Comprehensive evaluation of serum HE4 and CA125 with HE4 immunohistochemistry for ovarian tumor diagnosis

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ABSTRACT

Ovarian tumors present a critical health challenge due to high mortality. Current diagnostic modality like Cancer antigen 125 (CA125) in terms of low sensitivity or ultrasound in terms of cost and expertise have its own limitations. There is a crucial need for novel biomarkers such as Human epididymis protein 4(HE4) which may address their limitations. This study aimed to evaluate the magnitude of immunohistochemical expression of HE4 and correlate it with serum HE4 and CA125 levels in ovarian neoplasm further aiding in diagnosis and prognosis prediction.

Methods and Materials: A cross-sectional study, included 81 patients. Pre- and post-operative serum HE4, CA125 and immunohistochemistry were evaluated.

Results: Out of 81, 40(49.38%) were benign, 4 (4.93%) borderlines and 37(45.67%) malignant. In those benign tumors, 39(97.5%) cases showed S.HE4 level < 140 pmol/L whereas only 17 (42.5%) cases showed CA125 <35U/ml preoperatively. This shows that about 23cases (57.5%) of CA125 positive benign cases emerged as HE4 negative. Elevated preoperative CA125 levels in 59 patients (72.8%) demonstrated variable values in malignant, borderline and benign tumors, highlighting its complexity. Elevated preoperative HE4 levels were observed in 24 patients (92.3%) in malignant ovarian tumors. Combining serum HE4 and CA125 improved sensitivity (88%), specificity (91.56%), and accuracy (82.33%). Immunohistochemistry revealed strong HE4 tissue expression in 18 out of 22 cases (81.8%) of high grade serous and endometrioid carcinoma showing significant correlation with malignancy status.

Conclusions: HE4 emerges as a promising biomarker, both at tissue and serum levels ascribing its specificity to distinguish benign from malignant cases. The combined evaluation of serum HE4 and CA125 levels with immunohistochemical overexpression of HE4 at tissue enhances screening, diagnostic accuracy and prognostic outcomes in patients with ovarian tumors. This approach vouches for improving patient care and outcomes in the management of ovarian cancer.

Introduction

Ovarian tumors present a critical health challenge due to high mortality [1]. Surface epithelial tumors form two thirds of all ovarian neoplasm and 90% of all ovarian cancers are surface epithelial carcinomas [2].Cancer antigen 125 (CA125) has long been utilized as a serological marker for ovarian cancer detection and monitoring [3]. However, its sensitivity in early-stage disease and tendency for false positivity in certain conditions like pregnancy and infection limit its clinical utility.The International Ovarian Tumor Analysis (IOTA) Group introduced ultrasound-based algorithms to differentiate between benign and malignant ovarian neoplasms [4-8].

Human Epididymis protein 4 (HE4) has emerged as a promising novel biomarker for ovarian cancer. HE4 gene is present on 20q12-13.1 chromosomal region which is the most frequently amplified regions in ovarian carcinoma according to Comparative Genomic Hybridization (CGH) Studies [9]. Researchers have demonstrated its association with cancer cell invasion and metastasis, particularly in surface epithelial ovarian cancer [10]. Many authors have documented similar/increased sensitivity and specificity of HE4 as compared with CA125 in detecting early-stage ovarian cancers [11]. It has been incorporated as potential

biomarker in many proposed algorithms for triage patients with adnexal masses. [12] Its recent approval by the US FDA underscores its significance in early-stage ovarian cancer detection. An immunohistochemistry method was used to measure the expression of HE4 in different tissue. Drapkin *et al.* conducted an IHC study on tissue HE4 expression, revealing its presence in specific histologic subtypes of surface epithelial tumors but none in mucinous tumors [12]. HE4 addresses biomarker limitations, reducing morbidity and mortality while limiting unnecessary surgeries.
In this study, we evaluated the magnitude of immunohistochemical expression of HE4, and investigated its relationship with serum HE4 and serum CA125 levels in ovarian neoplasm, which will not only be helpful in diagnosis but also in differentiation of histological subtypes of ovarian neoplasm and prognosis prediction.

Materials and Methods
A prospective observational study was conducted at the Department of Pathology. 81 cases of ovarian tumors undergoing surgical procedures between January 2021 to June 2022 were studied. Patients above 16 years of age having clinical and radiological evidence of ovarian mass/cyst clinically with no significant underlying disease that may affect tumor markers were included. Those who had received/receiving chemotherapy or any other therapy for ovarian or any other malignancy before surgery and those who were unwilling to get enrolled in the study were excluded.
Preoperative and postoperative HE4 and serum CA125 estimations were performed on all patients by electrochemical luminescence immune assay (ECLIA) method using the Human epididymis protein 4 and CA125 assay kit at the Department of Biochemistry. Histopathological examination and HE4 immunohistochemistry were conducted on all samples received during the study period. The reference value of Serum HE4 is 0-140 pmol/L, and that of CA125 is 0-35 IU/ml, HE4 >140 pmol/L and CA125>35IU/ml were taken as positive. Serum HE4 diagnostic cut off point was obtained through the ROC curve. Normal epididymis was used as control. The interpretation of cytoplasmic staining was done on grading for intensity as **0-3.** 0- negative ,1- weak,2-moderate, 3- strong positive. as mentioned in Table 1.

TABLE 1: Percentage of positive cells

0	0%
1	1-24%
2	25-49%
3	50-100%

The grades were multiplied to determine H score. Minimum value of IRS score is 0 and maximum value 9. Protein expression was then further classified as Negative 0, Weak 1-2, Strong 3.
Percentage was calculated for categorical variable. Sensitivity, specificity, positive predictive value and negative predictive value was calculated for each test. All p value<0.05 is considered statistically significant. Software used for data analysis was Epi Info.

Results
Out of 81 cases analyzed, 40 (49.38%) were histologically benign, 4 (4.93%) borderline and 37(45.67%) malignant. Chief complaints primarily included abdominal pain and mass, followed by ascites. Most of the patients with benign tumors were <40 years of age while the ones with malignancy belonged to 41- 50 years of age. The youngest and oldest patient recorded were 17 and 66 years respectively. Premenopausal women were more than postmenopausal constituting 53(65.4 %) of the total cases.Majority of benign tumors were unilateral, while majority of borderline and malignant tumors were bilateral. In the study, majority of the patients with malignant ovarian tumors were Para1 and 2. Out of 13 females who were P1, 12 (92.3%) were diagnosed with malignant neoplasm on histology whereas 24 females of P2 showed malignancy in 12 cases (50%). Benign cases were mostly P3 and P4.On the contrary, 04(4.9%) nulliparous womenhadbenign tumors on histology. No malignancies were observed in para 5 and 6. The correlation between malignancy and parity was found statistically significant resonating with multiparity being preventive against malignancy as mentioned in Table *2*.

TABLE 2: Distribution of socio-demographic and gynecological characteristic of study participants for ovarian tumors

Characteristics	Frequency	P- value
Age (in years)		
< 20	03	0.778
21-30	12	
31-40	19	
41-50	21	
51-60	19	
>60	07	
Menopausal status		
Pre	53	0.219
Post	28	
Parity		
Nulliparous	04	0.032
1 - 4	71	
≥ 5	06	
Laterality		
Unilateral	48	0.037
Bilateral	33	

Of all the benign tumors, serous cystadenoma was most common tumor accounting 16% (13) of the total cases and 32.5% of the total (40) benign tumor. Mucinous cystadenoma was the second most common constituting 8(9.9%) of the total and 20% among benign category. Other benign tumors were mature teratoma, fibroma and Brenner tumor. Ovarian non neoplastic lesions included 04(10.0%) cases of ovarian endometriotic cyst, 02(5.0%) cases of corpus luteal cyst and 01(2.5%) case of xanthogranulommatus inflammation. 06(15.0%) cases showed no significant underlying pathology as mentioned in Table3(a.). In borderline tumor 03(75.0%) serous and 01(1.2%) case of mucinous were diagnosed as discussed in Table *3*(b). The most common malignant tumor was high grade serous carcinoma constituting 19 cases (23.5%) of total registered cases and 51.4% among malignant category followed by mucinous carcinoma and metastatic deposit accounting for 4.9% (4) each of the total. In the metastatic group 3(3.7%) were adenocarcinoma and 1(1.2%) was squamous cell carcinoma from cervix amongst the total cases. There were 03(8.1%) cases of ovarian endometrioid adenocarcinoma and 02(5.4%) of low-grade serous carcinoma of the malignant tumors. In non-epithelial malignancies dysgerminoma, fibrosarcoma and immature teratoma were diagnosed. One case showed evidence of residual tumor post chemotherapy which has been summarized in Table *3*(c).

TABLE 3: (a) Histological distribution of benign ovarian tumors. (b) Histological distribution of borderline ovarian tumors. (c.) Histological distribution of malignant ovarian tumors.

TUMOR TYPES	TUMOR SUBTYPES	Frequency	Percent
(a.) BENIGN	BENIGN BRENNER TUMOR	1	2.5%
	CORPUS LUTEAL CYST	2	5.0%
	ENDOMETRIOTIC CYST	4	10.0%
	FIBROMA	1	2.5%
	MATURE TERATOMA	4	10.0%

	MUCINOUS CYSTADENOMA	8	20.0 %
	SEROUS CYSTADENOMA	13	32.5%
	UNREMARKABLE	6	15.0%
	XANTHOGRANULOMATOUS INFLAMMATION	1	2.5%
	Total	40	100.0%
(b.) BORDERLINE	BORDERLINE MUCINOUS TUMOR	1	25.0%
	BORDERLINE SEROUS TUMOR	3	75.0%
	Total	4	100.0%
(c.) MALIGNANT	ENDOMETRIOID CARCINOMA	3	8.1%
	FIBROSARCOMA	1	2.7%
	HIGH GRADE SEROUS CARCINOMA	19	51.4%
	IMMATURE TERATOMA	1	2.7%
	LOW GRADE SEROUS CARCINOMA	2	5.4%
	METASTASIS	4	10.8%
	MUCINOUS CARCINOMA	4	10.8%
	RESIDUAL TUMOR CELLS	1	2.7%
	DYSGERMINOMA	2	5.4 %
	Total	37	100.0%

Serum HE4 and CA125 were evaluated pre and post operatively in all 81 cases. Out of 40(49.3%) benign tumors, 39 (97.5%) cases showed S.HE4 level < 140 pmol/L whereas only 17(42.5%) cases showed CA125 <35 U/ml preoperatively. This shows that about 57.5% of CA125 positive benign cases emerged as HE4 negative. High-grade serous and ovarian endometroid carcinomas in particular show serum HE4 levels >140pmol/l. 19 out of 26 cases (73.07%) showed significant fall of serum HE4 level after surgery except in 7 (26.9%) cases where it remained high. The correlation of malignancy status with pre and post-operative serum HE-4 levels were found to be statistically significant as mentioned in Table *4*.

TABLE 4: Correlation of pre- and post-operative serum HE4 level

Inpmol/l		**MALIGNANCYSTATUS**							**Pvalue**	
		BENIGN		**BORDERLINE**		**MALIGNANT**		**TOTAL**		
		Count	Row N%	Count	Row N%	Count	Row N %			
PREOP	**<140**	39	70.9 %	3	5.5%	13	23.6%	55	**<0.0001**	**Mean - 384.46**
SERUM HE4	**>140**	1	3.8%	1	3.8%	24	92.3%	26		**S.D-702.14**
POST-OPSERUM	**<140**	40	54.1 %	3	4.1%	31	41.9%	74	**0.02**	**Mean - 81.50**

HE-4	**>140**	0	0.0%	1	14.3 %	6	85.7%	07		**S.D-148.61**

Elevated serum CA-125 levels were present in 72.8% patients (>35) pre- operatively and in 43.2% patients post-operatively irrespective of their origin and histological subtypes. Although the relation of malignancy status with pre-operative as well as post-operative S. CA-125 levels were found statistically significant as mentioned in Table *5*.

TABLE 5: Correlation of pre- and post-operative serum CA-125 level

In U/mL		**MALIGNANCY STATUS**						**TOTAL**	**P value**	
		BENIGN		**BORDERLINE**		**MALIGNANT**				
		Count	Row N %	Count	Row N %	Count	Row N %			
PRE- OP SERUM CA-125	**<35**	17	77.3%	1	4.5%	4	18.2%	22	**0.008**	**Mean-339.71**
	>35	23	39.0%	3	5.1%	33	55.9%	59		**S.D-519.54**
POST- OP SERUM CA-125	**<35**	30	65.2%	2	4.3%	14	30.4%	46	**0.004**	**Mean-66.39**
	>35	10	28.6%	2	5.7%	23	65.7%	35		**S.D-74.63**

Among preoperative combined increased serum HE4 and CA125 in different histological subtypes, High grade serous carcinoma constituted maximum percentage (73.9%). The relation of preoperative combined increased serum HE4 and CA125 was found to be statistically significant as mentioned in Table *6*.

TABLE 6: Preoperative combined increased serum HE4 and CA125 correlation with malignant tumor subtypes

		INCREASEDPRE-OPSERUMHE4 +SERUMCA125				**P value**
		NO		**YES**		
		Count	**Column N%**	**Count**	**Column N%**	
MALIGNANTTUMOR SUBTYPES	**DYSGERMINOMA**	2	14.3%	0	0.0%	**<0.0001**
	ENDOMETRIOID CARCINOMA	0	0.0%	3	13.0%	
	FIBROSARCOMA	1	7.1%	0	0.0%	
	HIGHGRADESEROUS CYSTADENOCARCINOMA	0	0.0%	19	73.9%	
	IMMATURE TERATOMA	1	7.1%	0	0.0%	
	LOWGRADESEROUS CYSTADENOCARCINOMA	1	7.1%	1	4.3%	
	METASTATIC	2	14.3%	2	8.7%	
	MUCINOUSCARCINOMA	4	28.5%	0	0.0%	

	RESIDUALTUMOR CELLS	1	7.1%	0	0.0%	

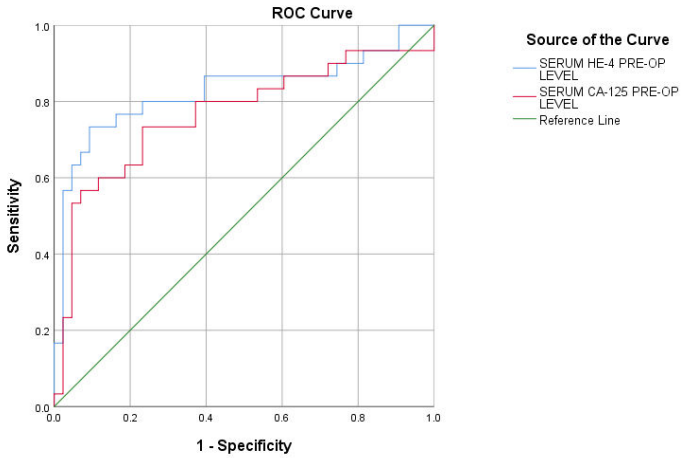
Out of 19 cases, 16 showed strong tissue expression for HE4 constituting 84.2% of positivity whereas 03 cases showed weak tissue expression. In low grade serous carcinoma, cases showed weak tissue expression. Out of 03 cases of ovarian endometrioid adenocarcinoma, 2 had strong and 01 had weak tissue expression. Correlation of tissue expression of HE4 with malignant ovarian types found to be significant mentioned in Table *7.*

TABLE 7: Relation of immunohistochemical HE4 expression H score with malignant ovarian types

		H SCORE							**Pvalue**
		NEGATIVE		**STRONGPOSITIVE**		**WEAKPOSITIVE**			
		Count, I*P	Column N%	Count, I*P	Column N %	Count, I*P	Column N %	TOTAL	
MALIGNANT TUMOR SUBTYPES	**DYSGERMINOMA**	02	16.7%	0	0.0%	0	0.0%	2	**<0.0001**
	ENDOMETRIOID CARCINOMA	0	0.0%	02, 2*3	11.1%	1	14.3 %	3	
	FIBROSARCOMA	1	8.3%	0	0.0%	0	0.0%	1	
	HIGH GRADE SEROUS CARCINOMA	0	0.0%	16, 3*2	88.9%	3	42.9 %	19	
	IMMATURE TERATOMA	1	8.3%	0	0.0%	0	0.0%	01	
	LOWGRADE SEROUSCARCINOMA	0	0.0%	0	0.0%	2	28.6 %	02	
	METASTATIC	4	33.3%	0	0.0%	0	0.0%	04	
	MUCINOUS CARCINOMA	3	25.0%	0	0.0%	1	14.3 %	01	
	RESIDUAL TUMOR CELLS	1	8.3%	0	0.0%	0	0.0%	01	

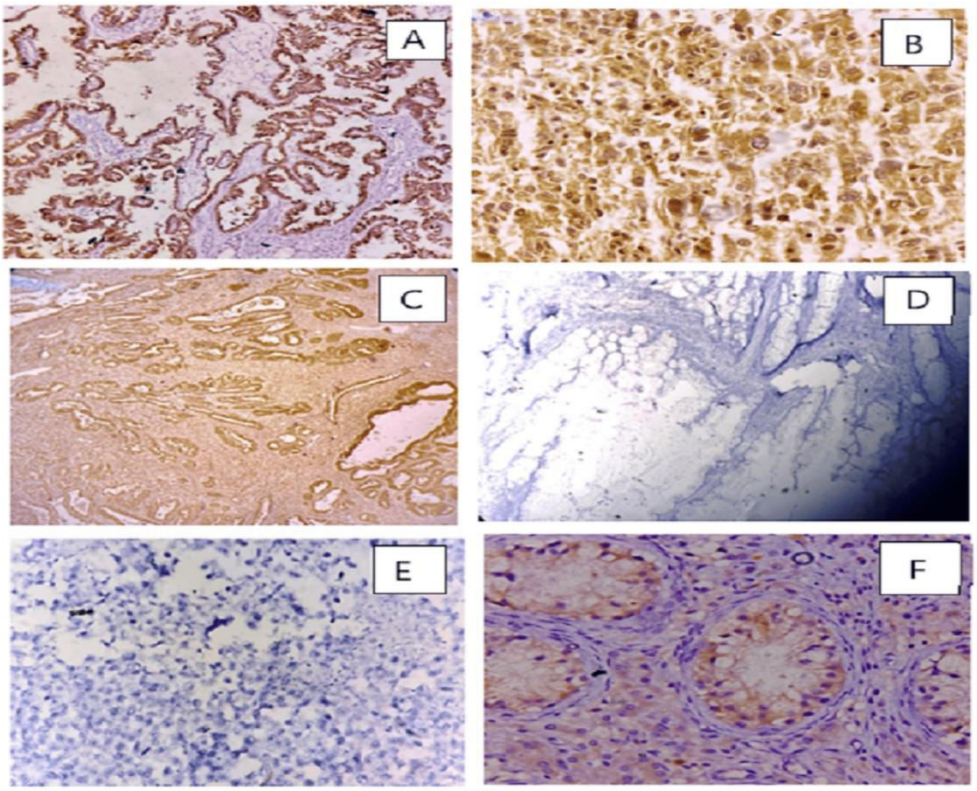
ROC Curve analysis - An ROC curve (figure 1) was created which showed that the area under curve was 0.827(95%CI, P<0.0001). The maximum diagnostic value occurred when the cut off value of HE4 for ovarian cancer was 81.8 pmol/l. Specificity and sensitivity were 80.00% & 78.26% respectively. Similarly, sensitivity for CA-125 levels was 77.14% and specificity was 76.09%. In this study both sensitivity and specificity of serum HE4 was found to be higher than serum CA125 as a marker of ovarian carcinoma. P value in both the parameters were found to be statistically significant. Combining Serum HE4 with serum CA125, improved both the sensitivity and specificity and was also found to be significant.

Figure 1: ROC curve of serum HE4 and serum CA125



HE4 IHC was applied on all enrolled cases irrespective of histological subtypes. The H- score and intensity was varied for different tumor types as illustrated in Figure 2.

FIGURE 2: Expression of HE4 in ovarian tissue



A. Borderline serous carcinoma 10X, HE4 stain (moderate intensity). High grade serous carcinoma 40X, HE4 stain (strong intensity)., C. Endometroid adenocarcinoma of ovary 10X, HE4 stain (strong intensity). D. Mucinous cystadenoma 10 X, HE4 stain(negative), E. Dysgerminoma 40 X, HE4 stain(negative), F. Epididymis tissue as positive control, HE4 stain

Discussion

HE4 is a N- glycosylated protein secreted from many normal tissues as well as in ovarian carcinoma, though there are significant differences between the two group. Molecular weight of HE4 is 25Kda and that of CA-125 is about 200-1000Kda which elucidate why HE4 is easily secreted into the blood compared to CA -125 in early stage of ovarian cancer [11]. We found increase Serum HE4 levels in all ovarian carcinomas except

with mucinous carcinoma and borderline mucinous tumor which had normal range of serum HE4 level. All benign lesions showed normal level of HE4 except 1 case of endometriotic cyst where serum HE4 was 273pmol/L. On comparative analysis with other researchers, *Wei SU et al.* in 2016 found mean preoperative Serum HE4 levels in benign tumors 54.76pmol/L and in malignant tumors 739.03 pmol/Lwhich was concordant with present study with mean of 60.14pmol/L in benign and 763.04pmol/L in malignant tumours [13]. We found high level of CA125 even in corpus luteal cyst, endometriosis and in benign serous cystadenoma. Mean S.CA125 in benign tumor was 125.82 IU/mL which was in sharp contrast with other investigators like *Wei SU et al* (49.07 IU/mL) and *Morimoto A et al* (21.6 IU/mL) [13,14]. Though mean value of CA125 in malignant tumors were concordant with observations made by other researchers, however, we observed markedly variable values of CA125 in few malignant tumors. Our result showed that in the malignant ovarian group there were about 29.7% of HE4 negative cases having high CA125 value, all of which were non-epithelial tumor and 8.1% cases of serum CA125 negative cases were HE4 positive which belonged to low grade serous carcinoma and ovarian endometroid adenocarcinoma. Hence clinical application of CA-125 to identify benign and malignant ovarian cancer lacked specificity. Though HE4 and CA-125 have different distribution, they were found complimentary. Collective detection of HE4 and CA-125in serum can minimize the rate of misdiagnosis, improve the accuracy and can limit unnecessary surgeries and morbidity in benign cases. We applied HE4 immunohistochemistry to all enrolled 81 cases and observed strong tissue expression of HE4 in 22.2%, weak in 13.6% and negative in 64.2% cases. All benign tumors were negative for HE4 immunoexpression, except for 1 case of mature teratoma. Borderline Mucinous tumorshowed no expression for HE4 immunohistochemistry. Therefore, we reckoned that the serum level of HE4 was positively linked with the HE4 expression in the tissue.As per American College of Obstetricians and Gynecologist 2017 [15], the sensitivity of CA125 in distinguishing benign from malignant tumors ranged between 61% and 90%, while specificity ranged between 35% and 91%. The positive predictive value (PPV) in women with an adnexal mass ranged from 35% to 90%, and the negative predictive value (NPV) ranged from 67% and 90%. In the current study, sensitivity for pre-operative HE4 levels was 80% and specificity was 78.26%. Similarly, sensitivity for CA-125 levels was 77.14% and specificity was 76.09%. The positive predictive value (PPV) of HE4 was calculated 73.68% and CA125 was 71.05%. The negative predictive value (NPV) of HE4 was calculated 83.72% and CA125 was 81.40%. Accuracy for HE4 was 79.01% and CA125 was 76.54%. The sensitivity, specificity, PPV, NPV and accuracy further improved by combining serum HE4 and CA125. The combined increased preoperative serum CA125 and serum HE4 had sensitivity 88%, specificity 91.56%, PPV74.34%, NPV 84.55% and accuracy of 82.33% as summarized in table *8*.

TABLE 8: Sensitivity, specificity, PPV and Accuracy of serum HE4 and serum CA125 level

	SERUM HE4	SERUM CA-125	SERUM HE4 + SERUM CA125
Cut off value	**81.8**	**128.05**	**78.9**
AUC	**0.827**	**0.767**	**0.868**
P value	**<0.0001**	**<0.0001**	**<0.0001**
Sensitivity	**80.00%**	**77.14%**	**88.00%**
Specificity	**78.26%**	**76.09%**	**91.56%**
PPV	**73.68%**	**71.05%**	**74.34%**
NPV	**83.72%**	**81.40%**	**84.55%**
Accuracy	**79.01%**	**76.54%**	**82.33%**

The present study showed higher sensitivity and specificity for HE4 in contrast to CA125 except for Van Grop T et al [16] who observed same sensitivity for HE4 and CA125 but higher specificity for CA125. Comparative results of sensitivity and specificity of HE4 and CA125 in relevance to this study by various researchers have been summarized in tabulated manner in Table *9.* This comparison table showed that HE4 is more sensitive as well as specific than CA-125 which was in accordance to other studies [17,18,19,20].

TABLE 9: Comparative table of Sensitivity and specificity of HE4 &CA125 marker in various studies

	HE4 (%)	CA125 (%)
1.Van Gorp T et al [16].		

Sensitivity	73.9	73.9
Specificity	85.1	89
2. Hamed et al [17].		
Sensitivity	90	83.3
Specificity	95	85
3. Terlikowaska et al [18].		
Sensitivity	84.1	83.1
Specificity	86.3	82.4
4. Verma N et al [19].		
Sensitivity	90	85
Specificity	96	75
5. Barr CE et al [20].		
Sensitivity	90.2	80.5
Specificity	75.6	92.2
6. Present study		
Sensitivity	80	77.14
Specificity	78.26	76.09

We acknowledge that small sample size, incomplete neoplasm representation were few limiting factors; although long-term studies can validate findings and assess benefits and costs.

Conclusions

Preliminary evidence supports HE4's as a better biomarker in diagnosing ovarian carcinoma and in distinguishing benign lesions. HE4 is notably up-regulated in ovarian tumor tissues, especially in serous and endometrioid carcinoma variants. Combined assessment of CA125 and HE4, along with consideration of HE4 tissue expression, is proposed as a more effective diagnostic approach than previous algorithms. It's important to note that diagnostic tools and algorithms may evolve over time, and ongoing research contributes to refining and improving their accuracy.

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