accomplished with diaminobenzidine substrate solution. The sections were then counterstained with methyl green, cleared, and mounted. Sections treated with DNAse I enzyme were used as positive controls (Amersham, Cleveland, OH, USA), and sections from which TdT enzyme was omitted were used as negative controls. Adjacent sections were used for Ki-67 immunohistochemical staining. Sections were deparaffinized, hydrated, incubated with proteinase K, washed in distilled water and PBS, and incubated with 3% H_2O_2 as above. Blocking serum was applied for 20 min and sections were then sequentially incubated in primary mouse anti-human Ki-67 antibody (1:100, clone Ki-S5; Dako, Carpinteria, CA, USA) for 1 hour, biotinylated rabbit anti-mouse IgG (Dako LSAB kit) for 12 min, and ABC reagent (avidin and biotinylated horseradish peroxidase complex) for 30 min (ABC kit; Vector, Burlingame, CA, USA). Detection was accomplished with diaminobenzidine substrate solution until the desired staining intensity was obtained (3 to 5 min). The sections were then counterstained with hematoxylin and eosin, cleared, and mounted.

Quantitative evaluation of Ki-67 expression and TUNEL positivity was performed in epithelial cells from the anatomically normal mammary gland (without any fibrocystic changes), including central and peripheral ducts within the lobules; myoepithelial and lymphoid cells were excluded. Cells were counted as apoptotic only if they were TUNEL-positive and showed characteristic nuclear morphology typical of apoptosis (that is, cells containing pyknotic nuclei plus apoptotic bodies). Successive counts, performed by individuals blinded to the groups, were made until 1,000 cells per tissue sample had been examined. Two indices were thus obtained: the proliferation index (PI), defined as the number of Ki-67-positive nuclei per 1,000 epithelial cells, and the AI, defined as the number of TUNEL-positive cells per 1,000 epithelial cells counted. From these values the cell renewal index (CRI = PI/AI) was obtained.

Statistical analysis

Statistical analyses were performed with analysis of variance for continuous data followed by Student's *t*-test, and with non-parametric tests (Mann–Whitney *U* test) for non-continuous data (gestation and parity). Fisher's exact test was used to verify the homogeneity of the samples relative to nursing history. The variation in frequency of proliferation and apoptosis relative to variations in the menstrual cycle as well as the variation in frequency of proliferation and apoptosis relative to the progesterone levels were measured with linear regression models and fourth-degree polynomial curves (Polnom Program; University of Manchester, Manchester, UK) as indicated. All data are expressed as means ± SD; $P < 0.05$ was considered significant.

Results

There were no differences between patients in group A (follicular phase) and group B (luteal phase) in terms of age of menstruation onset (12.8 ± 1.3 and 12.7 ± 1.4, respectively), number of pregnancies, parity, and lactation history.

The mean PI for group A was 13.5 ± 9.8, significantly smaller than in group B, which had a PI of 30.5 ± 22 ($P = 0.003$, by Student's *t*-test). Photomicrographs from representative patients showing Ki-67 expression for both groups are shown in Fig. 1a,b. The AI for both groups was very similar: group A (4.4 ± 1.8) and group B (5.2 ± 2.4; $P = 0.21$). TUNEL-positive cells from representative patients for each group are given in Fig. 1C–E; Fig. 1f shows a highly magnified TUNEL-positive cell displaying the characteristic features of chromatin condensation and apoptotic bodies. The CRI for group A (3.8 ± 3.4) was also significantly smaller than that for group B (7.4 ± 6.6; $P = 0.03$).

The data show that, in a 28-day interval, the number of proliferative, apoptotic, and cell renewal events vary as a function of time. The linear regressions for each one of the indices are shown in Fig. 2a,c,e. The cyclical variability shows that the highest proliferation values occur near the end of the menstrual cycle, whereas the AI is greatest at the beginning and the end of the menstrual cycle. The CRI shows cyclical variations, with significantly greater values near the 28th day of the menstrual cycle ($P = 0.033$). The fourth-degree polynomial curves show that neither the PI ($P = 0.6$; Fig. 2b) nor the CRI ($P = 0.25$; Fig. 2f) is statistically different throughout the course of the menstrual cycle. In contrast, there is a statistically significant cyclical variability in the AI ($P = 0.038$; Fig. 2d). The mathematical equations that best represent the PI and AI are the linear regression and the fourth-degree polynomial curve, respectively. When these are superimposed, after mathematical adjustment of the median visible time of Ki-67 expression with that of apoptosis, it is clear that the maximum values for both indices (PI and AI) coincide at about the 24th day of the menstrual cycle. At this point, the linear regression for the PI is tangential to the fourth-degree polynomial (Fig. 2g).

Furthermore, progesterone levels, irrespective of the stage of the cycle, correlate with proliferation. In apoptosis this is not so, because we found a decrease in apoptosis at progesterone levels higher than 15 ng/ml (Fig. 2h).

It is important to note that, relative to the total number of cells present, the number of epithelial cells undergoing proliferation or apoptosis at any given time during the menstrual cycle was quite small, and the cells were distributed throughout the mammary lobules.