A Multicenter prospective coHort study of controlled Ovarian stimulation in newly diagnosed breast cancer PatiEnts: the fAMHOPE study

Protocol version 1.0: 10 February 2018

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Contents

SYNOPSIS

Title of the study	A Multicenter prospective coHort study of controlled Ovarian stimulation in newly diagnosed breast cancer PatiEnts: the fAMHOPE study
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Sponsor	CUB-Hôpital Erasme, Brussels, Belgium
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Study Objectives	**Primary objective:** - To evaluate the efficacy of performing a controlled ovarian stimulation with or without letrozole in young women with newly diagnosed breast cancer who are candidates to receive (neo)adjuvant chemotherapy in terms of mature oocytes collected. **Secondary objectives:** - To evaluate the safety of performing controlled ovarian stimulation with or without letrozole in young women with newly diagnosed breast cancer and who are candidates to receive (neo)adjuvant chemotherapy in terms of complications associated with any of the procedure used for oocyte/embryo cryopreservation, invasive disease-free survival (iDFS), breast cancer-free interval (BCFI), overall survival (OS), kinetic of circulating tumor DNA (ctDNA) before and after stimulation. - To evaluate baseline characteristics of young women with newly diagnosed breast cancer who undergo controlled ovarian stimulation with or without letrozole.

	- To evaluate the characteristics of the ovarian stimulation cycle with or without letrozole in young women with newly diagnosed breast cancer who are candidates to receive (neo)adjuvant chemotherapy. - To evaluate the efficacy of performing a controlled ovarian stimulation with or without letrozole in young women with newly diagnosed breast cancer who are candidates to receive (neo)adjuvant chemotherapy in terms of number of oocytes collected, maturation rate, number of oocyte/embryo cryopreserved, number of patients with poor response, and number of patients with stimulation failure. - To evaluate the type and outcomes of assisted reproductive technology procedures in patients who return to the fertility clinic after the end of treatment. In patients with a clinically confirmed pregnancy obtained with assisted reproductive technology procedures after the end of treatment, pregnancy, fetal and obstetrical outcomes of the pregnancies will be evaluated. - To evaluate the impact of anticancer therapies (chemotherapy with or without endocrine therapy with or without anti-HER2 agents) on patients' ovarian function.
Number of patients	A total of 113 patients are expected to be included in the standard-stimulated cohort, 113 in the letrozole-stimulated cohort, and 339 in the non-stimulated cohort (1:1:3).

Eligibility criteria	**Inclusion criteria:** - Diagnosis of invasive non-metastatic breast cancer (i.e. stage I to III); - Breast cancer diagnosis ≥18 and ≤ 40 years; - No prior history of gonadotoxic treatments; - Fertility preservation counseling for fertility preservation; - Written inform consent; - FSH < 20 UI/L and/or antra-follicular count ≥ 6 (follicles of 2-9 mm) and/or AMH ≥ 6 pmol (only applicable for patients who undergo controlled ovarian stimulation for embryo/oocyte cryopreservation). **Exclusion criteria:** - Newly diagnosed stage IV breast cancer (i.e. de novo metastatic breast cancer); - Prior diagnosis of other malignancies before breast cancer.
Design of the trial	Multicenter hospital-based prospective cohort study
Statistics	Three cohorts of patients will be prospectively included in the study: **1) Letrozole-stimulated cohort:** this includes all newly diagnosed breast cancer who are candidates to receive (neo)adjuvant chemotherapy and undergo oocyte/embryo cryopreservation at the Belgian participating centres. **2) Standard-stimulated cohort:** this includes all newly diagnosed breast cancer who are candidates to receive (neo)adjuvant chemotherapy and undergo oocyte/embryo cryopreservation at the French participating centres. **3) Non-stimulated cohort:** this includes all newly diagnosed breast cancer who are candidates to receive (neo)adjuvant chemotherapy who have access

	to the Fertility Clinics in all the participating centres but are not willing to undergo oocyte/embryo cryopreservation. All the efficacy analyses will be perfomed to compare the letrozole-stimulated cohort and the standard-stimulated cohort. All the safety analyses will be conducted to compare separately the letrozole-stimulated cohort with the non-stimulated cohort and the standard-stimulated cohort with the non-stimulated cohort. The sample size has been estimated based on the study primary endpoint (i.e. number of mature oocytes collected). With 113 patients in the standard-stimulated cohort and 113 in the letrozole-stimulated cohort, assuming a mean difference of 1.5 in the number of mature oocytes collected between the standard-stimulated cohort and the letrozole-stimulated cohort, the study will have a power of 0.80 to reject the null hypothesis of equal means with a standard deviation of 4.0 and with a significance level (alpha) of 0.05 using a two-sided two-sample equal-variance t-test. Considering that approximately one out of three (1:3) eligible patients counseled to undergo controlled ovarian stimulation for oocyte cryopreservation before starting anticancer treatments will accept the procedure, it is estimated to enroll 339 patients in the non-stimulated cohort. Patients demographic, clinical and pathologic baseline characteristics will be compared in the three cohorts using chi-square test or Fisher Exact test for categorical variables and Wilcoxon rank-sum test or t-test for continuous variables, as appropriate. Dicothomous outcomes will be compared using

	univariate and multivariate logistic regression models. Time-to-event outcomes will be estimated and plotted using Kaplan-Meier methods. Survival rates will be compared using log-rank test and Cox proportional hazards models for univariate and multivariate analysis, respectively. All tests will be two-sided and p-values of < 0.05 will be considered statistically significant.

1. BACKGROUND AND SCIENTIFIC RATIONALE

Breast cancer is the most common tumor type arising in women of reproductive age [1]. Breast cancer in young women is considered a distressing and far-reaching disease requiring personalized approaches to manage the specific age-associated needs related not only to optimal anticancer treatments but also to other crucial quality of life issues [2]. As compared to patients diagnosed at an older age, young women have an increased risk of developing biologically aggressive forms of breast cancer with a higher incidence of poorly differentiated tumors and aggressive subtypes [3]. Hence, the majority of these patients are candidates to receive aggressive multimodality anticancer therapies that include chemotherapy. The use of systemic cytotoxic therapy in premenopausal patients can be associated with significant long-term negative side effects such as premature ovarian failure [4]. Ovarian function impairment has a negative impact on global health of young breast cancer survivors being associated with several side effects including infertility [5]. Concerns about fertility preservation and future chance of achieving a pregnancy are prevalent issues affecting newly diagnosed breast cancer patients [6].

Approximately 40% to 50% of young women with breast cancer wish to have a subsequent pregnancy [7]. However, the percentage of breast cancer patients reported in the literature with at least one full-term pregnancy after breast cancer diagnosis is very low. Of note, among cancer survivors, breast cancer patients have the lowest pregnancy rate with an overall 67% reduction in the chance of giving birth after cancer treatment as compared to the general population [8]. This observation reflects the possible damage to ovarian reserve due to the use of gonadotoxic anticancer treatments [9]. Recent data support the safety of having a pregnancy in breast cancer survivors after adequate treatment and follow-up [10,11]; hence, pregnancy after breast cancer should not be discouraged including among patients with endocrine-sensitive disease [12]. These data highlight the importance of preserving

fertility in young women with newly diagnosed breast cancer for improving the chance to complete their family plan after the end of treatment.

As suggested by professional guidelines, health care providers have the responsibility to inform patients of the potential development of treatment-related premature ovarian failure and infertility, discuss the available strategies for fertility preservation and refer concerned patients to fertility clinics [2,13–15]. Hence, oncofertility counseling should be considered now as routine clinical practice before the patients start chemotherapy [16].

In young cancer patients, including those with breast cancer, embryo/oocyte cryopreservation are effective and standard options to preserve fertility [2,13–15]. Oocyte cryopreservation requires 3 different phases: induction of multiple follicular growth through controlled ovarian stimulation, oocyte collection performed by ultrasound-guided transvaginal needle aspiration, and evaluation, selection, and vitrification of the oocytes [17,18]. Embryo cryopreservation requires an additional phase consisting in *in vitro* fertilization (IVF) of the collected oocytes [17,18]. Embryo/oocyte cryopreservation must be concluded before starting gonadotoxic anticancer treatments; these strategies are contraindicated in patients already exposed to cytotoxic therapy since a prior exposure to chemotherapy may result in morphologic/genetic abnormalities in the retrieved oocytes and reduced ovarian response to controlled ovarian stimulation [19].

In infertile women without cancer, embryo cryopreservation following controlled ovarian stimulation demonstrated the most reliable results in terms of subsequent pregnancies. The success of the procedure is strongly dependent of patients' age at the time of the procedure: the pregnancy rates according to the 2012 European registers by the European Society of Human Reproduction and Embryology (ESHRE) ranged from approximately 29% in women younger than 35 to 13% in those

older than 40 years [20]. In experienced centers, similar results can be obtained after oocyte cryopreservation [21,22]. Of note, so far, limited data are available on the success of embryo/oocyte cryopreservation after controlled ovarian stimulation in cancer patients. To date, the largest series of pregnancy and fertility outcomes reported in the specific subgroup of breast cancer patients was described by Oktay and colleagues in 2015 [23]. In this single-center prospective cohort study, 131 women diagnosed with stage I-III breast cancer and aged ≤ 45 years, underwent controlled ovarian stimulation (with a protocol that included letrozole) followed by embryo cryopreservation before the initiation of anticancer therapy. Oocytes were fertilized by intracytoplasmic sperm injection, and all embryos were cryopreserved via a vitrification method. After a median time of 5.25 years following oocyte retrieval, 33 breast cancer survivors returned to undergo frozen embryo transfer to self or used a gestational carrier. Embryo post-thaw survival rate was 84.4%; 40 frozen embryo transfers were performed with the embryos from these 33 breast cancer survivors (mean number of embryos transferred = 1.97). The authors observed an overall clinical pregnancy rate (defined as the presence of at least one gestational sac at the time of the first ultrasound examination by the eight week of gestation) of 65.0% (26/40), a live birth rate per embryo transfer of 45.0% (18/40), and an implantation rate (defined as the total number of pregnancy sacs per total number of embryos transferred) of 40.7% (33/81). These data did not differ from those reported by the American Society of Assisted Reproductive Technology in a comparable non-oncologic age group (age 35 to 37 years) and time period (2009). A total of 25 children were born from 18 pregnancies without fetal anomalies or malformations, developmental problems or any medical complications during pregnancies or the postpartum period. Out of the 33 breast cancer survivors attempting pregnancy, 20 women had at least one pregnancy: three patients had spontaneous abortions, bringing the fertility preservation rate (defined as the percentage of women obtaining at least one live birth among all who attempted a frozen embryo transfer) to 51.5% per attempting woman. The self transfer and gestational carrier groups had similar pregnancy outcomes, clinical pregnancy, and live birth rates [23]. Although these

results support the efficacy of letrozole-associated controlled ovarian stimulation followed by embryo/oocyte cryopreservation in breast cancer patients, the numbers remain low to draw solid conclusions.

In young breast cancer patients, specifically in those with endocrine-sensitive disease, the need for controlled ovarian stimulation raises specific issues to be taken into account during oncofertility counseling related to its possible negative impact on their prognosis [12]. During standard controlled ovarian stimulation, estradiol levels rise up to 10-20 times higher than in natural cycles. Although the high estradiol levels during controlled ovarian stimulation cycles is temporarily limited to a couple of weeks, many oncologists and breast cancer patients are concerned about the use of these techniques fearing that the high estrogenic state can promote cancer growth or recurrence [9]. Therefore, controlled ovarian stimulation protocols with the addition of letrozole have been specifically designed for breast cancer patients to limit the potential negative impact of the short-term increase in estradiol levels while maintaining a good oocyte yield [18]. To date, only one single-center prospective study investigated the safety of controlled ovarian stimulation before chemotherapy initiation in breast cancer patients [24,25]. Patients interested in embryo cryopreservation underwent controlled ovarian stimulation with letrozole while women who did not receive any fertility-preserving procedure served as controls. The analysis after approximately 2 years of median follow-up included 79 patients who underwent controlled ovarian stimulation for embryo cryopreservation and 136 controls [24]. Patients in the controlled ovarian stimulation group had a trend for higher estrogen-receptor positivity ($p = 0.08$). The study showed no difference in survival outcomes, with a hazard ratio (HR) for recurrence after embryo cryopreservation of 0.56 (95% confidence intervals [CI], 0.17-1.9; $p = 0.36$) [24]. A recent update of the study included 337 women, of whom 120 underwent controlled ovarian stimulation with letrozole prior to chemotherapy and 217 did not undergo any fertility-preserving procedure [25]. Patients in the control group had significantly more frequent lymph-node

involvement than women in the controlled ovarian stimulation group (p = 0.02). After a mean follow-up of approximately 5 years, the study confirmed the absence of survival difference between the two groups, with an HR for recurrence after controlled ovarian stimulation of 0.77 (95% CI, 0.28-2.13; p = 0.61). Although the numbers were limited, the authors performed several subgroup analyses showing that, apparently, neither *BRCA* mutation status (p = 0.57), nor hormone-receptor status (p = 0.75) nor undergoing controlled ovarian stimulation before or after breast surgery (p = 0.44) seemed to affect survival in the controlled ovarian stimulation group [25]. Taking into account these results, all major international guidelines consider this strategy as standard in breast cancer patients [2,13–15]. However, during oncofertility counseling, it should be made clear that limited data are available to strongly support the safety of this procedure in breast cancer patients.

Of note, the first studies investigating the use of letrozole for assisted reproductive technology (ART) showed a potential increased risk of congenital cardiac and musculoskeletal anomalies in the newborns [26]; hence, its manufacturer issued a warning to physicians to stop using letrozole for this purpose in pre-menopasual women. However, subsequent studies did not confirm these findings [27–29]. Recently, a large Japanese study including 3,136 natural cycles and 792 letrozole-induced cycles investigated the risk of major congenital anomalies, pregnancy and neonatal outcomes with the use of letrozole in patients undergoing ART [30]. The study showed that the risk of miscarriage was significantly lower in women treated with letrozole (adjusted odds ratio [OR], 0.37; 95% CI, 0.30-0.47; p < 0.001). Furthermore, the overall risk of major congenital anomalies was similar between the two groups (natural cycle 1.5% vs. letrozole 1.9%; adjusted OR, 1.24; 95% CI, 0.64-2.40; p = 0.52), as well as the risk for any specific organ system malformation. Finally, no difference in all the other pregnancy and neonatal outcomes was observed between the two groups [30]. Hence, although these results support the safety of letrozole for assisted reproductive technology (ART), in some countries like France this compound cannot be used as part of controlled ovarian stimulation.

Despite the last years have brought many safety and efficacy data on the use of embryo/oocyte cryopreservation in cancer patients, numerous challenges remain for young women with breast cancer who consider undergoing controlled ovarian stimulation before chemotherapy initiation. Thus, further research is needed in the field. Particularly, prospective reproduction studies to address the safety and efficacy of controlled ovarian stimulation and exploring potential differences between the different protocols including or not letrozole are lacking and should be considered a research priority. Therefore, due to the limited data on this regard, the present study aims at refining the understanding of the safety and efficacy of performing controlled ovarian stimulation with or without letrozole in young women with newly diagnosed breast cancer who are candidates to receive (neo)adjuvant chemotherapy. This is crucial information to be acquired to further improve the oncofertility counseling of young cancer patients.

2. STUDY OBJECTIVES AND ENDPOINTS

Primary objective:

- To evaluate the efficacy of performing a controlled ovarian stimulation with or without letrozole in young women with newly diagnosed breast cancer who are candidates to receive (neo)adjuvant chemotherapy in terms of mature oocytes collected.

Secondary objectives:

- To evaluate the safety of performing controlled ovarian stimulation with or without letrozole in young women with newly diagnosed breast cancer and who are candidates to receive (neo)adjuvant chemotherapy in terms of complications associated with any of the procedure used for oocyte/embryo cryopreservation, invasive disease-free survival (iDFS), breast cancer-free interval (BCFI), overall survival (OS), kinetic of circulating tumor DNA (ctDNA) before and after stimulation.

The safety of performing controlled ovarian stimulation with or without letrozole will be evaluated in various subgroups of young women with newly diagnosed breast cancer who are candidates to receive (neo)adjuvant chemotherapy according to different patient, tumor or treatment characteristics.

- To evaluate baseline characteristics of young women with newly diagnosed breast cancer who undergo controlled ovarian stimulation with or without letrozole.

- To evaluate the characteristics of protocols with or without letrozole used for controlled ovarian stimulation in young women with newly diagnosed breast cancer who are candidates to receive (neo)adjuvant chemotherapy.

- To evaluate the efficacy of performing a controlled ovarian stimulation with or without letrozole in young women with newly diagnosed breast cancer who are candidates to receive (neo)adjuvant chemotherapy in terms of number of oocytes collected, maturation rate, number of oocyte/embryo cryopreserved, number of patients with poor response, and number of patients with stimulation failure. The efficacy of performing controlled ovarian stimulation with or without letrozole will be also evaluated in various subgroups of young women with newly diagnosed breast cancer who are candidates to receive (neo)adjuvant chemotherapy according to according to different patient, tumor or treatment characteristics.

- To evaluate the type and outcomes of assisted reproductive technology procedures in patients who return to the fertility clinic after the end of treatment. In patients with a clinically confirmed pregnancy obtained with assisted reproductive technology procedures after the end of treatment, pregnancy, fetal and obstetrical outcomes of the pregnancies will be evaluated.

- To evaluate the impact of anticancer therapies (chemotherapy with or without endocrine therapy with or without anti-HER2 agents) on patients' ovarian function.

The impact of anticancer therapies on patients' ovarian function will be also evaluated in various subgroups of young breast cancer patients according to different patient, tumor or treatment characteristics.

3. METHODOLOGY

This is a multicenter hospital-based prospective cohort study conducted in institutions with known expertise in performing controlled ovarian stimulation for embryo/oocyte cryopreservation. The study aims at refining the understanding of the efficacy and safety of controlled ovarian stimulation with or without letrozole in young women with newly diagnosed breast cancer who are candidates to receive (neo)adjuvant chemotherapy.

3.1. Eligibility criteria

To be eligible for the present study, patients should fulfill the following inclusion and exclusion criteria:

Inclusion criteria:

- Diagnosis of invasive non-metastatic breast cancer (i.e. stage I to III);
- Breast cancer diagnosis ≥18 and ≤ 40 years;
- No prior history of gonadotoxic treatments;
- Fertility preservation counseling for fertility preservation;
- Written inform consent;
- FSH < 20 UI/L and/or antra-follicular count ≥ 6 (follicles of 2-9 mm) and/or AMH ≥ 6 pmol (only applicable for patients who undergo controlled ovarian stimulation for embryo/oocyte cryopreservation).

Exclusion criteria:

- Newly diagnosed stage IV breast cancer (i.e. de novo metastatic breast cancer);
- Prior diagnosis of other malignancies before breast cancer.

3.2. Data collection

For all consecutive eligible patients included in the study, anonymized data will be entered into ad hoc electronic Case Report Forms (eCRF). The following information will be collected:

1) Baseline characteristics (at study inclusion): BMI; smoking habit; age at menarche; use of birth control pills; prior children before breast cancer diagnosis; history of spontaneous or induced abortion; prior treatment for infertility; prior gynecological surgery and/or medical history with possible impact on fertility; baseline AMH value.

2) Breast cancer history: date of diagnosis; age at diagnosis; disease stage; type of breast and axillary surgery; histology; tumor size and nodal status (for patients who underwent primary systemic therapy, both clinical tumor size and nodal status before treatment initiation and pathological tumor size and nodal status at the time of surgery will be collected); grade; hormone receptor status (estrogen and progesterone receptors); HER2 status.

3) Treatment of the primary tumor: timing of systemic therapy administration (adjuvant or neoadjuvant); use of chemotherapy (type and number of cycles); use of endocrine therapy (type and duration); use of anti-HER2 targeted therapy (type and duration); use of radiotherapy; use of breast reconstruction surgery.

4) Survival status: any recurrence of invasive breast cancer including date of the event; type of recurrence of invasive breast cancer (locoregional or distant event); any second primary contralateral breast cancer including date of the event; any second primary malignancy including date of the event; date of last follow-up and/or death.

5) Controlled ovarian stimulation protocol: type of stimulation (follicular or random); total FSH dose; number of stimulation days; number of days with antagonist; estradiol and progesterone levels at triggering and during luteal phase (L3-L8); number of oocyte/embryo collected; number of mature oocytes collected; maturation rate; number of oocyte/embryo cryopreserved; number of patients with poor response; number of patients with stimulation failure.

6) Ovarian function before and after chemotherapy: treatment-induced amenorrhea; age at menopause; AMH values.

7) Pregnancy after breast cancer: any pregnancy including date of the event; pregnancy outcome; gestational age at delivery; pregnancy/fetal/obstetrical outcomes; breastfeeding and its duration; use and type of assisted reproductive technology.

8) Presence of germline *BRCA* mutation or other type of germline mutations (if available): date of genetic testing; type of mutation; use and date of breast prophylactic surgery; use and date of gynecological prophylactic surgery.

4. PROTOCOLS FOR CONTROLLED OVARIAN STIMULATION AND LABORATORY PROCEDURES

The patient population of this trial is unique and rather homogenous; this represents a precious opportunity to study different parameters related to fertility, pregnancy and also breast cancer biology. As a part of signing the study informed consent and their participation in this study, patients consent to perform the examination specified in Table 1.

Table 1. Schedule of assessment for correlative research

	Inclusion (diagnosis)	**M 0-1 (start of COS)**	**M 0-1 (end of COS**	**M4-6 (end of CT)**	**M 16-18 (12 M after CT)**	**M 28-30 (24 M after CT)**	**M 60 (5 y after diagnosis)**	**Relapse**
Serum (AMH, FSH, E2)	x			x	x	x	x	
Serum (Progesterone/E2)		x*	x**					
Plasma for ctDNA	x		x					x
Whole blood	x							
1 block of FFPE of primary tumor $	x							

x: mandatory
$: 15 representative slides of 10 µm should be provided.
*: Can be performed at the inclusion if COS start within 5days
**: serum samples at L3 and L8 will be collected

4.1. Schema of the two controlled ovarian stimulation protocols

1) Letrozole-stimulated breast cancer cohort

There are a few options to start letrozole-associated controlled ovarian stimulation, depending on the cycle phase at the time of patient referral for fertility preservation.

During early follicular phase, letrozole is usually administered on the second day of menstruation. The controlled ovarian stimulation (recombinant FSH or HMG, 75-300 IU/d) is started the following day. GnRH antagonist is added to the controlled ovarian stimulation at day 7 or when the leading follicle reaches 12-14 mm and/or when estradiol reaches 250 pg/ml to prevent premature luteinizing hormone (LH) surge. In the late follicular phase, controlled ovarian stimulation with letrozole can be started before ovulation or ovulation can be induced with GnRHa, before starting controlled ovarian stimulation.

During the luteal phase, antagonist may be administered for a few days before controlled ovarian stimulation to induce luteolysis, or controlled ovarian stimulation can be started at the same time as letrozole, and antagonist will be added when leading follicle has reached 12-14 mm.

all situations, GnRH agonist (Décapeptyl or Gonapeptyl 0.2 mg) is used for ovulation trigger when at least 3 follicles reach 18 mm.

2) Standard-stimulated breast cancer cohort

During early follicular phase, controlled ovarian stimulation is started on the second-third day of the cycle (with recombinant FSH or HMG, 75-300 IU/d). GnRH antagonist is added to the controlled ovarian stimulation at day 7 or when the leading follicle reaches 12-14 mm and/or when estradiol reaches 250 pg/ml to prevent premature LH surge. In the late follicular phase, controlled ovarian stimulation can be started before ovulation or ovulation can be induced GnRHa, before starting controlled ovarian stimulation.

During the luteal phase, antagonist may be administered for a few days before controlled ovarian stimulation to induce luteolysis, or controlled ovarian stimulation can be started, and antagonist will be added when leading follicle has reached 12-14 mm.

In all situations, GnRH agonists (Décapeptyl or Gonapeptyl 0.2 mg) are used for ovulation trigger.

Random start protocol

Standard protocol

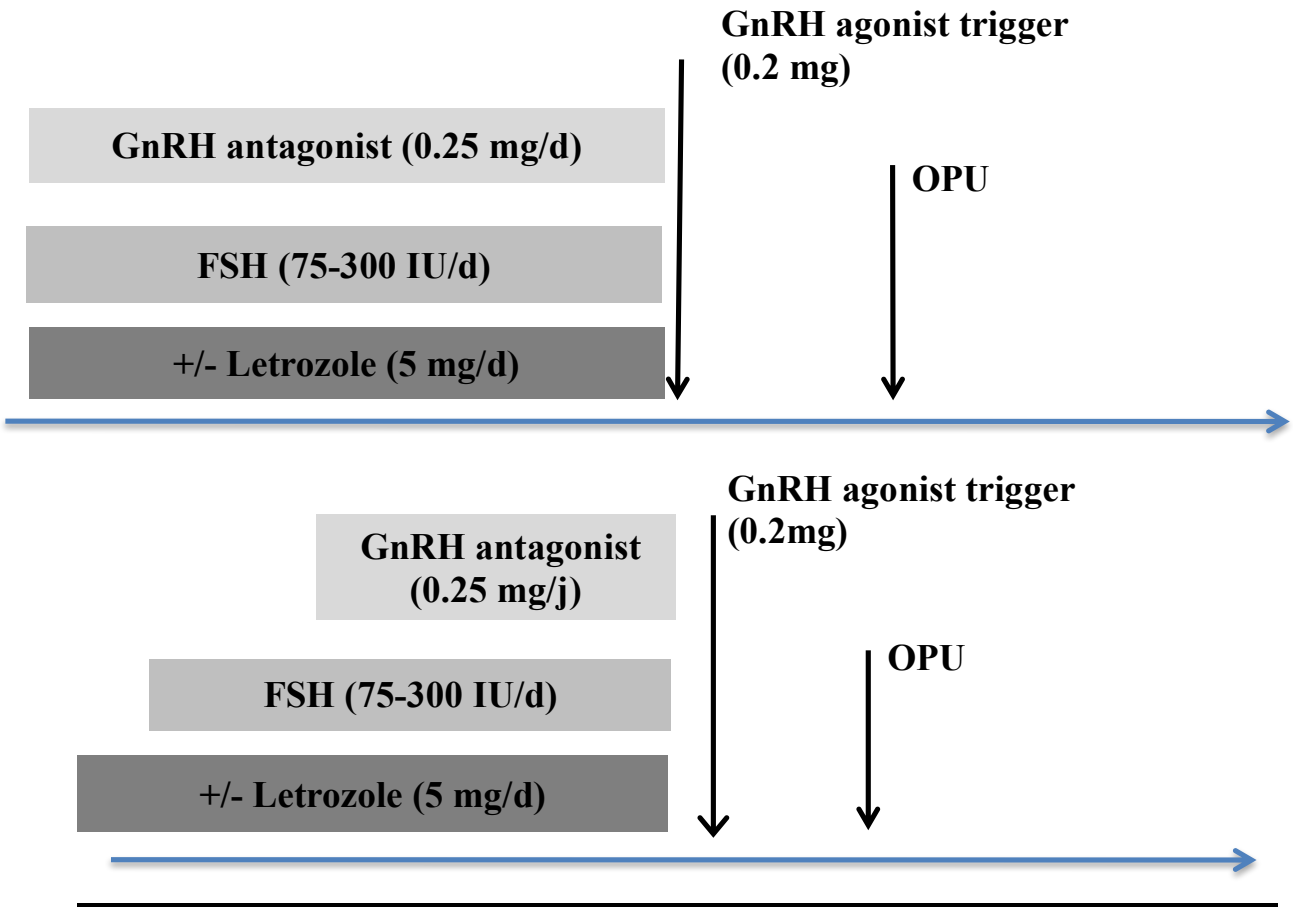

Figure. Random-start or standard controlled ovarian stimulation protocols.

4.2. Laboratory procedures for oocyte/embryo cryopreservation

Oocytes and embryos are vitrified in the equilibration and vitrification solutions according to the manufacturer's instructions of the vitrification Vit Kit®-Freeze (Irvine Scientific, California) or Rapid-I-kit® (Vitrolife, Sweden). The oocytes/embryo are then loaded on in vitrification high security closed system (VHS, CryoBioSystem, France or mediprene straw RapidStraw, Vitrolife, Sweden) in a volume of <1µl, and immediately submerged into liquid nitrogen.

During warming, the cryoprotectants are removed according to the manufacturer's instructions of the devitrification kit Vit Kit®-Thaw (Irvine Scientific, California) or devitrification Kit Rapid Warm (Vitrolife, Sweden).

4.3. Laboratory procedures for ovarian reserve and function biomarker analysis

This constitutes serum evaluation of anti-mullerian hormone (AMH), follicle stimulating hormone (FSH), estradiol (E2) and serum progesterone. Hormonal measurement for the study will be centrally performed at the LHUB (Laboratoire Hospitalier Universitaire de Bruxelles). Elecsys® AMH module (Roche Diagnostics, Vilvoorde, Belgium) is used for AMH measurement. Estradiol and progesterone are assayed by electrochemiluminescence immuno-assay using a competitive immunoassay (Modular E170—Roche diagnostics, Germany). The inter-assay coefficient of variation was less than 5% for all assays.

The evaluation of AMH, FSH, and E2 will be done at enrollment (i.e. at the time of oncofertility counseling/breast cancer diagnosis) before starting chemotherapy (and before starting controlled ovarian stimulation in patients who access oocyte/embryo cryopreservation). Then, these analyses will be repeated at the end of chemotherapy, 12 and 24 months after the end of chemotherapy, and finally 5 years after diagnosis. At all time points, a 5ml of blood sample should be collected. After blood clotting, each tube should be centrifuged at 2600g for 10 minutes and serum should be collected

in 1mL aliquots. For each sample, two 1mL aliquots are required. Aliquots should be stored at -80^{C}. The aim of these analyses is to evaluate the impact of anticancer treatment on ovarian reserve (i.e. AMH) and ovarian function parameters (FSH, E2).

Serum progesterone and E2 will be evaluated the first day and at the end of controlled ovarian stimulation (trigger day). A 5ml of blood sample should be collected. After blood clotting, each tube should be centrifuged at 2600g for 10 minutes and serum should be collected in 1mL aliquots. For each sample, one 1mL aliquot is required. Aliquots should be stored at -80^{C}.

All samples will be shipped to the central laboratory (Erasme hospital) at fixed time points for analysis.

4.4. Laboratory procedures for circulating tumor DNA

Three blood samples of 10 ml should be collected in EDTA tubes; two samples will be centrifuged within 1 hour from collection, to separate plasma from peripheral blood cells, at 820g for 10 minutes at 4°C. Recover and transfer plasma (the top yellowish layer of the tube content) into new micro tubes, compatible with high-speed centrifuge. Centrifuge the plasma at maximum speed (if available 20000g is recommended) for 10 minutes at 4°C. Transfer all plasma into cryovals and stored at -80°. The 3rd sample of whole blood will be stored directly at -80°C. Blood and plasma samples will be shipped on dry ice to the central laboratory (Erasme hospital) at fixed time points for evaluation.

Plasma samples will be collected at enrollment (i.e. at the time of oncofertility counseling/breast cancer diagnosis) before starting chemotherapy (and before starting controlled ovarian stimulation in patients who access oocyte/embryo cryopreservation), Then, this analysis will be repeated at the end of controlled ovarian stimulation, and at relapse or end of follow-up.

In this study, evaluation of the circulating tumor DNA (ctDNA) in the plasma will be performed. The aim is to 1) evaluate the effect of controlled ovarian stimulation on the levels of ctDNA and correlate

with risk of recurrence and 2) to evaluate the pattern of somatic mutations in young breast cancer patients.

Before performing the evaluation of ctDNA, primary tumor analysis is required. Hence, 15 representative slides (10µm) must be collected instead. Samples will be shipped to the central laboratory (Erasme hospital) at fixed time points for evaluation.

The aim is to elucidate the biology of breast cancer in young women. Techniques will comprise but not restricted to immunohistochemistry, in situ hybdridization, expression profiling and sequencing. Extraction of nuclei acids (DNA and RNA) may be required to perform these analyses.

5. STATISTICS

Three cohorts of patients will be prospectively included in the study (Figure 1 in the Appendix):

1) Letrozole-stimulated cohort: this includes all newly diagnosed breast cancer who are candidates to receive (neo)adjuvant chemotherapy and undergo oocyte/embryo cryopreservation at the Belgian participating centres.

2) Standard-stimulated cohort: this includes all newly diagnosed breast cancer who are candidates to receive (neo)adjuvant chemotherapy and undergo oocyte/embryo cryopreservation at the French participating centres.

3) Non-stimulated cohort: this includes all newly diagnosed breast cancer who are candidates to receive (neo)adjuvant chemotherapy who have access to the Fertility Clinics in all the participating centres but are not willing to undergo oocyte/embryo cryopreservation. Patient who underwent another fertility preservation procedure (ovarian tissue cryopreservation, GnRH analogues,…) are eligible in this cohort.

All the efficacy analyses will be perfomed to compare the letrozole-stimulated cohort and the standard-stimulated cohort. All the safety analyses will be conducted to compare separately the letrozole-stimulated cohort with the non-stimulated cohort and the standard-stimulated cohort with the non-stimulated cohort.

To evaluate the efficacy of performing a controlled ovarian stimulation with or without letrozole in young women with newly diagnosed breast cancer who are candidates to receive (neo)adjuvant chemotherapy, the following endpoints will be assessed:

- Number of mature oocytes collected (primary endpoint).
- Number of oocytes collected;
- Maturation rate: defined as ratio between the number of mature oocyte collected and total number of oocyte collected;
- Number of oocyte/embryo cryopreserved;
- Number of patients with poor response: defined as retrieval of ≤ 4 oocytes;
- Number of patients with stimulation failure: defined as no follicular growth after 1 week of adequate controlled ovarian stimulation administration.

The efficacy of performing controlled ovarian stimulation with or without letrozole will be evaluated also in various subgroups of young women with newly diagnosed breast cancer who are candidates to receive (neo)adjuvant chemotherapy according to the following characteristics:

- Type of stimulation: follicular vs. random;
- Hormone receptor status: positive vs. negative;
- Presence of *BRCA* mutation: *BRCA*-mutated vs. *BRCA*-not mutated.

To evaluate the safety of performing a controlled ovarian stimulation with or without letrozole in young women with newly diagnosed breast cancer who are candidates to receive (neo)adjuvant chemotherapy, the following endpoints will be assessed:

- Invasive disease-free survival (iDFS) defined as the time from study inclusion to the first appearance of the following invasive events: local recurrence, distant metastases, contralateral or ipsilateral breast tumor, second primary malignancy, or death from any cause;
- Breast cancer-free interval (BCFI) defined as the time from study inclusion to the first appearance of the following invasive events: local recurrence, distant metastases, contralateral or ipsilateral breast tumor;
- Overall survival (OS) defined as the time from study inclusion to death from any cause;
- Kinetic of circulating tumor DNA (ctDNA) assessed before, after stimulation and during study follow-up (if disease relapse).
- Complications associated with any of the procedure used for oocyte/embryo cryopreservation.

The safety of performing controlled ovarian stimulation with or without letrozole will be evaluated in various subgroups of young women with newly diagnosed breast cancer who are candidates to receive (neo)adjuvant chemotherapy according to the following characteristics:

- Stage at diagnosis: I vs. II vs. III;
- Hormone receptor status: positive vs. negative;
- HER2 status: positive vs. negative;
- Type of exposure to chemotherapy: prior to surgery (neoadjuvant) vs. post surgery (adjuvant);
- Exposure to endocrine therapy: exposure vs. no exposure;
- Presence of *BRCA* mutation: *BRCA*-mutated vs. *BRCA*-not mutated;
- Baseline ctDNA level: present vs. absent.

The following baseline characteristics of young women with newly diagnosed breast cancer who undergo controlled ovarian stimulation with or without letrozole will be assessed:

- Body-Mass Index (BMI) and body surface;
- Smoking history;
- Age at menarche;
- Prior pregnancies (completed, induced or spontaneous abortion);
- Prior treatment for infertility;
- Prior use of birth control pills;
- Prior gynecological diseases with potential impact on reproductive potential;
- Baseline ovarian reserve and function analysis.

Furthermore, the following characteristics of protocols with or without letrozole used for controlled ovarian stimulation in young women with newly diagnosed breast cancer who are candidates to receive (neo)adjuvant chemotherapy will be evaluated:

- Type of stimulation: follicular or random;
- Total follicle-stimulating hormone (FSH) dose;
- Number of stimulation days;
- Number of days with antagonist;
- Progesterone and Estradiol levels at trigger;
- Progesterone level at triggering and during luteal phase (if available).

In patients who return to the fertility clinic after the end of treatment, to evaluate the type and outcomes of assisted reproductive technology procedures used, the following endpoints will be evaluated:

- Number of patients who returned to fertility clinic;
- Type of assisted reproductive technology procedures used;
- Number of embryos transferred;
- Pregnancy rate: defined by ß-hCG determination 14 days after embryo transfer;
- Clinical pregnancy rate: defined as the presence of at least one gestational sac at the time of the first ultrasound examination by the eight week of gestation;
- Implantation rate per embryo transfer: defined as the ratio between the number of visible sacs 4 weeks after embryo transfer and the number of embryos transferred.

In patients with a clinically confirmed pregnancy obtained with assisted reproductive technology procedures after the end of treatment, pregnancy, fetal and obstetrical outcomes of the pregnancies will be evaluated. The following endpoints will be evaluated:

- Miscarriage rate;
- Live birth rate per embryo transfer: defined as the number of births of live infants beyond viability (> 24 weeks);
- Twinning rate;
- Premature delivery: <37 weeks of gestation;
- Malformation rate with number and types of malformations;
- Obstetrical complication rate with number and types of complications.

Finally, to evaluate the impact of anticancer therapies (chemotherapy with or without endocrine therapy with or without anti-HER2 agents) on patients' ovarian function, the following endpoints will be evaluated:

- Incidence of treatment-induced amenorrhea one year after the end of chemotherapy;
- Incidence of treatment-induced amenorrhea two years after the end of chemotherapy;

- Incidence of treatment-induced amenorrhea five years after the end of chemotherapy;
- Age at menopause defined as no menstrual period for more than 12 months irrespectively of menstrual resumption after chemotherapy;
- AMH values one year after the end of chemotherapy;
- AMH values two years after the end of chemotherapy;
- AMH values five years after the end of chemotherapy.

The impact of anticancer therapies on patients' ovarian function will be evaluated in various subgroups of young breast cancer patients according to:

- Age: <30 vs. 30-35 vs. > 35;
- Type of chemotherapy received: anthracycline- and taxane-based vs. taxane-based vs. others;
- Number of months of chemotherapy received: <4 months vs. > 4 months;
- Exposure to platinum agents: use vs. no use.
- Exposure to endocrine therapy: use vs. no use.
- Exposure to anti-HER2 therapy: use vs. no use.
- Presence of *BRCA* mutation: *BRCA*-mutated vs. *BRCA*-not mutated.

The sample size has been estimated based on the study primary endpoint (i.e. number of mature oocytes collected). With 113 patients in the standard-stimulated cohort and 113 in the letrozole-stimulated cohort, assuming a mean number of mature oocytes collected of 6.1 in the standard-stimulated cohort, the study will have a power of 0.80 to reject the null hypothesis of equal means when the population mean difference is 1.5 mature oocytes collected (estimating a mean number of mature oocytes collected of 7.6 in the letrozole-stimulated cohort) with a standard deviation for both groups of 4.0 and with a significance level (alpha) of 0.05 using a two-sided two-sample equal-variance t-test. Considering that approximately one out of three (1:3) eligible patients counseled to

undergo controlled ovarian stimulation for oocyte cryopreservation before starting anticancer treatments will accept the procedure, it is estimated to enroll 339 patients in the non-stimulated cohort.

Patients demographic, clinical and pathologic baseline characteristics will be compared in the three cohorts using chi-square test or Fisher Exact test for categorical variables and Wilcoxon rank-sum test or t-test for continuous variables, as appropriate.

Dicothomous outcomes will be compared using univariate and multivariate logistic regression models.

Time-to-event outcomes will be estimated and plotted using Kaplan-Meier methods. Survival rates will be compared using log-rank test and Cox proportional hazards models for univariate and multivariate analysis, respectively.

All tests will be two-sided and p-values of < 0.05 will be considered statistically significant.

6. QUALITY CONTROL AND QUALITY ASSURANCE

6.1. Quality control

Throughout the study the dedicated sponsor study team members verify the data to ensure that:

- the rights and well-being of subjects are protected
- the reported trial data are accurate, complete and verifiable from source documents
- the conduct of the trial is compliant with the International Conference on Harmonisation (ICH) – Good Clinical Practice (GCP), the applicable regulatory requirements, the study protocol and the study guidelines.

Quality control activities combine central monitoring and clinical site monitoring. Monitoring activities may be performed remotely and/or on site according to the study needs.

6.2. Quality assurance

To ensure compliance with the protocol, study documentation, GCP and all applicable regulatory requirements, the sponsor may conduct a quality assurance audit on participating site. Regulatory agencies may also conduct a regulatory inspection of the study. The principal investigators must inform the sponsor in case an inspection from Regulatory agencies has been scheduled at their sites.

Such audits/inspections can occur at any time during or after completion of the study. If an audit or inspection occurs, the investigator and institution must agree to allow the auditor/inspector direct access to all relevant documents and to allocate his/her time and the time of his/her staff to the auditor/inspector to discuss findings and any relevant issues in the presence of the sponsor, as needed or required.

7. REGULATORY AND ETHICAL CONSIDERATIONS

7.1. Regulatory considerations

The study is submitted by the principal investigators or the national coordinator or the sponsor (or its legal representative), in accordance with local regulations, to and approved by an appropriate Independent Ethical Review Committee (IEC) / Institutional Review Board (IRB) and a Regulatory Authority if required by the national laws of the countries where the study is conducted. Local regulatory approval may also be required.

The study cannot start in a participating site before written approval by corresponding Ethics Committee(s) has been obtained and the local regulatory requirements have been complied with.

The sponsor provides a copy of the final protocol, subject information sheets, consent forms and all other relevant study documentation for local required submissions.

The principal investigators and the sponsor ensure that the study is conducted in full conformance with the principles of the "Declaration of Helsinki" 1964, as revised from time to time and with the laws and regulations of the country in which the research is conducted, whichever affords the greater protection to the individual. The study must fully adhere to the principles outlined in "Guideline for Good Clinical Practice" ICH-E6 Tripartite Guideline (January 1997) and with national laws.

For the Study conducted in the EU/EEA countries, the principal investigators ensure compliance with the ICH GCP and with the EU Data Protection Directive (95/46/EC).

7.2. Informed consent form

It is the responsibility of the principal investigators or a person designated by the principal investigators (if acceptable by local regulations) to obtain a signed written informed consent from each potential subject prior any study related procedure being carried out. The informed consent is applicable throughout the subject's participation to the study.

The Informed Consent form (ICF), approved by an appropriate IEC / IRB, is provided by the sponsor to the principal investigators as a separate document dated and version controlled to this protocol.

The ICF describes in details the aims, the procedures, the methods, the interventions, the anticipated benefits, the possible adverse events and the potential hazards of the study. Many events are likely to occur which meet the definition of serious adverse events and which are due explicitly to the patients' underlying malignancy management or chemotherapy treatment. For this study, these adverse events or SAE will be exempt from report or declaration in the CRF, except if unexpected or suspected to be linked to the ovarian stimulation.

The principal investigators or designee also explain to the subject that the participation to the study is voluntary and that the subject is free to refuse to enter the study or to withdraw from it at any time, for any reason without any impact in the subject's subsequent care.

All subjects receive the appropriate version of the written information and are asked to read and review it.

For subjects not qualified to give or incapable of giving consent, written consent must be obtained from the legal representative. In the case where both the subject and his or her legal representative are unable to read, an impartial witness should be present during the entire informed consent discussion. After the subject and/or legal representative has/have orally consented to participation in

the trial, the witness's signature on the form will attest that the information in the consent form was accurately explained and understood.

The written ICF must be dated and personally signed by the principal investigators or authorised sub-investigator and the subject taking consent (or subject legal representative).

The original copy of the signed ICF will be retained in investigator's study file (ISF) and must be made available for monitoring, audit or inspection. A copy of the signed ICF is given to the subject. In case of new information that might affect the subject's willingness to continue participating in the study or results in significant changes in the risk/benefit assessment, the ICF should be reviewed and updated if necessary. All subjects, including those already being treated, should be informed of the new information, given a copy of the revised form, and give their consent to continue in the study.

7.3. Subject identification

The principal investigators or any authorised study member staff must assure that subjects' identity is maintained confidential and that their identities are protected from unauthorised parties. Personal medical information is always treated as confidential.

On CRFs or any other study document, subjects shall not be identified by their names or initials or social security number or patient chart number, but always by the assigned subject study identification code and their date of birth (either complete or partial as allowed by national laws) in order to avoid identification errors.

The principal investigators should create and maintain up-to-date in the ISF the Subject screening/enrolment/identification log. This ICH-GCP log is required for documenting chronically

any screened subject, their name, their assigned study number in case of study enrolment or the reason in case of non-enrolment. A note is made in the hospital medical records that the subject is participating in the study.

The name of a subject is neither asked for nor recorded by the sponsor. If subjects' names or any other confidential subject information are included by error on copies of documents submitted to the sponsor, they are obliterated and the assigned subject study identification code added to the document by the sponsor. Moreover, the sponsor asks to the principal investigators or any authorised study member staff to also obliterate this information on the documents that he/she has submitted.
Documents (e.g. subjects' written ICFs) not for submission to sponsor should be maintained by the principal investigators in strict confidence.

8. DATA HANDLING AND RECODING KEEPING

8.1. Investigator's files and subject's clinical source documents

The principal investigators must maintain adequate and accurate records to enable the conduct of the study to be fully documented and the study data to be subsequently verified. These documents should be classified into two separate categories: (1) ISF; and (2) subject clinical source documents.

The ISF should be established according to the ICH-GCP E6 and should include, among other documents, the site staff delegated study task log established by the principal investigators, the subject screening/enrolment and identification log and signed ICF.

Subject clinical source documents (paper or electronic) are the documents where the data are recorded for the first time and include, but are not limited to, subject hospital/clinic records; physician's and

nurse's notes; appointment book; original laboratory reports; consultant letters and pharmacy dispensing records.

8.2. Case Report Forms (CRF)

Data for this study are captured by using an electronic CRF (eCRF) via a sponsor designated electronic data capture system for each subject enrolled (REDcap software). The site receives training for appropriate eCRF handling and completion and personal access code by the sponsor.
The eCRF should only be completed by designated and trained site staff and submitted electronically to sponsor.

The principal investigators are responsible for creating and maintaining up to date the site staff delegated study task log (named Signature Log in ICH GCP) mentioning the responsible persons completing, updating and/or signing the eCRF.

Once completed, the eCRF must be reviewed and electronically signed by the principal investigators or an authorised delegate from the study staff. This also applies to records for those subjects who fail to complete the study. If a subject withdraws from the study treatment or does not complete the follow-up, the reason must be noted in the eCRF. If a subject withdraws from the entire study, the withdrawal of consent should be noted in the eCRF.

The principal investigators should ensure the accuracy, completeness, legibility and timeliness of the data reported in the eCRF and in all required reports. Data reported in the CRF must be derived from source documents and should be consistent with the source documents.

An audit trail will maintain a record of the initial entries and changes made, reasons for change (if necessary), time and date of entry, and user name of the person adding or changing an entry.

8.3. Retention of documents

Following closure of the study, the principal investigators must maintain all site study records, except for those required by local regulations to be maintained by someone else, in a safe and secure location. The records must be maintained to allow easy and timely retrieval, when needed (e.g., audit or inspection), and, whenever feasible, to allow any subsequent review of data in conjunction with assessment of the facility, supporting systems, and staff. Where permitted by local laws/regulations or institutional policy, some or all of these records can be maintained in a format other than hard copy (e.g., microfiche, scanned, electronic); however, caution needs to be exercised before such action is taken. The principal investigators must ensure that all reproductions are legible and are a true and accurate copy of the original and meet accessibility and retrieval standards, including re-generating a hard copy, if required. Furthermore, the principal investigators must ensure there is an acceptable back-up of these reproductions and that an acceptable quality control process exists for making these reproductions.

The sponsor informs the principal investigators of the time period for retaining these records to comply with all applicable regulatory requirements. The minimum retention time meets the strictest standard applicable to that participating site for the study, as dictated by any institutional requirements or national laws or regulations; otherwise, the retention period will default to 20 years after the completion of the study and/or after approval by relevant Health Authorities, whichever is longer.

Should the principal investigators wish to assign the study records to another party or move them to another location, the sponsor must be notified in advance. If the principal investigators cannot

guarantee this archiving requirement at the participating site for any or all of the documents, special arrangements must be made between the principal investigators and the sponsor to store these in a sealed container(s) outside of the participating site so that they can be returned sealed to the principal investigators, in case of a regulatory audit. Where source documents are required for the continued care of the subject; appropriate copies should be made before storing outside of the participating site.

9. PUBLICATION POLICY

A manuscript summarizing the results of the analysis will be prepared for submission to and publication in a peer-reviewed scientific journal. Regular emails, teleconferences and/or face-to-face meetings will be organized to discuss progress of the data analysis. The manuscript will be prepared by the study chairs and the study steering committee along with the statistical team and will be submitted to the all collaborators for review according to the signed agreement form.

Any other publication arising from this project will be made on behalf of the study chairs and the study steering committee.

All authors will be given sufficient time to provide comments and attempts will be made to come to mutual agreement. No financial benefits will be pursued or derived by the study and the corresponding results.

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