Original Investigations

"Identification and characterization of endometrial carcinoma with tumor markers HE4 and CA125 in serum and endometrial tissue samples and systematic review of the literature"

Tatiana et al. Diagnosis of endometrial cancer with HE4 and CA125

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DOI: 10.4274/jtgga.galenos.2020.2020.0120

Received: 26 September, 2019 **Accepted:** 04 June, 2020

Abstract

Objective: the diagnosis of endometrial cancer is made by biopsy sampling with pathological analysis, but it is extremely important to make an accurate diagnosis in order to plan the specific treatment, and we suggest that HE4 in the endometrial tissue and in serum could be tools to make the diagnosis more precise.

Material and Methods: our prospective study compared patients with endometrial cancer against non-endometrial cancer ones, matched with several variables. The inclusion criteria were females older than 18 years old that accepted to participate in the research study but that had never underwent surgery for other oncological pathologies, whether for ovarian, colon, cervical carcinoma or uterine sarcoma, and none of them had received preoperative chemo or radiotherapy; moreover, they could not have any severe renal or liver pathology. All of them had hysterectomy surgery and the endometrium was studied by a pathologist who compared the regular staining with HE4-antibody staining, in addition, there were collected the serum samples previous to the surgery.

Results: suggest bad correlation between the tissue HE4 in patients with and without carcinoma, however, the serum HE4 is statistically significant in the diagnosis of endometrial carcinoma (median EC= 123.1 U, median NE=64.67 U, p=0.002), although the CA125 level is not significant (p=0.208).

Conclusion: compared to previous studies our results are quite different in the pathological side, but the serum conclusions are positive and very hopeful as the tumor marker HE4 seems to be able to diagnose endometrial cancer.

Keywords: CA125, diagnosis, endometrial cancer, HE4, tissue

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Introduction

Human Epididymis Protein 4 (HE4), also known as acid protein (WFDC2), was first identified in the epithelial cells of the epididymal duct and plays a role in natural immunity and in sperm maturity [1-3]. In 2001, FDA approved HE4 as a serum tumor marker of ovarian cancer. Uterus, fallopian tubes and ovaries come from the urogenital crest and, in turn, the first two come from the paramesonephric tissue; therefore, they have similar embryological properties suggesting that they could be related.

Currently, only serum CA125 (125 Carbohydrate Antigen) is being used as a biomarker in endometrial cancer, although serum HE4 has shown good results [4]. Some positive results have already been shown in metaanalysis assessment of serum HE4 in relation to EC [5], though the sensitivity is not high enough to make it evident. So far, there are few publications that relate HE4 in tissue samples [6]. The published studies provide statistically significant relation between Human Epididymal Protein 4 and worse prognosis results or adverse clinicopathological variables [7-9].

The primary objective of this study is to identify and characterize HE4 in endometrial tissue samples obtained from patients diagnosed with endometrial cancer. Moreover, there have been comparisons with samples of endometrial tissue from non-endometrial cancer patients and of the levels of biomarkers in tissue versus serum. Finally, there has been a comparison among the HE4 staining or serum HE4 and several prognostic variables.

Material and Methods

Our prospective study is a case and control one nestled in a hospital-based cohort. There has been a comparison between EC patients (cases) and healthy ones (controls). Each sampling case was matched with one control selected from the group of patients with hysterectomy for other non-oncological reasons.

The study was conducted at the tertiary hospital in Spain, during the period from July 2017 to April 2018. All the targeted patients (in both arms) who fulfilled the following points: diagnosed of endometrial cancer, older than 18 years, wants to participate in the research study and sign consent forms voluntarily. They accepted that their serum sample as their tissue sample were used equally with the standards and recommendations declared in the Declaration of Helsinki. Their participation is not related to their medical treatment, and it is highlighted that there is not going to be identification or characterization of the patients in any step of the researching. The Ethics Committee of Clinical Research in Leon approved the design of the study.

The excluded criteria were the following: patients whom underwent surgery for other oncological pathologies, whether for ovarian, colon, cervical carcinoma or uterine sarcoma, and none of them had received preoperative chemo or radiotherapy. Moreover, they could not have any severe renal or liver pathology.

The inclusion and exclusion criteria for the control patients were exactly the same except because they did not have diagnosis of cancer.

The recruitment of patients took place when the diagnosis of endometrial cancer was done thanks to an endometrial biopsy during a previous visit. Furthermore, the controls were females who were planned for an hysterectomy due to another reason different from cancer. Every patient that fulfilled the criteria, and whom we were able to request the preoperative test from, were selected. There was ongoing recruitment from July 2017 to April 2018. After accurate diagnosis we would make contact and explain the study. If the patient understood, accepted and signed the informed consent, then we would include her in the study and the analysis of CA125 and HE4 were realized preoperatively. As usual, after surgery, the

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surgical piece was analyzed in the pathologic laboratory. Furthermore, the techniques to study HE4, Ki67 and p53 in endometrial tissue were carried out.
Variables considered for matching patients were: parity, hypertension, obesity and diabetes. Data were collected from the medical record and the personal interview: date of surgery, preop image tests and its results, preoperative staging according to FIGO guidelines, surgical procedure and whether lymphadenectomy was realized or not. Pathological outcomes: histological type, cell differentiation, size of the tumor, myometrial invasion, vascular or lymphatic invasion, perineural or stromal invasion, invasion of other tissues and final FIGO staging with node metastasis.
The main variable is the tissue HE4 (H-Score determination). To analyze the H-Score tissue samples were routinely processed and paraffin embedded. Sections of 3µm thick were cut with a microtome and stained with hemotoxilin-eosin, HE4, Ki67 and some samples with p53. The calibration of the technique was designed according to the optimal result of the human epididymis. The definitive dilution was 1:20.000, as it was necessary to modify it from the trading house, which was used at the beginning, to set it with the epididymis.
Immunohistochemistry to the endometrial tissue sample was measured using recombinant rabbit monoclonal Anti-HE4 antibody [EPR16658] of Abcam® on a Ventana Benchmark IHC processor. Representative areas were chosen from hematoxylin and eosin stained sections. Immunohistochemistry results were assessed by a semi quantitative approach used to assign an H-score to tumor samples. Cytoplasmic staining was graded for intensity (0-weak, 1-moderate and 2-strong) and the percentage of positive cells was scored as 0 (0-33%), 1 (34-66%) and 2 (67-100%).
A single scale with scores 0-4 will be obtained by multiplying the intensity and the percentage staining score, and a total score will be calculated by grouping score 0 in total score 0, 1-2 in total score 1, and 3-4 in total score 2.
Serum HE4 was determined using HE4 enzyme immunometric assay using a monoclonal antibody. Measuring range is 15-900 ppmol/L.
Serum CA125 was identified by electrochemiluminescence immunoassay using two monoclonal antibodies. Measuring range is 0.6-5000 U/mL.
Immunohistochemistry results were assessed by a semi quantitative approach used to assign an H-score (Figure 1) to tumor samples. Cytoplasmic staining was graded for intensity (0-negative, 1-weak and 2-strong) and the percentage of positive cells were scored as 0 (0%), 1 (1-50%) and 2 (51-100%).
Tissue Ki67 determination was carried out in a semi quantitative way as it is recommended in the “International Ki67 in Breast Cancer Working Group”. The measure of Ki67 was conducted through the counting of stained cores in the studied area (200 cores) without taking into account the intensity of the immunostaining and excluding the counting of cores of other cells. The cell proliferation index was established as the average of the values obtained in three different areas (including areas of more and less proliferation). Ki67 was analyzed as a continuous variable setting the cutoff point at 25% [10].

Statistical analysis

Continuous variables were reported as mean ± standard deviation or median (25th - 75th percentiles), according to the normality of their distribution, which was assessed by the Kolmogorov-Smirnov test. Categorical variables are reported as count (percentage). Comparisons of categorical variables between case-control groups were assessed using the Fisher's exact test. For comparing the continuous variables between the groups, the Student t test was used if the samples were normally distributed or their variances were homogeneous; otherwise, the Mann-Whitney U test was used. Correlations between continuous variables were assessed using the Pearson (r) or the Spearman (ρ) rank correlation test. Possible biomarkers and H-score were compared using ROC curves and the corresponding Area Under

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de Curve (AUC), whose differences were assessed using the DeLong test. Statistical analysis was performed using SPSS software (IBM corp., version 20) and p-values $p<0.05$ (two-sided) were considered significant.

Results

There were 34 cases collected and 35 controls. There were patients whose serum HE4 we could not measure because the recruitment was made after the preoperative analysis, so definitively we had results of the preoperative HE4 in 45 patients, 33 controls and 12 cases. The possible confusion factors could be the menopausal status (which is significantly higher in the cases group), the age at treatment because patients with EC are older, and other variables related to endometrial cancer which are also more frequent in the cases group. Demographic features of both cases and controls are shown in table 1, and the cohort of patients with endometrial cancer treated, including the matching variables. Our sample is the 38% of all the endometrial cancer cases during these two years. As we can see in this table, there are no differences among any of the variables studied in our cases and all the endometrial cancer patients.

Our result in the principal outcome shows that the expression of HE4 in endometrial tissue from patients with cancer is significantly weaker than in those without cancer ($p=0.035$). However, the difference between serum HE4 levels in no-EC compared with all ECs is statistically significant (median EC= 123.1 U, median NE=64.67 U, $p=0.002$), although the CA125 level is not significant ($p=0.208$) as is described in Table 2.

As displayed in Table 2 too, the comparison between the modified H-score and different variables measured in pathological terms only shows statistical difference in few variables related to the staining that are part of the staging itself. We do not find any difference among G1-G2-G3 cellular differentiation, with Ki67 or comparing p53.

HE4 showed a considerably higher sensitivity compared with CA125 for detecting EC, 38.5% vs 7.7% with CA125 and similar specificity of 84.8% compared with 90.9% for CA125 (Figure 2). However, this calculation is made based on the reference ranges of normality in our laboratory which are 0-35 UI/mL for CA125 and in HE4: 0-70 pmol/L in postmenopausal women and 0-140 pmol/L in premenopausal ones.

The relationship between serum HE4 levels and the clinicopathological features of the EC patients are shown in Table 3. Higher serum HE4 levels are not significantly associated with any of the variables collected, however, serum CA125 in associated with menopause state.

Discussion

Endometrial carcinoma (EC) is the most frequent malignant tumor of the female reproductive system in the developed countries, although not the one associated to the highest mortality rate, which is approximately 20% in 5 years. In USA, the estimated incidence of EC is 26.5 for every 100.000 per year, the 3.6% of all diagnosed cancer in the said country. Actually, prognostic factors are histologic differentiation, deep myometrial invasion, non-endometrioid histologic subtype, lymphovascular invasion, lymph node status, cervical involvement, and the presence and extent of extrauterine disease.

Localized EC treatment is hysterectomy and double adnexectomy. Moreover, the identification of those patients that should undergo a pelvic and/or paraaortic lymphadenectomy depends on several parameters.

Therefore, an endometrial biopsy and an abdominopelvic imaging technique (MNR as first election) are necessary for diagnosis and local extension prediction. Despite this, there is a significant percentage of cases in which an extension of the scheduled surgery is required. That is the reason of our interest in finding an efficient diagnostic test.

However, the H-score did not have any significant result compared to any important variable studied, because the ones with p-value<0.05 were pathological descriptions of the staining

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itself, and it is very confused compared to the studies we focused on our design. The results are even negative, the controls score more that the cases.
Nevertheless, the serum HE4 gives us a positive result and it is a possible future marker in endometrial cancer. It is currently being studied by many research groups [22-26] and the outcomes are positive, so that, it is necessary to increase the level of the studies and the number of cases in order to manage having it as a confident tumoral marker in serum. Moreover, from our point of view, it could even move the CA125 as a prospective and prognostic marker because it would have better results.
The articles analyzed are homogeneous in terms of the number of patients, the H-Score method and their general results. Furthermore, the prognosis with the tissue HE4 was studied in Li et al study and Deng et al study, but the article of Bignotti et al only mentions the serum HE4. The follow-up of their patients, as well as their survival results are very unequal (survival of 14%, 18% and 33% respectively).
As we described in the first Table, our sample is a good one, as there are no differences between the sample and the total of cases during a long period of time. About our study itself it is necessary to highlight that we had to change the classical H-score, described in the previous articles, to a new classification, similar in the bases but with less grades. This is because the staining did not result as used and we were not able to distinguish more than 3 grades (Modified H-Score 0, 1 and 2), so, we designed a "Modified H-Score" which is the one we described and used in our study.
Regarding to our bias, the patients were interviewed in the office by different professionals fact that worsen the quality of the collection and there is not a randomized selection of cases as the pathology's incidence is not high enough. As it is a cases and controls design there are several typical bias of selection and information as well as not be able to determine the incidence or prevalence of the EC.
Albeit, our main problem is that the sampling size is too small. This is because our results were so negative in the tissue HE4 in the preliminary analysis, that we decided to stop the collection before the expected recruitment number was obtained. Despite this, the results of the HE4 in blood are encouraging.
In conclusion, the sensibility and specificity of our results in serum HE4 was not good enough, but our opinion is that it is essential to calculate a correct cutoff at endometrial cancer and not to use the ovarian cancer one, then it would be a proper level to compare positive and negative cases. In any case, it would be necessary to carry out a larger study in order to test the validity of our theory.
Acknowledgments: Carmen Marín-Vieira, Margot Marqués-Martinez, Esther Fraile López and Vania Kant.

Conflict of Interest: No conflict of interest is declared by the authors.
Financial Disclosure: The authors declared that this study received no financial support.

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Table 1. Demographic features

	Total cases during 2017/2018 n=92	**Cases (n=35)**	**Controls (n=34)**	**p-value between C-C**	**p-value between total-cases**
Age at treatment (years)	67.2 ± 12.7	66.6 ± 13.3	57.4 ± 13.9	**0.006**	0.448
Parity	1.8 ± 1.3	1.9 ± 1.2	1.7 ± 1.2	0.592	0.181
Menopause	85 (92.4%)	32 (91.4 %)	20 (58.8 %)	**0.002**	0.738
Hypertension	37 (40.2%)	12 (34.3 %)	7 (20.6 %)	0.282	0.241
Obesity	15 (16.3%)	8 (22.9 %)	5 (14.7 %)	0.540	0.153
Diabetes	18 (19.6%)	6 (17.1 %)	2 (5.9 %)	0.259	0.724
Other related to endometrial cancer	29 (31,5%)	9 (25.7 %)	2 (5.9 %)	**0.045**	0.250

Table 2. Principal variable. HE4 expression in endometrial tissue with pathological parameters

		Cases	**Controls**		**p-value**
H-score					
0		5 (7.2%)	0		**0.035**
1		8 (11.6%)	5 (14.7%)		
2		22 (31.9%)	29 (42%)		
Serum HE4		123.1 (63.7-156.2)	62.05 (54.5-74.6)		**0.002**
Serum CA125		21.04±11.27	17.08 ± 8.678		0.208
		Modified H-score			
		0	**1**	**2**	
Nuclear grade	G1	2 (5.7%)	2 (5.7%)	9 (25.7%)	0.729
	G2	1 (2.9%)	4 (11.4%)	9 (25.7%)	
	G3	2 (5.7%)	2 (5.7%)	4 (11.4%)	
Ki67	<25%	2 (6.1%)	2 (6.1%)	11 (33.3%)	0.586
	>25%	2 (6.1%)	5 (15.2%)	11 (33.3%)	
Staining macroscopic intensity	1	5 (7.2%)	0	0	**<0.001**
	2	0	8 (11.6%)	10 (14.5%)	
	3	0	5 (7.2%)	41 (59.4%)	

Cellular Staining area	Cytoplasm	3 (4.3%)	2 (2.9%)	3 (4.3%)	**0.005**
	Cytoplasm + Nucleus	2 (2.9%)	11 (15.9%)	48 (69.6%)	
Staining tissue area	Apical	2 (2.9%)	1 (1.4%)	4 (5.8%)	0.124
	Diffuse	3 (4.3%)	12 (17.4%)	47 (68.1%)	
Staining	Homogeneous	2 (2.9%)	2 (2.9%)	51 (73.9%)	**<0.001**
	Heterogeneous	3 (4.3%)	11 (15.3%)	0	
P53	Normal	2 (5.7%)	6 (17.1%)	17 (48.6%)	0.267
	Aberrant	3 (8.6%)	2 (5.7%)	5 (14.3%)	

Table 3. Serum HE4 and CA125

Variable	**n**	**Serum HE4 median (Q1-Q3)**	**p-value**	**Serum CA125 (□ ± SD)**	**p-value**
Age (years)	45	67.3 (54.9-101.4) Rho= 0.247	0.102	19.1 ± 9.9 r=- 0.107	0.519
Diabetes					
Yes	6	71.4 (54.7-142.9)	0.616	19.0 ± 13.4	0.991
No	39	64.9 (54.7-101.3)		19.1 ± 95	
HTA					
Yes	14	70.6 (57.9-126.9)	0.384	19.3 ± 11.9	0.924
No	31	64.4 (54.5-86.9)		19.0 ± 8.9	
Menopause					
Yes	30	70.6 (55.9-111.8)	0.354	16.4 ± 9.4	**0.029**
No	15	61.7 (54.7-82)		23.4 ± 9.4	
Obesity					
Yes	8	66.6 (55.9-156.7)	0.449	20.0 ± 7.8	0.783
No	37	67.3 (54.6-94.1)		18.9 ± 10.4	
Parity					
Yes	40	69.2 (54.8-101.45)	0.448	18.6 ± 9.5	0.373
No	5	58.8 (47.3-103.5)		23.3 ± 13.3	
Other related to EC					
Yes	6	65.3 (57.2-121.6)	0.726	17.8 ± 11.4	0.731
No	39	67.3 (54.7-101.3)		19.3 ± 9.8	
Pelvic lymphadenectomy					
Yes	5	101.3 (62.3-137.5)	0.181	15.6 ± 6.5	0.402
No	40	64.7 (54.6-85.7)		19.6 ± 10.3	
Paraaortic lymphadenectomy					
Yes	4	109 (67.6-147.3)	0.151	16.9 ± 6.6	0.653
No	41	64.9 (54.6-84.5)		19.3 ± 10.2	
Myometrial invasion >50%					
Yes	6	112.2 (55.1-156.0)	0.361	19.3 ± 6.1	0.875
No	5	142.5 (93.1-210.3)		20.4 ± 16.2	
Vascular, lymphatic or perineural invasion					
Yes	2	84.7 (51.4-)	0.182	12.5 ± 4.1	0.182
No	6	132.8 (93.0-182.6)		18.4 ± 10.6	
Adnexal affection					
Yes	1		0.333		0.660
No	11	138.2 (101.3-156.8)		20.1 ± 11.0	

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Lymph node status					
Affected	2	106.7 (56.3-)	1	20.2 ± 7.5	0.958
Not affected	9	123.1 (84.8-156.2)		19.7 ± 12.2	
Final FIGO stage					
I-II	6	132.8 (93.0-182.6)	0.286	18.4 ± 10.6	0.490
III-IV	2	84.7 (51.4-)		12.5 ± 4.1	
Second surgery					
Yes	1	-	0.397	-	0.891
No	44	66.1 (54.9-94.2)		19.1 ± 10.0	

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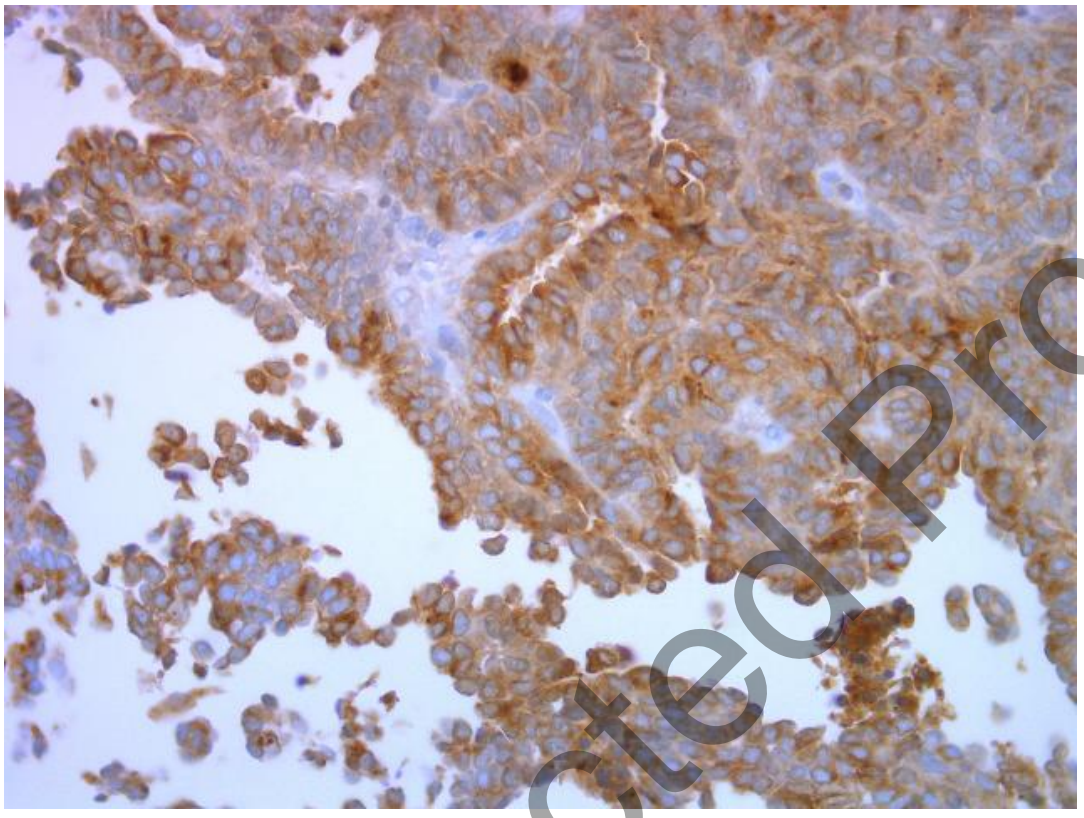
Figure 1: Representative immunohistochemical staining for HE4 in tissue microarrays of endometrial carcinoma: (1a) H-Score 0 in endometrial endometrioid type adenocarcinoma FIGO G1; (1b) H-Score 1 (endometrioid carcinoma G1 case); (1c) H-Score 2 (FIGO G1 area of an endometrioid carcinoma G3).



(a)

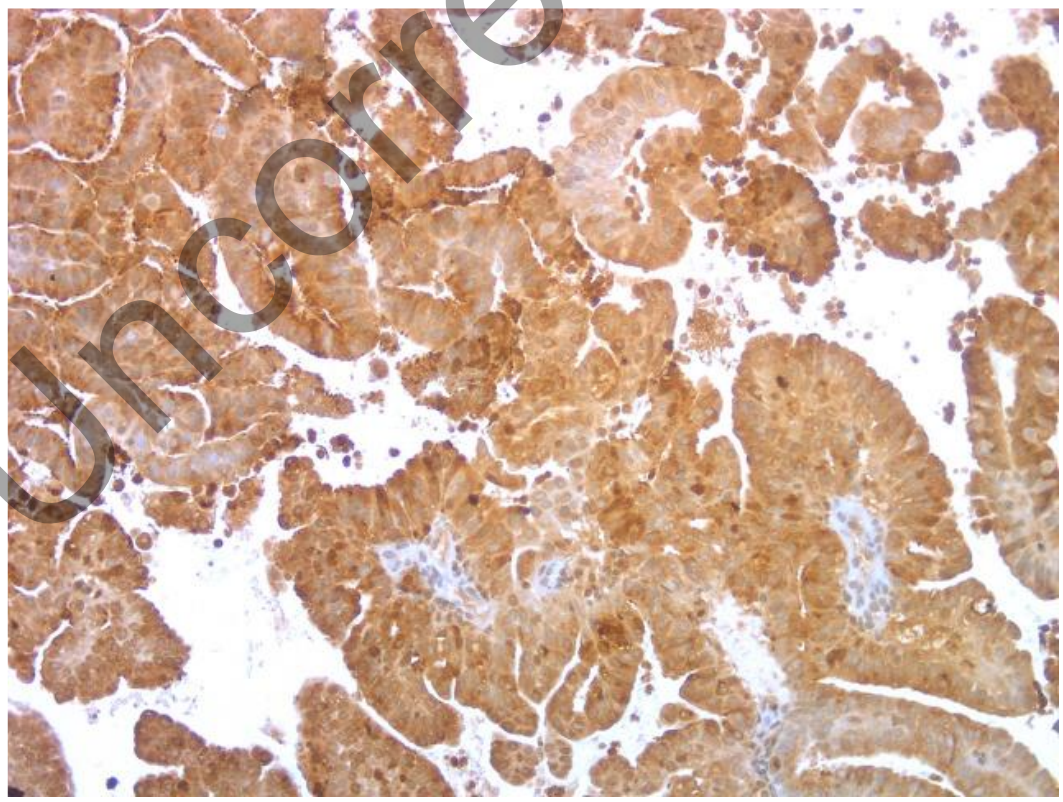


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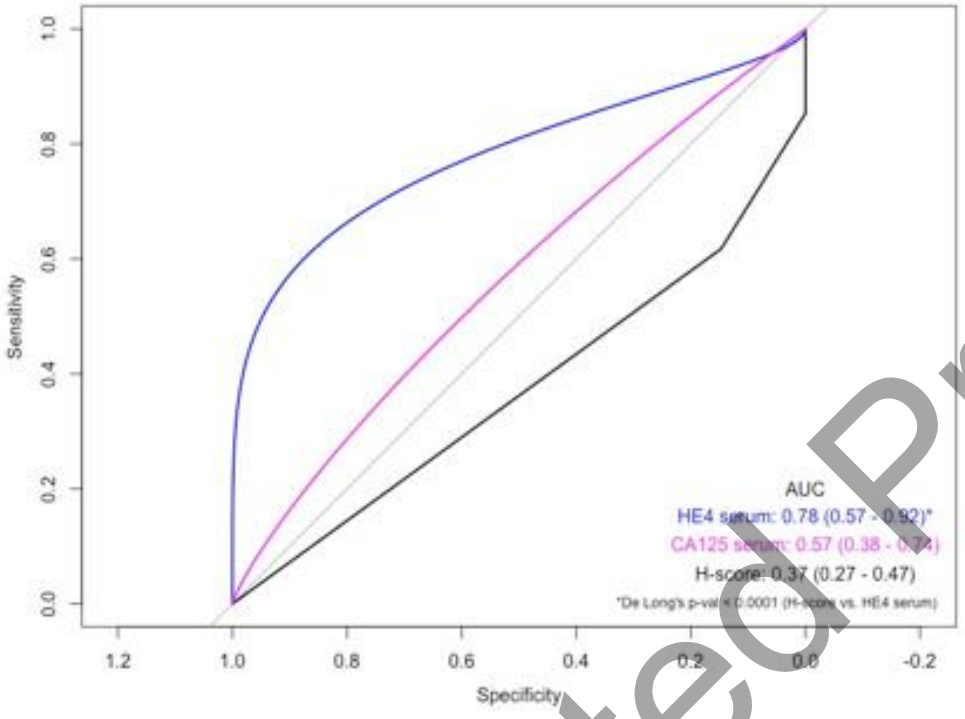
Figure 2. ROC Curve: Modified H-Score, serum HE4 and serum CA125



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