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# Ovarian reserve biomarkers usefulness for optimization of counselling in a public network for fertility preservation in oncological patients

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## ABSTRACT

**Objective**: the aim of the present study was to investigate the strength and reliability of biomarkers to optimize counselling in cancer patients.

**Methods**: retrospective observational study of fertility preservation cycles, performed in cancer patients referred to Pathophysiology of Human Reproduction, Semen and Oocyte Bank (Pordenone, Italy).

Ovarian reserve was evaluated with antral follicle count by 2D ultrasound and AMH determination by Beckman Coulter Generation II ELISA and also Roche Elecsys fully automated method, when available.

All patients were stimulated with antagonist protocol and modified ovulation triggering and when indicated according to random start protocol, to reduce the lag between referral and fertility preservation.

Aromatase inhibitors were added to gonadotropin in oestrogen receptor positive patients.

**Results**: numbers of retrieved/vitrified oocytes were used to evaluate fertility preservation efficiency. Fertility preservation was completed in 17 breast cancer oestrogen receptor positive patients, 6 breast cancer oestrogen receptor negative patients, 10 lymphomas patients and one gastrointestinal stromal tumour, one colon adenocarcinoma, one thyroid sarcoma, one medulloblastoma and one Ewing's sarcoma. The mean number of vitrified oocytes was 8.18 ± 5.22 in breast cancer oestrogen receptor positive, 11 ± 6.26 in breast cancer oestrogen receptor negative and 11.1 ± 8.81 in lymphomas. AMH was the most effective biomarker as predictor for fertility preservation outcomes and correlation was comparable with the two methods.

**Conclusions**: AMH seems to be the best biomarker to predict FP efficiency in cancer patients. Aromatase inhibitor introduction for ovarian stimulation seems to reduce its reliability.

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## SOMMARIO

**Obiettivo**: lo scopo dello studio è stato quello di indagare l'affidabilità dei biomarkers per ottimizzare il counselling in pazienti affetti da patologie neoplastiche.

**Metodi**: studio retrospettivo dei cicli di preservazione della fertilità eseguiti presso la "Fisiopatologia della Riproduzione Umana e Banca del Seme e degli Ovociti" dell'ospedale di Pordenone.

La riserva ovarica è stata valutata tramite conta dei follicoli antrali e determinazione dell'AMH.

I pazienti sono stati stimolati con protocollo antagonista e triggering ovulatorio modificato e, quando indicato, secondo il protocollo "random start stimulation" per ridurre il periodo tra riferimento e pick up. Gli inibitori dell'aromatasi sono stati aggiunti alle gonadotropine nei pazienti affetti da carcinoma mammario recettore positivo.

**Risultati**: il numero di ovociti recuperati/vitrificati è stato utilizzato per valutare l'efficienza dei cicli di preservazione della fertilità. Questa è stata portata a termine in 17 pazienti affetti da carcinoma mammario recettore positivo, 6 da carcinoma mammario recettore negativo, 10 da linfoma, uno da tumore stromale gastrointestinale, uno da adenocarcinoma del colon, uno da sarcoma della tiroide, uno da medulloblastoma ed uno da sarcoma di Ewing.

Il numero medio di ovociti vitrificati è risultato 8,18 ± 5,22 nei carcinomi mammari recettore positivi, 11 ± 6.26 nei carcinomi mammari recettore negativi e 11,1 ± 8,81 nei linfomi.

**Conclusioni**: l'AMH è risultato il migliore biomarker per predire l'efficienza della Fertility Preservation in pazienti affetti da patologie neoplastiche.

L'introduzione di inibitori dell'aromatasi per la stimolazione ovarica sembra ridurre la sua affidabilità.

**Keywords**: oocytes, vitrification, fertility preservation, AMH, breast cancer, lymphomas, aromatase inhibitor.

## INTRODUCTION

The combined effect of reproductive program deferral, increased incidence of cancers in childbearing age women and the huge improvement of their quod vitam prognosis, induce a growing demand for fertility preservation (FP) through oocytes vitrification. This trend was highlighted in a recent statement of the main Italian scientific societies in this field[1]. Moreover, a recent international statement highlighted also the need for early counselling about fertility preservation opportunities for all women affected by cancer[2]. Fertility Preservation substantially contributes to improve the quality of life for patients whose survival is now guaranteed, in the large majority of cases, by new cancer therapies. The introduction of oocyte vitrification into our daily laboratory practice, in the light of its non-experimental nature[3], has substantially contributed to the implementation of fertility preservation programs, finally ensuring gender equity in reproductive potential preservation in cancer patients. Even if artificial gametogenesis from both embryos and induced pluripotent stem cells has been recently validated in mouse[4], we think that a long time is needed until it could be implemented in human reproduction. So, at present an effective FP can be applied only with gametes cryopreservation before gonadotoxic therapies.

## MATERIALS AND METHODS

With the aim of optimizing fertility preservation counselling, we have retrospectively analysed all the cycles performed at Pathophysiology of Human Reproduction, Semen and Oocyte Bank at Pordenone Hospital, from October 2012 until now. The correlations between FP cycles outcomes, humoral and biophysical markers, and methods of ovarian stimulation were evaluated.

The 38 patients referred by neighbouring centers (Clinical Hematology University of Udine, Oncology Department of Udine and CRO of Aviano) mostly belong to two main groups: 23 breast cancer patients stimulated before or after surgery, according to oestrogen receptor status and 10 patients with Hodgkin's (HL) or non-Hodgkin's lymphomas (NHL). In addition to these two main groups, a gastrointestinal stromal tumour (GIST), a colon adenocarcinoma, a thyroid sarcoma, a medulloblastoma and an Ewing's sarcoma were referred for FP. Patients affected by breast cancers were mainly oestrogen receptor positive (17 cases), while only a minority (6 cases) were oestrogen receptor negative.

Multiple follicular growth was induced with different protocols according to oestrogen receptor status. In fact, in patients with oestrogen receptor positive neoplasm, the ovarian stimulation was carried out by co-administration of aromatase inhibitors (AI) (letrozole 5 mg daily) starting two days before gonadotropins introduction, in order to keep oestrogen levels as low as possible during multifollicular growth. Notwithstanding the lack of prospective randomized trials comparing aromatase inhibitors plus gonadotropins with the standard protocol with gonadotropins alone evaluating cancer recurrence as primary endpoint, this approach is generally adopted according to a precautionary principle suggesting to minimize the oestrogen exposure of the potentially residual neoplastic cells in oestrogen receptor positive tumours. Moreover, the safety of this protocol seems to be preliminarily confirmed by retrospective studies showing no higher recurrence rates of neoplastic diseases in patients undergoing fertility preservation, than those who do not[5-6].

In all our patients, a protocol with antagonist was adopted to minimize the risk of ovarian hyperstimulation syndrome (OHSS)[7] by inducing final oocyte maturation by GnRH agonists instead of human chorionic gonadotropin administration. This complication could be particularly undesirable in patients who need to start chemotherapy (CT) as soon as possible, due to the risk of worsening prognosis with its deferral. Moreover, in order to minimize the lag between the start of fertility preservation program and the beginning of the chemotherapy, an approach with random start stimulation (RSS) has been adopted when necessary. With this approach, the stimulation can start in any phase of the ovarian cycle, in accordance with the most recent evidences on the existence of multiple waves of folliculogenesis in each ovarian cycle. Oocytes were vitrified on open devices (Cryotop) according to Kuwayama protocol[8].

Antral follicle count (AFC) was performed immediately before the start of ovarian stimulation and therefore not always in the early follicular phase, considering follicles of 2-9 mm in diameter observed with 2D transvaginal ultrasound. AMH values were determined using random sampling and each sample was stored at -20 °C before assay with Beckman Coulter Generation II ELISA (BC Gen II) system and, after its recent implementation, Elecsys Roche automated method.

## RESULTS

We evaluated FP cycles outcomes (oocytes retrieved and vitrified), humoral and biophysical markers and methods for ovarian stimulation **(table 1)**.

Overall, the mean number of oocytes retrieved at pick-up was 12.95 ± 8.35, 80% of which was mature (10.37 ± 7.34 oocytes). The mean value of AMH was 4.6 ± 3.85 ng/ml. The parameters for which we assessed the correlation with retrieved

**Table 1.**
*Overview of all Fertility Preservation cycles*

| | Age | Type of neoplasm | AMH BC Gen II (ng/ml) | AMH Elecsys Roche (ng/ml) | AFC | Starting dose (IU) | Gn total dose (IU) | Peak E2 (pg/ml) | Oocytes retrieved | Oocytes vitrified |
|---|---|---|---|---|---|---|---|---|---|---|
| **1** | 38 | NHL | 2.1 | 1.67 | 8 | 300 | 3300 | 2928 | 14 | 12 |
| **2** | 39 | K breast ER + | 1.6 | 0.96 | 10 | 200 | 3000 | 282 | 13 | 8 |
| **3** | 37 | GIST | 2.2 | - | 7 | 225 | 2475 | 2972 | 8 | 6 |
| **4** | 35 | K breast ER - | 1.8 | - | 29 | 150 | 1875 | 2173 | 16 | 15 |
| **5** | 37 | K breast ER - | 2.2 | - | - | 225 | 2850 | 882 | 8 | 8 |
| **6** | 32 | K breast ER + | 7.2 | - | 37 | 150 | 1350 | 1999 | 11 | 10 |
| **7** | 32 | K breast ER + | 11.5 | 7.91 | 40 | 100 | 1100 | 490 | 4 | 2 |
| **8** | 29 | K breast ER - | 9.6 | - | 20 | 150 | 1500 | 1802 | 22 | 22 |
| **9** | 33 | HL | 1.7 | - | 11 | 225 | 2700 | 4763 | 16 | 12 |
| **10** | 32 | HL | - | - | 16 | 150 | 1125 | 1825 | 6 | 6 |
| **11** | 33 | K breast ER + | 6.1 | - | 36 | 150 | 1200 | 496 | 12 | 8 |
| **12** | 30 | Meulloblast. | 9.3 | - | 22 | 150 | 2625 | 3894 | 16 | 14 |
| **13** | 32 | K breast ER - | 3.4 | 2.38 | 24 | 150 | 2000 | 1050 | 14 | 7 |
| **14** | 39 | K breast ER + | 0.2 | - | 5 | 150 | 2625 | 121 | 4 | 3 |
| **15** | 39 | K breast ER + | 1.8 | - | 13 | 150 | 1425 | 273 | 6 | 5 |
| **16** | 27 | K breast ER + | 4.1 | - | 20 | 150 | 1650 | 172 | 12 | 9 |
| **17** | 33 | Colon adenok | 17.8 | - | - | 150 | 2337 | 213 | 10 | 6 |
| **18** | 26 | K breast ER + | 2.1 | - | 15 | 150 | 1562 | 171 | 2 | 0 |
| **19** | 22 | HL | 1.9 | 1.07 | 25 | 112.5 | 1050 | 637 | 12 | 10 |
| **20** | 27 | K breast ER + | 1.9 | 1.73 | 7 | 225 | 4125 | 269 | 10 | 6 |
| **21** | 37 | K breast ER - | 3.3 | 3.13 | 20 | 150 | 1650 | 1211 | 9 | 8 |
| **22** | 31 | K breast ER + | 11.9 | - | - | 150 | 1050 | 312 | 31 | 16 |
| **23** | 27 | HL | 2.5 | - | 15 | 150 | 1425 | 2055 | 7 | 6 |
| **24** | 25 | NHL | 2.2 | 1.38 | 12 | 150 | 2175 | 655 | 8 | 4 |
| **25** | 29 | HL | 2.5 | - | 14 | 300 | 2850 | 2825 | 12 | 9 |
| **26** | 26 | HL | 5 | - | - | 150 | 1300 | 326 | 12 | 10 |
| **27** | 32 | K breast ER + | 1.5 | - | 3 | 150 | 1350 | 424 | 12 | 6 |
| **28** | 26 | Thyroid sarc. | 7.7 | - | 20 | 150 | 1687 | 4483 | 26 | 22 |
| **29** | 34 | K breast ER + | 4.8 | - | 20 | 150 | 1400 | 265 | 14 | 13 |
| **30** | 26 | HL | 2 | - | 20 | 150 | 2400 | 939 | 7 | 7 |
| **31** | 28 | K breast ER + | 3.5 | - | 20 | 150 | 1200 | 438 | 9 | 8 |
| **32** | 35 | K breast ER + | 5.1 | - | - | 150 | 1650 | 503 | 14 | 12 |
| **33** | 32 | K breast ER + | 1.1 | 0.93 | 7 | 300 | 2550 | 124 | 4 | 4 |
| **34** | 16 | Ewing's sarc. | 6.2 | - | 20 | 150 | 1500 | 1084 | 32 | 30 |
| **35** | 38 | K breast ER + | 1.9 | - | 27 | 150 | 1800 | 357 | 8 | 8 |
| **36** | 36 | K breast ER + | 9.1 | - | 25 | 150 | 1725 | 677 | 23 | 21 |
| **37** | 23 | K breast ER - | 2.5 | 1.94 | 12 | 250 | 2100 | 619 | 7 | 6 |
| **38** | 31 | HL | 8.9 | 8.75 | 25 | 150 | 1500 | 2694 | 41 | 35 |

**K breast ER +**: receptor positive breast cancer; **K breast ER -**: receptor negative breast cancer; **Medulloblast.**: medulloblastoma; **Colon adenok**: colon adenocarcinoma; **Thyroid sarc.**: thyroid sarcoma; **Ewing's sarc.**: Ewing's sarcoma; **Gn**: gonadotropin.

and vitrified oocytes were: AMH measured with the Beckman Coulter Generation II ELISA and, when available, Roche Elecsys method, AFC, starting-dose, total dose of gonadotropins and peak oestradiol. The values which best correlated with the number of vitrified oocytes were oestradiol peak and AMH, although overall correlations were low **(table 2)**.

**Table 2.**
*Correlation r Pearson indexes between retrieved/vitrified oocytes and the different parameters studied*

| r | AMH BC Gen II (ng/ml) | AFC | Starting dose (IU) | Gn total dose (IU) | Peak E2 (pg/ml) |
|---|---|---|---|---|---|
| OOCYTES RETRIEVED | 0.46 | 0.22 | -0.14 | -0.18 | 0.35 |
| OOCYTES VITRIFIED | 0.39 | 0.25 | -0.13 | -0.18 | 0.4 |

Both, the starting-dose and gonadotropins total dose, negatively correlated with the number of vitrified oocytes. In table 3 are reported the mean values of retrieved and vitrified oocytes in receptor positive breast cancers, receptor negative breast cancers and lymphomas **(table 3)**.

**Table 3.**
*Retrieved and vitrified oocytes in the different subgroups*

| | BREAST CANCER ER + | BREAST CANCER ER - | LYMPHOMAS |
|---|---|---|---|
| AMH (ng/ml) | 4.44 ± 3.63 | 3.8 ± 2.91 | 3.2 ± 2.35 |
| OOCYTES RETRIEVED | 11.12 ± 7.23 | 12.67 ± 5.79 | 13.5 ± 10.22 |
| OOCYTES VITRIFIED | 8.18 ± 5.22 | 11 ± 6.26 | 11.1 ± 8.81 |
| MATURE PERCENTAGE | 74% | 87% | 82% |

The differences observed in retrieved and vitrified oocytes in the three subgroups were not significant. Correlation values reported in table 2 were calculated also in the different sub-groups: receptor positive breast cancers (table 4), receptor negative breast cancers (table 5) and lymphomas (table 6). This group includes the patients treated with the aromatase inhibitor, due to receptor positive status. In these patients with mean AMH value 4.44 ± 3.63 ng/ml, a mean of 11.12 ± 7.23 oocytes was retrieved, 8.18 ± 5.22 of which vitrified (74%). In this sub-group, the biomarker which best correlated with vitrified oocytes was the AMH level, even if the correlation value was low **(table 4)**.

**Table 4.**
*Correlation r Pearson indexes between retrieved/vitrified oocytes and different parameters in receptor positive breast cancers patients*

| r | AMH BC Gen II (ng/ml) | AFC | Starting dose (IU) | Gn total dose (IU) | Peak E2 (pg/ml) |
|---|---|---|---|---|---|
| OOCYTES RETRIEVED | 0.6 | 0.16 | -0.14 | -0.21 | 0.14 |
| OOCYTES VITRIFIED | 0.53 | 0.26 | -0.14 | -0.21 | 0.27 |

In these patients, with mean AMH value 3.8 ± 2.91 ng/ml, a mean of 12.67 ± 5.79 and 11 ± 6.26 (87%) were respectively retrieved and vitrified. In this subgroup, as expected, peak oestradiol highly correlated with the number of both retrieved and vitrified oocytes, due to the well-known relationship between the number of growing follicles and oestradiol output, in absence of aromatase inhibitors administration **(table 5)**.

**Table 5.**
*Correlation r Pearson indexes between retrieved/vitrified oocytes and different parameters in receptor negative breast cancer patients*

| r | AMH BC Gen II (ng/ml) | AFC | Starting dose (IU) | Gn total dose (IU) | Peak E2 (pg/ml) |
|---|---|---|---|---|---|
| OOCYTES RETRIEVED | 0.76 | 0.51 | -0.69 | -0.61 | 0.79 |
| OOCYTES VITRIFIED | 0.77 | 0.34 | -0.5 | -0.54 | 0.81 |

However, in this group, the statistical evaluation is only preliminary and not conclusive, due to the small number of observations presently available. The second largest group of patients was that of lymphomas. Even for this group the parameter with the best correlation was the AMH value, with very high Pearson index (0.87). In these patients with mean AMH value 3.2 ± 2.35 ng/ml, a mean of 13.5 ± 10.22 oocytes were retrieved, of which an average of 11.1 ± 8.81 vitrified (82%) **(table 6)**.

**Table 6.**
*Correlation r Pearson indexes between retrieved/vitrified oocytes and different parameters in lymphoma patients*

| r | AMH BC Gen II (ng/ml) | AFC | Starting dose (IU) | Gn total dose (IU) | Peak E2 (pg/ml) |
|---|---|---|---|---|---|
| OOCYTES RETRIEVED | 0.87 | 0.43 | 0.02 | -0.05 | 0.35 |
| OOCYTES VITRIFIED | 0.87 | 0.48 | -0.01 | -0.09 | 0.33 |

Moreover, means of patients' age, AMH, AFC, starting dose, peak oestradiol, total units of gonadotropin, retrieved and vitrified oocytes between different sub-groups were compared by Student's t-test.

A significant difference of peak oestradiol was observed between the patients with oestrogen receptor positive breast cancers and patients with both lymphomas (433.71 ± 431.40 vs 1967.2 ± 1387.13; p= 0.7x10-2) and oestrogen receptors negative breast cancers (433.71± 431.40 vs 1289.5±586.92; p= 0.01). In addition, a significant difference between lymphomas and breast cancers patients' ages was observed (28.9 ± 4.68 vs 32.94 ± 4.34; p= 0.04).

Recently, in our laboratory a fully automated method for AMH determination (Elecsys Roche)

has been implemented. After this implementation, we tried to compare AMH reliability for the number of retrieved and vitrified oocytes prediction with both non-automated and automated method.

Due to the observation that AI introduction for ovarian stimulation could reduce AMH reliability, we arbitrarily choose to compare AMH performance with both methods, only in patients stimulated without AI co-treatment. The correlation between oocytes retrieved/vitrified and AMH value was comparable with BC Gen II and Elecsys methods **(table 7)**.

**Table 7.**
*Correlation r Pearson indexes between retrieved/vitrified oocytes and AMH levels with BC Gen II and Elecsys Roche methods in AI-cycles*

| r | AMH BC Gen II | AMH Elecsys Roche |
|---|---|---|
| Oocytes retrieved | 0.95 | 0.93 |
| Oocytes vitrified | 0.93 | 0.93 |

We also determined the delay from diagnosis and start of chemotherapy. Time to chemotherapy was determined adding times between diagnosis and surgery, time between surgery and FP referral and between referral and pick-up.

For our breast cancer patients, the mean delay was 67.67 days ± 21.10 **(table 8)**.

**Table 8.**
*Delays to chemotherapy for breast cancer patients*

| | Diagnosis-surgery | Surgery-referral | Referral-pick up | Cumulative Delay |
|---|---|---|---|---|
| **Mean days ± S.D.** | 22.44 ± 11.94 | 27.05 ± 12.62 | 22.48 ± 9.02 | 67.67 ± 21.10 |

In lymphomas patients, due to the lack of surgical therapy, the delay consists of only two periods **(table 9)**.

**Table 9.**
*Delays to chemotherapy for lymphomas patients*

| | Diagnosis-referral | Referral-pick up | Cumulative delay |
|---|---|---|---|
| **Mean days ± S.D.** | 21.88 ± 9.72 | 14.3 ± 2.95 | 34.25 ± 11.23 |

For lymphomas patients the mean delay was 34.25 days ± 11.23.

The length of controlled ovarian stimulation was comparable in RSS and conventional protocol (9 days ± 0.82 with RSS vs 9.11 ± 2.27). Moreover in patients treated with random start stimulation protocol, the average number of vitrified oocytes was 15.9 ± 9.87 vs 9.43 ± 7.13 in conventional protocol in front of comparable ovarian reserves as evaluated with mean AMH level (3.78 ± 2.49 vs 4.69 ± 3.36; p=n.s.) with comparable starting and cumulative dose of gonadotropins **(table 10)**.

**Table 10.**
*Overview of our 10 RSS Fertility Preservation cycles*

| | Day of the cycle | AMH BC Gen II (ng/ml) | AMH Elecsys Roche (ng/ml) | AFC | Starting dose (IU) | Gn total dose (IU) | Peak E2 (pg/ml) | Oocytes retrieved | Oocytes vitrified |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 9 | 2.1 | 1.67 | 8 | 300 | 3300 | 2928 | 14 | 12 |
| 2 | 19 | 1.6 | 0.96 | 10 | 200 | 3000 | 282 | 13 | 8 |
| 4 | 15 | 1.8 | - | 29 | 150 | 1875 | 2173 | 16 | 15 |
| 8 | 48 | 9.6 | - | 20 | 150 | 1500 | 1802 | 22 | 22 |
| 13 | 19 | 3.4 | 2.38 | 24 | 150 | 2000 | 1050 | 14 | 7 |
| 21 | 13 | 3.3 | 3.13 | 20 | 150 | 1650 | 1211 | 9 | 8 |
| 25 | amenorrhea | 2.5 | - | 14 | 300 | 2850 | 2825 | 12 | 9 |
| 29 | 26 | 4.8 | - | 20 | 150 | 1400 | 265 | 14 | 13 |
| 34 | 23 | 6.2 | - | 20 | 150 | 1500 | 1084 | 32 | 30 |
| 38 | 36 | 8.9 | 8.75 | 25 | 150 | 1500 | 2694 | 41 | 35 |

Three patients were not included in the statistical evaluation, due to previous treatments with gonadotoxic drugs (for sarcoma, neuroblastoma and NHL). In these patients, six MII oocytes, four oocytes all lysated during decoronization and zero oocytes were respectively retrieved. These numbers are significantly lower than those observed in other patients and considered as needed for an acceptable probability of pregnancy[9]. Previous chemotherapy with alkylating agents therefore substantially impaired fertility preservation cycle efficacy.

On the contrary, in patient 9 (table 1) previously treated with six cycles of ABVD for HL, 16 oocytes were retrieved, of which 12 were mature. This patient was included in the statistical evaluation of the group of lymphomas, as the therapy with ABVD, considered less gonadotoxic than protocols carried out in the other three patients[10], allowed an oocyte recovery compatible with a good chance of pregnancy.

## DISCUSSION

We tried to find the most predictive parameters of FP programs effectiveness and to compare results with those of previous studies reporting AMH as the best biomarker for this purpose[11-12].

AMH predictability for FP outcomes in terms of retrieved and vitrified oocytes was excellent in oestrogen receptor negative breast cancers and lymphomas, but markedly lower in oestrogen receptor positive breast tumours. As expected, due to aromatase inhibitors co-administration, a significant difference of peak oestradiol was observed between the patients with oestrogen receptor positive breast cancers and patients with both lymphomas and oestrogen receptors negative

breast. In the small subgroup of the oestrogen receptor negative breast cancers, even peak oestradiol showed a good predictive power even if its usefulness is limited by its post hoc availability. Surprisingly, peak oestradiol correlated very well with retrieved and vitrified oocyte in oestrogen receptor negative breast cancers, but not in the subgroup of lymphomas, even if both subgroups were stimulated without aromatase inhibitors co-treatment.

In addition, lymphomas patients were significantly younger than breast cancers one. AMH as FP efficiency biomarker predictor was more reliable than AFC in each subgroup.

Moreover, both the starting dose, estimated on the basis of individual patient ovarian reserve and the total dose of gonadotropins were negatively correlated with the number of oocyte retrieved. This seems to suggest that the major determinant of oocyte recovery is ovarian responsiveness, in comparison with starting or cumulative dose of gonadotropins.

Concerning the efficacy of different protocols for ovarian stimulation the number of vitrified oocytes, with and without aromatase inhibitors, was not significantly different (p=0.08). However we cannot exclude that this trend will become statistically significant increasing the number of observations. So at present, a type β statistical error due to the small number of observations cannot be excluded at all. So a definitive costs/benefits evaluation of letrozole co-treatment for ovarian stimulation in oestrogen dependent tumours is not presently possible. Indeed, different opinions regarding this protocol exist in the literature with both positive[13] and negative effects reported as well[14]. Moreover, recent evidences from other authors show that the timing of letrozole introduction also seems to influence ovarian response and mature oocytes number, more than its use per se[15]. The advantage in terms of prognosis of the underlying disease, without adequately statistically powered studies evaluating the frequency of relapses with traditional stimulation or with AI addition, is not exactly quantifiable. Presently, the prescription of AI in oestrogen sensitive cancers is suggested only in accordance with a general principle of precaution but a detrimental effect on both number and percentage of mature oocytes cannot be ruled out. Moreover, our observations suggest that peak oestradiol does not correctly predict the number of retrieved oocytes if ovarian stimulation is performed with aromatase inhibitor use.

Regarding the overall interval before chemotherapy, the delay introduced by fertility preservation per se was extremely shorter than that before referral. However, overall delay in breast cancer patients was longer than that recently suggested as acceptable[16].

Our preliminary observations seem to suggest that ovarian stimulation with random start approach allows to retrieve at least the same or even an higher number of mature oocytes, if compared with standard protocol. These data are in agreement with previous observations by other authors[17-18]. Considering a possible detrimental effect of AI use on the number of retrieved/vitrified oocytes a realistic explanation of this observation could be that the percentage of patients treated with RSS and AI was lower (20%) than that of patients treated with gonadotropin alone (80%). Anyway even in AI+ protocol the number of vitrified oocytes was suitable for a good chance of pregnancy, according to what suggested in recent elective fertility preservation programs[9].

However, even using RSS to minimize the delay of CT, we urgently need to sensitize both oncologists and haematologists to a fast patient referral for fertility preservation, even before surgery, if indicated. The possibility that clinical prognosis of receptor negative cancers could be improved by pre-surgery fertility preservation due to shortened time to chemotherapy should be addressed by future studies.

In our experience, 12 patients refused fertility preservation due to the advanced stage of breast cancers or lymphomas, or due to the perceived physical burden of the procedure.

With antagonist protocol and modified triggering, no cases of ovarian hyperstimulation syndrome were observed even in patients with an high number of retrieved oocytes. Thus, ovarian stimulation with antagonist protocol and modified triggering seems to be safe for our daily clinical practice of FP.

Moreover our preliminary data show that fertility preservation can be done with a limited further delay of chemotherapy if compared with that between diagnosis and patient referral for FP.

Anyway, several burning questions still remain unanswered in FP programs. Today how many patients are presently referred for FP? The ASCO guidelines suggest that in the past only a small percentage of cancer patients were referred to the centers performing fertility preservation[19]. Which is the best protocol for ovarian stimulation? Gonadotropins alone or gonadotropin plus AI in oestrogen receptor positive cancers? Which is the best drug for ovarian stimulation in such patients:

classic gonadotropins or the more user friendly corifollitropin needing only one injection for the first seven days of stimulation? Moreover, the problem of how FP can be matched with ovarian protection by pharmacological treatment by GnRH analogue administration is still an open question. To avoid OHSS we need to do ovarian stimulation using antagonist protocol without GnRH receptors down regulation, allowing ovulation triggering by GnRH agonist. Therefore, GnRH analogue administration before ovarian stimulation for FP is not possible. However, with GnRH analogue administration after oocytes retrieval, immediately before chemotherapy a flare effect, due to gonadotropins mediated angiogenic effect, could theoretically increase post chemotherapy ovarian damage. At present, we don't know neither the percentage of utilization of vitrified oocytes, nor the ART outcome in cancer patients with vitrified oocytes. Presently data regarding ART outcomes with vitrified oocytes in patients with history of cancer are not completely consistent. Some studies show comparable results with that of infertile couples[20], while others show that the number of retrieved oocytes is related to the type of cancer. Anyway, ART outcome also in such case are clearly related to the mean age of the patients[21] which is variable in different types of cancers. It could be that in older patients, emergency IVF with embryo cryopreservation could be a more effective approach if compared with oocyte vitrification notwithstanding ethical qualms. However, such an approach is presently not allowed by the Italian law on reproduction, due to limitations for embryo cryopreservation.

FP cost effectiveness, acceptability and clinical outcomes in comparison with post hoc ovodonation should also be addressed by future studies.

The last but not least problem is the presently limited affordability of FP in our public health insurance system due to shortage of centres offering such practices.

In conclusion, our preliminary data seem to confirm that antagonist protocol with modified agonist triggering of ovulation is a good approach to avoid OHSS. RSS seems equally effective than conventional protocol. AI introduction for receptor positive breast cancer stimulation, reduced the number of retrieved/vitrified oocytes, and AMH level correlation with the number of retrieved/vitrified oocytes was lower in cycles with AI if compared with both oestrogen receptor negative cancers and lymphomas stimulated with gonadotropins alone. Aromatase inhibitors co-treatment is able to significantly reduce peak oestradiol level but presently the impact of this protocol on cancers recurrence is not clear and a detrimental effect on FP effectiveness cannot be excluded.

Both automated AMH and BC Gen II AMH correlated well with both oocytes retrieved and vitrified in cycles without AI co-treatment. AMH BC Gen II correlation with both retrieved and vitrified oocytes in FP cycles with ovarian stimulation without AI was lower. Our data confirm previous observations of lower AMH values with the new automated assay[22].

The mean delay to chemotherapy in our patients was different according to the kind of neoplasm: this lag was shorter in lymphomas if

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