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RESEARCH ARTICLE

Ovarian tumor marker HE4 is differently expressed during the phases of the menstrual cycle in healthy young women

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Abstract The objective of the present study was to investigate in healthy young women the fluctuations in serum concentration of human epididymal secretory protein human epididymis-specific protein 4 (HE4) and CA125 during the phases of the menstrual cycle and the correlation between HE4 and CA125 values and age. Forty women with regular menstrual cycles were included in the study. Pelvic and transvaginal ultrasound were performed in order to exclude ovarian pathologies. Blood samples were collected at follicular (FP), ovulatory (OP), and luteal (LP) phases of the hormonal cycle. The values of HE4 (expressed as picomoles per liter) observed were (mean ± SEM) 39.1±1.1 (FP), 45.3± 1.19 (OP), and 42.0±1.3 (LP). The difference between FP and OP was statistically significant (p=0.0002). By contrast, serum CA125 levels (expressed as units per milliliter) were 14.35±0.66 (FP), 13.15±0.54 (OP), and 13.70±0.54 (LP), respectively. The levels of HE4 observed in serum samples of women below 35 years were 37.5±1.28 in the FP, 46.6± 1.4 in the OP, and 42.8±1.49 in the LP. In this group, a statistically significant difference was observed in the FP compared with the OP (p<0.0001), whereas no statistically significant difference was observed during the different hormonal phases in the group of women over 35. In conclusion, the correct interpretation of laboratory data is essential to define a threshold of normality, and for what concerns HE4 levels, the menstrual cycle phase-dependent variability appears indicated in the interpretation of the results.

Keywords HE4 · Hormonal cycle · Age

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Introduction

The most important function of serum tumor markers in oncologic pathologies is detecting recurrence or monitoring progression of disease and its response to therapy [1, 2]. To date, none of the known tumor markers is tumor specific since all biomarkers may also be present in physiological conditions [3]. The discrimination between a neoplastic and a nonneoplastic entity occurs prevalently on a quantitative basis, with a definition of a threshold value rather than on a qualitative basis [4].

In healthy women, during the menstrual cycle, a coordinated interplay of feedback mechanisms between pituitary and ovarian steroid secretion is present. Recent studies demonstrated that the physiological monthly variation of hormones influence the release of many growth factors and peptides including ovarian tumor markers [5, 6].

Human epididymis-specific protein 4 (HE4) is a new ovarian biomarker initially identified in the epithelium of the distal epididymis and originally predicted to be a protease inhibitor involved in sperm maturation [7–9]. It is also called WFDC2 because it contains two whey acid

protein domains and a "four disulphide bond core" made up from eight cysteine residues [10]. HE4 is commonly overexpressed in ovarian neoplastic tissue, and it is elevated in the serum of patients with cancer of the ovary. It is important to highlight that HE4 is not only expressed in the early stages of the disease, but it is an early indicator of disease recurrence [11, 12].

CA125 was originally identified following the development of the OC125 antibody and was found to be elevated in about 80% of patients with ovarian cancer and in 30% of patients with any primary cancer with extensive intra-abdominal disease [13]. Therefore, even if CA125 has a high sensitivity, its clinical use in confirming ovarian cancer is limited because it is also frequently increased in women with benign gynecological diseases as well as benign diseases associated with inflammatory cells of the pleura, pericardium, and peritoneum [14–16]. Several authors have reported data on serum tumor markers level fluctuations including CA125, during the menstrual cycle, whereas less is known about HE4. In this study, we aimed to evaluate, in healthy young women, the expression of HE4 vs CA125 during the follicular, ovulatory, and luteal phases of the menstrual cycle and the possible correlation with age.

Material and methods

Subjects

Forty female members of the Sapienza University of Rome (aged 20–49 years, mean ± SEM; 30.75±1.2) with regular menstrual cycles were included in the study. They were subdivided into two groups below and over 35 years, respectively. In the subjects below 35 years, the mean age was 27±0.67, whereas in the group of healthy women over 35, the mean age 41.8±1.85. Pelvic and transvaginal ultrasonography was performed in order to confirm the absence of ovarian pathologies. Progesterone level at day 21 confirmed ovulation. Pregnancy, childbearing, and hormonal contraceptives were considered an exclusion criteria. An informed consent was required from all subjects before sampling.

Serum collection

Three blood samples were collected from each subject at days 7–14–21 (from the beginning of the menstrual cycle). Sera were acquired following a standard collection protocol. Briefly, samples were collected in a Red Top Vacutainer, clotted 60–90 min and centrifuged for 10 min at 1,300×*g*. The serum fractions were aliquoted and stored at −80°C until analysis.

Biomarker assays

HE4 and CA125 determination

HE4 levels were determined using the HE4 EIA assay (Fujirebio Diagnostics). The HE4 EIA is a solid phase, noncompetitive immunoassay based upon the direct "sandwich" technique using two monoclonal antibodies, 2H5 and 3D8, directed against two epitopes in the C-WFDC domain of HE4. Serum samples and standards were incubated with biotinylated anti-HE4 monoclonal antibody 2H5 aliquots in streptavidin-coated microstrips. HE4 present in standards or serum samples was adsorbed to the streptavidin-coated microstrips by the biotinylated anti-HE4 monoclonal antibody during the incubation period. The strips were then washed and incubated with HRP-labeled anti-HE4 monoclonal antibody 3D8. After washing, buffered substrate/chromogen reagent was added to each well, and the enzyme reaction was allowed to proceed. During the enzyme reaction, a blue color developed if the antigen was present. The color intensity was directly proportional to the amount of HE4 present in the samples. Normal values of HE4 were considered to be less than 150 pmol/L, according to the manufacturer's indications.

CA125 levels were evaluated by a one-step "sandwich" radioimmunoassay (Radim, Netherlands). Polystyrene beads coated with M11 capture antibody reacting with molecules containing OC 125 reactive determinants were incubated with serum samples, standards, and tracer (^{125}I-labeled mouse monoclonal OC 125 antibody) aliquots. The bound radioactivity observed was proportional to the concentration of the OC 125 reactive determinant (antigen). Normal levels of CA125 were considered to be less than 35 U/mL. The intra-assay coefficients of variation (sera measured ten times in the same assays) were 6.1% for HE4 and 5.9% for CA125 assays, respectively, whereas the inter-assay coefficients of variation (sera measured in five different assays) were 9.3% for HE4 and 10.1% for CA125 assays, respectively.

Statistical evaluation

Statistical analyses were performed using SPSS statistical software, version 13 (SPSS, Chicago, IL, USA). Data were presented in mean ± SEM. Statistical analysis was performed using the Student's *t* test. The level of statistical significance was set at $p<0.05$.

Results

HE4 and CA125 expression during the follicular, ovulatory, and luteal phases of the menstrual cycle

The following serum concentration levels of HE4 (mean ± SEM) 39.1±1.1, 45.3±1.19, and 42.0±1.3 were obtained at

the follicular, ovulatory, and luteal phases of the menstrual cycle, respectively; the difference observed between the follicular and the ovulatory phases was statistically significant ($p<0.0002$). Follicular, ovulatory, and luteal CA125 levels were (mean ± SEM) 14.35±0.66, 13.15±0.54, and 13.70±0.54, respectively, and the differences between the three phases of the hormonal cycle were not statistically significant (Fig. 1).

Expression of HE4 and CA125 values into age groups

The serum concentration levels of HE4 (mean ± SEM) observed in patients below 35 years were 37.5±1.28 in the follicular phase, 46.6±1.4 in the ovulatory phase, and 42.8±1.49 in the luteal phase. The different values observed in the follicular phase compared to the ovulatory phase were highly statistically significant ($p<0.0001$).

The group of women over 35 showed the following values of HE4: 45±0.81 follicular phase, 41.0±1.8 ovulatory phase, and 39.7±3.0 luteal phase. No statistically significant difference was observed in the different phases of the hormonal cycle (Fig. 2).

Figure 2 shows that the levels of HE4 in women below 35 showed a peak of HE4 concentration during the ovulatory phase. A linear expression of HE4 concentration during the three phases of the menstrual cycle was observed in the group of women over 35 years.

The levels of CA125 concentration (mean ± SEM) observed in women below 35 years (age mean, 27±0.67) were 12.9±0.67 in the follicular phase, 12.6±0.65 in the ovulatory phase, and 13.3±0.69 in the luteal phase, whereas the group of over 35 years (age mean, 41±1.85) showed the following values of CA125 concentration: 18.7±0.74 in the follicular phase, 14.7±0.73 in the ovulatory phase, and 14.8±0.75 in the luteal phase. For what concerns the CA125 concentration, we have not observed a statistically significant difference in the levels of CA125 concentration in the different phases of the hormonal cycle in both the age groups (Fig. 3).

Discussion

Cancer is a threat to global health; early diagnosis of the disease, confined to the tissue of origin, followed by surgical removal, represents the best chance of cure [17]. Consequently, the identification of new serum markers enabling early detection of cancer has a high level of priority [18]. The term tumor marker defines that substance, molecule, or molecular profile that can indicate the presence of cancer, detectable in the patient sera or other body fluids. To date, none of the known tumor marker is tumor specific because they are substances present in many other conditions, not excluding the normal [19, 20]. The dosage of tumor markers, through the definition of a threshold value, allows a distinction between cancer patients and healthy subjects on a quantitative basis rather than on a qualitative basis [1]. Tumor marker CA125 is commonly used in the surveillance of patients with advanced stage ovarian cancer and in monitoring response to treatment. However, its application has limitations because high serum levels of CA125 are also observed in other oncologic and nononcologic diseases [14]. Recently, a new ovarian cancer tumor marker called HE4 has been identified, with a high sensitivity in the early stages of disease and a high specificity in the differential diagnosis between malignant and benign diseases [12]. Numerous studies have shown that the hormonal changes that characterize the menstrual cycle may be responsible of oscillations of different tumor markers [5, 21, 22]. The aim of this study was to evaluate whether the new ovarian

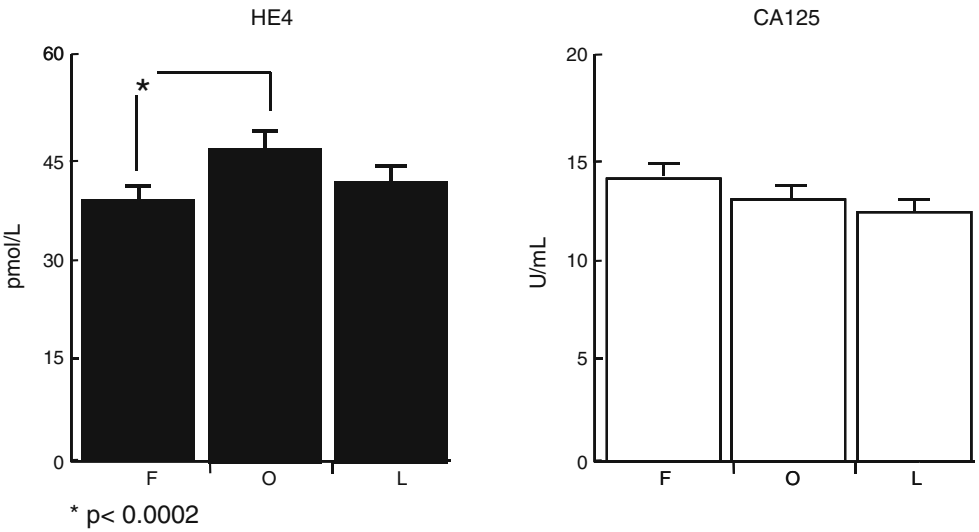

Fig. 1 Levels of HE4 (*right*, *black bars*) and CA125 (*left*, *white bars*) observed in healthy women during the phases of the hormonal cycle: *F* follicular, *O* ovulatory, and *L* luteal. A statistically significant difference was observed between the values of HE4 detected in the follicular phase compared to values observed in the ovulatory phase (*$p<0.002$)

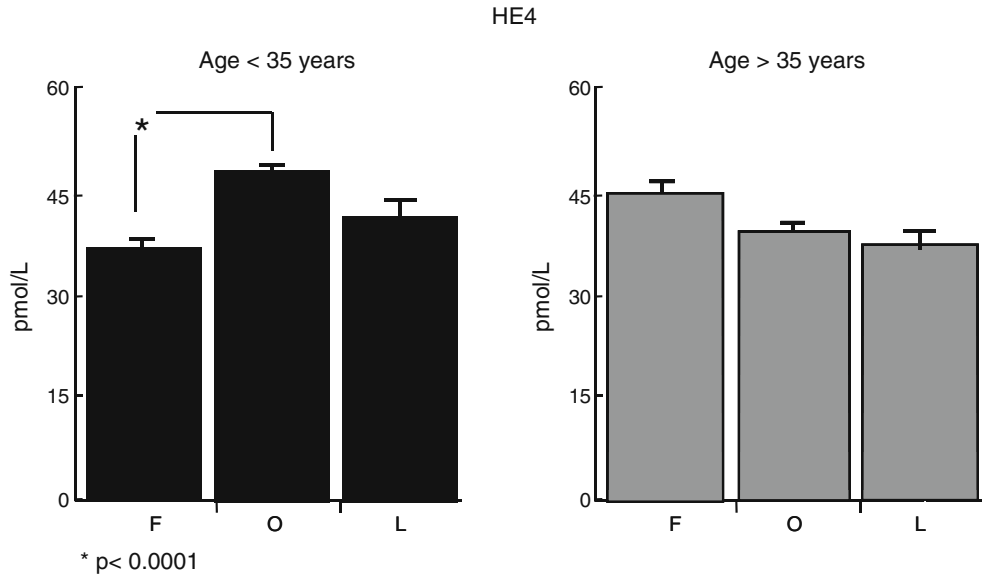

Fig. 2 Levels of HE4 observed in healthy women during the different phases of the hormonal cycle (*F* follicular, *O* ovulatory, *L* luteal). The values were grouped by age less than 35 years (*left*, *black bars*) and over 35 years (*right*, *gray bars*), respectively. A highly statistically significant difference was observed between the follicular and ovulatory phases in women under 35 years (**p*<0.0001)

tumor marker HE4 was differently expressed in the phases of the hormonal cycle. In particular, we observed a lower level of HE4 during the follicular phase, in comparison with the values observed in the ovulatory and the luteal phases. No change in CA125 levels during the follicular, ovulatory, and luteal phases of menstrual cycle was observed. Although the literature is somewhat contradictory on this subject, several authors, including Zweers et al., have also shown no fluctuation of CA125 during the menstrual cycle [23]. It is likely that the CA125 fluctuations observed by other authors in childbearing women may be associated with concomitant clinical or asymptomatic conditions such as colic or inflammation diseases [24–26].

The lower serum concentration HE4 during the follicular phase is consistent with other studies showing fluctuations in levels of some tumor markers during the phases of the menstrual cycle and, in particular, with a reduction during the follicular phase [14, 21, 22]. There may be different explanations, and in our opinion, the most intriguing is that there may be a role played by follicular steroidogenesis. To evaluate this hypothesis, we divided the group of women included in the study in two age groups, respectively, above or below 35 years. This subdivision was made to assess whether a more intense follicular activity, which occurs in younger women, could be involved in different levels of HE4. We have thus observed that the significant reduced

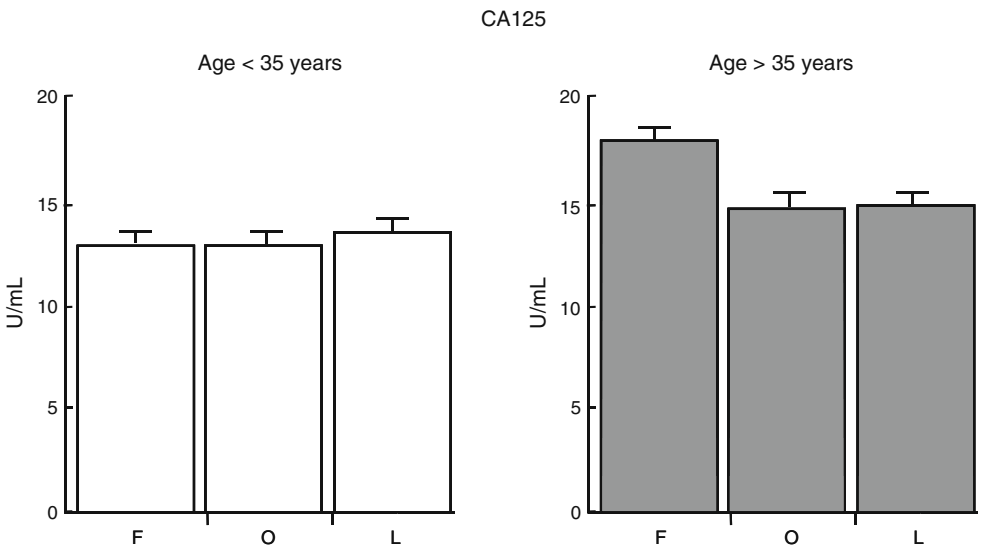

Fig. 3 Levels of CA125 observed in healthy women during the different phases of the hormonal cycle (*F*, follicular, *O* ovulatory, *L* luteal). The values were grouped by age below 35 years (*left*, *black bars*) and over 35 years (*right*, *gray bars*), respectively. No significant difference was observed in both groups of women (under 35 years old or over 35 years) during ovulatory, follicular, or luteal phases of the menstrual cycle

level of HE4 observed during the follicular phase was only observed in the group of women below 35 years.

Gonadotropins and ovarian hormones are produced and secreted during all phases of the hormonal cycle. The abundant release of these hormones could lead to greater stimulation of these antigens, including HE4, closely associated with the ovary. On the other hand, in younger women, a more appropriate production of ovarian hormones such as estradiol and progesterone could be responsible for an inhibitory action on the production of tumor antigens including HE4. In conclusion, it is essential to define a threshold of normality of the HE4 marker for ovarian cancer.

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Conflict of interest statement None declared.

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