Ex vivo measurement of cell apoptosis and proliferation in breast tissue of healthy women: Influence of age and steroid status. An exploratory study

Dear Editors,

Hormonal conditions such as early menarche, late menopause, and postmenopausal hormone therapy have been associated with an increased risk of developing breast cancer in epidemiological as well as clinical studies [1,2]. Non breast feeding mothers have also been reported to suffer an increased risk [2], whereas studies on oral contraceptive use so far have not demonstrated an increased risk [3]. The most plausible cause of the elevated risk associated with these conditions is the level of oestrogen exposure, either due to a higher level or to a longer duration of exposure. Some evidence suggests that oestrogens are not only mitogenic but also have mutagenic effects [4].

Randomised controlled trials found no increased breast cancer risk after unopposed oestrogen therapy [5], whereas continuous combined oestrogen plus progestogen therapy increased breast cancer risk [6] in postmenopausal women. These observations strongly suggest that progestogens, when combined with oestrogens, play a crucial role in hormonally induced breast cancer.

The delicate balance between apoptosis and proliferation determines uncontrolled growth expansion or inhibition of normal breast and breast cancer cells. Previously, we demonstrated that some progestogens, alone or combined with oestradiol, stimulate proliferation of breast cancer cells, whereas others may induce apoptosis [7].

The present study was designed to explore the influence of different hormonal factors as well as age on both apoptosis and proliferation rates in breast tissue in healthy women. Therefore, normal mammary tissue was obtained from 46 healthy women (mean age 33.5 years, standard deviation (S.D.) 13.0 years), undergoing breast reduction surgery.

One to two cubic centimeter of breast tissue was collected from mammary tissue and kept in RNA *later* (RNA stabilization reagent, Qiagen, Hilden, Germany). The tissue was cut in very small pieces and left overnight at 4 °C in RNA *later*. Thereafter, RLT buffer (Qiagen), containing β-mercaptoethanol (Sigma; Saint Louis, MO, USA) was added to the small pieces of breast tissue, and samples were stored at −80 °C until isolating RNA.

Cell proliferation was measured by quantification of the expression of Cyclin D1 mRNA in quadruplicate. The mRNA was measured quantitatively using a competitive reverse transcription-polymerase chain reaction (RT-PCR) technique as described by Marx et al. [8]. RNA was isolated using the QIAamp RNA Blood Kit (Qiagen). cDNA was synthesized and the amount of Cycline D1 mRNA was determined using a TaqMan based real-time RT-PCR technique. The intra-assay variability was 3.1% and the inter-assay variability was 4.6%.

Apoptosis was measured by quantification of the expression of tissue transglutaminase (tTG) mRNA in quadruplicate. tTG is a transamidating enzyme which catalyses the cross-linking reactions of intracellular proteins. tTG is activated during the late stages of apoptosis and plays a key role in the formation of apoptotic bodies [9]. The mRNA was measured quantitatively using the RT-PCR technique. RNA was isolated using the QIAamp RNA Blood Kit (Qiagen). cDNA was synthesized and the amount of tTG mRNA was determined using a TaqMan based real-time RT-PCR technique. The intra-assay variability was 2.9% and the inter-assay variability was 3.7%.

Standard statistical tests were used to test comparisons for age, menstrual cycle day and cycle phase, parity, history of breast feeding, oral contraceptive use, and menopausal status. We used a linear regression analysis model to assess the potential influence of the hormonal variables on rates of apoptosis and proliferation and the calculated ratio.

Descriptive characteristics of the study population are given in Table 1. No statistically significant influence of any of the variables studied was found in relation to cell apoptosis and proliferation rates or the apoptosis/proliferation ratio. Nevertheless, our observations suggest a two-fold higher median apoptosis/proliferation ratio

Table 1
Descriptive characteristics

	Mean ± S.D.	Median	Range
Age (total population) (years)	33.5 ± 13.0	33.0	17–57
Premenopausal women, *n*	38		
Age (years)	29.2 ± 9.9	26.5	17–47
Menstrual cycle days 1–14[a], *n*	6		
Menstrual cycle days 15–28[a], *n*	12		
Hormonal contraceptive users[b], *n*	15		
Postmenopausal women, *n*	8		
Age (years)	53.8 ± 2.1	53.5	51–57
Parous women, *n*	24		
AP ratio	1.47 ± 1.34	0.98	0.17–5.64
Premenopausal women	1.49 ± 1.37	1.04	0.51–2.34
Postmenopausal women	1.38 ± 1.28	0.97	0.25–3.94
Menstrual cycle days 1–14	0.71 ± 0.44	0.51	0.35–1.28
Menstrual cycle days 15–28	1.55 ± 1.37	1.10	0.27–4.51
Parous women	1.65 ± 1.51	0.98	0.25–5.64
Non-parous women	1.34 ± 1.15	1.09	0.17–4.44
OC users[c]	1.41 ± 1.51	0.72	0.17–5.64
OC non-users[d]	1.43 ± 1.20	1.26	0.27–4.51
Breast feeders	1.77 ± 1.81	0.96	0.35–5.64
Non breast feeders	1.32 ± 1.10	1.09	0.17–4.44

Total population: $n = 46$; S.D. = standard deviation; n = number; AP ratio = apoptosis/proliferation ratio.

[a] Missing data cycle phase for four women not on hormonal contraception.

[b] Oral contraceptive: $n = 14$; intramuscular contraceptive: $n = 1$.

[c] 14 premenopausal women.

[d] 22 premenopausal women.

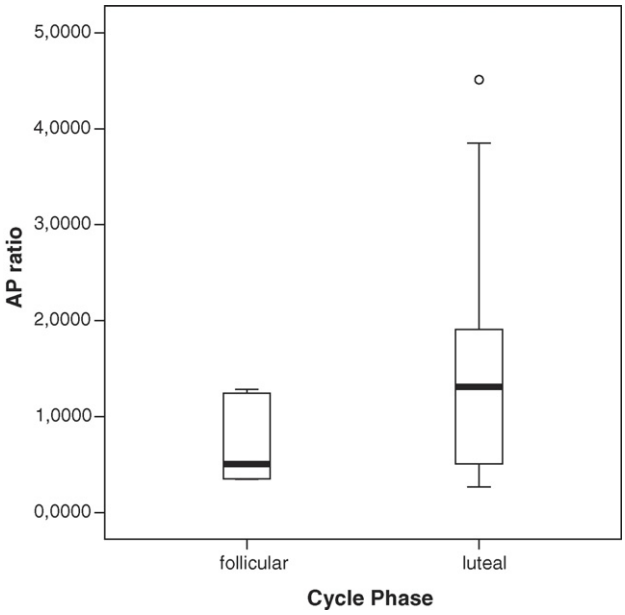

Fig. 1. Apoptosis/proliferation (AP) ratio for menstrual cycle phase. Box and Whisker plots: boxes represent 25th and 75th percentile with the median, ends of whiskers indicate 10th and 90th percentile of the AP ratio. Dots are outliers. Follicular: menstrual cycle days 1–14; luteal: menstrual cycle days 15–28. Menstrual cycle days 1–14 vs. menstrual cycle days 15–28: $p = 0.111$ (Mann–Whitney U test).

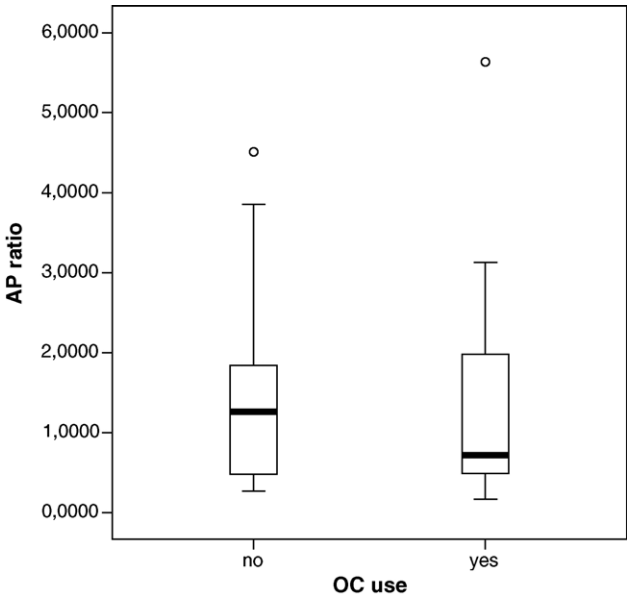

Fig. 2. Apoptosis/proliferation (AP) ratio for oral contraceptive (OC) use. Box and Whisker plots: boxes represent 25th and 75th percentile with the median, ends of whiskers indicate 10th and 90th percentile of the AP ratio. Dots are outliers. OC users versus non-users: $p = 0.673$ (Mann–Whitney U test).

during days 15–28 compared with days 1–14 of the menstrual cycle (1.10 versus 0.51; $p = 0.111$; Fig. 1). A slightly lower ratio was observed in oral contraceptive users compared with non-users (0.72 versus 1.26; $p = 0.673$; Fig. 2). None of these observations was however statistically significant.

The small number of subjects as well as the heterogeneity of the study population probably explains the lack of statistical significance of our findings. Other shortcomings of this exploratory study are the lack of information on cycle length, and on hormonal status of the individual cases (serum gonadotrophin, oestradiol and progesterone concentrations). For future well-powered studies it would be most useful to extend the investigations with (semi)quantitative determination of receptors in tissue samples, and including other groups of women, e.g. with benign and premalignant breast pathology.

The observed trend toward a higher apoptosis/proliferation ratio during the menstrual cycle days 15–28 fits well with previous observations showing a higher cell turnover in breasts of healthy women operated during the luteal phase of the menstrual cycle [10]. This is also in line with our previous in vitro experiments in which we demonstrated that natural progesterone, in contrast with several synthetic progestogens, induces apoptosis when combined with oestradiol [7]. The observed small difference between users and non-users of oral contraceptives is also in line with this.

To conclude, in this small exploratory study we found some indication for an increase in apoptosis/proliferation ratio when oestradiol is combined with natural progesterone, whereas synthetic progestogens as used in oral contraceptives have no, or even a negative, impact. No influence of age or menopausal status was observed.

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Pelvic echinococcosis in differential diagnosis of pelvic masses

Dear Editors,

Hydatid disease, or echinococcosis, is a parasitic infection caused by echinococcus larvae. Although echinococcosis is endemic in certain regions of the world, it can be found almost anywhere due to migration and increased travelling. Echinococcus granulosus is the most common type and endemic in the Mediterranean, Middle East, Eastern Europe and South America.

Humans are intermediate hosts with sheep, cattle, goats and camels which develop cysts after ingesting the parasite's eggs. The definitive hosts are dogs, wolves, foxes and jackals passing the eggs of the parasite in their feces. Echinococcus cysts are found mostly in the liver (60%) and lung (15%), but they can be located in any part of the body. Pelvic echinococcosis is rare with an incidence between 0.2% and 0.9% [1]. Nearly 80% of all pelvic cases involve the genital area, the ovary being the most frequent location, followed by the uterus [1]. These cases are usually secondary to the accidental rupture of a cyst in other areas of the body [2].

A 50-year-old, gravida 3, para 3 woman was admitted to our hospital with chronic pelvic and abdominal pain. In her medical history, she had hypertension for 5 years treated by ACE inhibitor and ovarian cancer history in her family. Her physical examination revealed tenderness in right lower quadrant of the abdomen. She was in menopause for 6 years. On bimanual examination, a right adnexial mass approximately 15 cm in diameter was palpated. In laboratory tests, CBC and tumor marker levels were normal. Also biochemical parameters were within normal ranges except a slight increase in ALP (ALP: 308 U/L (<240)).

Transvaginal ultrasound examination showed a well-defined, multicystic mass with hyperechogenic solid components in right adnexial region 18 cm × 9 cm × 6 cm in size.

On exploratory laparotomy, a 15 cm × 10 cm × 7 cm in size cystic mass was found adhering to the right pelvic side wall and pushing the right ureter to the medial. When exploring the cyst, the cyst ruptured due to adhesions and the cyst content was sent to the pathology for frozen-section examination. Frozen-section reported scolexes consistent with cyst hydatic (Fig. 1). Then the cavity was filled with a 0.9% NaCl solution to kill any remaining alive scolexes. Before the operation, the patient was informed about the fact that the mass might be benign or malign. However, she insisted on total abdominal hysterectomy and bilateral salpingooopherectomy because of ovarian cancer history in her family. So total abdominal hysterectomy and bilateral salpingooopherectomy was done.

Pathology report confirmed the diagnosis of cyst hydatic. In microscopical findings, chronic cervicitis, nabothian cysts, atrophic endometrium, one intramural leiomyoma and adenomyozis were detected.

Postoperative abdominal CT showed a hypodense cyst in the right lobe of the liver with septates and calcifications,

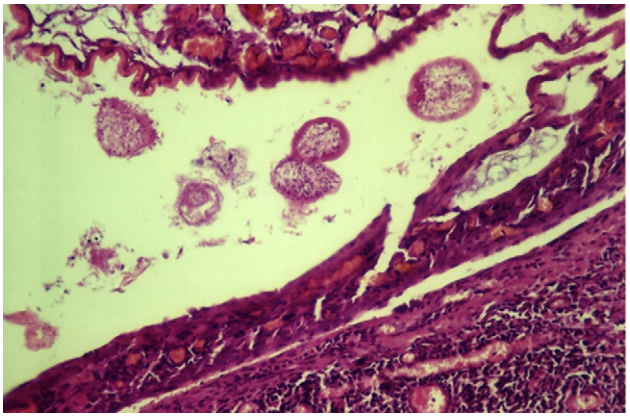

Fig. 1. Scolexes around ovarian tissue suggesting pelvic echinococcosis. HE 100×.