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Serum concentrations of the biomarkers CA125, CA15-3, CA72-4, tPSA and PAPP-A in natural and stimulated ovarian cycles

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ABSTRACT

Objective: Biomarkers associated with cancer screening (CA125, CA15-3, CA72-4, total prostate specific antigen [tPSA]) and the monitoring of pregnancy (pregnancy associated plasma protein-A [PAPP-A]) were measured during natural and stimulated ovarian cycles in disease-free non-pregnant women to determine if they could reflect normal events relating to ovulation and/or endometrial changes. **Methods:** A total of 73 blood samples (10 women) collected throughout the natural menstrual cycle, and 64 blood samples (11 women) taken during stimulated ovarian cycles, were analysed on the Roche Cobas e411 automated analyser. **Results:** Detectable levels of tPSA were measured in at least one point in the cycle in 6/10 of women in the natural cycle and 10/11 of women in stimulated cycles, and CA72-4 was detected in only 12/21 women tested. Concentrations of CA125, tPSA, CA15-3 and CA72-4 showed no significant difference between the natural and stimulated ovarian cycle groups. On average the mean PAPP-A of the natural group was (2.41±0.58) mIU/L higher than the stimulated group (t=4.10, P< 0.001). CA125 and CA15-3 results were both significantly influenced by the stage of the cycle (P<0.0001), whereas tPSA and PAPP-A concentrations revealed no significant changes (P≥0.65). CA72-4 was not affected by the stage of the cycle nor ovarian stimulation. **Conclusion:** Ovarian stimulation reduced serum PAPP-A levels, CA125 and CA15-3 levels were generally unaffected by ovarian stimulation but displayed cyclical changes throughout both natural and stimulated cycles, whilst tPSA and CA72-4 were not affected by the stage of the cycle or ovarian stimulation.

1. Introduction

Molecular biomarkers are rarely passive and specific end-products of a single tissue but are more often potent compounds involved in a range of biological processes. Pregnancy-associated plasma protein-A (PAPP-A) is a good example of such a biomarker in reproductive medicine, and it is now known to be a protease specific for the cleavage of insulin-like growth factor binding proteins[1]. Having been described in normal pregnancy [2, 3], PAPP-A is useful as a biomarker in a combined test with free β-hCG and fetal nuchal translucency in the identification of increased risk of Down's syndrome[4] and miscarriage[5]. Although most of the research surrounding PAPP-A has been performed during pregnancy[6-12], there are recent studies which indicate differences in PAPP-A concentrations during ART treatment cycles[13-16]. Furthermore, PAPP-A is produced by granulosa cells, having a role in follicle selection through its effect upon IGF availability[17].

PAPP-A is not the only enzyme whose name is misleading: prostate specific antigen (PSA) was originally thought to be produced exclusively by prostatic tissue and was therefore used to monitor prostate cancer[18]. PSA is

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actually a serine protease that is also known as kallikrein-3 (KLK3)[19], and has been associated with a number of tissues and biological events in women[20] such as in the breast[21], during the ovarian cycle[22, 23], and in pregnancy[24].

Another tumour biomarker, CA15-3, is a mucin-like glycoprotein encoded by the MUC1 gene and has a clear association with reproduction. MUC1 is heterogeneously expressed on the surface of epithelial cells, including those in the breast and upper reproductive tract and is thought to prevent embryo implantation[25]. In addition, expression of MUC1 has been shown to be progesterone dependent and is up-regulated in endometrial epithelial cells in the luteal phase of the menstrual cycle[26, 27]. However, the main clinical use of the assay is in the monitoring of women with breast cancer[28-30]. CA125, a high molecular mass mucin-type molecule, is a tumour biomarker that is used extensively to monitor epithelial ovarian cancer [31], but it also is expressed elsewhere such as during the ovarian cycle[32], in association with endometriosis[33], in pregnancies that are destined to miscarry[34], and with pelvic inflammatory disease[35].

CA72-4 was once described as a useful tumour marker for all epithelial derived tumours and gastric carcinomas[36]. This research demonstrated that the sensitivity of CA72-4 for gastric carcinoma was 38%, which is greater than the tumour markers CA19-9 which is 33%, CEA at 31% and CA125 at 21%[36, 37]. However, CA72-4 has also been proposed as a complimentary biomarker to CA125 in the screening of ovarian cancer, where it was found that by combining the biomarkers the sensitivity for detecting early stage disease increased from 45% to 70%[38].

The aim of the present study was to measure five serum biomarkers (PAPP-A, tPSA, CA15-3, CA125 and CA72-4) in women during periods of ovarian and endometrial activity, namely in natural ovarian cycles and stimulated cycles. Results were analysed to (i) compare the concentrations between the two reproductive situations, and (ii) identify any temporal changes that may have occurred relating to follicular development.

2. Materials and methods

Patient information and consents were approved by both the Joondalup Health Campus Research Ethics Committee and the Edith Cowan University Human Research Ethics Committee. All blood samples were taken as part of the routine management of the women at Fertility North, but consent was obtained for the analysis of additional compounds not indicated medically.

2.1. Patients

Women were recruited during their routine clinical management, and none of the women had evidence of cancer or endometriosis. Blood samples collected during natural cycles were from women (n=10) who were undergoing assessment of their natural cycle prior to commencing fertility treatment. These women were on no medications that affect ovarian and uterine function such as the oral contraceptive pill or hormone replacement therapy. Cycle length was normalised for the purpose of statistical analysis according to Hadlow *et al*[39] using the following formula to calculate the day of the cycle:

Adjusted day = Actual day × (14/Actual day of ovulation)

The cycle was also divided into phases using the adjusted day of the cycle[40]. Women providing blood in stimulated cycles (n=11) were undergoing IVF using standard clinical protocols[41].

2.2. Sample processing and analysis

Blood was collected using syringes and transferred into 5 mL Vacutainer SST™ tubes (Becton Dickinson, UK) before delivery to the laboratory. The blood was allowed to clot at room temperature and then centrifuged at 1 300 g for 4 minutes, with the tubes then being ready for loading directly onto the automated analyser upon removal of the lids. Serum oestradiol, luteinising hormone, progesterone, and human chorionic gonadotrophin (hCG) were measured on a Siemens Centaur CP automated analyser (Siemens, Bayswater, Victoria 3053, Australia) within 1 hour of the blood being collected, and all between-run coefficients of variation were <5%. The serum was then stored in secondary tubes at −80 ℃ before being analysed in one batch on a Roche Cobas e411 automated analyser (Roche Diagnostics, Germany) for the biomarkers PAPP-A, CA125, CA15-3, CA72-4 and total PSA (tPSA). Assay variability for the biomarkers was determined by analysing pooled patient serum in the analytical range for this study, sometimes close to the limit of detection, and the within-run variability at these concentrations for the biomarkers (CA125 <3%; CA15-3 <2%; CA72-4 <5%; PAPP-A <3%; tPSA <13%) was invariably less than the between-run variability (CA125 <5%; CA15-3 <7%; CA72-4 <24%; PAPP-A <27%; tPSA <39%). Assay sensitivity for CA125, CA15-3, CA72-4, PAPP-A, and tPSA were 0.6 U/mL, 1.00 U/mL, 0.2 U/mL, 4.00 mIU/L, and 0.003 ng/mL respectively.

2.3. Statistical analysis

The ovarian cycle data were analysed with a linear mixed effects model to compare the marker concentrations in stimulated and natural cycles across the cycle phases. For each model, the response variable (CA125, tPSA, CA15-3, CA72-4 and PAPP-A) was log transformed before analysis. Group ("natural" and "stimulated") and cycle phase were included as fixed effect factors and 'Subject' and 'Time' were modelled as random effects as in some subjects there were multiple time points measured within phases. The interaction between group and phase was also modelled. The analyses were performed using the R version 3.0.0

computing software[42].

3. Results

3.1. Ovulation and reproductive hormones

The day of ovulation for the 10 natural cycles is shown in Figure 1 (a). It was extremely variable, ranging from day 10 to day 25. The day of ovulation in the 11 cycles stimulated with exogenous gonadotrophin is shown in Figure 1 (b), and was less variable than the natural cycles ranging between day 12 and day 15 of the cycle. The reproductive hormones oestradiol and progesterone that were measured as part of routine patient management are shown in Table 1. They follow classical patterns of change throughout the natural cycle, confirming that the modelling and expression of results according to the stage of cycle is appropriate. Differences between the natural cycles and stimulated cycles were noted and include higher oestradiol values in the mid-follicular and late follicular phases of the stimulated cycles, and higher progesterone in the luteal phase of the stimulated cycles as a consequence of multiple corpora lutea and the continued administration of progesterone luteal support.

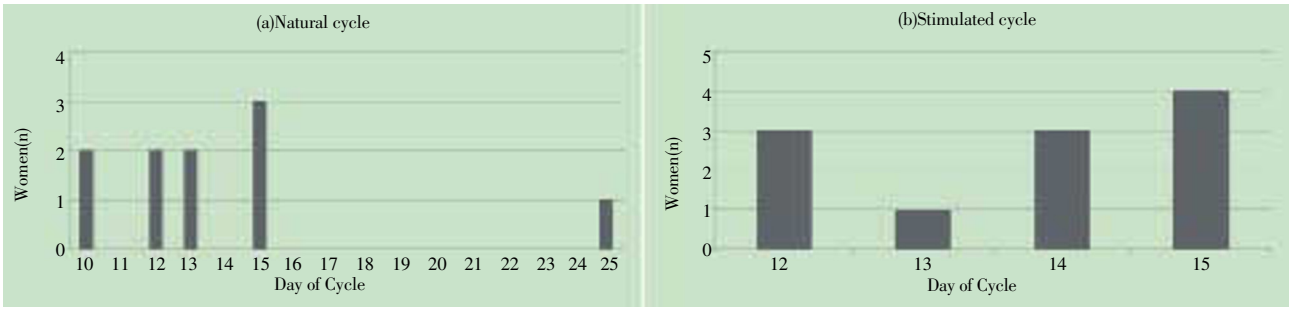

Figure 1. The distribution of the day of ovulation for women during (a) natural cycles (n=10), and (b) stimulated cycles (n=11).

Table 1
Reproductive hormone concentrations (mean±sem) in natural (n=10) and stimulated (n=11) ovarian cycles.

Stage	Oestradiol (pmol/L)		Progesterone (nmol/L)	
	Natural	Stimulated	Natural	Stimulated
EF	165.6±11.8	156.0±6.6	1.9±0.3	2.3±0.6
MF	265.0±43.0	1 133.7±241.1	1.4±0.2	–
LF	528.0±51.2	3 980.0±435.8	1.3±0.2	–
ML	484.8±68.4	2 187.0±301.1	36.5±4.6	197.2±30.0

The stage of the cycle was classified as early follicular (EF), mid-follicular (MF), late follicular (LF) and mid-luteal (ML).

3.2. Biomarkers during ovarian cycles

The concentrations of the serum biomarkers CA125, CA15-3 and CA72-4 during natural and stimulated cycles are shown in Table 2. CA125 concentrations showed no significant difference between natural and stimulated ovarian cycles (P=0.5989) but results were significantly influenced by the stage of the cycle (P<0.0001). Concentrations of CA125 were on average highest during the early follicular phase of the cycle which is concurrent with menstruation (25.92±4.45) U/mL and lowest in the late-follicular phase before ovulation (16.76±2.38) U/mL in natural menstrual cycles. In stimulated ovarian cycles, concentrations of CA125 were highest during the mid-luteal phase (22.89±13.45) U/mL and lowest at the mid-follicular phase (13.41±1.90) U/mL. All samples in the ovarian cycles had detectable levels of CA15-3. There was no overall significant difference in the concentration of CA15-3 between the natural and stimulated ovarian cycles (P=0.8694). However there was an overall significant difference within each cycle between phases, suggesting CA15-3 levels significantly change between phases (P<0.0001). There were 6/10 (60%) of the individuals

Table 2
The concentrations (mean±sem) of the serum biomarkers CA125, CA15-3 and CA72-4 measured during natural (10 women) and stimulated (11 women) ovarian cycles.

Stage of cycle	CA125 (U/mL)		CA15-3 (U/mL)		CA72-4 (U/mL)	
	Natural	Stimulated	Natural	Stimulated	Natural[a]	Stimulated[b]
EF	25.92±4.45	17.06±2.37	15.59±1.67	15.10±2.00	1.86±0.92	2.33±1.12
MF	21.36±3.21	13.41±1.90	17.52±2.31	14.18±2.09	1.21±0.50	1.13±0.39
LF	16.76±2.38	13.62±1.64	15.91±1.69	14.60±1.90	1.10±0.33	0.98±0.27
ML	20.39±1.83	22.89±13.45	16.85±1.94	16.99±2.15	1.04±0.3	1.72±0.88

The stages of the cycles were early follicular (EF), mid-follicular (MF), late follicular (LF), and mid-luteal (ML).[a]Detectable concentrations were only seen in 6/10 women; [b]Detectable concentrations were seen in 10/11 women.

in natural menstrual cycles and 6/11 (54.5%) of individuals in the stimulated cycles that had detectable levels of CA72-4 for at least one of the samples. When detectable, CA72-4 concentrations were overall on average (1.47±0.31) U/mL in natural cycles and (1.58±0.35) U/mL in stimulated ovarian cycles. There was a larger degree of variation between the individuals than there was at different phases of the cycles.

The concentrations of the serum biomarkers tPSA and PAPP-A are shown in Table 3. Detectable levels of tPSA were measured in at least one point in the cycle in 6/10 of women in the natural cycle and 10/11 of women in stimulated cycles. Concentrations of tPSA were low during natural and stimulated cycles and there was no significant difference either between natural cycles and stimulated cycles (P=0.9193), or between different stages of the cycle (P=0.8769). On average the mean PAPP-A of the natural group was (2.41±0.58) mIU/L higher than the stimulated group (t=4.10, P<0.001). For PAPP-A, there was no evidence for an interaction effect between PAPP-A concentrations and the phase of the cycle (t=-0.08, P=0.93), or for a change in PAPP-A within natural and stimulated ovarian cycles across the phases of the cycle (t= -0.44, P=0.65).

Table 3

The concentrations (mean±sem) of the serum biomarkers tPSA and PAPP-A measured during natural (10 women) and stimulated (11 women) ovarian cycles.

Stage of cycle	tPSA (ng/mL)		PAPP-A (mIU/L)	
	Natural[a]	Stimulated[b]	Natural	Stimulated
EF	0.012±0.009	0.004±0.004	8.97±0.79	6.24±0.54
MF	0.007±0.004	0.009±0.002	8.91±1.04	6.73±0.59
LF	0.009±0.006	0.004±0.003	8.59±0.64	6.57±0.35
ML	0.004±0.002	0.015±0.001	8.40±0.81	6.19±0.53

The stages of the cycles were early follicular (EF), mid-follicular (MF), late follicular (LF), and mid-luteal (ML); [a]Detectable concentrations were only seen in 6/10 women; [b]Detectable concentrations were seen in 10/11 women.

4. Discussion

4.1. Assays

The use of immunoassays allows precise quantitative measurements to be made when measuring analytes. However, different assays often have different characteristics due to the choice of reagents or their calibration, resulting in different numerical values. This is important when comparing work from various laboratories or over a range of time frames. For example, the expression of PAPP-A results in mIU/mL in the present study compared to μg/L elsewhere[43] reflects the change in methodology and the move to a different standardisation. Between-assay differences have been reported when measuring CA15-3 with commercial kits from different companies, resulting from differences in calibration rather than specificity[44]. This is perhaps not too surprising as many of the companies used similar capture and signal antibodies in their sandwich immunometric assays. CA125 can also show between-supplier variability, and large differences have been reported between assays supplied by Siemens and Panomics [45].

4.2. Changes during ovarian cycle

There were cycle dependent changes seen in CA125 concentrations for both natural and stimulated ovarian cycles. This study showed that ovarian stimulation had no effect on CA125 levels and that both natural and stimulated ovarian cycles showed similar changing patterns. The results from the natural group agreed with the literature in that the highest CA125 levels were found during menstruation [46-50]. The stimulated group results also agreed with the literature where the highest CA125 levels were found in the luteal phase of the cycle[51]. It is thought that the endometrium is responsible for the cyclical changes in CA125 concentrations and it is the disruption of the endometrium during menses that allows increased amounts of CA125 to enter the blood stream[46, 52, 53]. It was also proposed that pregnancy outcomes following ART treatment could be predicted by measuring CA125 on the day of oocyte retrieval and that levels >10IU/mL were correlated with an 86.6% positive pregnancy rate based on a prospective study of 75 ART cycles[54]. Of the 8 participants in this study that had a CA125 of >10 U/mL before oocyte retrieval, only 3 of those became pregnant (37.5%) which was markedly lower than the literature had suggested. There was also one participant who had a CA125 level <10 U/mL that did become pregnant. Although our results seem to suggest that CA125 levels >10 IU/mL are not as strongly correlated to positive pregnancy rate as the previous study, they are limited by the relatively small sample size. Nonetheless the results of this study do warrant further investigation.

Serum concentrations of CA15-3 in natural menstrual cycles were not statistically different to those found in stimulated cycles. This suggests that ovarian stimulation *per se* for the purposes of IVF and ICSI procedures does not affect circulating serum CA15-3 levels. The CA15-3 concentration did however show some interaction with the phases of the

cycles which is in agreement with the literature[55]. The MUC1 gene, which encodes the CA15-3 glycoprotein, is expressed in the upper female reproductive tract and its function has been suggested to be to prevent ectopic embryo implantation[25]. Other studies have shown that MUC1 expression is progesterone dependent whereby it is up regulated in the luteal phase of the menstrual cycle [26, 27]. These findings are significant because if MUC1 is up-regulated by progesterone in the luteal phase, it would suggest that it is a part of the body's mechanisms to avoid ectopic pregnancy. This current study has shown that serum concentrations of CA15-3 are at their highest in the mid-follicular phase of the natural menstrual cycle, which is at a time that progesterone is at its lowest levels, suggesting that although CA15-3 is encoded by the MUC1 gene, it does not appear to be progesterone dependent. This being said, the previous research on MUC1 expression was carried out on tissue samples whereas this is an analysis of serum concentrations so the 'lag' in peak CA15-3 concentrations may not reflect activity at a local level.

Ohuchi et al[36] described CA72-4 as a useful tumour marker for all tumours derived from epithelial cells highlighting the tumour markers increased sensitivity to gastric carcinoma compared to other tumour markers such as CA19-9, CEA and CA125. It was also proposed that when CA72-4 is used as a complimentary biomarker to CA125, the sensitivity for detecting early stage ovarian cancer increased from 45% to 70%[38]. The CA72-4 assay used in this research failed to register any results above the assays lower limit of detection in 40.0%-45.5% of individuals from both natural and stimulated ovarian cycles. Of those individuals that did have detectable levels of CA72-4, the results showed an extremely high degree of variability both within each individual (whereby each sample from the same cycle was vastly different to the others) and between patients where the difference was so large that there were no obvious patterns of change. It was for these reasons that statistical analysis was not carried out and it was concluded that the assay was too unreliable for use as a diagnostic measure in the clinical setting.

Total prostate-specific antigen (tPSA) was detectable in 60% of women in natural cycles and 91% of women during stimulated ovarian cycles. The range of mean results from each phase was between 0.004 μg/L-0.012 μg/L for both natural and stimulated ovarian cycles. There was no significant relationship between tPSA concentration and phase of the cycle, nor was there any significant difference between tPSA concentrations in natural and stimulated cycles. Zarghami *et al.* [56] has indicated that tPSA in the menstrual cycle followed the progesterone concentration peak with a 10-12 day delay. This finding is suggestive of tPSA concentrations changing in a cyclical manner. In our present study, we found that tPSA concentrations were highest in the early follicular and late luteal phase, which is relative to menstruation, although this did not reach statistical significance. Total prostate specific antigen concentrations are very low in female serum and it is not known why some women have measurable levels of tPSA and others do not. The physiological function of tPSA in females is yet to be determined.

Finally, we found a significant difference in the mean concentration of PAPP-A between natural and stimulated ovarian cycles, where stimulated ovarian cycles were on average (2.41±0.58) mIU/L lower than natural cycles. The present study also showed that throughout each of the two types of cycles there were no significant changes in PAPP-A levels. Findings of lower serum PAPP-A concentrations in women during stimulated ovarian cycles confirms previous work where women were shown to have lower serum PAPP-A levels with higher oocyte number after oocyte retrieval, leading to the proposal that differences in PAPP-A concentrations may be due to the presence of multiple follicles in the ovaries[16]. Amor *et al.* [13] found that PAPP-A levels were reduced in both fresh and frozen-thawed embryo transfers when compared to naturally conceived pregnancies. However, fresh transfers did have significantly lower PAPP-A levels than frozen-thawed transfers, providing evidence for the multiple follicle theory where the ovaries in frozen-thawed embryo transfer cycles are not hyperstimulated to create multiple follicle development like those of fresh cycles.

In summary, batch analysis of all samples from each of the participants was conducted to maximise the possibility that any changes seen in biomarker concentrations were due to biological fluctuations and not because of assay variability. Ovarian stimulation reduced serum PAPP-A levels, whilst CA125 and CA15-3 were generally unaffected by ovarian stimulation but did have cyclical changes throughout both natural and stimulated cycles.

Conflict of interest statement

We declare that we have no conflict of interest.

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