# HUMAN EPIDIDYMIS PROTEIN (HE4) AS A NOVEL BIOMARKER FOR DIFFERENTIAL DIAGNOSTIC POTENTIAL FOR BENIGN OVARIAN CYSTS IN CORRELATION WITH THE PUTATIVE CA125 IN ULTRASONOGARPHICALLY (USG) CONFIRMED PATIENTS

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## ABSTRACT

**Objective:** The aim of this population-based study was to elucidate differential diagnostic potential of human epididymis protein (HE4) correlation with the putative CA125 in Ultrasonogarphically (USG) confirmed patients.

**Study Design:** The study design included 15 ultrasonographically confirmed patients with benign ovarian cysts and 9 self-reported apparently healthy subjects.

**Materials and Methods:** In this study, nine control samples and fifteen samples from patients suffering from ultrasonographically diagnosed benign ovarian cysts were collected. Samples were collected by considering Inclusion and Exclusion criteria.

**Results:** The mean concentration of HE4 and CA125 were analyzed in Ultrasonogarphically (USG) confirmed patients. Receiver Operator Curve (ROC) was insignificant for both the biomarkers, ( HE4 P= 0.42; CA 125 P= 0.70),but AUC ( Area Under the Curve) predicted HE4 as a better biomarker (AUC HE4:0.65 ; AUC CA125 :0.55).Moreover, combining ROMA ( Risk of Malignancy Algorithm) with HE4 or CA 125 showed HE4 as a promising biomarker than CA 125, $P< 0.0001$, $P=0.2833$ respectively

**Conclusion:** Ultrasonography is a gold standard to diagnose pelvic masses/gynecological diseases, in correlation with HE4 and CA125.

**Keywords: HE4, CA125, Pelvic masses, Benign ovarian cysts, Conventional Biomarker**

## INTRODUCTION

Gynecological diseases including both malignant and benign are the posing health problem. In the USA, only 1,638,910 new cancer cases and 577,190 deaths due to cancer are expected in 2012 **[1]**. Asymptomatic nature and poor prognosis are the fundamental factors if ignoring all other aspects. As benign disease mostly lead to malignancy, early diagnosis remains the core priority **[2]**.

Till date ultrasonography is considered as gold standard for pelvic masses but need of developing non-invasive biomarker is equally important. Transvaginal ultrasound is mostly preferred over transabdominal ultrasound as the former has 100% sensitivity and around 83% specificity **[3]**, in comparison with the later which has sensitivity and specificity about 80% **[4]**. Color flow Doppler technique when combined with ultrasound analysis more specifically distinguish between benign and malignant tumors by studying the vascularization of the tumor. Vascular flow is normal in benign tumors and its neovascularized in malignant masses **[5]**.

As far as ovarian cysts are concerned, commonly occurring ones are sebaceous or fluid filled called physiological cysts which can be easily cured on treatment, while endometriomas are the cysts occurring in women with endometriosis- formed by the growth of some glands and stroma, identical to the lining of the uterus, appearing in an unusual location, have more tendency to get malignant, and they occur around in two third of the women **[5]**.

Biomarkers are such type of diagnostic tools, which could refer to any feature that can be independently

determined as a gauge to discriminate between normal and pathological biological processes, or to show in what way a therapy will respond **[6]**. Typical biomarkers in oncology include vital clinical features such as patient's age, gender, body weight and smoking status. Also including different types of inherited gene mutations, that could make a patient susceptible to cancer or assist in choosing the right treatment. Moreover, the pathology and molecular nature of the tumor also becomes evident **[7]**.

For ovarian cancers and gynecological diseases, CA 125 is a conventional marker with a cutoff value of 35 U/ml for malignancy. It is highly increased in cancerous diseases **[8, 9]**. However, its nonspecificity and non-linear behavior has prompted scientific community to find an alternative. In this context HE4 (Human Epididymus protein 4) is a novel biomarker under extensive study. HE4 was identified and characterized for the first time by **Cheng *et al*., in 1991 [9]**. It is also called WFDC2 (Wap-type four disulphide core) protein. HE4 is also found among various WAP proteins localized on human chromosome 20q12-13.1 **[10]**. It is reported to be a 10 KDa secretory protein with an initial prediction to exhibit protease inhibitor properties, but later on was studied to have a probable role in natural immunity **[8-10]**.

Although HE4 has been considered by researchers as a novel biomarker for discriminating benign from malignant gynecological diseases, still there are avenue where diagnostic potential of HE4 has yet to be explored.

**[11]** has reported HE4 as a new tumor biomarker for OC. It is specificity in benign gynecological and nongyne-cological diseases is better than CA 125. In addition, it is a better tool for differential diagnosis of OC from non-ovarian diseases. In addition to this finding a number of false positive results were also found for HE4 and CA 125 alone .Thus the coupling of the two biomarkers has been suggested for improvement of the diagnosis.

The aim of this study is to compare HE4 & CA 125 for diagnostic values in ultrasonographically confirmed patients. This is a pilot study with small sample size ideally aiming to indicate a cut off between benign ovarian cystic patients and healthy controls. So far, there is not much work about such distinctive study, and in local population, this is to be reported for the first time.

## MATERIALS AND METHODS

### 1. Experimental Design

In this study, nine control samples and fifteen samples from patients suffering

from ultrasonographically diagnosed benign ovarian cysts were collected.

## 2. Sample Collection

Samples were collected by considering the following criterion:

### Inclusion Criteria

Patients diagnosed with Benign Ovarian cysts & Premenopausal Status were included in the study.

### Exclusion Criteria

Patients with Gynecological tumors, Ovarian Cancer & Postmenopausal Status were excluded from the study.

### Point of Collection

Blood samples were collected from Gilani Ultrasound Centre, Ferozpur road, Lahore and Nawaz Sharif Social Security Hospital, Lahore.

### Serum Collection

5 cc blood was withdrawn from the patients suffering from ovarian cysts and were collected in BD Vacutainer Serum Separator Tube,( BD SST TM II).Serum tubes were centrifuged at 3000rpm for 5 mins at $23^0$ C. Serum was then separated and transferred to micro centrifuge tube and stored at -20 0 C. All the samples were thawed before use.

## 3. Ultrasonography

At the Gilani Ultrasound Centre, the following colour Doppler Ultrasound machines were used for diagnosis.

- Mindray De-7-Colour doppler Ultrasound machine with 3.5-5 MHz convex transabdominal transducer.
- GE-C-5 colour doppler Ultrasound machine with 3.5-5 MHz convex transabdominal transducer.

## 4. Quantitative ELISA for HE4 & CA 125

Kit method was followed at INMOL (Institute of Nuclear Medicine and Oncology Lahore) by using Fujiribio Diagnostic HE4 EIA kit (ref no: 404-10 ) with the cutoff value of 150pM established by the kit method for HE4 analysis and Serum CA125 concentrations were measured using the CanAg CA125 EIA assay (Fujirebio Diagnostics, ref no 400-10), with the Cutoff of 35 U/ml as a predefined value.

A double-determinant (sandwich) solid-phase, non-competitive immunoassay ELISA was performed by using two monoclonal antibodies 2H5 & 3D8, for determining serum concentrations of HE4.Similarly the amount of soluble CA125 in serum was also measured by using double determinant ELISA tests that engage two monoclonal antibodies (mAb) Ov197 & Ov187, which could attach with two epitope groups M11 and OC125.

## 5. Statistical Analysis

All the clinical data was statistically analysized by using Medcalc (Medical

Calculation) version 12.2.1.0 software. For determining the threshold values, sensitivity and specificity for HE4 and CA125 ROC AUC analysis was performed. To determine the significance of both the biomarkers descriptive test was carried out showing the coefficient of variation. The Screening test was performed to find out the sensitivity, specificity, Positive Predicted Value (PPV) and Negative Predictive Value (NPV) for HE4 & CA125 alone and also in combination. The magnitude of variation in levels of both the biomarkers was shown by Box and Whisker Test.

A Correlation of ROMA (Risk of Ovarian Malignancy Algorithm) with HE4 and CA125 was used to evaluate which is the better biomarker HE4 or CA125.

## RESULTS

### Samples Collection

For the study 24 samples were collected which were divided in two groups. Group 1 consisted of control group (n=9) and Group 2 consisting of pretreated patients suffering from ovarian cysts (n=15). The collected data was statistically analyzed.

### Ultrasonography Result

All the patients were diagnosed ultrasonographically with an ovarian cyst with different sizes and types. Some of the ultrasound images are as under **Figures 1 and 2**.

### Estimation of HE4 & CA 125 Levels

As from **Table 1** it is clear that there is very less difference in the average values of ELISA based levels of HE4 & CA 125 between the control and benign groups, indicates the insignificance of both the biomarkers in differential diagnosis of benign ovarian cysts from healthy control. Whereas the insignificance of CA125 is clear from the descriptive test in **Table 2** on the bases of CV (Coefficient of Variation) which is largely different in the two groups (CV for Control Group= 0.296; for patient group=1.45) whereas for HE4 CV = 0.24 was equal for both the groups. This difference can be attributed to the non-specificity of CA 125 for discriminating benign from malignant.

Equal distribution and variation in the levels of both the biomarkers are shown by applying Box and Whisker test as in the **Figures 3 and 4**.

The sensitivity and specificity of HE4 & CA125 alone and in combination was determined by generating ROC AUC curve and obtaining various thresholds. **Table 3**, shows the particular threshold values for HE4 and CA125 at which their specificity and sensitivity was determined.

According to the ROC analysis the optimal threshold value for HE4 is >36.728. The sensitivity and specificity was 66.67% (95% CI 38.4- 88.2) and 66.67% (95% CI

29.9 - 92.5) respectively. AUC was calculated as 0.652 at 95% CI=0.432-0.833 which is statistically insignificant i.e. P= 0.4224 as standard significant levelis P<0.05 (**Figure 5**).

According to the ROC analysis the optimal threshold value for CA 125 is >13.38. The sensitivity and specificity were40.0% (95% CI 16.3-67.7) and 100.0% ( 95% CI 66.4-100.0) respectively. AUC was calculated as 0.548 at 95% CI=0.334 to 0.750 which is statistically insignificant i.e P= 0.6955 as standard significant level is P< 0.05 **(Figure 6)**.

The AUC for CA 125 was 0.548 at 95% CI= 0.334 to 0.750 and HE4 was 0.652 at 95% CI=0.432 to 0.833. This was statistically insignificant i.e P = 0.5210 as standard significant level is P<0.05. AUC for HE4 is greater than CA 125 indicating it a better serum biomarker **(Figure 7)**.

When screening test was performed based on ROC data for HE4 and CA 125 alone shown in **Table 4**, CA 125 appears to be more specific, i.e CA 125 ( spec 90.91%); HE4 ( spec 66.67%). Whereas PPV and NPV for HE4 shows it a better marker than CA 125 (HE4 PPV= 78.92%, NPV 54.55%; CA 125 PPV= 65.71%, NPV=45.45%).

The correlation between ROMA and the two biomarkers are shown in **Table 5**, indicates that ROMA correlates better with HE4 and their combination will increase the differential diagnostic ability of HE4 as the Significance level P< 0.0001 was obtained, suggesting HE4 as a novel biomarker.

Whereas combining ROMA and CA 125 does not enhance CA 125 ability for differential diagnosis as the relationship was insignificant P=0.5517.



**Figure 1: Follicular Cyst size 2.8cm x 3.0cm**
**Transabdominal ultrasound showing a follicular cyst measuring 2.8 x 3.0cm. No septa/ nodules seen, walls are thin, on Doppler no flow seen- simple benign follicular cyst.**

**Figure 2: Endometriotic cyst size 5.2cm x 4.5 cm**
**Transabdominal scan showing bilateral Endometriotic cyst & nodules, on Doppler flow shows benign cyst.**

**Table 1: HE4 & CA 125 average levels in Control and Patient Groups**

| HE4 Levels pmol/L | | CA 125 Levels U/ml | |
|---|---|---|---|
| **Control Group Average** | **Patient Group Average** | **Control Group Average** | **Patient Group average** |
| 36.4526 (28.209~57.381) | 42.63397 ( 29.568~63.330) | 8.3938 (6.08~13.38) | 24.1213 ( 0.55~107.53) |

**Table 2: Diagnostic test for both HE4 and CA 125 showing CV (Coefficient of Variation)**

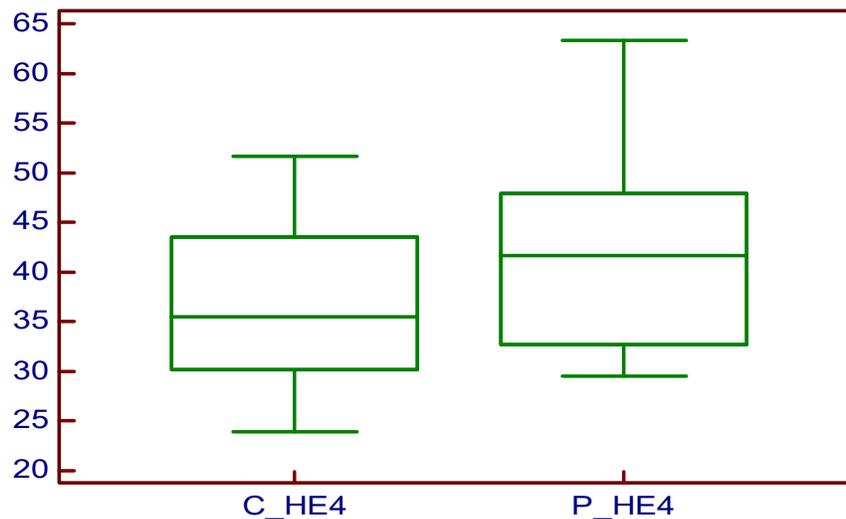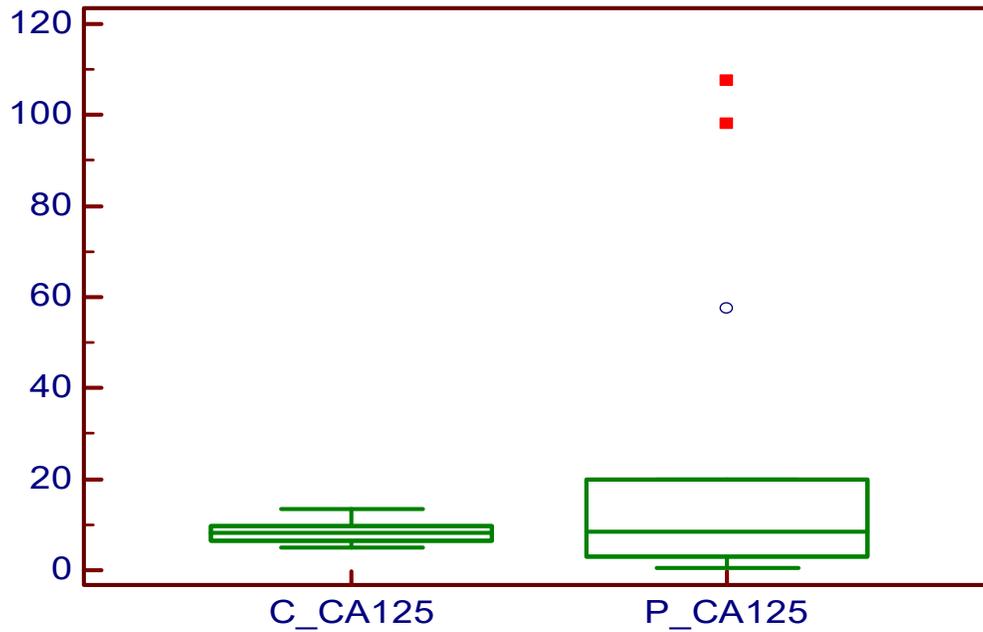| Biomarkers | Diagnosis | | |
|---|---|---|---|
| | **Parameter** | **Control** | **Patient** |
| **CA 125** | SD<br>Median<br>95% CI<br>CV% | 2.4846<br>8.300<br>6.172 - 9.740<br>0.296 | 34.9442<br>8.525<br>3.022 - 19.805<br>1.45 |
| **HE4** | SD<br>Median<br>95% CI<br>CV% | 9.0646<br>35.541<br>28.567 - 46.339<br>0.24 | 10.4610<br>41.670<br>32.736 - 47.859<br>0.24 |



**Figure 3: Showing CV for HE4 = equal distribution**

**Figure 4: Showing CV for CA125 with outliers in blue and red dots**

**Table 3: Sensitivity & Specificity of HE4 and CA125 at certain threshold value**

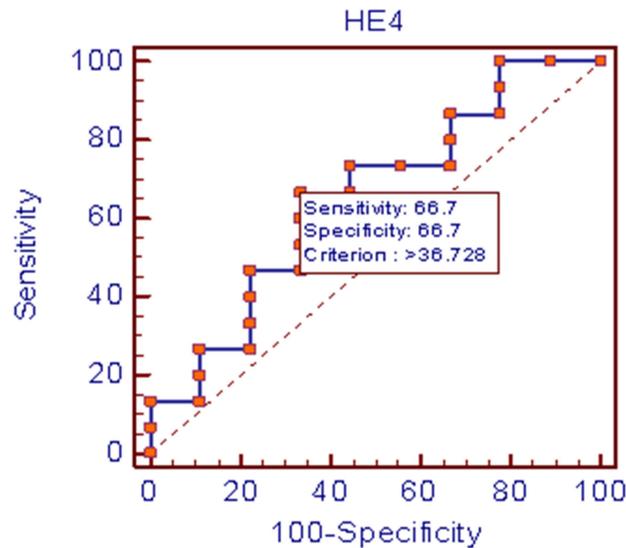| Biomarkers | Threshold Value | Sensitivity | Specificity |
|------------|-----------------|-------------|-------------|
| HE4 | >36.728* | 66.67 | 66.67 |
| CA125 | >13.38* | 40.00 | 100.00 |



**Figure 5: ROC analysis of HE4 as a novel biomarker for the detection of Benign Ovarian Cysts.**
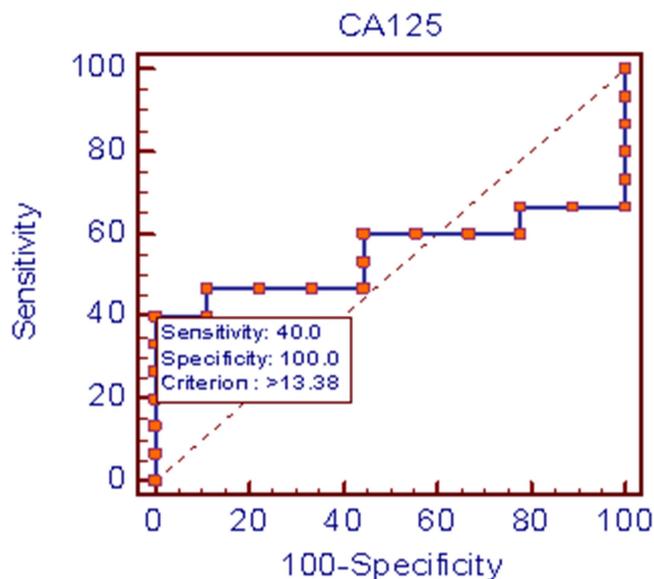
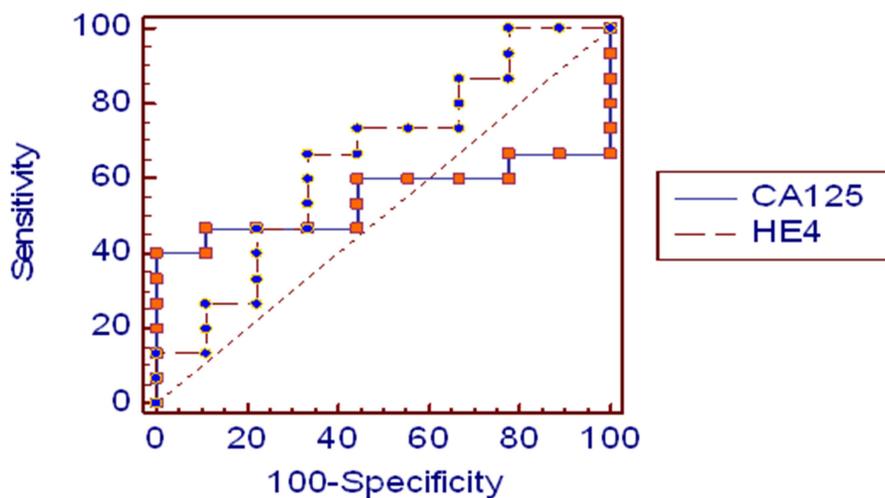**Figure 6: ROC analysis of CA 125 for the detection of Benign Ovarian Cysts**



**Figure 7: ROC analysis of HE4 and CA 125 combined**

**Table 4: Comparison of Sensitivity, Specificity, PPV & NPV of HE4 and CA 125.**

| Parameters | HE4 alone | CA 125 alone | HE4 & CA 125 Combined |
|---|---|---|---|
| Sensitivity | 66.67% | 33.33% | 35.71% |
| Specificity | 66.67% | 90.91% | 90.00% |
| Positive Predictive Value | 78.92% | 65.71% | 83.33% |
| Negative Predictive Value | 54.55% | 45.45% | 50.00% |

**Table 5: Correlation& Significant levels between ROMA, HE4 & CA 125**

| Variable | ROMA & HE4 | ROMA & CA 125 |
|---|---|---|
| Sample Size | 24 | 24 |
| Correlation | 0.986 | 0.128 |
| Significance Level | P< 0.0001 | P=0.5517 |
| 95% Confidence Interval | 0.967 to 0.994 | 0.967 to 0.994 |

## DISCUSSION

HE4 has emerged as a novel biomarker promisingly able to discriminate malignant from non-malignant gynecological conditions [11, 12]. Comparatively lesser data is available on its behavior in benign conditions [11, 13, 14]. Our study aimed to explore the behavior of CA 125 & HE4 as differential diagnostic biomarkers for self-reported healthy controls (n=9) and ultrasonogarphically confirmed benign ovarian cystic patients (n=15). For this purpose, sera of all subjects were assayed for HE4 and CA125 quantitatively and the resultant data was then statistically analyzed.

The sensitivity and specificity of HE4 and CA 125 by applying Receiver Operator Curve (ROC) analysis was 66.7%, 66.7% and 40.0%, 100.0%, respectively. Although ROC indicated both biomarkers to be insignificant (HE4 P= 0.4224; CA 125 P= 0.6955; as standard insignificant level P<0.05.), but AUC predicted HE4 as a better marker (AUC HE4 0.652; AUC CA 125 0.548) as also suggested by **Lenhard et al., 2011 [15]**. When it comes to specificity though ROC has marked CA 125 to be more specific than HE4, but as ROC calculates specificity at a shorter intervals of all values but cannot recognize any limits so it's not reliable as a final point as three of benign patients crossed international cutoff value 35 U/ml for CA 125 but HE4 didn't show any such non specificity.

Overall descriptive statistical analysis indicated better CV (coefficient of variation) for HE4 than that of CA 125. CV was 0.24 for HE4 in both the patient and control group whereas for CA 125 larger magnitude of variation was observed as indicated by **Table 2**. Since CA 125 false positively predicted three of the benign patients as malignant, while HE4 did not. This observation is in concordance with previous data suggesting non-specific behavior of CA 125 [16] and vice versa for HE4 when it comes to discriminate benign and malignant cases [12]. Later on Box Whisker test **Figure 6 & 7** also confirmed it by indicating the presence of outlier values for CA 125. Furthermore when ROMA was applied, it also correlated well with HE4 as compared to CA 125, P< 0.0001,P=0.2833 respectively. On the whole positive predictive values for both the markers alone are not sufficient for differential diagnosis but in combination PPV (HE4 PPV=78.92%, CA125 PPV=65.71% and HE4 CA125 combine PPV=83.33%) increases as well as the sensitivity. When it comes to be alone, HE4 is more specific than CA125.

Though statistically overall data is lesser significant to derive a clear cut off value

between the healthy and patient group; partially it could be attributed to the presence of outlier values which for sure interfere with statistical presentation of data. The recent data also suggest that HE4 could increase with age [17], and other physiological conditions might also interfere with final serum HE4 values [14]. Therefore, the overlapping between levels of HE4 in patients and apparently healthy controls could be attributed to undefined factors as none of healthy control was ultrasonographically confirmed for absence of any kind of disease.

Moreover, in patient group highest values of HE4 were observed for those with a potential risk of malignancy as they had hemorrhagic & endometriotic cysts, while lower values were estimated for low risk follicular cystic patients. HE4 values make it an important biomarker for discriminating patients suffering from ovarian malignancies from those showing benign ovarian endometriotic cysts [13]. On the other hand, CA 125 could not establish such a correlation.

## CONCLUSION

In conclusion ultrasonography is a gold standard to diagnose pelvic masses/ gynecological diseases, at the very same time we suggest a large cohort study with ultrasonographically confirmed healthy subjects and patients both before the final verdict about strength of HE4 is made.

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