Minimal residual disease detection in cryopreserved ovarian tissue by multicolor flow cytometry in acute myeloid leukemia

Approximately 85% of patients aged under 45 years will survive for at least five years after chemo- and/or radiotherapy.[1] Despite the gonadotoxicity of these treatments, the possibility for women to conceive a child after surviving cancer is a reality that can be envisaged thanks to advances in fertility preservation methods. Ovarian tissue cryopreservation (OTC) is one of the available options to preserve fertility before receiving treatment that could result in sterility.[2] Currently, frozen/thawed ovarian tissue can only be re-used by autograft, and more than twenty live births have already been reported.[3,4] However, there is legitimate concern about the risk of cancer re-seeding by transplanting the tissue in cases of cancer.[5] In acute leukemia (AL), real-time quantitative polymerase chain reaction (RT-qPCR)[6] or multicolor flow cytometry (MFC) based on leukemia-associated immunophenotype (LAIP)[7] can be used to identify persisting leukemic cells at low level in ovarian tissue. Two studies have investigated minimal residual disease (MRD) in ovarian tissue in the setting of acute myeloid leukemia (AML) by RT-qPCR.[8,9] MFC makes it possible to differentiate leukemic cells from normal progenitors and hematopoietic stem cells, based on a specific LAIP profile in 95% of AML patients in bone marrow (BM).[10] MFC has been used before by our team to quantify MRD in the ovarian tissue of acute lymphoid leukemia (ALL) patients.[11] The objective of the current study was to develop a technique for MRD detection using 8-color flow cytometry in ovarian tissue from AML patients.

Ovarian cortical fragments, obtained by oophorectomy or biopsies, were cryopreserved using slow cooling, stored in liquid nitrogen, thawed, and then underwent mechanical and enzymatic dissociation according to previously described protocols.[11] Healthy reference ovarian tissue samples were obtained from women undergoing laparoscopic surgery for polycystic ovary syndrome (PCOS). AML blood or BM cells were used for validation of an experimental model of serial AML cell dilutions in ovarian cell suspensions. Cryopreserved ovarian tissue from 4 AML patients was used for MRD assessment. All 4 patients had received chemotherapy before harvesting of ovarian tissue. The use of human ovarian tissue for this study was approved by the Clinical Ethics Committee (9 June 2010) and by the Ethical Research Committee of Besançon University Hospital (5 March 2013). All patients gave written informed consent. MFC was performed using a FACSCanto II™ flow cytometer and FACSDiva™ software v.6.1.3 (BD Biosciences, San Jose, CA, USA). The freezing/thawing and dissociation steps did not modify cell surface marker expression.[11] MFC gating strategy was based on the elimination of debris by an initial morphological gate using forward (FSC) and side

Table 1. MRD detection in 4 AML patient ovarian tissues by MFC and RT-qPCR.

Patient n., age at ovarian tissue cryopreservation (years)	AML subtype (FAB classification)	AML patient immunophenotype at diagnosis	Specific antibody combinations used for MRD assessment	N. of viable events	N. of LAIP+ events	Maximum sensitivity (S_{max})	MRD	Suitable molecular marker used for MRD assessment	MRD
#1, 22	M1	CD11b+, CD13+, CD15+, CD33+, CD34+, CD38+, CD43+, CD117+, HLA DR+, CD10−, CD20−, CD24−, CD2−, CD4−, CD5−, CD16−, CD56−, CD14−, CD36−, CD64−, CD41a−, CD42b−, CD61−, cCD3−, cMPO−, cCD22−, cTDT−, cCD79a−	CD11b, CD15, CD43	300 000	56	$4x10^{-5}$	**Positive $2x10^{-4}$**	–	–
#2, 33	M4	CD13+, CD15+, CD33+, CD34+, CD38+, CD43+, CD117+, CD361+, cMPO+, CD11b−, CD14−, CD64−, CD65−, CD36−, CD71−, CD10−, CD19−, CD20−, CD24−, CD2−, CD4−, CD5−, CD7−, CD16−, CD56−, cCD3−, cCD22−, cTDT−	CD34, CD43, CD117, CD361	255 000	7	$8x10^{-5}$	Negative $< S_{max}$	CBFB-MYH11	Negative
#3, 15	M5	CD11b+, CD15+, CD33+, CD34+, CD38+, CD43+, CD117+, CD361+, CD3−, CD14−, CD19−, CD20−, CD22−	CD11b, CD33, CD43, CD117	332 000	1	$6x10^{-5}$	Negative $< S_{max}$	–	–
#4, 15	M1	CD13+, CD33+, CD34+, CD38+, CD43+, CD117+, HLA DR+, CD2−, CD3−, CD4−, CD10−, CD14−, CD19−, CD22−, CD56−, CD64−	CD33, CD34, CD43, CD117	133 000	34	$2x10^{-4}$	**Positive $3x10^{-4}$**	–	–

Age, AML subtype, immunophenotype and molecular marker at diagnosis are indicated for each patient. The antibody combinations used for MRD assessment include fixed mAbs (7AAD/SYTO13^{FITC}/CD45^{V500}/CD3^{V450}) and variable mAbs displayed in this table. Each specific antibody combination allows us to determine the number of LAIP+ in the sample among viable events quantified by MFC. The maximum sensitivity was calculated by dividing the number of viable events acquired for each patient by 20. MRD was calculated dividing the number of LAIP+ events by the number of viable events. Antibodies were used according to the manufacturer's instructions: 7AAD (Beckman Coulter), SYTO13 (Invitrogen), CD11b (Bear 1; Beckman Coulter) and CD34 (581; Beckman Coulter) coupled with phycoerythrin cyanin 7 (PE-Cy7), CD361 (MEM-216; Exbio) and CD33 (P67.6; BD Biosciences) coupled with phycoerythrin (PE), CD15 (HI98; BD Biosciences) and CD117 (104D2; BD Biosciences) coupled with allophycocyanin (APC), CD3 (UCHT1; BD Biosciences) coupled with V450, CD43 (IG10; BD Biosciences) with allophycocyanin H7 (APC-H7) and CD45 (HI30; BD Biosciences) coupled with V500.
MRD: minimal residual disease; AML: acute myeloid leukemia; MFC: multicolor flow cytometry; RT-qPCR: real-time quantitative polymerase chain reaction; FAB classification: French-American-British classification.

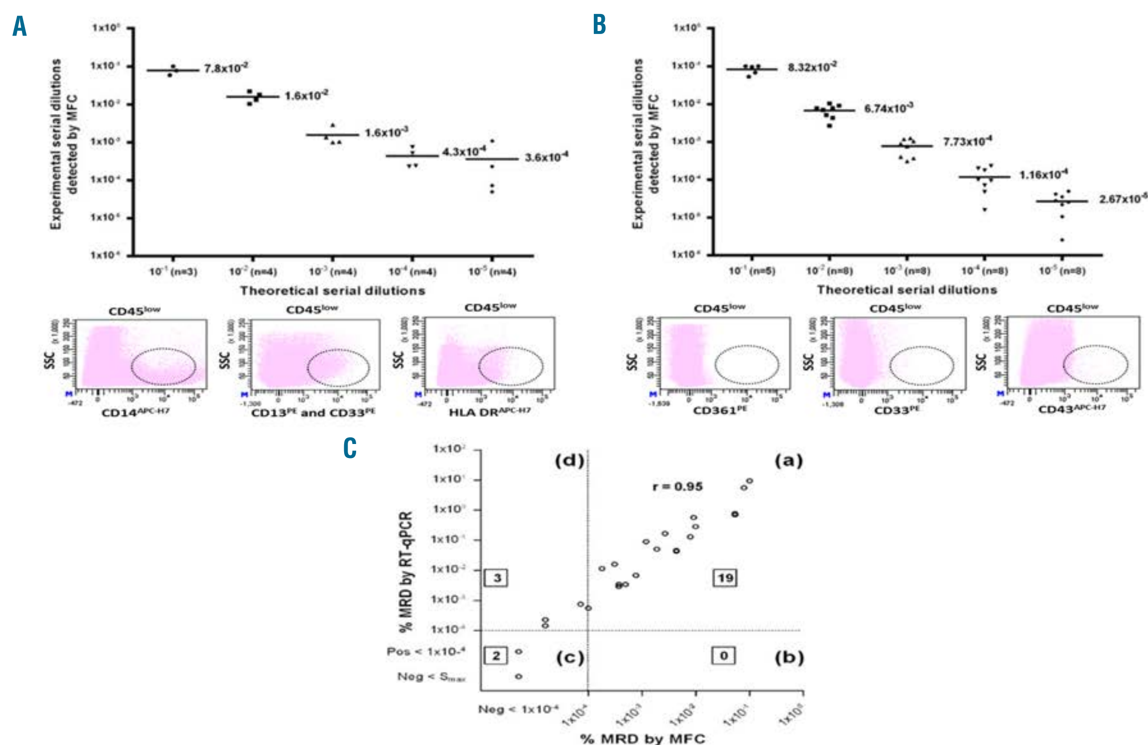

Figure 1. Validation of an experimental model of AML cell dilutions within reference ovarian cells. (A and B) The dilution experiments consisted in adding a decreasing number of AML leukemic cells to non-contaminated ovarian single-cell population. n: number of experiments per dilution point performed using AML blood or BM cells from independent patients. The X-axes represent theoretical values of AML cell dilution in reference ovarian cells and the Y-axes represent experimental values quantified by MFC (axes in the log scale). The mean value for each dilution level is represented by a horizontal bar. (A) Modeling of AML cell detection in reference ovarian cell suspension by MFC using CD13-CD33, CD14 or HLA DR and expression of these AML markers used in hematology on reference ovarian cells. (B) Modeling of AML cell detection in reference ovarian cell suspension by MFC using CD361, CD33 alone or CD43 and expression of these more appropriate AML markers for MRD assessment on reference ovarian cells. (C) Comparison of MRD results obtained by MFC and RT-qPCR in dilution experiments. MRD results obtained with MFC were compared with RT-qPCR for the 10-fold serial dilutions of leukemic cells from 6 AML patients with NPM1 mutation A in reference ovarian cells. The number of samples is indicated for each part of the graph. (a) Concordance of 19 positive MRD values ($> 1x10^{-4}$) obtained by MFC and RT-qPCR. The correlation coefficient was calculated with the Spearman's rank correlation test. (b) No samples were MFC positive ($>1x10^{-4}$) and RT-qPCR negative ($<1x10^{-4}$). (c) For one sample, RT-qPCR was positive below $1x10^{-4}$ whereas MFC was negative ($< 1x10^{-4}$) and for one other sample, RT-qPCR was below the maximum sensitivity (Smax) and MFC was negative. (d) Samples for which RT-qPCR was positive ($> 1x10^{-4}$) and MFC was negative ($< 1x10^{-4}$). MRD: minimal residual disease; MFC: multicolor flow cytometry; RT-qPCR: quantitative polymerase chain reaction; AML: acute myeloid leukemia; BM: bone marrow; MRD: minimal residual disease; Smax : maximum sensitivity.

(SSC) light scatter characteristics. Nucleated viable cells were then selected by their SYTO13$^+$/7AAD$^-$ phenotype, giving the total events used for MRD quantification. Within these cells, we identified CD45$^+$ hematopoietic cells and CD3$^+$ T lymphocytes. The myeloid blasts were gated with 4 additional antibodies. Acquisition of at least 200,000 viable ovarian cells was performed as required in BM MRD assessment, except for one case where only 130,000 events were acquired (range 130,000-570,000). A significant abnormal cell population was defined as a homogeneous cluster of at least 20 LAIP$^+$ events.[7] To assess the specificity of the 8 antibody combinations used and the maximum sensitivity corresponding to the limit of detection that can be achieved for MRD, a quantitative study of ovarian cells from reference ovarian tissue was performed in the same conditions. The sensitivity of the MFC method was confirmed by 10-fold serial dilutions of AML cells in reference ovarian cell suspensions (10^{-1} to 10^{-5}).[11] Molecular quantification of NPM1 mutation A and CBFB-MYH11 were performed as previously described.[12,13] ABL1 was used as housekeeping gene.

Markers used in routine clinical practice for AML MRD detection by MFC in blood or BM (CD13 and CD33, CD14, HLA DR) were applied to AML cell detection in our experimental model of blood or BM leukemic cell dilutions (10^{-1} to 10^{-5}) within reference ovarian cells. MRD quantification by MFC reached a plateau above the level of 10^{-4} (mean $4x10^{-4}$) (Figure 1A). This was explained by background noise due to the expression of CD13 and CD33, CD14 or HLA DR on ovarian cells, thus precluding achievement of sufficient sensitivity for MRD detection by MFC (Figure 1A). We therefore tested CD33 alone and other potentially useful AML markers (such as CD43 or CD361), and did not observe any background signal on ovarian cells with these new antibody combinations. We obtained robust sensitivity of 10^{-4} (Figure 1B), with a good correlation between experimental and theoretical MRD values (r=0.97, $P<0.0001$, n=37). Molecular MRD quantification was performed in dilution experiments using AML cells originating from patients carrying NPM1 mutation A. On analysis of 24 dilution points by both MFC and RT-qPCR, one sample was negative by both techniques and 19 were positive at or

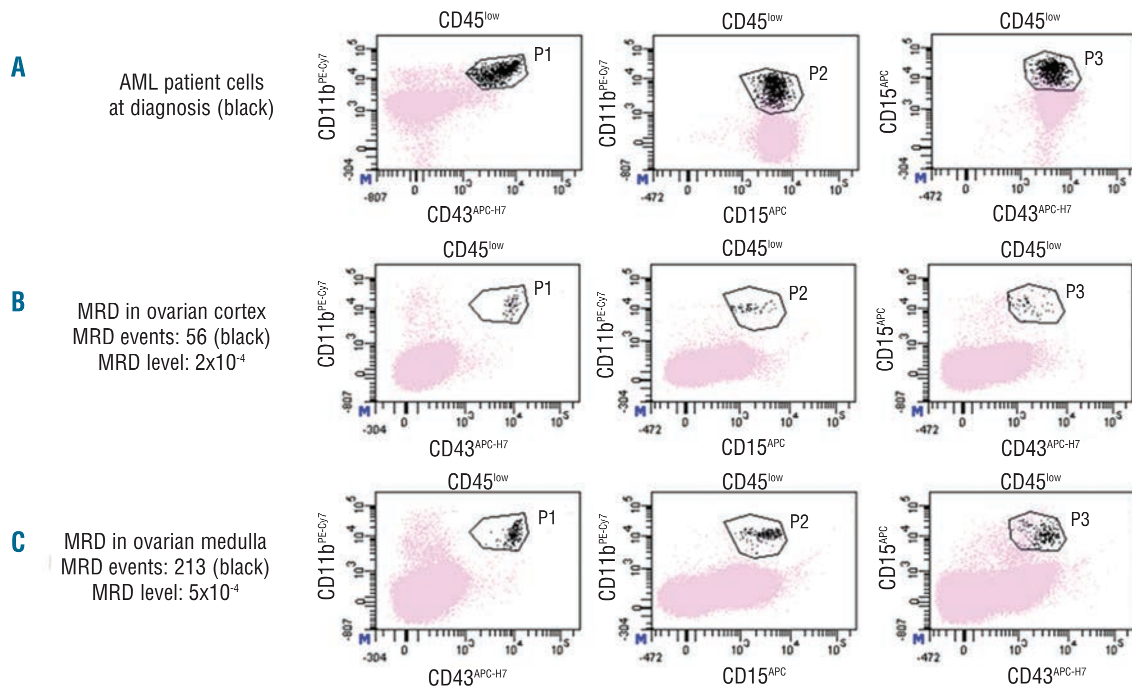

Figure 2. Positive MRD detection by MFC in the cortical ovarian tissue and medulla of Patient #1. Only viable cells ($SYTO13^+/7AAD^-/CD45^{low}$) are displayed in all dot plots (pink). (A) AML patient cells at diagnosis express the following markers: $CD11b^+/CD43^+/CD15^+$ (P1∩P2∩P3, black). (B) Cortical ovarian cells from the same patient at hematologica remission: among 300,000 events acquired, we identified 56 $LAIP^+$ events with the same phenotype as AML cells at diagnosis: MRD level is quantified at $2x10^{-4}$. (C) Medulla ovarian cells from the same ovarian sample: among 460,000 events acquired, we identified 213 $LAIP^+$ events, corresponding to an MRD level of $5x10^{-4}$. MRD: minimal residual disease; MFC: multicolor flow cytometry.

above $1x10^{-4}$ by both techniques with good correlation (r=0.95) (Figure 1C). Three dilution points were positive (>$1x10^{-4}$) by RT-qPCR but negative by MFC (<$1x10^{-4}$, between 5 and 10 $LAIP^+$ events), and one sample was considered positive below $1x10^{-4}$ by RT-qPCR but negative by MFC. Thus, these results seem to indicate that there is a good correlation between MFC and RT-qPCR for positive MRD levels up to 10^{-4}, and an increased sensitivity of RT-qPCR in cases of low positive MRD levels. Finally, for 4 AML patients, we investigated ovarian MRD by MFC (n=4) and RT-qPCR (CBFB-MYH11 quantification; n=1) (Table 1). Among these 4 patients, ovarian MRD results were positive by MFC in 2 cases. For Patient #1, 56 $LAIP^+$ events presenting the same phenotype as the patient's own AML cells at the time of diagnosis (Figure 2A) were detected among $3x10^5$ viable ovarian cortical cells; MRD was quantified at $2x10^{-4}$ (Figure 2B). Moreover, AML cells were also found in the ovarian medulla of this patient at $5x10^{-4}$ (Figure 2C). For Patient #4, MRD was positive in the ovarian cortical tissue at a level of $3x10^{-4}$. We were not able to analyze more than 133,000 viable nucleated ovarian cells. However, we quantified 34 $LAIP^+$ events, corresponding to positive MRD of $3x10^{-4}$. Patient #2 was MRD-negative by both MFC and CBFB-MYH11 RT-qPCR while Patient #3 was MRD-negative by MFC (Table 1).

This study demonstrates that MFC is a powerful tool for MRD detection in ovarian tissue from patients who undergo OTC before gonadotoxic treatment. Two studies published about ovarian MRD detection in AML patients[8,9] suggest that immunohistochemistry is unable to identify a very low number of AML cells in ovarian tissue. Although RT-qPCR is known to have good sensitivity for MRD detection (10^{-4} to 10^{-5}), this technique was only applied in one of 10 AML patients in these studies. Xenotransplanted ovarian tissue with positive MRD may have failed to induce AML development in mice because too few AML cells were present in the transplanted ovarian pieces. The xenograft model may not be accurate for ovarian AML MRD detection, but is currently the only available method to evaluate potential *in vivo* proliferation of leukemic cells. We describe here the use of MFC to quantify AML cells in ovarian tissue. Ovarian MRD detection by MFC can be applied to all AML patients using a single standardized myeloid MRD tube. The experimental model of serial dilutions of AML cells in an ovarian cell suspension made it possible to validate robust sensitivity of 10^{-4} (20 events with abnormal phenotype among 200,000 viable events). The determination of the appropriate antibody combination for MRD assessment is very important to achieve good specificity. In this study, switching two antibodies enabled us to obtain approximately one log increase in sensitivity, and we observed a good correlation between MFC and RT-qPCR MRD results in the dilution experiments. MRD detection by MFC was applied to cryopreserved ovarian tissue from 4 AML patients. MRD was positive in 2 AML patients at very low levels (10^{-3} to 10^{-4}). The clinical significance of these positive MRD results must be interpreted with caution, since the ovarian MRD level that can induce a relapse after ovarian tissue transplantation is currently unknown. An MRD threshold mandatory for ovarian tissue transplan-

tation should be specified in the future, and for this purpose ovarian tissue xenograft could be an interesting option. Two other options are currently under investigation to eliminate the risk of re-introducing disease during ovarian tissue autograft, namely in vitro maturation of oocytes[14] and grafting of isolated ovarian follicles.[15]

In conclusion, we validate a technique to detect the presence of live AML cells in the ovarian cortex by MFC with high specificity and sensitivity. Improved MFC sensitivity could be achieved by performing the analysis on a larger number of cells. This MFC approach for ovarian MRD detection is currently the only available technique for ovarian MRD assessment when no molecular markers are available. MFC should promote the safe re-use of cortical ovarian fragments by autograft, without risk of re-introducing disease, in patients who have undergone gonadotoxic treatments.

Tristan Zver,[1,2,3] Magalie Alvergnas-Vieille,[1,4]
Francine Garnache-Ottou,[2,3,4,5] Christophe Ferrand,[2,5]
Christophe Roux,[1,2,3,4] and Clotilde Amiot[1,2,3,4]

[1]Service de Génétique Biologique, Histologie, Biologie du Développement et de la Reproduction, CHRU Jean Minjoz; [2]INSERM UMR1098; [3]Université de Franche-Comté, SFR FED4234; [4]INSERM CIC-1431, CHRU Jean Minjoz; and [5]EFS Bourgogne Franche-Comté, Besançon, France

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Correspondence: clotilde.amiot@univ-fcomte.fr
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