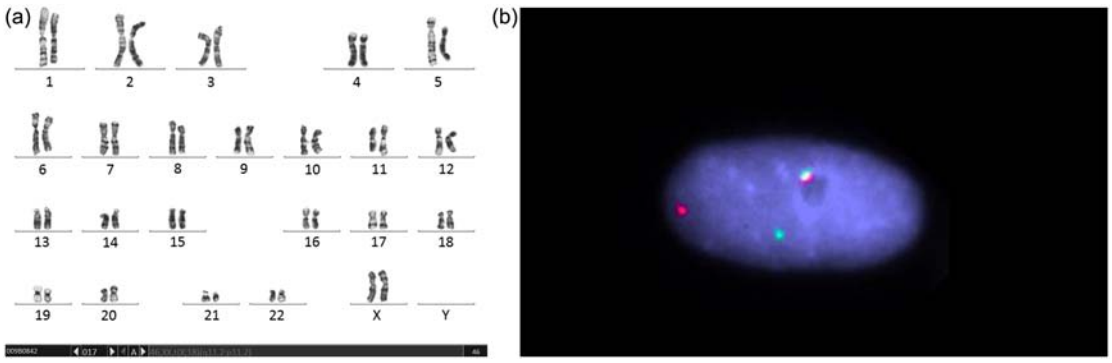


**Figure 4** Karyotyping and FISH hybridization with the LSI SYT/SS18 (Abbot) probe on a tumor imprint in case of synovial sarcoma (patient 22). (**a**) The karyotype exhibits the characteristic t(X;18)(p11.2;q11.2) translocation in the 20 analyzed mitoses. (**b**) FISH with a SYT/SS18 break-apart probe (mixture of a 5′/telomeric probe labeled with the SpectrumOrange fluorochrome and a 3′;/centromeric probe labeled with the SpectrumGreen fluorochrome). On this nucleus, one fusion signal corresponds to the normal SYT/SS18 allele, and the two separate dots (one green and one red) to the rearranged SYT/SS18 allele.

(Lawrence *et al.*, 1997), 2 of our patients had advanced-stage disease (stage III and IV).

Thanks to their high sensitivity for RMS in our study, both myogenin and MyoD1 antibody were used to test all primary RMS tumors by IHC. In 2001, Cessna *et al.* (Cessna *et al.*, 2001) analyzed 32 primary RMS tumors using both myogenin and MyoD1 antibody. All 32 RMS tumors expressed immunoreactivity for both antibodies, although MyoD1 often showed cytoplasmic and nonspecific background staining (Cessna *et al.*, 2001). In our series, all primary tumors expressed myogenin, and all but one (in a patient who had received chemotherapy before ovarian tissue cryopreservation) were positive for MyoD1. However, histology and IHC failed to demonstrate MDD in any samples of analyzed ovarian tissue.

Cytogenetic and molecular analyses of ARMS have demonstrated a specific chromosomal aberration involving the *FOXO1*/13q14 locus, either reciprocal translocation t(2;13)(q35;q14) or the variant t(1;13)(q36;q14), involving PAX3-FOXO1 and PAX7-FOXO1 fusion genes respectively. These fusion genes are specific for ARMS and our 2 ARMS patients were indeed found to have these genes.

As Sartori *et al.* (Sartori *et al.*, 2006) demonstrated that MyoD1 is specific for RMS cells, we selected the MyoD1 gene as a marker to detect MDD in ovarian tissue (in both ARMS and ERMS patients). However, in our study, normal ovaries used as controls also showed very weak expression of Myo-D1 by qPCR, indicating that this molecular marker cannot be used to detect MDD in ovarian tissue due to an obvious lack of specificity. Other mesenchymal neoplasms were also found to express MyoD1 (Krskova *et al.*, 2006, 2010).

*Non-rhabdomyosarcomatous soft tissue sarcoma*

Non-rhabdomyosarcomatous STS is a rare and heterogeneous form of disease that includes SVS, CCS and MPNSTS.

In our series, ovarian tissue from SVS 2 patients tested negative by IHC (Bcl-2) and qPCR (SYT-SSX). Greve *et al.* (Greve *et al.*, 2013) reported that frozen-thawed ovarian tissue from two SVS patients was xenotransplanted to SCID mice for 20 weeks, after which time the removed grafts did not show any MDD by histology. Ovarian tissue from two CCS patients also tested negative by IHC (HMB-45, melanoma A, MART-1, S-100 protein) (Wexler *et al.*, 2001) and qPCR (t(12;22)(q13;q12). As for MPNSTs, according to the literature NF1 is a common autosomal dominant disorder that has been mapped to 17q11.2, the site of the NF1 tumor suppressor gene. In addition, p53 point mutations (17p13) occur in MPNSTs (Wexler *et al.*, 2001). Based on these reports, we attempted to identify molecular markers using primary tissues, but were unsuccessful.

## Conclusion

In conclusion, of 48 women affected by sarcoma who had their cryopreserved ovarian tissue stored in our tissue bank, 26 could be assessed for the presence of MDD. We did not detect MDD in any of our analyzed samples. Our study is therefore quite reassuring regarding the risk of malignant sarcomatous cells in the ovary, as it involves a large series that includes different types of sarcomas. We believe this will help clinicians in their patient counseling. Nevertheless, the study has some limitations, as relevant and sensitive molecular markers could only be found for sarcomas with characterized fusion genes.

Detailed genetic characterization of the primary tumor is essential to be able to identify biomarkers to be tested by sensitive techniques on frozen ovarian tissue. At this stage in our understanding, we recommend performing histological analyses, IHC, FISH and qPCR (if markers are available), and xenotransplantation to SCID mice (if no markers are available), before any tissue is reimplanted. Only if the available analyses prove negative and show reasonable sensitivity can reimplantation go ahead. Research will now focus on use of digital droplet PCR in order to increase the sensitivity of molecular detection even further, and gathering data from large series to refine our knowledge of MDD in cryopreserved ovarian tissue, before contemplating transplantation in the safest conditions.

## Supplementary data

Supplementary data are available at http://humrep.oxfordjournals.org/.

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## Authors' roles

MMD was the principal investigator, designed the research program, and participated in writing the manuscript; YI performed laboratory investigations and participated in writing the manuscript; CAA and JD evaluated results and reviewed the manuscript; MS participated in laboratory investigations, including PCR and data collection; JLV gave advice for the technical parts of PCR and evaluated data; HP developed markers for detection, performed FISH and karyotyping, evaluated the results and participated in writing the manuscript.

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## Conflict of interest

The authors declare no competing financial interests.

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