ONCOLOGY

Serum levels of the ovarian cancer biomarker HE4 are decreased in pregnancy and increase with age

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OBJECTIVE: The purpose of this study was to establish normal ranges for human epididymis protein 4 (HE4) serum levels in healthy women.

STUDY DESIGN: HE4 levels were measured in healthy women and analyzed by age, menopausal status, and pregnancy status. Upper 95th percentiles were determined for normal ranges.

RESULTS: Serum samples from 1101 healthy women and 67 pregnant women were analyzed. Above the age of 40 years significant elevations in HE4 concentrations emerged with advancing age. The upper 95th percentile for HE4 levels was 89 pmol/L for premenopausal women, 128 pmol/L for postmenopausal women, and 115 pmol/L for all women. There was a significant difference in the median serum HE4 levels in premenopausal women (46.6 pmol/L) compared with postmenopausal women (57.6 pmol/L; $P < .001$). In pregnant women, median HE4 concentrations were significantly lower than their premenopausal counterparts ($P < .001$).

CONCLUSION: HE4 serum concentrations vary significantly on the basis of age. These variations must be considered when the upper limit of normal for HE4 is determined.

Key words: biomarker, HE4, healthy women, pregnancy

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Over the past 3 decades cancer antigen 125 (CA125) has provided a biomarker for the monitoring of women who receive a diagnosis of ovarian cancer during treatment and before disease recurrence.[1,2] CA125 has also been studied extensively for a possible role in early detection and screening for ovarian cancer. Although promising, the role of CA125 in this area has yet to be defined.[3-6]

Although CA125 is the current standard biomarker for the management of ovarian cancer, it is not without limitations. CA125 is elevated in only 50-60% of early stage cases and is not expressed by up to 20% of all ovarian cancers.[7] CA125 specificity is also limited because levels can be elevated in several benign gynecologic disorders, such as endometriosis, pelvic inflammatory disease and benign neoplasms of the ovaries and uterus.[7-11] CA125 can be elevated in many common nongynecologic conditions such as congestive heart failure, hepatic disease, and inflammatory diseases that affect pleural, peritoneal, and pericardial surfaces.

The novel serum biomarker human epididymis protein 4 (HE4) has been shown to be over-expressed in serous, endometrioid, and clear cell epithelial ovarian cancers.[12] HE4 has also been demonstrated to be a sensitive and specific serum biomarker for ovarian cancer that is elevated less frequently by benign conditions that occur in premenopausal women.[13,14] Recently it was shown that the addition of HE4 to CA125 increased the sensitivity and specificity of either marker alone for the detection of ovarian cancer.[14-17]

The HE4 protein is a whey acid protein with a 4 disulfide core that originally was isolated in epithelial cells of the human epididymis and is expressed in numerous tissues throughout the body, including the female reproductive tract.[18] Importantly, HE4 circulates in the bloodstream and can be detected through an immunosorbent assay (EIA) with a monoclonal mouse antibody directed at an HE4 epitope.

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In 2009, the United States Food and Drug Agency (FDA) approved HE4 for the monitoring of women who have received a diagnosis of epithelial ovarian cancer with similar indications to the use of CA125. To date, however, there are no large trials that are examining serum HE4 levels in healthy premenopausal and postmenopausal women and healthy pregnant women. The upper 95th percentile of 150 pmol/L for both premenopausal and postmenopausal women is reported in the FDA package insert for the HE4 EIA Kit (Fujirebio Diagnostics Inc, Malvern, PA). This value does not take into consideration patient age or menopausal status; what actually constitutes normal levels in healthy women and whether these levels vary by subgroups have not been clearly evaluated and published. The purpose of this study was to examine serum levels of HE4 in healthy women on the basis of age, menopausal status, and pregnancy status to refine normative data for this novel biomarker.

MATERIALS AND METHODS

A metaanalysis was performed with data collected in 3 independent trials that measured HE4 levels in healthy women with the use of the HE4 EIA kit (Fujirebio Diagnostics Inc). Data from the following studies were included: (1) An institutional review board–approved study at Women and Infant's Hospital (WIH) that obtained residual serum from healthy premenopausal women (n = 101) and postmenopausal women (n = 91) and residual serum samples from women during their first, second, and third trimesters of pregnancy (n = 67); (2) an institutional review board–approved trial through M.D. Anderson Cancer Center that enrolled postmenopausal women in a multicenter low-risk ovarian cancer screening trial through an ovarian Specialized Programs of Research Excellence grant (SPORE P50), from whom 143 samples were obtained; and (3) Fujirebio Diagnostics Inc obtained serum samples that had been collected from institutional review board–approved repositories that included samples from 374 premenopausal and 392 postmenopausal healthy women that had been banked (protocol FDI-53). Institutional review board review of the FDI-53 protocol found that the data were unlinked and deidentified and therefore did not require approval. All blood samples were centrifuged, and the serum was collected and frozen at −80°C until testing.

Menopausal status was determined with the following criteria for each of the individual studies: For the WIH samples, women who were ≥55 years old were considered postmenopausal, and women who were ≤45 years old were considered premenopausal. No samples were obtained from WIH for women between the ages of 46 and 54 years. All women who were entered from the M.D. Anderson Cancer Center trial were postmenopausal, which was determined by a medical interview and a history of amenorrhea for >1 year. The menopausal status for the patient samples that were obtained from the Fujirebio clinical trial sample banks was determined through medical history or chart review, which was reported by the serum banks that supplied these samples. Serum levels for HE4 were tested at each institution with an HE4 EIA assay kit (Fujirebio Diagnostics Inc).

Statistical analysis

The primary endpoint of this study was to describe serum concentrations of HE4 (picomoles per liter) to determine the normal ranges in healthy premenopausal and postmenopausal women and pregnant women. In each group, the median, range, mean, standard deviation, percent coefficient of variation, and the 90th, 95th, 97.5th and the 99th percentile for serum HE4 levels were determined. Normal serum levels for HE4 were defined with a cut point at the upper 95th percentile. Probability values for medians were derived with a continuity corrected Pearson's chi-square median test and the Wilcoxon rank sum method. Log base 2–transformed scatter plots also were generated for HE4 levels by decadal age group and menopausal status; standard scatter plots were generated for HE4 levels in pregnant women. All HE4 values were derived by cubic spline interpolation.

RESULTS

This study included serum samples from 1168 women with a total of 1101 healthy, nonpregnant women and 67 pregnant women. There were 475 premenopausal women (101 women from WIH Rhode Island; 374 women from Fujirebio Diagnostics Inc), with a mean age of 34.3 years (range, 15–57 years) and 626 postmenopausal women (91 women from WIH; 143 women from M.D. Anderson Cancer Center; 392 women from Fujirebio Diagnostics Inc), with a mean age of 62.8 years (range, 34–94 years). In addition, the study included 67 healthy pregnant women from WIH, with 25 women in the first trimester, 25 women in the second trimester, and 17 women in the third trimester of pregnancy.

HE4 levels by menopausal status

The mean, standard deviation, median and ranges for serum HE4 levels by age groups and menopausal status are shown in Table 1.

The combined median serum HE4 levels for all premenopausal women (n = 475) was 46.6 pmol/L. A comparison of the median HE4 serum levels for premenopausal women by decade of age showed no significant differences between the age group <30 years (n = 170), median level of 46.2 pmol/L, and age 30-39 years (n = 159), median of 43.5 pmol/L (median probability, .204; rank sum probability, .1600). When the age group 30-39 years (n = 159) was compared with the age group of ≥40 years (n = 146), there was a statistically significant difference in the median HE4 serum levels (43.5 vs 50.5 pmol/L, respectively; median probability, .007; rank sum probability, < .0001). When we compared premenopausal women at <40 years old (n = 329) to those ≥40 years old (n = 146), a statistically significant difference in the median HE4 levels was observed (44.9 vs 50.5 pmol/L, respectively; median probability, .010; rank sum probability, < .0001).

The median serum HE4 levels for all postmenopausal women combined (n = 626) was 57.6 pmol/L. A comparison of the HE4 serum levels for postmenopausal women by decade of age showed statistically significant differences in median serum HE4 levels between each age group, with increasing HE4 levels up to the age of 80 years. The postmenopausal

TABLE 1
Serum HE4 levels for premenopausal and postmenopausal women by age group (n = 1101)

	Age group, y						
	Premenopausal			Postmenopausal			
Variable	<30	30-39	≥40	<60	60-69	70-79	≥80
n	170	159	146	256	236	102	32
Mean, pmol/L	55.5	49.9	61.4	56.0	66.9	76.6	137.8
SD, pmol/L	66.8	28.6	54.6	26.1	49.3	34.1	87.7
Median, pmol/L	46.2	43.5	50.5	50.7	59.9	66.9	113.4
Range, pmol/L	24.5-656.4	22.4-293.5	22.6-645.6	18.7-285.8	12.0-690.8	21.7-228.9	48.5-430.8

HE4, human epididymis protein 4.

Moore. Serum HE4 levels in healthy women. Am J Obstet Gynecol 2012.

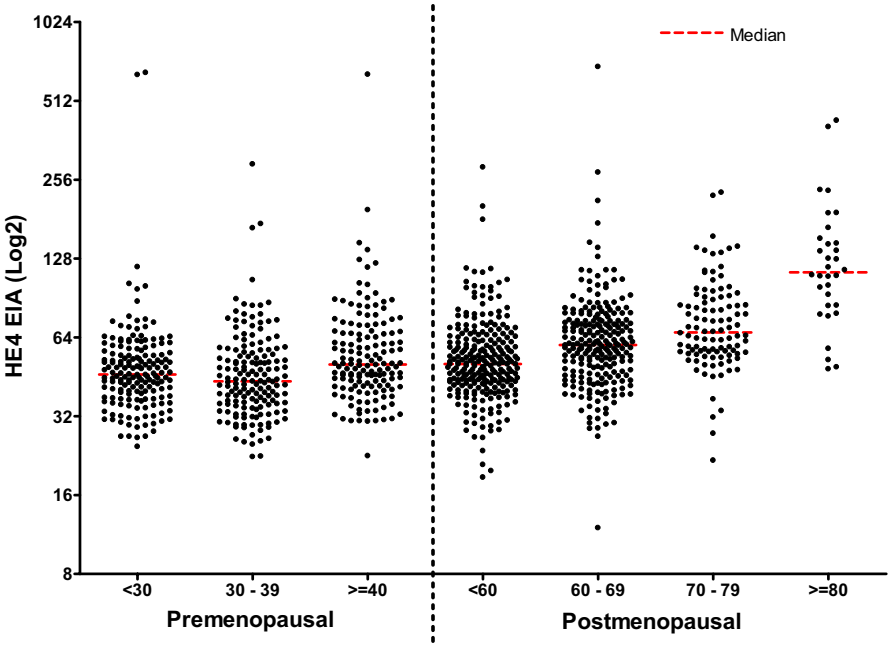
age group <60 years (n = 256) had a median HE4 level of 50.7 pmol/L, which was significantly lower compared with the age group of 60-69 years (n = 236) that had a median HE4 level of 59.9 pmol/L ($P < .001$). The age group 60-69 years had a significantly lower median serum HE4 level compared with a median of 66.9 pmol/L for the age group 70-79 years (n = 102; median probability, .024; rank sum probability, .0005). Comparison of the age group ≥80 years (n = 32) showed significant elevation of the median serum HE4 level (113.4 pmol/L) when compared with all other postmenopausal age groups ($P < .001$). The median serum HE4 level for all premenopausal women was 46.6 pmol/L, which was significantly lower compared with the median HE4 level for all postmenopausal women (57.6 pmol/L; $P < .001$) and to the median HE4 level for postmenopausal women who were <80 years old (n = 594; 56.8 pmol/L; $P < .001$). However, the median HE4 level for premenopausal women ≥40 years old (50.5 pmol/L) and postmenopausal women <60 years old (50.7 pmol/L) showed no significant difference, which indicated that differences in HE4 levels may not be related to menopausal status but age. Taking this into consideration the most significant age cut off, with regard to the difference between the HE4 concentrations, was at the age of 60 years. For all women <60 years old, the median HE4 level was 48.2 pmol/L; for all women ≥60 years old, the median HE4 level was 63.4 pmol/L ($P < .001$).

Figure 1 displays a scatterplot of the serum HE4 levels for all 1101 samples, which were grouped by menopausal status and age. The median serum HE4 levels clearly rise consistently with age, regardless of menopausal status (Figure 2).

HE4 upper limits of normal

The upper 90th, 95th, 97.5th and 99th percentiles for premenopausal, postmenopausal, and all women combined were determined and shown in Table 2. The 95th percentile is often used as the upper limit of normal in biomarker analysis. With the 95th percentile as the upper limit of normal cut point, premenopausal women had a cut point 89.1 pmol/L, and postmenopausal women had a cut point of 128.0 pmol/L. When we combined the data for all women (pre- and postmenopausal), the 95th percentile for serum HE4 was 114.8

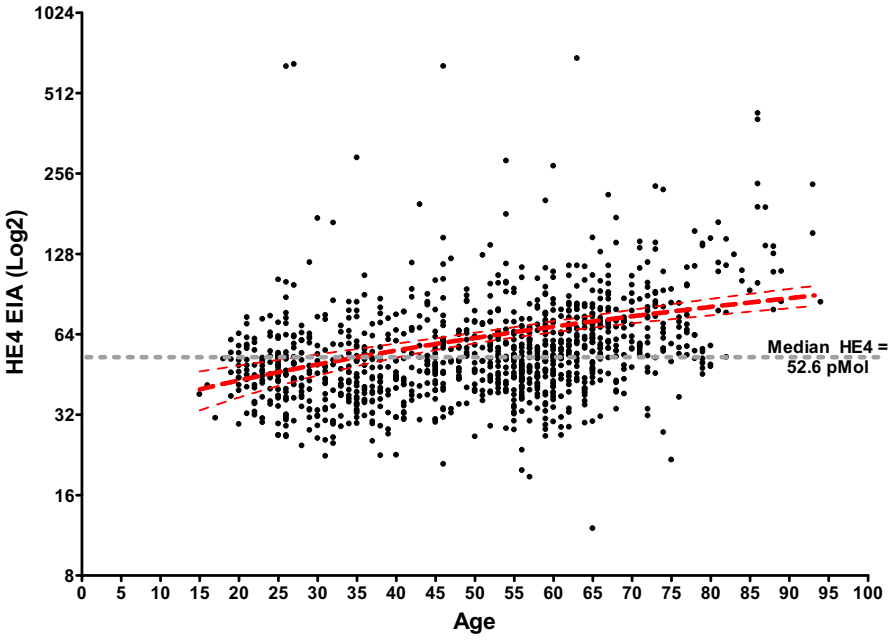
FIGURE 1
Scatterplot of the serum HE4 levels for pre- and postmenopausal women by age group



EIA, immunosorbent assay; *HE4,* human epididymis protein 4.

Moore. Serum HE4 levels in healthy women. Am J Obstet Gynecol 2012.

FIGURE 2
Serum HE4 levels increase with age and are independent of menopausal status



EIA, immunosorbent assay; *HE4*, human epididymis protein 4.

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pmol/L. Examination of all women (pre- and postmenopausal) with the use of an age cut point of 60 years, women <60 years old had an 95th percentile cut point of 93.3 pmol/L and women >60 years of age had a 95th percentile cut point of 141.7 pmol/L.

The International Federation of Clinical Chemistry and Laboratory Medicine and the Clinical and Laboratory Standards Institute have described methods for determining biomarker cut points.[19] This method uses the upper 95% reference limit of the 95th percentile reference interval with 90% confidence intervals. These values for serum HE4 levels are displayed in Table 3. With this method, the upper limit for premenopausal women was 118.9 pmol/L (90% confidence interval [CI], 97.7–167.4); for postmenopausal women, the upper limit is 167.8 pmol/L (90% CI, 140.8–212.7). When data for both pre- and postmenopausal women were combined, the upper limit of normal is 146.3 pmol/L (90% CI, 138.0–191.5; Table 3), which is equivalent to the 150 pmol/L threshold reported in the FDA package insert for the HE4 EIA assay. When we examined the cut point of 60 years for all women (pre- and postmenopausal), women who were <60 years old had an upper limit of normal of 116.9 pmol/L (90% CI, 102.6–138.0) and women >60 years old had an upper limit of normal of 212.7 (90% CI, 152.7–234.3).

TABLE 2
Distribution of serum HE4 levels in women, stratified by menopausal status

Variable	All women	Premenopausal women	Postmenopausal women
n	1101	475	626
Percentile			
90th	90.9	75.1	101.2
95th	114.8	89.1	128.0
97.5th	145.8	118.6	160.1
99th	228.9	179.7	231.8

HE4, human epididymis protein 4.

Moore. Serum HE4 levels in healthy women. Am J Obstet Gynecol 2012.

HE4 levels by pregnancy status

In a separate group of 67 pregnant women, no statistically significant differences were noted in median HE4 levels by trimester (Table 4). However, the difference in median HE4 levels between the second and third trimesters approached statistical significance (30.0 vs 35.0 pmol/L; $P = .059$). Moreover, the distribution of the HE4 serum values between the second and third trimester groups did differ significantly (Wilcoxon rank sum probability, .0116), most likely because of a larger number of higher HE4 values in the third trimester group. The distributions of the first and second and the first and third trimesters did not differ significantly (Wilcoxon rank sum probability, .0990 and .1826, respectively). The 95th percentile HE4 cutoff for all pregnant women was 49.7 pmol/L and ranged from 35.1 to 50.2 pmol/L in the second and 3st trimesters, respectively.

When women in any trimester of pregnancy were compared with all premenopausal women, median HE4 values were significantly lower in pregnant women (30.5 vs 46.6 pmol/L; $P < .001$), and serum sample distributions differed significantly (Wilcoxon rank sum probability, < .0001; Figure 3).

COMMENT

HE4 has demonstrated utility as a marker for the detection and management of ovarian cancer, especially in combination with CA125, which is the current gold standard marker for this malignancy.[14-16,20] However, to date no clear normative values have been published for the serum biomarker in healthy women and pregnant women. The results of our study elucidate

TABLE 3
Serum HE4 levels for premenopausal women, postmenopausal women, and all women

Variable	All women	Premenopausal women	Postmenopausal women
n	1101	475	626
95% reference interval	28.2–146.3	26.8–118.9	29–155.4
Lower 95% reference limit (90% CI)	28.2 (26.5–29)	26.8 (25.7–29)	29 (27.5–30.9)
Upper 95% reference limit (90% CI)	146.3 (138–191.5)	118.9 (97.7–67.4)	167.8 (140.8–212.7)
Average, pmol/L	62.4	55.4	67.6
SD, pmol/L	49.2	52.9	45.6
Variance, pmol/L	2424.5	2796.5	2082.2
Median, pmol/L	52.6	46.6	57.6

CI, confidence interval; *HE4,* human epididymis protein 4.

Moore. Serum HE4 levels in healthy women. Am J Obstet Gynecol 2012.

normal HE4 serum levels in healthy women without malignant or benign gynecologic disorders and provide insight into how HE4 levels vary in different populations of healthy women.

Our findings show a significant difference in serum HE4 concentrations by age, with a significant rise that starts at age 60 years. When we combined data for all menopausal women with a comparison to all premenopausal women, there was a significantly higher serum HE4 level in the postmenopausal patient population. However, interestingly when we compared data for premenopausal women ≥40 years old with data for postmenopausal women ≤60 years old, there was no statistical difference in the median serum HE4 levels for the 2 groups. This suggests that menopausal status does not play a role in the increasing median serum levels; rather, age is the critical factor. These findings are consistent with a study that examined a cohort of women at high risk for ovarian cancer that showed increasing HE4 levels with age.[21] Among premenopausal healthy women, no statistically significant differences in median HE4 serum concentrations emerged that were based on decadal age when we compared data for women <30 years old with women 30-39 years and women ≥40 years old. However, when we compared data for premenopausal women <40 years old with women ≥40 years old, a statistically significant difference emerged, which again supported age as a determinant for the increasing HE4 levels in healthy females. Among postmenopausal women, steady increases in median serum HE4 concentrations were seen across the different age groups: <60, 60-69, 70-79, and ≥80 years old. In postmenopausal women, increasing CA125 levels also have been linked to advancing age in healthy women.[22,23] In our study, the most notable increase occurred after age 79 years. Increases in HE4 that were observed in our study of aging postmenopausal women elevates the risk for false-positive tumor marker findings, which underscores the clinical importance of recognizing these trends in normal values when HE4 is used for cancer diagnosis. Although the precise reason for these increases remains uncertain, they are likely the result of age-related declines in renal function or perhaps an increased prevalence of comorbid conditions.

An upper limit of normal for serum HE4 levels of 150 pmol/L has been reported in the FDA package insert for the HE4 EIA kit (Fujirebio Diagnostics Inc) for the combined data for pre- and postmenopausal women. The current study achieved similar results with the use of the 97.5th percentile that resulted in a threshold of 145.8 pmol/L. Also, the use of the International Federation of Clinical Chemistry and Laboratory Medicine and the Clinical and Laboratory Standards Institute method for the determi-

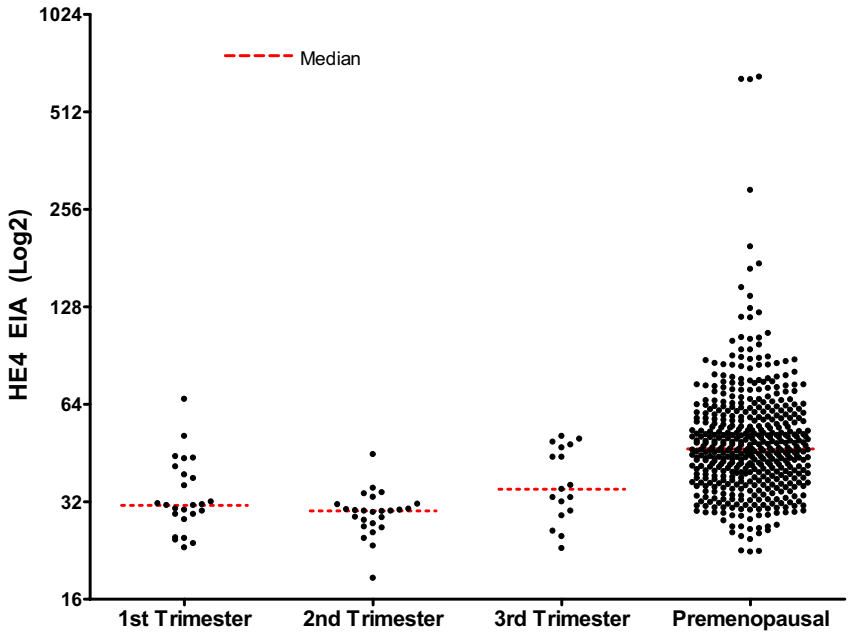
TABLE 4
Serum human epididymis protein 4 (*HE4*) levels during pregnancy by trimester

	Trimester			
Variable	1	2	3	All
n	25	25	17	67
Mean, pmol/L	34.3	29.7	37.4	33.4
Median, pmol/L	31.2	30.0	35.0	30.5
SD, pmol/L	9.9	4.8	9.6	8.7
Percentile				
95th	49.6	35.1	50.2	49.7
97.5th	57.2	39.1	50.6	51.0
99th	62.7	42.5	50.8	56.2

HE4, human epididymis protein 4.

Moore. Serum HE4 levels in healthy women. Am J Obstet Gynecol 2012.

FIGURE 3
Scatterplot of pregnant women by trimester and all premenopausal women



	1st Trimester	2nd Trimester	3rd Trimester	Premenopausal
N	25	25	17	475
Avg + StdDev	34.3 ± 9.9	29.7 ± 4.8	37.4 ± 9.6	55.5 ± 52.9
Median	31.2	30.0	35.0	46.6
Range	23.1 - 66.4	18.6 - 44.8	23.0 - 51.0	22.4 - 656.4

AVG, average; *HE4,* human epididymis protein 4; *EIA,* immunosorbent assay; *StdDev,* standard deviation.

Moore. Serum HE4 levels in healthy women. Am J Obstet Gynecol 2012.

nation of cut points that uses the upper confidence interval for the 95th percentile, a threshold of 146.3 pmol/L was observed in this study, which is similar to that reported in the FDA package insert. However, a single cut point for both menopausal groups may lead to inaccurate patient evaluations. Clinically important variations in HE4 serum concentration occur on the basis of age among healthy women, which underscores the need to use normative data that addresses these specific subgroups. In our study, normal HE4 serum levels, which were defined with the use of a cut point at the 95th percentile, were 114.8 pmol/L for all women, 89.1 pmol/L for premenopausal women, and 125.6 pmol/L for postmenopausal women, which illustrates the need to use age- or menopausal-specific cut points. When specific cut points for biomarkers are used, clinicians must be familiar with the patient population, the laboratories, and the methods by which the biomarkers are being measured and reported. For instance, with a cut point at the 99th percentile, HE4 values for healthy premenopausal women were 180 pmol/L, for postmenopausal women were 232 pmol/L, and for all women combined were 229 pmol/L. With this in mind, when HE4 levels are obtained in otherwise healthy individuals, it is important to realize that values higher than the FDA-approved normal of 150 pmol/L can occur. The same holds true for CA125 levels; some manufacturers' platform CA125 assays have a 95th percentile cut point of 21 U/mL, and others have a 35 U/mL upper limit of normal. When known ovarian cancer is monitored, it will be important to use individual patient baselines, particularly in postmenopausal women. Further studies will be required to determine whether the trend of HE4 values provides even greater specificity, as is the case with CA125.[24]

Median HE4 concentrations were not significantly different among trimesters but were significantly lower when compared with their premenopausal counterparts ($P < .001$). Only slight increases in HE4 serum levels were noted between the second and third trimesters. In pregnant individuals, the 95th percentile upper limits of normal for HE4 serum concentration were 49.6 pmol/L, 35.1 pmol/L, and 50.2 pmol/L during the first, second, and third trimesters, respectively, with an overall upper limit of 49.7 pmol/L. Moreover, median HE4 serum levels were significantly lower (approximately 16 pmol/L) in pregnant women vs premenopausal women. In contrast, serum levels of CA125 and other tumor markers (such as CA19-9, carcinoembryonic antigen, squamous cell carcinoma antigen, mucin-like carcinoma-associated antigen, and tissue polypeptide-specific antigen) increase notably during pregnancy.[25] Elevations in CA125 during pregnancy occur predominantly during the first trimester, perhaps because of its role in early fetal development.[26,27] The lower concentrations of serum HE4 in pregnancy may be due to increased renal clearance that is associated with pregnancies. In our study, no elevations in median HE4 serum concentrations were seen during the first trimester vis-à-vis the second and third trimesters or all trimesters combined. These findings suggest that, in pregnant women, HE4 will remain a relatively reliable and robust marker of ovarian cancer and may be useful for the evaluation of ovarian cysts and pelvic masses in pregnant patients; CA125 serum measures could yield an increased number of false-positive results. Our results further bolster the rationale for the use of the dual marker combination of HE4 and CA125 to maintain optimal levels of specificity.[14,16,28]

The current study was limited by the absence of demographic data, which would allow conclusions about HE4 concentration variability based on such factors as race and ethnicity. Nonetheless, our findings show that HE4 serum concentrations display clinically relevant variability by age in healthy women that should be weighed when considering what constitutes the upper limit of normal for HE4 serum concentrations. Furthermore, our findings demonstrate that, in healthy pregnant women, HE4 concentrations do not manifest clinically

important variability among trimesters. Indeed, HE4 concentrations were not elevated during any trimester of pregnancy. Additionally, all HE4 serum levels in this study were measured with an HE4 EIA assay kit from Fujirebio Diagnostics Inc. Currently the only other available platforms to measure HE4 serum levels are provided by Roche and Abbott Diagnostics. Although these platforms were designed to provide comparable measurements, direct comparative studies must be performed, and the findings of the present study should be limited to the HE4 EIA assay.

Our findings should provide clinically relevant normative HE4 serum concentrations for healthy women that are based on age and menopausal and pregnancy status. ■

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