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Differences in the Molecular Species of CA125 Across the Phases of the Menstrual Cycle

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Abstract

Background: CA125, a tumor-associated antigen, is primarily used to monitor epithelial ovarian cancer. There is evidence that different species of CA125 exist; however, it is not known if any of these species are present in healthy women during the menstrual cycle and if they are associated with serum concentrations of CA125. The purpose of this study was to determine if the molecular species of CA125 differ across the three phases of the menstrual cycle in healthy women.

Methods: Healthy, Caucasian women between the ages of 18 and 39 were enrolled using strict criteria to exclude factors known to contribute to CA125 fluctuations. Menstrual cycle regularity was determined using calendars maintained by participants for 3 months. After cycle regularity was established, blood was drawn at three time points for Western blot analysis.

Results: Western blot analysis yielded 17 distinct profiles (i.e., patterns of species) of CA125, with 80% of the sample exhibiting 5 common profiles. No differences in demographic characteristics and serum CA125 values were found among the various CA125 profiles.

Conclusions: Different molecular species of CA125 exist in healthy women with regular menstrual cycles. These data provide evidence that CA125 is not a homogeneous molecular species. Future research should evaluate the molecular composition and the clinical importance of these species.

Keywords

CA125, molecular speciation, menstrual cycle

Molecular speciation is the classification of distinct groups of similar entities (King & Stansfield, 2002). Members of a distinct molecular species must have at least one common precursor (Tiollais, Galibert, & Boiron, 1971), exhibit differences in electrophoretic mobility from other species (Tiollais et al., 1971), show different responses to the same experimental conditions as compared to members of other species (Stewart et al., 1975), or contain a different number of residues or nucleotides from other species (Stewart et al., 1975; Tiollais et al., 1971). These original criteria continue to be valuable for current molecular speciation studies (Andersch-Bjorkman, Thomsson, Holmen-Larsson, Ekerhovd, & Hansson, 2007; Bouanèè et al., 2012; Jankovic & Milutinovic, 2008; Jankovic & Tapuskovic, 2005; Nustad, Onsrud, Jansson, & Warren, 1998; Saldova et al., 2013).

Molecular speciation studies of prostate-specific antigen (PSA) have allowed clinicians and researchers to distinguish which species of PSA are associated with prostate cancer, benign conditions of the prostate, or normal metabolic changes associated with aging (Meany, Zhang, Sokoll, Zhang, & Chan, 2009). PSA values were rescaled when the different molecular species of PSA (i.e., PSA, free PSA, and percent free PSA) were identified in the serum of healthy men and survivors of prostate cancer. The primary objective in rescaling PSA values was to decrease the number of unnecessary prostate biopsies and diagnostic scans, as well as to decrease anxiety in men with elevated PSA levels that were reflective of benign conditions (Gelmann et al., 2001).

CA125 is a tumor-associated antigen used to monitor epithelial ovarian cancer (OC; Bast et al., 2005; Bast & Spriggs, 2011). However, the molecular species of CA125 are not well defined (Weiland, Martin, Oehler, & Hoffmann, 2012). CA125 is secreted by all of the tissues of the female reproductive tract as a large glycoprotein and is degraded by proteolysis during transport throughout the circulatory system (Bidart et al., 1999; Hollingsworth & Swanson, 2004; Jankovic & Milutinovic, 2008; Maeda et al., 2004; Nustad et al., 1998).

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Tumor-derived CA125 is degraded in serum, ascites, and the supernatant of tumor cell lines, which results in subunits of varying sizes (Bouanène et al., 2012; Jacobs & Bast, 1989; Nustad et al., 1998). In laboratory studies, researchers have observed that the oligosaccharides of tumor-derived and pregnancy-associated CA125 are added to the peptide core of CA125 as posttranslational modifications, which results in differences in size and glycosylation profiles (Biskup et al., 2013; Chen et al., 2013; Saldova et al., 2013;). MUC16, the gene that encodes for the peptide core of CA125 (O’Brien et al., 2001; O’Brien, Beard, Underwood, & Shigemasa, 2002), has been observed to be alternatively spliced in OC tumor samples, ascites, and supernatant from tumor cell lines (Maeda et al., 2004; O’Brien et al., 2001, 2002; Yin & Lloyd, 2001).

Serum CA125 levels fluctuate during the menstrual cycle in healthy women, with the highest levels observed during menses (Bon, Kenemans, Verstraeten, van Kamp, & Hilgers, 1995; Bouanène et al., 2012; Kafali, Artunc, & Erdem, 2007; McLemore et al., 2012). We hypothesized that these fluctuations in CA125 levels might be due in part to different species being present at each phase of the menstrual cycle. Despite evidence that different species of CA125 exist in OC and pregnancy (Bidart et al., 1999; Hamilton et al., 2002; Jankovic & Tapanoskovic, 2005; Maeda et al., 2004; Nustad et al., 1998), it is not known if these species are (1) present in healthy women, (2) present at specific time points in the menstrual cycle, or (3) associated with serum concentrations of CA125. Therefore, the purpose of the present study was to determine whether the molecular species of CA125 differ across the three phases of the menstrual cycle within and across healthy women with regular menstrual cycles and to evaluate for differences in demographics and menstrual cycle characteristics as well as serum levels of CA125 by CA125 profile.

Materials and Methods

Study Design and Participants

In a previous study examining changes in CA125 over time (McLemore et al., 2012), our power analysis showed that 60 participants were sufficient. We used data from these same 60 participants for this analysis. We enrolled healthy, Caucasian women between the ages of 18 and 39 using strict criteria to exclude factors known to contribute to fluctuations in serum CA125. Specifically, participants were excluded if they (1) had a previous personal history of ovarian, endometrial, lung, or colorectal cancer; (2) had a first-degree relative with a history of ovarian, endometrial, lung, or colorectal cancer; (3) were pregnant or had been postpartum for less than 6 months, were lactating, or had an abortion or miscarriage within the last 3 months; (4) had endometriosis, ovarian cysts, polycystic ovarian syndrome (PCOS), or a recent history of pelvic inflammatory disease (PID); (5) were participating in infertility treatment or ovum donation currently and were on hyperstimulatory medications or gonadotropins; (6) had severe premenstrual syndrome/prenomenstrual dysphoric disorder; (7) had chronic illnesses requiring routine nonsteroidal anti-inflammatory medications (NSAIDs); (8) were using hormonal contraceptives; (9) were taking herbal supplements (i.e., black cohosh, red clover). The Institutional Review Boards at the University of California, San Francisco, and San Francisco General Hospital (SFGH) approved all procedures, and eligible participants provided written informed consent.

We determined menstrual cycle regularity using calendars that participants maintained for 3 months. After we established cycle regularity, we collected blood samples for Western blot analysis of CA125 at three different phases of the menstrual cycle: menses (T1), follicular (T2; on Day 10 of the cycle, ±3 days), and luteal (T3; on Day 20 of the cycle, ±3 days) and transported them to the research laboratory. The same member of the research team (M.M.) collected all of the blood samples at locations convenient to participants (primarily at their homes or places of employment). Participants notified the researcher by phone at the start of menstruation and set up appointments for the T2 and T3 blood draws.

We obtained CA125 enzyme-linked immunosorbent assay (ELISA) values for all samples using a common commercially available assay (Seimens, catalog number 01678114). Plasma was isolated and aliquots were placed in −80°C storage within 48 hr of the blood draw. We obtained plasma samples from an OC patient with a CA125 ELISA value of 2,700 units per milliliter (U/ml) from the UCSF Cancer Center Ovarian Cancer Tumor Bank and used them as a positive control.

Measurements and Materials

We purchased the primary antibody (1° Ab), X75, from Novus Biologicals (Littleton, CO, cat. no. NB600-1468). The secondary Ab (2° Ab), conjugated to horseradish peroxidase for visualization, was a goat anti-mouse antibody purchased from Jackson ImmunoResearch Laboratories (West Grove, PA, cat. no. 115-035-062). We purchased Immobilon-P 45 μm polyvinylidene fluoride (PVDF) membranes from Millipore (Bedford, MA, cat. no. TM151-1), HiMark pre-stained high molecular weight protein standard (ladder) from Invitrogen (Carlsbad, CA, cat. no. L5699), and Super Signal West Femto electrochemiluminescence reagent from Thermo Scientific (Rockford, IL, cat. no. 34096).

Gel Electrophoresis and Immunoblotting. For each participant (N = 60) at each of the three time points, 0.5 μl of plasma was mixed with 2 μl of sample buffer and 5 μl of denoized water and heated for 30 min at 65°C. After 10 min of cooling under nonreducing conditions, sample mixtures were loaded onto the gel lanes in the following order: molecular weight standard, positive control sample, empty lane, Participant 1 sample at T1, empty lane, Participant 1 sample at T2, empty lane, Participant 1 sample at T3, empty lane until all lanes were filled.

We loaded 26-well, 3–8% gradient Tris-Acetate Criterion XT precast gels, XT-Tricine buffer, and 4X-XT sample buffer for sample resolution (Bio-Rad, Hercules, CA, cat. no. 345-0131). Gels were subjected to electrophoresis at 100 volts until...
the dye front exited the bottom of the gel (i.e., approximately 115 min). Gels were removed from cassettes and equilibrated in Tris-Glycine, 20% methanol (MeOH) transfer buffer for 10 min. Proteins were transferred to the PVDF membrane for 1.5 hr at 400 milliamps at 4°C. After labeling, the blots were immediately immersed in 100% MeOH and placed on filter paper to dry for a minimum of 30 min.

Blots were rehydrated prior to immunoblotting in 100% MeOH and rocked for 5 min in Tris-buffered magnesium chloride. Blots were blocked using 5% milk (generic powdered, non-fat) in 25 ml Tris-buffered saline (TBS) and 0.1% Tween. Blots were incubated with 1st Ab at 4°C overnight. The 1st Ab preparation was X75 at a 1:1850 dilution (i.e., 8.11 μl in 5% milk in 15 ml TBS and 0.1% Tween). After incubation, blots were washed quickly in TBS-0.1% Tween twice and then 3 times on a rocking apparatus for 5 min. Once washed, the blots were then incubated with the 2nd Ab for 45 min at room temperature. The 2nd Ab preparation was goat anti-mouse IgG at a 1:500,000 dilution in 5% milk in 25 ml TBS and 0.1% Tween. Blots were rinsed quickly in TBS and 0.1% Tween twice and then 3 times on a rocking apparatus for 15 min. Blots were immediately treated with electrochemiluminescent reagents per the manufacturer’s instructions and were exposed for four different durations: 30 s, 1 min, 4 min, and 10 min.

Image Acquisition. The radiographic films of all autoradiogram exposures of the Western blots were scanned using an Epson Perfection 4990 flatbed photo scanner. The resolution for all scans was 300 dots per inch (DPI) and all files were imported into Fireworks 4™ software and converted into 24-bit, portable network graphic images (.png). Radiographic films were annotated using the ladder included on each blot, allowing the visible bands to be documented at a kilodalton (kDa) level resolution. All visible bands were included in the annotation, and the darkest (i.e., most abundant) band was chosen as the referent band for each film.

Profile Recognition and Counting. Scanned images were compared on screen using referent bands. Initial banding patterns were documented using the location of the referent band. An iterative process was used to determine distinct patterns, and a template schematic for each pattern was created (Figure 1). The primary investigator (M.M.) performed the initial profile assignment with final adjudication conducted by two investigators (M.M. and B.E.). This process was repeated until consensus was reached. Samples that represented the exemplars of each profile were selected and used as a referent to confirm assignment for each sample. Final adjudication of the profiles was conducted with the research team using the digital and printed images of each profile. The number of samples exhibiting a specific profile was counted.

Statistical Analyses
Data were analyzed using Stata Version 11. Descriptive statistics were generated on demographic characteristics. Frequencies for each CA125 profile were calculated. Exploratory analysis of variance (ANOVA) was used to examine for differences in age, body mass index (BMI), menstrual cycle length, and prior pregnancy status by CA125 profile. Negative binomial regression models were used to examine for differences in mean CA125 levels at each time point by CA125 profile due to the skewed distribution of serum CA125 values and high dispersion. A p value of <.05 was considered statistically significant.

Results

Demographic Characteristics
As shown in Table 1, the mean age of the 60 participating women was 32 years (SD = 4.5 years). Only 15 participants (25%) had ever been pregnant. Menstrual cycle length ranged from 25 to 35 days (mean = 28 days, SD = 1 day) and bleeding during menses ranged from 3 to 9 days (mean = 5 days, SD = 1.5 days). The mean values of CA125 ranged from 12.2 U/ml to 15.9 U/ml.

Profiles of CA125 Species
As shown in Figure 1, Western blot analysis yielded 17 distinct CA125 profiles in healthy women with regular menstrual cycles, with 5 of these occurring in more than one participant. More than 80% of the participants exhibited one of these 5 profiles (i.e., A = 15 participants, B = 12 participants, C = 11 participants, D = 6 participants, and E = 4 participants). The 12 participants with rare profiles (Profile R) represent 20% of the overall sample (Figure 2).

Differences in Demographics and Serum CA125 Values by Profile
We conducted exploratory ANOVA, excluding Profiles E and R (n = 16 participants). We excluded Profile E due to the low number of participants (n = 4) and Profile R because each participant had a unique profile (n = 12). Age at enrollment, age at menarche, BMI, menstrual cycle length, and ever-pregnant status did not differ significantly among Profiles A through D (Table 2). Additionally, within each profile, CA125 values did not differ significantly across menstrual phase (Table 3).

Discussion
In the present study, we used CA125 profiles, based on size in kDa, to identify molecular species. We identified 17 CA125 profiles using Western blot analysis in healthy women with regular menstrual cycles. Consistent with previous studies of molecular speciation of CA125, the species identified in our study exhibited reproducible differences in electrophoretic mobility. Also, consistent with previous studies that included Western blot analysis of CA125 (Andersch-Bjorkman et al., 2007; Jankovic & Tapuskovic, 2005 Kobayashi, Tamura, Satoh, & Terao, 1993; Nustad et al., 1998; O’Brien et al., 2001, 2002), our data show species ranging from 31 kDa to
460 kDa. Most of the referent bands in our data appear at the level of 117 kDa or higher, indicating that CA125 may primarily be a higher molecular weight species in healthy women with regular menstrual cycles. Of note, only two participants had changes in CA125 profiles across the menstrual cycle and they both had unique profiles. Within the five common profiles, CA125 appeared unchanged across the menstrual cycle. In other words, regardless of the phase of the menstrual cycle
The profiles appeared identical. This finding suggests that the species of CA125 does not change over time associated with a single menstrual cycle. Our previous work examining the same cohort of women showed a statistically significant decrease of absolute serum concentration of CA125 from the menses to the follicular phases (McLemore et al., 2012). However, we did not detect a statistically significant difference in CA125 serum concentration by menstrual cycle phase in the current study. We speculate that there are two possible explanations for this finding. The first is that, while differences in the relative abundance of each species exist for each profile, the antibody detects all species and was not able to differentiate among the individual species. The second possibility is that the effects were more subtle than we were able to detect in the available sample size.

It is important to note two major differences between our data and other published studies that investigated the molecular species of CA125: the type of biospecimens we evaluated and the more stringent criteria we used to determine the sample. Investigators have used five tissue types for CA125 molecular speciation studies: ascites (Jacobs & Bast, 1989; Nustad et al., 2002; Whitehouse & Solomon, 2003), cell lines in culture available commercially for purchase (Nustad et al., 1998,

### Table 1. Demographic Characteristics of Study Participants.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Mean ± SD</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>32.0 ± 4.5</td>
<td>21–39</td>
</tr>
<tr>
<td>Age at menarche (years)</td>
<td>12.6 ± 2.0</td>
<td>8–19</td>
</tr>
<tr>
<td>Menstrual cycle bleeding (days)</td>
<td>5.0 ± 1.5</td>
<td>3–9</td>
</tr>
<tr>
<td>Menstrual cycle length (days)</td>
<td>28.0 ± 1.0</td>
<td>25–35</td>
</tr>
<tr>
<td>Height (in.)</td>
<td>64.7 ± 2.6</td>
<td>58.5–70.0</td>
</tr>
<tr>
<td>Weight (lb)</td>
<td>139.0 ± 17.5</td>
<td>105–179</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>23.2 ± 2.4</td>
<td>17.5–29.2</td>
</tr>
</tbody>
</table>

### Table 2. Exploratory Analysis of Variance of Age, Body Mass Index (BMI), and Menstrual cycle Length by CA125 Profile.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Sum of squares</th>
<th>df</th>
<th>Mean square</th>
<th>F</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at enrollment</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Between groups</td>
<td>166.48</td>
<td>5</td>
<td>33.29</td>
<td>1.78</td>
<td>.132</td>
</tr>
<tr>
<td>Within groups</td>
<td>1009.98</td>
<td>54</td>
<td>18.70</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>1176.46</td>
<td>59</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age at menarche</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Between groups</td>
<td>9.42</td>
<td>5</td>
<td>1.84</td>
<td>.44</td>
<td>.821</td>
</tr>
<tr>
<td>Within groups</td>
<td>203.26</td>
<td>47</td>
<td>4.33</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>212.68</td>
<td>52</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMI</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Between groups</td>
<td>16.66</td>
<td>5</td>
<td>3.33</td>
<td>.57</td>
<td>.726</td>
</tr>
<tr>
<td>Within groups</td>
<td>317.92</td>
<td>54</td>
<td>5.88</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>334.58</td>
<td>59</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Table 3. Cross-Sectional Negative Binomial Regression Models of Serum CA125 Levels by CA125 Profile.

<table>
<thead>
<tr>
<th>CA125 Profile</th>
<th>Observed coefficient</th>
<th>Bootstrap SE</th>
<th>z</th>
<th>p &gt; (z)</th>
<th>95% Confidence interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>T₁</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Profile B</td>
<td>−0.015</td>
<td>.196</td>
<td>−0.08</td>
<td>.937</td>
<td>[−.399, .369]</td>
</tr>
<tr>
<td>Profile C</td>
<td>.072</td>
<td>.198</td>
<td>0.36</td>
<td>.719</td>
<td>[−.317, .460]</td>
</tr>
<tr>
<td>Profile D</td>
<td>−0.267</td>
<td>.193</td>
<td>−1.39</td>
<td>.165</td>
<td>[−.645, .010]</td>
</tr>
<tr>
<td>Profile E</td>
<td>−.336</td>
<td>.212</td>
<td>−1.58</td>
<td>.114</td>
<td>[−.753, .081]</td>
</tr>
<tr>
<td>T₂</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Profile B</td>
<td>−.043</td>
<td>.147</td>
<td>−0.29</td>
<td>.769</td>
<td>[−.333, .246]</td>
</tr>
<tr>
<td>Profile C</td>
<td>−.036</td>
<td>.151</td>
<td>−0.24</td>
<td>.812</td>
<td>[−.332, .260]</td>
</tr>
<tr>
<td>Profile D</td>
<td>−.130</td>
<td>.219</td>
<td>−0.59</td>
<td>.552</td>
<td>[−.561, .300]</td>
</tr>
<tr>
<td>Profile E</td>
<td>−.161</td>
<td>.189</td>
<td>−0.85</td>
<td>.394</td>
<td>[−.532, .209]</td>
</tr>
<tr>
<td>T₃</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Profile B</td>
<td>−.027</td>
<td>.122</td>
<td>−0.22</td>
<td>.823</td>
<td>[−.267, .212]</td>
</tr>
<tr>
<td>Profile C</td>
<td>−.267</td>
<td>.137</td>
<td>−1.95</td>
<td>.051</td>
<td>[−.535, .001]</td>
</tr>
<tr>
<td>Profile D</td>
<td>−.139</td>
<td>.184</td>
<td>−0.76</td>
<td>.450</td>
<td>[−.501, .222]</td>
</tr>
<tr>
<td>Profile E</td>
<td>−.274</td>
<td>.164</td>
<td>−1.67</td>
<td>.096</td>
<td>[−.596, .048]</td>
</tr>
</tbody>
</table>

2002; Whitehouse & Solomon, 2003), serum or plasma from healthy women with or without OC but with no documentation of menstrual cycle (Bouané et al., 2012; Jacobs & Bast, 1989; Nustad et al., 1998, 2002; Tuzun et al., 2009). In the present study, we used serum samples derived from human participants. Only three previous studies have used samples derived from healthy human participants (Andersch-Bjorkman et al., 2007; Bouané et al., 2012; Jankovic & Tapuskovic, 2005). The most recent study (Bouané et al., 2012) used serum from healthy women and patients with epithelial OC. The authors measured serum CA125 using enzyme immunoassay, but do not report the values. They do report abundant CA125 bands at 40 kDa; however, two participants had bands at >2,000 kDa. The next most recent study (Andersch-Bjorkman et al., 2007) used cervical mucus collected at different phases across the menstrual cycle, and the oldest study (Jankovic & Tapuskovic, 2005) obtained samples from first-trimester human placentas at between 6 and 12 weeks’ gestation. While the authors did not report ELISA values for the cervical mucus samples, they did note distinct bands between 50 kDa and 300 kDa across all participants, and banding patterns changed within each woman across the menstrual cycle (Andersch-Bjorkman et al., 2007), where the menstrual cycle was confirmed using transvaginal ultrasound and midluteal-phase serum progesterone levels. The authors did report ELISA values for the placental samples, with an average value of 6,000 U/ml and a single broad band at 205 kDa (Jankovic & Tapuskovic, 2005). Our data include bands ranging from 41 kDa to 460 kDa. Despite the use of healthy human samples, each study used a different tissue type, which could explain the variation in the observed species.

Studies of the molecular species of CA125 have been difficult to interpret because of the heterogeneity of tissue types and selection criteria of those tissues included in previous studies. For example, researchers previously used ascites, which is abdominal fluid removed from OC patients during tumor reduction surgery, for molecular speciation studies (O’Brien et al., 2001, 2002). In their 2001 study, O’Brien and colleagues blotted the extracellular repeat region of CA125 using three antibodies (i.e., OC125, K95, and M11), which yielded similar bands of varying intensities all at 32 kDa. However, in the 1998 O’Brien study (O’Brien, Hanimoto, Konishi, & Gee, 1998), investigators blotted all of the isolated CA125 and observed bands at 200 kDa, between 60 and 55 kDa, and at 30 kDa. In both of these studies, the ascites was taken from patients with Stage 3 or 4 tumors, and the data were not stratified by stage of tumor, which may contribute to the variation observed in the profiles of CA125.

Additionally, two studies examining the molecular species of CA125 used cell culture medium for CA125 analysis. The first study used media from the Henrietta Lacks (HeLa) cell line (the cells that differentiate into amnion are called the WISH cell line; Nustad et al., 1998). The researchers initially used gel filtration to separate CA125, which resulted in fractions of 750, 210, 55, and 15 kDa. This analysis showed banding patterns of CA125 above 200 kDa with no banding occurring under 200 kDa. The second study used cell culture media from the HOC-I cell line (Kobayashi et al., 1993). In the aggregate, these researchers found species with masses ranging from 30 kDa to 750 kDa. Given the multivalent nature of CA125 (Jankovic & Milutinovic, 2008; Jankovic & Tapuskovic, 2005; McLemore & Aouzierat, 2005; Nustad et al., 1998) and the documented variation of its measurement in serum (Andersch-Bjorkman et al., 2007; Bidart et al., 1999; Bon et al., 1995; Jankovic & Milutinovic, 2008; Wong et al., 2003), stringent selection criteria for the samples used for molecular speciation studies may provide crucial information about tissue-associated or tissue-specific CA125 species.

**Limitations**

Two limitations need to be acknowledged. First, we observed two of the patterns identified in this study (D and E) in 10 or fewer participant samples, which only allowed for exploratory statistical analyses. Second, it is unknown what the differences in the species represent and it remains to be determined whether these species have different compositions (e.g., residues, nucleotides, and amino acids) that result in different functions.

**Conclusions**

These data provide concrete evidence that CA125 is not a homogeneous molecular species in the serum of healthy women across the menstrual cycle. Future research needs to validate these observations and evaluate the clinical importance and molecular compositions of these species as well as the relationship between these species and the serum ELISA values of CA125.

**Author Contribution**

MML contributed to conception and design, acquisition, analysis, and interpretation; drafted and critically revised the manuscript; gave final approval; and agrees to be accountable for all aspects of work ensuring integrity and accuracy. CM contributed to conception and design, analysis, and interpretation; drafted and critically revised the manuscript; gave final approval; and agrees to be accountable for all aspects of work ensuring integrity and accuracy. KL contributed to design, analysis, and interpretation; critically revised the manuscript; gave final approval; and agrees to be accountable for all aspects of work ensuring integrity and accuracy. L-mC contributed to the acquisition, analysis, and interpretation; critically revised the manuscript; gave final approval; and agrees to be accountable for all aspects of work ensuring integrity and accuracy.
ensuring integrity and accuracy. BA contributed to conception and design, acquisition, analysis, and interpretation; drafted and critically revised the manuscript; gave final approval; and agrees to be accountable for all aspects of work ensuring integrity and accuracy.

Declaration of Conflicting Interests
The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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