

Ovarian hormones and reproductive risk factors for breast cancer in premenopausal women: the Norwegian EBBA-I study

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BACKGROUND: Ovarian hormones, parity and length of ‘menarche-to-first birth’ time interval are known risk factors for breast cancer, yet the associations between 17 β -estradiol, progesterone and these reproductive factors remain unclear.

METHODS: A total of 204 women (25–35 years) who participated in the Norwegian EBBA-I study collected daily saliva samples for one complete menstrual cycle, and filled in a reproductive history questionnaire. Anthropometry was measured and saliva samples were analyzed for ovarian hormones. Associations between parity, the interval and ovarian hormones, and effects of hormone-related lifestyle factors were studied in linear regression models.

RESULTS: Mean age was 30.7 years, and age of menarche 13.1 years. Parous women had on average 1.9 births, and age at first birth was 24.5 years. No association was observed between parity and ovarian steroids. In nulliparous women, higher waist circumference (≥ 77.75 cm) and longer oral contraceptive (OC) use (≥ 3 years) were associated with higher levels of 17 β -estradiol. Short (< 10 years) versus long (> 13.5 years) ‘menarche-to-first birth’ interval was associated with higher overall mean ($P_{\text{trend}} = 0.029$), 47% higher maximum peak and 30% higher mid-cycle levels of 17 β -estradiol. We observed a 2.6% decrease in overall mean salivary 17 β -estradiol with each 1-year increase in the interval.

CONCLUSIONS: Nulliparous women may be more susceptible to lifestyle factors, abdominal overweight and past OC use, influencing metabolic and hormonal profiles and thus breast cancer risk. Short time between ‘menarche-to-first birth’ is linked to higher ovarian hormone levels among regularly cycling women, suggesting that timing of first birth is related to fecundity.

Key words: 17 β -estradiol / progesterone / menarche / age at first birth / parity

Introduction

Ovarian function plays a fundamental role in female fecundity and fertility (Lipson and Ellison, 1996), and ovarian hormones are major risk factors for breast cancer initiation and progression (Jasienska and Thune, 2001; Endogenous Hormones and Breast Cancer Collaborative Group, 2002; Eliassen *et al.*, 2006; McTiernan *et al.*, 2006). Furthermore, it is well established that early age at menarche, late age at first birth and low

parity increases breast cancer risk, and it is generally thought that reproductive events and their timing may influence breast cancer risk through their effects on differentiation of breast tissue and on hormonal and immunological profiles (Verkasalo *et al.*, 2001; Ma *et al.*, 2006; Li *et al.*, 2008). Given the large changes in both the timing of sexual maturation and childbearing pattern, and the rise in breast cancer incidence worldwide (Kaplowitz, 2006; World Cancer Research Fund/American Institute for Cancer Research, 2007), defining the relationship between

reproductive history and levels of circulating estradiol and progesterone is of particular importance.

Younger age at menarche is associated with higher cumulative exposure to ovarian hormones throughout life (Apter et al., 1989; Bernstein et al., 1991; Emaus et al., 2008a). Both early age at menarche and delayed first full-term birth translate into a longer 'menarche-to-first birth' time interval, which is recognized as a susceptible period for breast cancer development as the undifferentiated breast tissue is exposed to mitogenic estrogen and progesterone (Russo et al., 1982; Li et al., 2008). Moreover, overweight and obesity are established risk factors for breast cancer among postmenopausal women (Ballard Barbash et al., 2006), and girls with excessive body fat tend to experience early age at menarche (Emaus et al., 2008a) and reduced fecundity (Crosignani et al., 2003).

To our knowledge, data regarding levels of ovarian hormones throughout a menstrual cycle in relation to parity and timing of first birth are very limited. Our previous studies show that 17 β -estradiol profiles are associated with age at menarche (Emaus et al., 2008a), body composition from birth to adult life (Jasienska et al., 2006a; Finstad et al., 2009a), metabolic profile in adult life (Furberg et al., 2005; Emaus et al., 2008b) and energy balance throughout the menstrual cycle (Ziomkiewicz et al., 2008). These associations point to further studies of ovarian hormones in relation to both fecundity and fertility, and reproductive risk factors for breast cancer.

Thus, we chose to study the variation in the primary endogenous ovarian hormones in premenopausal years, 17 β -estradiol and progesterone. A unique aspect of this study is the daily assessments of salivary 17 β -estradiol and progesterone, which represent the free, unbound, biologically active fraction of these hormones (Ellison and Lipson, 1999). Therefore, the main aim of the present study was to elucidate whether daily levels of free and biologically active 17 β -estradiol and progesterone throughout an entire menstrual cycle are associated with parity and the 'menarche-to-first birth' time interval.

Materials and Methods

Participants and study design

In the Norwegian EBBA-I study (2000–2002), women aged 25–35 years and living in the municipalities of Tromsø and Balsfjord were recruited by local announcements in media and public meeting places (Furberg et al., 2005). Among those who volunteered to participate, 214 women met the inclusion criteria (age: 25–35 years, self-reported regular menstruation, normal cycle length within the previous 3 months, no use of steroid contraceptives, no pregnancy or lactation over the previous 6 months, no history of gynecological disorder and no chronic disorders, e.g. diabetes and hypo-/hyperthyroidism). Suitable respondents were subsequently enrolled in the study, and a total of 204 healthy women completed the study.

Questionnaires and interview

We used questionnaires to collect information including age at menarche, reproductive history, marital status, education, physical activity, previous use of hormonal contraceptives, smoking and alcohol consumption. Data from a 7-day pre-coded food diary were used to estimate daily energy intake (Furberg et al., 2005; Lillegaard et al., 2005). All questionnaires were checked for inconsistencies, and interview by one trained nurse was performed. Recall and memory-probing aids including a lifetime

calendar and a list of examples of milestones, were used to date the reproductive history events (Furberg et al., 2005; Emaus et al., 2008a).

Clinical parameters

All clinical procedures and measurements were conducted by trained nurses at the Clinical Research Center, University Hospital of North Norway (UNN), Tromsø. Each study participant came to the research center three times for clinical examination: first visit (Days 1–5 of the menstrual cycle), second visit (Days 7–12) and third visit (Days 21–25). The first visit was conducted on the first day possible after the onset of menstrual bleeding. Anthropometric measurements were taken with participants wearing light clothing and no footwear (Furberg et al., 2005; Finstad et al., 2009b). Body height was measured to the nearest 0.5 cm, and body weight to the nearest 0.1 kg on an electronic scale. BMI (kg/m²) was used to estimate relative weight. Waist circumference (WC, cm) was measured in a horizontal line 2.5 cm above the umbilicus, and hip circumference (HC, cm) was measured at the largest circumference of the hip (Finstad et al., 2009a). WC and HC (measured to the nearest 0.5 cm) were used to calculate Waist-to-Hip Ratio (WHR = WC/HC). Blood pressure (BP) was measured three times (PROPAQ 104), with the participants sitting in a resting position, and the mean of the final two measurements was used in the analysis.

Saliva hormone samples and analysis

Women collected daily morning saliva samples at home for one entire menstrual cycle starting on the first day of bleeding. Previously established collection protocols (Lipson and Ellison, 1996) were modified and developed for use (Furberg et al., 2005). Hormone assays were run in the Reproductive Ecology Laboratory at Harvard University, USA.

In each cycle, 17 β -estradiol was assayed for 20 days (reverse cycle days: –5 to –24) and progesterone was assayed for 14 days (reverse cycle days: –1 to –14), and all values were used in calculation of overall mean hormone concentrations for all participants. Salivary 17 β -estradiol and progesterone measurements were made using I-125 based radioimmunoassay (RIA) kits (Diagnostic Systems Laboratories, Webster, TX, USA) with published modifications to the manufacturer's protocols (Furberg et al., 2005). All samples were run in duplicate, and all samples from an individual were run in the same assay, with women randomly assigned to assays.

Saliva pools characterized by high or low hormone values (appropriate to the range of each steroid) were run in each assay. The sensitivity of the 17 β -estradiol assay (lowest value measurable by assay) was 4 pmol/l. Average intra-assay variability was 9% and inter-assay variability ranged from 23% for low pools to 13% for high pools. For progesterone, the sensitivity of the assay was 13 pmol/l. Average intra-assay variability was 10%, and inter-assay variability ranged from 19% for low pools to 12% for high pools.

Before statistical analysis of daily hormonal levels, all cycles were aligned at mid-cycle following published methods (Lipson and Ellison, 1996). Alignment was based on the identification of the mid-cycle 17 β -estradiol drop (aligned cycle Day 0), which provides a reasonable estimate of the day of ovulation. Identification of the mid-cycle 17 β -estradiol drop could not be made for 14 women, and they were not included in subsequent analyses. For the remaining 99 nulliparous and 91 parous women with aligned cycles, the following follicular and luteal hormonal indices were calculated: 'mid-follicular' (defined as the average of values for aligned cycle Days –10 to –6); 'late-follicular' (defined as the average of values for aligned cycle Days –5 to –1); 'luteal' (defined as the average of values for aligned cycle Days +2 to +9); 'mid-menstrual' (defined as the average of values for aligned cycle Days –7 to +6); 'mid-cycle' (defined as the average of values for aligned cycle Days –4 to +2). Maximum peak

level refers to the highest measured hormone value during the mid-menstrual index.

The 17 β -estradiol levels in saliva represent the free, unbound, biologically active fraction of the circulating steroid only, rather than the levels of both free and protein-bound 17 β -estradiol as in serum [i.e. bound to sex-hormone-binding globulin (SHBG) and albumin] (Ellison and Lipson, 1999). Furthermore, as saliva can readily be collected from individuals on many occasions, it is possible to compare 17 β -estradiol levels across entire menstrual cycles among different women, rather than relying on one or a few timed blood samples (Jasienska *et al.*, 2006b).

Serum lipid samples and analysis

Fasting serum blood samples were drawn from an antecubital vein in the morning on each of the three visits. Lipids were measured at the Department of Clinical Chemistry, University Hospital of North Norway using fresh sera from the first visit (Furberg *et al.*, 2005). Serum triglycerides were assayed by enzymatic hydrolysis with lipase. Serum cholesterol was determined enzymatically using cholesterol esterase and cholesterol oxidase. High-density lipoprotein cholesterol (HDL-C) was quantified by a direct assay using polyethylene glycol modified enzymes and dextran sulfate.

Ethical considerations

All participating women signed an informed consent form, and the study was approved by the Regional Committee for Medical Research Ethics and the Norwegian Data Inspectorate.

Statistical analysis

We used multivariable linear regression models to study whether parity and the timing of births in relation to age at menarche were associated with levels of salivary 17 β -estradiol and progesterone (STATA version SE 11.0). All hormone data were log transformed prior to the linear regression analyses: for presentation, all hormone values were transformed back to the original scale (geometric Means and 95% Confidence Intervals).

To study the associations between parity and 17 β -estradiol and progesterone, nulliparous women were compared with parous women and potentially confounding factors were taken into account on the basis of biological plausibility. Parity was included as a dichotomous (nulliparous versus parous), and a continuous (number of full-term childbirths) predictor variable in separate linear regression models. The following potentially confounding factors were assessed on the basis of biological plausibility: BMI, cycle length, age at menarche, smoking, alcohol, physical activity and previous use of oral contraceptives (OCs). However, only minor changes in the regression coefficient of parity were observed for each of these covariates in the models. Thus, age was the only covariate included in the final models. We elucidated possible effect modification and thresholds for variables of importance for the association between parity and ovarian hormones. Thus, we stratified by number of children (0, 1–2, ≥ 3), body composition: BMI (overweight, ≥ 25 kg/m²) and WC (Median, ≥ 77.75 cm), and previous OC use (Median ≥ 3 years). Generalized estimating equation (GEE) regression models were used to assess the association between daily salivary 17 β -estradiol concentrations and parity groups.

The parous women were further categorized into tertiles of the interval between age at menarche and age at first full-term birth: <10 years, 10–13.5 years and >13.5 years. The tertile groups were compared according to characteristics associated with breast cancer risk and/or fecundity. The 'menarche-to-first birth' time interval was included as a continuous and categorical predictor in different regression models. The same potentially confounding factors were evaluated as in the parity models, and only

minor changes in the regression coefficient of the 'menarche-to-first birth' interval were observed. Thus, age was the only covariate included in the final models. Linear and logistic regression analyses were used to assess linear trends over tertiles of the 'menarche-to-first birth' interval.

To study whether variation in age at participation and BMI modified the associations between ovarian hormones and the 'menarche-to-first birth' interval (tertiles), age and BMI were dichotomized by median split (33 years) and the cut off for overweight (≥ 25 kg/m²), respectively. Possible two-way interactions between the 'menarche-to-first birth' interval and age, BMI, WC, and OC use were assessed in separate models. GEE regression models were used to assess the associations between daily salivary 17 β -estradiol concentrations and groups of women in different 'menarche-to-first birth' intervals.

Area under the curve (AUC) for the time–salivary hormone concentration curves was calculated using the trapezium rule (Matthews *et al.*, 1990). Measurements of 17 β -estradiol for the 14 mid-menstrual days (aligned cycle Days –7 to +6), the 10 late follicular days (aligned cycle Days –10 to –1), the 7 mid-cycle days (aligned cycle Day –4 to +2) and of progesterone for the 8 luteal days (aligned cycle Days +2 to +9) were used in AUC calculations. Linear interpolation (i.e. the mean of the days immediately prior and following) was used to assign a value to days with missing values. If the missing value appeared at the end of the interval, the value from the day next to the missing value was used. One cycle for 17 β -estradiol and two cycles for progesterone were excluded from calculations due to two or more missing days at one of the ends of the interval. Among the 189 women included in the AUC analysis, the average number of missing values per cycle was 0.6 days for 17 β -estradiol in both parous and nulliparous women and 0.5 days (parous women) and 0.3 days (nulliparous women) for progesterone; both hormones had a range of 0–4 missing days per cycle. Linear regression was used to assess the differences in AUC between parous groups and tertiles of the 'menarche-to-first birth' interval.

Results

Parity and hormonal levels

The average age of nulliparous women ($n = 106$) was 29.2 years (range: 25.0–35.3) and the average age of parous women ($n = 98$) was 32.4 years (range: 24.9–35.9). Mean reported age at menarche was 13.2 years (range: 10.5–19.5) for nulliparous and 13.1 years (range: 9.20–17.0) for parous women (Table I). Mean age at first full-term pregnancy was 24.5 years (range: 16.0–32.0), and parous women had on average 1.9 children (range: 1–5). Compared with nulliparous women, parous women were older ($P < 0.001$), with a higher BMI ($P = 0.012$), larger WC ($P < 0.001$), lower HDL-C ($P = 0.049$) and had lower alcohol consumption ($P < 0.001$) (Table I).

There was no difference in overall mean salivary 17 β -estradiol level ($P = 0.31$) or overall mean salivary progesterone level ($P = 0.91$) between nulliparous and parous women (Table II) or between women who had given birth to one child compared with women who had given birth to multiple children (results not presented in table). We observed no difference in average daily level of salivary 17 β -estradiol throughout the entire menstrual cycle among three parity groups (nulliparous, 1–2 children, 3–5 children; $P = 0.57$, adjusted for age; Fig. 1A).

When subjects were stratified by BMI and parity, there was a difference in average salivary 17 β -estradiol levels throughout the entire

Table 1 Characteristics of the study population according to parity. The Norwegian EBBA-I study ($n = 204$)^a.

Characteristic	Parous ($n = 98^a$)		Nulliparous ($n = 106^a$)		P-value ^b
	Mean	(SD)	Mean	(SD)	
Age, years	32.4	(2.56)	29.2	(2.71)	<0.001
Education, total years	15.1	(3.09)	17.0	(2.63)	<0.001
Partnership, total years	8.16	(5.49)	2.95	(3.69)	<0.001
Body composition ^c					
Height, cm	167.1	(6.51)	166.6	(6.51)	0.58
BMI, kg/m ²	25.1	(3.83)	23.7	(3.58)	0.010
WC, cm	81.9	(10.1)	77.3	(8.97)	0.001
Waist to hip ratio	0.79	(0.06)	0.76	(0.06)	<0.001
Serum lipids, fasting					
Total cholesterol, mmol/l	4.51	(0.79)	4.39	(0.77)	0.25
HDL cholesterol, mmol/l	1.49	(0.34)	1.58	(0.32)	0.049
Triglycerides, mmol/l	0.87	(1.08)	0.87	(1.01)	0.99
Clinical measurements					
Systolic blood pressure (mmHg)	113.3	(12.1)	113.3	(10.3)	0.99
Diastolic blood pressure (mmHg)	71.0	(8.24)	70.8	(7.83)	0.87
Reproductive history					
Age at Menarche, years	13.1	(1.42)	13.2	(1.32)	0.67
Cycle length, days	27.8	(2.96)	28.6	(3.31)	0.61
Number of children	1.88	(0.88)			
Previous use of oral contraceptives, years	4.44	(3.85)	3.24	(3.46)	0.020
Time since last use of oral contraceptives, years	7.13	(4.47)	3.84	(3.47)	<0.001
Energy intake, kJ/day	8007	(1991)	8173	(1917)	0.53
Alcohol, units/week	2.85	(1.10)	3.56	(1.24)	<0.001
Current smokers, %		26.5		17.9	0.14
Physical activity in leisure time, %					
Sedentary activity		13.3		17.9	0.20
Moderate activity		64.3		54.7	
Regular activity		22.4		27.4	

Values are means (standard deviation, SD) and percents.

WC, waist circumference.

^aNumbers may vary due to missing information.

^bStudent's *t*-test or χ^2 test.

^cMeasurements at Day 1–5 after onset of menstrual cycle.

menstrual cycle between the four groups of women ($P = 0.016$, age-adjusted): parous women with BMI ≥ 25 kg/m² had a higher average daily level of salivary 17 β -estradiol throughout the entire menstrual cycle compared with both nulliparous women ($P = 0.021$, age-adjusted) and parous women ($P = 0.018$, age-adjusted) with BMI < 25 kg/m² (Fig. 1B). Also, nulliparous women with BMI ≥ 25 kg/m² had a higher average daily level of salivary 17 β -estradiol throughout the entire menstrual cycle when compared with nulliparous women with BMI < 25 kg/m² ($P = 0.039$, age-adjusted). Nulliparous women with WC ≥ 77.75 cm had higher average daily levels of salivary 17 β -estradiol throughout the entire menstrual cycle compared with nulliparous women with lower WC ($P = 0.017$, age-adjusted). There was a tendency of higher average daily levels of salivary 17 β -estradiol throughout the entire menstrual cycle also among

parous women with WC ≥ 77.75 compared with nulliparous women with lower WC ($P = 0.068$, age-adjusted; Fig. 1C).

Finally, when we stratified women by parity and OC use, we observed no difference in average levels of salivary 17 β -estradiol between the four groups of women defined by median split of the two variables ($P = 0.19$, age-adjusted). However, in subgroup analysis, nulliparous women with ≥ 3 years of OC use had higher average daily levels of salivary 17 β -estradiol throughout the entire menstrual cycle compared with nulliparous women with fewer years of OC use ($P = 0.050$, age-adjusted; Fig. 1D).

In an analysis using AUC, women with three to five full-term pregnancies had a tendency of higher 17 β -estradiol values for the mid-menstrual cycle and mid-cycle intervals, compared with nulliparous women and women with less than two full-term pregnancies

Table II Salivary ovarian hormone concentrations (age-adjusted geometric means, 95% CI) among parous and nulliparous women. The Norwegian EBBA-I study ($n = 204$)^a.

Ovarian steroids	Parous ($n = 98^a$)		Nulliparous ($n = 106^a$)		P-value ^b
	Mean	95% CI	Mean	95% CI	
17 β -estradiol, pmol/l					
Overall 17 β -estradiol	14.6	(13.0,16.4)	13.4	(12.0,14.9)	0.31
Mid-follicular 17 β -estradiol ^c	11.3	(9.53,13.3)	9.69	(8.27,11.4)	0.23
Late follicular 17 β -estradiol ^d	17.3	(15.2,19.7)	17.5	(15.5,19.8)	0.91
Overall Progesterone, pmol/l	94.2	(80.9,109.8)	95.6	(82.1,111.2)	0.91

CI, confidence interval.

^aNumber may vary due to missing information.

^bLinear regression with log transformed hormones as dependent variable.

^cAligned cycle Day -10, -6.

^dAligned cycle Day -5, -1.

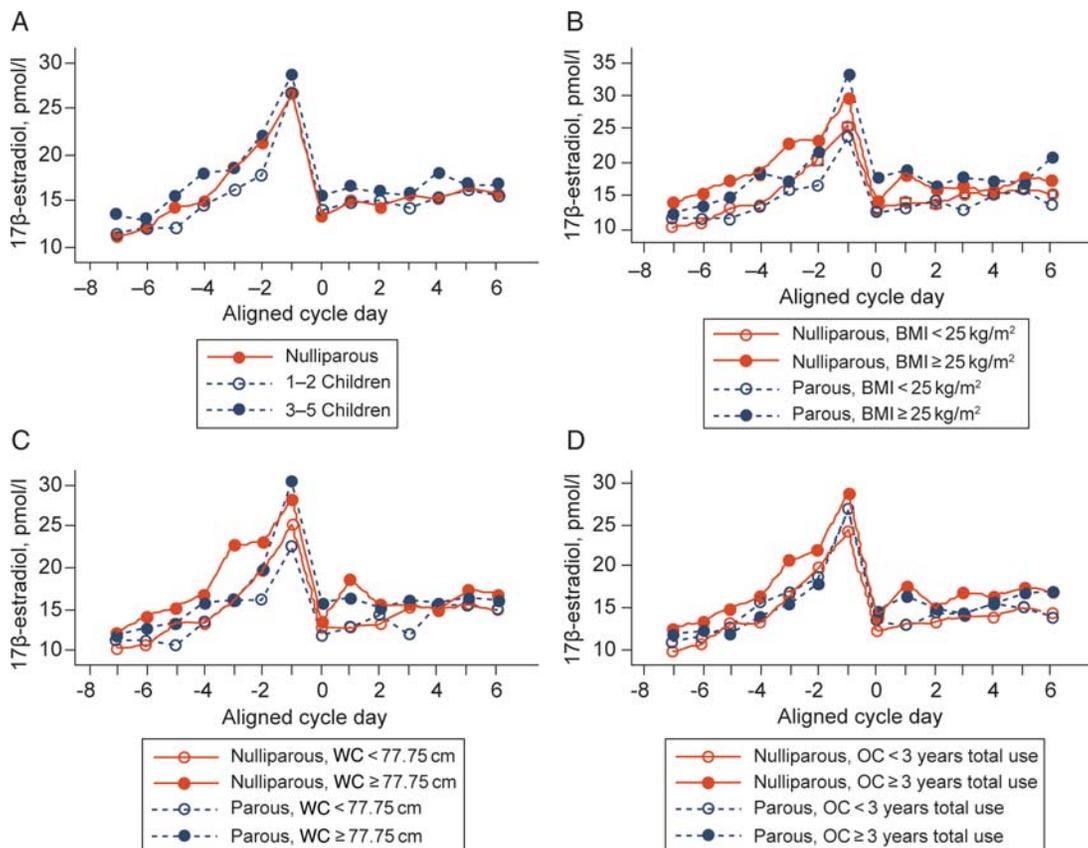


Figure 1 Daily salivary 17 β -estradiol concentrations (geometric means) in mid-menstrual cycle for women categorized by (A) number of children; nulliparous ($n = 99$), 1–2 children ($n = 74$), 3–5 children ($n = 17$), (B) BMI; nulliparous and BMI <25 kg/m² ($n = 70$), nulliparous and BMI ≥ 25 kg/m² ($n = 29$), parous and BMI <25 kg/m² ($n = 54$), parous and BMI ≥ 25 kg/m² ($n = 37$), (C) WC (median split); nulliparous and waist circumference <77.75 cm ($n = 61$), nulliparous and waist circumference ≥ 77.75 cm ($n = 38$), parous and waist circumference <77.75 cm ($n = 36$), parous and waist circumference ≥ 77.75 cm ($n = 55$), (D) OC use (median split); nulliparous and OC <3 years total use ($n = 55$), nulliparous and OC ≥ 3 years total use ($n = 44$), parous and OC <3 years total use ($n = 39$), parous and OC ≥ 3 years total use ($n = 52$).

Table III Estimated cumulative load of salivary ovarian steroids during menstrual cycle according to parity. The Norwegian EBBA-I study ($n = 189$)^a.

Ovarian steroids	Number of children								P-trend ^c
	0 ($n = 99$)		1 ($n = 36$)		2 ($n = 37$)		3–5 ($n = 17$)		
	Mean ^b	(95% CI)	Mean ^b	(95% CI)	Mean ^b	(95% CI)	Mean ^b	(95% CI)	
17 β -estradiol pmol/l									
Mid-menstrual ^d , 14 days	213	(192,236)	220	(205,236)	228	(202,257)	236	(195,286)	0.41
Mid-cycle ^e , 6 days	108	(97,120)	112	(104,121)	116	(102,130)	120	(98,145)	0.43
Late follicular ^f , 5 days	74	(63,93)	75	(69,81)	76	(67,86)	76	(63,93)	0.79
Progesterone, pmol/l									
Luteal ^g , 8 days	969	(866,1083)	934	(860,1013)	900	(789,1026)	867	(703,1070)	0.42

Values are area under curve (AUC, pmol/l).

CI, confidence interval.

^aNumber may vary due to missing information.

^bAge-adjusted geometric mean.

^cLinear regression with log transformed hormones as dependent variable.

^dAligned cycle Day -7,+6.

^eAligned cycle Day -4,+2.

^fAligned cycle Day -5,-1.

^gAligned cycle Day +2,+9.

(Table III). Nulliparous women also tended to have higher luteal progesterone values compared with all other women in the study, but the association was not statistically significant.

'Menarche-to-first birth' interval and hormonal levels

A shorter interval between menarche and first full-term pregnancy was associated with later age at menarche ($P_{\text{trend}} = 0.010$), younger age at first and last full-term pregnancy (both: $P_{\text{trend}} < 0.001$), higher parity ($P_{\text{trend}} = 0.002$), longer time since last birth ($P_{\text{trend}} < 0.001$) and fewer years of education ($P_{\text{trend}} = 0.001$) (Table IV).

Overall mean salivary level of 17 β -estradiol was inversely related to the length of the time interval between menarche and first birth ($P_{\text{trend}} = 0.029$, age-adjusted, Table V). Overall mean salivary progesterone level was not related to the length of the 'menarche-to-first birth' interval ($P_{\text{trend}} = 0.34$, age adjusted). The inverse relationship between 'menarche-to-first birth' interval and 17 β -estradiol was observed across different weight categories (BMI cut-off for overweight, 25 kg/m²), age groups (median split, 33 years) and age at menarche (median split, 13.0 years) (data not shown). The age-adjusted geometric mean for maximum peak salivary 17 β -estradiol level was 33.0 pmol/l (95% CI, 27.3–39.9) in the lower tertile of 'menarche-to-first birth' interval, 27.2 pmol/l (95% CI, 24.4–30.4) in the mid tertile and 22.5 pmol/l (95% CI, 19.0–26.6) in the upper tertile, equaling a 47% higher maximum peak level of 17 β -estradiol for women with the shortest 'menarche-to-first birth' interval when compared with women with the longest interval (data not shown in tables).

When analyzing the 'menarche-to-first birth' interval as a continuous predictor variable in age- and BMI-adjusted linear regression models, we observed a 2.6% ($P = 0.039$) decrease in overall average salivary 17 β -estradiol with each 1-year increase in the interval

(results not shown). We examined the mean salivary 17 β -estradiol concentrations by cycle day and observed a difference among the three 'menarche-to-first birth' interval groups (lower tertile: <10 years; middle tertile: 10–13.5 years; upper tertile: >13.5 years; $P = 0.010$, age-adjusted; Fig. 2).

In analysis of AUC, women with the shortest time interval between menarche and first birth (<10 years) had ~30% higher mid-cycle 17 β -estradiol levels ($P_{\text{trend}} = 0.050$, age-adjusted) compared with women with the longest intervals (>13.5 years) (Table VI). There was no difference in progesterone values between women in different 'menarche-to-first birth' tertiles ($P_{\text{trend}} = 0.99$, age-adjusted).

Discussion

In our study of full cycle profiles of free 17 β -estradiol and progesterone among healthy regularly cycling women, we observed no overall association with parity. Interestingly, however, larger waist and longer-term use of OCs were associated with higher daily levels of 17 β -estradiol throughout the entire menstrual cycle among nulliparous women. Furthermore, a strong inverse association between the time interval from menarche to first full-term birth and daily salivary 17 β -estradiol levels over an entire menstrual cycle among young healthy women with regular menstrual cycles was observed. Women with the shortest 'menarche-to-first birth' interval had 47% higher maximum peak level and 30% higher mid-cycle 17 β -estradiol levels compared with the women with the longest interval.

Several previous studies have documented that positive energy balance (Furberg et al., 2005), low physical activity (Verkasalo et al., 2001; Jasienska et al., 2006c; Tworoger et al., 2007) and higher energy resources (Ziomkiewicz et al., 2008) have a positive effect on levels of reproductive ovarian steroids, which in turn improve chances for conception (Lipson and Ellison, 1996; Venners et al., 2006). There are however very limited data on the association

Table IV Characteristics of the study population according to length of the 'menarche-to-first birth' interval (tertiles). The Norwegian EBBA-study (n = 98)^a.

Characteristic	Interval between menarche and age at first birth, years						P- trend ^b
	<10 years (n = 30)		10–13.5 years (n = 35)		>13.5 years (n = 33)		
	Mean	(SD)	Mean	(SD)	Mean	(SD)	
Age, years	32.0	(3.10)	32.0	(2.54)	33.0	(1.90)	0.12
Education, total years	13.3	(2.93)	15.6	(2.33)	16.0	(2.30)	0.001
Partnership, total years	9.54	(6.78)	7.35	(5.10)	7.80	(4.51)	0.26
Body composition ^c							
Height, cm	165.9	(5.26)	169.5	(6.96)	165.8	(6.37)	0.76
BMI, kg/m ²	24.8	(3.31)	25.1	(4.10)	25.2	(4.04)	0.77
WC, cm	81.4	(8.11)	83.0	(10.90)	81.2	(11.15)	0.94
Waist to hip ratio	0.80	(0.06)	0.79	(0.06)	0.77	(0.07)	0.07
Reproductive history							
Age at Menarche, years	13.4	(1.22)	13.3	(1.27)	12.5	(1.61)	0.010
Cycle length, days	27.5	(3.00)	28.0	(3.38)	27.8	(2.48)	0.62
Number of children	2.30	(0.95)	1.80	(0.90)	1.61	(0.66)	0.002
Age at first birth, years	20.1	(2.05)	24.8	(1.71)	28.2	(2.26)	<0.001
Age at last birth, years	25.6	(3.29)	27.4	(2.96)	29.7	(2.35)	<0.001
Time since last birth, years	6.41	(3.37)	4.61	(3.12)	3.31	(1.76)	<0.001
Total breastfeeding, months	21.3	(12.76)	17.8	(14.73)	18.9	(9.27)	0.46
Previous use of oral contraceptives, years	4.00	(3.71)	4.71	(3.77)	4.56	(4.13)	0.58
Time since last use of oral contraceptives, years	7.58	(5.42)	7.10	(4.13)	6.71	(3.86)	0.47
Energy intake, kJ/day	7866	(1760)	8145	(2131)	7988	(2084)	0.82
Alcohol, units/week	2.67	(1.01)	3.08	(1.28)	2.77	(0.91)	0.76
Current smokers, %		43.3		20.0		18.2	0.043
Physical activity in leisure time, %							
Sedentary		20.0		8.60		12.1	0.95
Moderate		53.3		74.3		63.6	
Regular		27.7		17.1		24.3	

Values are means (standard deviation, SD) and percents.

WC, waist circumference.

^aNumber may vary due to missing information.

^bLinear regression or logistic regression.

^cMeasurements at Day 1–5 after onset of menstrual cycle.

between parity and ovarian hormone levels. The present observation supporting no overall associations between levels of 17 β -estradiol and progesterone with parity is in agreement with those of former studies (Verkasalo *et al.*, 2001) and importantly, this may lead to further questions related to interacting predisposition and the need of more detailed studies.

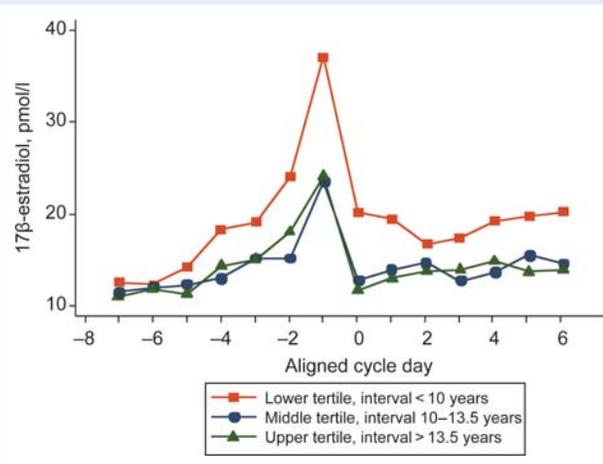
Interestingly, nulliparous women with larger waist circumference had higher salivary 17 β -estradiol levels compared with nulliparous women with a more narrow waist circumference, supporting that nulliparous women may be more susceptible to lifestyle factors influencing energy balance, abdominal overweight, and metabolic and hormonal profiles. Correspondingly, a positive linear relationship between body fat and estradiol levels throughout an entire menstrual cycle was observed in a parallel study among premenopausal Polish

women (Ziomkiewicz *et al.*, 2008). Furthermore, regulation of ovarian hormone levels by nutritional status has been suggested, for example, in studies of women with anorexia nervosa (Miller *et al.*, 2004), and in studies of women in rural communities with seasonal variation in workload (Panter-Brick *et al.*, 1993). Accumulation of excessive abdominal fat is related to insulin resistance with hyperinsulinemia. Insulin stimulates ovarian steroidogenesis and inhibits the hepatic synthesis of SHBG, leading to increased levels of free estradiol (Verkasalo *et al.*, 2001; IARC, 2002; Finstad *et al.*, 2009b). This may explain the positive relation between waist circumference and free 17 β -estradiol levels seen in our study, contrary to other studies that have reported inverse associations between waist circumference and total estradiol and its main binding protein, SHBG. However, adjustment for serum SHBG measured at the first visit did not change our

Table V Salivary ovarian hormone concentrations (age-adjusted geometric means, 95% CI) among women in groups of 'menarche-to-first birth' interval (tertiles). The Norwegian EBBA-I study ($n = 98$)^a.

Ovarian steroids	Interval between menarche and age at first birth, years						P-trend ^b
	<10 years ($n = 30$)		10–13.5 years ($n = 35$)		>13.5 years ($n = 33$)		
	Mean	(95% CI)	Mean	(95% CI)	Mean	(95% CI)	
17 β -estradiol, pmol/l							
Overall 17 β -estradiol	16.2	(13.8,19.3)	14.1	(12.7,15.6)	12.2	(10.4,14.3)	0.029
Mid-follicular 17 β -estradiol ^c	11.0	(8.68,14.0)	10.3	(8.98,11.8)	9.63	(7.67,12.1)	0.47
Late follicular 17 β -estradiol ^d	18.7	(15.2,23.0)	16.8	(15.0,18.9)	15.1	(12.7,18.1)	0.17
Overall Progesterone, pmol/l	99.2	(77.9,126.3)	90.6	(78.7,104.3)	82.8	(65.9,103.9)	0.34

CI, confidence interval.

^aNumber may vary due to missing information.^bLinear regression with log transformed hormones as dependent variable.^cAligned cycle Day $-10, -6$.^dAligned cycle Day $-5, -1$.**Figure 2** Daily salivary 17 β -estradiol concentrations (geometric means) in mid-menstrual cycle for women categorized by tertiles of interval length from menarche to age at first full term birth. Lower tertile; interval <10 years ($n = 26$). Middle tertile; interval 10–13.5 years ($n = 32$). Upper tertile; interval >13.5 years ($n = 33$).

estimates (analysis not presented). In addition, obesity may lead to anovulatory cycles and increased conversion of androgens to estrone in adipose tissue, while the EBBA women were mostly non-obese with normal levels of serum lipids (Furberg et al., 2005). Therefore, our findings support that long-term positive energy balance reflected by larger waist circumference may increase level of free 17 β -estradiol in women with regular ovulations, particularly in nulliparous women.

In our study, longer-term use of OCs was associated with higher levels of 17 β -estradiol throughout the entire menstrual cycle among nulliparous women. Current use of OCs influences endogenous sex hormone levels, while the association between former use of OCs and circulating levels of 17 β -estradiol in adult women is poorly documented. Our findings suggest that the ovarian function in nulliparous women may be more susceptible to long-term suppression by

exogenous hormones and possible boosting of 17 β -estradiol production after cessation of the pill—we hypothesize that OC exposure may change the physiological set point for the regulation of endogenous hormone levels especially among women that have not experienced a full-term pregnancy. Prospective studies are needed to determine the stability of the hypothalamic–pituitary–ovarian axis phenotype. It might also be that OC use itself is a behavior partially determined by endogenous hormone levels or hormone-related factors.

The present observations that parous women with shorter 'menarche-to-first birth' intervals had a higher parity, lower age at first birth, and higher salivary 17 β -estradiol levels than women with longer intervals are indirectly supported by others. Associations between early age at menarche and higher estradiol levels (Bernstein et al., 1991; Emaus et al., 2008a), and higher frequencies of ovulation (Apter et al., 1989) have been observed. Higher energy resources associated with higher estrogens within normal range improve chances for conception (Lipson and Ellison, 1996; Venners et al., 2006). Higher follicular levels of estradiol have been observed in healthy women's menstrual cycles resulting in conception, compared with cycles without conception (Lipson and Ellison, 1996; Venners et al., 2006). Furthermore, the pattern of human sexual behavior is complex but may be explained partly by estradiol levels (Pawlowski and Jasienska, 2005; Durante and Li, 2009). An elevated level of estradiol may lead to more frequent sexual activity, thereby increasing the likelihood of fertilization and parity (Durante and Li, 2009). In the EBBA women, early age at first birth is the main determinant of a shorter menarche-to-first-birth interval rather than late age at menarche—this could be the consequence of conscious choice or a consequence of higher fecundity, or both. On the basis of our observations, we hypothesize that childbearing pattern (i.e. delayed child-births) in this female population is partly determined by variation in fecundity which again is partly determined by genetic variation in proteins regulating ovarian function as well as gene–environment (i.e. socio-cultural) interactions. Further studies are needed to explore this hypothesis.

Several studies have documented an increase in risk of breast cancer with elevated serum estradiol levels in post-menopausal

Table VI Estimated cumulative load of salivary ovarian steroids during menstrual cycle according length of the 'menarche-to-first birth' interval (tertiles). The Norwegian EBBA-I study (n = 90)^a.

Ovarian steroids	Interval between menarche and age at first birth						P-trend ^c
	<10 years (n = 29)		10–13.5 years (n = 31)		>13.5 years (n = 30)		
	Mean ^b	(95% CI)	Mean ^b	(95% CI)	Mean ^b	(95% CI)	
17 β -estradiol, pmol/l							
Mid-menstrual ^d , 14 days	241	(205,286)	218	(197,242)	197	(167,232)	0.11
Mid-cycle ^e , 6 days	125	(106,148)	110	(99,122)	96	(82,114)	0.050
Late follicular ^f , 5 days	80	(67,96)	72	(65,80)	65	(55,77)	0.12
Progesterone, pmol/l							
Luteal ^g , 8 days	857	(700,1049)	858	(757,971)	859	(703,1048)	0.99

Values are area under curve (AUC, pmol/l).

CI, confidence interval.

^aNumber may vary due to missing information.

^bAge adjusted geometric mean.

^cLinear regression with log transformed hormones as dependent variable.

^dAligned cycle Day -7,+6.

^eAligned cycle Day -4,+2.

^fAligned cycle Day -5,-1.

^gAligned cycle Day +2,+9.

women (Endogenous Hormones and Breast Cancer Collaborative Group, 2002; Prentice *et al.*, 2008). There is evidence for a similar relationship also in premenopausal women (Eliassen *et al.*, 2006) even though the fluctuating level of hormones during the menstrual cycle complicates testing of the hypothesis in premenopausal women (Kaaks *et al.*, 2005).

Our observations suggest that nulliparity in combination with previous OC use and positive energy balance increases the 17 β -estradiol levels associated with increased breast cancer risk. Greater BMI and obesity are associated with decreased breast cancer risk in studies of premenopausal women (Friedenreich, 2001; Berstad *et al.*, 2010) while both current and former OC users have an increased risk of breast cancer in recent studies (Lund *et al.*, 2007; Hunter *et al.*, 2010). Low parity is a well-established risk factor for breast cancer (MacMahon *et al.*, 1970; Braaten *et al.*, 2004; World Cancer Research Fund/American Institute for Cancer Research, 2007). A few studies have suggested that giving birth before age 20 is associated with lower risk of breast cancer (MacMahon *et al.*, 1970), whereas first birth at ages above 35 increases breast cancer risk (Trichopoulos *et al.*, 1983), possibly due to increased likelihood of malignant transformations in breast cancer cells in older women. In general, massive differentiation of epithelial breast cells in response to the increased release of ovarian hormones during pregnancy has been proposed as an explanation for both the elevated short-term breast cancer risk after pregnancy, and the extended protective effect of pregnancy (Lambe *et al.*, 1994; Albrektsen *et al.*, 2010).

Furthermore, the longer period of time during which immature breast epithelium cells are exposed to estradiol may cause an elevated risk (Russo *et al.*, 1982; Pike *et al.*, 1993) pointing to the interval between age at menarche and age at first birth, relevant for breast cancer risk (Li *et al.*, 2008; McDougall *et al.*, 2010), and estradiol-receptor-positive tumors in particular (Li *et al.*, 2008). A strong inverse association between the time interval 'menarche-to-first birth' and levels of these hormones suggests that there may

be biological mechanisms other than the ovarian steroid pathway that underlie the associations between parity, age at first birth and breast cancer risk. A recent study suggested that a single full term pregnancy decreased the levels of circulating growth hormones (GH), shown to play a role in breast carcinogenesis with insulin-like growth factor I (IGF-I), thus pointing at another potential biological mechanism for protection from breast cancer (Dearth *et al.*, 2010). Further studies are required to fully understand the mechanisms underlying the association between reproductive history and risk of breast cancer.

Our study has the benefit of having collected samples every day over an entire menstrual cycle, rather than only on selected days within a cycle. This allows for estimation of daily free, unbound and biologically active 17 β -estradiol and progesterone, and for high quality estimates of full cycle ovarian hormone profiles (Jasisenska and Jasienski, 2008). Furthermore, well-developed and validated methods and assays were used to characterize the women's exposure to free biologically active ovarian steroids and compare levels by aligned cycle days (Lipson and Ellison, 1996). In addition, salivary levels of 17 β -estradiol were shown to be quite stable within participants over time (Ellison and Lipson, 1999). Salivary estradiol represents about 1% of the total circulating estradiol, and therefore the absolute values are small in comparison to the serum estradiol levels used in clinical practice. The salivary 17 β -estradiol profiles for the EBBA women closely resemble the well-known physiological pattern for cyclic variation in estradiol (Speroff and Fritz, 2005), and large relative differences between individuals were observed (i.e. 47% higher maximum peak level of 17 β -estradiol for women with the shortest 'menarche-to-first birth' interval as compared with women with the longest interval). Furthermore, a study evaluating estradiol levels in daily saliva samples for two consecutive menstrual cycles among 12 women observed higher variance in mean estradiol levels between women than between cycles from the same woman, suggesting that a

single menstrual cycle will give reliable estimates in the analysis of interindividual differences (Gann et al., 2001).

The main independent variables in our study (i.e. age at menarche, age at first full-term birth and parity) represent reproductive life milestones, and the women were 25–35 years at participation—these aspects facilitate high reliability of the self-reported data. Furthermore, to minimize false memory of exposures, each woman was interviewed by a trained nurse actively using a detailed lifetime calendar and a list of examples of milestones. In a previous study of the EBBA women, we have shown that age at menarche is associated with 17 β -estradiol levels (Emaus et al., 2008a). Also, self-reported birthweight showed to be nearly identical with the birthweight registered at the Medical Birth Registry of Norway (Emaus, 2009).

Our results indicate that lifestyle factors including OC use, excess weight and timing of first birth are associated with ovarian steroid levels among regularly cycling women. Interestingly, long-term positive energy balance may increase 17 β -estradiol levels in women with regular ovulations, particularly in nulliparous women. Furthermore, fecundity seems to play a significant role in timing of first childbirth. These findings demonstrate the complexity of the relationships among reproductive factors, levels of ovarian hormones, fecundity and breast cancer risk.

Authors' roles

A.-S.F. participated in collecting the data, suggested the hypotheses, supervised the statistical analyses and manuscript writing. A.M. played a role in data analysis, interpretation and manuscript writing. G.J. was involved in designing the study (in collaboration with Dr I Thune and Dr P. Ellison) and manuscript writing. P.E. designed the study in collaboration with Dr I. Thune and Dr G. Jasienska. T.W. Statistical advisor: supervised the statistical analyses and manuscript writing. V.F., S.E.F. and S.L. commented on the hypotheses and manuscript writing. A.E. participated in collecting the data and manuscript writing. I.T. (the principal investigator of the EBBA-I) designed the study, supervised the statistical analyses and drafted the manuscript. A.I. developed the hypotheses (in collaboration), cleaned the data, performed the statistical analyses and drafted the manuscript.

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