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# Comparison of ovarian function markers in users of hormonal contraceptives during the hormone-free interval and subsequent natural early follicular phases

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**OBJECTIVE:** The aim of this study was to evaluate whether values of FSH, LH, estradiol, anti-Müllerian hormone (AMH), inhibin B, antral follicle count (AFC) and ovarian volume (OV) determined on day 7 of the hormone-free interval are similar to values measured on days 2–5 of two subsequent natural menstrual cycles. In addition, values measured on day 7 of the hormone-free interval were examined for the purpose of predicting values measured on days 2–5 during the second natural cycle.

**METHODS:** In this study, 25 women using hormonal contraception provided a blood sample and underwent transvaginal ultrasound measurements on day 7 of the hormone-free interval and on cycle days 2–5 of two subsequent natural cycles. Changes were compared by repeated measures ANOVA and multivariate linear regression was used for prediction purposes.

**RESULTS:** Mean (SD) age of the participants was 26.3 (6.2) years. Overall significant decreases in FSH and inhibin B and significant increases in AMH, AFC and ovarian volume values were measured after discontinuation of hormonal contraception ($P < 0.001$, $P = 0.04$, $P = 0.01$, $P < 0.001$ and $P = 0.004$, respectively). Significant changes occurred both from day 7 of the hormone-free interval to natural cycle 1 as well as from natural cycle 1 to natural cycle 2. FSH, AMH and AFC values measured during days 2–5 of natural cycle 2 could be predicted by the corresponding values measured on day 7 of the hormone-free interval.

**CONCLUSION:** Hormonal and ultrasound markers of ovarian function in hormonal contraception users measured at the end of the hormone-free interval do not seem to represent subsequent natural early follicular phase values. However, these values can, in some cases (FSH, AMH and AFC), be used to predict early follicular phase values using calculated prediction equations, which need to be validated in future research.

## Introduction

A fully functional hypothalamic-pituitary-ovarian axis is one of the prerequisites for an adequate female reproductive function. Hypothalamic-pituitary-ovarian activity is traditionally assessed by reproductive hormones [FSH, LH and estradiol (E2)], and transvaginal ultrasound measurements (antral follicle count (AFC) and ovarian volume (OV)) during the early follicular phase of a natural menstrual cycle in women not using hormonal contraceptives. In addition, new hormonal markers, such as anti-Müllerian hormone (AMH) and inhibin B, are emerging and are increasingly used, but mainly in research settings. However, the majority of women of reproductive age use hormonal contraceptives (Skouby, 2004) and it would be of practical value to be able to accurately assess reproductive function under such conditions.

The contraceptive effect of hormonal contraception is predominantly established by suppression of gonadotrophin secretion by the

pituitary, which in turn inhibits ovarian activity resulting in arrested follicle growth and reduced hormone production (Mishell *et al*., 1977; Dericks-Tan *et al*., 1992). The standard regimen of oral contraceptives includes 21 days of estrogen/progestin pills followed by a 7-day hormone-free interval in which a withdrawal bleeding is induced. During this interval recovery of pituitary-ovarian activity occurs as the pituitary gland begins to secrete gonadotrophins (van der Spuy *et al*., 1990; Vandever *et al*., 2008) resulting in follicular development and hormone production (van Heusden and Fauser, 1999; Baerwald *et al*., 2004; Schlaff *et al*., 2004). Indeed, it has been demonstrated that FSH, LH, E2 and inhibin B values increase during the hormone-free interval (Willis *et al*., 2006) and that follicle growth is established, even to dominance in some cases (van Heusden and Fauser, 1999; van Heusden *et al*., 2002; Baerwald *et al*., 2004). Although the events during the hormone-free interval have shown to resemble those during the early follicular phase of a natural cycle (van Heusden and Fauser, 2002; van Heusden *et al*., 2002), it remains unclear whether hormone and ultrasound values on day 7 of the hormone-free interval resemble natural early follicular phase values.

A few studies have shown that FSH levels at the end of the hormone-free interval are similar to early follicular phase levels, although differences in maximum levels have been observed between different types of oral contraceptives (Cohen and Katz, 1979; Fauser and van Heusden, 1997; van der Spuy *et al*., 1990; van Heusden and Fauser, 2002). Similarly, LH levels were also found to be comparable (Cohen and Katz, 1979; van der Spuy *et al*., 1990). However, E2 levels were found to be significantly lower at the end of the hormone-free interval compared with early follicular phase values but were comparable with midfollicular phase values at the day of dominant follicle selection (van der Spuy *et al*., 1990; Fauser and van Heusden, 1997). Values of AMH, AFC and ovarian volume were unfortunately not included in the aforementioned studies and results were not obtained from measurements performed prospectively in the same group of women. This may be a substantial limitation of these studies since the large inter-individual variability of the reproductive markers and the relatively small study groups may have made it difficult to adequately determine whether levels of reproductive markers at the end of the hormone-free interval are comparable to those measured during the early follicular phase.

To our knowledge only one study has compared reproductive markers in the hormone-free interval and in the early follicular phase within one group of women. These women, who served as a control group, were first measured during the early follicular phase of a natural menstrual cycle and subsequently started using oral contraception (Somunkiran *et al*., 2007). Data showed that FSH, LH, E2, AFC and ovarian volume values measured on days 3–5 of the natural cycle decreased significantly after six cycles of oral contraceptives and were not comparable to values measured during the hormone-free interval. AMH values, however, remained unchanged. This implies that, except for AMH, values of reproductive markers obtained during the hormone-free interval do not resemble early follicular phase values.

However, no data are available on short-term changes in values of reproductive markers in women using hormonal contraceptives who subsequently discontinue this usage. This information is relevant to clinicians who counsel young women of reproductive age who are in need of information about their reproductive function, sometimes without an immediate wish to have children. It is known that a large proportion of these women use oral contraception as this is the most widely used method of contraception among women of reproductive age (Skouby, 2004; de Graaf, 2009). Therefore, from a clinical point of view it would be of interest to obtain a decisive answer as to whether women, who use oral contraceptives and who would like their reproductive function to be evaluated without having an immediate wish to have children, should stop taking these contraceptives for a period of time in order to adequately assess their reproductive function.

Therefore, we designed the current exploratory study to evaluate whether values of FSH, LH, E2, AMH, inhibin B, AFC and total ovarian volume determined on day 7 of the hormone-free interval are comparable to values measured on days 2–5 of two subsequent natural menstrual cycles. In addition, if these values were not similar, we investigated whether values measured on days 2–5 of the second natural cycle could be predicted from the corresponding values measured on day 7 of the hormone-free interval.

# Patients and methods

## Subjects

Study participants were recruited through advertisements on blackboards and in hallways of the VU University Medical Center, the VU University and several family practitioners in Amsterdam. To those interested in participating in the study, study information was given orally and in writing.

Inclusion criteria were female gender, age 18–40 years at study entry, hormonal contraceptive use for at least 3 months (either using the standard 21- /7-day regimen or an extended regimen) prior to the start of the study, an initial contraceptive indication for use of hormonal contraceptives, willingness to discontinue hormonal contraceptives use for at least two natural menstrual cycles and willingness to use methods of contraception other than hormonal contraception during these cycles. Exclusion criteria were virginity, history of endocrine disease such as thyroid dysfunction and history of ovarian or cranial surgery. During the study period, condoms were provided free of charge as an alternative method of contraception. All participants gave written informed consent. Furthermore, they were compensated financially for their participation.

## Study design

The study was designed as a longitudinal prospective study and was approved by the Medical Ethics Committee of the VU University Medical Center in Amsterdam. Data were collected by a one time only questionnaire and by blood sampling and transvaginal ultrasound measurements of the reproductive organs on three occasions. Subjects who agreed to participate were first sent the questionnaire. After having received the filled-out questionnaire, an appointment was made for the first measurement. Since for the purpose of this study this measurement had to take place on the day of the hormone-free interval on which the influence of the hormonal contraception would be the least, this measurement was performed on the last day of the hormone-free interval, i.e. day 7. The second and third hospital visit were planned on days 2, 3, 4 or 5 of the two subsequent natural menstrual cycles.

If the first natural menstrual bleeding did not occur within 3 months of the first day of the withdrawal bleeding (following the discontinuation of hormonal contraception), participants were contacted by the researcher and referred to a gynaecologist when desired. These amenorrhoeic participants were excluded from the study.

Participants were asked to monitor their basal body temperature (BBT) during the first natural menstrual cycle following discontinuation of

hormonal contraception. An experienced gynaecologist (C.B.L.) identified ovulation based on an obvious biphasic shift of around 0.3°C in the BBT. If ovulation could not be verified by the BBT chart, an elevated mid-luteal phase progesterone level (≥10 nmol/l) was confirmative for ovulation. Cycle length of the first completely evaluable natural cycle (i.e. the second natural cycle) was determined by counting the number of days from the first day of bleeding until the day before the next bleeding period.

## Data collection

*Questionnaire*

The questionnaire was an adaptation of a well-tested questionnaire used by the Department of Epidemiology of the Netherlands Cancer Institute in a large-scale Dutch cohort study on long-term effects of ovarian stimulation for *in vitro* fertilization (de Boer *et al.*, 2003). It addressed the following issues relevant to our study: socio-demographic characteristics, menstrual history and type and duration of current and past usage of hormonal contraceptives.

*Hormonal assays*

Blood samples were centrifuged for 10 min at 4°C (3000 rpm) within 30 min after venipuncture and frozen (−20°C) for storage until assayed. Laboratory screening was performed by the endocrine laboratory of the VU University Medical Center. All samples of one individual were analysed in the same run for each hormone.

Plasma FSH levels were analysed by an immunometric assay (Delfia, Wallac, Turku, Finland), with a lower detection limit of 0.5 IU/l. The intra- and inter-assay coefficient of variation (CV) was 5 and 7%, respectively, at a concentration of 2 IU/l and 3 and 6%, respectively, at a concentration of more than 4 IU/l. Plasma LH levels were determined by an immunometric assay (Delfia, Wallac, Turku, Finland), with a lower detection limit of 0.3 IU/l. The intra- and inter-assay CVs were 3 and 7%, respectively. E2 was measured by radioimmunoassay (Daisorin, Sallugia, Italy) with a lower limit of quantification of 18 pmol/l and an intra- and inter-assay CV of 5 and 10%, respectively.

An ultra-sensitive immuno-enzymometric assay kit (Diagnostic Systems Laboratories, Webster, TX, USA) was used to measure the AMH in duplicate (Al-Qahtani *et al.*, 2005). The limit of quantification was 0.1 μg/l. Intra- and inter-assay CV was 5 and 8%, respectively. Inhibin B was measured in duplicate by ultra-sensitive two-site enzyme immunoassays (Serotec, Oxford, UK). The lower limit of quantification was 15 pg/ml and the intra- and inter-assay CV was 5 and 9%, respectively.

*Ultrasound*

All transvaginal ultrasound measurements were performed by a specifically trained investigator (MvdB) using a 6 MHz transvaginal probe (EnVisor HD, Philips Medical Systems, Eindhoven, The Netherlands). An AFC was performed, counting the number of follicles sized 2–10 mm in both the right and left ovary. Furthermore, the volume of the left and right ovary was estimated from its length (L) and width (W) using the formula $(\pi LW^2)/6$, which assumes the ovaries to have a prolate ellipsoid shape. Ovarian volume was defined as the mean value of the right and left ovary.

## Statistical analysis

Data were analysed using SPSS for Windows-version 14.0 (SPSS Inc., Chicago, IL, USA). Data which were not normally distributed were log-transformed prior to analysis and normality was checked again after this transformation. Changes in hormone levels (FSH, LH, E2, AMH, inhibin B) and ultrasound measurements (AFC and OV) were analysed by repeated measures ANOVA. When the overall change over time was significant, 'within subjects contrasts' analyses were used to detect which changes between two time points reached statistical significance. Furthermore, Pearson correlation coefficients were used to evaluate whether changes in hormone and ultrasound values were dependent on age, ethinyl estradiol dose of hormonal contraception or BMI. In order to assess whether hormone levels during the hormone-free period can act as markers for the number of ovarian follicles, we analysed whether AMH and AFC were correlated in conditions of hormonal contraceptive use as well as in conditions of natural cycles. Since AMH is currently considered to be the most promising marker of ovarian function (de Vet *et al.*, 2002; La Marca *et al.* 2009), Pearson correlations with AFC were calculated for this variable only. In addition, by calculating Pearson correlations between levels of AMH at all three time points, the inter-cycle stability of AMH was evaluated. Finally, multivariable linear regression was used to predict hormone or ultrasound values in natural cycles from the corresponding values on day 7 of the hormone-free interval. In addition, it was evaluated whether the interaction between the age and the variable to be evaluated significantly contributed to the regression model. A *P*-value $<0.05$ was considered to be statistically significant.

For the sample size calculation, the AFC variable was used. The sample size required to detect at least a change of four antral follicles (Somunkiran *et al.*, 2007) was calculated. The power to detect this change was set at 90% and the significance level at 0.05. Based on the formula of Twisk *et al.* (2007), it was estimated that a minimum of nine female participants would be needed to detect these changes in the number of follicles. Since other outcomes are also included in this study and to compensate for an expected dropout rate of 15% and an 'exclusion rate' after the first measurement of 10% (because of non-occurrence of first natural bleeding), we planned to enrol at least 30 women in the study.

# Results

Thirty women were recruited for the study and five dropped-out: one because of repeatedly not showing up, three as a result of polycystic ovarian syndrome induced amenorrhoea, and one due to the presence of a dermoid cyst in the ovary which made it not possible to perform an AFC. As a result, the study population consisted of 25 women who all appeared to have ovulatory cycles (23 established by BBT curves and 2 by additional mid-luteal progesterone assessment). The characteristics of the participants are summarized in Table I.

The mean (SD) hormone and ultrasound values on day 7 of the hormone-free interval and on days 2, 3, 4 or 5 of the two subsequent natural menstrual cycles are shown in Table II. Repeated measures ANOVA showed an overall significant decrease in FSH and inhibin B values and a significant increase in AMH, AFC and OV values after discontinuation of the pill ($P<0.001$, $P=0.04$, $P=0.005$, $P<0.001$ and $P=0.004$, respectively). The overall effect of time regarding the other hormone values (LH and E2) appeared non-significant. Additional within subjects contrast analysis indicated that significant changes were measured from day 7 of the hormone-free interval to natural cycle 1 but also from natural cycle 1 to natural cycle 2 and from day 7 of the hormone-free interval to natural cycle 2 (Table II). None of the changes appeared to significantly correlate with age, ethinyl estradiol dose or BMI (data not shown). Correlations between AMH and AFC values measured at day 7 of the hormone-free interval, at the first natural cycle and the second natural cycle all appeared to be significant ($r=0.50$ ($P=0.02$); $r=0.47$ ($P=0.02$); $r=0.67$ ($P=0.001$), respectively). Furthermore, the correlation between AMH at day 7 of the hormone-free interval and AMH at the first natural cycle was 0.75 ($P<0.001$), whereas the

**Table I Socio-demographic characteristics of the 25 study participants.**

| | |
|---|---|
| Age, years; mean (SD) | 26.3 (6.2) |
| Body mass index, kg/m$^2$; mean (SD) | 22.0 (1.8) |
| *Type of hormonal contraception last used* | |
| 20 μg estrogen monophasic pill | 3 (12) |
| 30–35 μg estrogen monophasic pill | 17 (68) |
| 50 μg estrogen monophasic pill | 1 (4) |
| 30–40 μg estrogen triphasic pill | 2 (8) |
| Other[#] | 2 (8) |
| Duration of hormonal contraception last used, months; median (IQR**) | 20 (57) |
| *Regimen of last used hormonal contraception* | |
| Standard 21- /7-day regimen | 21 (84) |
| Extended regimen | 4 (16) |
| Cycle length, days; mean (SD) | 28.8 (4.8) |

Values are the number (%) of women, unless indicated otherwise.
[#]Ortho-Evra Patch ($n = 1$) and NuvaRing ($n = 1$).
**IQR, interquartile range.

correlations between AMH at the first and the second natural cycle and between AMH at day 7 of the hormone-free interval and AMH at the second natural cycle were 0.80 ($P < 0.001$) and 0.85 ($P < 0.001$), respectively.

Results of linear regression analysis (Table III) revealed that FSH, AMH and AFC values measured on day 7 of the hormone-free interval significantly contributed to the prediction of values measured during the early follicular phase of natural cycle 2, all explaining about 72% of the variance. For FSH it was found that adding age and the interaction between age and FSH at day 7 to the model revealed that the interaction with age was significant ($P = 0.02$), increasing the explained variance to 82%. This analysis led to the following regression equation: FSH at NC2 = 5.922 − (0.347 × FSH at day 7 HFI) − (0.112 × age) + (0.026 × FSH at day 7 HFI × age), where HFI is the hormone-free interval and NC2, the natural cycle 2.

For example, when FSH values of a 30-year old woman measured on day 7 of the hormone-free interval is 7.0 U/l, the expected value during the early follicular phase of the second natural menstrual cycle is 5.922 − (0.347 × 5.0) − (0.112 × 30) + (0.026 × 5. × 30) = 5.6 U/l.

Calculated regression equations for AMH and AFC were: [AMH at NC2 = 0.636 + (0.945 × AMH at day 7 HFI)] and AFC (AFC at NC2 = 8.629 + (0.778 × AFC at day 7 HFI), with HFI = hormone-free interval and NC2 = natural cycle 2). Neither inhibin B nor OV on day 7 of the hormone-free interval could predict the corresponding values during the early follicular phase of natural cycle 2.

Correlations between values of FSH, AMH and AFC measured on day 7 of the hormone-free interval and those obtained during the second natural menstrual cycle were high (see $R^2$ values in Table III). These correlations are illustrated in the graphs in Fig. 1. The correlation coefficient stated in the FSH-graph remained significant when the elevated FSH extreme (>12 U/l) was excluded ($r = 0.53$, $P = 0.01$).

# Discussion

This study demonstrates that most hormonal and ultrasound markers of ovarian function measured at the end of the hormone-free interval in women using hormonal contraception do not seem to represent natural early follicular phase values. FSH and inhibin B values appear to decrease significantly when contraception use is discontinued, whereas values of AMH, AFC and OV increase and LH and E2 did not change significantly. These changes were not dependent on age, ethinyl estradiol dose or BMI. Furthermore, regression models allow FSH, AMH and AFC values measured on day 7 of the hormone-free interval to predict the corresponding values during the early follicular phase of the second natural cycle.

The results of this study may provide clinicians and researchers knowledge on whether ovarian function can be assessed in women who are using hormonal contraception and in need of information on their fertility potential without having an immediate wish to have children. This for example could be the case for young women of reproductive age who have previously been treated for cancer. It is

**Table II Mean (SD) values of hormone levels and ultrasound characteristics at three chronological time points and significance of change in scores within subjects between different time points.**

| | Day 7 HFI | NC1 | NC2 | *P*-value | | |
|---|---|---|---|---|---|---|
| | | | | NC1–HFI | NC2–NC1 | NC2–HFI |
| FSH (U/l) | 6.9 (3.0) | 5.6 (2.0) | 5.4 (2.1) | 0.003* | 0.18 | <0.001* |
| LH (U/l) | 3.5 (1.7) | 4.1 (1.6) | 3.9 (1.5) | 0.23 | 0.74 | 0.28 |
| E2 (pmol/l) | 90.4 (41.2) | 95.4 (38.0) | 106.6 (45.6) | 0.63 | 0.27 | 0.30 |
| AMH (μg/l) | 2.0 (1.2) | 2.0 (1.3) | 2.6 (1.3) | 0.91 | 0.01* | 0.001* |
| Inhibin B (ng/l) | 88.3 (47.5) | 61.9 (32.1) | 68.5 (36.8) | 0.002* | 0.62 | 0.09 |
| AFC (no.) | 19.9 (8.1) | 22.0 (7.8) | 23.9 (7.7) | 0.02* | 0.01* | <0.001* |
| OV (cm$^3$) | 3.0 (1.2) | 3.2 (1.3) | 4.0 (1.4) | 0.35 | 0.01* | 0.01* |

Analysed with repeated measures ANOVA.
HFI, hormone-free interval; NC1, natural cycle 1; NC2, natural cycle 2.
*$P < 0.05$.

**Table III** Results of regression analysis.

| Dependent variable | Independent variable | Regression coefficient (95% CI)[#] | *P*-value | $R^2$ |
|---|---|---|---|---|
| FSH at NC2 | FSH at day 7 HFI | 0.589 (0.420, 0.758) | <0.001* | 0.71 |
| AMH at NC2 | AMH at day 7 HFI | 0.945 (0.680, 1.210) | <0.001* | 0.72 |
| Inhibin B at NC2 | Inhibin B at day 7 HFI | −0.004 (−0.347, 0.340) | 0.98 | <0.001 |
| AFC at NC2 | AFC at day 7 HFI | 0.778 (0.543, 1.014) | <0.001* | 0.72 |
| OV at NC2 | OV at day 7 HFI | 0.349 (−0.167, 0.866) | 0.17 | 0.09 |

HFI, hormone-free interval; NC2, natural cycle 2.
[#]Crude regression coefficients are reported.

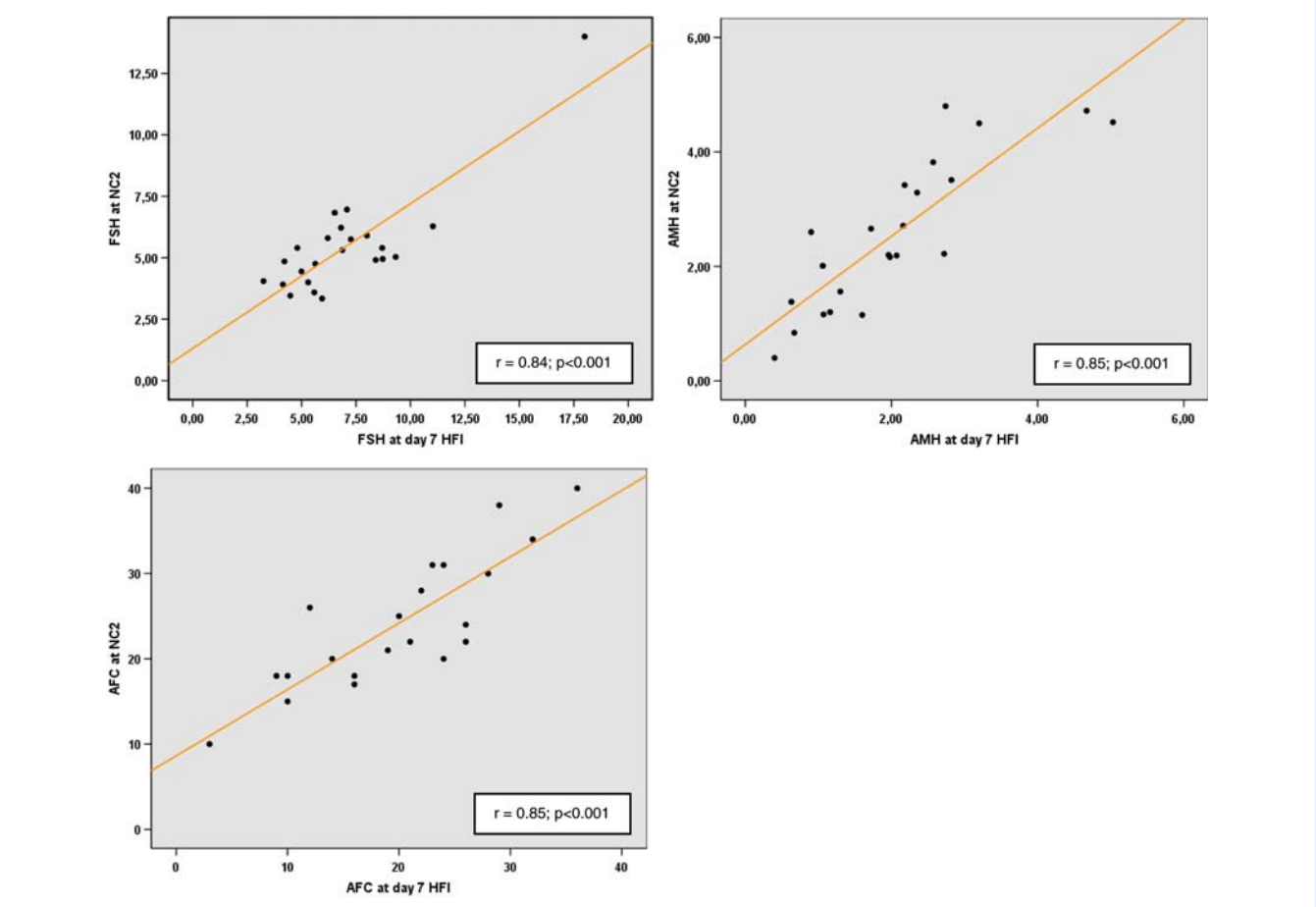


**Figure 1** Scatter plot of FSH, AMH and AFC values measured on day 7 of the hormone-free interval (HFI) versus those measured during the second natural menstrual cycle (NC2).

known that these women are in need of information about their reproductive function as most of them are aware that former treatment regimes might have caused damage to their reproductive system (Zebrack, 2009; Zebrack *et al.*, 2007; Rosen *et al.*, 2009). Since a large proportion of these females use oral contraceptives it could be decided to measure reproductive markers on day 7 of the hormone-free interval. However, the results of our study show that in that case caution should be taken when interpreting values of these markers measured at this point in time. Moreover, future research in which female cancer survivors are included is needed in order to establish the exact value of reproductive markers measured on day 7 of the hormone-free interval for this group of women.

The increased level of FSH in this study on day 7 of the hormone-free interval implies a limited negative feedback during this interval. This is most likely to occur during the first few days of this interval since high FSH levels coincide with elevated inhibin B levels on day 7 compared with early follicular phase values. Most probably, this increased level of inhibin B on day 7 is a reflection of a pronounced ovarian

response to the earlier generated increase in FSH, although at this stage multiple follicle growth could not be confirmed by our data. However, it is known that multiple pregnancy can occur as a consequence of multiple follicle growth shortly after interrupting hormonal contraception (Jernstrom *et al*., 1995; Lambalk and Schoemaker, 1997).

Our results differ to some extent with the published data. In contrast to our study, previous studies report maximum levels of FSH at the end of the hormone-free interval to be similar to early follicular phase levels in the natural cycle, although maximum levels differ between different types of contraceptive pills due to a varying ethinyl estradiol component (Cohen and Katz, 1979; Fauser and van Heusden, 1997; van der Spuy *et al*., 1990; van Heusden and Fauser, 2002). Furthermore, previous studies found levels of E2 to be significantly lower at the end of the hormone-free interval compared with early follicular phase values (Fauser and van Heusden, 1997; van der Spuy *et al*., 1990), whereas LH values were similar (Cohen and Katz, 1979; van der Spuy *et al*., 1990). We found no changes in both E2 and LH and a higher value of FSH at the end of the hormone-free interval. This may be explained by methodological differences between the previous studies in which between-group comparisons were used, whereas we measured changes within subjects.

To our knowledge, only one study has compared tests for measuring ovarian responsiveness within a group of subjects (Somunkiran *et al*., 2007). In this study these tests were performed among 15 healthy women before and after 6 months of oral contraceptive use. We found comparable results with respect to AFC and OVs, i.e. lower values during the hormone-free interval compared with early follicular phase values. However, with respect to hormone values contradicting results were found. In the study by Somunkiran *et al*., no changes in the AMH values were found, while we found AMH to be significantly lower during the hormone-free interval. Furthermore, we found FSH values to be significantly higher during this interval and LH and E2 values to be unchanged while they found FSH, LH and E2 to be significantly lower during the hormone-free interval. Apparently, it either seems to make a difference whether measurements during the early follicular phase were performed prior to or following measurements during the hormone-free interval or whether the measurements in the hormone-free interval were performed after a relatively short (6 months in the study of Somunkiran *et al*.) or a prolonged period of contraceptive use (20 months on average in our study). It should, however, be noted that the results in the study of Somunkiran *et al*. are based on a relatively small sample size.

In addition, a possible explanation for the contradicting results regarding FSH, LH and E2 may be found in the timing of the measurement during the hormone-free interval. It has been demonstrated that during the 7-day hormone-free interval FSH, LH and E2 values increase significantly. However, the start of this increase varies between different hormones and the type of oral contraceptives used (Renier *et al*., 1998; van Heusden and Fauser, 1999; van Heusden *et al*., 2002). It has been shown that the suppressive effect of oral contraceptives is prolonged with higher doses of ethinyl estradiol. Despite this, levels of reproductive hormones on day 7 of the hormone-free interval appear to be higher with increasing doses of ethinyl estradiol (van der Spuy *et al*., 1990; van Heusden and Fauser, 1999). All *women* in the study of Somunkiran *et al*. (Somunkiran *et al*., 2007) used 35 μg E2 pills while in our study the majority of the *women* used either 30 μg E2 pills ($n = 17$) or 20 μg E2 pills ($n = 3$). Therefore, the rise in hormone levels during the hormone-free interval is expected to occur later in the Somunkiran study compared with our study. Moreover, measurements were performed on day 5 of the hormone-free interval in the study by Somunkiran while this was done on day 7 in our study. This might explain why significantly lower FSH values during the hormone-free interval compared with natural cycle values were found in the study by Somunkiran *et al*., while we found the opposite.

Recent studies indicate that serum levels of AMH are relatively constant throughout the menstrual cycle or that at least large physiological fluctuations are not seen (Hehenkamp *et al*., 2006; La Marca *et al*., 2006; Tsepelidis *et al*., 2007; Streuli *et al*., 2008). This is likely to be a reflection of the rather constant size of smaller FSH-sensitive antral follicles that typically produce AMH (Weenen *et al*., 2004). Development of these follicles is not yet under full control of FSH but when they enter the stage of FSH-responsiveness AMH production drops (Durlinger *et al*., 2002; Broekmans *et al*., 2008). Furthermore, several studies show a drop in the AMH levels concurrent with stimulation with exogenous FSH for example in women undergoing IVF treatment (Fanchin *et al*., 2003; La Marca *et al*., 2004). This drop in AMH is currently interpreted as an indication that the number of smaller pre-antral follicles temporarily decreases. Therefore, the most plausible explanation for our observation that AMH increases after discontinuation of hormonal contraception is that the cohort of smaller antral follicles increases monthly and that prolonged suppression of FSH by means of hormonal contraception to some extent prevents pre-antral and small antral follicle formation, i.e. the class of follicles that under normal circumstances typically contributes to serum AMH levels. So, somehow previous use of hormonal contraceptives influences AMH values. However, even in our study inter-cycle AMH correlations remained extremely high indicating that AMH seems to be a reliable marker for measuring ovarian function, a statement supported by other studies as well (de Vet *et al*., 2002; La Marca *et al*., 2009; Streuli *et al*., 2009). Moreover, the significant correlations between AMH and AFC found in our study as well as in other studies (van Disseldorp *et al*., 2009) seem to endorse this statement.

In our study it was shown that, except for E2 and LH, all markers of reproductive function changed significantly after discontinuation of hormonal contraception. One can, however, argue that the individual differences between values measured during the hormone-free interval and the natural cycle are not relevant from a clinical point of view since changes occurred within normal reference values in most cases. However, future research involving more and different types of subjects (e.g. women with proven sub- or infertility, or older women) should further examine this clinical relevance. It should for example be investigated to what extent women would be diagnosed differently regarding their fertility status when measurements on day 7 of the hormone-free interval are compared with measurements during natural cycles. It might be the case that measuring these women on either one of these time points would not bring on any different clinical consequences. However, from a research point of view, differences measured at the two time points indeed might be of relevance, since one has to be cautious in interpreting mean values of study groups, especially if these groups include both hormonal contraception users and non-users. If values of hormonal contraception users and non-users are combined and reported as representative of a study

group, this value could represent an over- or underestimation of the true value, depending on the number of hormonal contraception users in the study group concerned. Therefore, discontinuation of hormonal contraception use seems justified in research involving women on hormonal contraception, particularly when comparing mean values of two or more study groups.

Thus, results of our study indicate that at this time, to assess ovarian function by a full panel of reproductive markers, it seems most appropriate to discontinue oral contraceptive use for at least two months. However, this study does provide regression equations from which values of FSH, AFC and AMH during the natural menstrual cycle can possibly be predicted from values measured during the hormone free interval. These equations should however be validated first in future research in larger groups of women (including women with apparently normal ovarian function as well as women with a decreased ovarian function) before they can be used for clinical or research purposes.

There are some limitations to the present study. First of all, different types of hormonal contraception were used. This might have influenced our results since maximum levels of reproductive hormones measured on day 7 of the hormone-free interval seem to depend on the type of contraceptives used (Renier *et al*., 1998; van Heusden and Fauser, 1999; van Heusden *et al*., 2002). Previous studies have shown that higher doses of ethinyl estradiol lead to prolonged periods of hormonal suppression but also to significantly higher levels of FSH on day 7 of the hormone-free interval (van der Spuy *et al*., 1990; van Heusden and Fauser, 1999). Unfortunately, due to the small sample size in our study we were not able to reliably evaluate the effect of the different types of hormonal contraception used. Future research should investigate whether oral contraceptive pills with different doses of ethinyl estradiol bring on different changes in reproductive markers after discontinuation of these pills.

Secondly, the values of the reproductive markers observed in this study may have been subject to inter-cycle variability. Indeed, it has been demonstrated that inter-cycle variability is present both in hormonal as well as ultrasound markers of ovarian function (Lenton *et al*., 1983; Scheffer *et al*., 1999; Kwee *et al*., 2004; Penarrubia *et al*., 2004; Elter *et al*., 2005; Fanchin *et al*., 2005; van Disseldorp *et al*., 2009). However, the statistical analyses used in this study take into account this type of variability. Since we were able to detect significant changes despite the acknowledged inter-cycle variability, it can be stated that the significant differences found in this study are likely to reflect true biological changes.

Thirdly, in this study significant changes in reproductive markers did not only occur between the first two time points, i.e. day 7 of the hormone-free interval and first natural cycle, but for some markers values continued to change significantly between the first and second natural menstrual cycle. Therefore, it could be that the values of reproductive markers of each new cohort of follicles might continue to increase, decrease or revert to baseline even after the second natural menstrual cycle. Future research in which longer follow-up protocols are used is therefore recommended and is currently under way. Moreover, data obtained from such research can be used to validate the regression equations provided by this study, enabling clinicians and researchers to reliably predict early natural cycle values from values measured during the hormone-free interval. However, it should be noted that the results of our study do not contribute to the question whether or not ovarian function or the occurrence of primary ovarian insufficiency can be accurately predicted from values of reproductive markers measured on day 7 of the hormone-free interval. We merely studied whether values of reproductive markers were similar during the pill-free period and natural menstrual cycles. Even when these markers are measured during the early follicular phase (as this is clinical practice in normal circumstances), it is still a matter of debate whether these markers can indeed reliably be used for these kind of prediction purposes (Broekmans *et al*., 2009; Lambalk *et al*., 2009).

Finally, in our study all participants were ostensibly healthy and appeared to have normal ovarian function values. Only one (older-aged) woman had elevated FSH concentrations with corresponding lower AFC values when compared with the other participating women. It is of importance to investigate in future studies if the results we found in this study also apply to women with primary ovarian insufficiency.

In conclusion, this study has demonstrated that hormonal and ultrasound markers of ovarian function in hormonal contraception users measured at the end of the hormone-free interval do not seem to represent subsequent natural early follicular phase values. These results are of importance when measuring ovarian function of women who use hormonal contraception as they indicate that, when reproductive markers are measured at day 7 of the hormone-free interval, caution should be taken when interpreting the values of these markers measured at this point in time.

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