Minimal residual disease detection of leukemic cells in ovarian cortex by eight-color flow cytometry

Clotilde Amiot¹,²,³,* , Fanny Angelot-Delettre¹,²,⁴,† , Tristan Zver¹,²,† , Magalie Alvergnas-Vieille¹,³ , Philippe Saas¹,²,⁴ , Francine Garnache-Ottou¹,²,⁴ , and Christophe Roux¹,²,³

¹INSERM UMR 1098, 1 Bd A Fleming, Besançon Cedex F-25020, France ²Université de Franche-Comté, SFR FED4234, Besançon Cedex F-25000, France ³CHU Besançon, Biologie de la reproduction, CIC-BTS06, Besançon Cedex F-25030, France ⁴EFS Bourgogne Franche-Comté, Besançon Cedex F-25020, France

*Correspondence address. E-mail: clotilde.amiot@univ-fcomte.fr

Submitted on July 17, 2012; resubmitted on February 28, 2013; accepted on April 2, 2013

STUDY QUESTION: How can leukemic cells be detected in cryopreserved ovarian tissue?

SUMMARY ANSWER: Multicolor flow cytometry (FCM) is useful to evaluate the presence of viable leukemic cells in the ovarian cortex with a high specificity and a robust sensitivity.

WHAT IS KNOWN ALREADY: Storing ovarian tissue is an option to preserve fertility before gonadotoxic radiotherapy or chemotherapy treatments. However, transplantation of cryopreserved ovarian cortex to women cured of leukemia is currently not possible due to the risk of cancer re-seeding.

STUDY DESIGN, SIZE, DURATION: We developed an automated ovarian cortex dissociation technique and we used eight-color FCM to identify leukemic cells with a series of dilutions added to ovarian single cell suspensions obtained from healthy cortex.

PARTICIPANTS/MATERIALS, SETTINGS, METHODS: Healthy ovarian cortex originated from women between 23 and 39 years of age undergoing laparoscopic ovarian drilling for polycystic ovary syndrome. Blood or bone marrow cells were collected in acute lymphoblastic leukemia (ALL) patients at diagnosis.

MAIN RESULTS AND THE ROLE OF CHANCE: The tissue dissociation technique yield was $1.83 \pm 1.49 \times 10^6$ viable nucleated cells per 100 mg of ovarian cortex. No cell exhibiting a leukemic phenotype was present in the normal ovarian cortex. Added leukemic cells were detected using their leukemia-associated phenotype up to a dilution of $10^{-4}$. When specific gene rearrangements were present, they were detected by real-time quantitative PCR at the same dilution. The ovarian cortex from two leukemia patients was then used, respectively, as positive and negative controls.

LIMITATIONS, REASONS FOR CAUTION: Making available minimal residual disease (MRD) detection techniques (multicolor FCM, PCR and xenograft), that can be used either alone or together, is essential to add a fail-safe oncological dimension to pre-autograft monitoring.

WIDER IMPLICATIONS OF THE FINDINGS: This approach can be performed on fresh ovarian tissue during cryopreservation or on frozen/thawed tissue before reimplantation and it is currently the only available technique in cases of ALL where no molecular markers are identified. This new perspective should lead to studies on ovarian tissue from leukemia patients, for whom the presence of MRD should be established before autograft.

STUDY FUNDINGS/COMPETING INTEREST(S): The study was supported by the BioMedicine Agency, the Committee of the League against Cancer, the Besançon University Hospital, DGOS/INSERM/INCa and the regional Council of Franche-Comté. There were no conflicts of interest to declare.

Key words: ovarian tissue / cryopreservation / leukemia / minimal residual disease / flow cytometry

† These authors have contributed equally to this work.

© The Author 2013. Published by Oxford University Press on behalf of the European Society of Human Reproduction and Embryology. All rights reserved.
For Permissions, please email: journals.permissions@oup.com
Introduction

Early detection and aggressive chemotherapy and/or radiotherapy treatments have considerably increased the long-term survival rates of young women with various types of cancer (Desandes et al., 2006; Howlader et al., 2012). Unfortunately, these cytotoxic treatments generally result in the loss of both endocrine and reproductive functions, leading to premature ovarian failure and infertility (Donnez et al., 2006). Loss of fertility has a major impact on the quality of life of young cancer survivors who, having overcome their disease, expect to have a normal reproductive life.

Progress in assisted reproductive technology has resulted in the availability of several options for preserving fertility in females prior to such potentially sterilizing treatments (Rodriguez-Wallberg and Oktay, 2012). For women of childbearing age, fertility can be preserved by embryo or oocyte cryopreservation. However, as these techniques generally require ovarian stimulation, they are not suitable for women requiring immediate treatment and they may be contraindicated in cases of estrogen-sensitive tumors. Furthermore, embryo cryopreservation is not possible unless the woman has a male partner. For prepubertal patients and women who cannot delay the start of chemotherapy, cryopreservation of ovarian tissue is the main option available to preserve their fertility before cancer treatment (Salama et al., 2013).

In the current absence of other techniques such as in vitro maturation or injection of isolated ovarian follicles (Smitz et al., 2010), cryopreserved ovarian tissue can only be re-used by autograft and the prospect of re-developing cancer post-graft is by in vivo amplification of MRD by xenografting human ovarian tissue into an immunodepressed mouse (Kim et al., 2001; Dolmans et al., 2010; Greve et al., 2012). It therefore seems essential to make available techniques and markers that prove the absence of MRD in cryopreserved ovarian tissue. We will then be able to re-use ovarian tissue for reproductive purposes entirely safely using the autograft technique, especially for AL survivors (Rodriguez-Wallberg and Oktay, 2012).

Two methods are used in hematology laboratories to access MRD quantification: PCR amplification of specific transcripts (for example BCR-ABL in CML) or IgH gene rearrangements using real-time clonal-specific strategies (Gabert et al., 2003) and quantification of MRD by multicolor flow cytometry (FCM) (Campana, 2012). To detect live leukemic cells specifically, by differentiating them from ovarian cells based on the identification of markers defining a leukemia-associated phenotype (LAP), which is expressed on leukemic lymphoblasts, but absent on normal hematopoietic cells and ovarian cells. However, because FCM had never been used previously in this setting, we needed to identify markers that clearly differentiate leukemic cells from ovarian cells.

Using a standardized protocol of tissue dissociation and FCM, we developed and validated a new technique to detect live leukemic cells in the ovarian cortex. We designed an experimental model of dilution of leukemic cells in cell populations isolated from the ovarian cortex, to evaluate the specificity and sensitivity of our method and the clinical feasibility of this approach. This is, to our knowledge, the first time such a model has been developed.

Materials and Methods

Collection of tissue and cells

Healthy ovarian tissue

The use of human ovarian tissue for this study was approved by the clinical ethics committee of Besançon University Hospital on 9 June 2010, and all patients gave their informed consent. Ovarian biopsies were obtained from women between 23 and 39 years of age (mean = 29.94 years) undergoing laparoscopic ovarian drilling for polycystic ovary syndrome. Ovarian cortical fragments were obtained using biopsy grasping forceps from an avascular portion of the ovary devoid of visible follicles or luteal tissue, and before electrocoagulation of the puncture site. The specimens were immediately transported to the laboratory in Leibovitz L-15 medium (Sigma, Saint-Quentin Fallavier, France) at 4°C and they were used either immediately or after freezing–thawing.

Leukemic cells

Acute lymphoblastic leukemia (ALL) blood or bone marrow cells were collected at diagnosis frozen and stored at −196°C (n = 10) (biological collection authorization no. DC-2008-713, department of research and innovation; CRB F. Cabanne, Dijon-Besançon). Cells with the known LAP were used for leukemic cell dilution experiments in ovarian cell suspension.
Peripheral blood mononuclear cell
Peripheral blood mononuclear cells (PBMCs) from healthy donors were obtained after written informed consent (EFS Bourgogne Franche-Comté) and isolated on Ficoll-Hypaque (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) centrifugation.

Freezing and thawing procedure for ovarian tissue
Cortical biopsies were cryopreserved in cryovials containing freezing solution (1.5 mol/l dimethyl sulfoxide) (DMSO; Sigma, Saint-Quentin Fallavier, France) and 0.1 mol/l sucrose (Sigma) in Leibovitz L-15 medium supplemented with 10% complemented patient serum), according to a protocol using slow cooling with manual seeding (Gosden et al., 1994; Fauque et al., 2007). After freezing, the vials were stored in liquid nitrogen.

Ovarian cortical biopsies were thawed according to the technique previously described (Roux et al., 2010). The vials were air warmed for 30 s, then immersed in a 37°C heat chamber (5 min), and the ovarian tissue pieces were washed in decreased solutions of DMSO 1.5 M (5 min), 1 M (5 min) and 0.5 M (10 min) and in a solution of 0.05 mol/l sucrose in Leibovitz L-15 medium supplemented with 10% complemented AB serum. The pieces were then rinsed in medium containing 20% serum only.

Isolation procedure for ovarian cells
A fragment of the ovarian cortex (weight: mean = 96.8 ± 68.3 mg; range = 21–276.4) was cut into small pieces of ~1–2 mm³. These small pieces underwent mechanical and enzymatic dissociation by collagenase Ia (1.6 U/ml; Sigma) with DNase I (0.1 mg/ml; Roche Diagnosis, Meylan, France) in 5 ml of RPMI (PAA laboratories, Les Mureaux, France), using a defined number of calibrated 3-µm latex beads (Flowcount beads; Beckman Coulter) was added to each sample, as an internal standard in order to obtain the absolute value of different cells.

Isolation of viable nucleated cells and all events were recorded. A significant abnormal cell population was identified using FCM by allowing the identification of the B lymphoblastic population (CD19+ CD10−/− CD38−/− CD58−/− CD123−/− CD304−/−) or CD19+ CD10+ CD123−/− CD304−/− CD38−/− or CD19− CD10−/− CD123+ CD304−/− CD38−/− or CD19− CD10−/− CD123−/− CD304−/− CD38+ or CD19− CD10−/− CD123−/− CD304+ CD38−/− or CD19− CD10−/− CD123−/− CD304−/− CD38+.

Morphological characterization of the isolated cells
Optical microscopy
Smears were made from isolated cells obtained from the ovarian cortex, fixed with alcohol ether and colored with Harris Shorr.

Electron microscopy
The cell suspension recovered from frozen/thawed tissue was centrifuged at 400g for 5 min. The pellet was fixed for 3 h at 4°C in 2.5% glutaraldehyde (Sigma) in 0.1 M sodium cacodylate at pH 7.3, washed three times for 10 min in the same buffer, post-fixed in 1% osmium tetroxide (EMS; Euromedex, Souffleveyersheim, France) in 0.1 M sodium cacodylate at pH 7.3, washed three times for 10 min in the same buffer, treated in 1% tannic acid in cacodylate for 10 min, dehydrated in ascending series of ethanol and embedded in araldite (araldite®, accelerator and hardener, Fluka; Sigma Aldrich, Lyon, France). Ultrathin sections (60 nm) laid on nickel grids and stained with uranyl acetate and lead citrate were observed with a Philips CM10 electron microscope at 80 kV and images were taken on an Olympus SIS Morada camera.

Multicolor FCM detection of MRD
Eight-color FCM was performed using a BD CANTO II flow cytometer (BD Biosciences, San José, CA, USA) and data were analyzed using Diva 6 software (BD Biosciences, Le Pont de Claux, France). The compensation matrix was set up using compbeads® (BD Biosciences) according to the manufacturer’s instructions. Daily quality control was performed using compbead setup and tracking beads® (BD Biosciences).

Antibodies
The same combinations of eight monoclonal antibodies (mAbs) were applied to ALL cells, to ovarian cell suspension and to different leukemic cell dilution experiments in ovarian cell suspension.

Isolation of viable nucleated cells
Peripheral blood mononuclear cells (PBMCs) from healthy donors were obtained after written informed consent (EFS Bourgogne Franche-Comté) and isolated on Ficoll-Hypaque (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) centrifugation.

Modelization of MRD assessment
For leukemic cell dilution experiments, the percentage and phenotype of leukemic cells were assessed after thawing by FCM. An initial dilution in ovarian cell suspension was made (dilution 1) to obtain a suspension with a leukemic cell concentration of ~10−1 (10%). From this dilution, three or four suspensions were obtained by serial 1/10 dilutions (10−2 to 10−5).

As previously described (Robillard et al., 2005; Solly et al., 2012), total cells (1 × 10⁶/tube) were stained with the eight-color combination chosen for each ALL case. The acquisition was performed for a minimum of 0.2 × 10⁶ viable cells and all events were recorded. A significant abnormal cell population was defined as a homogeneous cluster of at least 20 LAP+ cells. MRD values were obtained by dividing the number of cells in the LAP gate by the total number of nucleated viable cells (without debris) analyzed.

RNA isolation and quantitative PCR analysis
Total RNA was isolated from the ovarian cell suspension containing increasing dilutions of leukemic cells, using RNeasy mini kit according to the manufacturer’s instructions and quantified using a TD-700 NanoDrop spectrofluorometer (Thermo Scientific, Waltham, MA).
manufacturer’s instructions (Qiagen, France). cDNA was synthesized from total ovarian RNA using High Capacity RNA to cDNA MasterMix® kit (Applied Biosystems, France) at 42°C for 30 min. Each cDNA sample (5 μl) was used for real-time (RT)-PCR amplification by using TaqMan polymerase from Applied Biosystems. In all cases, the quality of the cDNA products was assessed by monitoring human cRaf gene expression. Analysis of leukemia transcripts, BCR-ABL was performed by RT-quantitative (qPCR). For this purpose, each cDNA sample was added in triplicate to the PCR plate. The following program was used: enzyme activation cycle at 37°C, 10 min; 50°C, 2 min, a denaturation step at 95°C for 15 min and 50 cycles of PCR (denaturation, 95°C, 15 s; annealing, 62°C, 1 min), then one cycle at 15°C for 1 min. Negative controls (i.e. no template cDNA) were added to every experimental plate. The quantitative expression of mRNA was estimated using standard ABL and GAPDH as housekeeping genes.

Ovarian control patients
The ovarian tissue controls originated from deceased ALL patients who had undergone ovarian cryopreservation and had given their informed consent for the research.

A T-ALL patient (positive control) had a cell invasion of abnormal lymphoblasts demonstrated at systematic histopathological examination performed at the time of ovary removal. Immunohistochemistry showed a positive CD3 membrane immunostaining of these infiltrating lymphoblasts within the ovarian tissue. The immunophenotype, performed on blood and bone marrow at the time of diagnosis, revealed CD2, CD7, CD13, CD10 and cyCD3 positive expression and absence of surface CD3 expression (sCD3), which is frequent in immature T-ALL. The following eight-color FCM tube was used for the analysis of this patient’s ovarian tissue: 7-AAD/SYTO13/CD45/sCD3/cyCD3/CD7/CD10/CD13. All the available molecular markers were negative and no gene rearrangement was detected.

A B-ALL patient (negative control) had no ovarian invasion of abnormal lymphoblasts at histopathological examination. The immunophenotype, performed on blood and bone marrow at the time of diagnosis, revealed CD22, CD19, CD20 and CD10 expression. We performed the next FCM tube to analyze this patient’s ovarian tissue using: 7-AAD/SYTO13/CD45/CD19/CD20/CD22/CD10.

For each patient, the amount of tissue used for the FCM detection represented less than 8% of the total amount of cryopreserved tissue.

Statistical analysis
Data are expressed as the mean, coefficient of variation and/or standard deviation. The populations were compared using the student’s t-test (P-values of 0.05 were considered to be significant). The comparison between the theoretical and experimental values of leukemic cell dilution modelization included regression analysis by the method of the least square.

Results
Isolation of ovarian cortex cells and quality assessment
The single-cell suspension obtained after ovarian cortex dissociation was analyzed by FCM according to its size (forward scatter, FSC) and granularity/nucleus–cytoplasm ratio (side scatter, SSC). In this population, we identified nucleated cells (SYTO13+ or P2) and viable cells (7-AAD2 or P3, pink) to determine the percentage of viable nucleated isolated cells in each sample (Fig. 1A), which was similar regardless of whether fresh ovarian cortex (46±18%; n = 12) or frozen/thawed cortex (33±16%; n = 20) was used (P = 0.042).

The yield of the tissue dissociation technique, obtained for fresh or frozen ovarian tissue, was 1.83±1.49×10^6 (range = 0.2×10^6–6.85×10^6) viable nucleated cells per 100 mg of cortex on average.
Morphological controls performed on stained smears illustrated that
this dissociation technique essentially produced single follicular and
stromal cells, with only a few clusters of cells and dissociated collagen
fibers observed after the isolation procedure (Fig. 2). Transmission elec-
tron micrographs showed that cells isolated from both fresh and frozen/
thawed tissue had a well-preserved cytological architecture of the plasma
membrane, the cytoplasmic organelles (membranous system and dis-
crete particulate organelles) and the nuclear structures (Fig. 3).

**Sensitivity and specificity of the experimental model of minimal residual disease detection by eight-color FCM**

Before performing our experimental model for dilutions of leukemic cells
in the ovarian cell suspension, we first checked that both isolated ovarian
cells and added leukemic cells exhibit the same size and granularity profile
allowing us to detect these cells by FCM in the same gate (Fig. 1B and C).
We then ensured that freezing-thawing and enzymatic dissociation pro-
tocols had no effect on the expression of cell surface markers used to
identify leukemic cells (i.e. CD19, CD45 and CD10). We compared
the mean fluorescence intensity (MFI) of these markers before and
after the freezing/thawing/dissociation protocols on PBMCs (n = 5),
leukemic cells alone (n = 5) or added to the ovarian cortex before isola-
tion procedure (n = 1) and we did not find any statistically difference
in the MFI of each marker (data not shown). Therefore, the freezing/
thawing/dissociation steps did not modify cell surface marker expression
on leukemic cells.

The modelization consisted in adding a decreasing number of ALL leuk-
emtic cells to the non-contaminated ovarian single-cell population. Ten
different ALL cells were used (one per experiment of serial dilutions). All
the different leukemic cells were well characterized by the expression of
specific markers, defining their LAP and ensuring the specificity of their
detection by FCM. Abnormal ALL cells were detected from the dilution
of $10^{-4}$ to $10^{-5}$ (Fig. 4). At the dilution of $10^{-5}$, we detected fewer than
the 20 abnormal events required (range 3–16), in relation to the insuffi-
cient number of total events analyzed. However, at the dilution of $10^{-4}$,
the number of abnormal events was equal to 48 ± 13 (min = 23), there-
fore conferring a sensitivity of $10^{-4}$ to this detection technique (Fig. 4).

The comparison between theoretical and experimental values of leu-
emtic cell quantification in the different dilutions showed a linear regres-
sion, with an excellent correlation between the theoretical and experi-
mental cell numbers ($r = 0.9597$, $P < 0.001$, $n = 45$) (Fig. 5).
When specific gene rearrangements (BCR-ABL) were present (n = 5),
the quantification of leukemic cells was performed both by FCM and
RT-qPCR for each dilution ($10^{-1}$ to $10^{-5}$). For this purpose, the
ovarian cell suspensions containing the different dilutions of leukemic
cells were all divided into two equal parts.

If we consider a threshold of $10^{-4}$ leukemic cells to define MRD posi-
tively, no discordant results were observed between FCM and RT-qPCR
and all detections attained the critical level of $10^{-4}$ with both assays. Fur-
thermore, we observed a good correlation for abnormal leukemic cell
quantification between RT-qPCR and FCM ($r = 0.8840$, $P < 0.0001$,
$n = 21$) (Fig. 6). At the dilution of $10^{-5}$, RNA identification was negative
by RT-qPCR in three cases out of five, while viable leukemic cells
detected by FCM were under the 20 cell limit considered necessary
for MRD positivity (3, 7 and 8 events, respectively). As evidence, an ana-
lysis of more total cells could allow us to obtain a sensitivity of less than

**Figure 2** Smear of human cells isolated from the ovarian cortex. Different
cell types can be observed, such as cells with an oval or elongated,
dark nucleus demonstrating the thecal or stromal cells (arrow) and cells
with a round and clear nucleus demonstrating the granulosa cells (arrow-
head). Clusters of cells are rare (C). Some dissociated collagen fibers are
present and colored in green.

**Figure 3** Electron microscopic assessment of human cells isolated
from the ovarian cortex. Cells were well identified. They exhibit a
round or more irregularly shaped nucleus. The nucleus, organelles
and plasma membranes are well preserved. Some cytoplasmic frag-
ments or debris are present between the cells.

(n = 32). The effectiveness of the cell extraction was not significantly
different ($P = 0.038$) for fresh ovarian cortex $[1.54 \pm 1.02 \times 10^6$
(range = 0.56 – 4.28 × 10^6), n = 12] compared with frozen ovarian
cortex $[2.01 \pm 1.71 \times 10^6$ (range = $0.2 \times 10^5$– 6.85 × 10^6), n = 20].

We first had to ensure that the isolated cell suspension obtained from
healthy ovarian cortex did not contain any cells coexpressing specific leu-
emtic markers. Nucleated ALL cells showing a typical immunopheno-
type are displayed in Fig. 1B. Of the healthy ovarian viable nucleated
cells, we selected CD45 and CD19 positive cells (i.e. B lymphocytes),
and none of these non-contaminated ovarian cells had an LAP (Fig. 1C).
In all the molecular biology analyses, the efficiency of mRNA extraction carried out from ovarian cell suspensions contaminated by leukemic cells (dilutions $10^{-2}$ to $10^{-5}$), was satisfactory, with $20.69 \pm 8.98$ ng of mRNA obtained per ml.

Positive and negative control patients tested by FCM

For the first patient (positive control), we detected 365 MRD positive events among $1.36 \times 10^6$ total alive nucleated events: MDR is therefore positive in the ovarian cortex at a level of $2.7 \times 10^{-4}$ (Fig. 7A).

For the second patient (negative control), no MRD positive event was detected within the ovarian cell suspension. The maximum sensitivity obtained in this MRD setting is $7 \times 10^{-5}$ indicating that MRD is negative in the ovarian cortex for this patient at this maximum of sensitivity (Fig. 7B).

Discussion

Although it has been successfully carried out in a rodent model, in vitro follicular culture technology and transplantation of frozen/thawed isolated primordial follicles are far from fully developed in humans (Carroll and Gosden, 1993; Picton et al., 2008). Autograft is therefore currently the only available option to restore fertility by using cryopreserved ovarian cortex.

However, ovarian cortex autograft has limitations, notably in cases of cancer, where the main concern of this procedure is the risk of reintroducing metastatic tumor cells with the implant. The risk of reintroducing a malignant condition when transplanting the tissue depends on the
particular disease and a careful selection of patients for this procedure helps ensure that no relapse occurs after transplantation of ovarian cortex (Rosendahl et al., 2013). Indeed, in cases of neoplastic pathology, the malignancy type and the activity of the disease are taken into account. Autografts are performed in patients with solid tumors (such as Hodgkin and non-Hodgkin lymphoma, sarcoma, breast cancer) presenting a low risk of ovarian localization. Only one case of successful autotransplantation of frozen/thawed ovarian tissue resulting in the birth of a healthy baby has been reported in a patient with metastatic disease (neuroectodermal tumor of the orbit), but no malignant cells were identified on serial sections of the frozen/thawed tissue (Donnez et al., 2011a,b).

**Figure 5** Modeling of ALL cell detection in an ovarian cell suspension by FCM. The X-axis represents theoretical values of the MRD dilutions realized and the Y-axis the experimental MRD values of the same samples quantified by FCM dilutions of ALL cells in an ovarian cell suspension (axes in the log scale). The mean value for each dilution level is represented by a horizontal bar. ‘n’ indicates the number of experiments per dilution.

**Figure 6** Correlation of MRD levels measured by FCM and by RT-qPCR. BCR-ABL transcripts were quantified on the same sample dilution. This figure is representative of five independent experiments (axes in the log scale).
When ovarian tissue is thawed, investigative studies to detect MRD must obviously be performed: they include conventional histological evaluation at macroscopic and microscopic levels and immunohistochemical staining (Meirw et al., 1998; Poirier et al., 2002; Seshadri et al., 2006; Meirw et al., 2008; Sanchez et al., 2009; Abir et al., 2010; Dolmans et al., 2010; Rosendahl et al., 2010, 2011; Bittinger et al., 2011; Greve et al., 2012). If cancer cells are detected in ovarian tissue harvested for cryopreservation, the ovarian tissue cannot be reimplanted, as described by Bittinger et al. (2011) for Hodgkin lymphoma. However, the sensitivity of these techniques is limited (Bockstaele et al., 2012).

AL is the most frequent type of malignancy in children and it usually requires highly gonadotoxic treatments. Cryopreservation of the ovarian cortex is often the only fertility preservation technique we can offer these patients, because we have little time for ovarian stimulation and because of the prepubertal age of these patients (Wallace et al., 2005; Donnez et al., 2006). Immature oocyte retrieval in the luteal

**Figure 7** MRD detection by FCM in the ovarian tissue from ALL patients. Only live nucleated cells are displayed (Syto13^+7-AAD^- = P3, see Fig. 1). (A) T-ALL patient with positive ovarian MRD. (1) T-ALL cells at diagnosis express the following phenotype: cyCD3^+sCD3^+/CD13^low/CD10^low/CD7^high. (2) Healthy ovarian tissue: there is no event expressing a T-ALL phenotype. A.2 is representative of two independent experiments. (3) Ovarian cells from the same T-ALL patient: in 1 360 815 events acquired (corresponding to P3), we identify 365 events presenting the same phenotype as the T-ALL cells: the MRD level is quantified at 2.7 x 10^-4. (B) B-ALL patient with negative ovarian MRD. (1) B-ALL cells at diagnosis express the next phenotype: CD19^+CD3^-CD22^low/CD20^low/CD10^high. (2) Healthy ovarian tissue: there is no event presenting a B-ALL phenotype. B.2 is representative of two independent experiments. (3) Ovarian cells from the same B-ALL patient: in 287 049 events acquired, we identify no event presenting the same phenotype as the B-ALL cells. The ovarian MRD is negative.
phase has been proposed to preserve fertility (Demirtas et al., 2008), but so far no pregnancies have been reported with this technique in cancer patients.

As leukemia is a disease involving the bone marrow and the blood, malignant cells may be present in the blood-filled organs, including the ovaries. In cases of leukemia [ALL, acute myeloid leukemia (AML), chronic lymphoid leukemia, CML] or in the leukemic phase of lymphoma, malignant cells could be transferred during transplantation if leukemic cells are present in the ovarian cortex during freezing.

It has been reported that transplantation of testicular cells from leukemic donor rats transmits AL to healthy recipients. The number of leukemic cells sufficient to induce the disease in the rodent is, respectively, 20 and 200 cells by allograft and xenograft (Jahnukainen et al., 2001; Hou et al., 2007).

Neither prior chemotherapy, nor preparing ovarian tissue in relation to cryopreservation, can completely eliminate ALL or CML cells (Dolmans et al., 2010; Rosendahl et al., 2010). On the other hand, these cytotoxic therapies may decrease the number of oocytes and they may be deleterious for oocyte quality, leading to a lower functional potential of the graft.

Until recently, residual leukemia or lymphoma in bone marrow, blood or lymph node cells was detected using different methods such as morphology, immunocytoLOGY, karyotype analysis or fluorescence in situ hybridization techniques or FCM using a single antibody. However, these techniques cannot detect small numbers of malignant cells. Molecular techniques (such as PCR) and FCM, seem to offer highly sensitive detection, with a high specificity from 10^{-2} up to 10^{-6} (Campana, 2012; Gaipa et al., 2012).

In the ovarian cortex, MRD detection for leukemia cannot be achieved using cytogenetic techniques, and histology and immunohistochemistry fail to locate leukemic cells in this tissue. Rosendahl et al. analyzed cryopreserved ovarian cortex of 26 patients diagnosed with different types of leukemia (AML, ALL, CML, juvenile myelomonocytic leukemia): immunohistology did not reveal malignant cell infiltration in the ovarian cortex of any of the 26 patients. In 6 of the 8 patients (75%), disease-specific genetic markers (fusion transcript), originally found in the patients’ bone marrow and blood during active disease, were detected in the ovarian cortex (Rosendahl et al., 2010). Dolmans et al. evaluated the presence of leukemic cells in cryopreserved ovarian tissue from 18 leukemic patients: 12 with ALL and 6 with CML. If histology failed to identify malignant cells in the ovarian tissue, PCR analysis (detection of fusion gene transcripts or gene rearrangements) was positive for 7 of the 10 ALL with available molecular markers and for 2 of the 6 CML. Of these 7 ALL MRD positive patients, 3 had already received chemotherapy before ovarian tissue cryopreservation (Dolmans et al., 2010). In cases of leukemia, PCR positivity contraindicates ovarian autograft (Meir et al., 2008; Courbiere et al., 2010; Dolmans et al., 2010; Rosendahl et al., 2010).

Dolmans et al. also evaluated the presence of leukemic cells in cryopreserved ovarian tissue by achieving long-term (6 months) intraperitoneal xenografts in immunodeficient mice (SCID) (one mouse per patient). After grafting of ovarian tissue from the 12 ALL patients, 4 mice showed macroscopic intraperitoneal proliferation and another mouse presented a microscopic invasion. These five diseased mice had been transplanted with the ovarian cortex from ALL patients whose molecular markers were available and positive in four cases and unavailable in one case. However, the six mice grafted with ovarian tissue from CML patients remained healthy (Dolmans et al., 2010). Greve et al. recently complemented the study published by Rosendahl et al. (2010) by performing subcutaneous xenotransplantations of the ovarian cortex into nude mice for 20 weeks. The transplanted pieces originated from 25 patients with leukemia, with the majority of the patients in complete remission. No sign of disease was found in any of the mice according to macroscopic analysis, histological analysis and RT-qPCR, although the ovarian cortex showed a positive RT-qPCR result in four of the seven patients with a known molecular marker prior to transplantation (Greve et al., 2012). Xenograft is currently the only available technique to assess the proliferative potential of malignant cells in the ovarian cortex. However, the technique has limitations. First, the xenograft should be kept for at least 6 months. Sixteen weeks seems to be too short a period for mice to develop disease when grafted with ovarian tissue from lymphoma patients (Kim et al., 2001). Secondly, xenograft may not be a valid model for detecting MRD in cases of CML (Dolmans et al., 2010).

The technique we propose here uses an original and standardized protocol for dissociating fragments of the ovarian cortex. It involves population of isolated viable ovarian cells that can be analyzed by FCM, and by molecular technology depending on the availability of markers, to ensure MRD detection.

The use of an automated cell dissociator combining mechanical and enzymatic cycles and using a program previously adapted to ovarian tissue provide optimized conditions to obtain a satisfactory yield of live cells. On average, 100 mg of the dissociated ovarian cortex allowed us to obtain enough live cells to perform the entire FCM modelization procedure, itself containing five dilutions. We therefore used five times as much ovarian tissue than the amount required for MRD detection at 10^{-4} for one patient (20 positive events out of 200 000 total events).

Furthermore, when this cell dissociator is used in combination with C Tubes, tissue can be dissociated in a closed system, allowing for sterile sample handling. It was also useful for evaluating the results of the dissociation technique in terms of viability. For this purpose, trypan blue staining (Fauque et al., 2007) and a cell counting chamber are usually recommended. They could not be used here because of the presence of tissue and cell debris in cell suspensions, which interfered with the reading and the results themselves. Therefore, we used FCM and 7-AAD to identify viable cells, as this technique can detect a higher number of events than a microscopic count.

Leukemic cell dilution techniques in ovarian tissues have enabled us to find abnormal leukemia cells from ALL up to a dilution of 10^{-4} (1 cell in 10,000 normal ovarian cells) conferring on the technique a robust sensitivity of 10^{-4}. FCM can detect one positive cell with certainty, but according to the literature, a cluster of at least 10 events with an LAP and back-gating light scatter is recommended to define a sample as ‘MRD-positive’ (Dworzak et al., 2008). In our study, the minimum target sensitivity for qualifying MRD was defined as the ability to detect 20 MRD cluster events in 2 x 10^{3} cellular events. This is because, unlike with PCR, the more positive events we detect with FCM the higher is the sensitivity. The sensitivity we obtained is similar to the standard threshold for MRD in bone and bone marrow in ALL (Coustan-Smith et al., 2011). The level of MRD threshold mandatory for ovarian tissue analysis should be specified in the future.

In ovarian tissue, in which we search for the persistence of leukemic cells within non-hematopoietic cells, the FCM approach has the advantage of having many surface markers that can differentiate between
ALL and ovarian cells. This approach can thus potentially be applied to 100% of AL patients, which is not the case for the molecular approach, especially when using transcript detection. Furthermore, the protocol we have developed allows for the absolute value quantification of leukemic cells using beads.

By analyzing viable cells, FCM may exclude dead leukemic cells, which could potentially yield false-positive results by PCR. Courbière et al. reported one case of CML, where RT-qPCR detected a low but positive level of BCR-ABL transcripts in blood and ovarian medulla, but the level was below the limit of quantification of the technique in the ovarian cortex (Courbière et al., 2010). Furthermore, there was no parallel in the transcript levels between the ovarian cortex and bone marrow or blood. Indeed, Dolmans et al. (2010) reported that the expression of the BCR-ABL fusion gene was higher in the ovarian cortex than in bone marrow in one patient with ALL and lower in two patients with CML. Rosendahl et al. (2010) showed variable levels of molecular marker expression according to the fragments of ovarian tissue analyzed, which may partly be a result of insufficient fibrous ovarian tissue disruption and homogenization leading to a variability in RNA yield (Rosendahl et al., 2010). In our modelization, RNA extractions were performed on isolated ovarian cell populations, which provided a homogeneous yield of RNA extraction and a level of RNA that was high enough to allow for cDNA amplification.

In conclusion, we used FCM with beads to quantify the presence of live leukemic cells from ALL in the ovarian cortex with a high specificity and sensitivity. This approach can be performed on fresh ovarian tissue during cryopreservation or on frozen/thawed tissue before reimplantation, and it’s currently the only available technique in cases of ALL where no molecular markers are identified.

The technique we propose here could also be transposed to testicular tissue from prepubertal cancer patients.

Making available MRD detection techniques (FCM, PCR and xenograﬁ) that can be used either alone or together, is essential to add a fail-safe oncological dimension to pre-autograft monitoring. Controlling the cancer risk will, on the one hand, allow indication for cryopreservation of ovarian tissue to be broadened in young women presenting with a neoplastic pathology; on the other hand, it will encourage fragments of cryopreserved cortical ovarian tissue to be re-used by the autograft technique in patients at risk.

**Acknowledgements**

The authors thank Germain AGNANI, MD (Service de Gynécologie-Obstétrique, Centre Hospitalier Universitaire de Besançon) and Catherine POIROT, MD PhD (Service de Biologie de la Reproduction, Hôpital Tenon, Paris) for their help in the ovarian tissue collection; Christophe FERRAND, PhD (UMR1098, biomonitoring platform EFS B/FC, CIC-BTS06) for helpful discussions and for providing the primers; Graham KNOTT, PhD (Interdisciplinary Center for Electron Microscopy, EPFL, Lausanne, Suisse) for his contribution to the collection of the Electron Microscopic observations and Frances Sheppard (Inserm CIC: Clinical Investigation Center of Besançon) for proofreading the article.

**Authors’ roles**

C.A. drafted the manuscript. C.A. and C.R. designed the study, F.A.-D. and T.Z. contributed equally to this work. C.A., F.A.-D., T.Z. and M.A.-V. conducted the biological experiments. C.R. and F.G.-O. contributed to analysis of the data. C.R., F.G.-O. and P.S. contributed to critical revision of the manuscript. All authors approved the final manuscript.

**Funding**

This study was supported by the BioMedicine Agency (Agence de la Biomédecine, 2010 call for tender: assisted reproductive technology, prenatal diagnosis and genetic diagnosis), the Committee of the League against Cancer (Comité de la lutte contre le Cancer, Haut-Rhin, 2011), Besançon University Hospital (APICHIU call for tender, Franche-Comté Regional council, 2011) and the DGOS/INSERM/INCa (Appel d’offre Recherche translationnelle 2012, to C.A. and P.S.). Funding for a doctoral thesis (T.Z.) has been awarded by the regional Council of Franche-Comté (Conseil Régional de Franche-Comté, 2010).

**Conflict of interest**

None declared.

**References**


