Post print version. Published in International Journal of Gynecological Cancer, 2017, vol. 27, no. 7, pp. 1360-1366. The final publication is available http://dx.doi.org/10.1097/IGC.0000000000001031.

Urinary polyamines as biomarkers for ovarian cancer

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Acknowledgments: The authors wish to thank Mrs. Tuula Reponen for the technical assistance.

Funding: This project was financially supported by the Competitive Research Funding of the Tampere University Hospital (Grant X51001 for T.L., Grant 9S040 for J.M., Grant 9S045 for N.O.) and by the strategic funding from University of Eastern Finland (for M.H., T.K. and J.V.), Cancer Foundation of Finland (J.M., T.L. and N.O.), Elna Kaarina Savolainen's Cancer Research Fund (N.O.), Seppo Nieminen's Testament Fund (N.O.), Tampere Tuberculosis Foundation (N.O.) and Finnish Funding Agency for Technology and Innovation (TEKES) (N.O.)

Word count: 2 322

Abstract

Objectives: Elevated concentrations of polyamines have been found in urine of patients with malignant tumors, including ovarian cancer. Previous research has suffered from poorly standardized detection methods. Our liquid chromatography tandem mass spectrometry (LC-MS/MS) -method is capable of simultaneous standardized analysis of the majority of known polyamines. LC-MS/MS has not previously been used in the differential diagnostics of ovarian tumors in postmenopausal women.

Materials and Methods: In this prospective study, postmenopausal women (N = 71) presenting with an adnexal mass, and as controls, women with genital prolapse or urinary incontinence scheduled for surgery (N = 22), were recruited to the study. For analysis of the polyamines, a morning urine sample was obtained before surgery. Preoperative serum CA125 concentrations were determined in the study group.

Results: Twenty-three women with benign and 37 with malignant ovarian tumors were eligible. Of all analyzed polyamines, only urinary N^1,N^{12}-diacetylspermine (DiAcSpm) showed statistically significant differences between all groups except controls vs. benign tumors. DiAcSpm was elevated in malignant vs. benign tumors ($p < 0.001$), in high grade vs. low malignant potential tumors ($p < 0.001$), in stage III-IV vs. stage I-II cancers ($p < 0.001$), and even in early-stage cancer (stage I-II) vs. benign tumors ($p = 0.017$). DiAcSpm had better sensitivity (86.5 %) but lower specificity (65.2 %) for distinguishing benign and malignant ovarian tumors than CA125 with cut-off value of 35 kU/L (sensitivity 75.7 % / specificity 69.6 %).

Conclusions: Urinary DiAcSpm seems to be able to distinguish benign and malignant ovarian tumors as well as early and advanced stage, and low malignant potential and high grade ovarian cancers from each other, respectively.

Keywords: LC-MS/MS, ovarian tumor, ovarian cancer, DiAcSpm, polyamine

Introduction

Ovarian cancer is the leading cause of deaths due to gynecological cancer in the U.S. and Europe. The lack of specific symptoms makes the diagnosis of ovarian cancer difficult, with ultrasound being typically the first imaging test used to distinguish benign and malignant tumors from each other. Ultrasound features combined with CA125 and menopausal status have been used to calculate the Risk of Malignancy Index (RMI).[1] A major problem in ultrasound imaging is the relatively large number of false positive results. Unfortunately, CA125 antigen lacks sensitivity even in the postmenopausal setting, as it is not expressed by approximately 20 % of early stage ovarian cancers.[2]

Polyamines are molecules essential for nearly all living organisms with critical roles in cell signaling and growth in vertebrates. Putrescine, spermidine and spermine are the most common polyamines detected in mammals. Polyamines exist in urine mainly in acetylated forms.[3] Increased polyamine concentrations are found during active cellular proliferation, like in patients with cancer.[4] In fact, ovarian cancer was one of the first cancers linked to polyamines.[5] These early studies were hampered by methodological problems, but subsequently, the development of gas chromatography-mass spectrometry enabled quantitation of both free and acetylated polyamines in human urine without hydrolysis of polyamines.[6] Recently, an optimized liquid chromatography tandem mass spectrometry (LC-MS/MS) -method has been developed, which allows for a simultaneous analysis of up to 14 polyamines in different forms in urine.[7]

The present study was undertaken as a proof of concept study to explore the usefulness of LC-MS/MS in differential diagnostics of ovarian tumors.

Materials and Methods

Patients

From May 2013 through March 2016, 71 patients presenting with an abnormal adnexal mass and scheduled for surgery at the Department of Obstetrics and Gynecology of Tampere University Hospital, were prospectively recruited to the study. They had to be at least 50 years old, and the only exclusion criterion was an ongoing treatment for cancer. Besides pelvic examination, a vaginal ultrasound scan was performed, and a serum sample for CA125 was obtained from each patient. The final sample size was 60 patients, after exclusion of ineligible patients (Fig. 1).

Twenty-two postmenopausal women scheduled for surgery because of genital prolapse or urinary incontinence from May 2013 through April 2014, were recruited as controls. One patient of the control group had to be excluded because of an incidental endometrial adenocarcinoma. All subjects provided a morning urine sample at the day of operation. The samples were stored at -70° C until analysis for polyamines.

All participants gave their informed consent to the study, which was approved by the Ethics Committee of Tampere University Hospital (ETL R10066).

LC-MS/MS-analysis

The LC-MS/MS-analysis was conducted at the University of Eastern Finland (Kuopio, Finland) and was performed using the previously described method.[7] In short, the chromatographic separations were carried out using a reversed phase column (Phenomenex Kinetex C18 150 mm × 2.1 mm, 2.6 µm) and 0.1% heptafluorobutyric acid as the evaporative ion-pairing reagent. Detection was performed with Agilent 6410 triple quadrupole mass spectrometry equipped with an electrospray ionization source, and using selected reaction monitoring method. Deuterated polyamines were used as stable internal standards in quantification. Polyamine concentrations were normalized using creatinine concentration. The concentrations are expressed as µmol of polyamine per gram of creatinine.

The creatinine concentrations of the urine samples were determined enzymatically using Cobas 6000, C 501-module (Roche diagnostics GmbH, Mannheim, Germany) at Fimlab Laboratories.

Statistical analysis

No formal sample size calculations were made due to the preliminary nature, of the study. The statistical analysis was performed by using MATLAB version R2016b. The normality of the distributions of continuous variables was evaluated by Kolmogorov-Smirnov tests. Due to the skewness of the distributions, the differences in polyamine concentrations between the distinct groups were analyzed by Wilcoxon rank sum test. The differences in the concentrations of polyamines are visualized by box plots. A probability value of < 0.05 was considered as statistically significant. All tests were two-sided. To compare the predictive performance of DiAcSpm and CA125, a receiver operating characteristics (ROC) analysis was accomplished.

Results

Patients and tumors

All 81 women in the final study population were over 50 years old and postmenopausal. The median age (75 years; range 55-83) in the control group was significantly higher than in the groups with benign (65 years; range 51-73) and malignant tumors (64 years; range 51-86), respectively ($p = 0.02$). Three of the controls and four of the cases had used systemic hormonal therapy before surgery. Five patients (one in the control group, one with a benign and three with a malignant ovarian tumor) had previously been treated for breast cancer and one patient (with a malignant ovarian tumor) for thyroid cancer, but all of them were disease-free at the time of sampling.

Of the ovarian tumors, 23 were benign and 37 were malignant (Fig. 1). The benign group consisted of 11 serous and two mucinous cystadenomas, two fibromas, five simple and two endometriotic cysts, and one necrotized cyst. Eighteen of the malignant tumors were of low malignant potential (four serous and five mucinous borderline tumors, three mucinous adenocarcinomas, four granulosa cell tumors, one Sertoli-Leydig cell tumor, and one endometrioid adenocarcinoma). Eighteen of the high grade tumors were serous carcinomas (HGSC) and one was a carcinosarcoma. One of the patients with HGSC had two primary tumors, or the stage IIIC ovarian cancer and an invasive ductal grade 3 breast cancer, which were operated on at the same session.

The median serum concentrations of CA125 were 18.0 kU/L (range, 5-1,984), 53.5 kU/L (range, 7-841), and 546.0 kU/L (range, 22-8,152) in the patients with benign tumors, with low malignant potential tumors, and with high grade tumors, respectively. The difference of the tumor marker concentration between the groups was statistically significant ($p < 0.001$).

Urinary polyamines and their metabolites

The polyamines and their metabolites detected by the LC-MS/MS are given in Table 1. Six polyamines, or, acetylated putrescine (AcPut), acetylated cadaverine (AcCad), N^1-acetylated spermidine (N^1AcSpd), N^8-acetylated spermidine (N^8AcSpd), DiAcSpd, and DiAcSpm were constantly found in all urine samples, but only DiAcSpm showed statistically significant differences between all groups except between controls and benign ovarian tumors (Fig. 2, Fig. 3). The polyamine concentrations were independent of body mass index (BMI; data not shown).

A detailed description of the urinary concentrations of DiAcSpm in different settings is given in Table 2. There were no differences between the women with genital prolapse or urinary incontinence and the women with

benign tumors. Of the women with malignant tumors, the ones with high grade and advanced stage tumors had the highest urinary concentrations of DiAcSpm. However, the concentrations were significantly higher also in women with low malignant potential and early stage tumors than in the controls.

Besides DiAcSpm, there was also a significant difference in concentrations of DiAcSpd, N^1AcSpd and N^8AcSpd between low malignant potential and high grade malignant ovarian tumors (*p* values 0.02, 0.004 and 0.013, respectively), and between benign and malignant ovarian tumors in concentrations of DiAcSpd and N^8AcSpd (*p* values 0.015 and 0.042).

Performance of CA125 and urinary DiAcSpm

In distinguishing benign tumors from the malignant ones, the specificity and sensitivity of CA125 (using the standard used cut-off value 35 kU/L) were 69.6 % and 75.7 %. Correspondingly, the specificity of DiAcSpm (cut-off value 0.097 μmol/g creatinine) was 65.2 % and sensitivity 86.5 % (Fig. 4).

Discussion

The main finding of this preliminary study was that urinary concentrations of DiAcSpm are elevated among postmenopausal women with malignant ovarian tumors as compared to women with benign ovarian tumors or genital prolapse. Although DiAcSpm concentrations were higher in the case of high grade and advanced stage cancers than among low malignant potential and early-stage cancers, respectively, there was still a statistically significant difference in DiAcSpm levels between early-stage (and low malignant potential) and benign tumors.

Previous studies utilized methodology with limited polyamine array and were unable to measure all polyamines in a single run, which deteriorated repeatability and increased variability of different studies. Another limitation was that acetylated polyamines, which are highly specific for malignant disease, could not be measured. Consequently, the earlier research focused on less specific unacetylated polyamines.[8] Many methods rely on derivatization techniques where polyamines are conjugated with other molecules to allow extraction and analysis.[9] This technique is not suitable for the analysis of diacetylated polyamines due to acetylation of active amino-groups. Our LC-MS/MS method optimized for noninvasive and standardized single-run analysis of urine can be utilized for simultaneous analysis of up to 14 polyamines in free, mono- and diacetylated forms in human urine, without any derivatization.[7] In the present study, however, 1,3-diaminopropane and putrescine were not

detectable. Additionally, results concerning diacetylated cadaverine and diacetylated putrescine should be interpreted with caution, since their analysis is only semiquantitative.[7]

While we are not aware of any subsequent studies since the report by Suh et al.[10] , on the urinary polyamine profile in ovarian cancer, our results are in accordance to previous experience with other tumors. Patients with non-Hodgkin's lymphoma, or urogenital cancer (testicular, prostatic, renal and bladder carcinoma) have been found to have elevated urinary concentrations of DiAcSpm, and those with urogenital cancer also of DiAcSpd.[6, 11-13] In breast cancer, even early stage disease seems to be associated with elevated levels of DiAcSpm in the urine.[13] In colon cancer, urinary DiAcSpm may be a better tumor marker than carcinoembryonic antigen (CEA) or cancer antigen 19-9 (CA 19-9).[13, 14] Besides being predictive markers, there is a possibility that DiAcSpm and DiAcSpd could be used also as prognostic markers, at least in testicular or prostatic cancer.[11] Urinary DiAcSpm has been shown to be both diagnostic and prognostic marker also for non-small-cell lung cancer.[15, 16]

In the present study, the performance of CA125 used in the preoperative diagnostic work-up of pelvic tumors, was poorer than in published large screening studies.[1, 17-20] The reason may be by chance only, due to the rather limited sample size, but the fact that a large portion of the malignant tumors were of low malignant potential or borderline epithelial, granulosa and Sertoli-Leydig cell tumors, may have contributed to the less than optimal performance. In any case, urinary DiAcSpm was a more sensitive marker of malignant ovarian tumors than CA125 with a cut-off value 35 kU/L, while being less specific.

Although the mechanism underlying elevated concentrations of DiAcSpm in the urine of cancer patients is not completely understood, an increased synthesis of polyamines in proliferating cells, including malignant cells is well documented. It has been suggested that the increased secretion of DiAcSpm may reflect a defense mechanism by the organism to lower intracellular polyamine concentrations by acetylating and excreting polyamines and hence, decreasing the growth rate of the tumor.[8, 13, 21] It has also been purported that the production of DiAcSpm in tumorigenesis may be a result of host macrophage activation.[22]

The results of the present study are to be considered as preliminary, to be confirmed in larger patient cohorts. Chronic inflammatory diseases may influence DiAcSpm concentrations. However, in the study of pancreatico-biliary diseases the levels of DiAcSpm in urine were lower in benign inflammatory conditions than in cancers.[23] Presence of urinary tract infection may also have a confounding influence on urinary polyamines, increasing especially the level of putrescine.[24] The impact of infection cannot totally be ruled out in the present study, because a routine urine sample for diagnosing urinary infection was taken only from the patients of the control

group. Moreover, cadaverine in human urine is suggested to be derived from the gut microbial flora.[24] A limitation of the study is that there were no patients with a benign teratoma, because among benign tumors, teratomas have been associated with elevated levels of urinary polyamines.[5] On the other hand, teratomas are typically tumors of adolescents and young adults and not of postmenopausal women, the risk population for ovarian cancer.

Ornithine decarboxylase is one of the key enzymes in polyamine synthesis and is stimulated by estrogens in estrogen-responsive tissues.[25] A previous study found that urinary DiAcSpm values are higher in women than in men, while postmenopausal women had lower concentrations than premenopausal ones.[26] Accordingly, Buyn et al. showed that premenopausal breast cancer patients had higher (free and monoacetylated) polyamine levels in serum than postmenopausal ones, whose polyamine levels were similar to healthy controls.[27] In our study all women were postmenopausal, but seven of them used hormone replacement therapy, creating a potential bias. However, excluding these patients did not alter the results. The control women were older than the ones with tumors, which may have had impact on the results. However, there was no significant difference between DiAcSpm levels of control group and women with benign ovarian tumors. One patient in the malignant tumor group had been treated for breast cancer over five years ago, but she had no signs of recurrence. Another patient had a grade 3 breast cancer concurrently with the stage IIIC HGSC, which may have affected her DiAcSpm level. However, her urinary level of DiAcSpm was of similar magnitude as in the patients with advanced high grade tumors in general.

We used a morning urine sample in our study, which however may not have been necessary. According to a study on healthy people, a spot urine sample at any time of the day represents the true excretion rate of DiAcSpm after creatinine normalization.[26] Although the LC-MS/MS method is very accurate, it is as such not suitable for routine clinical use, at least not for time being.[7] However, if only one (DiAcSpm) or a few polyamines need to be analyzed, the analysis could probably be made faster and less expensive to perform. Alternative methods to measure only urinary DiAcSpm in routine clinical practice include an enzyme-linked immunosorbent assay (ELISA)[13, 28] or a colloidal gold aggregation procedure.[29]

In conclusion, urinary levels of DiAcSpm were elevated in patients with malignant ovarian tumors, even in patients with tumors of low malignant potential and early-stage tumors. These preliminary findings suggest that urinary DiAcSpm may in the future become a useful diagnostic marker for malignant ovarian tumors in the postmenopausal setting.

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Table 1. The polyamines studied and their urinary concentrations (μmol/g creatinine) in patients with ovarian tumors and in controls

Polyamine	Controls (*N* = 21)		Benign ovarian tumors (*N* = 23)		Low grade malignant tumors (*N* = 18)		High grade malignant ovarian tumors (*N* = 19)	
	Range μmol/g creatinine	Detection rate	Range μmol/g creatinine	Detection rate	Range μmol/g creatinine	Detection rate	Range μmol/g creatinine	Detection rate
Cad	NA-2.65	13/21	NA-3.94	19/23	NA-20.19	15/18	NA-48.7	9/19
AcCad	0.04-5.64	21/21	0.08-14.26	23/23	0.11-13.08	18/18	0.17-5.57	19/19
DiAcCad	NA-0.48	13/21	NA-0.28	18/23	NA-1.79	12/18	NA-0.63	8/19
AcPut	6.57-19.28	21/21	5.60-28.1	23/23	9.15-41.45	18/18	8.54-50.16	19/19
DiAcPut	NA-0.24	17/21	NA-0.62	18/23	NA-0.75	16/18	NA-0.25	17/19
Spd	NA-0.30	20/21	0.08-0.27	23/23	0.10-0.28	18/18	0.09-0.56	19/19
N^1AcSpd	1.78-8.92	21/21	1.04-8.17	23/23	1.39-6.50	18/18	2.52-18.50	19/19
N^8AcSpd	1.84-4.75	21/21	1.15-4.69	23/23	1.65-5.03	18/18	2.18-5.99	19/19
DiAcSpd	0.18-0.65	21/21	0.14-1.32	23/23	0.16-1.34	18/18	0.23-1.20	19/19
Spm	NA-0.55	8/21	NA-0.45	12/23	NA-0.25	12/18	NA-0.32	14/19
AcSpm	NA-0.01	3/21	NA-0.01	4/23	NA-0.004	1/18	NA-0.04	7/19
DiAcSpm	0.06-0.20	21/21	0.04-1.02	23/23	0.06-0.37	18/18	0.14-2.37	19/19

NA; analyzed concentration was below the lowest calibration point
Cad, cadaverine; AcCad, acetylated cadaverine; DiAcCad, diacetylated cadaverine; AcPut, acetylated putrescine; DiAcPut, diacetylated putrescine; Spd, spermidine; N^1AcSpd, N^1-acetylated spermidine; N^8AcSpd, N^8-acetylated spermidine; DiAcSpd, N^1,N^8-diacetylspermidine; Spm, spermine; AcSpm, acetylated spermine; DiAcSpm, N^1,N^{12}-diacetylspermine

Table 2. The urinary concentrations of N^1,N^{12}-diacetylspermine (DiAcSpm) (μmol/g creatinine) in patients with ovarian tumors and in controls and comparisons between the groups

	N^1,N^{12}-diacetylspermine (DiAcSpm) (μmol/g creatinine)						
	Mean	SD	Median	Min.	Max.	Comparison	*p* value
Controls	0.10	0.04	0.09	0.06	0.20	vs. benign tumors	NS
Benign tumors	0.15	0.21	0.08	0.04	1.02		
All malignant tumors	0.54	0.62	0.26	0.06	2.37	vs. benign tumors vs. controls	< 0.001 < 0.001
Low malignant potential tumors	0.17	0.09	0.16	0.06	0.37	vs. benign tumors	0.020
High grade tumors	0.89	0.72	0.67	0.14	2.37	vs. low malignant potential tumors	< 0.001
Stage I-II tumors	0.19	0.15	0.16	0.06	0.69	vs. benign tumors	0.017
Stage III-IV tumors	0.87	0.72	0.64	0.14	2.37	vs. stage I-II tumors	< 0.001

Fig. 1 Study population

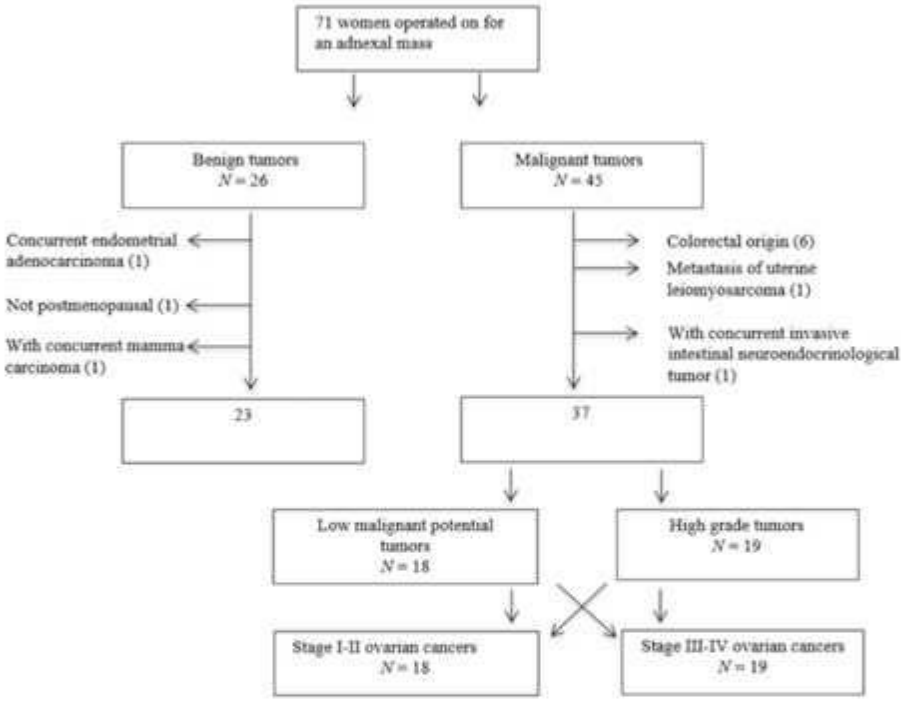

Fig. 2 The urinary concentrations of N^1,N^{12}-diacetylspermine (DiAcSpm) (µmol/g creatinine) in patients with ovarian tumors and in controls and comparisons between the groups

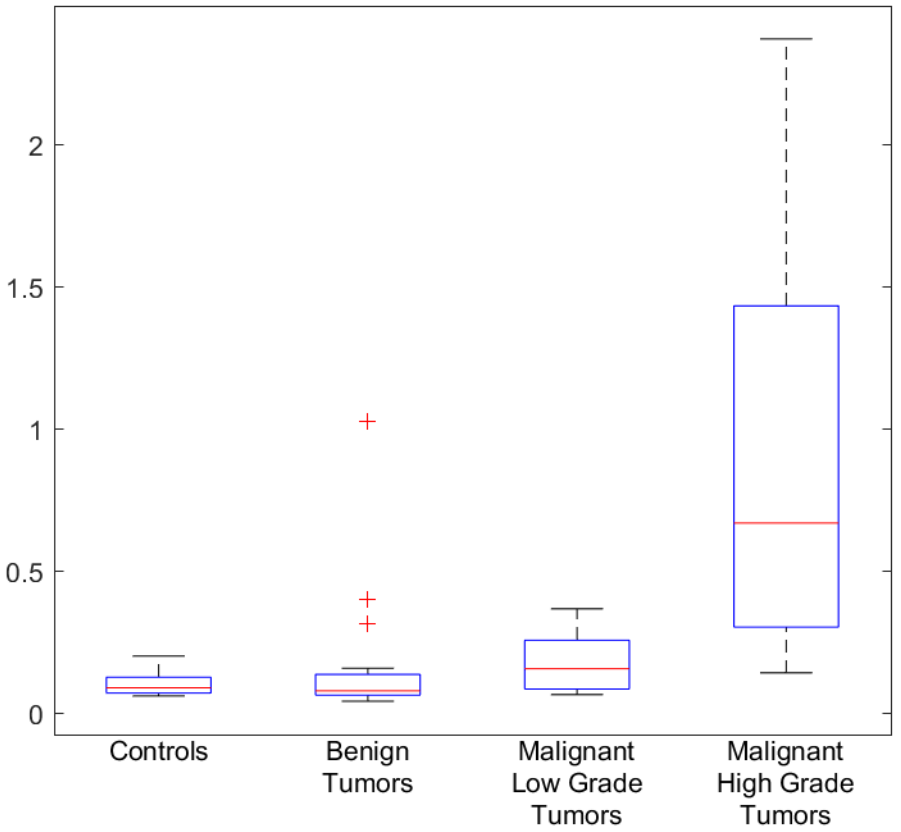

Fig. 3 The urinary concentrations of N^1,N^{12}-diacetylspermine (DiAcSpm) (µmol/g creatinine) in the women with stage I-II and stage III-IV ovarian tumors

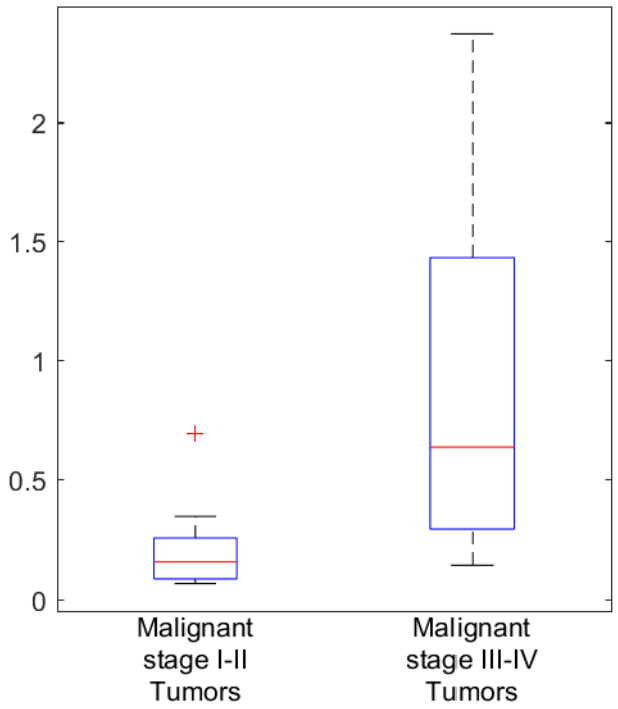

Fig. 4 ROC-curves for urinary N^1,N^{12}-diacetylspermine (DiAcSpm) and CA125 in benign and malignant ovarian tumors with optimal (DiAcSpm 0.097 µmol/g creatinine) and typically used (CA125 35 kU/L) thresholds