



# Reference values of human epididymis protein 4 in the Bulgarian population – assessment of the influence of age, menopause, pregnancy, and renal function

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## Abstract

**Introduction:** Human epididymis protein 4 (HE4) is increasingly utilized in diagnosing pelvic masses and monitoring patients with ovarian or endometrial carcinoma. However, various publications and diagnostic kit manufacturers report a wide range of reference and threshold values, complicating accurate clinical interpretation and reducing the marker's clinical utility. This study aimed to establish reference values for HE4 in the Bulgarian female population and investigate the impact of physiological factors such as age, menopause, and pregnancy, as well as pathological conditions like impaired renal function, on marker levels.

**Materials and methods:** The study included 338 women: 246 healthy, nonpregnant women aged 20–82 (124 of whom were premenopausal); 52 pregnant women; and 40 women with reduced renal function, as determined by serum creatinine levels. Data were collected on gynecological status, renal and hepatic function, diabetes, and other clinically significant chronic or oncological diseases. Serum HE4 concentrations were measured using an indirect chemiluminescent microparticle immunoassay (CMIA) on the Architect ci8200 system (Abbott Diagnostics). Statistical analyses were performed using SPSS version 25.0, and values were considered statistically significant at  $p < 0.05$ .

**Results:** The upper reference limit for HE4 in healthy women (95th percentile with 95% confidence interval) was 62.43 (59.02–65.45) pmol/L. For menopausal status, upper reference limits were 53.22 (50.80–60.82) pmol/L for premenopausal women and 65.31 (61.57–68.35) pmol/L for postmenopausal women. In pregnant women, the upper HE4 limit was 50.90 pmol/L, with values by gestational age of 57.73 pmol/L (first trimester) and 46.35 pmol/L (second trimester). None of the women with serum creatinine within the reference range had elevated HE4 levels, whereas all patients with elevated creatinine had HE4 levels above the 95th percentile for their respective age and menopausal status.

**Conclusions:** The established reference limits for HE4 in this study significantly differ from those proposed by Abbott Diagnostics that are used to date. Implementing these limits in clinical practice could enhance the marker's sensitivity and enable earlier detection of gynecological malignancies. The data confirm that HE4 levels depend on age, particularly after 60 years, and on reduced renal function, necessitating cautious interpretation in these patients. No significant differences in HE4 concentrations were observed between pregnant and non-pregnant women, supporting the use of uniform reference values regardless of pregnancy or gestational stage.

## Keywords

age, gynecological malignancies, HE4, healthy women, pregnancy, renal function

## Introduction

The development of cDNA and oligonucleotide microarray analyses in studying ovarian carcinoma (OC) biology has identified numerous overexpressed genes in primary tumors and ovarian cancer cell lines. Among the most frequently and significantly overexpressed genes in ovarian tumors compared to normal ovarian tissue is the gene encoding human epididymis protein 4 (HE4). First described in 1991 by Kirchoff et al.<sup>[1]</sup> using Northern blot analysis and in situ transcript hybridization in human epididymal tissue, HE4 expression was later shown to be elevated in ovarian carcinoma tissue by Schummer et al.<sup>[2]</sup> in 1999. In 2003, Hellström et al.<sup>[3]</sup> reported the initial use of HE4 as a serum biomarker for ovarian cancer. The HE4 gene, located on chromosome 20q12-13.1—a region frequently amplified in ovarian carcinoma—encodes a low-molecular-weight glycoprotein (25 kDa, compared to >200,000 kDa for CA125) belonging to the whey acidic protein family. Due to its two whey acidic protein domains and four disulfide cores formed by eight cysteine residues, it is also designated WFDC2 (Whey Acidic Protein with Four Disulfide Core protein 2). This glycoprotein is expressed by epithelial cells of normal female reproductive tissues (fallopian tubes, endometrium, and endocervix), as well as oral and respiratory epithelium, but is minimally expressed in healthy ovarian tissue. HE4 is absent in the gastrointestinal tract, liver, pancreas, spleen, lymph nodes, and mesenchymal tissues such as heart, skeletal muscle, mammary gland, or brain. Consequently, Drapkin et al.<sup>[4]</sup> concluded in 2005 that HE4 expression is highly restricted in normal tissues. They also demonstrated that HE4 is overexpressed not only in serous and endometrioid epithelial ovarian carcinoma but also in other neoplastic conditions, including endometrial carcinoma, lung cancer, and oral epithelial cancer. The physiological role of HE4 remains unclear, though its presence in epididymal tissue suggests a role in spermatogenesis (sperm maturation). HE4 likely functions as a protease inhibitor, although its target protease remains unidentified. It may contribute to the tumor's anti-inflammatory response, similar to protease inhibitors such as elafin, eppin, and SLPI.<sup>[5]</sup> In a 2012 study, Lu et al.<sup>[6]</sup> reported that HE4 plays a key role in the adhesion and migration of ovarian cancer cells, observing HE4 overexpression in SKOV-3 ovarian cancer cells. Inhibition of HE4 suppressed tumor growth in vitro, suggesting its involvement in cancer cell adhesion, migration, and tumor growth, likely through its influence on signaling pathways. Over the past decade, increasing evidence has highlighted changes in HE4 concentrations in various carcinomas, fostering optimism for its use as a serum tumor marker. However, despite extensive research, data on HE4's biological variation and levels in healthy individuals remain limited.

A large study by Park et al.<sup>[7]</sup> established reference ranges for HE4 among women aged 20–65 years in Asia, but its reliability is limited due to insufficient participant selection details. Additionally, as the incidence of ovarian carcinoma

increases with age, the exclusion of women over 65 limits the study's applicability.

Another large study on HE4 reference values and biological variation, conducted in Norway by Bolstad et al.<sup>[8]</sup>, analyzed 1,591 serum samples from a donor bank using two methods: 802 samples with the manual EIA test by Fujirebio Diagnostics Sweden and 789 samples with the automated Architect HE4 CMIA test by Abbott Diagnostics. Variables considered included age, sex, BMI, smoking, alcohol consumption, physical activity, serum creatinine, time of blood draw, and whether the sample was taken fasting or postprandial. Results showed that age had the strongest influence on serum HE4 levels, increasing by 2% at age 30, 9% at 40, 20% at 50, 37% at 60, 63% at 70, and 101% in individuals over 80 compared to 20-year-olds. Smokers had approximately 29% higher HE4 concentrations than non-smokers, with this effect becoming more pronounced with age due to the accumulation of multiple factors. Greater standard deviation in HE4 levels was also observed in smokers. Although the exact mechanism is unknown, inflammatory changes in bronchial epithelium due to smoking are hypothesized to increase HE4 expression, similar to the tumor marker CEA. Sex had a minor effect: men had about 7% lower HE4 levels than women, though the age-related increase was more pronounced in men. The minimal differences between sexes suggest that the primary source of this glycoprotein in healthy individuals is likely not reproductive organs. An inverse relationship was found with BMI: higher BMI was associated with lower HE4 levels (5% lower at BMI 25 and 10% lower at BMI 30 compared to BMI 20). A strong positive correlation was observed between serum creatinine and HE4 concentrations; when creatinine rose from 50  $\mu\text{mol/L}$  to 100  $\mu\text{mol/L}$ , HE4 increased by 27%. No associations were found between HE4 levels and time of sampling, fasting status, physical activity, or seasonality. The reported reference values were significantly lower than those declared by the ELISA test manufacturer (up to 150 pmol/L regardless of menopausal status) and those published by Molina et al. (138 pmol/L for premenopausal and 132 pmol/L for postmenopausal women).<sup>[9]</sup> These differences may stem from varying sample sizes and insufficient demographic data in the studies. In no age group did the 97.5th percentile exceed 70 pmol/L with a 90% confidence interval, aligning with findings by Park et al.<sup>[7]</sup> for the Asian population and Lenhard et al.<sup>[10]</sup>, who studied 109 healthy individuals.

Anastasi et al.<sup>[11]</sup> reported higher HE4 concentrations during the ovulatory phase of the menstrual cycle compared to the follicular phase in young women. Moore et al.<sup>[12]</sup>, in a U.S.-based study of 1,101 healthy women and 67 pregnant women, established 95th percentile values of 89 pmol/L for premenopausal women, 128 pmol/L for postmenopausal women, and 115 pmol/L overall. They confirmed age dependence (median values: 46.6 vs. 57.6 pmol/L;  $p < 0.001$ ) and reported significantly lower HE4 levels during pregnancy.

These studies highlight the lack of unified HE4 reference ranges and consistent data on biological variability. Col-

lecting such data and verifying the applicability of manufacturer-recommended reference values to the Bulgarian population are crucial for accurate HE4 result interpretation and evaluating its diagnostic utility, particularly in early-stage malignant disease.

## Aim

This study aims to establish reference values for HE4 in the Bulgarian female population and evaluate the influence of physiological factors (age, menopause, pregnancy) and pathological conditions (reduced renal function) on marker levels.

## Materials and methods

The study included 338 women aged 20–82 years: 246 healthy women (124 premenopausal), 52 pregnant women, and 40 with reduced renal function (based on serum creatinine concentration) but no gynecological disease. A retrospective analysis was conducted using data from the laboratory information system of Acibadem City Clinic Tokuda Medical Center and Acibadem City Clinic Tokuda University Hospital. Health status was assessed regarding gynecological status, renal and liver function, diabetes, and other clinically significant chronic or oncological conditions. Serum samples were processed according to the laboratory's standard operating procedures. HE4 concentrations were measured using a chemiluminescent microparticle immunoassay (CMIA) on the Architect ci8200 analyzer (Abbott Diagnostics, USA) with linearity up to 1500 pmol/L. Since in some cases HE4 concentrations exceeding the upper limit of linearity were not re-analyzed following dilution, and the result was reported as >1500 pmol/L, such values were equated to 1500 pmol/L for the purpose of evaluating the correlation with creatinine concentration. During the method verification process, the within-run coefficient of variation (CV) was calculated to be in the range of 1.93–2.63%, and the between-run CV ranged from 7.09% to 9.39% (for low, medium, and high HE4 concentrations), with bias (d%) ranging from 2.08% to 2.69%. Serum creatinine concentration was measured using the Jaffe kinetic assay without deproteinization on the Architect ci8200 analytical platform, calibrated with the ConCC calibrator, traceable to the NIST SRM967 reference material. For each creatinine result, the estimated glomerular filtration rate (eGFR) was also calculated using the CKD-EPI 2021 equation. Statistical analysis was performed using SPSS v. 25.0 with significance set at  $p < 0.05$ . Tests included Shapiro-Wilk and Kolmogorov-Smirnov for normality, Spearman's rho and Pearson's R for correlation, regression analysis for continuous variables with near-normal distributions, and the Kruskal-Wallis test for differences in means among independent samples. Percentile methods were used to determine reference values and to test statistical hypotheses for

significant differences in specific tested thresholds. For this purpose, 95% confidence intervals (95% CI) for the 95th percentile were constructed, and the threshold values were compared against these intervals to determine the significance of any observed differences.

## Results

To establish HE4 reference ranges in the Bulgarian population, 246 healthy women aged 20–82 years (mean±SD: 48.20±13.04) were analyzed, divided by menopausal status into premenopausal ( $n=124$ , aged 20–54 years, mean±SD: 37.77±8.13) and postmenopausal ( $n=122$ , aged 35–82 years, mean±SD: 58.80±7.26) subgroups. Normal distribution tests and visual evaluations (histograms and P-P plots) showed that HE4 values did not follow a Gaussian distribution. The Kolmogorov-Smirnov test rejected the null hypothesis of normality ( $p < 0.05$ ). Data were therefore analyzed using nonparametric methods following transformation ( $\alpha=0.05$ ). After statistical processing and normalization, a percentile method was used to calculate reference ranges. The upper reference limit corresponds to the 95th percentile with a 95% confidence interval. Median and interquartile range (IQR) for HE4 were 38.90 (33.50–47.43) pmol/L for the entire cohort, 36.65 (31.70–42.35) pmol/L for premenopausal women, and 44.15 (37.20–54.33) pmol/L for postmenopausal women. The Mann-Whitney test showed significantly higher HE4 levels in postmenopausal women compared to premenopausal women ( $U=4391.5$ ,  $p < 0.001$ ,  $r=-0.362$ ). Reference limits were defined as follows: total population—62.43 (59.02–65.45) pmol/L; premenopausal—53.22 (50.80–60.82) pmol/L; postmenopausal—65.31 (61.57–68.35) pmol/L (**Fig. 1**).

A Spearman's Rho correlation coefficient was used to assess the relationship between age and HE4 levels. A significant positive correlation was found ( $\rho=0.430$ ,  $p < 0.001$ ). Pearson's method also confirmed this linear relationship ( $r=0.43$ ,  $p < 0.0001$ ). To determine whether age-specific reference ranges were necessary, the cohort was divided into four age groups:  $\leq 39$  years ( $n=68$ ), 40–49 ( $n=56$ ), 50–59 ( $n=69$ ), and  $\geq 60$  years ( $n=52$ ). The medians and IQRs were:  $\leq 39$ —36.50 (31.83–41.00) pmol/L; 40–49—37.35 (32.05–44.70) pmol/L; 50–59—41.40 (35.50–47.70) pmol/L; and  $\geq 60$ —47.70 (37.65–61.50) pmol/L. The Kruskal-Wallis test revealed significant differences among age groups ( $\chi^2(3)=33.51$ ,  $p < 0.001$ ), with post-hoc tests identifying differences particularly between younger and older groups. The upper reference limits (95% CI) by age were:  $\leq 39$ —54.86 (48.93–73.16) pmol/L; 40–49—57.64 (51.47–60.82) pmol/L; 50–59—60.85 (56.06–63.15) pmol/L; and  $\geq 60$ —68.84 (63.84–71.63) pmol/L. Due to overlapping confidence intervals, age-decade-specific reference ranges were not deemed necessary. (**Fig. 2**)

To evaluate the combined effect of age and menopausal status, women were stratified by age within premenopausal and postmenopausal groups. Due to limited sample siz-

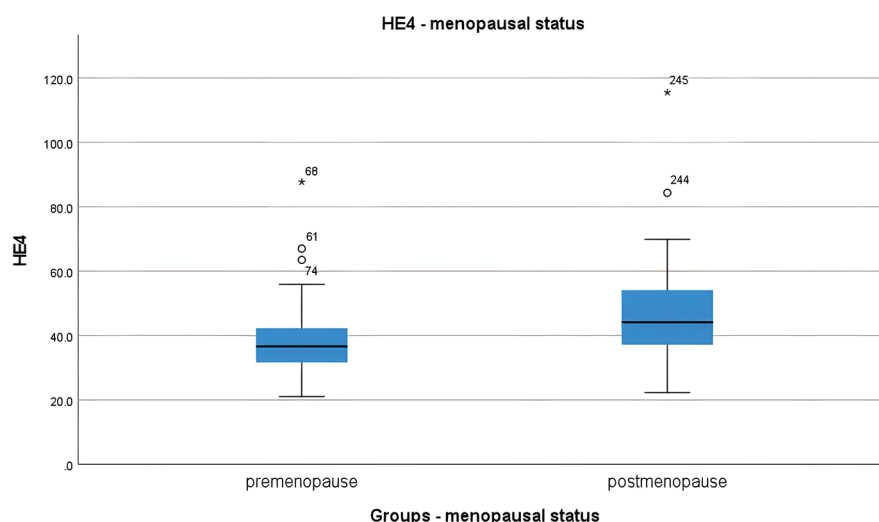
es, only certain subgroups were compared. In premenopausal women, Spearman's correlation showed no significant relationship between age and HE4 levels ( $p>0.05$ ). In postmenopausal women, a significant correlation was observed ( $\rho=0.316$ ,  $p<0.001$ ), with higher HE4 levels in those aged  $>60$  years compared to 50–59 years ( $U=1195.0$ ,  $p=0.012$ ,  $r=-0.235$ ). Age-specific upper reference limits in postmenopausal women were: 50–59 years—61.27 (56.44–

64.06) pmol/L;  $>60$  years—68.84 (63.76–71.63) pmol/L. **Table 1** summarizes HE4 reference ranges based on age and menopausal status.

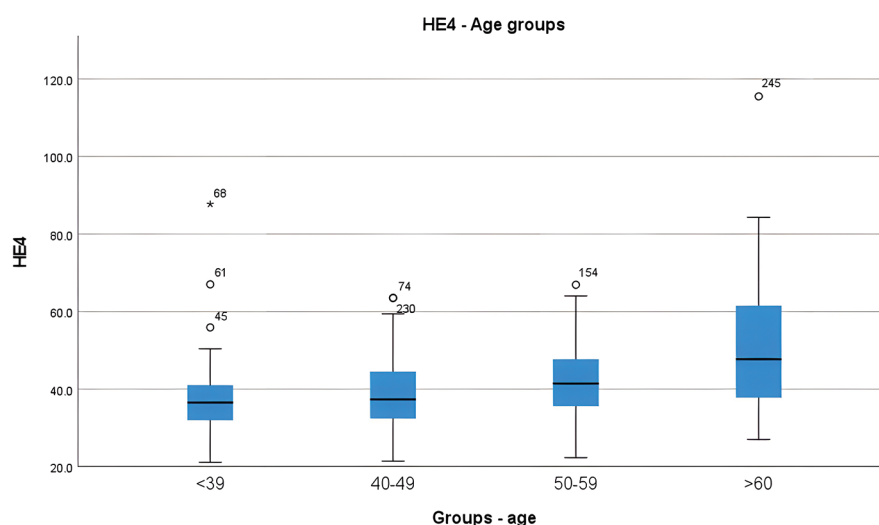
The results indicate that HE4 concentration increases with advancing age. However, no statistically significant differences were observed between the individual subgroups of premenopausal women. This supports the use of a single upper reference limit for this group. The proposed

**Table 1.** Reference ranges for HE4 based on age and menopausal status

Group	Reference range (P95)	95% Confidence interval		Units
HE4 premenopausal	53.22	50.8	60.82	pmol/L
HE4 postmenopausal, total	65.31	61.57	68.35	pmol/L
HE4 postmenopausal, $<59$ years	61.27	56.44	64.06	pmol/L
HE4 postmenopausal, $>60$ years	68.84	63.76	71.6	pmol/L



**Figure 1.** Distribution of the HE4 concentrations in the groups of premenopausal and postmenopausal women.



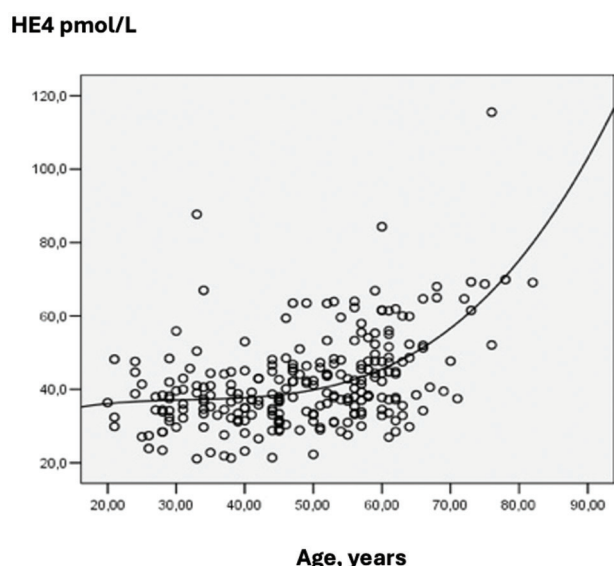
**Figure 2.** Distribution of HE4 concentration in the age-differentiated groups.

reference limit of 53.22 pmol/L (95% CI: 50.8 to 60.82) differs significantly from the manufacturer's recommended threshold of 70 pmol/L.

In the group of postmenopausal women, a statistically significant difference was observed between the 95th percentile for the overall group (65.31 pmol/L) and the manufacturer's proposed limit (140 pmol/L), as well as between the two age subgroups—those aged  $\leq 59$  and  $>60$  years (61.27 pmol/L vs. 68.84 pmol/L, respectively).

These findings demonstrate that age is the primary factor contributing to increased HE4 concentrations, rather than the hormonal status of the woman.

In addition to the identified linear correlation between HE4 and age, non-linear relationships were also explored. **Fig. 3** illustrates both quadratic and cubic trends, revealing different rates of marker increase across age periods—a relative plateau in HE4 levels up to the age of 50, followed by

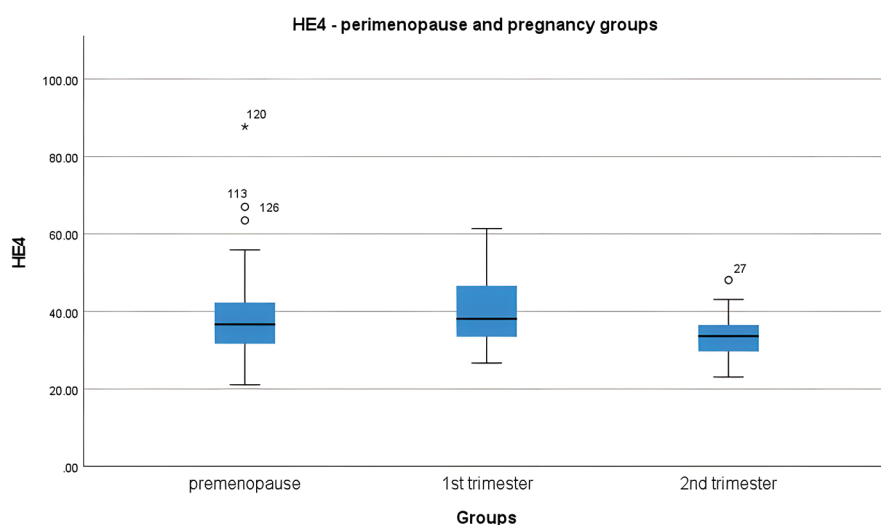


**Figure 3.** Nonlinear relationship between HE4 concentration and age.

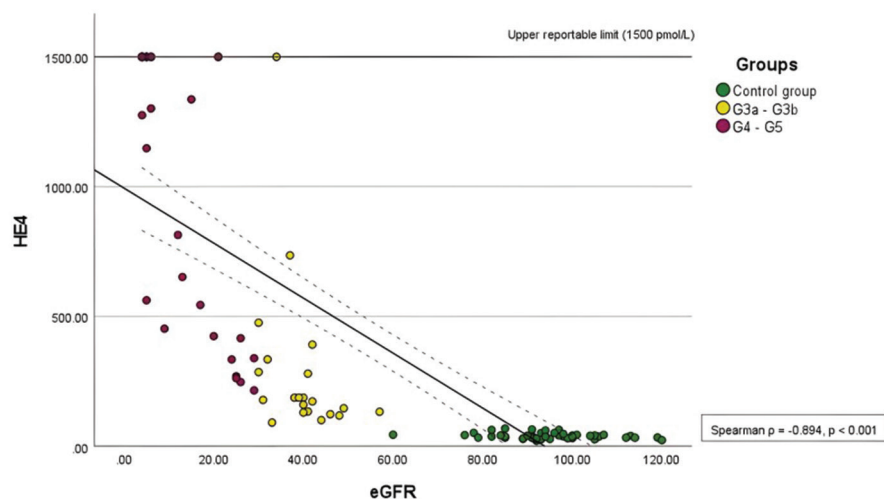
a rapid rise, particularly pronounced in advanced age, i.e., above 70 years.

Among 52 healthy pregnant women in the first ( $n=26$ ) and second ( $n=26$ ) trimesters (gestational age  $\leq 26$  weeks), mean age was  $30.98 \pm 4.18$  years, Shapiro-Wilk's test confirmed normal distribution ( $p > 0.05$ ), allowing parametric methods. Median (IQR) and mean (SD) HE4 values were overall—35.65 (29.83–42.90) and 36.73 (8.20) pmol/L; first trimester—38.10 (33.03–46.80) and 39.66 (8.95) pmol/L; second trimester—33.65 (29.55–36.80) and 33.80 (6.26) pmol/L. The overall upper reference limit was 50.90 pmol/L. Student's t-test showed significant differences between the two trimesters ( $t=2.735$ ,  $p=0.009$ ): first trimester— $<57.73$  pmol/L; second trimester— $<46.35$  pmol/L (**Fig. 4**). To assess the effect of pregnancy on HE4, comparisons were made with healthy non-pregnant premenopausal women aged 21–43 years ( $n=82$ , mean  $\pm$  SD:  $33.22 \pm 5.92$ ). No significant differences were found in HE4 levels between pregnant and age-matched non-pregnant women ( $p > 0.05$ ). Significance was reached only between first and second trimester groups ( $p=0.012$ ), rather than between premenopausal and pregnancy subgroup. The study observed lower HE4 levels in the second trimester compared to the first, but not significantly different from those in age-matched non-pregnant women. Further data are needed to assess physiological HE4 changes during pregnancy and to determine if trimester-specific reference ranges are necessary.

Given that HE4 is primarily cleared via glomerular filtration, impaired renal function may cause elevated serum HE4, risking misinterpretation. Therefore, this study evaluated the relationship between HE4 levels and impaired renal function, indicated by elevated creatinine or decreased estimated glomerular filtration rate (eGFR) (**Fig. 5**). Among 94 women (40 with elevated creatinine and 54 within reference range), linear correlations were assessed. None of them had any gynecological or oncological disease at the time of testing. HE4 values ranged from 21.3 to 3782.4 pmol/L and creatinine from 56 to 1060  $\mu$ mol/L. No



**Figure 4.** Distribution of HE4 concentration in healthy nonpregnant and pregnant women.



**Figure 5.** Association between concentration of HE4 and eGFR in patients with chronic kidney disease and healthy controls.

women with normal creatinine had HE4 >70 pmol/L, while all with elevated creatinine had HE4 >95th percentile for age and menopause status. Pearson's correlation between HE4 and serum creatinine was strong ( $r=0.703$ ,  $p<0.0001$ ), confirming a positive linear relationship. Spearman's rho analysis yielded similar results ( $\rho=0.773$ ,  $p<0.0001$ ), confirming a strong correlation between HE4 and creatinine concentrations. Among 15 patients with end-stage renal disease (eGFR <15 mL/min), all HE4 values exceeded the linearity of the assay (>1500 pmol/L). Even slight increases in serum creatinine were associated with significantly increased HE4 levels.

## Discussion

For accurate assessment of diagnostic performance and for the purpose of achieving optimal sensitivity and specificity of any biomarker or diagnostic algorithm, the correct choice of threshold value is of utmost importance. This is particularly true for biomarkers used in oncological disease management. Initially, reference ranges or threshold values recommended by the test manufacturer are commonly used. According to the Abbott Diagnostics insert<sup>[13]</sup>, HE4 values are considered normal up to 70 pmol/L for premenopausal women and up to 140 pmol/L for postmenopausal women. These values were established in a US-based study involving 210 premenopausal and 190 postmenopausal clinically healthy women. Ninety-five percent of premenopausal women had HE4 concentrations below 70 pmol/L, and 95% of postmenopausal women had values below 140 pmol/L. These reference limits differ significantly from those found in our study, likely due to differences in the studied cohorts. Other studies have also reported varying reference limits based on population differences. For example, Moore et al.<sup>[12]</sup> reported reference values of 89 pmol/L and 128 pmol/L for pre- and postmenopausal women, respectively. A 2015 study by Tian et al.<sup>[14]</sup> found

broad ranges of 29.3–68.79 pmol/L for premenopausal and 35.96–114.43 pmol/L for postmenopausal Chinese women. Gasiorowska et al.<sup>[15]</sup> reported upper limits of 73 pmol/L and 93 pmol/L for Central European pre- and postmenopausal women. Our study found lower upper limits: 53.2 pmol/L for premenopausal and 66.8 pmol/L for postmenopausal women. In contrast, Molina et al.<sup>[9]</sup> reported much higher values—132 pmol/L and 138 pmol/L, respectively—but small-numbered cohort of 66 women may explain the discrepancy, especially given our own findings of non-normal distribution of HE4 values. Analytical platform differences may also contribute to variations. During method verification, we compared the two main analytical systems used worldwide—Abbott and Roche—and found slightly higher HE4 values when measured using Roche reagents. The Polish study also used Roche's Cobas 6000 platform, which typically yields higher HE4 concentrations than Abbott's Architect platform. Median values from other publications are close to ours: Moore et al. reported a median HE4 of 43.5 pmol/L for women aged up to 40 and 66.9 pmol/L at age 70. Gasiorowska et al. observed 48 pmol/L at 40 years and 62 pmol/L at 70 years in Polish women. In our study, the corresponding values were 36.5 pmol/L ( $\leq 39$  years) and 47.7 pmol/L ( $\geq 60$  years). This confirms the age dependence of HE4, even though the correlation coefficient was only 0.43 ( $p<0.001$ ,  $N=246$ ). This raises some key questions: should age-specific reference ranges be used? Should HE4 results be interpreted differently in longitudinal follow-up of high-risk patients? Furthermore, it is important to distinguish the effect of age from menopausal status. Our data show overlapping confidence intervals across age groups, making age-decade-specific HE4 reference ranges unnecessary.

To determine the impact of pregnancy, comparisons between pregnant and non-pregnant women of the same age group revealed no statistically significant differences. These findings align with existing literature<sup>[16]</sup>, supporting the use of a unified reference range for both pregnant and

non-pregnant women. This is especially relevant in the second trimester, when CA 125 levels may rise and risk misinterpretation. Pregnant women in the third trimester were not included in our study. During the late stage of pregnancy, laboratory testing is typically conducted in a case of any complication – such as suspected preeclampsia, anemia, cholestasis, gestational diabetes, etc. This impedes collecting enough samples from healthy pregnant women in the third trimester. On the other hand, if there is an unexpected finding of a pelvic mass this late in the pregnancy, the usual clinical approach is limited to monitoring, while further diagnostic evaluation is postponed to after delivery. The absence of a statistically significant difference in HE4 concentrations between healthy non-pregnant women and pregnant women in the first and second trimesters in our study, as well as previous published data showing no significant difference between healthy non-pregnant women and those in the third trimester, provided the rationale for reporting our results without this patient cohort.

Since renal clearance is the primary elimination pathway for HE4, it is essential to understand how renal impairment affects HE4 levels. Analysis of 84 patients with varying degrees of kidney dysfunction—from normal to end-stage renal disease—revealed a strong correlation between creatinine and HE4. Spearman's  $\rho$  showed a significant positive correlation ( $\rho=0.871$ ,  $p<0.001$ ), and a strong negative correlation with eGFR ( $\rho=-0.894$ ,  $p<0.001$ ). These findings are consistent with other reports and highlight potential errors when interpreting HE4 results in patients with impaired renal function.<sup>[17]</sup> We consider proposals to use HE4 as a marker for glomerular function assessment in women without gynecological pathology<sup>[18]</sup> to be speculative.

## Conclusions

This study established that HE4 reference limits in the Bulgarian female population differ significantly from those recommended by Abbott Diagnostics for both premenopausal and postmenopausal women. A positive correlation between HE4 levels and age was confirmed. In pregnant women, HE4 concentrations did not differ significantly from those in age-matched non-pregnant women, regardless of gestational stage, indicating that pregnancy-specific reference ranges are unnecessary. A strong linear relationship between renal impairment and elevated HE4 levels was observed, necessitating cautious interpretation in patients with reduced renal function, which limits HE4's clinical applicability in this group.

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This research received no external funding.

## Conflict of interest

The authors declare no conflict of interest.

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