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The effects of chemotherapy and long-term gonadotrophin suppression on the ovarian reserve in premenopausal women with breast cancer

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BACKGROUND Reproductive function following cancer treatment is of increasing importance with improving survival rates. We therefore assessed the markers of the ovarian reserve in premenopausal women, to investigate and compare the effects of chemotherapy and long-term gonadotrophin withdrawal on ovarian function. METHODS Fifty premenopausal (age range 28–52 years) women with early breast cancer were recruited. Serum hormone and ovarian ultrasound measurements were taken before treatment and at intervals up to 1 year during chemotherapy or gonadotrophin suppressive therapy. RESULTS Pretreatment samples indicated a fall in anti-Müllerian hormone (AMH) concentration with age before changes in other hormone concentrations. AMH concentration showed a rapid and marked fall during chemotherapy, with undetectable concentrations in many women ($P < 0.0001$). Inhibin B concentration showed a lesser fall ($P < 0.0001$), whereas estradiol (E_2) concentrations were maintained. Both antral follicle count (AFC) and ovarian volume fell ($P < 0.0001$ and $P < 0.05$ respectively). Regimens containing taxanes in addition to cyclophosphamide showed increased gonadotoxicity. Gonadotrophin suppression resulted in expected falls in E_2 ($P < 0.05$) and inhibin B ($P < 0.001$) levels, but also resulted in a delayed fall in AMH level after 6 months ($P < 0.0001$). CONCLUSIONS These data confirm the value of AMH concentration as an early indicator of ovarian ageing including assessment of chemotherapy-induced ovarian follicle loss. FSH and AMH concentration measurements may be useful for the comparison of ovarian toxicity of different chemotherapy regimens.

Key words: anti-Müllerian hormone/chemotherapy/gonadotrophin/ovary

Introduction

Ovarian failure is a common sequela of the treatment of malignant disease in women (Reichman and Green, 1994; Bines *et al*., 1996; Meirow and Nugent, 2001). Increasing survival rates have raised the importance of the long-term consequences of treatment, and new strategies are also being developed to circumvent the adverse effect of chemo- and radiotherapy on ovarian function (Sonmezer and Oktay, 2004; Wallace *et al*., 2005). These concerns all require detailed understanding of the effects of these therapies on the ovary, but there have not been detailed prospective studies.

Chemotherapy causes depletion of the primordial follicle pool in a drug- and dose-dependent manner (Himelstein-Braw *et al*., 1978; Chapman *et al*., 1979; Sonmezer and Oktay, 2004). Analyses of ovarian function following cancer therapy have mostly described the prevalence of ovarian failure following treatment, although follicular depletion may occur despite maintenance of regular menstrual cycles (Bath *et al*., 2003; Larsen *et al*., 2003). Chemotherapy can therefore be considered to cause a form of accelerated ovarian ageing. The number of growing follicles is related to the number of primordial follicles (Gougeon *et al*., 1994), but the latter cannot be quantified directly *in vivo*. Hormonal and ultrasound-based surrogates are therefore used, including FSH, inhibin B and ovarian volume and antral follicle count (AFC) (Seifer *et al*., 1997; Syrop *et al*., 1999; Creus *et al*., 2000; Bancsi *et al*., 2002; Scheffer *et al*., 2003; Yong *et al*., 2003). Anti-Müllerian hormone (AMH, or Müllerian-inhibiting substance, MIS) is expressed by granulosa cells of follicles on initiation of growth until the early antral stages (Baarends *et al*., 1995; Durlinger *et al*., 2002b; Weenen *et al*., 2004), thus potentially more closely reflecting the follicular reserve than other hormones. AMH concentrations in the circulation decline with age (de Vet *et al*., 2002; van Rooij *et al*., 2005) and show good prediction of the ovarian response to FSH (van Rooij *et al*., 2002; Fanchin *et al*., 2003). We have demonstrated reduced AMH concentrations, but not reduced inhibin B concentrations, in young women treated for cancer during childhood in whom ovarian function is maintained (Bath *et al*., 2003).

Women being treated for cancers provide an opportunity for the investigation of ovarian function during treatments that would not be possible in volunteer studies. In the case of breast cancer, this includes prolonged gonadotrophin suppression (Robertson and Blamey, 2003) and gonadotoxic chemotherapy. The gonadotrophins are without doubt essential regulators of the later stages of folliculogenesis (Gougeon, 1996), but understanding of early folliculogenesis is more limited. Prolonged gonadotrophin suppression for the treatment of premenopausal breast cancer provides an opportunity to investigate this.

The main objectives of this prospective study were (i) to investigate relationships between ovarian hormones in a population of women at ages leading up to the menopause, (ii) to investigate and contrast the effects of chemotherapy with those of prolonged gonadotrophin suppression on ovarian function and (iii) to compare different chemotherapy regimens, using current markers of the ovarian reserve.

Subjects and methods

This was a prospective observational study of ovarian function in premenopausal women with newly diagnosed breast cancer. Inclusion criteria included operable breast cancer with no evidence of metastases and either regular spontaneous menstruation or normal gonadotrophin concentrations if recently discontinued from oral contraception.

A total of 56 women were recruited to the study and gave informed consent in writing. Patients attended for venesection in the early follicular phase (days 2–5) of the menstrual cycle before any treatment for their breast cancer. Additionally, 34 of these women consented to transvaginal ultrasound examination of the ovaries (7 Mhz probe; Toshiba Eccocee, Stirling, UK) to determine ovarian volume and AFC (follicles 2–10 mm diameter), performed by a single observer at the same time as venesection. Data were calculated as the mean for the two ovaries, with ovarian volume calculated using the volume for a prolate ellipsoid. At all examinations, the absence of a dominant follicle was confirmed.

Patients reattended 3, 6, 9 and 12 months after starting chemotherapy or hormonal treatment for their breast cancer. Return visits were scheduled to be in the early follicular phase if patients continued to have menstrual cycles, and patients kept a menstrual diary throughout. Menstrual data were subsequently coded as regular (all cycles between 23 and 35 days), irregular if outwith that range, and amenorrhoea when there was no further bleeding over the subsequent 6 months.

LH and FSH levels were measured by time-resolved immunofluorometric assay (Brady *et al*., 2004), sensitivity was 0.03 IU/l for FSH and 0.15 IU/l for LH, and both intra-assay and inter-assay coefficients of variation were less than 10%. Inhibin B level was measured by methods previously described (Groome *et al*., 1996) with sensitivity 7.8 pg/ml. AMH level was measured using a newly developed AMH immunoassay (Al-Qahtani *et al*., 2005). Sensitivity was 0.078 ng/ml, and the mean inter- and intra-assay coefficients of variation were 4.0 and 3.6%, respectively. Estradiol was measured by established in-house RIA with coefficients of variation <8%.

Treatment regimens

Nineteen women were enrolled in multicentre national adjuvant chemotherapy trials (17 in trail of accelerated chemotherapy (TACT) and 2 in paclitaxel, anthracycline, gemcitabine, cycophosphamide (TANGO)), and the remaining 23 women received chemotherapy outwith a trial. Choice of systemic therapy was made on clinical grounds independent of recruitment to this study. In addition to surgery, treatment protocols are based on risk of recurrence and potential endocrine sensitivity, such that higher risk women were offered chemotherapy. Ovarian suppression was requested by some women following chemotherapy and offered to women with moderate risk disease for whom chemotherapy was either inappropriate or declined. Details of treatments are given in Table I. Four women received chemotherapy pre-operatively and 38 post-operatively 3–4 weeks later. Twenty women were enrolled in multicentre national adjuvant chemotherapy trials (18 in TACT and two in TANGO), and the remaining 22 women received chemotherapy outwith a trial. Chemotherapy regimens were generally of 6 months duration, following which most women received endocrine therapy.

Of the 14 women who did not receive chemotherapy, nine were treated with goserelin (3.6 mg s.c. at 4 week intervals; Zoladex; AstraZeneca, Luton, Beds, UK) for at least 1 year. Eight of these also received tamoxifen, with one woman treated with goserelin plus the aromatase inhibitor anastrozole (Arimidex; AstraZeneca). A further five women were treated with tamoxifen only. For the purposes of this study, the eight women treated with goserelin plus tamoxifen were analysed together (gonadotrophin suppression group) for comparison with 42 women receiving adjuvant chemotherapy as their initial treatment.

Statistical analysis

Changes in hormone concentration with time were analysed by analysis of variance (ANOVA) for repeated measures with Bonferroni post-hoc testing when appropriate, to investigate at what time points significant treatment effects were evident. ANCOVA was used to compare hormone concentrations after 6 months chemotherapy to investigate differences between treatment groups with age as covariate and Bonferroni post-hoc testing when appropriate, and two-way ANOVA was used to compare hormone concentrations in the chemotherapy group with those in the suppression group. Fisher's exact test was used to compare the prevalence of amenorrhoea. For all comparisons, $P < 0.05$ was considered significant.

Table I. Details of chemotherapy regimens

Regimen	Component drugs	Number of women	Duration (weeks)	Cyclophosphamide regimen	Cycles of taxane
Non-trial					
AC (pre-operative)	Adriamycin + cyclophosphamide	3	18	3 600 mg/m^2 over 18 weeks	0
CMF	Cyclophosphamide + methotrexate + 5-fluorouracil	3	18	4 500 mg/m^2 over 18 weeks	0
A-CMF (Bonadonna)	Adriamycin followed by CMF	7	36	4 800 mg/m^2 over 24 weeks	0
E-CMF	Epirubicin followed by CMF	10	24	3 000 mg/m^2 over 12 weeks	0
Trial					
E-CMF (TACT)	Epirubicin followed by CMF	8	28	4 800 mg/m^2 over 16 weeks	0
FEC-T (TACT)	5-Fluorouracil + epirubicin + cyclophosphamide followed by docetaxel	8	24	2 400 mg/m^2 over 12 weeks	4
TANGO	Epirubicin + cyclophosphamide followed by paclitaxel ± gemcitabine	2	24	2 400 mg/m^2 over 12 weeks	4

Results

Pretreatment

Median age at recruitment was 41 years (age range 28.6–52.7 years). Analysis of pretreatment hormone and ultrasound measurement showed significant negative correlations between age and serum AMH (r = –0.45, P = 0.004) and estradiol (E_2) (r = –0.35, P = 0.016) levels. There were positive correlations between age and FSH (r = 0.41, P = 0.002) and LH (r = 0.37, P = 0.005) levels, but there was no significant correlation between age and inhibin B (r = –0.092) level. Ovarian volume and AFC were closely correlated (r = 0.47, P = 0.004), but neither showed a significant relationship with age.

To explore changes in hormone concentrations with age, we grouped subjects into 5-year cohorts (Figure 1). These data show marked differences in the pattern of change of the various hormones. AMH and FSH levels showed significant differences between age cohorts (ANOVA: AMH P = 0.02; FSH P = 0.03), whereas inhibin B and E_2 levels did not. AMH concentrations declined early, with significant differences only between the ≤35-year-old and older cohorts. FSH concentrations showed the opposite pattern, with the oldest group having significantly higher concentrations than the three youngest groups. Multiple regression analysis between FSH and the three ovarian hormones demonstrated a highly significant negative correlation with inhibin B (r = –0.37, P = 0.005) but no relationship with AMH or E_2.

Pretreatment characteristics of the groups receiving chemotherapy or gonadotrophin suppression are summarized in Table II. The chemotherapy group patients were an average of 4 years younger, but this was not statistically significant. AMH concentrations tended to be higher in the chemotherapy group, although none of the hormonal measurements were significantly different between groups.

Chemotherapy group

Chemotherapy regimens were generally of 6–9 months' duration, following which hormonal treatment was introduced in all but

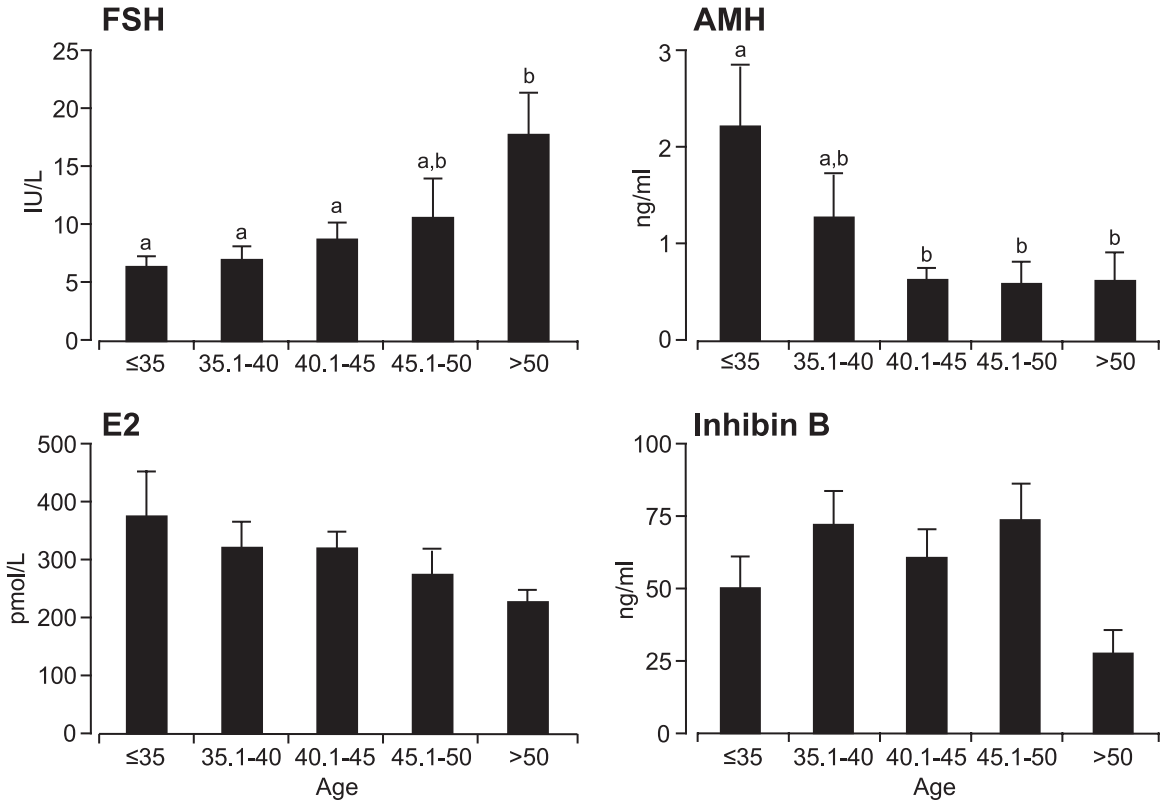

Figure 1. Serum concentrations of FSH, anti-Müllerian hormone (AMH), estradiol (E_2) and inhibin B before treatment, grouped by age cohort. Mean ± SEM, n = 7, 12, 18, 11 and 8 in the ≤35, 35.1–40, 40.1–45, 45.1–50 and >50-year-old cohorts, respectively. Different superscripts indicate significant (P < 0.05) differences between groups.

Table II. Pretreatment characteristics of women in the gonadotrophin suppression and chemotherapy groups

	n	Age (years)	FSH (IU/l)	E_2 (pmol/l)	Inhibin B (ng/ml)	AMH (ng/ml)	Ovarian volume	Antral follicle count
Gonadotrophin suppression	8	45.3 ± 2.1	6.3 ± 0.6	335 ± 57	64.7 ± 13.8	0.51 ± 0.14	3.0 ± 0.7	3.4 ± 0.5
Chemotherapy	42	41.3 ± 0.9	9.7 ± 1.2	297 ± 24	58.2 ± 6.5	1.11 ± 0.20	3.1 ± 0.4	5.8 ± 0.6

E_2, estradiol; AMH, anti-Müllerian hormone. Mean ± SEM. There were no significant differences between groups in any of these variables.

seven of the 42 women in this group. This consisted of tamoxifen in 34 women, combined with goserelin in eight. A further two were treated with anastrozole, given in combination with goserelin in one woman. Thus, for most women only the initial 6 months show the effect of the chemotherapy alone, and analysis was therefore primarily confined to this treatment period.

There was a marked increase in FSH and LH (both $P < 0.0001$) concentrations within 3 months of starting chemotherapy treatment, which was sustained at 6 months (Figure 2). There was no significant change in E_2 concentrations over the first 6 months of treatment; however, the median concentration fell from 319 to 144 pmol/l, with 13 women having E_2 concentrations below 100 pmol/l at 6 months compared with only one before treatment.

Inhibin B and AMH concentrations showed more consistent and rapid falls during chemotherapy. Inhibin B concentration fell to approximately 50% of pretreatment concentration by 3 months ($P < 0.0001$) and became undetectable in 20/42 subjects at 6 months. AMH concentration also showed a rapid fall ($P < 0.0001$), with concentrations close to the limit of detection in many women.

Only one woman (of the 42 in the group) maintained regular menses during the first 6 months of treatment. All other women in the chemotherapy group had irregular menstrual bleeding at least initially during this time, with 23 (55%) becoming amenorrhoeic by 6 months. Women who became amenorrhoeic were older (44.4 ± 0.9 versus 36.7 ± 1.2 years, $P < 0.0001$) and, before treatment, had lower AMH concentrations (0.58 ± 0.14 versus 1.9 ± 0.4 ng/ml, $P = 0.0007$) and higher FSH concentrations (12.9 ± 1.7 versus 5.2 ± 0.5 IU/l, $P = 0.0007$) than those who maintained menses but had similar E_2 (265 ± 21 versus 350 ± 49 pmol/l, $P = 0.07$) and inhibin B (51 ± 8 versus 68 ± 10 ng/ml) concentrations.

Comparison of treatment regimens

As chemotherapy treatment regimens generally lasted approximately 6 months, hormone data were compared between treatment groups at that time point. There were four main treatment groups (Table I): FEC-T (TACT), E-CMF (TACT), E-CMF and A-CMF (Bonadonna). Of these, only FEC-T (TACT) includes a taxane, docetaxel. Other treatment regimens were only represented by ≤3 patients each and hence were not included in this analysis. There were no significant differences in age between the four main treatment groups, nor in FSH, AMH or E_2 concentrations before treatment (Table III), but nevertheless age was included as a covariate in the statistical analysis. Inhibin B concentrations showed a pretreatment difference ($P = 0.01$), with the E-CMF group having lower concentrations than the others, but there were no other differences between groups. ANCOVA demonstrated significant treatment group effects for FSH ($P = 0.0001$), AMH ($P = 0.05$) and E_2 ($P = 0.002$, Figure 3). The FEC-T (TACT) group showed highest FSH and lowest E_2 and AMH concentrations after treatment. The FEC-T (TACT) group was the only one which showed a fall in E_2 concentration, to 77 ± 11 pmol/l at 6 months. The E-CMF (TACT) group showed

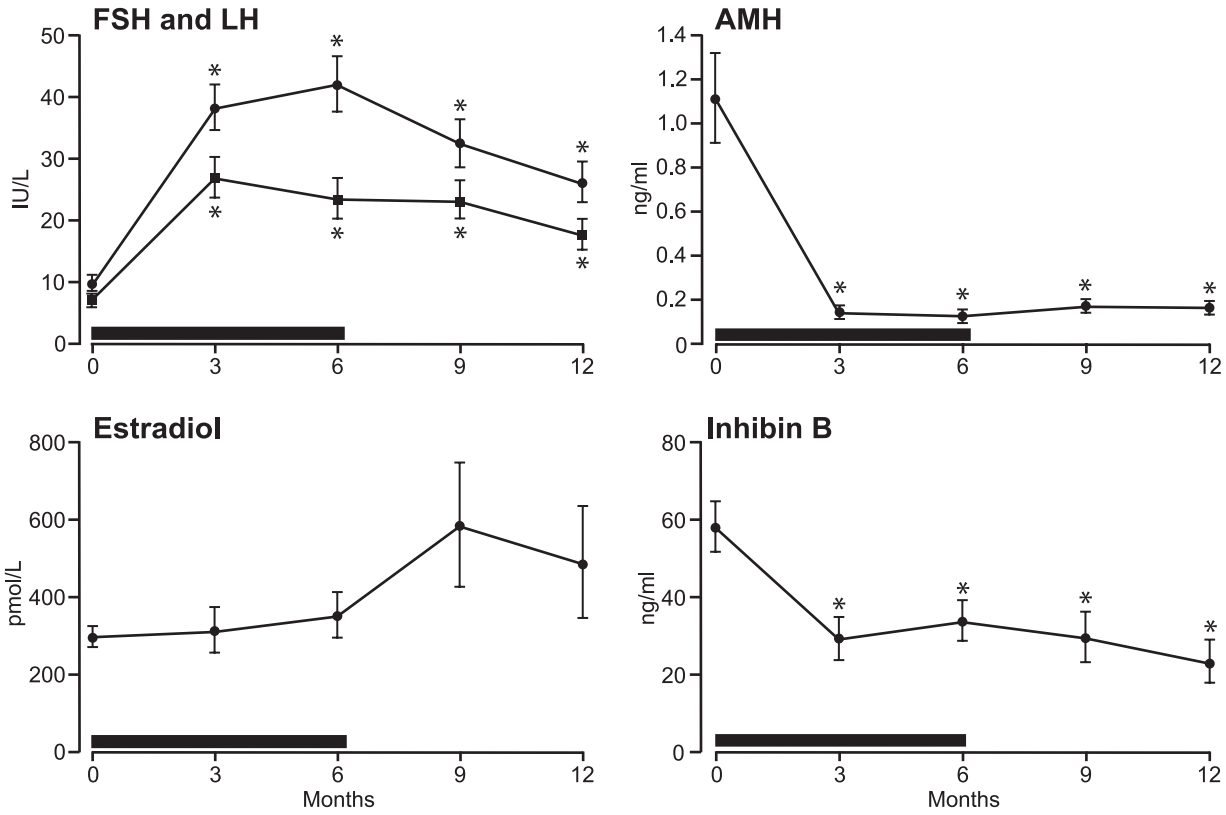

Figure 2. Serum concentrations of LH (squares) and FSH (circles), AMH, E_2 and inhibin B during 12 months of treatment in the chemotherapy group. Mean ± SEM, $n = 42$. Asterisk (*) indicates significant difference ($P < 0.05$) from pretreatment. Note that chemotherapy treatments lasted 6 months in most women, as indicated by the shaded bar.

Table III. Pretreatment hormone concentrations in women subsequently treated with the main chemotherapy regimens

Regimen	Age (years)	FSH (IU/l)	AMH (ng/ml)	E_2 (pmol/l)	Inhibin B (ng/ml)
FEC-T (TACT)	44.5 ± 2.5	14.8 ± 4.0	1.13 ± 0.63	372 ± 63	58 ± 12
E-CMF (TACT)	40.1 ± 1.0	6.2 ± 0.8	1.09 ± 0.37	384 ± 32	65 ± 15
E-CMF	42.5 ± 1.8	14.6 ± 3.7	0.69 ± 0.19	377 ± 84	26 ± 10[a]
A-CMF	39.3 ± 2.2	6.5 ± 0.5	1.43 ± 0.69	292 ± 29	89 ± 14

Mean ± SEM. FECT-T (TACT) $n = 9$, E-CMF (TACT) $n = 8$, E-CMF $n = 10$, A-CMF $n = 7$.
[a]Inhibin B concentrations were significantly lower in the E-CMF group than in each of the other three groups ($P = 0.01$).

similarly low AMH concentrations as the FEC-T (TACT) group (i.e. at the limit of detection in all women) but was similar to the other two groups in FSH and E_2 concentrations. Mean inhibin B concentrations in the four groups showed the reciprocal order to that for FSH concentrations, and there was a highly significant inverse correlation between FSH and inhibin B concentrations ($P = 0.001$). ANCOVA of inhibin B concentrations by treatment group did not reach statistical significance, but there was a significant linear trend between treatment groups ($P = 0.02$), and pairwise comparison showed a significant difference between the FEC-T (TACT) and A-CMF (Bonadonna) groups ($P = 0.019$).

These hormonal differences were also reflected in the proportions of women who became amenorrhoeic within 6 months: eight of nine women in the FEC-T (TACT) group, compared with four of eight in the E-CMF (TACT) group, five of 10 in the E-CMF group and two of seven in the A-CMF (Bonadonna) group [FEC-T (TACT) versus other groups: $P = 0.047$].

Further evidence for additional gonadotoxicity of taxane administration was provided by analysis of whether those women treated with a taxane [FEC-T (TACT) and TANGO groups] showed higher FSH concentrations at 6 months (57.3 ± 5.7 versus 36.5 ± 5.1, $P = 0.03$, $n = 11$ versus 33). This difference remained when cyclosphosphamide dose was included as a covariate and was more prominent in women aged ≤ 40 years: 48.7 ± 11.5 versus 17.7 ± 5.3 ($P = 0.01$, $n = 5$, taxane treated; $n = 16$, no taxane), whereas there was no difference in women aged >40 years, and the difference in women ≤40 years remained when cyclophosphamide dose was included as a covariate. In the ≤40 years group, pretreatment FSH concentrations and age did not differ between those who were or were not treated with a taxane (FSH 5.4 ± 1.3 versus 7.1 ± 1.0; age 36.0 ± 2.7 versus 36.6 ± 0.8, respectively).

Gonadotrophin suppression group

In this group of women who did not receive any chemotherapy, goserelin (usually plus tamoxifen) administration resulted in the expected rapid and sustained declines in LH, FSH, E_2 and inhibin B concentrations (Figure 4). E_2 concentrations showed a similar marked fall ($P = 0.04$), from 335 ± 57 to 65 ± 14 pmol/l at 6 months, and inhibin B concentrations fell from 64.7 ± 13.8 to 20.2 ± 5.6 ng/ml ($P < 0.001$). AMH concentrations also

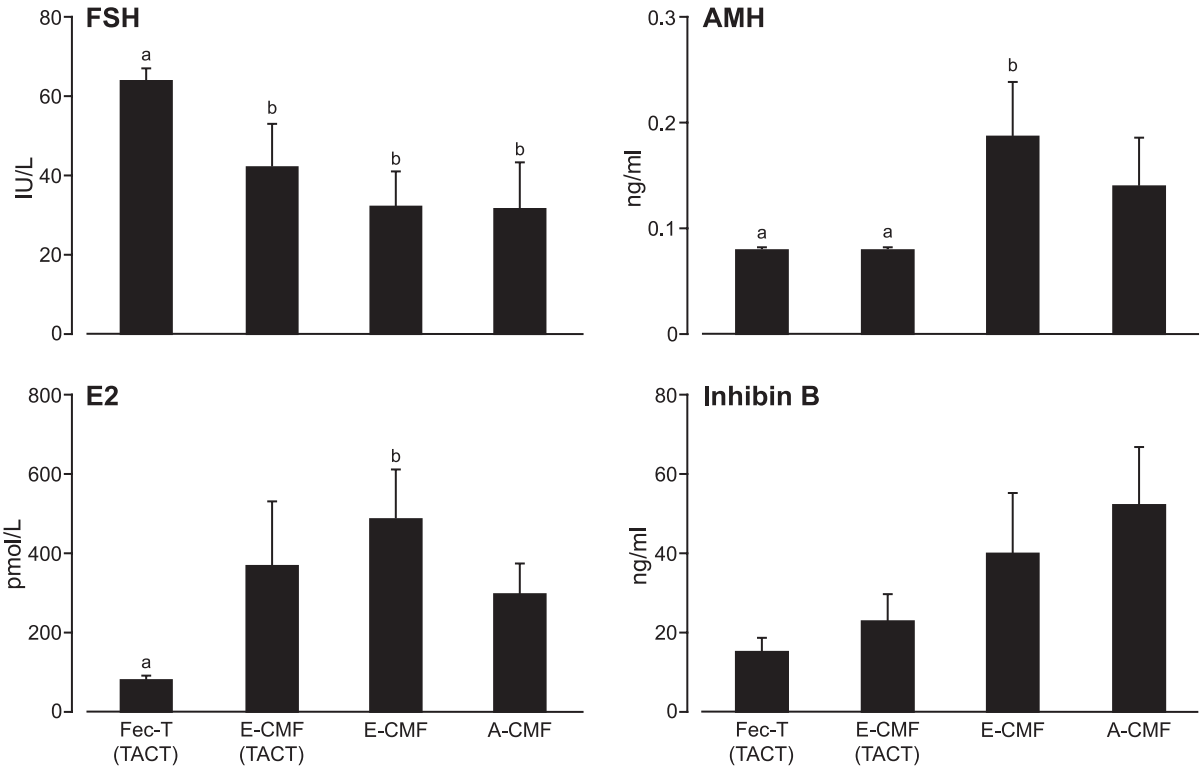

Figure 3. Serum concentrations of FSH, AMH, E_2 and inhibin B in women in the chemotherapy group after 6 months treatment, by treatment regimen. Mean ± SEM. Groups sizes: $n = 9$, FEC-T (TACT); $n = 8$, E-CMF (TACT); $n = 10$, E-CMF; $n = 7$, A-CMF (Bonadonna). Different superscripts indicate significant ($P < 0.01$) differences between groups. FEC-T (TACT): 5-fluorouracil + epirubicin + cyclophosphamide followed by docetaxel; E-CMF (TACT): trial group of epirubicin followed by CMF (CMF = cyclophosphamide + methotrexate + 5-fluorouracil); E-CMF: non-trial epirubicin followed by CMF; A-CMF: adriamycin + cyclophosphamide.

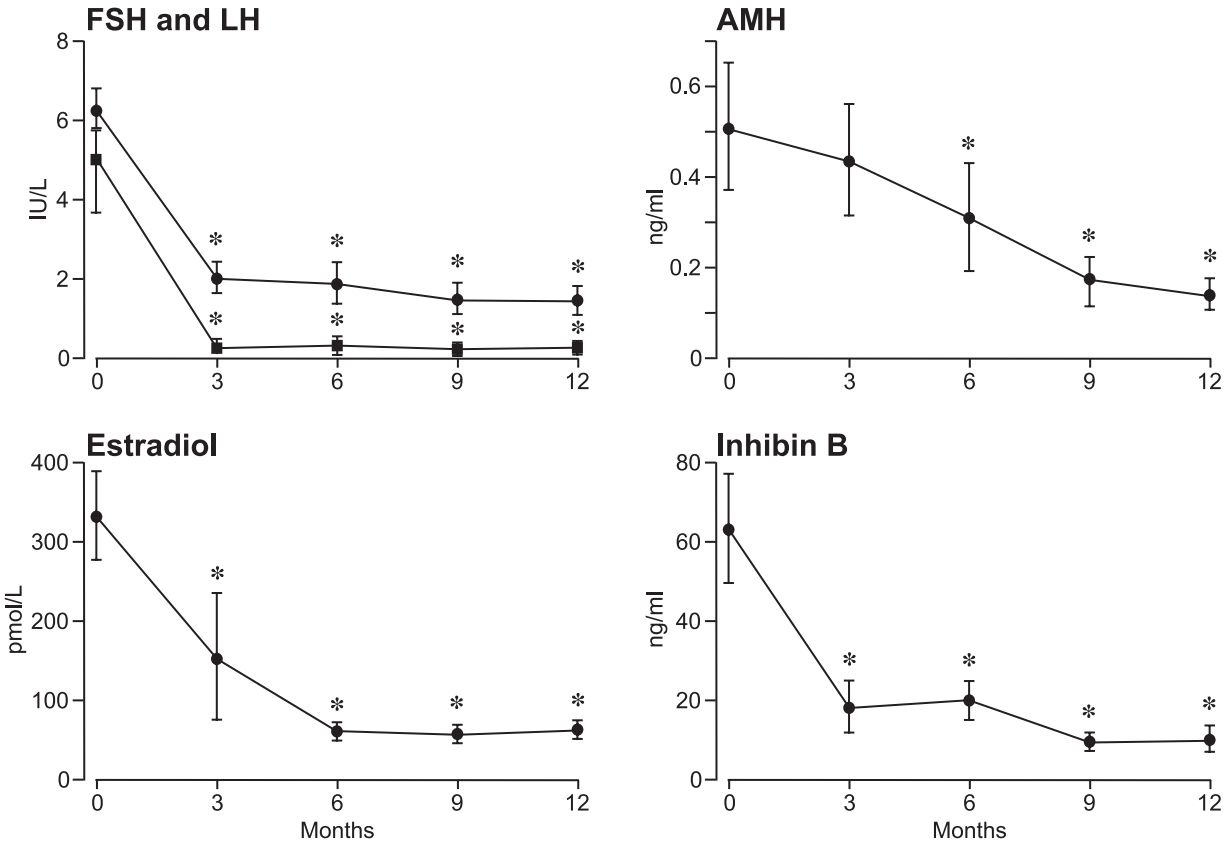

Figure 4. Serum concentrations of LH (squares) and FSH (circles), AMH, E_2 and inhibin B in the gonadotrophin suppression group. Mean ± SEM, $n = 8$; '*' indicates significant difference ($P < 0.05$) from pretreatment.

declined in all women during treatment ($P < 0.0001$) but showed a very different pattern. AMH concentrations were not significantly different from pretreatment levels at 3 months, but thereafter a gradual decline was observed. This reached statistical significance at 6 months (0.51 ± 0.14 to 0.34 ± 0.13 ng/ml, $P = 0.02$) and fell further to 0.15 ± 0.04 ng/ml at 12 months.

The patterns of change in E_2 and AMH concentrations, but not in inhibin B concentration, therefore differed markedly between the chemotherapy and gonadotrophin suppression groups. ANOVA demonstrated significant differences in hormone concentrations between the chemotherapy and suppression groups for AMH ($P = 0.02$) and E_2 ($P = 0.03$) concentrations but not inhibin B concentration. This is illustrated in Figure 5, where mean data for the two groups are expressed as percentages of pretreatment concentrations.

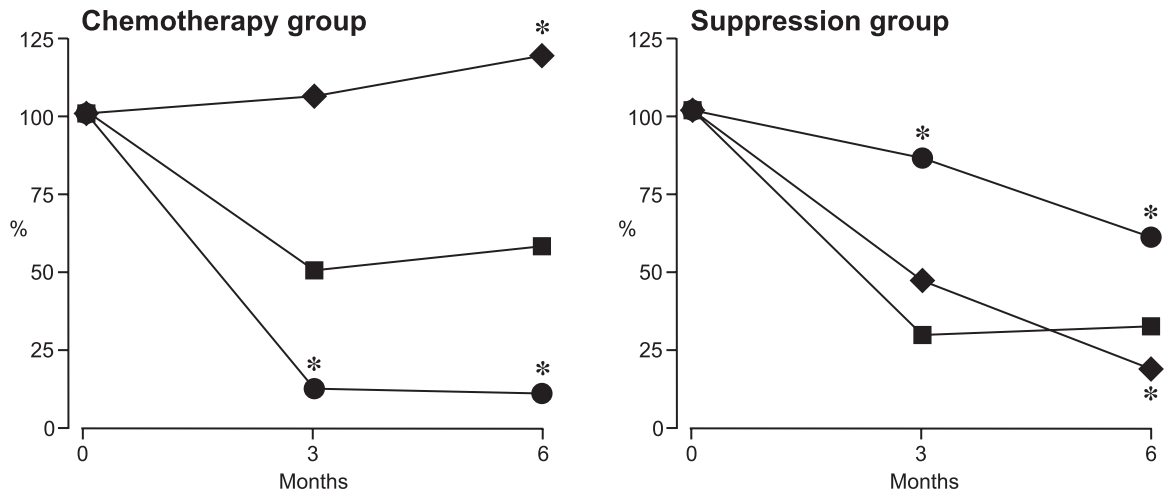

Figure 5. Serum hormone concentrations in the chemotherapy and gonadotrophin suppression groups, expressed as percentage of pretreatment concentrations. E_2, diamonds; inhibin B, squares; AMH, circles; $n = 42$ and $n = 8$, chemotherapy and gonadotrophin suppression groups, respectively. *$P < 0.01$ comparison of the two treatment groups by analysis of variance of hormone data before transformation.

AFC and ovarian volume

AFC concentrations fell in the chemotherapy group from 5.8 ± 0.7 to 2.1 ± 0.3 at 12 months ($P < 0.0001$, Figure 6). The fall was predominantly in the first 6 months of treatment, with the decline in the second 6 months not reaching statistical significance. Ovarian volume also fell in the chemotherapy group over 12 months, from 4.3 ± 1.3 to 3.0 ± 0.2 ml ($P = 0.047$), with the fall in neither 6 month interval achieving statistical significance.

There were no significant changes in either AFC or ovarian volume in the gonadotrophin suppression group.

Discussion

The data presented here further explore the validity of AMH as a marker of reproductive ageing, both physiological and as a result of chemotherapy. Changes in AMH concentrations were shown to indicate gonadal toxicity during chemotherapy for breast cancer more clearly than those in E_2 or inhibin B, supporting a role for AMH as a marker of ovarian damage during such therapies (Bath *et al*., 2003) and allowing comparison of the gonadotoxicity of different regimens.

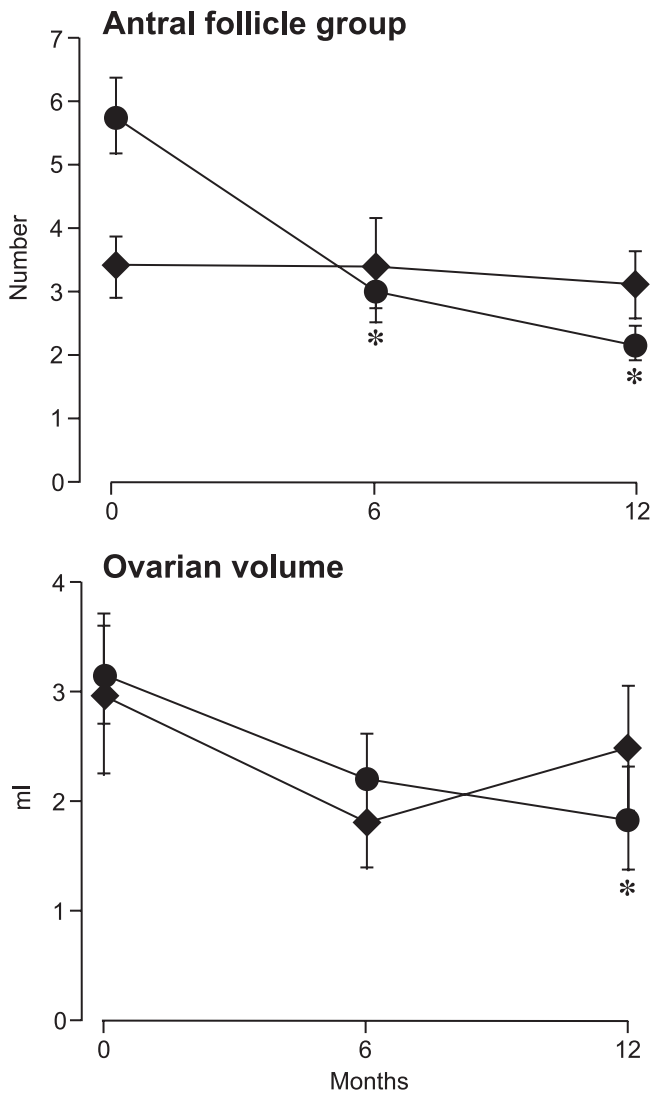

Figure 6. Antral follicle count (AFC) and ovarian volume in the chemotherapy group (circles, $n = 42$) and gonadotrophin suppression group (diamonds, $n = 8$) during 12 months of treatment. Mean ± SEM; '*' indicates significant difference ($P < 0.05$) from pretreatment. Note that chemotherapy regimens were generally of 6 months duration. Subjects thereafter received endocrine therapy, usually tamoxifen.

AMH is expressed by the granulosa cells of growing follicles (Baarends *et al*., 1995; Durlinger *et al*., 2002b; Weenen *et al*., 2004), with expression initiated in the smallest growing primary follicles and declining in the early antral stages as the follicle is selected for dominance or becomes atretic. AMH concentrations therefore vary little across the menstrual cycle (Cook *et al*., 2000) in marked contrast to E_2 and inhibin B concentrations. AMH concentrations decline with age (van Rooij *et al*., 2005), and these data confirm that a major decline has already occurred by the age of 40 years. The early decline in AMH concentrations demonstrated here occurs at an age similar to the age of acceleration in follicular loss found both in histological studies (Gougeon *et al*., 1994) and by mathematical modelling (Faddy *et al*., 1992). This occurred without a decrease in inhibin B concentration or increase in FSH concentration. These data therefore strongly support the proposed relationship between AMH concentrations and the follicular reserve and that AMH may be a useful marker of reproductive ageing (de Vet *et al*., 2002; Fanchin *et al*., 2003; van Rooij *et al*., 2005). In keeping with this, we have previously demonstrated a deficit in AMH concentration, but not in inhibin B concentration, in young women who had received chemotherapy for cancer during childhood, despite maintenance of regular menstrual cycles (Bath *et al*., 2003).

The women recruited to this study were all premenopausal and were treated with adjuvant chemotherapy (in the majority) or ovarian suppression. The primary analyses of changes in ovarian function were confined to the first 6 months, i.e. the duration of chemotherapy, and marked differences between the groups were detected. Few studies have prospectively investigated gonadal function in either men or women during such therapy (Chatterjee *et al*., 1994; Wallace *et al*., 1997). The major findings were that E_2 concentrations were largely maintained in the chemotherapy group, whereas inhibin B concentrations fell by approximately 50%, and AMH concentrations fell rapidly to the limit of detection. Gonadotrophins were raised. Both ovarian volume and AFC also fell, although these changes were modest. These results illustrate the timescale of gonadal toxicity of anti-cancer regimens. The differential effects on the three ovarian hormones allow deduction of follicle sizes most sensitive to chemotherapy. The severity and rapidity of the fall in AMH concentrations compared with the partial decline in inhibin B concentrations may reflect primordial and preantral follicles as the primary site of toxicity, with larger follicles (producing predominantly inhibin and E_2) being less affected. The elevated FSH concentrations would prevent atresia and support hormone production in any remaining antral follicles, resulting in the relative sparing of E_2 production. Consistent with continuing but irregular follicular development and E_2 production during chemotherapy, most women in that group showed irregular menstrual bleeding.

Recent meta-analysis has clearly demonstrated the survival benefit of adjuvant chemotherapy in premenopausal breast cancer (Early Breast Cancer Trialists' Collaborative Group, 2005). Advances in the management of patients with malignant diseases have led to greatly increased survival and greater interest on the long-term effects of treatment. Damage to reproductive function is common in cancer survivors, manifesting in women as infertility, premature ovarian failure and its consequences, such as osteoporotic fractures. The risk of premature ovarian failure varies with treatment regimens and age at diagnosis (Whitehead *et al*., 1983; Meirow and Nugent, 2001), clearly confirmed in the present analysis. The use of combination regimes however complicates the assessment of the effects of individual drugs, and regimens are constantly evolving. Premature ovarian failure is reported to occur in 60–80% of women treated with standard cyclophosphamide, methotrexate and 5-fluouracil (CMF) therapy but fewer in anthracycline-based regimens (Lower *et al*., 1999). The present study provided an opportunity to analyse changes in ovarian function in detail and compare the effects of regimens currently used for this common malignancy.

The majority of women receiving adjuvant chemotherapy in this study were treated with one of four regimens. As treatment was based on disease staging and pathological grading, patients were not randomized between treatments, potentially introducing bias into the analysis. However, pretreatment characteristics, i.e. age and hormone concentrations, were similar between treatment groups. Using a combination of changes in AMH, E_2, FSH and inhibin B concentrations and the development of amenorrhoea during the 6 months of chemotherapy, we detected differences between regimens. Most notably, there was evidence that FEC-T (TACT) was the most gonadotoxic regimen resulting in the lowest AMH and highest FSH concentrations and the only group showing a decrease in E_2 concentration, and the A-CMF Bonadonna and E-CMF regimens the least. FEC-T (TACT) was the only one of these four regimens to contain a taxane, with a relatively low dose of cyclophosphamide. This analysis, indicating additional gonadal toxicity associated with taxane administration, is supported by the finding that when analysis was confined to women under the age of 40 years, FSH concentrations after chemotherapy were markedly higher in those who had been treated with a taxane, despite pretreatment concentrations being similar. Conversely, the E-CMF (TACT) regimen involved the highest dose of cyclophosphamide per week, although the total dose was similar to the A-CMF Bonadonna regimen. Although alkylating agents such as cyclophosphamide are generally considered the most toxic drugs to the ovary (Meirow and Nugent, 2001; Sonmezer and Oktay, 2004), these data suggest that taxanes add to this toxicity, although the power of the present analysis is limited by the sample size and the lack of randomization and needs to be confirmed in larger studies. There are limited previous data on the effects of taxanes on menstrual function and ovarian endocrinology, although inhibitory effects on steroidogenesis has been reported (Chen *et al*., 1994). These data therefore support animal evidence of loss of primordial follicles after treatment with paclitaxel (Gucer *et al*., 2001). A recent study has reported that in women aged ≤40 years, treated with regimens very similar to those administered here, only 15% were amenorrhoeic for more than a year (Fornier *et al*., 2005).

Women who did not receive chemotherapy were generally treated with a combination of goserelin and tamoxifen. This combination resulted in the expected suppression of gonadotrophin and E_2 concentrations. Inhibin B concentrations also fell, with a similar time course to changes in E_2 concentrations, consistent with the secretion of both from gonadotrophin-dependent antral follicles. AMH concentrations however showed a delayed fall, which did not become significant until 6 months of treatment, and then continued to decline further. A fall of approximately 20% would be expected from the effects of increasing age alone over a 1-year period in women of this age group (van Rooij *et al*., 2005), compared with the 70% fall observed. The absence of an immediate decline is consistent with the predominant source being small, pre-antral follicles: growth of these is generally considered gonadotrophin-independent (Gougeon, 1996). This is also consistent with the stability of AMH concentrations during the menstrual cycle in the face of varying gonadotrophin concentrations (Cook *et al*., 2000; La Marca *et al*., 2004) and also between cycles in the same woman (Fanchin *et al*., 2005). The slow decline in AMH concentrations may be explained by a reduction in the stimulatory effect of FSH on early follicle growth, as recently suggested in the sheep (Campbell *et al*., 2004), taking some months to establish because of the protracted duration of folliculogenesis in the human and partial gonadotrophin independence at early stages together with changes in small antral follicles. Interpretation is complicated by the incomplete suppression of FSH in the women studied here, with readily detectable concentrations of 1.5–2 IU/l during treatment. AMH itself has an inhibitory effect on the initiation of follicle growth (Durlinger *et al*., 2002a; Gigli *et al*., 2005). As AMH is expressed by a range of size of follicles from primary to early antral stages, a decrease in contribution from the larger ones may be partially offset by an increase from smaller ones, leading to delayed overall changes. It is likely that the changes in circulating AMH during prolonged gonadotrophin suppression derive from alterations in more than one mechanism in the regulation of intraovarian folliculogenesis.

In contrast to the effect of chemotherapy, gonadotrophin suppression did not result in significant changes in either AFC or ovarian volume. As previously reported in normal women (Pavlik *et al*., 2000), both measures were however already low in this population of women who are considerably older than many of the populations previously studied (Syrop *et al*., 1999; Bancsi *et al*., 2002; Yong *et al*., 2003). This may also account for the lack of a significant correlation between pretreatment AMH concentration and AFC. Both AFC and ovarian volume were less than half the values we have previously reported in young healthy women (Bath *et al*., 2003). In that study, AFC in young women taking oral steroidal contraception (i.e. during prolonged gonadotrophin suppression) was maintained and also more than twice that found before treatment in our patients. These results contrast with the effect of chemotherapy on ovarian ultrasound measures, which particularly reduced AFC. These biophysical markers of ovarian ageing therefore appear to have inadequate sensitivity to reflect the effects of

gonadotrophin withdrawal in the smaller ovaries with fewer follicles of older premenopausal women, although still allowing demonstration of the effects of gonadal toxicity from chemotherapy.

In conclusion, these data confirm AMH as the first endocrine marker of ovarian ageing. Comparison of the effects of chemotherapy with gonadotrophin suppression on ovarian function showed markedly different patterns of change in ovarian hormones with AMH concentrations showing the greatest fall during chemotherapy. By contrast, gonadotrophin suppression resulted in a delayed fall in AMH concentrations, taking several months to become apparent. Differences were also found between regimens, such that taxanes may add to the ovarian toxicity of cyclophosphamide, hitherto considered the most toxic drug to the ovary.

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