Changes in urine autofluorescence in ovarian cancer patients

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Ovarian cancer is the type of cancer with the highest mortality rate among gynaecologic malignancies. Due to lack of screening tools, this disease is mainly diagnosed at a progressed stage, when it is too late to adequate therapy. Despite many attempts, enough sensitive and specific biomarker was not still uncovered. Fluorescence spectroscopy has proven to be a useful diagnostic tool with high efficiency. Fluorescence detection has three major advantages over other light-based investigation methods: high sensitivity, high speed, and reliability. Biological materials consist of a number of intrinsic fluorescent compounds –autofluorophores, which are associated with cardinal metabolic pathways. It is well known, that cancerous tissue metabolism is altered compared to healthy one, what influence also intrinsic fluorophores composition of bodily fluids. Urine is one of the biological fluids that could be obtained most easily and displays a blue – green fluorescence that can change in case of pathological process. Analysis of urine autofluorescence is non invasive and simple technique. Using fluorescent spectroscopy, ovarian cancer patients and healthy control group were discerned with high significance, so we predict that fluorescence analysis of urine could be a potential means of ovarian cancer screening.

Key words: fluorescence, cancer screening, urine, ovarian cancer

Ovarian tumors are concerned a very heterogeneous group of both, epithelial and non-epithelial tumors. Epithelial tumors go developed from the ovarian epithelium (mesothel) and non-epithelial tumors are germ – cell or sex-cord stroma origin, which results in a diversity of suitable diagnostic markers. To overcome this drawback, various tumor markers can be utilized and in combinations can offer better diagnostic results. Even though several tumor markers are already known, the search for an effective screening method is not over. The most used, best described and with dominant importance is carbohydrate antigen CA 125. The specificity and thus the screening value of CA125 are diminished, however, by the fact that many benign gynaecological conditions, as well as other malignancies, can result in elevated CA 125 levels. Also, while elevated CA 125 is diagnosed in up to 80 % of patients with epithelial ovarian cancer (EOC), it is elevated in only 50 – 60 % of patients with epithelial ovarian cancer in stage I [1]. The stage-independent sensitivity of serum CA125 as single marker is 70 % at a set specificity

of 95 %. For the early stage of ovarian cancer, the sensitivity is only 52 % [2] and there is still a subset of ovarian tumors that do not express CA125 [3]. Therefore, new ways of detecting early stage ovary cancer consisting of discovery of a new marker or by combining known markers (panels of markers) and leading to enhancement of sensitivity and specificity are still being sought. Markers incorporated into these analyses look at cancer from different points of view, such as metabolic change, immune response, proliferation signs, overproduction of secretions or hormones, etc. Mostly they are non-specific to any organ or tissue, or their exact function in the human body is not absolutely clear. Overviews of tumor markers associated with ovarian cancer and some tested algorithms are presented in Tables 1 and 2. However, neither of these methods is used in screening or is satisfactory enough for detection of early stage ovarian cancer to improve the prognosis of patients.

Body fluids are valuable biological materials and are more suitable than tissues for screening procedures because they

Table 1. Tumor markers associated with ovarian cancer (HE 4 – human epididymis protein 4, SMRP – soluble mesothelin-related peptide, GTCT – granulose-theca cell tumors, OGCT – ovarian germ cell tumors)

Marker	Sensitivity %	Specificity %	EOC expression	Non-EOC expression	Comment	Ref.
CA 125	70; for early stage 52	95	+	±	elevated in benign conditions and other malignancies	2
HE4	76.5	95	+		less in benign condition	
SMRP	53.7	95				
Total inhibin	93/94 (serous/mucinous)		+			4
Total inhibin			+	+ for GTCT	import in menopausal women	5
Inhibin A	67	100				6
Inhibin B	89	100			correlates with malignant disease	
B7-H4					+ in ½ of early cancer and 2/3 of advanced stage patients	7
αFP						8
ßhCG				+ for OGCT	+ only in advanced stage	9
LDH						
Oestrogen				+ for GTCT		10
Androstenedione						

Table 2. Combination of CA125 biomarker with others

Additional biomarker	Sensitivity %	Specificity %	Comment	Ref.
HE4	92.3	75	post-menopausal	11
HE4	76.5	74.8	pre-menopausal	
HE4 (ROMA, logaritmic values)	94.3	75	depending on menopausal state	12
			decreased results after the inclusion of border-line tumors, metastatic tumors, non-EOC	13
SMRP	56.8	95		2
Total inhibin	99/95 (serous/mucinous)			4
B7-H4	65	97	suitable for early stage, elevated in both benign and malignant	14
C-reactive protein, serum amyloid A, interleukin 6, interleukin 8	94	91		15
Leptin, prolactin, osteopontin, insulin-like growth factor II and macrophage migration inhibitory factor	95.3	98.7	positive predictive value 6.5 %	16

are less invasive. Their exact composition is not yet known and is still being studied. Proteomics and the use of robust analytical techniques (mass spectroscopy) to explore biological fluids such as serum, urine or ascites for possible markers offer new screening possibilities for early diagnostic of ovarian cancer. The comprehensive ascites proteome in advanced ovarian cancer has already been published and includes a list of 80 candidate markers [17]. Early attempt at profiling low-molecular-weight serum proteins has yielded surprisingly good results – sensitivity of 100 %, specificity of 95 % and a positive predictive value of 94 % [18]. Recent studies have continued to deliver similar results, with sensitivity, specificity and positive predictive value all above the 95 % threshold [19, 20]. Proteomics also seems to be useful in the classification of information on specific ovarian cancer subtypes, which can be used to tailor pre-surgical therapy [21]. The study of genes and regulatory proteins (genomics and proteomics) has focused mainly on diagnostics, but the study of small molecules must also not be forgotten and should be placed on the same level of importance. "While genomic or proteomic analysis cannot give any evidence of stress even if the person turns blue, metabolomics will indicate dramatic changes"

[22]. Fluorescence measurement of biological materials is a very sensitive, simple executable and cost-effective tool with great potential for clinical diagnostics. The use of fluorescent spectroscopy capable of studying molecular interaction could become a very useful method, even if is not specific to particular fluorophores.

With the aim of decreasing mortality in patients with ovarian cancer, management of such patients should be multidisciplinary. Team of professionals from different medical science disciplines should participate in both early detection (screening, markers, imaging methods) and multimodal therapy (surgical, oncological). Without a solid understanding of the origin of cancer, it becomes difficult to specify a clear strategy for effective management. For most genes the mutation rate is low, but cancer cells express many different types and kinds of mutations. New insights into cancer as a primarily metabolic disease will result in novel approaches to cancer management and prevention. Autofluorescence-based optical imaging technologies could be an inexpensive step forward in supporting the development *in vivo* screening for cancer diagnoses. The native fluorescence or autofluorescence of some molecules can provide useful information about the metabolism of normal cells. It is known, that endogenous fluorescence from aromatic amino acids, certain proteins as well as coenzymes in tissues, can be altered by carcinogenesis.

Most studies using the high sensitivity of fluorescence for cancer detection are focused on tumor tissue or cell materials and declare high diagnostic sensitivity and specificity. Screening suitability and cancer risk prediction are also mentioned often. Although changes in metabolism during pathological states can endure well beyond morphological changes, results from fluorescence measurements are almost always related to histological diagnoses in order to confirm the accuracy of the assumption. For screening, procedures they are as simple as possible and are the most suitable. Despite their suitability for screening and their less invasive collection, biological fluids are rarely used for similar experimental purposes. Recently, Masilamani et al. [23], in their study using urine for early cancer detection, described the case of a patient with lung cancer, for whom four biological materials (lung cancer tumor tissue, plasma, urine and pleural effusion) were examined, with the result, that the fluorescence spectrum of the urine was very similar to that of the tumor tissue. In addition, autofluorescence of urine was reported in conjunction to renal diseases [24] and urinary tract infection [25]. To our knowledge, only Lu et al. [26] has described urine autofluorescence as potential diagnostic tool for ovarian cancer patients, but without providing information about the number of tested groups of healthy and diseased patients.

The present study is focused on the applicability of one of most available biological materials – urine – for serious disease diagnostics in regard to the lack of a satisfactory screening method – for ovarian cancer detection.

Materials and methods

For the experiment, groups of patients were created as follows: healthy women without cancer anamnesis (N = 36, age 22 – 65, average 35.7 ± 9.7), patients with a benign ovarian tumor (N = 16, age 27 – 86, average 54.6 ± 17.8) and patients with malignant ovarian cancer (N = 21, age 33 – 84, average 59.9 ± 14.4). The group of patients with ovarian cancer also included border-line tumors (N = 3). Healthy women filled in the questionnaire about their age, health status and anamnestic information. Information about the benign or malignant origin of tumors was based on the results of the final histopathological examination after surgery. All patients signed the informed consent.

Morning urine samples from fasting subjects were withdrawn from middle stream and frozen at -18°C until. Thawed urine samples were centrifuged for 10 minutes at 3000 rpm. The autofluorescence of undiluted urines was measured at room temperature, using a Luminescence Spectrophotometer Perkin Elmer LS 55 (USA) in a 10 mm quartz cuvette. From every sample we measured synchronous spectra with ΔΛ 30 nm in the range 250 – 545, step 0, 5 nm, excitation/emission slits 5/5 nm, and a scan speed of 1200 nm/min. For better comparison and discrimination of groups we used both original and normalized spectra. A Student`s T-test was used to calculate the statistical significance.

Results

The fluorescence spectra of urines are the function of many overlapping chromophores present in the sample. The synchronous spectrum (ΔΛ 30 nm) of undiluted urine is characteristic with 2 peaks (Figure 1). The maximum of the first peak may vary over a wide range of wavelengths from 340 – 420 nm. The second peak´s maximum is typical and repeating at 480 nm. Due to the wide range of the wavelength, we can only speculate about what is hidden in the first peak, but the second peak is most likely given by the fluorescence of riboflavin and its derivatives.

The intensity of fluorescence in both areas mentioned above highly varies visually in all tested groups. Often (in all groups) a peak was present in various parts of the spectra which exceeded the detection limit and increased the dispersion of the fluorescence emission values. The standard deviation sometimes exceeded the average value. When matching the average spectra, some differences are seen, but statistical analysis differs from visual evaluation. When comparing the values of fluorescence intensity statistically in the whole range of spectra (250 – 545 nm, step 0.5 nm) between all three examined groups, no significant differences were found between the group of healthy patients and those with a benign tumor, however, a few significant differences were found between the group of patients with malignant tumors and both the healthy women and those with a benign tumor.
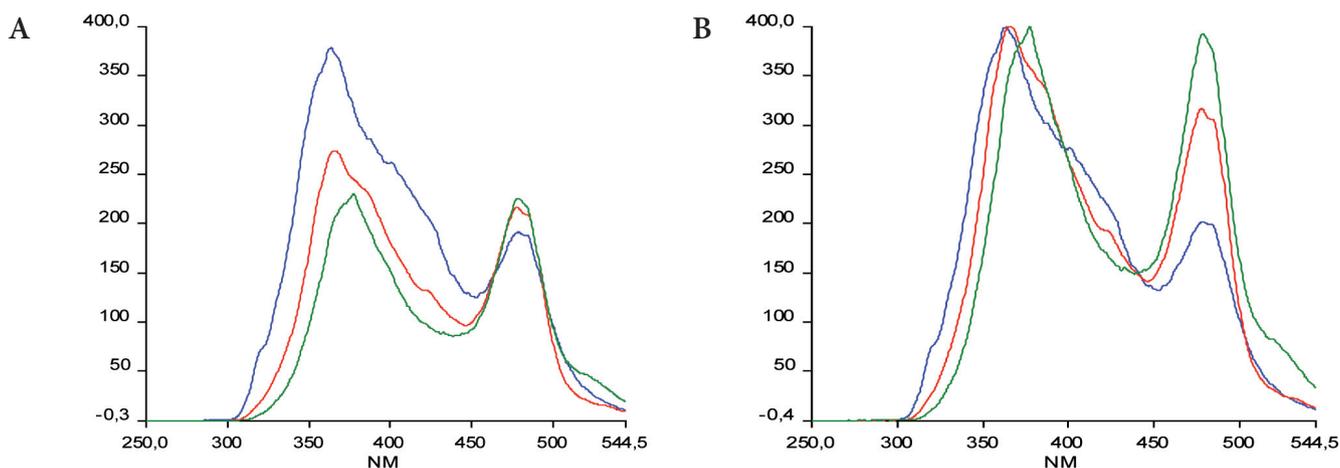
Figure 1. Average synchronous spectra of healthy control group (red lines), patients with benign tumors (blue) and patients with a malignant tumor (green). A) untouched synchronous spectra; B) normalized synchronous spectra.

The first difference was between healthy women and women with a malignant ovarian cancer at 312 – 317 nm (Figure 2A), with significance around p = 0.04 (0.039 – 0.048). The second difference was between groups with malignant and benign tumors, but not when compared with the healthy women in the range at 325 – 360 (p = 0.015 – 0.049). This spectral difference was most expressed at 350 nm (Figure 2B). The third difference appeared in the range 422 – 430 nm (p = 0.030
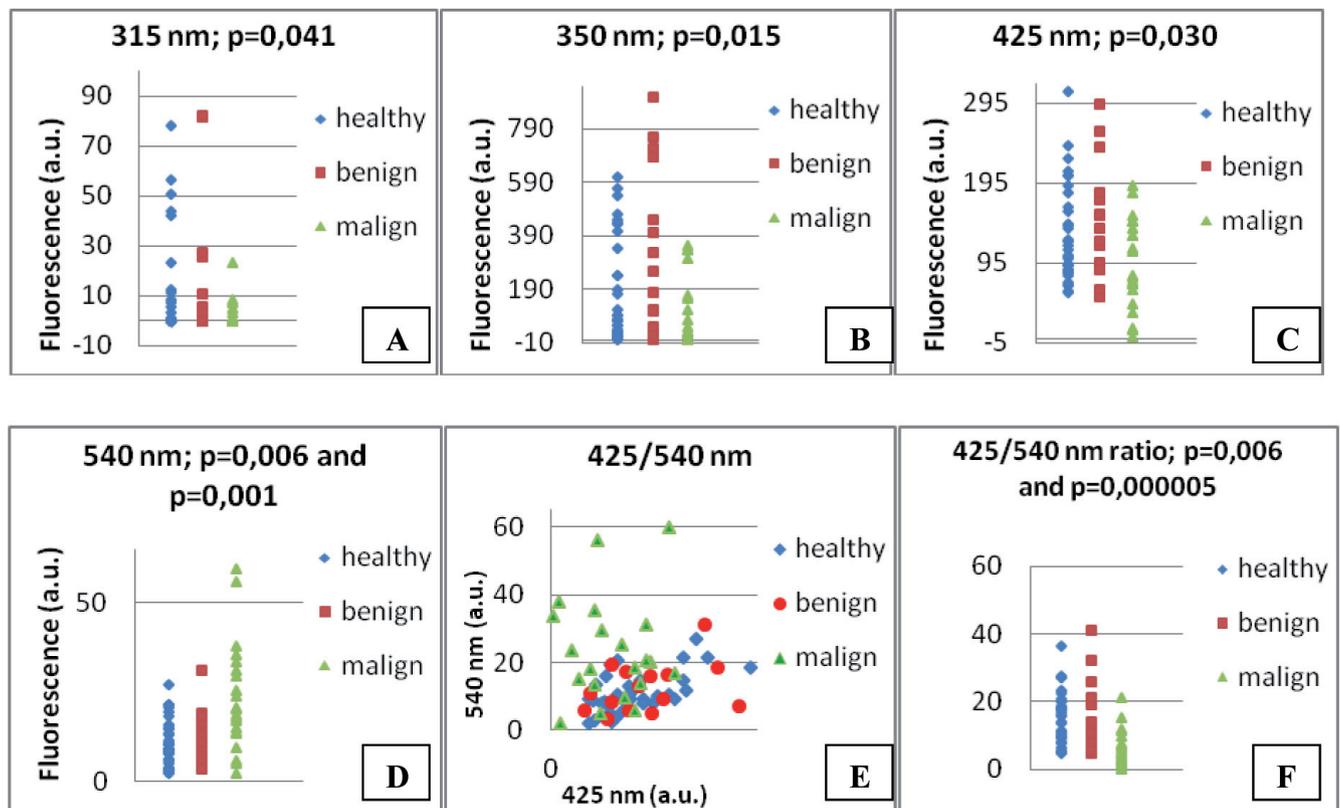

Figure 2. Fluorescence intensities at 315 (A), 350 (B), 425 (C) and 540 nm (D), the relation between 425 and 540 nm (E) and the ratio 425/540 nm values (F)

– 0.048) between the healthy group and the malignant ovarian cancer patients (Figure 2C). The spectra from women suffering from ovarian cancer differed with the highest significance in the part of spectra from 505 – 545 nm. The significant value when comparing healthy vs. malign is p = 0.011 – 0.047 in the range 505 – 509 nm and p = 0.001 – 0.009 in the range 510 – 545 nm. When comparing the benign vs. the malign group, significance was p = 0.006 – 0.049 in the range 525 – 545 nm. For both, the healthy women and those with benign tumors when compared with those having malign tumors the most striking distinction appeared at 540 nm (Figure 2D). The difference between the groups can be improved by calculating the 425/540 nm ratio with values p = 0.006 for benign vs. malign and p = 0.000005 for healthy vs. patients with malign ovarian cancer (Figures 2E and 2F). The results of statistical analysis are shown in Table 3.

Discussion

Until now many markers suitable for ovarian cancer have been reported, but with no satisfaction. There is great interest in decreasing the mortality of ovarian cancer and finding any marker suitable for early diagnostics, the best being when the disease is still in its curative stage (I-II). Only a few pilot studies have described and evaluated the differentiation efficacy between autofluorescence of ovarian tissues under normal, benign and malignant conditions. Both, the sensitivity and specificity they reported, are high and produce a real-time diagnosis. Kamath et al. used pulsed laser excitation at 325 nm to obtain fluorescence spectra in the range from 350–600 nm to discriminate 34 normal, 33 benign and 30 malignant ovarian tissues from 22 subjects, with 90.9 % sensitivity and 100 % specificity [27]. Also, Renkoski et al. reported similar experimental parameters (30 patients, excitation at 325 nm and 8 emission bands from 400–640 nm) to obtain fluorescence spectra, and their ability to discriminate ovarian cancer from normal tissue was with 100 % sensitivity and 51 % specificity [28]. George et al. carried out a study with a wider excita-

tion (270–550 nm) and also emission (290-700 nm) range. The number of patients was higher (49 patients undergoing oophorectomy) and when classifying malignant versus others samples, they achieved 88 % sensitivity and 93 % specificity. They described an increased performance of classifying cancer when the normal group excludes benign neoplasm and endometriosis samples [29]. All of the mentioned studies used sophisticated mathematical analysis for classification of the sample groups. McAlpine et al. reported changes of fallopian tube epithelium autofluorescence detected in tissues *ex vivo* in 47 patients with ovarian cancer or a history of hereditary breast-ovarian cancer, with a calculated sensitivity of 73 % and 83 % specificity. The results were better (87.5 % sensitivity and 92 % specificity) when the values in a subgroup that excluded non-serous histology were determined [30].

Many publications [23, 31, 32] report on the significant changes of flavines and NADH in tissues or body fluids in patients with oncologic disease. In our experiments with undiluted urine autofluorescence, we detected a peak at 540 nm, which was the most important feature in distinguishing with high significance the group of healthy women or those with benign tumors from those with malignant tumors. Some earlier reports exist describing a peak at a similar or the same wavelength in cancer. Dramićanin et al. (2006) similarly described in mammary cancer tissue an unidentified peak in synchronous spectra with the same wavelength difference as we found. Some authors attribute this wavelength specifically to excess flavine fluorescence (ex/em 450/520 – 530 nm). However, in our conditions with synchronous spectra of ΔΛ 30 nm, flavines we have a peak at 478 – 480 nm. As the increased fluorescence at 540 nm rather imitated the tail of the flavine peak or the new peak formation, not increased fluorescence at 478-480 nm, and we evaluated this wavelength as very variable part of spectrum in both healthy and diseased patients, we think that flavines alone do not account for the spectral difference when comparing healthy patients with those having cancer and anticipate another reason [33]. Masilamani et al. also used the synchronous spectra (Δλ 70 nm)

Table 3. Values of fluorescence intensity at 315, 350, 425, 540 nm, and the calculated ratio 425/540 nm

Patient	Parameter	315 nm (a.u.)	350 nm (a.u.)	425 nm (a.u.)	540 nm (a.u.)	425/540 nm
Healthy	Average	9.8	170.5	129.4	10.7	14.9
	SD	19.6	194.6	59.9	6.1	7.6
Benign	Average	40.9	313.4	201.4	12.5	15.32
	SD	103.5	305.7	223.9	7.2	10.6
	T-test healthy vs. benign	0.25	0.10	0.22	0.39	0.90
Malign	Average	2.4	97.4	93.9	23.5	6.1
	SD	5.4	127.3	56.2	15.1	5.4
	T-test healthy vs. malign	**0.04**	0.096	**0.030**	**0.001**	**0.000005**
	T-test benign vs. malign	0.16	0.016	0.079	**0.007**	**0.006**

reported in patients with cancer, with a typical red shift of the peak of 20 nm from 510 to 530 nm. They found an increased peak at 530 nm and a decreased peak at 515 nm, which the authors attributed to excessive concentration of flavines leading to self-quenching of original 515 nm peak. However, there is no self quenching of excess of flavines concentration in urine at our conditions (data not presented), and we detected an increased fluorescence shifted 60 nm from the flavines peak (from 480 to 540 nm), we therefore think that another change is responsible for the increasing of fluorescence at 540 nm, as do Masilamani et al. [23]. To our knowledge, Lu et al. were first who tested urine in ovarian cancer with a similar finding who considered the interplay of coproporphyrin, riboflavin and p-hydroxyphenol derivatives existing in urine [34]. Zvarík et al. used excitation-emission matrices of undiluted urine in ovarian cancer screening. They revealed significant differences (p < 0.001) between ovarian cancer patients and healthy donors at the emission wavelength from 400 nm to 460 nm when excited at 310 – 390 nm [35]. Norgaard et al. highlighted the importance of the fact, that results from fluorescent fingerprints should be interpreted as "un block", not only by considering one or a few concrete fluorophores, but also in relation to the environment in which the fluorescent compounds exist [36].

As urine is a very complex fluid, and since ovarian cancer is specific in its heterogeneous origin, we are not surprised that few changes in urine spectra were found in diseased patients compared with healthy women. There are many fluorophores present in urine which may possibly be associated with cancer, like molecules linked to oxidative stress and aging or immune response, for instance, or advanced glycation end-products, pterines, various tryptophan derivatives or certain proteins like collagen or elastin. Many of them are multifunctional in human organism and can accompany different health situations, not only cancer disease. In addition, we must not forget that previously unknown substances could also be present in urine in such a condition. Compounds responsible for changes in fluorescent spectra can be estimated only be the fluorescent fingerprints of urine, but this can be a very useful tool for detection of changes between groups, as our results show.

We evaluate as very interesting the area around 350 nm, where statistics show a significant difference between benign vs. malign groups, but not between healthy vs. malign or benign vs. healthy. This could be caused by a combination of the already mentioned incidental peaks and the small patient number, which is the greatest disadvantage of this study. Another is the fact, that the group with malignant ovarian cancer consisted mostly of women in stage III (N = 5) and IV (N = 9) and in three patients the stage could not be established. Only one patient was in stage I and three patients were with borderline tumors. If the fluorescent fingerprints of urine can help with screening for ovarian cancer, it is necessary to test more patients with ovarian cancer in the early stage, which is not an easy task, as the diagnostic capacity at this early stage are very poor. Also, the fact that we often detected unidentified peak excesses and tested such a small number of patients could have a significant impact on the results of the statistical analysis, it is therefore desirable to exclude from analysis certain factors on the fluorescence of urine, such as diet or pharmacotherapy influence, by advanced patient preparation and guidance before urine collection.

Conclusion

This small study was oriented on possible urine utilization as a biological material suitable as non–invasive screening procedure for ovarian cancer by a cost – effective and very simply executable autofluorescence measurement. Fluorescence is a very sensitive and suitable method for detecting unexpected changes, which can be of various origin. Significant or highly significant differences were detected in several areas of the spectrum between patients with malignant ovarian cancer and healthy women (at 315, 425 and 540 nm) or those with benign histology (350 and 540 nm). The statistical analysis of fluorescence intensities also showed no difference between the healthy group and women with benign ovarian tumors in the entire measured spectrum range. In further research there is a need to solve some problems, like enlarging the tested sample number with an emphasis on the early stage of ovarian cancer or better preparation of patients before urine collection. Based on results obtained in these measurements we can say there is some potential and perspective to using the fluorescent fingerprints of urine as a helpful method for ovarian cancer screening.

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