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Detection of free intraperitoneal tumor cells in peritoneal lavage fluid from patients with peritoneal metastasis before and after treatment with Pressurized IntraPeritoneal Aerosol Chemotherapy (PIPAC)

Martin Graversen[1, 2, 6], Claus Fristrup[1, 2], Thomas Kielsgaard Kristensen[4], Trine Rennebod Larsen[5], Per Pfeiffer[1, 3, 6], Michael B. Mortensen[1, 2, 6], Sönke Detlefsen[1,4,6]

[1]: Odense PIPAC Center (OPC) & Odense Pancreas Center (OPAC), Odense University Hospital, J.B. Winsloews Vej 4, 5000 Odense, Denmark

[2]: Department of Surgery, Upper GI and HPB Section, Odense University Hospital, Denmark

[3]: Department of Oncology, Odense University Hospital, Denmark

[4]: Department of Pathology, Odense University Hospital, Denmark

[5]: Department of Clinical Biochemistry, Svendborg Hospital, Odense University Hospital, Denmark

[6]: Department of Clinical Research, Faculty of Health Sciences, University of Southern Denmark, Odense, Denmark

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Correspondence to: Martin Graversen, MD

ORCID 0000-0001-5719-4916

Upper GI and HPB Section

Department of Surgery

Odense University Hospital

J.B Winsloews Vej 4

5000 Odense, Denmark

Email: Martin.Graversen@rsyd.dk

Telephone: +45 26172081

ABSTRACT

Aims

In this study, we investigated whether free intraperitoneal tumor cells (FITC) were detectable in ascites or peritoneal lavage fluid (PLF) from patients with peritoneal metastasis (PM) before and after treatment with Pressurized IntraPeritoneal Aerosol Chemotherapy (PIPAC).

Methods

Ascites or PLF retrieved at the first and third PIPAC procedure was analyzed by conventional cytology, carcinoembryonic antigen (CEA) and total protein concentration and quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) for mRNA expression of CEA, Epithelial Cell Adhesion Molecule (EpCAM) and cancer antigen 125 (CA-125). Conventional cytology and qRT-PCR were also performed in a negative control group (benign PLF specimens and inflammatory ascites). The treatment response was compared to the histological response based on repeated peritoneal biopsies evaluated by the Peritoneal Regression Grading Score (PRGS).

Results

Thirty five patients with PM of various origins were included from 2015 to 2016. At the first PIPAC procedure, FITC were detected by conventional cytology (sensitivity 0.58, specificity 1.00), CEA protein (cut off 0.4 µg/L, sensitivity 0.71), CEA mRNA (sensitivity 0.75, specificity 1.00), EpCAM mRNA (sensitivity 0.71, specificity 1.00) and CA-125 mRNA (sensitivity 0.43, specificity 1.00). The combination of CEA/EpCAM mRNA had a sensitivity of 0.88 and a specificity of 1.00.

The evaluation of ascites or PLF retrieved at the third PIPAC procedure failed to detect treatment response, when compared to the histological PRGS.

Conclusions

The evaluation of CEA and EpCAM mRNA detects FITC with a high sensitivity and an excellent specificity, but is not useful for response evaluation in patients treated with PIPAC.

Key words: Peritoneal metastasis, PIPAC, Free Intraperitoneal tumor cells, CEA, EpCAM, CA-125

INTRODUCTION

Free intraperitoneal tumor cells (FITC) may be detected by evaluation of ascites or peritoneal lavage fluid (PLF). For patients with colorectal or gastric cancer, it has been shown that FITC predict peritoneal recurrence and poor overall survival (1-3), but the detection of FITC by conventional cytology is hampered by a low sensitivity. Adding protein or PCR analysis to conventional cytology may improve detection rates of FITC (2, 4), but there is no standard definition of the techniques or cut off points used. Further, the treatment related consequences of FITC in otherwise resectable cancer patients are debated.(5) Systemic treatment may eradicate FITC in some patients, but the effect is ambiguous (6, 7), and new treatment strategies are mandated. Recent reports on repeated intraperitoneal drug delivery by Pressurized IntraPeritoneal Aerosol Chemotherapy (PIPAC) have shown positive outcomes in the palliative treatment of patients with peritoneal metastasis (PM).(8, 9) However, response is mainly documented by tumor regression grading systems based on histology, such as the Peritoneal Regression Grading Score (PRGS) (10), and the potential eradication of FITC is only reported through analysis of conventional cytology in one study.(9) It is unknown, if comprehensive analyses of ascites or PLF is useful in the detection and eradication of FITC in patients with PM treated by PIPAC.

Through comprehensive analyses of ascites or PLF retrieved at the index PIPAC procedure, the main objective of this study was to evaluate the ability of detecting FITC in patients with PM by conventional cytology, PCR and protein analyses. As a secondary objective, the same techniques and cut offs were applied in the analysis of ascites or PLF collected at the third PIPAC procedure, to evaluate if they, compared to PRGS, were able to detect a response to treatment.

METHODS

Study design

Ascites or PLF was collected as part of the PIPAC-OPC1 study, which is a prospectively controlled outcome study, investigating the feasibility and effect of PIPAC in patients with PM from gastrointestinal, pancreatic or ovarian malignancies or with primary peritoneal cancer.(9) As these patients had histological or cytological verified PM, they were perceived true positives in the analysis of FITC. As negative controls for the quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) analyses, PLFs were collected from 20 patients during laparoscopic repair of

hiatal hernias (benign) and from ten patients relieved of ascites due to decompensated liver cirrhosis (benign inflammation).

Data collection and analysis

Ascites was collected during each laparoscopy prior to peritoneal sampling and PIPAC directed treatment. If the patient had less than 200 ml of ascites, 500 ml of saline was irrigated into the peritoneal cavity and 150 ml of PLF was collected for cytological analysis, while the remaining PLF was disposed. If a spontaneous coagulum was present, it was fixed in formalin and embedded in paraffin. Three centrifuge tubes were filled with 50 ml of the fluid. The fluid in the three tubes was centrifuged. From the first tube, 5 ml of the supernatant was pipetted into a separate tube for CEA protein and total protein analysis, and from the sediment, two smears were produced, dried and stained according to Papanicolaou and May-Giemsa Grünwald. From the sediment of the second tube, a cell block was prepared after addition of three drops of plasma and two drops of thrombin. The cell block was fixed in formalin and embedded in paraffin. From each of the two paraffin blocks, a 4-5 μm thick section was cut with a microtome and stained with H&E for microscopic analysis. The sediment of the third tube was stored at -80°C in MagNA Pure LC Lysis Buffer (Roche), for subsequent qRT-PCR mRNA expression analysis.

Cytological and immunocytochemical analysis

The cytological diagnosis was based on two smears stained according to Papanicolaou and May-Giemsa Grünwald and the H&E stained sections from the paraffin embedded cell block preparation and/or the spontaneous coagulum. If necessary, as judged by the pathologist based on the findings at conventional cytology, additional sections were cut from the paraffin embedded material and used for immunocytochemical analyses for tumor markers such as CEA, EpCAM, CDX2 and/or CK20 as well as markers for mesothelial cells, such as calretinin and vimentin. Each specimen was classified as 1) no malignant cells, 2) atypical cells, 3) cells suspicious for malignancy or 4) malignant tumor cells. The cytological diagnosis was considered positive if either cells suspicious for malignancy or malignant tumor cells were detected. As this was a clinical study, the responsible cyto-pathologist was not blinded.

Protein Analysis

CEA was analyzed using an antibody sandwich principle, while total protein (TP) was analyzed using a turdidimetric method, using plasma and urine applications, respectively, on the Cobas8000 (Roche Diagnostics). In order to evaluate whether the applications were suited for PLF, linearity, detection limit and analytical variation were assessed. Linearity was established with dilution series using different amounts of two PLF samples with a high and a low CEA concentration, respectively, while detection limits were calculated as 0 + 5 standard deviations calculated from 10 measurements of a PLF sample with either a low CEA or a low TP concentration. Analytical variation was evaluated using patient PLF samples analyzed twice a day for 10 days.

CEA had analytical variations of 3.7%, 6.6% and 6.1% at concentrations of 2.8 µg/L, 36.3 µg/L and 175 µg/L respectively, and was found to be linear in the interval of 1.0 – 900 µg/L. Detection limit was 0.12 µg/L. TP using the urine application was found to have analytical variations of 10.4%, 1.7% and 1.4% at 0.07 g/L, 1.15 g/L and 2.37 g/L respectively, and to be linear in the interval of 0.03 – 4.0 g/L. Furthermore, linearity at higher concentrations was linear up to at least 40 g/L. Detection limit was 0.015 g/L. Hence, for both the CEA and TP (urine) applications, PLF was found to be an acceptable sample material and the manufacturer's information regarding range of measurement and detection limits could be used also for this type of specimens. In addition, for the application TP (urine), the range of measurement could be expanded. Protein analysis was solely performed in the study population without negative controls, and was considered positive if the CEA protein level was above 0.4 µg/L.(11)

qRT-PCR

RNA was extracted using the MagNA Pure LC Instrument (Roche) with the MagNA Pure LC RNA Isolation kit – High Performance (Roche) according to the manufacturer's recommendations. RNA was eluted with 100 µL elution buffer and up-concentrated to 10 µL using the RNeasy MinElute Cleanup Kit (Qiagen). Complementary DNA (cDNA) synthesis was performed in a total volume of 20 µL using the SuperScript VILO cDNA Synthesis Kit (Thermo Fisher) and diluted to 80 µL. qRT-PCR was performed using the QuantStudio 12K Flex Real-Time PCR System (Applied Biosystems) with the TaqMan Universal PCR Master Mix with AmpErase UNG (Applied Biosystems) and the following commercially available FAM-labelled TaqMan gene expression

assays; CEACAM5: Hs00944025_m1, EpCAM: Hs00901885_m1, CA-125: Hs01065175_m1 (Thermo Fisher). The Human GAPD (GAPDH) Endogenous Control (Thermo Fisher) was included as house-keeping gene. The total qRT-PCR volume was 25 µL, including 5 µL cDNA solution. Thermocycling conditions consisted of 50°C for 2 min, 95°C for 10 min followed by 50 cycles of 95°C for 15 seconds and 60°C for 1 minute. All qRT-PCR experiments were performed in triplicate and included a no template control (water) and a gene expression positive control (colorectal cancer) sample. A threshold of 0.1 was used to calculate cycle threshold (Ct)-values. The sensitivity of the detection of FITC by mRNA expression of CEA, EpCAM and CA-125 were found by ROC curve analyses at a specificity of 100%.

Histology

As previously described (9), the peritoneal biopsies were fixed in formalin (6-24 hours) and embedded in paraffin. Three step sections with a distance of 15 µm between each section were cut from the paraffin embedded tissue blocks and stained with hematoxylin-eosin (H&E), followed by a section immunostained for EpCAM and a final series of three step sections stained with H&E. For evaluation of the histological regression, the PRGS was used.(10)

PIPAC

The PIPAC procedure has been described previously.(9, 12, 13) In brief, PIPAC was performed during a standard laparoscopy. Following mapping of the peritoneum according to the Peritoneal Cancer Index (14) and evacuation of ascites or PLF, the parietal peritoneum was sampled by punch biopsies of the anterior abdominal wall in all four quadrants. The biopsy sites were marked by clips to reduce the risk of sampling errors during subsequent biopsies and PIPAC procedures. Patients with colorectal PM were treated with oxaliplatin 92 mg/m^2 body surface area (BSA), while patients with PM from other primary tumors were treated with cisplatin 7.5 mg/m^2 BSA and doxorubicin 1.5 mg/m^2 BSA. Following administration of chemotherapy, the patients were closed according to departmental guidelines and the PIPAC procedure was repeated every four-six weeks until 1) complete response (negative histology and cytology), 2) unacceptable treatment related toxicity or 3) progressive disease.

Statistics

Values are given as means or medians where appropriate. As data followed a non-normal distribution, comparisons were performed using Wilcoxon-Mann-Whitney test for continuous data and Fisher's exact test for categorical data. P-values were two-tailed and a p-value of 0.05 was considered statistically significant. The statistical software Stata version 13 (Stata Corp, Texas, USA) was used for statistical analysis. Missing data was excluded in the statistical analyses without imputation. Data processing and analysis was performed by an independent statistician.

Ethics

The study was conducted according to the Helsinki Declaration and approved by The Regional Committees on Health Research Ethics for Southern Denmark (Project-ID: S-20140211) and the Danish Data Protection Agency (Project-ID: 14/52603). The study was registered with the ClinicalTrials.gov identifier NCT02320448. All participants gave oral and written informed consent.

RESULTS

From March 2015 to October 2016, 35 patients with PM of any origin were treated with a total of 129 PIPAC procedures (median=3, range 1-9).(9) The last PIPAC procedure was completed in July 2017. Apart from three patients, who refused, all patients had received systemic chemotherapy before inclusion in this trial. Five patients (14%) received bidirectional chemotherapy, with a wash out period of two weeks between systemic chemotherapy and PIPAC (Table 1). Thirty and 27 patients received two and three PIPAC procedures, respectively, while 14 patients were scheduled for more than three PIPAC procedures.

Table 1. Baseline characteristics of patients with peritoneal metastasis, who were treated with Pressurized IntraPeritoneal Aerosol Chemotherapy (PIPAC) in the PIPAC-OPC1 study

Variable	**Value**
Number of patients	35
Age, median years (range)	65 (41-84)
Sex (male/female)	19/16
Previous systemic chemotherapy	32
Bi-directional treatment	5
Primary tumor origin	
Stomach	4
Small bowel	2
Bile ducts	2
Pancreas	3
Appendix	4
Mucinous adenocarcinoma	*1*
Goblet cell carcinoid	*1*
LAMN	*2*
Colorectal	13
MPM	1
Ovary	5
MUP	1
Primary tumor resected	21
Baseline ascites volume	
0 ml	22
1-500 ml	7
501-1000 ml	3
>1000 ml	3

LAMN: low grade appendiceal mucinous neoplasm, MPM: Malignant peritoneal mesothelioma, MUP: Metastasis of unknown primary

Detection of free intraperitoneal tumor cells

After exclusion of one patient with malignant peritoneal mesothelioma (MPM), two patients with pseudomyxoma peritoneii (PMP) from low grade appendiceal mucinous neoplasia (LAMN) and six patients due to missing data collection, ascites or PLF from the first PIPAC procedure was analysed by conventional cytology in 26 patients. qRT-PCR mRNA analysis (CEA, EpCAM, CA-125) was performed in 24 of these 26 patients and in 30 ascites / PLF specimens from patients without any malignant disease (20 benign PLF specimens and 10 benign ascites specimens with inflammation). Protein analysis (total protein and CEA protein) was performed in a subset of 21 patients.

At the first PIPAC procedure, conventional cytology found FITC in 15 of 26 patients, while CEA protein found FITC in 15 of 21 patients. The analysis of total protein or the CEA/total protein ratio did not reveal more patients with FITC. In combination, CEA mRNA and EpCAM mRNA expression (at a cut off of Ct = 32.90 and Ct = 31.98, respectively) showed a sensitivity of 0.88 and a specificity of 1.00 (Table 2). Based on a sensitivity of 0.43 at a specificity of 1.00, CA-125 mRNA expression was discarded in the subsequent analyses (Figure 1). The difference of CEA and EpCAM Ct values between the study population and the negative controls was statistically significant ($p<0.001$). The difference of CEA and EpCAM Ct values within the negative control group were also statistically significant, when stratified between the benign PLF specimens and the benign ascites controls ($p<0.01$) (Table 3). The combination of CEA and EpCAM mRNA expression identified all the specimens found positive by conventional cytology, and further revealed six patients with FITC not identified at conventional cytology.

Response evaluation at third PIPAC procedure

Based on paired data from ascites or PLF retrieved at the first and third PIPAC procedure, 3/19 (16%) patients converted from positive to negative conventional cytology, while 2/19 (11%) went from negative to positive cytology ($p=0.07$). CEA protein analysis was positive in 14/19 (74%) at the third PIPAC compared to 15/21 (71%) at the first PIPAC. The group of patients with FITC based on CEA mRNA and EpCAM mRNA expression was reduced from 20/24 (83%) at the first PIPAC to 16/21 (76%) at the third PIPAC ($p=0.3173$). When using the PRGS as a determinant of response to PIPAC (as described in (9), the mean PRGS score was reduced from 2.05 to 1.54 from

PIPAC 1 to PIPAC 3, $p < 0.001$) conventional cytology, CEA protein and CEA+EpCAM mRNA failed to detect responders to PIPAC.(10)

Figure 1.

Table 2. Detection of Free Intraperitoneal Tumor Cells based on conventional cytology, qRT-PCR for mRNA of CEA, EpCAM, CA-125 and CEA protein analysis at the first PIPAC procedure and at the third PIPAC procedure. The sensitivity and specificity of mRNA analyses are found by receiver operating characteristics shown in figure 1.

	Sensitivity (95% CI)	Specificity	Test positives PIPAC 1	Test positives PIPAC 3
Conventional cytology	X	X	15/26 (58%)	10/19(53%)
mRNA CEA	0.75 (0.53-0.90)	1.00	18/24 (75%)	12/21 (57%)
mRNA EpCAM	0.71 (0.49-0.87)	1.00	17/24 (71%)	14/21 (67%)
mRNA CA-125	0.43 (0.18-0.71)	1.00	X	X
mRNA CEA/EpCAM	0.88 (0.71-0.97)	1.00	20/24 (83%)	16/21(76%)
CEA protein	X	X	15/21 (71%)	14/19 (74%)

CA-125: Cancer antigen 125, CEA: Carcinoembryonic antigen, CI: confidence interval, EpCAM: Epithelial Cell Adhesion Molecule, PIPAC: Pressurized IntraPeritoneal Aerosol Chemotherapy, qRT-PCR: quantitative reverse transcriptase polymerase chain reaction, X: not analyzed

Table 3. Cycle threshold (Ct) values of qRT-PCR for mRNA expression of CEA and EpCAM in the study population (malignant) and the negative controls (n=30) that were included in the ROC curve analyses. The negative controls were divided into a benign (n=20) and a benign ascites (n=10) group. Data are presented as mean Ct (sd) values.

		Negative controls		
	Malignant	Benign PLF	Benign ascites	Benign PLF+ benign ascites
mRNA CEA	30.80 (10.07)	49.25 (2.39)	40.78 (4.49)	46.42 (5.14)
mRNA EpCAM	29.30 (6.91)	43.34 (6.24)	34.85 (1.96)	40.51(6.58)

CEA: Carcinoembryonic antigen, EpCAM: Epithelial Cell Adhesion Molecule, PLF: peritoneal lavage fluid, qRT-PCR: quantitative reverse transcriptase polymerase chain reaction

DISCUSSION

This appears to be the first study on a structured and comprehensive evaluation of ascites or peritoneal lavage fluid from patients with peritoneal metastasis treated by PIPAC. It showed that the detection of CEA mRNA and/or EpCAM mRNA in ascites or PLF from patients scheduled for the first PIPAC procedure was able to detect free intraperitoneal tumor cells without having any false positive tests. This was superior to conventional cytology and CEA protein analysis. Further, combining the expression of CEA and EpCAM mRNA detected FITC in all the patients that had malignant cells at conventional cytology, and in addition in 27% patients who based on conventional cytology were FITC negative. We also found that, while these methods could detect FITC, they were not useful in the PIPAC response evaluation, when compared to the PRGS that was based on histological peritoneal quadrant biopsies.(9, 10)

In small and mostly retrospective studies, PIPAC seems to improve survival rates and quality of life in patients with PM, but larger and prospective studies with long term follow-up are needed.(8, 15, 16) In such studies, comprehensive analysis of conventional cytology, the histology-based PRGS and PCR for tumor markers should be correlated to relevant clinical outcomes, to evaluate whether the PRGS or analyses of ascites/PLF specimens can determine a clinically meaningful treatment response. Further, there is no consensus on the number of PIPAC procedures necessary to have the optimal efficacy both clinically, histologically and cytologically. In the present study, the response evaluation was based on peritoneal quadrant biopsies and ascites or PLF retrieved at the third PIPAC compared to the first PIPAC, but perhaps this is not the optimal timing. Still, due to the severity of disease in these palliative patients, the number of eligible patients will rapidly decrease after three PIPAC procedures.(9)

Based on the wide indications for PIPAC treatment, a heterogenous study population was expected in this study, thus comprehensive analysis strategies were planned both in terms of conventional cytology with immunocytochemistry, protein and qRT-PCR based analyses. The protein data were analyzed according to cut offs from previous studies on gastric cancer patients, where elevated CEA protein levels were a negative prognostic factor regarding peritoneal recurrence and survival.(11, 17) CEA protein analysis did not reach the sensitivity of CEA/EpCAM mRNA analysis, but before

discarding this cheaper analysis, more data are needed, including negative benign and inflammatory controls to adapt the optimal cut off level.

While CEA mRNA is most often used in studies on gastric and colorectal cancer patients, this study also included mRNA of EpCAM and CA-125. CEA mRNA had the highest sensitivity, but the addition of EpCAM mRNA improved the detection of FITC, while still negative in the controls consisting of benign PLF and inflammatory ascites specimens. CA-125 may be useful in gastric cancer patients (11), but was included in this study mainly to evaluate whether it was better to detect FITC from ovarian cancer, compared to mRNA of CEA or EpCAM. At a specificity of 100%, CA-125 had a sensitivity of 75% in an ovarian cancer subgroup analysis (data not shown), but failed as an overall indicator of FITC. Of note, all ovarian cancer patients treated at Odense PIPAC Center had malignant cells based on conventional cytology, indicating that further in depth analysis might not be required.

Systemic chemotherapy and the investigated PIPAC treatment were only able to eradicate FITC in selected patients. Still, this study reports an optimized setup for detection of FITC that can be applied to staging laparoscopy before the final treatment decision is made. Moreover, our data indicate that mRNA based analysis may in fact be used as an adjunct to select patients for neoadjuvant treatment.

Despite the prospectively controlled design of this study, some methodological considerations have to be addressed. In lack of validated methods for monitoring the treatment response in patients with PM, the detection of FITC at PIPAC 1 and PIPAC 3 was compared to the response evaluation according to the PRGS, which is based on expert consensus. This study was not large enough to perform relevant subgroup analyses. The missing data might have an impact on the results and interpretations, and due to the heterogeneity of the study population, the optimized setup for detecting FITC must be used with caution, if used in subgroups of cancer patients. As all patients had visible biopsy proven PM, the study findings are based on the assumption, that all patients within the study population truly had FITC, regardless of findings at conventional cytology. This assumption may never be confirmed. If these analyses are to be included in routine clinical practice, the setup should be refined to minimize the associated costs and time. At best, these ancillary tests should be performed at the same time as the conventional cytology analyses, and the findings and cut off levels have to be investigated in larger series optimally stratified by tumor origin.

Based on this study, we conclude that qRT-PCR based analyses of CEA/EpCAM mRNA detect free intraperitoneal tumor cells in patients with peritoneal metastasis with a high sensitivity and specificity. This technique may be used in the evaluation of ascites or peritoneal lavage fluid specimens to define the optimal treatment strategy in patients at high-risk of developing peritoneal metastasis from various types of abdominal cancers. However, analysis of ascites and peritoneal lavage fluid did not seem to be useful for evaluation of response to PIPAC treatment, when compared to the histological PRGS.(9, 10)

Data Availability Statement

Participant data will be retained at the study facility for five years after completion of the trial according to Danish law. After this time period, it will be de-identified and made available for other researchers upon reasonable request to the corresponding author.

License for Publication

Declarations of funding and competing interests: None declared

Key messages

- Conventional cytology detects free intraperitoneal tumor cells at a low sensitivity, even in patients with known peritoneal metastasis

- Analysis of CEA and EpCAM mRNA can optimize the detection of free intraperitoneal tumor cells
- The treatment response of Pressurized IntraPeritoneal Aerosol Chemotherapy (PIPAC) cannot be documented by comprehensive analyses of ascites or peritoneal lavage fluid

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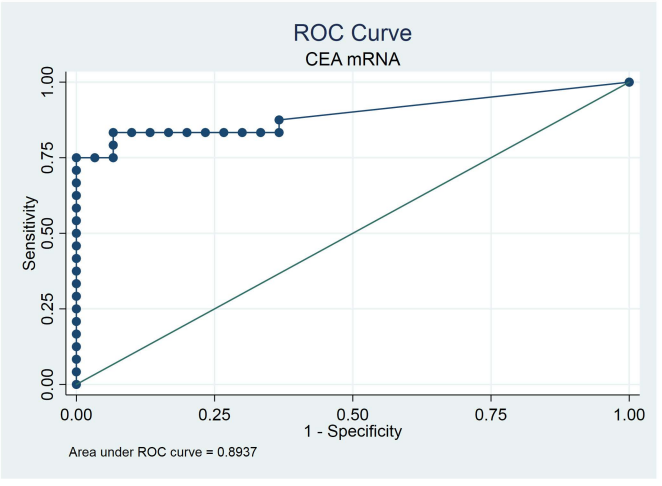
Figure 1. Receiver Operating Characteristics for mRNA of a) CEA, b) EpCAM and c) CA-125

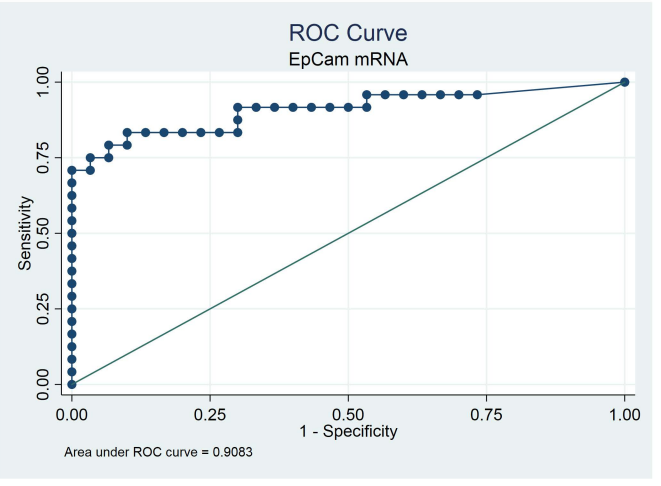
Below figure 1:

CA-125: Cancer antigen 125, CEA: Carcionoembryonic antigen, EpCAM: Epithelial Cell Adhesion Molecule

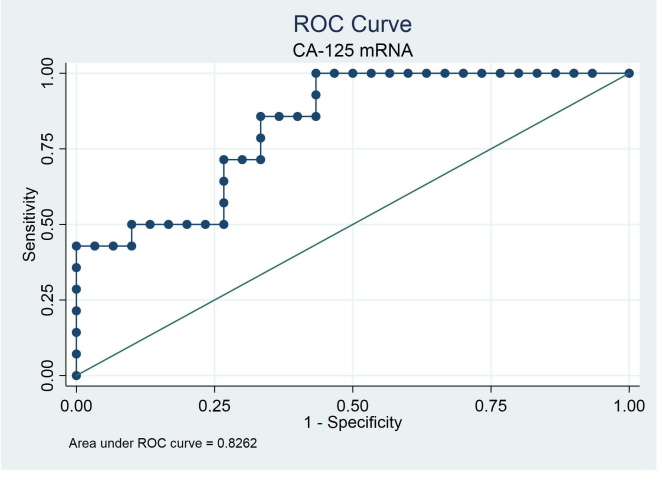
a)


ROC Curve
CEA mRNA
Sensitivity
0.00
0.25
0.50
0.75
1.00
1 - Specificity
Area under ROC curve = 0.8937

b)


ROC Curve
EpCam mRNA
Sensitivity
0.00
0.25
0.50
0.75
1.00
1 - Specificity
Area under ROC curve = 0.9083

c)


ROC Curve
CA-125 mRNA
Sensitivity
0.00
0.25
0.50
0.75
1.00
1 - Specificity
Area under ROC curve = 0.8262