

Differential Rapid Diagnosis of Endometrial Cancer and Its Benign Pathological Conditions Using Surface-Enhanced Raman Spectroscopy

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Abstract. The purpose of this study is to improve the efficiency of early diagnosis of endometrial cancer using the analysis of surface-enhanced Raman scattering (SERS) of blood plasma. Blood plasma of patients aged 22 to 79 years was investigated. The study included 95 women. All patients were divided into 3 groups: group 1 consists of 29 women with endometrial adenocarcinoma, group 2 – 31 patients with endometrial polyp, group 3 – 10 women with endometrial hyperplasia. A control group consisted of 25 healthy women. The SERS spectra of dried samples were studied on an experimental stand consisting of a Photon-Bio RL785 spectrometric system based on a charge-coupled device (CCD) detector and a laser radiation source with a wavelength of 785 nm and an ADF U300 microscope. In order to realize the effect of surface enhancement of the Raman signal from blood plasma, we used a silver substrate based on a dried silver colloid. In the result of the study, the spectral features and specific features which characteristic of adenocarcinoma, polyps and endometrial hyperplasia were determined. With the use of discriminant analysis by projection onto latent structures (PLS-DA) method, the accuracy of optical diagnostics of endometrial adenocarcinoma relative to the control group and endometrial hyperplasia for the calibration and verification sets of spectra was 87% and 85%, respectively. Class discrimination accuracy of the control group with respect to endometrial hyperplasia and adenocarcinoma was 85%, and endometrial hyperplasia relative to the control group and endometrial adenocarcinoma was 81% for the verification set of spectra. The study shows the possibility of using SERS for differential express diagnostics of endometrial cancer and its benign pathological conditions. © 2024 Journal of Biomedical Photonics & Engineering.

Keywords: blood plasma; polyp; hyperplasia; adenocarcinoma; surface-enhanced Raman spectroscopy; SERS; PLS-DA; projection onto latent structures; discriminant analysis.

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1 Introduction

The key step in the diagnosis of diseases of any organs of the female reproductive system, such as inflammatory, dystrophic, borderline, tumor and other diseases associated with tissue changes, is a histological (pathomorphological) study. The true correspondence to the diagnosis and the legitimacy of the conclusion depends on the subjective opinion and decision of the pathologist and are determined by the level, knowledge, work experience, etc. Clinical cases are often complex, controversial, questionable and cause difficulties in making a diagnosis, which requires third-party consultation by higher-level specialists. However, the fate of the patient often depends entirely on the pathologist.

The presence of a highly accurate and at the same time independent of the expert method of pathomorphological diagnostics, such as “optical biopsy” of tissues, would allow to avoid many difficulties and correctly determine the patient management plan.

Benign endometrial diseases and benign diseases of the uterus (endometrial polyps (PE), endometrial hyperplasia (HE)) are currently one of the most common pathologies among patients with gynecological diseases [1]. Often, they are considered as a background for further cell transformation, which is the beginning of an atypical process. Diagnosis of PE and HE is important for women planning pregnancy both naturally and in assisted reproductive technology (ART) programs. As a rule, obstetrician-gynecologists cure PE, HE before pregnancy. Patients with intrauterine pathology are not allowed to the ART program. Resection of PE, HE by curettage is accompanied by severe trauma to the endometrium, followed by a potential inflammatory process and a decrease in the structural and functional potential of the endometrium. Polypectomy under hysteroscopic guidance, dilation and curettage for diagnostic HE is accompanied by severe trauma to the endometrium, followed by a potential inflammatory process and a decrease in the structural and functional potential of the endometrium.

Among benign diseases of the endometrium, the most common pathology is PE: 21.7–27.3%. According to Ref. [2], PE are rare at a young age, but their number doubles to 45–53.8% during perimenopause and postmenopause. According to Ref. [3], the incidence of malignant PE is 3.57%. Research results show that the risk of malignancy is higher in postmenopausal patients and in patients with abnormal uterine bleeding [3]. HE without atypia is detected in 4–10% of patients and tends to recur [4]. According to Ref. [5], the overall incidence of HE was 144 cases per 100,000 women. At the same time, the incidence of HE without atypia most often occurs in patients aged 50 to 54 years (from 142 to 213 per 100,000 women), and HE with atypia most often occurs in patients aged 60 to 64 years (56 per 100,000 women) [6]. According to Kaprin et al. endometrial cancer ranks first among tumors of the female reproductive system from 2010 to 2020, and the incidence of endometrial cancer in the Russian

Federation increased by 28.79% [7]. It should be noted that cancer of the body of the uterus is on the 3rd place in terms of frequency of occurrence among all cancers in the female population and is 8.0% [7]. The peak incidence occurs at the age of 60 to 70 years, but according to the latest data, cancer of the uterine body “gets younger” and in 2.0–5.0% of cases is detected in patients younger than 40 years [8].

Pathological conditions of the endometrium often occur without any specific clinical picture, which makes it difficult to diagnose these conditions. The gold standard for diagnosing benign endometrial diseases is still hysteroscopy with Dilatation and Curettage (D&C) and subsequent histological examination. This diagnostic manipulation is highly invasive, while the degree of tissue traumatization largely depends on the qualification of the doctor. The quality of the histological conclusion also largely depends on the human factor, namely the volume of the endometrial tissue sample and the subjectivity of the assessment of histological specimens.

Discrepancies in histological findings occur even among expert-level pathologists, and immunohistochemical studies are expensive and not always possible [9]. According to Polyakova et al. 7% of the conclusions obtained as a result of diagnostic dilation and curettage are not informative, and a discrepancy in diagnoses occurs in 30.6–71.9% of patients [10]. Based on the fact that diagnostic curettage of the uterine cavity has insufficient diagnostic value, is highly invasive and associated with intra- and/or postoperative complications, experts recommend avoiding this diagnostic method in patients of reproductive age.

Thus, it is an expedient task to develop a new highly efficient and high-precision, minimally invasive and economically low-cost diagnostic method for early and preclinical differential diagnosis of cancer and benign diseases of the endometrium. This study was carried out jointly with experts from physical laboratories and subdivisions of the Institute of Solid State Physics RAS and Samara University. For the differential diagnosis of endometrial pathologies, we analyzed the surface-enhanced Raman scattering (SERS) spectra of the blood plasma of patients with various endometrial diseases and the comparison group. A multidimensional analysis of a large array of spectral data was performed using the method of the projections to latent structures discriminant analysis. It should be noted that the proposed diagnostic method is inexpensive, fast, highly accurate and minimally invasive.

There are studies showing the possibility of using volumetric and SERS for the diagnosis of malignant tumors [11–13]. In 2015, using Raman spectroscopy, a group of volunteers with an intact bladder and a group of patients with a confirmed diagnosis of bladder cancer were compared, the classification accuracy was

96% [14]. In 2021, Artemyev et al. conducted an analysis of prostate tissue with the allocation of two groups: benign hyperplasia and prostate cancer. The classification accuracy was from 80% to 100% [15]. Currently, a small number of studies have been published

that provide data on the study of endometrial tissues using Raman spectroscopy [16]. In the studies, the authors used laser radiation with a wavelength of 1064 nm. In this study, a laser source with a wavelength of 785 nm was chosen to study blood plasma. Patients were divided into 3 groups: 25 blood plasma samples in the comparison group, 29 samples of adenocarcinoma and 41 samples of benign endometrial diseases (31 blood plasma samples from patients with PE and 10 samples of HE). In previously published studies, the described diagnostic method was not used in the study of such nosological forms.

2 Materials and Methods

2.1 SERS Spectral Acquisition

Raman spectroscopy is a widely discussed and promising method of tissue morphological diagnostics. This type of optical spectroscopy is based on the interaction of laser radiation with matter and the detection of the optical response from matter in the form of Raman light scattering using matrix photodetectors. During the interaction of laser radiation and matter, spectral components appear in the spectrum of scattered light that are shifted relative to the frequency of the exciting laser radiation by the corresponding frequencies of intramolecular vibrations. These frequency shifts appear in the spectrum and unambiguously determine the chemical structure of the substance.

Registration and analysis of the results of SERS of blood plasma were carried out using silver nanoparticles and a microscopic system. The experimental stand diagram is shown in Fig. 1.

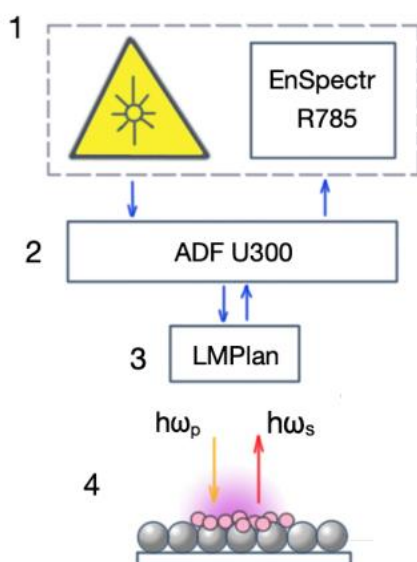


Fig. 1 Experimental setup: 1 – spectrometric system with laser module (785 nm central wavelength); 2 – microscope; 3 – objective; 4 – plasma sample on the silver substrate.

The study was made of the micro-Raman spectral characteristics with surface enhancement for plasma samples. The analysis of the spectral characteristics of the analyzed SERS substrate was carried out on an experimental stand consisting of a spectrometric system RL785 (LLC FOTON-BIO, Russia) based on a charge-coupled device (CCD) detector and a laser source with a wavelength of 785 nm and an ADF U300 microscope (ADF, China). An LMPlan objective with a magnification of 50× was used to focus the radiation on the sample and collect scattered radiation. The laser spot diameter at the focus was 5 μm. The spectra were recorded in the spectral range 380–1800 cm⁻¹ with a spectral resolution of 6–8 cm⁻¹. The limit of the permissible relative mean square deviation of wavenumber measurements was no more than 1%. The laser radiation power was 20 mW. The parameters for recording the spectra were as follows: the exposure time was 2 s, the number of averagings was 10. The spectra were recorded using the EnSpectr program. Immediately before recording the spectral characteristics of the studied plasma sample, a preliminary recording of the ambient background signal was made. After that, the background component was automatically subtracted from subsequent recorded spectra of the sample.

2.2 Colloidal Silver Nanoparticles Solution

A silver substrate based on a dried solution of colloidal silver was used as a substrate material to achieve the effect of SERS of the plasma [17]. A colloidal solution of silver was obtained by reduction from an aqueous solution of silver nitrate with sodium citrate at a temperature of 95 °C for 20 min. The absorption spectrum of the obtained colloidal silver solution has an absorption maximum at 410 nm with a half-width of 40 nm, which, according to a number of experimental studies of the dependence of the absorption spectrum on the geometric characteristics of silver nanoparticles, corresponds to spherical nanoparticles with a diameter of 30–40 nm. To form more complex and large structures, the resulting colloidal solution with a volume of 20 ml is applied to aluminum foil with an area of 75 mm × 25 mm and dried at room temperature until completely dry. As a result of drying, agglomerates of silver particles are formed on the foil. The resulting substrate is agglomerates of spherical particles with a size of about 200 nm.

The characteristics of the substrate are presented in more detail in Ref. [17]. It should be emphasized that an important feature of the approach to spectral analysis used is the insignificant spectral contribution of the silver substrate to the spectrum of the tested sample, which simplifies the subsequent analysis due to the absence of the need to take into account the substrate spectral contribution in the analysis. Integration of spectral characteristics from a sample region of several microns within 2 s provides a number of scattering events sufficient to level out blinking acts. Analysis of substrate stability demonstrated that the blinking effect is not

significant, the standard deviation of the sample spectral characteristics on a substrate is no more than 8% [17].

2.3 Plasma Samples Preparation

Not all available samples were included in spectral studies, since many of them were not intended for this due to blood hemolysis (violation of the biomaterial sampling technique and individual characteristics of patients' hemostasis), small sample volume, or thick consistency. As a result, the following groups were formed for the SERS analysis of blood plasma: the control group (25 blood plasma samples), PE (31 plasma samples) and HE (10 samples), which were combined into one group, and endometrial adenocarcinoma (29 plasma samples).

Spectral studies included 95 blood plasma samples taken from patients aged 22 to 79 years. All women gave written informed consent to participate in the study. To confirm the diagnosis, a histological examination of endometrial tissues was performed. These tissues were obtained during dilation and curettage, or tissue excision was performed immediately after hysterectomy. In the control group, we included 25 patients aged 22 to 40 years without clinical and laboratory signs of endometrial pathology. Histological confirmation of the absence of endometrial pathology became possible because of diagnostic dilation and curettage due to a cervical canal polyp in 19 patients (76%) and primary infertility in 6 patients (24%). In the second group, we included 10 patients aged 28 to 63 years with a histologically confirmed diagnosis of HE and 31 patients aged 28 to 63 years with a histologically confirmed diagnosis of an PE. Among these patients, 12 (29%) patients came to the gynecologist with complaints of abnormal uterine bleeding, and in 29 (71%) patients, endometrial pathology was suspected according to the planned ultrasound of the pelvic organs. Pipelle endometrial sampling in an outpatient setting was performed in 10 patients, but histological confirmation of the presence of endometrial pathology was obtained only in 3 (30%). The third group included 29 patients aged 51 to 79 years with a confirmed diagnosis of highly differentiated endometrial adenocarcinoma, all women were postmenopausal. Ten (34%) patients were diagnosed with cancer *in situ*, and 19 (66%) patients had a tumor that invaded less than half the thickness of the myometrium (T1a). Twenty two (76%) patients complained of abnormal uterine bleeding, and in 7 (24%) patients, endometrial pathology was suspected during a planned ultrasound of the pelvic organs. It is worth noting that 15 out of 19 patients (79%) with tumor invasion into the myometrium complained of abnormal uterine bleeding. Patients included in the study groups did not take hormonal drugs for 12 months, they were not diagnosed with acute infectious diseases and exacerbations of chronic somatic diseases.

Before the introduction of intravenous anesthesia, blood in a volume of 4 ml was taken into centrifuge vacuum tubes for hematological studies with EDTA-K2. Within 30 min after sampling the biological fluid, blood

plasma was separated by centrifugation at 3500 rpm for 5 min. The resulting plasma in a volume of 1 ml was transferred into a sterile test tube without filler using an automatic dispenser and frozen at a temperature of -18°C . Subsequently, three Raman scattering spectra were recorded for each sample.

2.4 Spectra Processing

The main method for improving the performance of statistical models is the optimal choice of a strategy for preprocessing spectral data. The task of preprocessing is to isolate a component of value for further analysis by eliminating third-party components, noise, correcting baseline, and normalizing the signal [18]. Therefore, before applying the multivariate analysis, the recorded spectral data of the plasma were freed from noise by the Savitsky-Golay method [18], and from the background radiation by correcting the reference line and baseline using the baseline correction with asymmetric least squares (baseline ALS) method [19].

Preprocessed blood plasma spectra, characterized by multidimensionality and multicollinearity, were analyzed using the method of discriminant analysis by projection onto latent structures (PLS-DA). The combination of PLS-based multivariate analysis and SERS is a powerful analytical tool and demonstrates the stability and promise of human plasma and serum analysis to discriminate patients by typhoid stage [20], to classify patients by the presence of the absence of colorectal cancer and precancerous neoplasms [21], to discriminate patients according to the severity of renal failure [22]. The PLS-DA method is a common approach for the chemometric analysis of spectral data from serum and plasma.

In this study, each plasma sample corresponds to a priori information about belonging to a particular group. Therefore, the data was analyzed through supervised learning. Based on the recorded intensity of the Raman spectra, a matrix was compiled, where each sample was assigned a value of 1, 2 or 3, depending on the class: comparison group (1), HE and PE (2) or endometrial adenocarcinoma (3). The application of the SIMPLS [23] algorithm makes it possible to calculate the PLS factors directly as linear combinations of the original variables. The PLS factors are defined in such a way as to maximize the covariance criterion, subject to certain orthogonality and normalization constraints. To avoid overfitting, the assessment of the stability of the experimental data analysis method and the choice of optimal parameters were implemented on the basis of k-fold cross-validation ($k = 7$). Cross-validation is a widely used technique for evaluating the quality of a model when analyzing spectral data. The general procedure is to split the data into subsets for training and testing. Training is the process of fitting a model, and testing is the process of checking the fitted model by measuring the prediction error. The training and test sets do not overlap, so the test data for model evaluation is not used in model fitting.

In order to avoid overestimation of any of the studied groups by the model, it is necessary to provide an

analysis of equally sized samples. To do this, 21 spectra were randomly selected from the total data set for each analyzed group. Based on the selected data, a classification model was built. The process of selecting samples of equal size and then obtaining a model was thus repeated 5 times. The final characteristic of the classification was obtained on the basis of averaging the results for 5 models. Sensitivity (Eq. (1)), specificity (Eq. (2)), accuracy (Eq. (3)), and error curve (ROC curve) [24] were used as parameters characterizing the resulting classification model.

$$\text{Sensitivity} = \frac{TP}{TP+FN}, \quad (1)$$

$$\text{Specificity} = \frac{TN}{TN+FP}, \quad (2)$$

$$\text{Accuracy} = \frac{TP+TN}{TP+TN+FP+FN}, \quad (3)$$

where TP – the number of true positive results, FP – the number of false positive results, TN – the number of true negative results, FN – the number of false negative results.

The PLS-DA method made it possible to reveal the spectral features of the classes associated with the presence of compounds and molecules determined as markers of endometrial diseases. All preprocessing methods and multivariate analysis were implemented in the RStudio (version 2023.03.0+386 for Windows).

Raman peaks belonging to certain classes of substances were determined using the database of Raman spectra of biological tissues [25–27].

3 Results and Discussion

During the study, spectral patterns were obtained for the control group in comparison with the HE and PE group, and the endometrial adenocarcinoma group (Fig. 2).

When studying the averaged spectra of SERS of blood plasma, the following spectral bands were identified: 596 cm^{-1} (phosphatidylinositol), 640 cm^{-1} (C-S stretching and C-C twisting in tyrosine), 726 cm^{-1} (C-S vibrations in proteins, CH_2 , adenine, hypoxanthine, coenzyme A), 766 cm^{-1} (compression of the pyrimidine ring), 815 cm^{-1} (C-C-O stretching vibrations, L-serine, glutathione, proline, hydroxyproline, tyrosine, PO_2 stretching in nucleic acids), 891 cm^{-1} (C-O-H bending vibrations, saccharide, glutathione, D-galactosamine), 933 cm^{-1} (proline, hydroxyproline), 956 cm^{-1} (stretching vibrations C-C, alpha helix, proline, valine), 1527 cm^{-1} (carotenoid), 1004 cm^{-1} (phenylalanine), 1055 cm^{-1} (C-C lipid stretch, collagen), 1132 cm^{-1} (tyrosine, D-mannose), 1208 cm^{-1} , 1265 cm^{-1} , and 1276 cm^{-1} (amide III, L-tryptophan, phenylalanine), 1314 cm^{-1} (CH_3 , CH_2 twisting in collagen, stretching vibrations, stretching vibrations of CH bases of nucleic acids), 1477 cm^{-1} (CH_2 , collagen, phospholipids, deoxyribose), 1617 cm^{-1} (tryptophan, phenylalanine, tyrosine).

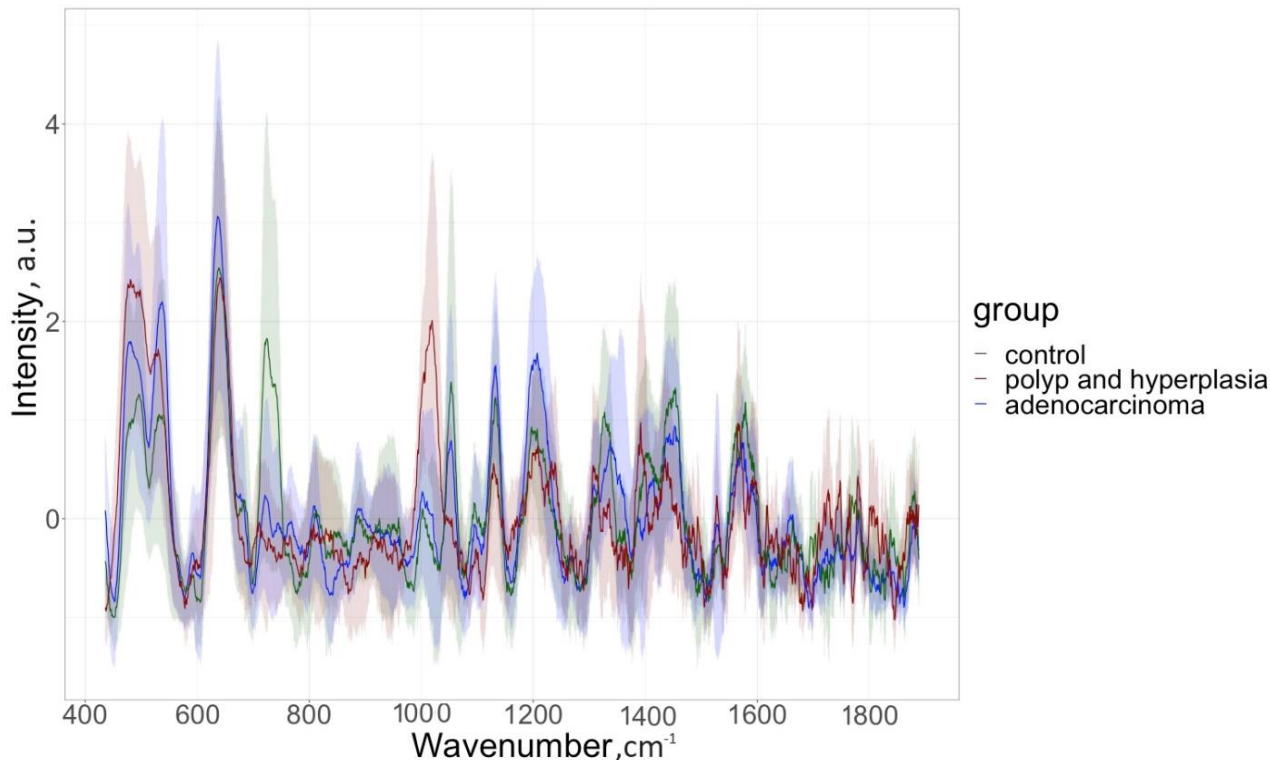


Fig. 2 Average processed SERS spectra of blood plasma from samples with endometrial hyperplasia and polyp, and endometrial adenocarcinoma in comparison with the control group. Filling with color indicates standard deviation.

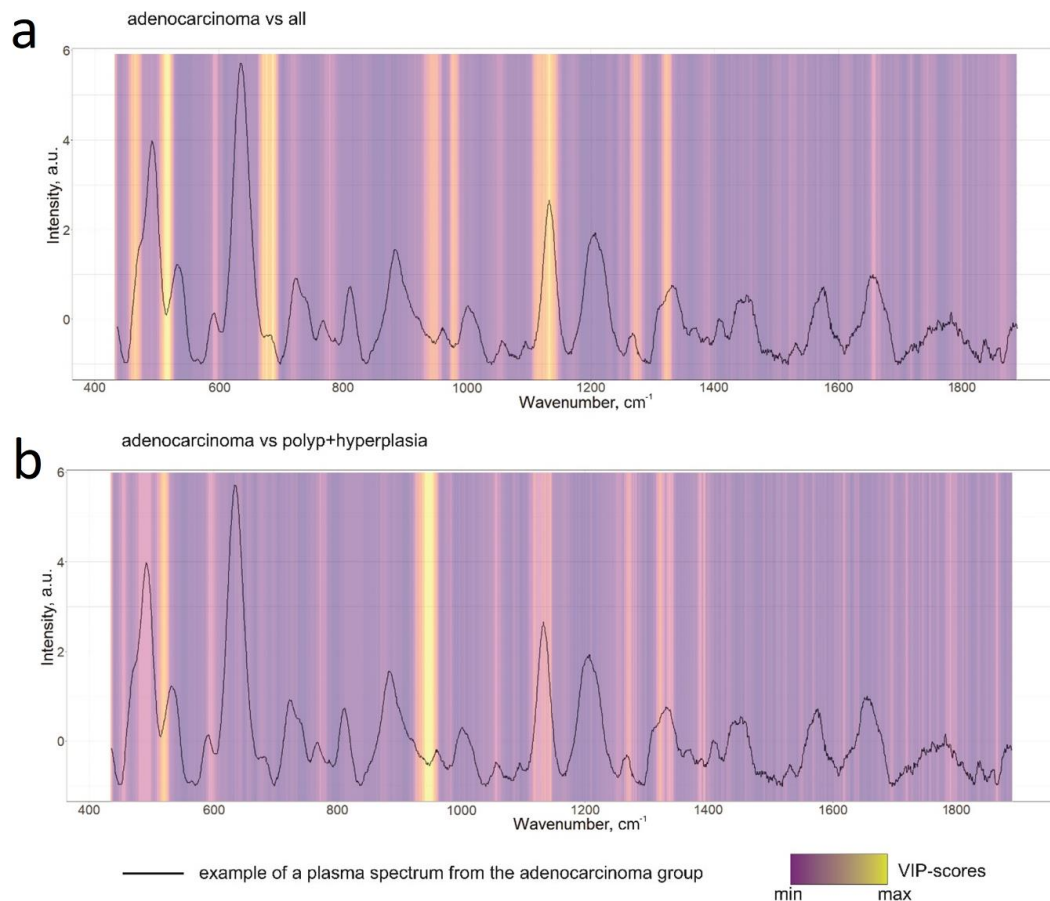


Fig. 3 The distribution of the variables importance for projection surface-enhanced Raman spectra of blood plasma for classifying samples according to a pathologically associated feature when implementing methods for analyzing experimental data based on PLS-DA. (a) Classes: control group, hyperplasia, adenocarcinoma, (b) classes: hyperplasia, adenocarcinoma.

Common spectral bands for PE and HE groups: 858 cm^{-1} (tyrosine, collagen), 917 cm^{-1} , and 974 cm^{-1} (stretching vibrations C-C: alpha helix, proline, valine), 1080 cm^{-1} (tensile vibrations C-N: collagen; phosphate vibrations: phosphodiester groups in nucleic acids) [28–32]. The band at 1242 cm^{-1} belongs to the group with PE; it is characteristic of the amide III vibration; for the other two groups, the band is absent. The band at 1418 cm^{-1} is the CH_2 oscillation [33] in lipids; it is typical for the group with polyps, for the control group it is shifted to 1416 cm^{-1} , and it is absent for the group with hyperplasia. The studied groups can also be detected not only by the shift of the bands, but also by the intensity, the most revealing were the bands at 465 cm^{-1} , 536 cm^{-1} , 816 cm^{-1} , 996 cm^{-1} , 1055 cm^{-1} , 1132 cm^{-1} , and 1208 cm^{-1} .

The distribution of the importance of variable SERS spectra of plasma (Fig. 3) when classifying samples according to a pathologically associated trait provides an opportunity to separate groups according to the most significant bands.

We have identified significant Raman peaks such as 724 cm^{-1} (C-H bending vibrations, adenine), 740 cm^{-1} (thymine, uracil), 1015 cm^{-1} (phenyl ring vibrations), 1055 cm^{-1} (proline), 1132 cm^{-1} (D-mannose), 1211 cm^{-1}

(phenyl ring vibrations), 1337 cm^{-1} (CH_2 vibrations of proline, purine bases of DNA), 1391 cm^{-1} (tryptophan), 1453 cm^{-1} (CH_2 bend in proteins), 1580 cm^{-1} (pyrimidine ring and hemoglobin proteins). The Raman peak at 724 cm^{-1} corresponds to the C-H bending vibrations of adenine, and the 740 cm^{-1} peak corresponds to nitrogenous bases such as thymine and uracil. According to studies, changes in the intensity of these peaks indicate an abnormal metabolism of DNA and RNA bases in blood serum [34]. Changes in the intensity of the 1015 cm^{-1} peak were also detected, these changes are of the greatest importance for the selection of a class of polyps, while the averaged spectra show an increase in the intensity of Raman scattering for polyps in this band. This may be related to the angular vibrations of the phenyl ring due to the higher content of phenylalanine [35]. A more intense value of the Raman peak at 1132 cm^{-1} , which indicates the content of D-mannose in the blood serum, may be associated with a violation of the processes of glycolysis and oxidation of pyruvic acid (Krebs cycle) [36]. The peak at 1211 cm^{-1} , as well as the peak at 1015 cm^{-1} , is probably associated with vibrations of the phenyl ring due to the higher content of phenylalanine.

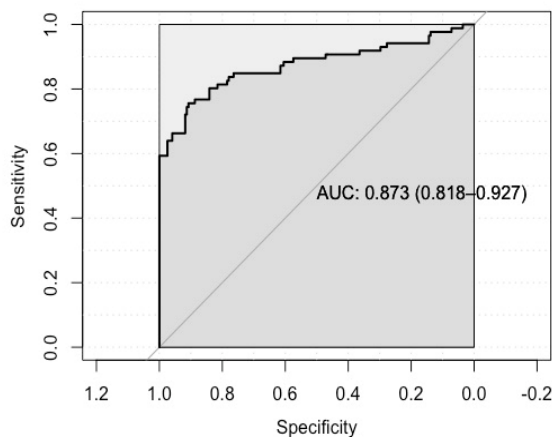


Fig. 4 The ROC curve of differentiation of endometrial adenocarcinoma relative to the control group and the group of HE+PE.

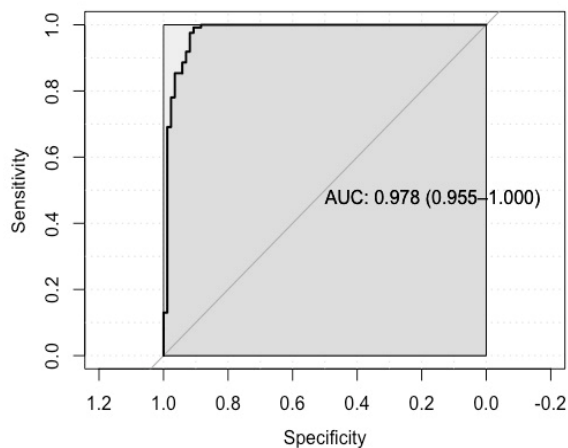


Fig. 5 The ROC curve of differentiation of endometrial adenocarcinoma relative to the group of HE+PE (without a control group).

As a result of building an analytical model using the PLS-DA method to further evaluate the effectiveness of the PLS-LDA classification model, ROC curves were obtained and differentiation was made between samples with adenocarcinoma and the other two groups – the control group and the hyperplasia group, which included PE (Fig. 4). The accuracy of differentiation of endometrial adenocarcinoma relative to the control group and the group of HE and PE for the calibration and validation datasets was 87% and 85%, respectively (66% sensitivity, 92% specificity for the validation set). The classification accuracy of the control group relative to the HE and PE group and the endometrial adenocarcinoma group was 86% and 85%, and the HE and PE group relative to the control group and the endometrial adenocarcinoma group was 81% for the calibration and validation datasets. At the same time, the accuracy of class discrimination of adenocarcinoma and HE (including PE) was 93% for the calibration data set (96% sensitivity, 90% specificity) and 91% for the

validation data set (93% sensitivity, 88% specificity) (see Fig. 5).

In 2020, a group of scientists conducted a study of endometrial pathologies using attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR). The study involved 652 women who were divided into 3 groups: endometrial cancer (342 samples), HE with atypical HE (68 samples), control (242 samples). The sensitivity and specificity of endometrial cancer detection by this method was 87% and 78%, respectively [37]. Based on the results of our study, we believe that the use of SERS is a more promising method for diagnosing endometrial cancer. Our findings are supported by a 2021 study in which endometrial structure was assessed using Fourier transform Raman spectroscopy and ATR-FTIR. The authors also concluded that Raman spectroscopy is more effective than FTIR in assessing the development of carcinogenesis in endometrial cancer [16].

Our research shows the ability to detect diseases and the potential of the SERS method to diagnose specific types of endometrial pathologies. In this study, the laser wavelength of 785 nm is optimal for the following reasons: (1) lower fluorescence from the samples and the SERS substrate (under potential excitation); (2) higher intensity of Raman scattering compared to the use of laser radiation with a wavelength of 1064 nm; (3) the possibility of using a cheaper silicon detector for 785 nm radiation, in contrast more expensive InGaAs detectors using laser radiation with a wavelength of 1064 nm. Due to the complex composition of blood, it is very difficult to distinguish the molecular species contributing to the overall SERS spectra, but there are certain cancer-associated metabolites that cause differences in the observed spectra of adenocarcinoma compared with normal tissue and hyperplasia. And SERS allows us to increase the signal-to-noise ratio of spectra, which leads to a reduction in registration time and an increase in the reliability of registration of spectral features.

4 Conclusion

In the present study, we have shown the potential value of using Raman spectroscopy in clinical practice for the differential diagnosis of benign and malignant pathologies of the endometrium. A literature review confirmed the absence of similar studies. The use of laser radiation with a wavelength of 785 nm in combination with SERS of blood plasma using a silver substrate showed a higher diagnostic efficiency.

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Disclosures

The authors declare that they have no conflict of interest.

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