

Antral Follicles Count and Anti-Müllerian Hormone Levels after Gonadotoxic Chemotherapy in Patients with Breast Cancer: Cohort Study

Contagem de folículos antrais e níveis de hormônio anti-Mülleriano após quimioterapia gonadotóxica em pacientes com câncer de mama: estudo de coorte

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Rev Bras Ginecol Obstet 2017;39:162–168.

Abstract

Aim To assess ovarian reserve (OVR) by means of follicle-stimulating hormone (FSH), anti-Müllerian hormone (AMH), and antral follicle count (AFC) measurement in eumenorrheic women with breast cancer, exposed to gonadotoxic chemotherapy.

Method Fifty-two women (35.3 ± 3.8 years old) with breast cancer and undergoing cyclophosphamide-containing chemotherapy were enrolled. The assessment was performed before chemotherapy (T1) and after 2 (T2) and 6 months (T3).

Results Six months after chemotherapy, the prevalence of regular cycles was 60%. Anti-Müllerian hormone decreased down to undetectable levels at T2 and T3 (T1: 2.53 [1.00–5.31]; T2 < 0.08; T3: < 0.08 [< 0.08 –1.07] ng/mL), ($p < 0.0001$). Antral follicle count was 11 [8.0–13.5] follicles at T1 and lower at T2 (5.50 [3.75–8.0]) and T3 (5.0 [2.5–7.0]) ($p < 0.0001$). In patients who remained with regular cycles during chemotherapy or resumed normal menses, FSH and estradiol levels remained unchanged.

Conclusion Anti-Müllerian hormone and AFC are useful as markers of OVR decline in women exposed to chemotherapy. Follicle-stimulating hormone is only adequate in women who become amenorrheic.

Keywords

- ▶ anti-Müllerian hormone
- ▶ antral follicle count
- ▶ ovarian reserve
- ▶ chemotherapy-induced amenorrhea
- ▶ anovulation

received
July 22, 2016
accepted after revision
January 16, 2017
published online
April 11, 2017

DOI <http://dx.doi.org/10.1055/s-0037-1601438>.
ISSN 0100-7203.

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Resumo

Objetivo Avaliar a reserva ovariana (OVR) através da contagem de folículos antrais (AFC), dosagem sérica de hormônio folículo estimulante (FSH) e hormônio anti-Mülleriano (AMH) em mulheres com câncer de mama submetidas a quimioterapia gonadotóxica.

Método Foram incluídas na pesquisa 52 mulheres ($35,3 \pm 3,8$ anos) com câncer de mama, em tratamento com quimioterapia com ciclofosfamida. As dosagens e medidas foram realizadas antes do início da quimioterapia (T1) e após 2 (T2) e 6 meses (T3).

Resultados Seis meses após quimioterapia, a prevalência de ciclos regulares foi de 60%. O AMH sérico diminuiu a níveis indetectáveis em T2 e T3 (T1: 2,53 [1,00–5,31]); T2 < 0,08; T3: < 0,08 [$< 0,08-1,07$] ng/mL) ($p < 0,0001$). A contagem de folículos antrais foi de 11 [8,0–13,5] folículos em T1, e ainda menor em T2 (5,50 [3,75–8,0]) e T3 (5,0 [2,5–7,0]), ($p < 0,0001$). Em pacientes que mantiveram ciclos regulares durante a quimioterapia ou retomaram a menstruação normalmente, os níveis de FSH e estradiol permaneceram inalterados.

Conclusão O AMH e a AFC são marcadores úteis do declínio da OVR em mulheres expostas à quimioterapia. O FSH só é adequado em mulheres que se tornam amenorreicas.

Palavras-chave

- ▶ hormônio anti-Mülleriano
- ▶ contagem de folículos antrais
- ▶ reserva ovariana
- ▶ amenorreia induzida por quimioterapia
- ▶ anovulação

Introduction

Ovarian reserve (OVR) is the measure used to assess the capacity of the ovary to produce oocytes. It can be inferred from serum levels of follicle-stimulating hormone (FSH), inhibin B, and anti-Müllerian hormone (AMH), as well as by the antral follicle count (AFC), which is the number of follicles 2–10 mm in size as assessed by transvaginal ultrasonography during the first phase of the cycle. Low OVR reflects ovarian “aging” and jeopardizes reproductive potential.^{1,2}

A systematic review of the literature assessing several markers of OVR in the assisted reproductive technology setting was published in 2006.² Levels of estradiol, the first marker assessed, were not shown to be effective predictors of OVR-related outcomes, even when combined with other markers.³ Follicle-stimulating hormone measurements are affected only when OVR is critically jeopardized.^{4,5} In a 2002 study of potential predictors of poor response to ovarian stimulation for in vitro fertilization (IVF) cycles, Bancsi et al³ showed that AFC alone provided the best prognostic value as compared with FSH, inhibin B, and estradiol.

More recently, AMH has been considered the best available marker of OVR, particularly as its levels do not vary over the course of the menstrual cycle, and become undetectable after menopause.^{6–9} Anti-Müllerian hormone is a dimeric glycoprotein produced by granulosa cells from the 3rd month of intrauterine life. Its production increases in puberty, and it is believed to exert autocrine and paracrine effects during follicle development. It modulates the primordial to primary follicle transition by inhibiting granulosa cell proliferation, aromatase activity, and luteinizing hormone (LH) receptor expression.^{7,10–13}

In the 1950s, investigators found that women exposed to chemotherapy (CTX) developed premature ovarian failure.¹⁴ This effect was later attributed to the gonadotoxicity of chemotherapeutic agents and found even in patients who

resumed regular menses after CTX.^{15,16} The main mechanism underlying CTX-induced anovulation, particularly in women treated with alkylating agents, are follicular depletion by apoptosis with loss of germ cells (oocytes), steroidogenic theca and granulosa cells.¹⁷

This study sought to assess OVR by means of AMH measurement in eumenorrheic women with breast cancer, exposed to gonadotoxic CTX. Other OVR markers (FSH and AFC) were compared with AMH in an attempt to ascertain which one is most sensitive for measurement of the decline in OVR after CTX.

Methods

Design

Cohort study.

Patients

The study sample comprised women with a diagnosis of breast cancer and indications for cyclophosphamide-containing CTX. From July of 2007 to November of 2009, 52 women aged 40 years or younger, with regular menses and no prior history of CTX (inclusion criteria), were recruited from six hospitals in Brazil. Previous ovarian surgery was an exclusion criterion. The study protocol (#07–061) was approved by the corresponding Research Ethics Committees and was conducted in accordance with the Brazilian guidelines and standards for human subject research.

Assessments were performed at baseline before the start of the CTX cycles (T1) and then at 2 (T2) and 6 months (T3) after completion of therapy. Each assessment consisted of an interview, blood sample collection for hormone measurement, and gynecologic ultrasonography for AFC quantification. The number of CTX cycles (4 or 6) and the dose of chemotherapeutic agent by total body surface area were also collected.

Anti-Müllerian Hormone Measurement

Blood samples were centrifuged at 3500 rpm for 15 minutes and the supernatant serum stored at -80°C for later analysis. Follicle-stimulating hormone and estradiol were measured by chemiluminescence performed with Siemens ADVIA CentaurXP Immunoassay System (Munich, Germany). Anti-Müllerian hormone was measured with a commercially available enzyme-linked immunosorbent assay (ELISA) kit made by Beckman Coulter - Immunosetech (Marseille, France), as described by Long et al.¹⁸

Ultrasonography

All sonograms were performed by the same examiner using the Sonoline Adara system by Siemens (Munich, Germany) and a 5 MHz transvaginal transducer. In view of the urgency of instituting CTX, ultrasonography was performed on any day of the menstrual cycle. Determination of the AFC took into account follicles with a mean diameter of 2–10 mm.^{19–21}

Statistics

The sample size required to detect a 1.4 ng/mL difference in AMH levels from baseline until 6 months after CTX, with a significance level of 0.05, and a statistical power of 90%, was 44 patients. Calculation was based on the findings of van Rooij et al.⁹

Data processing and analysis were performed in the SPSS 18 software (SPSS Inc., Chicago, IL, USA) environment. Data were asymmetrically distributed and are thus expressed as

medians. The following tests were used: Mann-Whitney U test and Kruskal-Wallis test for independent samples; Friedman test for related samples, and Wilcoxon test for comparisons. The significance level was set at $p > 0.05$.

Results

The mean age of the patients was 35.3 ± 3.8 years (range, 27–40 years). Ductal invasive carcinoma was the most common histologic tumor type (51/98%) and there was one case of Paget disease (1/2%). Most cases were treated by breast-conserving surgery with adjuvant (31/60%) or neoadjuvant (21/41%) CTX and 75% of the patients received local radiotherapy. The mean length of follow-up was 14 ± 3 months.

Over the course of the study, five patients were lost to follow-up (three were unable to keep appointments due to disease recurrence, one discontinued treatment, and one died). Complete follow-up was obtained from 49 patients in T2 and 47 in T3.

During CTX, 40% of the women developed irregular cycles (amenorrhea or oligomenorrhea); 2 months after completion of CTX (4 to 6 cycles of cyclophosphamide), 85% of the patients were irregular. At 6 months post-CTX, 60% of the patients remained with irregular menses or amenorrheic, whereas 40% had resumed normal menses.

► **Table 1** shows changes in the evaluated markers of OVR before and after CTX. Baseline assessment of OVR before CTX yielded values within the expected range for women of reproductive age (► **Table 2**).

Table 1 Markers of OVR before chemotherapy (T1), at 2 (T2) and at 6 months (T3) after completion of chemotherapy. Data expressed as median [interquartile range]. The limit of detection for AMH was 0.08 ng/mL

Marker	T1 <i>n</i> = 52	T2 <i>n</i> = 49	T3 <i>n</i> = 57
Estradiol (pg/mL)	79.42 (48.22–149)	27.55 (8.47–108.85)	95.50 (29.37–310.85)
FSH (IU/mL)	6.71 (3.87–8.64) ^{a,b}	47.67 (32.51–88.01) ^{a,c}	16.31 (7.41–41.16) ^{b,c}
AMH (ng/mL)	2.53 (1.0–5.31) ^{a,b}	< 0.08 ^{a,d}	< 0.08 (<0.08–1.07) ^b
AFC	11.0 (8–13.5) ^{a,b}	5.50 (3.75–8.0) ^a	5.0 (2.5–7.0) ^b

^aT1 versus T2; $p < 0.05$.

^bT1 versus T3; $p < 0.05$.

^cT2 versus T3; $p < 0.05$.

^dThere is no variation because all patients had values below the detection limit.

Abbreviations: AFC, antral follicle count; AMH, anti-Müllerian hormone; FSH, follicle-stimulating hormone; OVR, ovarian reserve.

Table 2 Markers of OVR 6 months after chemotherapy (T3), stratified by menstrual status. Data expressed as median [interquartile range]

	Eumenorrhea (40%)	Oligo/amenorrhea (60%)	<i>p</i>
Estradiol (pg/mL)	146.9 (70.1–515.6)	54.9 (9.87–302.9)	0.032
AMH (ng/mL)	1.46 (< 0.08–4.31)	< 0.08	< 0.0001
FSH (IU/mL)	7.24 (3.87–14)	34.91 (15.7–52.65)	< 0.0001
AFC	7.0 (5.5–10.0)	3.5 (2.0–6.0)	< 0.001

Abbreviations: AFC, antral follicle count; AMH, anti-Müllerian hormone; FSH, follicle-stimulating hormone; OVR, ovarian reserve.

There were no significant differences in estradiol levels at any of the time points of the assessment. Follicle-stimulating hormone was 6.71 [3.87–8.64] IU/mL at T1, significantly higher at T2 (47.67 [32.51–88.01] IU/mL), and significantly lower at T3 (16.31 [7.41–41.16] IU/mL), ($p < 0.001$). Anti-Müllerian hormone levels declined significantly between T1 (2.53 [1–5.31] ng/mL) and T2 (undetectable), with $p < 0.0001$. Six months after CTX (T3), AMH levels the same as in T2 ($p = 0.128$), even though some patients had resumed normal menses. The median AFC at T1 was 11 [8–13.5] follicles, significantly higher than at T2 and T3 ($p < 0.0001$). There were no significant differences between AFCs at T2 (5.5 [3.75–8]) and T3 (5 [2.5–7]) (► **Table 1**).

► **Table 2** shows the levels of OVR markers in eumenorrheic and oligo/amenorrheic patients (40 and 60% of the sample respectively) at 6 months post-CTX (T3). There were statistically significant differences in estradiol, FSH, AMH levels, as well as in AFCs. Median estradiol in the eumenorrhea group was 146.9 [70.1–515.6] pg/mL versus 54.9 [9.87–302.9] pg/mL in the oligo/amenorrheic group ($p = 0.032$). Median FSH in the eumenorrhea group was 7.24 [3.87–14] IU/mL versus 34.91 [15.71–52.65] IU/mL in the anovulatory group ($p < 0.0001$). Median AMH levels were 1.46 ng/mL (range, undetectable–4.31) in the eumenorrheic patients, whereas all oligo/amenorrheic women had levels below the threshold of detection ($p < 0.0001$). Antral follicle counts also differed between the groups, with a median of 7 [5.5–10] follicles in patients who had resumed normal menses versus 3.5 [2–6] in oligo/anovulatory patients ($p = 0.001$) (► **Table 2**).

Assessment of patients who remained eumenorrheic at T3 and comparison by pre-CTX OVR markers showed that AMH and AFC declined significantly, despite normal ovulation. In these patients, FSH and estradiol remained unchanged from baseline (► **Table 3**).

At T1, there were significant negative correlations between AMH and age ($r = -0.523$, $p < 0.0001$) and between AFC and age ($r = -0.469$, $p < 0.001$). Antral follicle count and AMH were positively correlated at T1 and T3 only (► **Fig. 1**).

Assessment of OVR markers was not influenced by the number of CTX cycles (4 or 6), nor by the dose of chemotherapeutic agent by total body surface area (mg/m^2), (data not shown). There was no difference in OVR markers between women who received adjuvant radiation therapy and those who did not.

Discussion

This study assessed the impact of chemotherapeutic agents on OVR in 52 young women with breast cancer. From a clinical standpoint, CTX exerts rapid and major impact on ovarian function, as 60% of the patients had become oligo/anovulatory by the end of the study. These findings are comparable to those of Stearns et al²², who reported amenorrhea as a common event in women exposed to CTX, although it is transient in 50% of cases. Goodwin et al²³ assessed women with breast cancer exposed to cyclophosphamide-containing CTX before the age of 40 and found that 40% of them became permanently amenorrheic.²⁴ Nevertheless, even patients who continue to have normal menses are not spared the negative effects of CTX on ovarian function.²⁵ In young patients, assessment of reproductive prognosis after cancer therapy required determination of OVR by reliable markers. In our study, higher AMH and AFC after CXT was associated with menstrual cyclicity. In a previous study, we have demonstrated that the age of 32 years presented 96% of sensitivity and 39% of specificity to predict amenorrhea or oligomenorrhea with receiver operating characteristic area under the curve (ROC AUC) of 0.77.²⁶

Partridge et al²⁷ showed that CTX-induced damage to ovarian function becomes dose-dependent only after the 6th treatment cycle; this corroborates our findings, in which no significant differences in OVR or menstrual pattern were observed after four or six CTX cycles with cyclophosphamide.

According to Petrek et al,²⁸ the time to resumption of menses in women with transient amenorrhea after CTX does not exceed 15 months. Therefore, assessment of OVR in our study, which was conducted on average 14 months after diagnosis and treatment of breast cancer, should accurately reflect OVR after CTX.

As shown elsewhere in the literature, estradiol was not a good marker of OVR.^{2,3} Follicle-stimulating hormone levels followed the change in the menstrual pattern of the patients at T2 and T3, but did not represent the actual decline in reproductive capacity, as oocyte depletion occurs even with normal menses. A reliable marker of OVR should exhibit changes before amenorrhea is established. As FSH reflects these changes too late, as reported by other authors, it is not an adequate marker of OVR.⁴

Anti-Müllerian hormone remained stable at T2 and T3 despite resumption of menstrual cycles in some patients

Table 3 Comparative analysis of OVR markers in eumenorrheic patients before chemotherapy (T1) and 6 months after completion of chemotherapy (T3). Data expressed as median [interquartile range]

	T1	T3	<i>p</i>
FSH (IU/mL)	5.03 (2.88–7.3)	7.24 (3.87–14)	0.125
Estradiol (pg/mL)	123.15 (49.55–185.12)	146.9 (70.1–515.62)	0.96
AFC	13 (11–15.5)	7 (5.5–10)	0.001
AMH (ng/mL)	6.17 (3.19–10.07)	1.46 (< 0.08–4.31)	0.001

Abbreviations: AFC, antral follicle count; AMH, anti-Müllerian hormone; FSH, follicle-stimulating hormone; OVR, ovarian reserve.

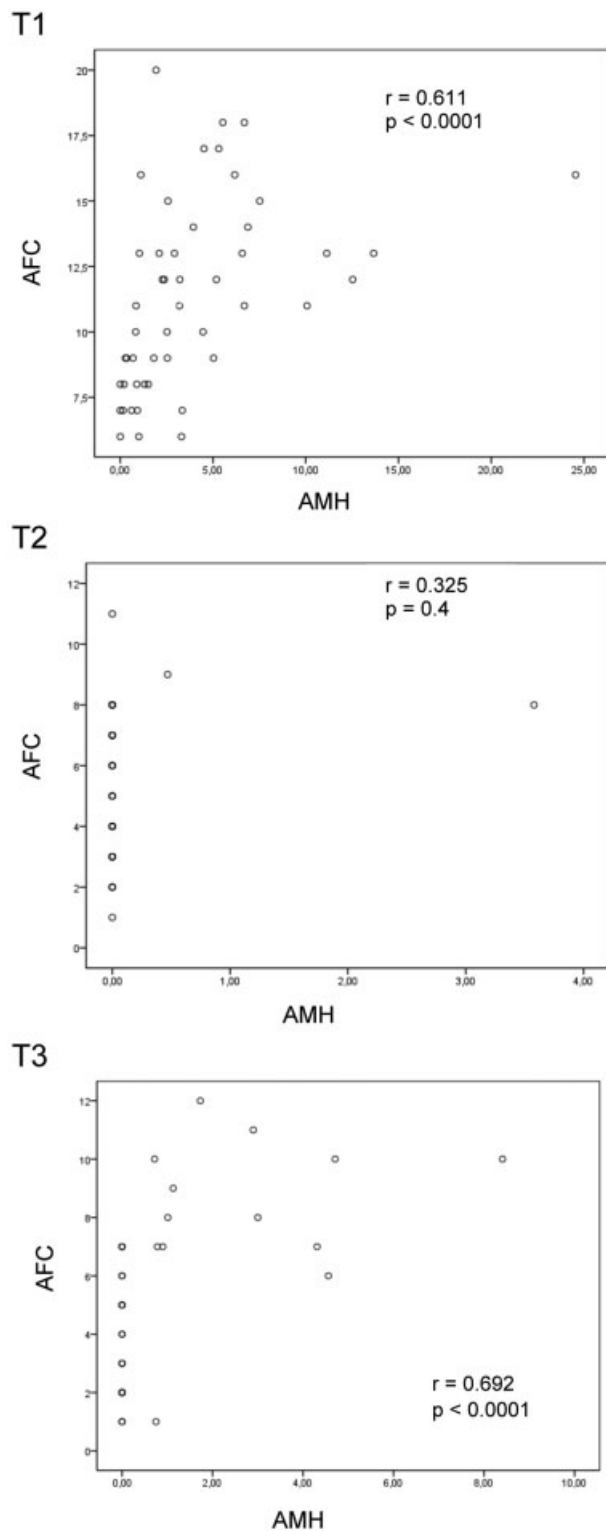


Fig. 1 Correlation between AFC and AMH levels at T1, T2, and T3. Abbreviations: AFC, antral follicle count; AMH, anti-Müllerian hormone.

who were oligo/amenorrheic at T2, which demonstrates that changes in AMH reflect the true impact of CTX on OVR. As the AMH is secreted by developing follicles, their depletion after exposure to chemotherapeutic agents leads to a decline in

levels of the hormone, even in eumenorrheic patients and, according to some authors,^{29,30} it seems to be of value in assessing ovarian function and advising patients before³¹ and after cancer treatment.

In 2006, Anderson et al³² published the 1-year results of a cohort of 50 women with breast cancer treated with CTX. Anti-Müllerian hormone was the earliest marker of chemotherapy-induced follicular depletion. Lie Fong et al³³ compared AMH levels in a cohort of 185 women with regular menses who had been exposed to CTX before the age of 18 (at least 5 years before the study) to those of 42 control subjects, and found that survivors with lower levels of AMH were under greater risk of irregular menses and ovarian failure.

Antral follicle counts decrease after CTX and, despite resumption of menstrual cycles in some patients at T3, counts remained unchanged as compared with T2. These findings suggest that AFC and AMH reflect the gonadotoxicity of chemotherapeutic agents on OVR regardless of menses. These markers demonstrate substantial changes even before clinically apparent menstrual pattern alteration and hence, they can be used to identify significant OVR reduction (whether induced by a gonadotoxic insult or by physiological changes, such as age) in women that are still ovulatory.

Although CTX damages the ovarian stroma and affects oocytes at any stage, it appears to be particularly deleterious to primary follicles and spare resting follicles, which explains the transient anovulation exhibited by some patients at T2.³⁴ Patients with a higher primordial follicle count (usually younger ones or those with a better pre-CTX ovarian reserve) still preserve a significant number of these follicles, which, upon development as part of the normal folliculogenesis process, lead to resumption of regular menses at T3. We believe the correlation between AMH and AFC is probably lost at T2 due to a decline in the number of AMH-producing follicles, which is expected to resume at T3, once the primordial follicles that remained unaffected at T2 reach the antral stage and start producing AMH.

As expected, on comparison between anovulatory (amenorrheic or oligomenorrheic) and eumenorrheic patients at T3, all OVR-related parameters showed significant differences. However, this finding does not appear relevant to determination of the optimal marker of OVR because clinical changes were already present.

Comparison of the AMH levels and AFCs of eumenorrheic patients at T3 with their T1 levels clearly showed the sensitivity of these parameters as markers of OVR, as both were decreased despite resumption of normal menses. Similar findings were reported by de Vet et al³⁵, who assessed ovarian aging over time among eumenorrheic patients not exposed to gonadotoxic agents. The authors found that in women whose cycles remained regular, only AMH decreased significantly over time, unlike the other markers assessed.³⁵ In a similar study, van Rooij et al^{9,36} concluded that AMH at any age and AFC, FSH and inhibin B in older women, accurately reflect changes in OVR among eumenorrheic women at 4-year follow-up.³⁷

Markers that allow early identification of a decline in OVR before clinical changes in the menstrual cycle become

apparent could help to determine reproductive prognosis.^{38,39} This study shows that, in cases of overt decline in ovarian function induced by a proven gonadotoxic agent, AMH and AFC are efficient markers of OVR. Anti-Müllerian hormone is readily measured in serum, not investigator-dependent, and provides more reproducible results, whereas AFC can be assessed immediately on ultrasound by a gynecologist or sonographer.

Declaration of Interest Section

The authors report no conflict of interest.

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