

### Absence of Conclusive Evidence for the Safety and Efficacy of Gonadotropin-Releasing Hormone Analogue Treatment in Protecting Against Chemotherapy-Induced Gonadal Injury

KUTLUK OKTAY,<sup>a</sup> MURAT SÖNMEZER,<sup>b</sup> ÖZGÜR ÖKTEM,<sup>a</sup> KEVIN FOX,<sup>c</sup> GÜNTER EMONS,<sup>d</sup> HEEJUNG BANG<sup>e</sup>

<sup>a</sup>Department of Obstetrics and Gynecology, Joan and Sanford I. Weill Medical College of Cornell University, New York, New York, USA; <sup>b</sup>Department of Obstetrics and Gynecology, Ankara University School of Medicine, Ankara, Turkey; <sup>c</sup>Rena Rowan Breast Center, Abramson Cancer Center of the University of Pennsylvania, Philadelphia, PA, USA; <sup>d</sup>Department of Obstetrics & Gynecology, University of Göttingen, Göttingen, Germany; <sup>e</sup>Division of Biostatistics and Epidemiology, Department of Public Health, Weill Medical College of Cornell University, New York, New York, USA

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#### ABSTRACT

Every year, an increasing number of women with malignant and nonmalignant diseases is successfully treated with cytotoxic chemotherapy and/or radiotherapy. Many of these patients suffer from infertility and gonadal failure as a result of these treatments. At present, these patients may resort to assisted-reproduction techniques to protect their future childbearing potential before the implementation of cytotoxic therapy. While embryo cryopreservation is an established technology, oocyte and ovarian tissue freezing techniques are still investigational. Nevertheless both

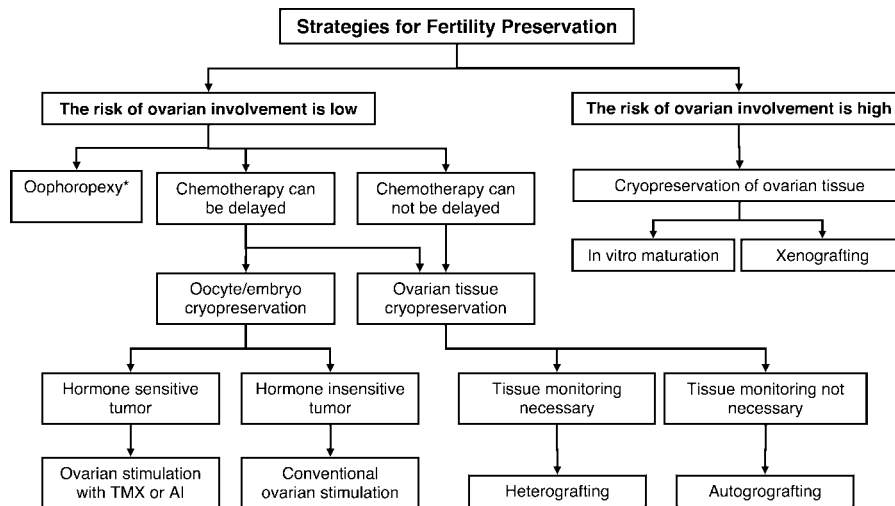
of these techniques have resulted in live births. Apart from assisted-reproduction techniques, it has been extensively debated whether administration of gonadotropin-releasing hormone (GnRH) analogues in conjunction with chemotherapy can protect ovarian reserve against cytotoxic insult. In this manuscript, we debate the rationale for the effectiveness of GnRH analogue coadministration in preservation of fertility by reviewing the literature, and provide preliminary data to support our views. *The Oncologist* 2007;12:1055–1066

#### INTRODUCTION

Fertility preservation has evolved within the past 10 years and an individualized approach has been developed, using mostly cryopreservation technologies (Fig. 1) [1]. While embryo cryopreservation is an established technology, oocyte and ovarian tissue freezing tech-

niques are still investigational. Nevertheless, both of these techniques have resulted in live births. Development of a medical prevention, on the other hand, would simplify the process by avoiding assisted-reproduction techniques and procedures. The role of gonadotropin-releasing hormone (GnRH) analogues in fertility preser-

Correspondence: Kutluk Oktay, M.D., Department of Obstetrics and Gynecology, Joan and Sanford I. Weill Medical College of Cornell University, 505 East 70th Street, HT-340, New York, New York 10021, USA. Telephone: 212-746-4292; Fax: 212-746-5929; e-mail: [Koktay@fertilitypreservation.org](mailto:Koktay@fertilitypreservation.org) Received January 16, 2007; accepted for publication July 20, 2007. ©AlphaMed Press 1083-7159/2007/\$30.00/0 doi: 10.1634/theoncologist.12-9-1055



**Figure 1.** Algorithmic approach to patients desiring fertility preservation.

\*The success of oophorectomy is diminished if radiosensitizing chemotherapy is performed.

Abbreviations: AI, aromatase inhibitor; TMX, tamoxifen.

vation is highly debated [2–5]. In this manuscript, we review the current data on the efficacy and safety of GnRH analogues in cancer patients when used for fertility preservation purposes.

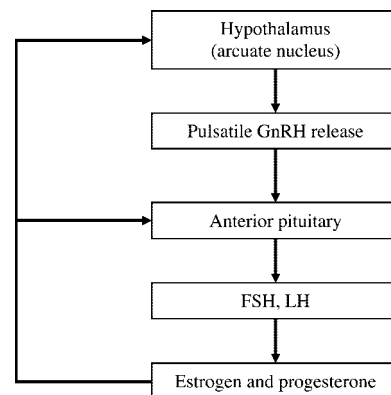
We believe that, for a new medical treatment to be proven effective, the following three conditions should be met:

1. There must be a biological plausibility of the effect of the drug or treatment.
2. Multiple prospective, controlled studies must show consistent results.
3. Potential risks of the treatment should not exceed potential benefits.

For the reasons that are discussed below, we do not believe that these conditions have been met for GnRH analogue treatment to be recommended for preservation of fertility in women undergoing chemotherapy or radiotherapy.

### BIOLOGICAL PLAUSIBILITY FOR GONADAL PROTECTION BY GnRH ANALOGUES IS LACKING

Regulation of the menstrual cycle and ovarian function requires complex interaction among the hypothalamus, anterior pituitary, and ovary (Fig. 2). GnRH, which is a decapeptide, is synthesized within the GnRH neurons located in the arcuate nucleus of the hypothalamus, and released in a pulsatile fashion from the nerve endings to the portal circulation and transported to the anterior hypothalamus to stimulate follicle-stimulating hormone (FSH) and luteinizing hormone (LH). Normal gonadotropin secretion requires pulsatile GnRH secretion within a critical range of frequency and amplitude. The half-life of GnRH is only



**Figure 2.** Regulation of ovarian function through the hypothalamic–pituitary–ovarian axis.

Abbreviations: FSH, follicle-stimulating hormone; GnRH, gonadotropin-releasing hormone; LH, luteinizing hormone.

2–4 minutes because of rapid cleavage of the amino acid bonds 5–6, 6–7, and 9–10. Agonistic GnRH analogues are produced by substitution of amino acids at the 6 position or replacement of the C-terminal glycine-amide. This results in sustained action of the GnRH analogues, which causes downregulation of GnRH receptors. Because of the initial sustained binding to GnRH receptors, there is an initial flare effect that increases gonadotropin levels and causes ovarian stimulation. To avoid ovarian stimulation, typically the GnRH analogues are administered in the days before menstruation. Following an initial flare-up effect, GnRH agonists produce a hypogonadal state within 1–3 weeks of administration [6].

In the adult ovary, >90% of the ovarian reserve is made up of primordial follicles in the resting stage that includes an oocyte arrested at prophase of the first meiotic division.

Even though the mechanism of primordial follicle growth initiation is still not understood, it is clearly an FSH-independent process; FSH receptors are not expressed until these follicles initiate growth and reach multilayer stages [7–10]. Because the main effect of GnRH analogues is to suppress pituitary gonadotropin secretion, it is not plausible that the presumed protective effect of GnRH analogues is gonadotropin dependent. Furthermore, because profound ovarian suppression may take several weeks to achieve, it is unlikely that sufficient lowering of gonadotropins will be achieved within the short time available before the initiation of chemotherapy [11]. Moreover, if GnRH analogues are given during the follicular phase of the cycle, they may actually cause a flare effect and create the opposite of the desired impact.

It has previously been misquoted that gonadotropin receptor mRNA is expressed in various stages of oocytes [3, 12]. In the study of Patsoula et al. [12], only mature oocytes that failed to fertilize with intracytoplasmic sperm injection were used in addition to embryos of various stages. If GnRH analogues were protective, oocytes of primordial follicles should express FSH receptors (FSHRs), but FSHRs have repeatedly been shown to be absent from these follicles [7, 13]. Moreover, gene expression does not necessarily imply that transcripts are translated in the protein or that these receptors are functionally involved in signal transduction. In the Patsoula et al. [12] study, reverse transcription-polymerase chain reaction (RT-PCR) products for the FSHR and LH receptor (LHR) in humans arose from the extracellular portion of the receptors, which has been shown to appear earlier than the transcripts for the full-length receptor protein [14, 15]. The presence of mRNA does not mean that functional receptors are expressed on cells, and does not prove a “physiological” role. In the study of Zheng et al. [16], which focused on FSH mRNA expression in tubal epithelium, only a weak focal mRNA signal was seen by *in situ* hybridization in primordial follicles, and the data were not shown. When using *in situ* hybridization, because of the minute size and flattened shape of primordial pregranulosa cells, it is technically challenging to distinguish background signals from signals in primordial follicles. By RT-PCR, we did not detect FSHR mRNA in isolated human primordial follicles [7]. Moreover, human ovarian follicles continue to initiate growth when xenografted in hypogonadal-immunodeficient mice [13], or in patients with FSHR mutation [9]. There has been no evidence for the presence of FSHR protein in primordial follicles. Primordial follicles continue to initiate growth through hypogonadal states such as prepuberty, pregnancy, and the use of oral contraception, and ovarian suppression by oral contraceptives does not prevent chemotherapy-

induced gonadal damage [17]. As the authors of the study pointed out, increased depletion of primordial follicles in LH-overexpressing mutant mice does not indicate a direct effect of LH [18]. In fact, in that study, the authors showed a reduction and not an increase in the fraction of follicles entering the growth pool (primary), and concluded that the effect of LH was indirect, and that their data did not prove that primordial follicles were gonadotropin responsive. As was discussed in that report, LHRs have never been detected in primordial or early preantral follicles, and the authors explained the loss of primordial follicles by the toxic effects of the local endocrine milieu stimulated by supra-physiologically high LH stimulation on stromal cells.

Moreover, as discussed below, because GnRH creates a hormonal milieu similar to the prepubertal state and because prepubertal children are not protected against the gonadal-damaging effects of chemotherapy, hypogonadism cannot be a means to preserve fertility.

Furthermore, GnRH analogue treatment is clearly ineffective in the setting of preconditioning chemotherapy for hematopoietic stem cell transplantation (HSCT). If GnRH analogues were truly protective by means other than hypogonadism, such as inhibition of apoptotic death, one would expect them to be protective against high-dose chemotherapy and radiation as well. For example, the antiapoptotic agent sphingosine-1-phosphate (S1P) blocks oocyte death against chemotherapy and radiation in mice [19, 20]. From the same logic, and considering that similar types of agents are toxic to the testis and ovary, that the testis is mitotically much more active than the ovary, and that germ cell production is more acutely dependent on FSH in the testis, the ineffectiveness of GnRH analogues in the testis can hardly be consistent with the claims that the same agents would protect the ovary against chemotherapy [11, 20].

Likewise, the claims that GnRH analogues may protect ovarian reserve by reducing blood flow are not supported by scientific evidence. Furthermore if GnRH analogues were to cause reduced blood flow to the ovary, one would expect this to happen with other organ systems, and even with the tumor, resulting in an overall lower effectiveness and organ toxicity of the drug.

#### **LACK OF RANDOMIZED CONTROLLED TRIALS**

Even though the effectiveness of GnRH analogues as fertility-preserving agents is highly debated, the only randomized study published thus far did not show a benefit, and nonrandomized studies provided mixed results (Table 1). In two recent studies, Recchia et al. [21] and Del Mastro et al. [22] made valuable contributions on this topic. In the first study, Recchia and colleagues demonstrated that gonadal function was protected in all breast cancer patients receiv-

**Table 1.** Summary of studies evaluating the value of GnRH analogue coadministration to protect against chemotherapy-induced ovarian failure

Study	Study group	Study design	n	Regimen	Age (mean)
Waxman et al. (1987) [11] <sup>a</sup>	Study (GnRHa)	Prospective, randomized	8	MVPP	28.5
	Control		10		25.9
Blumenfeld et al. (1996) [42] <sup>b,c</sup>	Study (GnRHa)	Study group prospective; control group retrospective	16	MOPP, ABV(D), CHOP C-MOPP/ABV(D), CHOP, C-MOPP, ABV	23.1
	Control		18		25.8
Blumenfeld et al. (2000) [43] <sup>d</sup>	Study (GnRHa)	Study group prospective; control group retrospective	6	Pulsed Cyc or chlorambucil	26.3
	Control		9		27.4
Pereyra Pacheco et al. (2001) [33] <sup>e</sup>	Group A	Group A and C retrospective; group B prospective	5	CAPVE, CVPP, ICE, CCOPP, ESHAP, CBV, BuCy	5.2
	Group B (GnRHa)		12		16.8
	Group C		4		17.8
Somers et al. (2005) [32]	Study (GnRHa)	Control group retrospective	20	Bolus Cyc	23.9
	Control		20		25
Dann et al. (2005) [37]	Group I (GnRHa)	Prospective, nonrandomized	7	Mega-CHOP	25.5
	Group II		6		26.5
Recchia et al. (2006) [21] <sup>f,g</sup>		Retrospective, single arm	26	CMF	42
			11	CEF	39
			54	CMF + E	42
			9	HSCT	37
Del Mastro et al. (2006) [22] <sup>h</sup>		Prospective, single arm	29	CEF	38
Ellis et al. (2006) [39] <sup>i</sup>		Retrospective, single arm	36	CHOP, VACOP-B	38

(continued)

ing cyclophosphamide-based chemotherapy under the age of 40 years, and in 56% of those aged >40 years. All of the 100 patients, almost 50% of whom were negative for estrogen receptors, were cotreated with a GnRH analogue for 2 years. The cumulative dose of cyclophosphamide ranged between 3,600 and 7,200 mg/m<sup>2</sup> and the mean follow-up was 75 months. The authors hypothesized that the GnRH analogue could preserve ovarian function against chemotherapy-induced gonadal damage by putting cells of the ovarian epithelium in G<sub>0</sub>. Moreover, they surmised that the administration of a GnRH analogue did not affect prognosis, because the projected recurrence-free survival rates were 84% and 76% at 5 and 10 years, and the projected overall survival rates were 96% and 91% at 5 years and 10 years, respectively.

While the above study reported a high incidence of resumption of menstruation, menstrual status is not a good index of residual fertility [23], and without a control group the success of GnRH analogue treatment cannot be validly assessed. Moreover, spontaneous retention of menstruation

can be significantly high depending on the chemotherapy regimen and the patient's age. Ovarian failure is diagnosed by at least two measurements of FSH >40 mIU/ml, regardless of menstrual bleeding. Continuation or resumption of menstruation is not a reliable indication of ovarian function and fertility, because pregnancy rates are extremely low when FSH measurements on the second or third day of the menstrual period exceed 12 mIU/ml (20 mIU/ml by radioimmunoassay) [24]. Likewise, elevation of estradiol levels above 75 pg/ml on the second or third day of the menstrual period is also associated with compromised fertility [25].

Schmidt et al. [26] evaluated ovarian function postchemotherapy in 22 cancer patients using ovarian reserve tests, including day-3 FSH, estradiol, and inhibin B levels, in addition to the assessment of menstrual pattern. GnRH analogues were not used during the courses of chemotherapy. When only menstrual cycle was considered, none of the eight breast cancer patients receiving cyclophosphamide, epirubicin, and fluorouracil (CEF) for six to eight cycles ( $n = 6$ ; total cumulative cyclophosphamide dose, 3,600–

**Table 1.** (Continued)

CT indication	Cumulative dose of alkylating agent (mean)	Mean follow-up (years)	POF rate (%)	Statistical analysis	Major weakness of study
Lymphoma	0.072 g/m <sup>2</sup> + 1.2 g/m <sup>2</sup>	2.3 2.0	75 80	Yes	Small number of subjects
Lymphoma	0.78 g/m <sup>2</sup> + 23.1 mg/m <sup>2</sup> 2.13 g/m <sup>2</sup> + 25.5 mg/m <sup>2</sup>	1.7 7	6.3 61	Yes	Longer mean and median follow-up in controls
SLE, nephrotic syndrome, juvenile rheumatoid arthritis	7.66 g 13.3 g	8.33 9	0 55.5	No	Small numbers in each group; dose of alkylating agent missing in 2 patients in study and 3 patients in control group; treatment was not specified in 2 of the control patients
Lymphoma, leukemia, thymoma	NA NA NA	18 5 6	0 0 100	No	Small number of patients; different follow-up between groups; cumulative dose not clear; no statistical analysis
SLE	12.9 g 12.9 g	4.6 10.3	5 30	Yes	Control group retrospective; follow-up longer in control group; E2 and P4 use during treatment; FSH threshold too high (<40) for fertility; disease more severe in controls
Lymphoma	11.7 g/m <sup>2</sup> 10.5 g/m <sup>2</sup>	5.34 6.23	0 16.6	No	Small numbers; nonrandomized; no statistical analysis
Breast cancer	7.2 g/m <sup>2</sup> 3.6 g/m <sup>2</sup> 4.8 g/m <sup>2</sup> 4.8 g/m <sup>2</sup> + 100 mg/m <sup>2</sup>	6.25	33 (none aged <40 yrs)	Yes	Single arm; hormonal assessment of ovarian function unclear; no statistical analysis
Breast cancer	3.6 g/m <sup>2</sup>	1	3	Yes	Single arm; no statistical analysis
Lymphoma	4.7 g	7	5	Yes	Single arm; no statistical analysis; fertility status determined according to menstrual cycle characteristics; with the aim of preserving fertility, only 3 patients received GnRH <sub>a</sub> , whereas 10 received OCP

The alkylating agent is cyclophosphamide if not specified.

<sup>a</sup>Cumulative dose of mechlorethamine and procarbazine.

<sup>b</sup>Cumulative dose of cyclophosphamide and mechlorethamine.

<sup>c</sup>Thirteen of 16 patients in the study group (mean, 2,320 ± 1,521 cGy) and 11 of 18 patients in the control group (mean, 1,882 ± 1,983 cGy) received mantle field irradiation (ovaries indirectly exposed).

<sup>d</sup>Cumulative dose of cyclophosphamide in the range of 6–11 g in the study group and 4–26.5 g in the control group. One patient in the control group and one in the study group received chlorambucil (totaling 5 g and 11 g, respectively).

<sup>e</sup>Group A, premenarchal girls; group B, postmenarchal girls who received a GnRH<sub>a</sub>; group C, postmenarchal girls who *did not* receive a GnRH<sub>a</sub>.

<sup>f</sup>Cumulative dose of cyclophosphamide and melphalan were given for HSCT.

<sup>g</sup>Follow-up and <sup>h</sup>age given as median.

<sup>i</sup>Patients also received doxorubicin at a mean dose of 466 mg. Ten patients were treated with additional radiotherapy. No statistical difference found in POF rates between those given and not given fertility-preserving measures.

Abbreviations: ABV(D), doxorubicin, bleomycin, and vinblastine (with or without dacarbazine); BuCy, busulphan and cyclophosphamide; CAPVE, cyclophosphamide, doxorubicin, vincristine, prednisone, and etoposide; CBV, BCNU, etoposide, and cyclophosphamide; CEF, cyclophosphamide, epirubicin, and fluorouracil; CCOPP, CCNU, cyclophosphamide, vincristine, procarbazine, and prednisone; CHOP, cyclophosphamide, doxorubicin, vincristine, prednisolone; CMF, cyclophosphamide, methotrexate, fluorouracil; C-MOPP, cyclophosphamide plus MOPP; CT, chemotherapy; CVPP, cyclophosphamide, vinblastine, procarbazine, and prednisone; Cyc, cyclophosphamide; E, epirubicin; E2, estradiol; ESHAP, etoposide, ara-cytidine, and platinum; FSH, follicle-stimulating hormone; GnRH<sub>a</sub>, gonadotropin-releasing hormone analogue; HSCT, hematopoietic stem cell transplantation; ICE, ifosfamide, carboplatin, and etoposide; MOPP, mechlorethamine, vincristine, prednisolone, and procarbazine; MVPP, mechlorethamine, vinblastine, procarbazine, and prednisone; NA, not available; OCP, oral contraceptive pills; P4, progesterone; POF, premature ovarian failure; SLE, systemic lupus erythematosus; VACOP-B, cyclophosphamide, doxorubicin, vincristine, bleomycin, etoposide, and prednisone.

4,800 mg/m<sup>2</sup>, cyclophosphamide, methotrexate, and fluorouracil (CMF) for three cycles plus 330 mg doxorubicin (*n* = 1; total cumulative cyclophosphamide dose, 1,800 mg/

m<sup>2</sup>), or CEF for three cycles plus CMF and doxorubicin (*n* = 1; total cumulative cyclophosphamide dose, 1,800 mg/m<sup>2</sup> + 4.4 g) experienced ovarian failure during a fol-

low-up that ranged between 21 and 45 months. However, despite resumption of menstruation, three breast cancer patients had irregular menstrual cycles and five had undetectable inhibin-B levels or FSH values  $>50$  IU/ml, suggesting impairment in ovarian reserve. The age of breast cancer patients ranged between 27 and 36, while the cumulative dose of cyclophosphamide ranged between 3,000 and 8,000 mg. The authors claimed that the cumulative dose of cyclophosphamide that was used in that study was not adequate to cause complete ovarian failure in that group of young breast cancer patients. However, in the same study, all of the five patients with leukemia and two with Hodgkin's disease undergoing HSCT, and one receiving high-dose chemotherapy with alkylating agents for Hodgkin's disease, had premature ovarian failure (POF) after completion of chemotherapy. Their ages ranged between 21 and 26.

It is known that anti-Müllerian hormone (AMH) is expressed by granulosa cells [27, 28]; its expression is initiated in the smallest growing follicles and declines in the early antral stages as one follicle is selected for dominance and the rest of them become atretic. An important finding from a very recent study was that, compared with estradiol and FSH, AMH showed a more rapid and sustained change after chemotherapy [29]. Moreover, the decrease in AMH occurred without a significant decrease in inhibin-B or increase in FSH concentrations. The authors concluded that the severity and rapidity of the fall in AMH concentrations compared with the partial decline in inhibin-B concentrations might reflect primordial and preantral follicles as the primary site of toxicity. This supports the observation that, even though there may be no clinical signs of ovarian failure, there is always damage to follicular reserve in proportion to the cumulative dose of exposed chemotherapeutic agents that might not be detectable with routinely used laboratory tests. Our recent findings are also in agreement with those of Anderson et al. [30].

Del Mastro and colleagues investigated the role of GnRH analogues to preserve fertility in 29 breast cancer patients receiving CEF for six cycles [22]. All except one patient received CEF for six cycles; the cumulative dose of cyclophosphamide was  $3,600$  mg/m<sup>2</sup>. The treatment was defined as successful if menstrual activity returned within 12 months after the last cycle of chemotherapy, or if an FSH level was  $<40$  IU/l between 3 and 12 months postchemotherapy. Based on that definition, the overall success rate was 97%, but there was no control group. Of the 17 patients under 40 years of age, 16 regained menstrual activity (94%), whereas only five patients aged  $>40$  years resumed menstruation postchemotherapy (42%), but it was not specified whether their periods were regular. Up to 97% of the patients suffered from GnRH analogue-related side effects,

including hot flushes, headaches, mood changes, and sweating. Unfortunately, the follow-up was too short to assess the impact on fertility. Moreover, fertility is impaired when FSH levels exceed 12 IU/ml, a much lower value than used by the authors [24]. Furthermore, the estradiol levels were also elevated in the patients reported by Del Mastro et al. [22]; baseline estradiol  $>75$  pg/ml is also associated with poor reproductive outcome regardless of FSH level [25]. The latter is a result of the accelerated follicle development induced by the rising baseline FSH levels [31]. Of the 25 patients in whom FSH was measured, 21 had a value  $<40$  IU/l; it would be interesting to know whether they were  $<12$  IU/l. Moreover, as an ovarian stimulant, tamoxifen raises estrogen levels that in turn would spuriously lower FSH levels because of negative feedback. Of the patients with menstrual resumption, one had an FSH value  $>40$  IU/l, and three had an estradiol level  $<20$  pg/ml; both are consistent with ovarian failure. Unreliability of menstruation as a marker of fertility is apparent from the authors' data, where FSH levels well above the menopausal range (up to 59.9 mIU/ml) were found in some patients who continued to menstruate. The authors did not specify whether the pattern of menstruation was similar to the prechemotherapy period; many perimenopausal women continue to menstruate at regular intervals. In their discussion, the authors stated that they could not rule out that age itself, rather than GnRH analogue treatment, is the main determinant of ovarian function preservation.

Somers et al. [32] evaluated the protective role of GnRH analogues in 20 systemic lupus erythematosus patients receiving monthly i.v. bolus cyclophosphamide, and compared them with a historical group of patients matched for age and cumulative cyclophosphamide dose. At a minimum follow-up of 3 years, ovarian failure developed in 1 of 20 GnRH analogue-treated patients (5%), compared with 6 of 20 controls (30%). There were several limitations to the study. The treatments were not randomized, and the study was not designed to assess preservation of ovarian function, as the authors pointed out. In any observational study, including this one, the comparability or homogeneity of comparison groups (via control group selection) in terms of various population characteristics should be preserved as much as possible in the absence of randomization. However, we noted that the control group and study group were not matched for menstrual status and hormonal profile prior to chemotherapy at study entry, among other conditions that could be potentially important. Furthermore, control patients were retrospectively selected from a group of patients who accrued significantly longer follow-up (evidenced by total person-years of 186.9 versus 100.2 or other summary measures such as median or maximum) that may

contribute to considerable imbalance and consequently unfair comparison. Even though the authors attempted to mitigate this by using time-to-event analysis and by only including patients with at least 3 years of follow-up, we do not feel that these strategies are of much help or a correct remedy. For example, it was not clear how many patients had irregular menstrual periods or elevated FSH levels ( $>12$  IU/l) at follow-up, and the definition of “normal ovarian function,” that is, “lack of amenorrhea of at least 12 months and an FSH level  $<40$  mIU/ml,” could not be used to assess fertility. Despite age matching, disease duration was longer in the control group than in the study patients (mean  $\pm$  standard deviation,  $5.1 \pm 1.5$  years versus  $3.6 \pm 1.2$  years), and as the authors acknowledged, the control patients tended to be more severely ill, all of which might have contributed to the higher ovarian failure rates in the control group. Furthermore, the patients received add-back estrogen treatment with i.m. progesterone, making the menstrual assessment unreliable. There were statistical weaknesses as well; the survival analysis tools beyond the Kaplan–Meier method should have considered or been accompanied by an attempt to adjust some prognostic factors that were differentially distributed between the two cohorts. Moreover, it was not known how many patients attempted pregnancy in either group, making it impossible to compare pregnancy rates. As such, much more information should have been ascertained and properly reported from study patients. In addition to the selection bias problem, another major methodological weakness of the study is the statistical power. Our own calculation shows that the given study provides approximately 40% power to detect the difference in the event rates observed. Let alone this post hoc assessment, it is hard to make a clinical or statistical argument from a study with only one event in one group. It is important to note that the number of events, not necessarily the number of the total sample, governs the power (equivalent, type II error) in the analysis of time-to-event data. This is indeed a fact that is quite intuitive, but that many practitioners and researchers often overlook in designing and operating their studies, although we fully understand that small samples in similar circumstances are difficult to overcome. Any covariate adjustment suggested above may not be feasible solely because of the extremely low number of events. These statistical pitfalls (especially, power and selection by indication in any nonrandomized studies) are relevant to virtually all of the biomedical studies generated by quantitative hypotheses, including our own.

In a case series including 12 patients aged 14.7–20 years (group B), Pereyra Pacheco et al. [33] addressed the effectiveness of GnRH analogues in fertility preservation. Control groups included five premenarchal children aged 3–7.5

years (group A) and four postmenarchal patients aged 15.9–20 years (group C). While the treatment group was followed up prospectively, control patients were chosen retrospectively. Moreover, the length of follow-up was clearly different among the groups: 18 years in group A, 5 years in group B, and 6 years in group C. Although three patients conceived during follow-up in group A, two to five patients had oligomenorrhea, which should have been considered as a clinical sign of diminished ovarian reserve. With group A, the authors argued that prepubertal children were protected from the gonadal effects of cancer treatments, in support of the benefit of GnRH analogue–induced hypogonadism in postpubertal individuals. Not only were the size and design of the study insufficient to reach such a conclusion, but a recent, large, prospective study showed that, when exposed to chemotherapy during childhood, the risk for POF is over 13-fold higher than in controls [34]. The preliminary report by Pereyra Pacheco et al. [33] was exploratory in nature, and no formal statistical analysis was attempted.

In the only prospective, randomized study, reported by Waxman et al. [11], albeit with small numbers, including 31 men and 18 women receiving chemotherapy for Hodgkin’s disease, GnRH analogues did not preserve fertility, as judged by sperm count and menstrual function. This was a well-designed study in which all patients underwent complete endocrine evaluation and GnRH stimulation tests before, during, and after chemotherapy. By performing GnRH stimulation tests, the authors determined the amount of ovarian suppression in each patient. They found that estradiol levels were consistently low in both men and women, but gonadotropin levels were more significantly suppressed in men than in women. Based on these observations, the authors pointed out that it is not realistic to expect GnRH analogues to result in complete suppression in women undergoing chemotherapy with the current doses used. Nevertheless, after 3 years of follow-up, all men in both the study (who used 0.6 or 1 mg busarelin/day) and control groups became oligo/azospermic, while four of the eight women treated with a GnRH analogue at a dose of 0.6 mg/day (50%) and six of nine female controls (66.6%) became amenorrheic. The authors concluded that GnRH analogues cannot offer gonadal protection in men or women at the clinically acceptable doses.

Some have cited a monkey study in support of the effectiveness of GnRH analogues, in which only three primates were investigated in each of the cyclophosphamide and cyclophosphamide plus GnRH analogue groups [35]. However, one animal in the cyclophosphamide group died prematurely, reducing the size of the treatment group to two. In contrast, a larger, rodent study did not show a ben-

efit of ovarian suppression [36]. Furthermore, that study did not look at the fecundity rates in these animals, and as shown previously, histological analysis is not sufficient to detect ultrastructural defects induced by chemotherapy in primordial follicles [37].

In another study by Dann et al. [38], the fertility impact of an alternative cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP) protocol (mega-CHOP) in 13 patients with non-Hodgkin's lymphoma on the risk for ovarian damage was studied. In that protocol, extremely high doses of cyclophosphamide consistent with the doses used in HSCT (range, 8,000–12,000 mg) were used with a modified time schedule and dose intensity. A similar proportion of patients developed ovarian failure in the GnRH analogue and non-GnRH analogue groups (zero of seven versus one of six) during a median follow-up of 70 months, but no statistical analysis was performed. All patients were  $\leq 31$  years of age, with the exception of a 40 year-old woman who developed POF. The authors' conclusion was that this mega-dose CHOP regimen resulted in a low incidence of POF regardless of GnRH analogue administration. Interestingly, in a more recent study by the same group, no differences were found between those who received hormonal suppression (the birth control pill or a GnRH analogue) and those who did not during chemotherapy with standard doses for non-Hodgkin's lymphoma [39]. This time, the authors' conclusion was that fertility preservation techniques were not needed for patients  $< 40$  years old. It thus appears that there is hardly a group of patients who might benefit from ovarian suppression.

In summary, most of the previous studies used control groups that were retrospectively selected from historical patients treated with similar regimens [40–42]. In some studies, the mean follow-up was longer in the control group than in the study group [43], and it was not clear from the study design whether cumulative doses of cyclophosphamide were similar between the treated and untreated patients [40–42, 44]. Similar concerns have also been raised by other authors previously [4].

### **GNRH ANALOGUES DO NOT PRESERVE GONADAL FUNCTION IN PATIENTS UNDERGOING HSCT**

Previous studies consistently showed a high risk for ovarian failure following preconditioning chemotherapy or radiotherapy for HSCT [45–47]. For example, Sanders et al. [45] reported that preconditioning with both chemotherapy and total body irradiation resulted in ovarian failure in 135 of 144 patients. Moreover, a GnRH analogue was not protective of the gonads in these groups of patients [43, 47]. In a study assessing the impact of total body irradiation and/or high-dose chemotherapy before undergoing HSCT in pre-

pubertal children, 12 of 14 patients who received grafting before 10 years of age resumed ovarian function; however, none of the four patients who underwent HSCT after the age of 11 regained ovarian function [48]. Interestingly, FSH concentrations showed a tendency to rise to menopausal levels after 10 years of age despite the occurrence of a timely menarche. When analyzing a similar study performed by Teinturier et al. [49], which investigated pubertal status and ovarian function in 21 girls, who underwent HSCT before puberty, Dr. Blumenfeld presented a different interpretation to argue that prepubertal ovaries are less likely to be affected by chemotherapy, in a debate article [3, 49, 50]. In that report, while 12 of 21 patients had clinical evidence of gonadal failure (57%), another patient had laboratory evidence. Moreover, two patients had gonadotropin levels at the upper limit of normal, bringing the percentage of subjects with *imminent ovarian failure* to 71%. Interestingly, all 10 patients who received busulfan developed persistent ovarian failure. Because the follow-up was performed at a very young median age (14.5; range, 11–21 years), it could not be concluded that the future reproductive performance of the remaining 29% was not affected by chemotherapy. As shown by Meirou et al. [51], in a rodent model, immediate reproductive endocrine performance is not a sign that ovarian reserve is not diminished. Similarly, Thibaud et al. [52] found elevated FSH levels in all 29 patients who were conditioned for HSCT at a mean age of 10.3 years. The authors observed at the last clinical evaluation, at a mean age of 16.3 years, that 23 girls had complete ovarian failure, two had partial ovarian failure, and six had normal ovarian function [52].

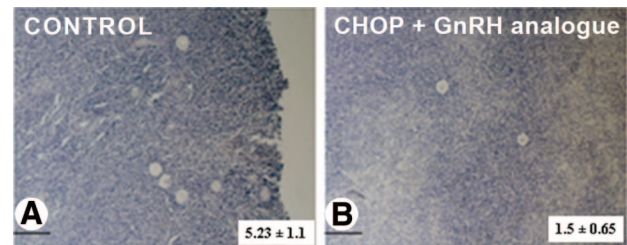
Finally, a recent study by Sklar et al. [34] clearly showed that children who receive chemotherapy are at an extremely high risk for POF. In that study, the authors followed a cohort of children who were diagnosed with a malignancy before the age of 21 and were menstruating for at least 5 years afterward. They compared 2,819 girls with these characteristics with their 1,065 siblings. The median age at diagnosis was 7 (range, 0–20) and the median age at study was 29 (range, 18–50). They found that those who received chemotherapy by age 21 had a 13.2 (range, 3.26–53.51) times higher likelihood of POF than their siblings.

### **THE SAFETY OF ADMINISTERING GNRH ANALOGUES DURING CHEMOTHERAPY IS NOT ESTABLISHED**

At the University of Pennsylvania, a project was carried out by Dr. Kevin Fox and colleagues in 1994–2001, wherein 24 patients with premenopausal, early-stage breast cancer received ovarian suppression with leuprolide during the course of adjuvant chemotherapy administration [53]. The

primary objective of that project was the measurement of maintenance of normal ovarian cycling, with actual fertility representing a secondary objective. The authors observed that 23 of 24 patients regained menstrual cycling. However, of the 11 patients who were actively attempting pregnancy, only two delivered healthy children, despite six pregnancies in five patients. Three spontaneous abortions were observed, with one elective abortion for a Down syndrome fetus. Although the number of patients in the project was small, the return of menstrual function occurred often enough to encourage the development of a randomized clinical trial by the Southwest Oncology Group (SWOG). In that clinical trial, premenopausal patients are being randomized to receive leuprolide or not during their chemotherapy, and preservation of menstrual function is being observed, as are fertility outcomes. However, the authors feel that there is insufficient evidence of the worth of GnRH ovarian suppression as a method of preserving fertility to recommend this technique outside a randomized clinical trial. Therefore, as of 2002, this method is no longer being offered to patients at the University of Pennsylvania, pending the results of this or any other randomized trial.

There is a biologically plausible explanation for Dr. Fox's findings. Because the developing follicles, but not the resting ones, are FSH sensitive [7], GnRH analogue treatment would halt the growth of those developing follicles. In the short term, protection of these growing follicles with a resultant resumption of menstrual function postchemotherapy, especially in young patients with a large ovarian reserve, might erroneously give the impression that ovarian function is preserved. Because those follicles have many mitotic granulosa cells and premeiotic oocytes, which would be amenable to residual DNA damage from chemotherapy, they are likely to result in abnormal conceptions [54, 55]. Because ovarian follicle development can take up to 6 months from the resting stage, it is expected that these women will begin menstruation upon discontinuation of GnRH analogues, as the damaged follicles resume their growth and are eventually cleared from the ovary. In support of our hypothesis, Familiari et al. [37] showed that, even though hormonal suppression with medroxyprogesterone acetate appeared to have protected against the acute follicle loss, these follicles were abnormal in ultrastructural analyses and were eventually lost, resulting in early ovarian failure. Thus, it is possible that GnRH analogues may be delaying the inevitable fate, the death of already damaged follicles, by slowing their growth. Moreover, we have histologically shown that, in two age-matched cancer patients, one of whom received gonadotoxic chemotherapy in parallel with a GnRH analogue and the other who did not,



**Figure 3.** Ovarian biopsies from two patients who underwent ovarian tissue freezing. The control was a 33-year-old breast cancer patient who underwent ovarian tissue cryopreservation before chemotherapy. The treatment patient was a 33-year-old woman who had received seven courses of cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP) chemotherapy along with a gonadotropin-releasing hormone (GnRH) analogue for the treatment of non-Hodgkin's lymphoma 90 days before ovarian freezing. The mean primordial follicle count is shown in the lower right corner.

primordial follicle density was significantly diminished despite GnRH analogue administration (Fig. 3A and B).

Moreover, alkylating agents are not cell-cycle specific and produce single- and double-stranded breaks regardless of *whether or not a cell is replicating* [56, 57]. Even if we assume that primordial follicles are responsive to gonadotropins, the fact that alkylating agents might also damage resting primordial follicles makes concomitant use of GnRH analogues to prevent chemotherapy-induced gonadal damage by holding follicles at the resting stage a risky approach. Furthermore, because ovarian germinal epithelium is extremely sensitive to ionizing radiation, gonadal protection is not achieved despite cotreatment with GnRH analogues [58, 59].

It was previously claimed that there is potentially no harm in giving GnRH analogues to cancer patients [3]. Not only are GnRH analogues expensive and they cause severe menopausal symptoms, but, in addition, the direct effects of GnRH agonists on human cancer cells are not sufficiently understood. A variety of human cancers, including those of the breast, ovary, and endometrium, express GnRH receptors. These receptors mediate several effects, such as inhibition of proliferation, induction of cell-cycle arrest, and inhibition of apoptosis, induced, for example, by cytotoxic drugs [60]. Thus, it cannot be excluded that GnRH agonist therapy concomitant with cytotoxic chemotherapy might reduce the efficacy of chemotherapy for breast cancer. In addition, for the adjuvant treatment of hormone-responsive breast cancer, a sequence of chemotherapy followed by endocrine manipulation is generally recommended, because concomitant chemoendocrine therapy might lead to inferior results [61]. Therefore, at least for breast cancer patients, it is advisable to investigate the protective effect of GnRH analogues on the ovary in the context of a well-designed, pro-

spective, clinical trial and to limit the concomitant use of GnRH agonists with adjuvant chemotherapy for those patients in whom no negative interaction between the two treatment modalities has to be considered, that is, patients with estrogen receptor- and progesterone receptor-negative tumors.

Direct effects of GnRH analogues on human ovaries are also not clearly understood. It is generally accepted that human granulosa cells, normal ovarian surface epithelial cells, and human ovarian cancer cells express GnRH receptors, which mediate antigonadotropic, antiproliferative, and antiapoptotic effects [60, 62]. In addition, however, proapoptotic effects of GnRH analogues on human granulosa lutein cells and cancer cells have been demonstrated [62]. Nevertheless, there is no evidence that GnRH receptors are expressed in primordial follicles.

A recent study postulated acute depletion of the murine primordial follicle reserve by GnRH antagonists [63], while in human granulosa lutein cells, a GnRH antagonist increased DNA synthesis and blocked the proapoptotic effect of a GnRH agonist [64]. These and a number of additional controversial findings of GnRH analogue effects on human ovaries make it advisable to test these compounds for use in ovarian protection in carefully designed clinical trials.

Another theoretical concern is increased gonadotoxicity. Gonadotropins induce a series of antioxidant enzymes called glutathione S-transferases [65]. These enzymes are present in granulosa cells of follicles of various stages in the ovary [66] and play a role in detoxifying chemotherapeutics [67]. Ovarian suppression can reduce the expression of these enzymes, in theory rendering follicles more vulnerable to the effects of chemotherapy. Moreover, if GnRH analogues protect the ovaries via the S1P route, as has been hypothesized by some authors, this would then raise the

possibility that they can also prevent cancer cell death by host immune defense as well as chemotherapy [3, 68, 69].

On a more practical level, up to 97% of patients suffer from hypoestrogenic symptoms when using a GnRH analogue along with chemotherapy [22]. Furthermore, when used for >4 months, patients may experience bone loss, which may not be reversible with longer durations of use [70].

### ETHICAL RESPONSIBILITIES TOWARD PATIENTS WHEN OFFERING UNPROVEN METHODS

Patients who wish to use GnRH analogues are motivated to conceive. When they are matched retrospectively to those who might not express an interest in fertility preservation, biased results are extremely likely in estimating subsequent pregnancy rates. Therefore, sufficiently powered randomized controlled trials are the best solution to resolve these issues in reality. Promisingly, various studies—including the SWOG study in the U.S., Zoladex Rescue of Ovarian Function study in Germany, Italian multicenter study for breast cancer patients, German Hodgkin Lymphoma group multicenter study, U.K. lymphoma multicenter study, Spanish Lymphoma multicenter study, and PREGO (Prevention of gonadal toxicity and preservation of gonadal function and fertility in young women with systemic lupus erythematosus treated by cyclophosphamide) study in Europe—are under way, and more rigorous evaluations will be performed and reported in the near future. In the meantime, offering GnRH analogues outside clinical trials to those groups of patients, while both oocyte and ovarian tissue freezing are offered under prospective trials [71], may violate the principle of *primum non nocere* [72], which we believe to be the fundamental precept in medicine.

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**Absence of Conclusive Evidence for the Safety and Efficacy of  
Gonadotropin-Releasing Hormone Analogue Treatment in Protecting Against  
Chemotherapy-Induced Gonadal Injury**

Kutluk Oktay, Murat Sönmezer, Özgür Öktem, Kevin Fox, Günter Emons and  
Heejung Bang

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