Increased chemotherapy-induced ovarian reserve loss in women with germline *BRCA* mutations due to oocyte deoxyribonucleic acid double strand break repair deficiency

Kutluk H. Oktay, M.D., Ph.D.,[a] Giuliano Bedoschi, M.D.,[a] Shari B. Goldfarb, M.D.,[b] Enes Taylan, M.D.,[a] Shiny Titus, Ph.D.,[a] Glenn E. Palomaki, Ph.D.,[c] Tessa Cigler, M.D.,[d] Mark Robson, M.D.,[b] and Maura N. Dickler, M.D.[b]

[a] Department of Obstetrics and Gynecology and Reproductive Sciences, Yale University School of Medicine, New Haven, Connecticut; [b] Memorial Sloan Kettering Cancer Center and [d] Weill Medical College of Cornell University, New York, New York; and [c] Department of Pathology and Laboratory Medicine, Women & Infants Hospital and Alpert Medical School at Brown University, Providence, Rhode Island

Objective: To assess whether woman who have *BRCA* mutations (WBM) experience more declines in ovarian reserve after chemotherapy treatment, as it induces oocyte death by deoxyribonucleic acid (DNA) damage, and *BRCA* mutations result in DNA damage repair deficiency.
Design: Longitudinal cohort study.
Setting: Academic centers.
Patient(s): The 108 evaluable women with breast cancer were stratified into those never tested (negative family history; n = 35) and those negative (n = 59) or positive (n = 14) for a pathogenic *BRCA* mutation.
Intervention(s): Sera were longitudinally obtained before and 12–24 months after chemotherapy treatment, assayed for antimüllerian hormone (AMH), and adjusted for age at sample collection.
Main Outcome Measure(s): Ovarian recovery, defined as the geometric mean of the after chemotherapy age-adjusted AMH levels compared with baseline levels.
Result(s): Compared with the controls, the before chemotherapy treatment AMH levels were 24% and 34% lower in those negative or positive for *BRCA* mutations, consistent with accelerated ovarian aging in WBM. The WBM had a threefold difference in AMH recovery after chemotherapy treatment (1.6%), when compared with *BRCA* negative (3.7%) and untested/low risk controls (5.2%). Limiting the analysis to the most common regimen, doxorubicin and cyclophosphamide followed by paclitaxel, showed similar results. These findings were mechanistically confirmed in an *in vitro* mouse oocyte *BRCA* knockdown bioassay, which showed that *BRCA* deficiency results in increased oocyte susceptibility to doxorubicin.
Conclusion(s): Women who have pathogenic *BRCA* mutations are more likely to lose ovarian reserve after chemotherapy treatment, suggesting an emphasis on fertility preservation. Furthermore, our findings generate the hypothesis that DNA repair deficiency is a shared mechanism between aging, infertility, and cancer.
Clinical Trial Registration Number: NCT00823654. (Fertil Steril® 2020;113:1251–60. ©2020 by American Society for Reproductive Medicine.)
El resumen está disponible en Español al final del artículo.

Key Words: *BRCA*, chemotherapy, ovarian insufficiency, fertility preservation, ovarian reserve

Received August 21, 2019; revised January 23, 2020; accepted January 24, 2020; published online April 22, 2020.
K.H.O. has nothing to disclose. G.B. has nothing to disclose. S.B.G. has nothing to disclose. E.T. has nothing to disclose. S.T. has nothing to disclose. G.E.P. has nothing to disclose. T.C. has nothing to disclose. M.R. has nothing to disclose. M.N.D. has nothing to disclose.
Supported by RO1 HD053112 from the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development and the National Cancer Institute (to K.H.O.).
Reprint requests: Corresponding Author: Kutluk Oktay, M.D., Ph.D., Department of Obstetrics, Gynecology and Reproductive Sciences, Yale University School of Medicine, New Haven, Connecticut (E-mail: info@fertilitypreservation.org).

Fertility and Sterility® Vol. 113, No. 6, June 2020 0015-0282/$36.00

https://doi.org/10.1016/j.fertnstert.2020.01.033

The *BRCA*1 and *BRCA*2 genes are key members of the kinase ataxia-telangiectasia mutated (ATM)-mediated deoxyribonucleic acid (DNA) double-strand break (DSB) repair pathway. Mutations in these genes are associated with an increased risk of breast, ovarian, and other types of cancers (1). While developing ovarian stimulation protocols with aromatase inhibitors in women with breast cancer we observed that those with *BRCA*1 mutations produced fewer oocytes compared with those who tested negative for these mutations (2). In subsequent studies (3–6), we and other investigators have shown that women with *BRCA*1 mutations have lower antiMüllerian hormone (AMH) levels, which is the most reliable serum marker of ovarian reserve (7).

We also completed laboratory and animal studies (3, 8, 9), which indicated for the first time, that age-related decline in oocyte DNA DSB repair capacity may be the root cause of ovarian aging and that the lower ovarian reserve in women with *BRCA* mutations is due to accelerated ovarian aging caused by deficient oocyte DNA repair. In other studies (10, 11), we showed that gonadotoxic chemotherapy induces DNA DSBs in human primordial follicle oocytes, which in turn activates apoptotic pathways and thereby cause chemotherapy-induced loss of ovarian reserve. Our studies suggested that the DNA repair mechanisms may also be important in shielding oocytes against chemotherapy-induced DNA damage.

Serum AMH is produced from replicating granulosa cells of developing follicles, which are destroyed by chemotherapy. Ovarian reserve is made up of quiescent primordial follicles, which do not produce AMH (12, 13). Because the size of the population of developing follicles is directly proportional to the number of primordial follicles, AMH is a biomarker of ovarian reserve (14–16). Although that there is no perfect marker to quantifying primordial follicles in human ovary noninvasively, AMH measurement provides the best accuracy for identifying damage to the ovarian reserve after chemotherapy treatment (7). Although the acute decline in circulating AMH after chemotherapy reflects the loss of developing follicles, those recovery levels would reflect the surviving primordial follicle reserve, if any. Considering our unpublished observations and limited published data, we estimated that it may take 3–6 months to repopulate the developing follicle pool to a level where AMH is measurable in the circulation. Hence, AMH levels measured using a sensitive measurement method serum should reach a steady level by 1 year, reflective of chemotherapy-induced ovarian damage (5, 6, 8, 9).

Whether women who are DNA DSB repair deficient because of pathogenic *BRCA* mutations would be more likely to lose their ovarian reserve as a side effect of chemotherapy has never been studied. These previous mechanistic findings suggest that chemotherapy accelerates ovarian aging and the mechanisms involved in age- and chemotherapy-induced ovarian aging seem to be shared. We therefore hypothesized that breast cancer chemotherapy would result in a larger loss of ovarian reserve, as determined by serum AMH levels, among reproductive age women with *BRCA* mutations compared with those who do not carry, or at low risk for carrying, such mutations (negative family history). These differences would be due to deficient DNA repair in their oocytes and result in lowering of AMH levels beyond what would be expected after chemotherapy, based on each woman's age at sampling.

If our hypothesis is confirmed, it would have strong implications in the fields of cancer and aging. If women with *BRCA* mutations are more likely to lose their ovarian reserve and hence become infertile after chemotherapy, they may have to be preferentially counseled on fertility preservation methods. Furthermore, if ovarian aging, as determined by faster loss of ovarian reserve in response to a genotoxic stress, such as chemotherapy, is accelerated in DNA repair-deficient individuals, it further establishes the critical role of intact DNA repair mechanisms in reproductive aging. Such a revelation may also suggest failing DNA repair as a connection among aging, infertility, and cancer.

MATERIALS AND METHODS

Patient enrollment, collection of family history, and *BRCA* mutation testing

This study was approved by the institutional review boards at all participating institutions. Enrollment began in January 2009, as part of a National Institutes of Health-funded parent translational research project to assess the impact of breast cancer chemotherapy on ovarian reserve. Women who had previous chemotherapy or ovarian surgery and those who did not have regular periods did not qualify for enrollment in the study. Enrollees provided blood samples before and after treatment. Our primary end point of serum AMH recovery was originally intended to be 12 months. However, due to lower than expected 12-month samples we modified the design to include the 18- and 24-month time points from the parent study, and combine these measurements using the geometric mean.

Information on treatment type (chemotherapy, hormonal therapy), age, and body mass index, as well as their family history of breast/ovarian cancer and other information relevant to assessing their risk of having a *BRCA* mutation (e.g., Jewish ancestry, relative with a known *BRCA* mutation) was recorded. Family history was collected at first visit with a medical oncologist and the National Comprehensive Cancer Network Guideline (version 1.2018) was followed for *BRCA* mutation testing decision (17).

Collection and testing of blood samples

Resulting sera were aliquoted and stored first at -80°C, then long term at -273°C. Frozen aliquots were sent on dry ice for testing at Ansh Labs where serum AMH was measured using a two-site enzyme-linked immunoassay (picoAMH ELISA) following the manufacturer's instructions. The AMH levels were expressed in nanograms per milliliter and all assays were performed within 3 days in mid-August 2017 with a single lot of reagents. Samples were initially diluted 1:10. The reportable range was 0.003–23 ng/mL. Initial values $<$0.03 ng/mL were retested without dilution and any samples with initial values $>$11.5 ng/mL were retested at a 1:20

dilution. All final optical densities were within the standard curve. The coefficient of variation for the four levels of pooled serum quality controls tested along with study specimens were all <7%.

Inclusion and exclusion criteria

Among all women enrolled, we excluded those that were aged ≥43 years as they may be approaching menopause during follow-up. Women who had recurrence and needed additional chemotherapy were also excluded. Also excluded were those women with a baseline AMH of <0.5 ng/mL, as these low baseline levels could limit the understanding of the magnitude of the impact of chemotherapy on the ovarian reserve, causing a dilution effect on the study findings. Levels <0.5 ng/mL were excluded according to the minimum AMH levels stipulated by the Bologna criteria (18). Also excluded were those women who did not have at least one observation at 12, 18, or 24 months and those who only received endocrine therapy (tamoxifen). The remaining patients were stratified into a study and two control groups: *BRCA*-mutation-positive (study), *BRCA*-mutation-negative and those that did not receive *BRCA* testing, generally due to a negative family history (low risk). To account for the possibility of breast cancer independently affecting ovarian reserve, both *BRCA* negative and low-risk untested women were combined to serve as controls.

Mouse bioassay

Three-month-old Friend Virus B (FVB) mice (N = 8) were superovulated to obtain supernumerary oocytes for the assay. For superovulation, mice aged 12–15 weeks were given an intraperitoneal injection of 5.0 IU pregnant mare's serum gonadotropin. Germinal vesicle oocytes were collected and were randomly assigned to *BRCA* small interfering ribonucleic acid (siRNA) (N = 128), scrambled siRNA (N = 118), or sham (N = 110) microinjections. After the microinjection, the oocytes were exposed to doxorubicin (100 μg/mL) in vitro for 1 hour in human tubal fluid media (Irvine Scientific California) (3, 11). After which time, the oocytes were transferred to fresh human tubal fluid media and evaluated for survival 8 hours later. An oocyte was considered to not have survived if it had a condensed nucleus dissociated from the zona pellucida.

The choice of doxorubicin (Adriamycin) was because this chemotherapeutic agent is one of the main components of the most common chemotherapy regimen (doxorubicin [Adriamycin] plus cyclophosphamide plus paclitaxel [Taxol] or AC+T) used in our study. In addition, cyclophosphamide is not bioactive in vitro. Furthermore, in previous in vivo human xenograft and in vitro human ovarian cortical culture studies we showed that doxorubicin induces oocyte death by DNA DSBs and that it activates the kinase ataxia-telangiectasia mutated-mediated repair pathway, in some cases resulting in repair and rescue from apoptosis (11). Hence doxorubicin is a suitable drug to test the role of impaired *BRCA* function on the chemotherapy sensitivity of oocytes.

Statistical analyses

Regression and inference testing was always performed on the log-transformed adjusted AMH values, percentages, or ratios. The AMH levels are dependent on the woman's age. A univariate log-linear regression analysis was performed on the referent population (those not tested for *BRCA* mutations) resulting in an expected AMH level given a woman's age. The AMH results at baseline (before cancer treatment) for all women were divided by their expected level based on age, to produce an "adjusted" AMH result with an expected median of 1.00 in the referent group. The adjusted baseline AMH levels were then compared between the three groups of women with breast cancer using analysis of variance (ANOVA) and *t*-tests, after a logarithmic transformation (AMH levels can vary by more than a factor of 1,000).

Adjusting AMH results for the woman's age at sample collection reduces variability and improves comparability in two ways. Each woman's results over time could be compared to her initial result without accounting for age. However, doing so would make it appear as though a ratio of 1.00 in one woman is the same as 1.00 in another woman, which would not be true if they were of different ages when enrolled. Accounting for age, a major covariate of AMH makes a direct comparison between women possible. In addition, accounting for age ensures that the observed levels of AMH 1 or 2 years later are not simply due to aging. The reductions we shown are independent of the women's age or time of sampling.

To estimate ovarian "recovery," the longitudinal AMH observations at 12, 18, and 24 months were also adjusted for the woman's age at the time of collection, as described earlier. The geometric mean adjusted AMH value during this time period was then computed for each woman and a ratio between this value and her baseline AMH was computed and reported as the percentage recovery. For example, if the recovery was to be <100% (average adjusted 12- to 24-month AMH levels equal that at baseline), it would indicate that woman's ovarian reserve had not completely recovered. These percentage recovery estimates were then compared between the three *BRCA* groups of women with breast cancer using ANOVA and *t*-tests, after a logarithmic transformation.

Statistical significance was two-tailed at the 0.05 level. Analyses and methods were specified by a formal analytic plan, but slightly modified as described. The original outcome measure was 12-month after chemotherapy AMH level recovery compared between the *BRCA*-mutation-positive study group and the two control groups (negative and untested). We planned to combine the control groups if they had similar AMH levels, as we have done in our previous studies (2).

Our initial power analysis based on expected 30% difference in serum AMH recovery estimated that approximately 40 subjects were needed in each of the three groups to have 80% power to detect these differences at a $P = .05$ level of significance. Oocyte survival in in vitro chemotoxicity assay was analyzed with contingency tables. Based on our previous publication (3), we determined that a minimum of 50 oocytes was needed in each group to detect 30% difference in survival with 90% power. Analyses were performed using BMDP

(Statsols US) with figures produced using GraphPad Software, Prism v7.04.

RESULTS

Patient population

Enrollment ended in November 2017. Among the 164 women who had a baseline sample and were followed, 21 were aged 43–45 years at enrollment, 16 had baseline AMH values under 0.5 ng/mL (0.02 to 0.48 ng/mL) and 19 had no blood samples in the 12- to 24-month time period used to define AMH recovery and were not eligible for the study. (Fig. 1). The remaining 108 evaluable women were stratified into three groups: 13 with a pathogenic or likely pathogenic *BRCA* mutation and 1 with a variant of unknown significance, 59 with no *BRCA* pathogenic mutations (including 2 with likely benign polymorphisms), and 35 with no *BRCA* mutation testing performed after breast cancer risk assessment. Table 1 shows demographic, raw AMH test results, estrogen receptor status, and treatments for the 108 included women, stratified by *BRCA* mutation status. Those with an identified mutation were somewhat younger (33.7 years) compared with the other groups (36.3 and 36.1 years), but the difference did not reach statistically significance ($P = .10$). There were no significant differences in body mass index, estrogen receptor status, or mean baseline unadjusted AMH result. The lowest average AMH levels occurred in those with an identified pathogenic *BRCA* mutation, despite their younger age, in concordance with earlier reported association of lower ovarian reserve in *BRCA* mutation carriers.

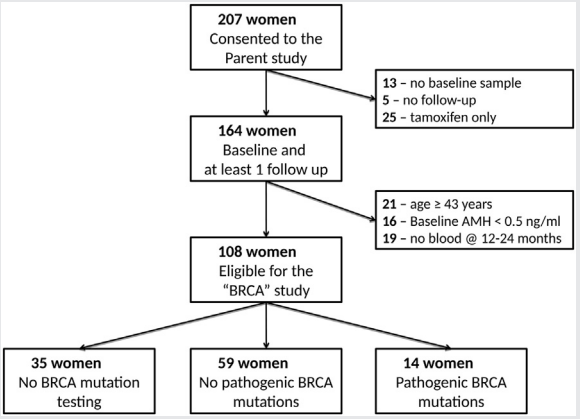
The 19 women who were excluded due to lack of a 12- to 24-month follow-up sample were similar to those included in terms of the type of chemotherapy used (doxorubicin and cyclophosphamide followed by paclitaxel use 84% vs. 77% in the study group; $P = .56$) and *BRCA* mutation status (16% vs. 13% in the study group; $P = .72$). However, they were older than those who completed the follow-up (39.8 ± 3.0 vs. 35.8 ± 4.0; $P < .001$). The latter could be explained because older women are more likely to experience menopause after chemotherapy and not see a need for continued follow-up.

Adjusted baseline AMH levels by *BRCA* mutation testing status

To account for the known dependence of AMH levels on the woman's age, a univariate linear regression on the log transformed AMH was performed for the 35 women without a known *BRCA* mutation (Supplemental Fig. 1, available online). The correlation was -0.28 ($P = .10$). For example, a 25-year-old referent woman (*BRCA* untested, low risk by National Comprehensive Cancer Network guidelines) has an expected AMH level of 5.87 ng/mL, whereas a 40-year-old woman has an expected AMH level of 2.82 ng/mL; a 52% reduction during the 15 years. The baseline AMH results in all 108 women were then adjusted by dividing by the expected AMH level according to each woman's age. The results are shown in Figure 2. The geometric mean ratios of observed to expected AMH for the *BRCA*-negative and *BRCA*-positive groups in relation to the referent untested group were 0.76 and 0.66, respectively. Thus, after accounting for age, women negative or positive for a *BRCA* mutation have adjusted baseline AMH levels that are 24% and 34% lower levels than in women who were not tested. Analysis of variance with a linear contrast (-1, 0, 1) showed that these differences approached statistical significance ($P = .07$). Although this was not the primary aim of this study and the study was not powered to assess this, the tendency for lower AMH levels in women with *BRCA* mutations is consistent with the recent data showing accelerated ovarian aging in these women.

As shown in Figure 2A, the baseline AMH levels are lower in those with a mutation, therefore it is possible that smaller ovarian reserves might correlate with heightened effect of chemotherapy. To explore this possibility further, we analyzed the relationship between the adjusted baseline AMH levels with the raw AMH levels found at end of the chemotherapy treatment in the 42 control women receiving doxorubicin and cyclophosphamide followed by paclitaxel (AC-T). Among the 21 women with the lowest AMH adjusted levels (0.21–0.91 ng/mL), 16 women (71%) had undetectable AMH levels and the median in the remaining 5 women was 0.024 ng/mL. Among the 21 women with the highest AMH adjusted levels (0.95–3.25 ng/mL), 12 women (57%) had undetectable AMH levels and the median in the remaining 9 was 0.025 ng/mL ($P = .52$, Fisher's exact, two-tailed test). Although this is not a large dataset, it presents relatively strong evidence that most of all controls, regardless of their initial AMH levels, are reduced to very low and essentially equivalent levels with a standardized chemotherapy.

FIGURE 1



Patient selection flow chart. From the parent study, 43 women did not qualify because they did not follow up after the initial encounter or received tamoxifen only. In the remaining 164 with at least one follow up, 19 did not have a sample 12-24 months after chemotherapy, 16 had a very low baseline AMH, and 21 were aged ≥43 years at baseline. The remaining 108 women were eligible to participate.

Oktay. BRCA *and chemotherapy-induced ovarian damage. Fertil Steril 2020.*

TABLE 1

Demographic information and unadjusted antiMüllerian hormone (AMH) results, stratified by *BRCA* mutation testing status.

	BRCA mutation status				
	Not tested	Negative	Positive	All	*P* value[a]
Number	35	59	14	108	
Age (y)[b]	36.3 (4.1)	36.1 (4.0)	33.7 (3.7)	35.8 (4.0)	.10
BMI (kg/m^2)	23.6 (4.2)	23.3 (4.8)	25.0 (4.6)	23.6 (4.5)	.49
AMH (ng/mL)[c]					
Baseline	3.4 (0.31) N = 35	2.6 (0.34) N = 59	2.5 (0.35) N = 14	2.8 (0.45) N = 108	.22
12 months after therapy	0.10 (0.78) N = 33	0.11 (0.87) N = 52	0.053 (0.95) N = 14	0.098 (0.85) N = 99	.44
18 months after therapy	0.11 (0.91) N = 30	0.12 (0.88) N = 47	0.029 (0.99) N = 9	0.10 (0.91) N = 86	.16
24 months after therapy	0.20 (0.83) N = 24	0.16 (0.86) N = 41	0.045 (0.90) N = 8	0.15 (0.86) N = 73	.18
ER +	30 (86%)	49 (83%)	11 (79%)	90 (83%)	.82
Treatments[d]					
AC-T	25 (72%)	46 (78%)	12 (86%)	83 (71%)	.54[e]
CMF-q2-3w	6 (17%)	5 (8%)	0 (0%)	11 (10%)	
Taxol only	4 (11%)	5 (8%)	1 (7%)	10 (9%)	
Other[f]	0 (0)	3 (5%)	1 (7%)	4 (4%)	

AC-T = doxorubicin + cyclophosphamide followed by paclitaxel; BMI = body mass index; CMF-q2-3w = cyclophosphamide + methotrexate + fluorouracil dosed every 2 or 3 weeks; EC-T = epirubicin + cyclophosphamide followed by docetaxel; ER = estrogen receptor.

[a] Comparing the three *BRCA* mutation status groups.

[b] Reported as mean (standard deviation).

[c] Reported as geometric mean (logarithmic standard deviation), number of observations. Note that these are unadjusted cross-sectional means and *P* values do not represent the final analysis. For comparison of adjusted values, please see Figure 2.

[d] Reported as N and column percent.

[e] χ^2 test of AC-T versus other treatments for the three groups.

[f] Includes EC-T / vineralbine.

Oktay. BRCA *and chemotherapy-induced ovarian damage. Fertil Steril 2020.*

Ovarian recovery at 12–24 months by *BRCA* mutation testing status

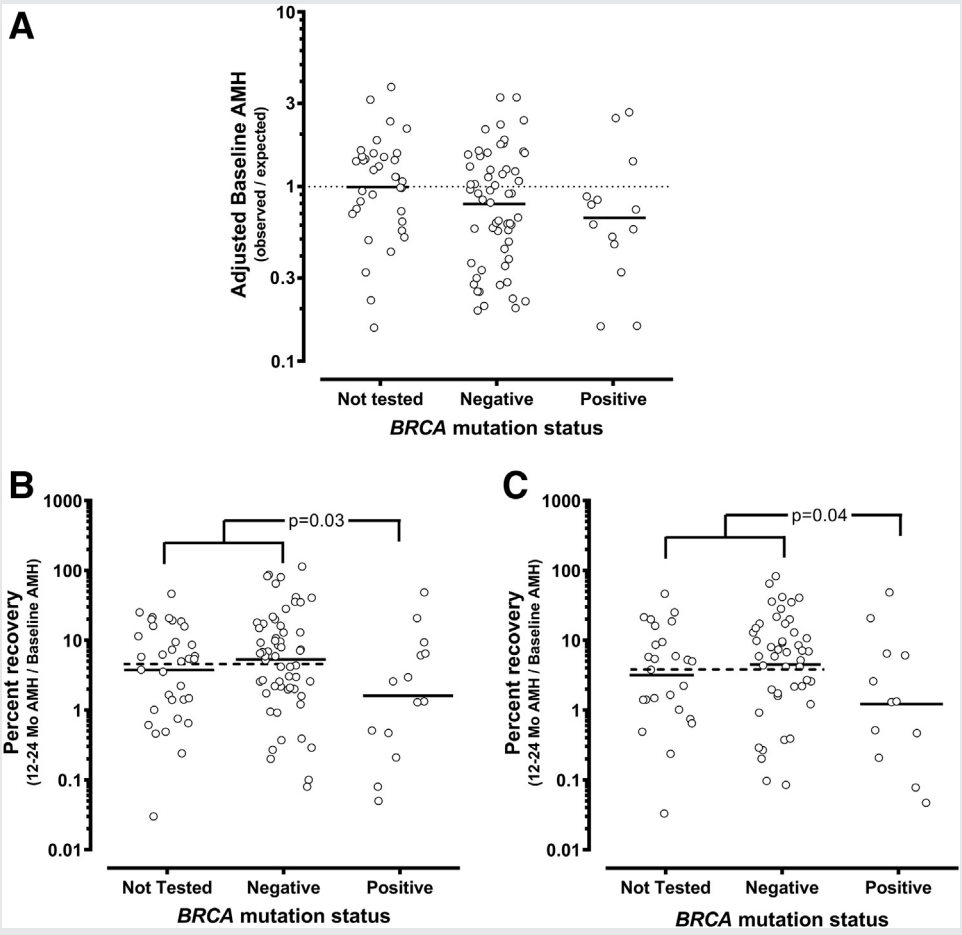
Among the 108 women, 63 (58%), 33 (31%), and 12 (11%) provided all three, two, or only one follow-up sample. The most commonly provided sample was at 12 months (99 of 108 women), whereas 86 and 79 provided samples at 18 and 24 months, respectively. Figure 2B shows the resulting distributions of percent AMH recovery, stratified by the three *BRCA* mutation testing groups (35 not tested, 59 *BRCA* negative, and 14 *BRCA* positive). Women with *BRCA* mutations lost significantly larger ovarian reserve than the *BRCA* mutation negative or untested (low risk) control groups. The geometric mean recoveries were 3.7%, 5.2%, and 1.6%, respectively. Stated differently, the mean recovery in the *BRCA* mutation positive group was about one-third the 4.6% recovery in the other two control groups combined (two-group ANOVA, $P = .03$, $F = 4.89$). A log-linear regression on all women with three AMH observations over time showed a slight tendency for the levels to increase but not sufficiently large or consistent to warrant adjustment. By that analysis, the geometric mean of the multiple measurements appeared to be a reliable indicator of ovarian recovery after chemotherapy treatment.

Given that the ovarian damage and recovery are dependent on the type and dose of chemotherapy, the data were reanalyzed after restriction to those treated with AC-T. Of the 108 women in the previous analysis, 83 (77%) were treated with AC-T; 25, 46, and 12 women in the three groups, respectively. The geometric mean AMH recoveries for these new groups were 3.2%, 4.7%, and 1.3% (Fig. 2C). When the *BRCA* mutation positive group was compared with other two groups, the former had significantly worse recovery of serum AMH levels (ANOVA, $P = .04$, $F = 4.2$). These data show increased liability to chemotherapy-induced ovarian reserve loss in women carrying pathogenic germline *BRCA* mutations.

Confirmation of biological basis in an in vitro mouse oocyte chemotoxicity assay

We have previously shown that an FVB mouse model replicates chemotherapy-induced DNA damage and repair mechanisms in human (3). We have also shown that doxorubicin induces DNA DSBs and activates *BRCA*-mediated DNA repair mechanisms in FVB mouse and human oocytes (10, 11). To demonstrate the biological basis of increased loss of ovarian reserve in women with *BRCA* mutations after chemotherapy, FVB mice oocytes were treated with doxorubicin (100 μg/mL) for 1 hour after sham, scrambled small interfering RNA, or small interfering RNA microinjection to silence *BRCA*1 in the oocytes. A significantly higher percentage of oocytes died in response to chemotherapy in the *BRCA* silenced group (58.59%; 75/128 injected) when compared with the sham (43.64%; 48/110 injected) and scrambled-siRNA-injected controls (40.68%; 48/118) ($P = .003$) (Fig. 3). These findings confirmed the biological basis of our clinical finding that the women with *BRCA* mutations are more susceptible of losing their ovarian reserve after chemotherapy treatment.

FIGURE 2



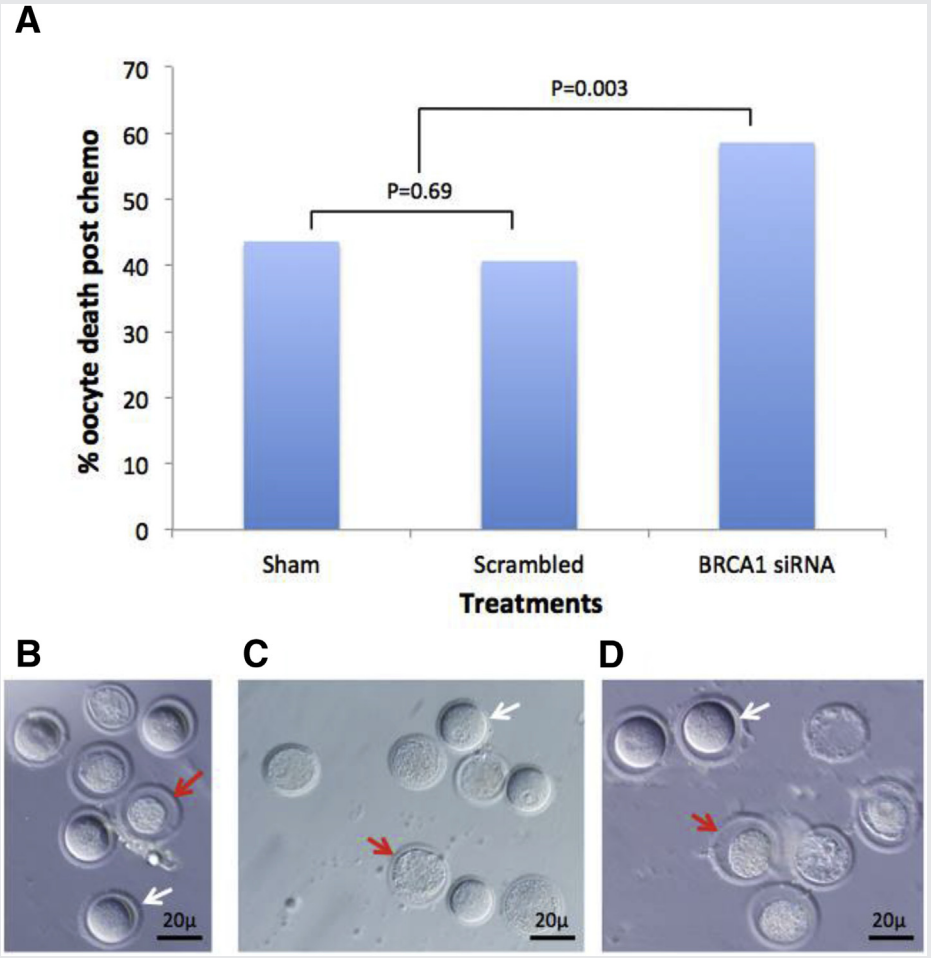
Adjusted baseline antiMüllerian hormone (AMH) and ovarian recovery in women with breast cancer after receiving gonadotoxic chemotherapy, as defined by the geometric mean AMH adjusted levels at 12–24 months, stratified by *BRCA* mutation testing status. (**A**) Each woman's AMH level was divided by the expected level based on her age at baseline testing. The expected value in the not tested group that were used for the regression analysis will, by definition, have an expected geometric mean ratio of 1.00 (dotted line). The *solid horizontal lines* indicate the geometric mean values for not tested and negative controls and the *BRCA* mutation positive group (1.00, 0.76, and 0.66). Analysis of variance with a linear contrast (-1, 0, 1) showed that these differences approached statistical significance ($P = .07$). (**B**) 108 women with all included chemotherapy regimens. Each woman's AMH levels at 12, 18, and 24 months (when available) were divided by the expected level based on her age at sample collection and plotted on the vertical logarithmic axis. The *solid horizontal lines* indicate the geometric mean values for the not tested and negative controls and the *BRCA* mutation positive group (3.7%, 5.2%, and 1.6%). The *dashed line* at 4.6% shows the geometric mean for the not tested and *BRCA* negative groups combined, which is significantly different from the recovery in women with an identified deleterious *BRCA*1 and/or *BRCA*2 mutation (two-group analysis of variance, $F = 4.89$, $P = .03$). (**C**) Same data with restriction to doxorubicin + cyclophosphamide + paclitaxel treatment, reducing the numbers from 108–83. The *solid horizontal lines* indicate the geometric mean values for the not tested and negative controls and the *BRCA* positive group (3.2%, 4.7%, and 1.3%). The *dashed line* shows the geometric mean for the not tested and negative groups combined (4.1%), which is significantly different from the recovery in women with an identified deleterious *BRCA*1 and/or *BRCA*2 mutation (two-group analysis of variance, $F = 4.2$, $P = .04$).

Oktay. BRCA *and chemotherapy-induced ovarian damage. Fertil Steril 2020.*

DISCUSSION

In this 8-year prospective longitudinal study, we have shown, for the first time, that women treated for breast cancer who carry pathogenic *BRCA* mutations lose significantly larger ovarian reserve than similarly identified women who do not carry or are at low risk for carrying such mutations. The loss of ovarian reserve was quantified using adjusted AMH levels between 12 and 24 months after chemotherapy treatment compared with baseline levels before chemotherapy treatment. Women with *BRCA* mutations lost significantly larger ovarian reserve than the *BRCA* mutation negative or untested (low risk) control groups. The mean recovery in the *BRCA* mutation positive group was about one-third the recovery in the other two groups combined (two-group ANOVA, $P = .03$, $F = 4.89$). Restriction of the analysis to single chemotherapy regimen (AC-T) did not change the results (ANOVA, $P = .04$, $F = 4.2$). AC-T tends to be the more commonly used chemotherapy regimen for *BRCA* mutation carriers and young women with breast cancer. That regimen contains two gonadotoxic drugs: doxorubicin (Adriamycin) and cyclophosphamide, both inducing DNA DSBs in primordial follicle oocytes as we have previously shown (10, 11).

FIGURE 3



*BRCA*1 deficiency results in oocyte sensitivity to chemotherapy in a mouse bioassay. FVB mice oocytes were treated with doxorubicin (100 µg/mL) for 1 hour after sham, scrambled small interfering ribonucleic acid (siRNA), or siRNA microinjection to silence *BRCA*1 in the oocytes. Oocyte survival was assessed 8 hours later. (**A**) Significant increase ($P = .003$) in the percentage of oocyte death was observed in the *BRCA* silenced group (128 oocytes from 8 mice) when compared to the sham (110 oocytes from 8 mice) and scrambled-siRNA-injected group (118 oocytes from 8 mice). Representative differential interference contrast images of the sham (**B**), scrambled siRNA (**C**) and *BRCA*1 siRNA-treated (**D**) oocytes are shown after doxorubicin exposure. *White arrows* point to representative viable and *red arrows* point to nonviable oocytes.

Oktay. BRCA *and chemotherapy-induced ovarian damage. Fertil Steril 2020.*

Although doxorubicin is not included in the second most common regimen (cyclophosphamide, methotrexate, and fluorouracil), its gonadotoxicity appears to be similar to the AC-T regimen because of the higher cumulative doses of cyclophosphamide used in the former (19). To strengthen our clinical findings and conclusion that *BRCA* deficiency results in increased ovarian reserve loss after chemotherapy treatment, we also performed a previously validated mouse bioassay. In that assay, interference with *BRCA*1 function resulted in significantly increased oocyte death after exposure to doxorubicin in vitro, confirming the biological basis of our clinical findings. These results show, for the first time, that women with pathogenic *BRCA* mutations have increased liability to chemotherapy-induced ovarian damage.

Our findings may have clinically significant implications for young women with *BRCA* mutations. Greater decline or lower recovery of AMH levels after chemotherapy treatment among women with *BRCA* mutations indicate that women with *BRCA* mutations may be more likely to lose their ovarian reserve after breast cancer treatment. In fact, we have previously shown that women with *BRCA* mutations may have accelerated ovarian aging. Compared with controls, they have lower serum AMH levels (3–6), fewer primordial follicles in their ovaries (8), and increased age-related DNA damage in their oocytes (9), as well as experiencing natural menopause at earlier age (20–22). Taken collectively with these data, our current findings warrant special attention to reproductive function of women with *BRCA* mutations. Having accelerated ovarian aging and increased liability to chemotherapy-induced ovarian reserve loss is a double whammy and underscores the importance of early intervention for fertility preservation. Women with *BRCA* mutations

can undergo oocyte or embryo cryopreservation with the aid of aromatase inhibitor supplemented ovarian stimulation protocols, as we have shown previously (23). Ovarian cryopreservation is no longer considered experimental by many professional societies in several countries. The American Society for Reproductive Medicine has also recently removed the ovarian tissue cryopreservation from the experimental category (24). However, the safety of early oophorectomy followed by ovarian cryopreservation for future ovarian autotransplantation as another fertility preservation strategy remains to be determined among women with pathogenic *BRCA* mutations (25, 26).

The strengths of our study include its novelty, prospective longitudinal nature, uniformity of treatments and relatively long term follow up post chemotherapy as well the mechanistic confirmation with a mouse bioassay. By showing in a mouse chemo-sensitivity assay that knock down of *BRCA*1 gene function results in significantly increased chemo-induced oocyte death, we provided the biological basis and mechanistic confirmation for our clinical findings.

Our study had some limitations. We had lower than expected accrual in the *BRCA* mutation-positive group. Our original power analysis indicated that approximately 40 subjects would be needed in each group; this was essentially met for two groups, but fell short for the group with deleterious *BRCA* mutations. However, because of the larger than expected difference between the ovarian recovery of women with *BRCA* mutations and controls, the results were still statistically significant. Although the low accrual did not have an impact on the primary outcome measure of serum AMH level recovery, it did affect the comparison of baseline serum AMH levels between the groups, which was not a primary outcome measure. Our baseline AMH values in mutation carriers were lower than the controls groups, approaching statistical significance ($P = .07$). Previous studies showed that the ovarian reserve is lower primarily in women with *BRCA*1 mutations (3, 9). Because we analyzed women who had *BRCA*1 and *BRCA*2 mutations together due to insufficient power for subgroup analysis in this study, the impact of *BRCA*1 mutations on ovarian reserve may have been underestimated. However, the finding of lower ovarian reserve in *BRCA* mutation carriers is strongly supported when these results are combined with our earlier work (3) and those of other investigators (4–6). Similarly, we did not have the power to analyze whether women who had *BRCA*1 mutations would be more susceptible to chemotherapy-induced ovarian reserve loss than those with *BRCA*2 mutations. It is also possible that we might have underestimated the impact of chemotherapy on ovarian reserve in women who had *BRCA*1 mutations.

This study was unique with its longitudinal nature. Not having every time point for each participant may have introduced additional variability. This was addressed by using age-adjusted geometric means of all available time points from each patient. We compared the geometric mean AMH levels at 12, 18, and 24 months after treatment. This had two advantages: it increased the effective sample size by about 11% and by providing multiple samples from the same individual, provided us with a more stable measure of ovarian reserve. Finally, although AMH levels strongly predict age at menopause (7, 27–29) and long-term ovarian function after chemotherapy treatment for early stage breast cancer (30–32), additional follow-up and/or larger cohort will be needed to determine whether women who have *BRCA* mutations are more likely to be infertile and menopausal after chemotherapy treatment. However, such studies may take decades to complete due to the challenges in obtaining protracted longitudinal follow-up in affected women with *BRCA* mutations. At present, a sharper decline in serum AMH levels after chemotherapy treatment is strongly suggestive of a shortened reproductive lifespan among women carrying *BRCA* mutations. Supporting this conclusion, limited published data, and the unpublished data from our reference laboratory (Ansh) indicates that serum AMH levels of <0.01 ng/mL is indicative of menopause (27, 31–33). Using this cutoff point, although this was not a preplanned analysis, a larger percentage of women with *BRCA* mutations were at risk for menopause versus those negative and untested controls combined (29% vs. 12.7%), but this difference did not reach statistical significance ($P = .12$, Fisher's exact test).

In conclusion, we showed that the presence of pathogenic *BRCA* mutations might put women at reproductive disadvantage when exposed to genotoxic stress such as the breast cancer chemotherapy treatment. These findings have significant bearing for fertility preservation counseling in women with *BRCA* mutations and the improved understanding of the mechanisms connecting aging, infertility, and cancer.

REFERENCES

1. Economopoulou P, Dimitriadis G, Psyrri A. Beyond *BRCA*: new hereditary breast cancer susceptibility genes. Cancer Treat Rev 2015;41:1–8.
2. Oktay K, Kim JY, Barad D, Babayev SN. Association of *BRCA*1 mutations with occult primary ovarian insufficiency: a possible explanation for the link between infertility and breast/ovarian cancer risks. J Clin Oncol 2010;28:240–4.
3. Titus S, Li F, Stobezki R, Akula K, Unsal E, Jeong K, et al. Impairment of *BRCA*1-related DNA double-strand break repair leads to ovarian aging in mice and humans. Sci Transl Med 2013;5:172ra21.
4. Wang ET, Pisarska MD, Bresee C, Chen YD, Lester J, Afshar Y, et al. *BRCA*1 germline mutations may be associated with reduced ovarian reserve. Fertil Steril 2014;102:1723–8.
5. Phillips KA, Collins IM, Milne RL, McLachlan SA, Friedlander M, Hickey M, et al. Anti-Mullerian hormone serum concentrations of women with germline *BRCA*1 or *BRCA*2 mutations. Hum Reprod 2016;31:1126–32.
6. Giordano S, Garrett-Mayer E, Mittal N, Smith K, Shulman L, Passaglia C, et al. Association of *BRCA*1 mutations with impaired ovarian reserve: connection between infertility and breast/ovarian cancer risk. J Adolesc Young Adult Oncol 2016;5:337–43.
7. Broer SL, Broekmans FJ, Laven JS, Fauser BC. Anti-Mullerian hormone: ovarian reserve testing and its potential clinical implications. Hum Reprod Update 2014;20:688–701.
8. Lin W, Titus S, Moy F, Ginsburg ES, Oktay K. Ovarian aging in women with *BRCA* germline mutations. J Clin Endocrinol Metab 2017;102:3839–47.
9. Oktay K, Turan V, Titus S, Stobezki R, Liu L. *BRCA* mutations, DNA repair deficiency, and ovarian aging. Biol Reprod 2015;93:67.
10. Li F, Turan V, Lierman S, Cuvelier C, De Sutter P, Oktay K. Sphingosine-1-phosphate prevents chemotherapy-induced human primordial follicle death. Hum Reprod 2014;29:107–13.
11. Soleimani R, Heytens E, Darzynkiewicz Z, Oktay K. Mechanisms of chemotherapy-induced human ovarian aging: double strand DNA breaks and microvascular compromise. Aging (Albany NY) 2011;3:782–93.

12. Grynnerup AG, Lindhard A, Sorensen S. The role of anti-Mullerian hormone in female fertility and infertility—an overview. Acta Obstet Gynecol Scand 2012;91:1252–60.
13. Gougeon A. Human ovarian follicular development: from activation of resting follicles to preovulatory maturation. Ann Endocrinol (Paris) 2010; 71:132–43.
14. Depmann M, Faddy MJ, van der Schouw YT, Peeters PH, Broer SL, Kelsey TW, et al. The relationship between variation in size of the primordial follicle pool and age at natural menopause. J Clin Endocrinol Metab 2015; 100:E845–51.
15. Fortune JE. The early stages of follicular development: activation of primordial follicles and growth of preantral follicles. Anim Reprod Sci 2003;78: 135–63.
16. Monniaux D, Clement F, Dalbies-Tran R, Estienne A, Fabre S, Mansanet C, et al. The ovarian reserve of primordial follicles and the dynamic reserve of antral growing follicles: what is the link? Biol Reprod 2014;90:85.
17. Kuleshova L, Gianaroli L, Magli C, Ferraretti A, Trounson A. Birth following vitrification of a small number of human oocytes: case report. Hum Reprod 1999;14:3077–9.
18. Ferraretti AP, La Marca A, Fauser BC, Tarlatzis B, Nargund G, Gianaroli L, et al. ESHRE consensus on the definition of 'poor response' to ovarian stimulation for in vitro fertilization: the Bologna criteria. Hum Reprod 2011;26: 1616–24.
19. Poorvu PD, Frazier AL, Feraco AM, Manley PE, Ginsburg ES, Laufer MR, et al. Cancer treatment-related infertility: a critical review of the evidence. JNCI Cancer Spectr 2019;3:pkz008.
20. Lin WT, Beattie M, Chen LM, Oktay K, Crawford SL, Gold EB, et al. Comparison of age at natural menopause in *BRCA*1/2 mutation carriers with a non-clinic-based sample of women in northern California. Cancer 2013;119: 1652–9.
21. Finch A, Valentini A, Greenblatt E, Lynch HT, Ghadirian P, Armel S, et al. Frequency of premature menopause in women who carry a *BRCA*1 or *BRCA*2 mutation. Fertil Steril 2013;99:1724–8.
22. Rzepka-Gorska I, Tarnowski B, Chudecka-Glaz A, Gorski B, Zielinska D, Tolóczko-Grabarek A. Premature menopause in patients with *BRCA*1 gene mutation. Breast Cancer Res Treat 2006;100:59–63.
23. Kim J, Turan V, Oktay K. Long-term safety of letrozole and gonadotropin stimulation for fertility preservation in women with breast cancer. J Clin Endocrinol Metab 2016;101:1364–71.
24. Practice Committee of the American Society for Reproductive Medicine. Fertility preservation in patients undergoing gonadotoxic therapy or gonadectomy: a committee opinion. Fertil Steril 2019;112:1022–33.
25. Rodriguez-Wallberg KA, Oktay K. Fertility preservation and pregnancy in women with and without *BRCA* mutation-positive breast cancer. Oncologist 2012;17:1409–17.
26. Oktay K, Karlikaya G. Ovarian function after transplantation of frozen, banked autologous ovarian tissue. N Engl J Med 2000;342:1919.
27. Visser JA, Schipper I, Laven JS, Themmen AP. Anti-Mullerian hormone: an ovarian reserve marker in primary ovarian insufficiency. Nat Rev Endocrinol 2012;8:331–41.
28. Aydogan B, Mirkin S. The utility of measuring anti-Mullerian hormone in predicting menopause. Climacteric 2015;18:777–89.
29. Broer SL, Eijkemans MJ, Scheffer GJ, van Rooij IA, de Vet A, Themmen AP, et al. Anti-mullerian hormone predicts menopause: a long-term follow-up study in normoovulatory women. J Clin Endocrinol Metab 2011;96: 2532–9.
30. Anderson RA, Cameron DA. Pretreatment serum anti-mullerian hormone predicts long-term ovarian function and bone mass after chemotherapy for early breast cancer. J Clin Endocrinol Metab 2011;96:1336–43.
31. Anderson RA, Mansi J, Coleman RE, Adamson DJA, Leonard RCF. The utility of anti-Mullerian hormone in the diagnosis and prediction of loss of ovarian function following chemotherapy for early breast cancer. Eur J Cancer 2017; 87:58–64.
32. Chai J, Howie AF, Cameron DA, Anderson RA. A highly-sensitive anti-Mullerian hormone assay improves analysis of ovarian function following chemotherapy for early breast cancer. Eur J Cancer 2014; 50:2367–74.
33. Smith IE, Dowsett M, Yap YS, Walsh G, Lonning PE, Santen RJ, et al. Adjuvant aromatase inhibitors for early breast cancer after chemotherapy-induced amenorrhoea: caution and suggested guidelines. J Clin Oncol 2006;24:2444–7.

Pérdida de reserva ovárica inducida por la quimioterapia incrementada en mujeres con mutaciones de la línea germinal BRCA debido a deficiencia en la reparación del ácido desoxirribonucleico de doble cadena en el ovocito

Objetivo: Evaluar si las mujeres con mutaciones BRCA (WBM) experimentan una mayor disminución en la reserva ovárica después de tratamiento con quimioterapia, debido a que este provoca la muerte ovocitaria debido a daño del ácido desoxirribonucleico (DNA), y las mutaciones BRCA resultan en deficiencia en la reparación de daños en el DNA.

Diseño: Estudio longitudinal de cohorte.

Ubicación: Centros académicos.

Paciente(s): Las 108 mujeres con cáncer de mama evaluables fueron estratificadas en aquellas que nunca habían sido testadas para mutación BRCA patogénica (historia familiar negativa; (n=35) y aquellas negativas (n=59) o positivas (n=14).

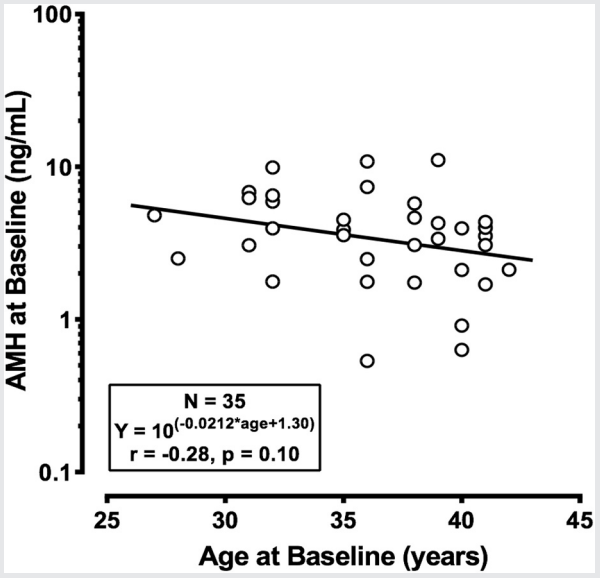
Intervención(es): Se obtuvo suero longitudinalmente antes y 12-24 meses después de tratamiento con quimioterapia, utilizado para medir hormona antimülleriana (AMH), y ajustado para la edad en el momento de la obtención de la muestra.

Principales Medidas de Resultados: Recuperación ovárica, definida como la media geométrica de los niveles de AMH ajustados a la edad después de la quimioterapia comparados con los basales.

Resultado(s): En comparación con los controles, los niveles de AMH antes del tratamiento de quimioterapia fueron 24% y 34% más bajos en aquellos negativos o positivos para mutaciones BRCA, consistente con el envejecimiento ovárico acelerado en WBM. El WBM tuvo una diferencia del triple en la recuperación de AMH después del tratamiento de quimioterapia (1.6%), en comparación con los controles BRCA negativos (3.7%) y no testados/ bajo riesgo (5.2%). Al limitar el análisis al régimen más común, doxorubicina y ciclofosfamida seguido de paclitaxel, se observaron resultados similares. Estos hallazgos se confirmaron mecánicamente en un bioensayo de eliminación de BRCA de ovocitos de ratón in vitro, que mostró que la deficiencia de BRCA resulta en un aumento de la susceptibilidad de los ovocitos a la doxorubicina.

Conclusión(es): Las mujeres que tienen mutaciones BRCA patogénicas tienen más probabilidades de perder reserva ovárica después del tratamiento de quimioterapia, sugiriendo un énfasis en la preservación de la fertilidad. Además, nuestros hallazgos generan la hipótesis de que la deficiencia de reparación del ADN es un mecanismo compartido entre envejecimiento, infertilidad y cáncer.

SUPPLEMENTAL FIGURE 1



Log-linear regression of adjusted baseline antiMüllerian hormone versus age, among the 35 referent women not tested for *BRCA* mutations who had a negative family history. The slope of the line is -0.0212 ($P = .07$) with an intercept of 1.30 and correlation of -0.28 ($P = .10$). The expected antiMüllerian hormone baseline value for a 25-year-old referent woman is 5.87 ng/mL; 2.82 ng/mL for a 40-year-old referent woman. AMH = antiMüllerian hormone.

Oktay. BRCA *and chemotherapy-induced ovarian damage. Fertil Steril 2020.*