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SENSITIZED FLUORESCENCE SPECTROSCOPY AS AN INVESTIGATION METHOD FOR PRE-MALIGNANT CERVICAL LESIONS DETECTION *IN VIVO*

Summary of Doctoral Dissertation

Biomedical Sciences, Medicine (06B)

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The study was carried out and doctoral dissertation was prepared during 2010-2014 at the National Cancer Institute (previous Institute of Oncology, Vilnius University).

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ABBREVIATIONS

5- ALA - 5–aminolevulinic acid

ASC-H - atypical squamuos cell suggestion for high grade squamous intraepithelial lesion

CIN 1 - I grade cervical intraepithelial neoplasia

CIN 2 - II grade cervical intraepithelial neoplasia

CIN 3 - III grade cervical intraepithelial neoplasia

CI - confidence interval

HSIL - high grade squamous intraepithelial lesions

HR - high risk cervical changes

LR - low risk cervical changes

NPV - negative prognostic value

n - number

PpIX - protoporphyrin IX

ROC - receiving operating curve

SFS - sensitized fluorescence spectroscopy

PPV - positive prognostic value

1. INTRODUCTION

Worldwide, cervical cancer is the third most common cancer among women, with an age-standardized incidence rate of 15.3 per 100,000 and mortality rate of 7.8 per 100,000 [Ferlay J. et al., 2008]. Cervical cancer mostly affects younger women aged 35–50 [Arbyn M et al., 2010]. In 2011, 452 new cases of cervical cancer were reported in Lithuania, which is one of the highest rates of morbidity for cervical cancer rate among the Baltic and Nordic countries [Lithuanian Cancer Registry; Arbyn M et al., 2011].

Cervical intraepithelial neoplasia (CIN) is the potentially premalignant transformation and abnormal growth of epithelial cells on the surface of the cervix. A premalignant lesion is always at a risk of malignant transformation if stimulated by certain exogenous factors or conditions. The main factor directly related to CIN development is persistent human papillomavirus infection; mainly with high-risk types 16, 18, 31 and 33 [Wheeler CM. et al., 2014; Nair S. et al., 2005]. If CIN is diagnosed at the appropriate time before cervical cancer manifestation, it may be cured and cervical cancer avoided [Singer A. et al., 2008; Tan JHJ. et al., 2011].

The main disadvantages of contemporary CIN detection methods are high false-negative rates and low sensitivity of cytology and low specificity of colposcopy. Some studies showed that, in most cases, patients with high-grade smear results and high-grade impression on colposcopy have an acceptable overtreatment rate [Bosgraaf RP. et al., 2013; Aue-Aungkul A. et al., 2011]. However conventional colposcopy demands long-term training and achieves an average ~48% (23–87%) specificity and has unsatisfactory accuracy even in trained hands [Singer A. et al., 2008; Palma PD. et al. 2008]. The low positive predictive value seen with conventional colposcopy results in unwarranted surgical procedures and an additional burden on cervical cancer screening programs. These data suggest that there is a need for new diagnostic methods to improve or replace colposcopy for a clearer CIN diagnosis and more individual approach.

Recently, there has been increased interest in optical biopsy to determine pathological diagnoses in various organs. Optical biopsy refers to any technique that uses the interaction of light and tissue to provide information about tissue morphology without

the need for excision [Alfano RR. 2011; Costas B. 2009; Rotomskis R. et al. 2008]. Premalignant and malignant tissue differs from healthy tissue in its morphology and cell growth rate, which results in altered optical characteristics [Costas B., 2009; Brown JQ. et al., 2009; Chang VT. et al., 2009;]. Most of the optical methods used in diagnostics are based on different types of spectroscopy: fluorescence, near infrared, Raman, diffuse reflectance, and similar techniques [Quan L. 2011; Badizadegan K. et al., 2004; Jerjes WK. et al., 2011]. However, the most widely used techniques in clinical practice are based on fluorescence [Sieroń A. et al., 2013; Moghissi K. et al., 2008]. When light interacts with the molecules in the tissue they become excited and may re-emit light of different colors (fluorescence). The fluorescence of the tissue can be traced by sensitive spectrometers and provide characteristic spectra that reflect carcinogenicity of the tissue [Badizadegan K. et al., 2004; Quan L., 2011; Moghissi K. et al., 2008]. The acceptance and suitability of these methods clinically are determined by the diagnostic effectiveness, simplicity and low cost of the procedures. Moreover, they are noninvasive and can be repeated many times.

The potential of the fluorescence method to identify normal and pathological tissues of the uterine cervix was raised in 1994 [Ramanujam N. et al., 1994; Ramanujam N. et al., 1996]. The possibility was investigated of autofluorescence being used for detection of cervical neoplasia. Despite the demonstrated diagnostic possibilities, tissue spectra vary both among patients and within each individual patient. That complicates application of the method, and it is difficult to maintain the exact measurement conditions that could additionally influence the intensity and spectral variations [Ramanujam N. et al., 1996]. The developed algorithms partially resolve this problem but the lack of sufficient contrast between fluorescence of healthy and neoplastic tissue encourages the use of sensitized fluorescence.

One of the most widely used precursor exogenous molecules in medical applications is 5-aminolevulinic acid (5-ALA). 5-ALA is a precursor of the fluorescent endogenous fluorophore, protoporphyrin IX (PpIX); an excess of which is produced in altered, especially cancerous tissue. This results in accumulation of intracellular porphyrins,

which increases tissue fluorescence in the red spectral region of cancerous tissue [Onizawa K. et al., 2003]. Subsequent irradiation of the lesion with visible light matching the highest absorption of PpIX (~405 or ~630 nm) leads to red fluorescence emission from PpIX, peaking at 635 nm. The different accumulation of endogenous fluorophores in cancerous and normal tissue causes differences in the red fluorescence ratio between healthy and premalignant/malignant tissue, which makes detection and analysis by fluorescence light more applicable for discrimination between malignant and nonmalignant tissues [Zheng W. et al. 2002]. This intensity is governed by the biological object itself, as well as many other conditions, such as variations in different preparations, doses and forms of application of 5-ALA, as well as different light sources and incubation times.

The aim of this research was to compare the ability of conventional colposcopy and 5-ALA-based fluorescence spectroscopy for detection of CIN. From the fluorescence measurements, we established low-risk and high-risk tissues for cancer development. We created a methodology to identify from the fluorescence spectra An independent threshold value for determining high-risk tissues for cancer development, eliminating possible measurement variations, depending on individual characteristics of the organism and technical errors.

1.1. Aim and objectives of the study

The aim of the study was to evaluate accuracy of sensitized fluorescence spectroscopy in diagnostics of pre-malignant cervical lesions *in vivo*.

The objectives of the study

1. To measure intensity of autofluorescence in studied tissues of the cervix *in vivo* and assess the threshold value for differentiation of high-risk pre-malignant changes of the cervix.

2. To measure intensity of sensitized fluorescence in studied tissues of the cervix *in vivo* and assess numerical threshold value (risk level point) for detection of high-risk pre-malignant changes of the cervix.

3. To estimate and compare the accuracy of sensitized fluorescence spectroscopy, colposcopy and combination of these methods and then to evaluate the relevance of sensitized fluorescence spectroscopy for diagnostics of high-risk pre- malignant changes of the cervix.

1.2. Statements to be defended

1. Autofluorescence spectroscopy is a sensitive method for diagnostics of high-risk pre-malignant changes of the cervix, however, the accuracy of the method is influenced by conditions of the measurement performed (fiber probe holding angle, pressure strength, body fluids on the measurements area, room lighting et al.).

2. The normalization of the values of sensitized fluorescence into values of autofluorescence enables to diminish dispersion of ratio of fluorescence intensity obtained for the patient and additional normalization of obtained ratio into the fluorescence value of healthy tissue enables to diminish dispersion of diagnostic coefficients among different patients.

3. Sensitized fluorescence spectroscopy is an objective method of examination that does not require large clinical experience; the accuracy of this method in detection of high-risk changes of the cervix is higher, in comparison with colposcopy.

1.3. Scientific novelty and practical relevance of the study

1. Scientific and clinical data concerning measurements of sensitized fluoroscopy of impaired cervix tissue *in vivo* are still insufficient and the methods of this promising examination are not universally accepted; the studies of sensitized fluoroscopy of impaired tissues of the cervix were carried out and accuracy of this method in differentiation of high-risk pre-malignant conditions was assessed. Therefore, the measurements of fluorescence spectrums of cervical tissues were performed prior and after administration of sensitizing preparation, and independent numerical threshold value enabling differentiation high-risk pre-malignant conditions of the cervix by means of sensitizing fluorescence spectroscopy was estimated.

2. Sensitizing fluorescence spectroscopy using sensitizing preparation 5-ALA for diagnostics of pre-malignant conditions of cervical cancer was initiated in Lithuania during this study and it was found out, that sensitizing fluorescence spectroscopy using preparation 5-ALA is an objective investigation, appropriate to be applied in clinical practice for identification of high-risk changes of the cervix, i.e. to perform an optical biopsy.

2. OBJECT AND METHODS OF THE STUDY

2.1. Study population and its characteristics

The study was carried out at the National Cancer Institute in 2012 - 2014. The investigation protocol was approved by the Vilnius Regional Research Ethics Committee and written informed consent was obtained from all women. Inclusion criteria were nonpregnant women aged >18 years with suspected high-grade changes in cervical cytology, with no allergies or history of porphyria, histological analysis planned and signed written consent. Exclusion criteria were unsatisfactory or absent histopathological analysis, pregnancy, no HSIL or ASC-H changes in cytology or inappropriate colposcopy or sensitized fluorescence spectroscopy measurements, not signed written consent. The scheme of this study is shown in Figure 1.

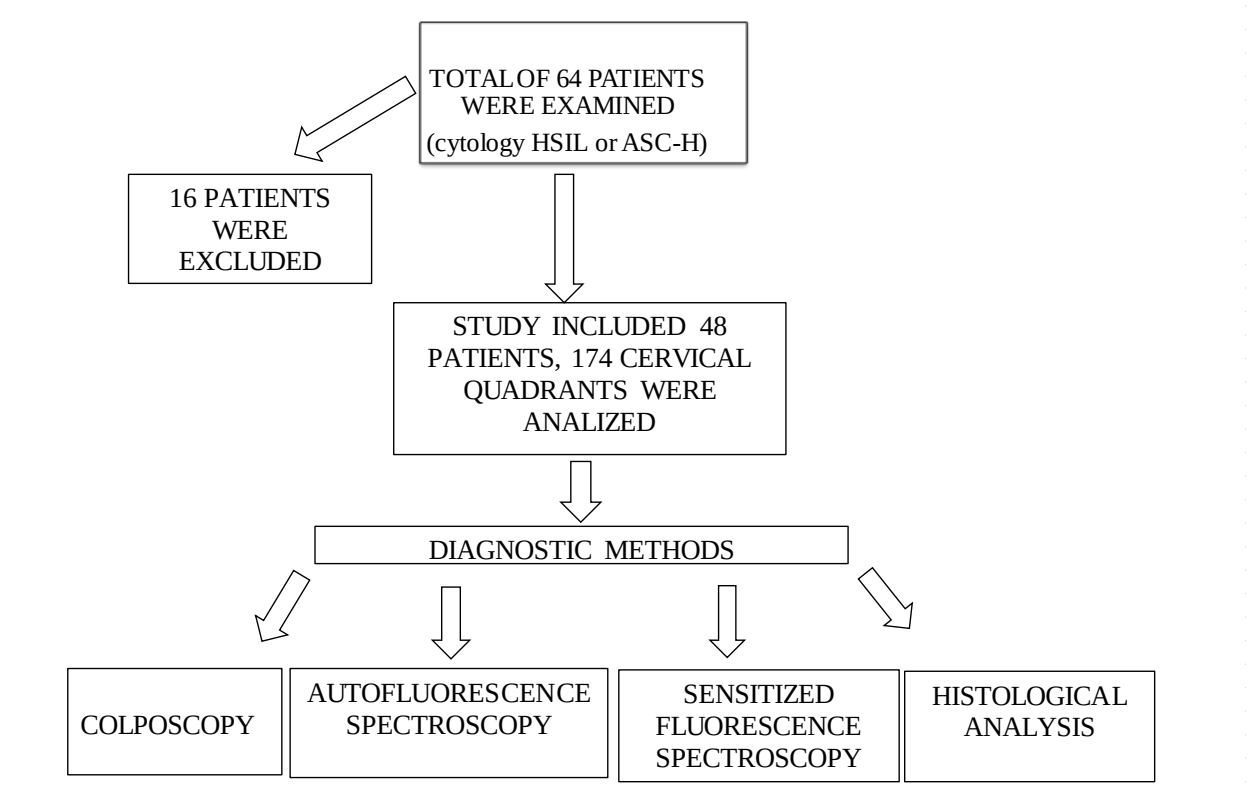


Figure 1. The scheme of this study

A total of 48 women with an average age of 36 years (range 23–57 years) were enrolled. Each patient underwent cytological smear, colposcopy, autofluorescence and sensitized

fluorescence spectroscopy. Every inspected cervix was divided into four quadrants clockwise (Fig. 2), and every quadrant was analyzed as a separate case. In total, 48 patients and 174 cervical quadrants were included.

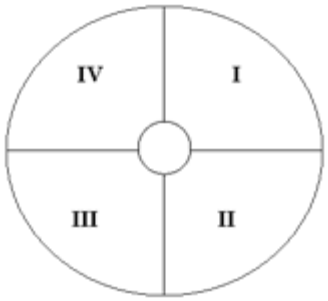


Figure 2. Colposcopic view of the cervix and conventional marking of the quadrants

Data of 18 cervical quadrants were excluded owing to absence of histopathological analysis (uncertain marking or absence of the quadrants after loop excision or biopsy [8 cases], coagulation defects [5 cases]) and inappropriate fluorescence spectroscopy measurements (bleeding from the cervical tissue during the investigation [4 cases], device failure during measurement [1 case]).

After histopathological analysis, we diagnosed 28% and 47% of low-risk cases and then 71% and 53% of high-risk cases per patient and per cervical quadrant, respectively (Tab. 1).

Table 1. Histopathological diagnosis of analyzed cases

Diagnosis (histopathology)		Total per patient n=48	Total per cervical quadrant n=174
CIN 0	LOW RISK	11 (23%)	80 (46%)
CIN 1		3 (6%)	2 (1%)
CIN 2	HIGH RISK	2 (4%)	7 (4%)
CIN 3		32 (67%)	85 (49%)

CIN 1-I grade cervical intraepithelial neoplasia; CIN 2- II grade cervical intraepithelial neoplasia; CIN 3- III grade cervical intraepithelial neoplasia.

2.2. Study methods

After colposcopy and fluorescence spectroscopy, punch biopsy or loop electrosurgical excision of the cervix for histopathological analysis was performed. These tissue samples were diagnosed as inflammatory (chronic cervicitis) (CIN 0), mild (CIN 1), moderate (CIN 2) or severe (CIN 3) neoplastic. All cases were divided in two clinically significant groups: low risk for cancer development (CIN 0-1) and high risk for cancer development (CIN 2-3). The histopathological diagnosis was provided for every quadrant of the cervix. However, the diagnosis of separate cervical quadrant (per quadrant) and subsequent final diagnosis of the whole cervix (per patient) was concluded according to the highest degree of the neoplasia discovered histopathologically.

Conventional colposcopy was performed using 3% acetic acid with the Zoomscope Trulight video colposcope. The colposcopic examination was performed by two experienced specialists. Fluorescence spectroscopy measurements and analysis were performed with a spectroscopy system incorporating a 405 nm laser diode, optical fiber probe, and filter, and spectrometer QE65000 (Ocean Optics) was used to record fluorescence spectra of a cervix *in vivo* (Fig. 3).

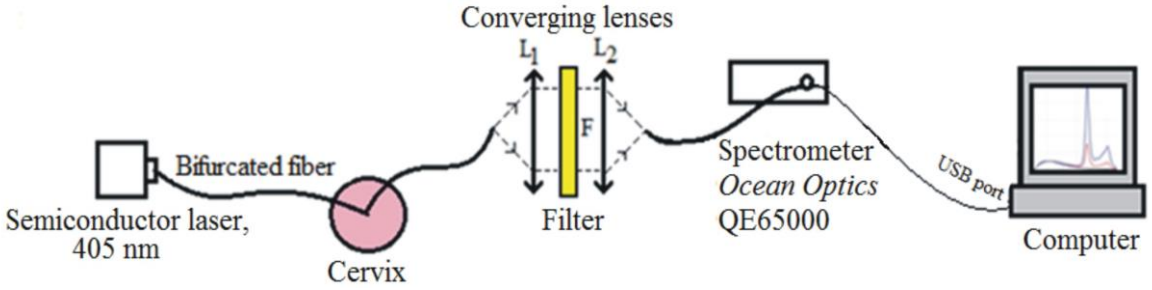
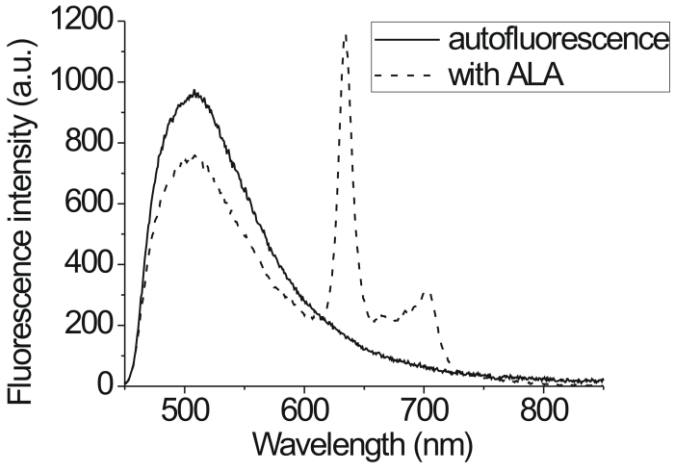


Figure 3. Scheme of the fluorescence spectroscopy system used for fluorescence detection of cervical lesions

Each woman received 3% 3 g 5-ALA cream (precursor of the endogenous fluorophore PpIX) topically as a visualizing agent on the cervix. The cream was prepared in a local pharmacy and used immediately afterwards. The incubation interval was chosen

according to the published scientific data that have been shown to be diagnostically efficient [Hillemanns P. et al. 2000; Collinet P. et al. 2007; Bogaards A. et al. 2002]. The minimum incubation time was 90 minutes and the maximum was 180 minutes. The average and median of incubation time was 135 minutes.

The fluorescence spectra measurements of cervical tissues were done before 5-ALA application and after a certain incubation time (Fig. 4).



a.u. arbitrary unit; ALA – 5-aminolevulinic acid.

Figure 4. Fluorescence spectra from cervical tissue after ALA application with PpIX fluorescence maximum at 634 nm and autofluorescence maximum at 510 nm plus autofluorescence spectra of the cervical tissue

The study was carried out in a darkened room. During the examination, fluorescence spectra were acquired from 3–5 sites of every cervical quadrant. Additional spectra corresponding to healthy cervical tissue were registered from places that were not typical of neoplastic development and that had no signs of inflammation. All fluorescence spectra were processed using Origin Pro software.

The fluorescence intensity ratio (R) was evaluated from fluorescence spectra of sensitized cervical tissue (a):

$$R = \frac{I_{(634nm)}}{I_{(510nm)}} \qquad \text{(a)},$$

where $I_{(634\ nm)}$ is fluorescence intensity at 634 nm (maximum of the fluorescence band of the endogenous fluorophore PpIX) and $I_{(510\ nm)}$ is at the tissue autofluorescence intensity maximum at 510 nm. Our previous studies revealed that it is not correct to determine the true state of the quadrant by averaging all fluorescence spectra measured from that quadrant [Vansevičiūtė R. et al. 2013]. Therefore, diagnosis according to fluorescence data was made on the basis of one fluorescence spectrum that had the highest R value. This value should correspond to the highest degree of neoplasia.

In our previous study, we observed that R values are scattered among patients [Vansevičiūtė R. et al. 2013], and the reliable threshold value for healthy and pathological tissue differentiation could not be estimated. Therefore, considering that every patient has individual fluorophore composition and metabolic characteristics (depending on age, menstrual cycle, metabolic and endocrine functions) that influence the intensity of fluorescence spectra [Chang VT. et al. 2009; Drezek R. et al. 2001; Richards-Kortum R. et al. 2003], the spectral measurements were additionally normalized for every patient. The normalized value D, diagnostic factor, was calculated as the proportion of R from individual quadrant and R from – healthy tissue of the particular women cervix (b):

$$D = \frac{R_{(quadrant)}}{R_{(healthy)}} \qquad \text{(b)},$$

where D is diagnostic factor, $R_{(quadrant)}$ is individual quadrant, and $R_{(healthy)}$ is healthy tissue of the particular cervix.

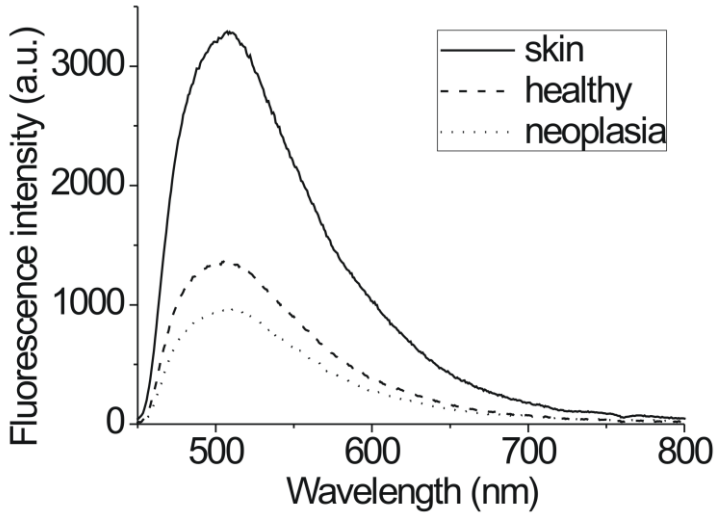
Statistical analysis. Using Origin Pro 8, we performed statistical analysis of the D values for all quadrants. To determine the normality of the distribution, we applied the Shapiro–Wilk test, when the significance level was 0.05. We identified that our data were not distributed normally, so for further analysis, we applied nonparametric tests. To inspect the raised hypotheses, we performed the Mann Whitney Wilcoxon two-independent-samples tests. The statistically significant difference was when $P(H_0=0)<0.05$. For proper grouping of LR and HR cases (D values), we calculated the

threshold value. For this reason Youden index (J) was assessed. According to this criterion, the threshold value is when J is the greatest. Statistical analysis of the collected material included sensitivity, specificity, positive and negative predictive values for fluorescence spectroscopy, colposcopy and combination of those methods, analyzing diagnosis per patient and per cervical quadrant. For evaluation of the method diagnostic efficiency, receiver operating characteristic curves (ROC) were plotted. The diagnostic value was obtained by assessing area under the curve (S_{ROC}). Area under the curve was 0.9–1.0, the study could be evaluated as excellent; 0.8–0.9, very good; 0.7–0.8, good; 0.6–0.7, tolerable; and 0.5–0.6, deficient. ROC analysis was performed using MedCalc.

3. RESULTS

3.1. Autofluorescence spectroscopy data analysis

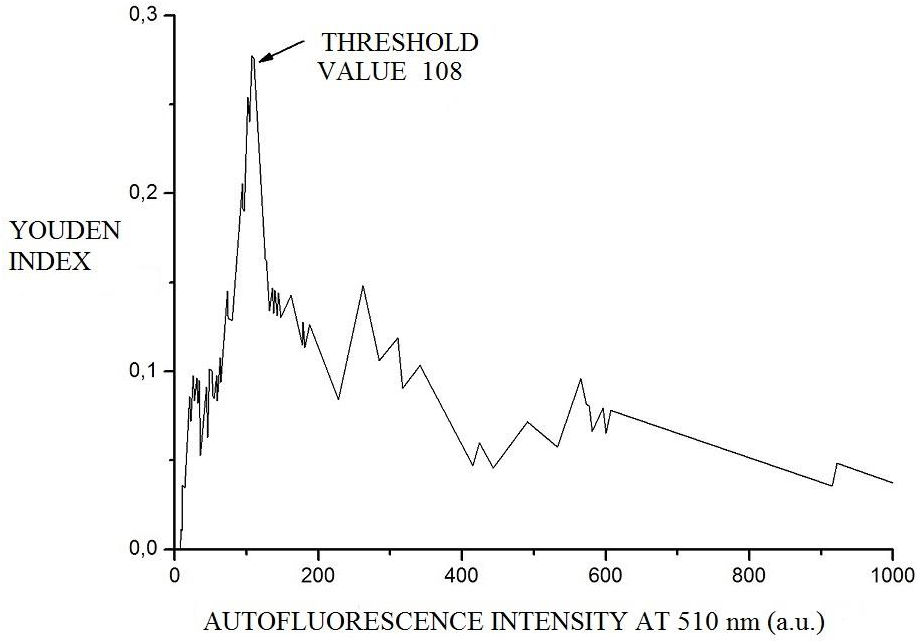
During examination, 1295 *in vivo* spectra were acquired (583 before 5-ALA application and 712 after). Before 5-ALA application, no notable PpIX fluorescence was observed in the cervical tissue. Typical autofluorescence spectra of different tissue types are presented in Figure 5.



a.u. arbitrary unit.

Figure 5. Autofluorescence spectra from neoplastic, healthy cervical tissue and skin (before ALA application) in the same patient

First of all autofluorescence data was analysed and after statistical analysis threshold value for high and low-risk cervical changes was obtained. The highest value of Youden index (J = 0,27) was reached, when autofluorescence intensity value was 108 (Fig.6).



a.u. - arbitrary units.

Figure 6. Determination of the threshold value from the calculated Youden indices for every cervical quadrant at autofluorescence intensity value 510 nm

This value was chosen as threshold value for high and low-risk cervical changes differentiation. Cervical quadrants, where threshold value was <108, were diagnosed as having high-risk changes, and all other quadrants were classified as low-risk. Autofluorescence spectroscopy and colposcopy data were compared with the gold standard - histopathological diagnosis. However, accuracy of this method as well as S ROC values, determined after data analysis per patient and per quadrant, revealed obviously lower results when comparing with colposcopy (Tab. 2, Tab. 3).

Table 2. Sensitivity, specificity, accuracy, positive and negative predictive values for autofluorescence spectroscopy and colposcopy, analyzing cervical quadrant diagnosis

Diagnostic method	Sensitivity, % (95% CI)	Specificity, % (95% CI)	PPV, %	NPV, %	S_{ROC} (95% CI)
Autofluorescence spectroscopy	**59,1** (48,1– 69,5)	**54,7** (43,5 – 65,4)	**57,6**	**56,2**	**0,569** (0,492 - 0,643)
Colposcopy	**67,4** (56,8-76,8)	**86,6** (77,3-93,1)	**84,9**	**70,3**	**0,770** (0,700-0,830)

PPV- positive prognostic value; NPV –negative predictive value; S ROC – study value; CI –confidence interval.

Table 3. Sensitivity, specificity, accuracy, positive and negative predictive values for autofluorescence spectroscopy and colposcopy, analyzing diagnosis per patient

Diagnostic method	Sensitivity, % (95% CI)	Specificity, % (95% CI)	PPV, %	NPV, %	S_{ROC} (95% CI)
Autofluorescence spectroscopy	**81,8** (64,5 – 93,0)	**46,7** (21,3 – 73,4)	**77,3**	**53,6**	**0,642** (0,491-0,775)
Colposcopy	**88,2** (72,5-96,6)	**78,6** (49,2-95,1)	**90,9**	**73,3**	**0,834** (0,699-0,925)

PPV- positive prognostic value; NPV –negative predictive value; S ROC – study value; CI –confidence interval.

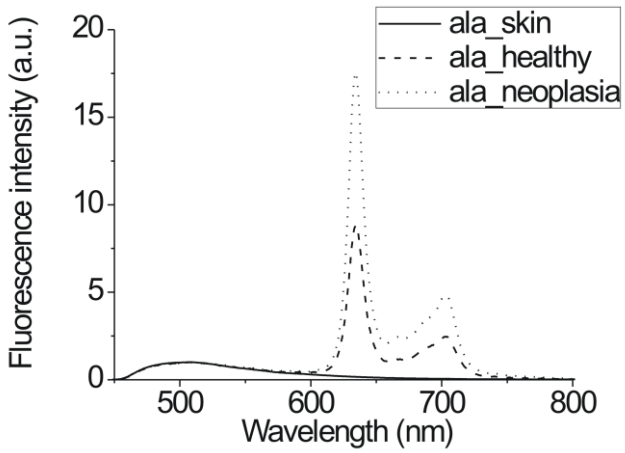
Whereas autofluorescence spectroscopy diagnostics is based only on the differences between fluorescence intensities, accuracy of this method is highly dependent on conditions (such as fiber probe holding angle, pressure strength, body fluids on the measurement area, room lighting et al.) constancy of the measurement performed. Our

study results revealed that autofluorescence spectroscopy is a sensitive method for diagnostics of high-risk pre-malignant changes of the cervix, however maintaining stable conditions during the investigation *in vivo* is complicated, consequently the accuracy of the method is not enough relevant for clinical practice.

While autofluorescence spectroscopy data showed insufficient contrast between the low and high-risk cervical changes, this encouraged us to look for other diagnostic methods and we chose sensitized fluorescence spectroscopy with 5-ALA.

3.2. Sensitized fluorescence spectroscopy data analysis

After topical application of 5-ALA, PpIX fluorescence was detected in the spectra of healthy tissues and tissues with neoplasia (peaks at 634 and 703 nm). The typical fluorescence spectra of the cervical tissues, normalized to the maximum of the tissue autofluorescence are presented in Figure 7.



a.u. arbitrary unit; ala – 5-aminolevulinic acid.

Figure 7. Sensitized fluorescence spectra from neoplastic, healthy cervical tissue and skin after topical ALA application in the same patient

The higher fluorescence intensity in the red spectrum region was detected from neoplastic tissue compared with healthy tissue. Normalization of fluorescence spectra to

autofluorescence peaks helps to overcome varying measurement conditions and allow unambiguous evaluation of fluorescence spectra from different places. The spectrum measured on the skin did not have a peak specific for PpIX, which means that ALA cream used topically on the cervix causes no PpIX production in other parts of the body, showing no systemic accumulation and therefore causing no notable side effects.

3.3. Calculation of the independent threshold value for sensitized fluorescence spectroscopy

To obtain the threshold value for low- and high-risk differentiation, D value was calculated for every quadrant and Youden index was calculated for every D value (Fig.8).

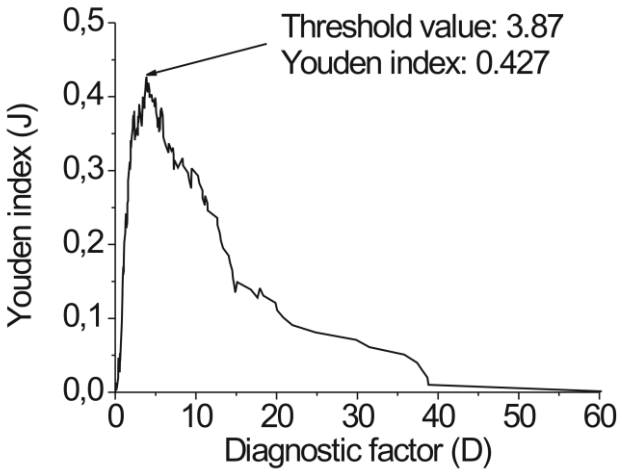


Figure 8. Youden index in dependence of numeric threshold value

The peak value of Youden index was 0.427, which was reached at a D value of 3.87. This value was chosen as the threshold value for low-risk and high-risk cervical tissue differentiation. Subsequently, D values split into two groups ($n_{(CIN0-1)}$ = 88; $n_{(CIN2-3)}$ = 86). After statistical analysis using the Shapiro–Wilk test, at the 0.05 level of significance, the D values of both groups were not normally distributed. To establish whether these two groups were statistically different, the Mann Whitney Wilcoxon test was performed for independent samples and showed at the 0.05 level of significance that the D values of the HR group were higher than those of the LR group, $P(H_0 = 0)$ = 2,53 x

10 [9]. After sensitized fluorescence spectroscopy and D factor calculation, cervical quadrants, where D was >3.87, were diagnosed as having high-risk changes, and all other quadrants were classified as low risk.

3.4. Feasibility of sensitized fluorescence spectroscopy for high/low risk cervical changes diagnosis

Further analysis was performed to evaluate the possibility of using sensitized fluorescence spectroscopy for differentiation of high- and low-risk cervical lesions. Sensitized fluorescence spectroscopy and colposcopy data were compared with the gold standard - histopathological diagnosis (Tab. 4).

Table 4. Comparison of sensitized fluorescence spectroscopy and colposcopy with histopathology (per quadrant)

Histopathology				
		HR	LR	Total
Sensitized fluorescence spectroscopy	HR	66	20	86
	LR	26	62	88
	Total	92	82	174
Histopathology				
SFS + colposcopy	HR	81	25	106
	LR	11	57	68
	Total	92	82	174
Histopathology				
Colposcopy	HR	62	11	73
	LR	30	71	101
	Total	92	82	174

HR – high-risk cervical changes; LR – low-risk cervical changes; SFS sensitized fluorescence spectroscopy.

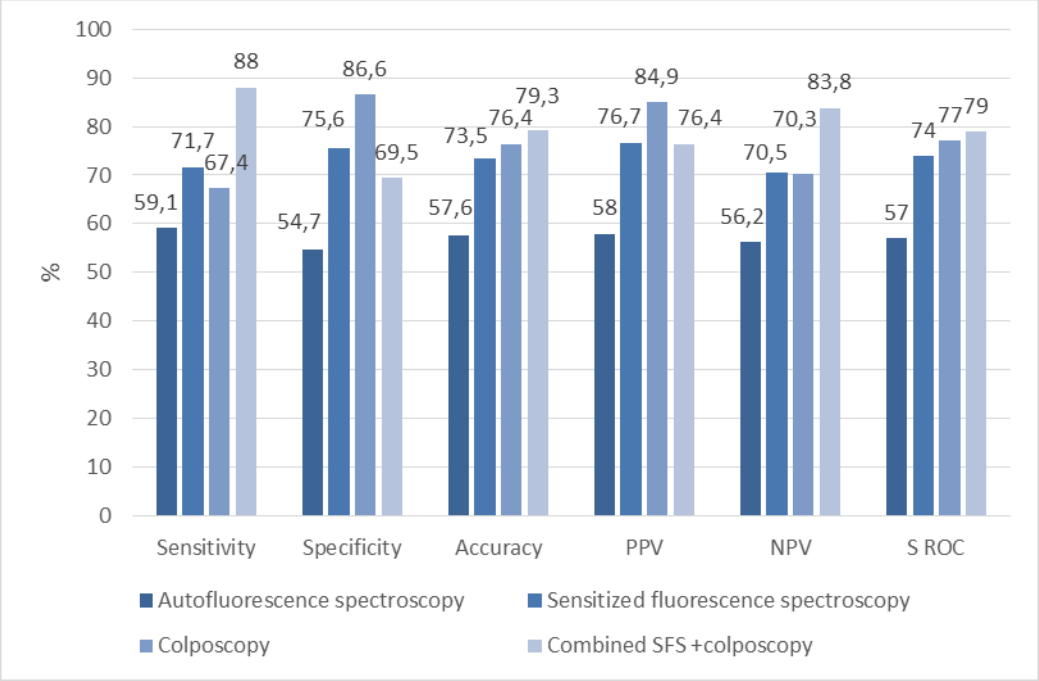
It was obvious that, based on sensitized fluorescence data, 20 low-risk quadrants were mistakenly diagnosed as high-risk CIN, and in 26 quadrants, no pathological changes were seen, and high-risk CIN was demonstrated histopathologically.

In determining the status of the cervical quadrants by colposcopy, 73 quadrants were high-risk and 101 were low-risk neoplastic. According to these results, the disease was missed in 30 quadrants, while only 11 cases were incorrectly diagnosed as high risk.

One of the potential applications of fluorescence diagnostics is as a complementary method to conventional techniques. High-risk neoplasia was considered if at least one method detected neoplastic changes.

The data in Table 4 show that when colposcopy and fluorescence were combined, only 11 cases were missed, and combination of methods gave the best results. However, the 25 low-risk quadrants were mistakenly identified as high risk, which led to unnecessary surgical procedures.

Following these results, the sensitivity, specificity, accuracy, test value, and positive and negative predictive values for sensitized fluorescence spectroscopy, colposcopy and combination of the methods were calculated.



PPV positive prognostic value; NPV negative prognostic value; SFS sensitized fluorescence spectroscopy.

Figure 9. Sensitivity, specificity, accuracy, positive and negative predictive values for autofluorescence, sensitized fluorescence spectroscopy, colposcopy and combination of methods, analyzing cervical quadrant diagnosis

Table 5. Sensitivity, specificity, accuracy, positive and negative predictive values for sensitized fluorescence spectroscopy, colposcopy and combination of methods, analyzing cervical quadrant diagnosis

	Sensitized fluorescence spectroscopy			Colposcopy			Colposcopy + SFS (combined)		
	n	%	95% CI	n	%	95% CI	n	%	95% CI
Sensitivity	66/92	71.7	61.4-80.6	62/92	67.4	56.8-76.8	81/92	88.0	79.6-93.9
Specificity	62/82	75.6	64.9-84.4	71/82	86.6	77.3-93.1	57/82	69.5	58.4-79.2
Accuracy	128/174	73.5	-	133/174	76.4	-	138/174	79.3	-
PPV	66/86	76.7	-	62/73	84.9	-	81/106	76.4	-
NPV	62/88	70.5	-	71/101	70.3	-	57/68	83.8	-
Total	174			174			174		

n - number of patients; PPV positive prognostic value; NPV negative prognostic value; 95% CI confidence interval; SFS sensitized fluorescence spectroscopy.

Table 5 and Figure 9 represents the statistical data for the cervical quadrants (per quadrant). Sensitized fluorescence spectroscopy had higher sensitivity than colposcopy (71.7% vs 67.4%), but specificity was higher for colposcopy (86.6% vs 75.6%). The combination of methods had higher sensitivity (88.0% vs 67.4%) but reduced specificity (88.0% vs 69.5%). As mentioned above, combination of the methods resulted in the highest number of correctly identified high-risk changes and the highest accuracy (79.3%). According to the ROC curves, the diagnostic values (S_{ROC}) were similar and

could be considered as good. The effectiveness of the combined diagnostic methods was the best (S_{ROC} = 0.788).

After quadrant analysis, data were summarized to provide the general diagnosis for the patient. The quadrant with the severest neoplasia determined the diagnosis of the whole cervix. Comparison of fluorescence and colposcopy data for each patient with the histopathological findings are presented in Table 4.

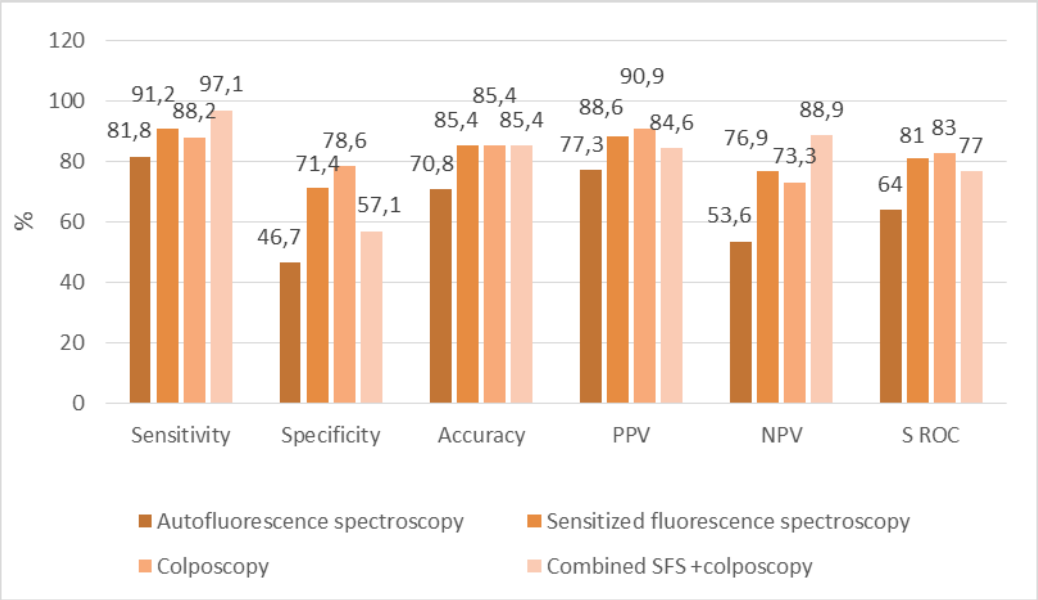
Table 6. Comparison of sensitized fluorescence spectroscopy and colposcopy with histopathology (per patient)

Histopathology				
		HR	LR	Total
Sensitized fluorescence spectroscopy	HR	31	4	35
	LR	3	10	13
	Total	34	14	48
Histopathology				
		HR	LR	Total
SFS + colposcopy	HR	33	6	39
	LR	1	8	9
	Total	34	14	48
Histopathology				
		HR	LR	Total
Colposcopy	HR	30	3	33
	LR	4	11	15
	Total	34	14	48

HR high-risk cervical changes; LR low-risk cervical changes; SFS sensitized fluorescence spectroscopy.

Per patient analysis showed that sensitized fluorescence spectroscopy failed to detect only three high-risk cases from 34, while during colposcopy, four cases were missed. However, colposcopy overdiagnosed three patients and sensitized fluorescence spectroscopy four. These results show that the two methods are comparable. Combination of the methods increases the number of correctly diagnosed neoplasia (Tab. 6) and only one high-risk case was missed. However, overdiagnosis also increased, with six patients being mistakenly diagnosed with high-risk changes.

Full statistical analysis for the per patient results is presented in Table 7 and Figure 10.



PPV positive prognostic value; NPV negative prognostic value; SFS sensitized fluorescence spectroscopy.

Figure 10. Sensitivity, specificity, accuracy, positive and negative predictive values for autofluorescence, sensitized fluorescence spectroscopy, colposcopy and combination of methods, analyzing diagnosis per patient

Table 7. Sensitivity, specificity, accuracy, positive and negative predictive values for sensitized fluorescence spectroscopy, colposcopy and combination of methods, analyzing per patient diagnosis

	Sensitized fluorescence spectroscopy			Colposcopy			Colposcopy + SFS (combined)		
	n	%	95% CI	n	%	95% CI	n	%	95% CI
Sensitivity	31/ 34	91.2	76.3-98.0	30/ 34	88.2	72.5-96.6	33/ 34	97.1	84.6-99.5
Specificity	10/ 14	71.4	41.9-91.4	11/ 14	78.6	49.2-95.1	8/ 14	57.1	28.9-82.2
Accuracy	41/ 48	85.4	-	41/ 48	85.4	-	41/ 48	85.4	-
PPV	31/ 35	88.6	-	30/ 33	90.9	-	33/ 39	84.6	-
NPV	10/ 13	76.9	-	11/ 15	73.3	-	8/9	88.9	-
Total	48			48			48		

n - number of patients; PPV positive prognostic value; NPV negative prognostic value; 95% CI confidence interval; SFS sensitized fluorescence spectroscopy.

Sensitivity of sensitized fluorescence spectroscopy was 91.2%, which was greater than 88.2% for colposcopy. Greater overdiagnosis resulted in decreased sensitivity for sensitized fluorescence spectroscopy—71.4% vs 78.6% for colposcopy. In both cases, the accuracy was 85.4%, and the effectiveness (S_{ROC}) was >80%, which means that the value in determining the high-risk CIN was good for both methods. On the contrary, the effectiveness of the combined methods was <80%. Nevertheless, the sensitivity of this combined diagnosis was 97.1%, indicating that it was best for detection of high-risk lesions.

4. DISCUSSION

We evaluated the use of 5-ALA-based sensitized fluorescence spectroscopy for the detection of pathological areas in the cervix. Identification of the diseased areas was estimated by measuring the fluorescence of PpIX, and more quantitative evaluation could be done by calculating the ratio (R) between porphyrin fluorescence and tissue autofluorescence. The additional normalization of ratios (R) for the inspected areas of the tissue to the ($R_{healthy}$) value of healthy tissue enabled us to calculate the diagnostic coefficients (D), and to obtain threshold values for low- and high-risk differentiation. The threshold value of 3.87 is the independent number that could be used for CIN diagnosis.

Examination of the cervical quadrants yields the possibility of specifying the localization of neoplastic regions. However, because they were observed during the study, the pathological areas could be small and missed during measurement. Therefore, for precise localization of all lesions, careful and fine scanning should be performed. In other cases, accurate diagnostics could only be performed per patient. The results of fluorescence spectroscopy per quadrant in some cases were influenced by difficulties during conization, which resulted in incorrect margins of the quadrants and mixed correct results. However, during routine procedures, such situations cannot be fully avoided, therefore, such inaccuracies must be incorporated when calculating the sensitivity and specificity as a random error. Nevertheless, these effects do not influence per patient diagnosis.

Per patient diagnosis showed 85.4% accuracy of fluorescence spectroscopy in identifying neoplastic changes, therefore, it has great diagnostic potential. However, to replace the conventional methods, the threshold value must be revised and, if necessary, corrected after data analysis from more patient cases.

Several studies show that the detection of porphyrin fluorescence can improve the identification of the cervical pre-malignancy. Only a few studies have been performed using fluorescence spectroscopy with 5-ALA. Hillemanns et al. showed that fluorescence imaging with 5-ALA after 60–90 minutes achieved similar sensitivity and

specificity compared with colposcopy in detecting CIN: 94% and 51% versus 95% and 50%, respectively. However, the specificity was markedly improved by fluorescence spectroscopy, achieving 75%. Sapoznikova et al. reported that sensitized fluorescence spectroscopy had a diagnostic efficiency of 79.5% for cervical neoplasia.

When comparing colposcopy with 5-ALA-based fluorescence spectroscopy, on the one hand we have the experience of physicians and on the other hand, we have the objectivity of the diagnostic method, and one also should decide between overestimated and underestimated CIN diagnosis. To date, it seems that this should be a compromise because there is still no single method or combination of methods that can perfectly fulfill all the desired requirements.

Nevertheless, fluorescence diagnostics appears promising despite non- ideal results. Fluorescence is an objective method, requiring only a short training for appropriate fluorescence measurements, while colposcopy is highly dependent on physicians' experience and might be more inaccurate when performed by less-trained hands. There is plenty of room for improving the precision and consistency of fluorescence measurement techniques. The additional burden for fluorescence spectroscopy was because the analysis was performed on patients who already had high-grade cytology results. Altered cytology could determine greater production of PpIX, which results in elevated fluorescence intensity and finally provide false-positive results and decreased specificity. Moreover, the women who were diagnosed as having no evidence or low risk neoplasia had significant signs of inflammation. The inflammation is not classified as malignancy, but it changes the optical properties of cervical tissue, therefore, the ability to differentiate inflammation from neoplastic changes should further increase the diagnostic value of fluorescence spectroscopy.

It is possible to define further directions for development. Sensitized fluorescence spectroscopy is a powerful technique for detecting altered tissues, however, additional attention should be paid to differentiation/identification of the neoplastic changes and inflammation.

By standardization of instrumentation and proper diagnostic algorithms it was possible to obtain an independent threshold value for low- and high-risk differentiation, $D_{threshold}$ = 3.87. Analysis using this threshold showed the potential of fluorescence spectroscopy for noninvasive identification of neoplastic changes at an early stage, and it could be used for differentiating high-risk changes. This method shows similar accuracy to currently used screening tests, but has the potential advantage of objective results. Fluorescence spectroscopy requires only a short time of training to achieve appropriate measurements, while colposcopy is highly dependent on physician experience, which is usually considered a major factor in its success.

5. CONCLUSIONS

1. The threshold value for autofluorescence spectroscopy method intended for diagnostics of high-risk pre-malignant changes of the cervix was assessed (the threshold value is 108), however accuracy of this method is not relevant for clinical practise.

2. The calculation algorithm of independent numerical threshold value (risk level point) used for sensitized fluorescence spectroscopy diagnostics of high-risk pre-malignant changes of the cervix, based on double normalization, provides possibility to compare results of different measurements for the same patient and for different patients. The threshold value ($D_{threshold} = 3.81$) for differentiation of high-risk pre-malignant changes of the cervix was assessed.

3. Comparing accuracy of sensitized fluorescence spectroscopy, colposcopy and combination of these methods in detection of high-risk changes of the cervix no statistically significant differences was found, all those methods are comparable. Sensitized fluorescence spectroscopy is an objective and not requiring large physicians experience method, appropriate for diagnostics of high-risk pre-malignant changes of the cervix.

6. PRACTICAL RECOMMENDATIONS

A possible clinical application could be a combination of a porphyrin fluorescence image viewer and an *in vivo* spectrum analysis system, which could be used for optical biopsies. With this system, high-grade pre-malignant changes could be diagnosed and treated in one session.

7. LIST OF DISSERTATION – RELATED SCIENTIFIC PUBLICATIONS AND PRESENTATIONS

Articles:

1. **Vansevičiūtė R,** Žukovskaja O, Venius J. COMPARISON OF 5-AMINOLEVULINIC ACID BASED FLUORESCENCE DIAGNOSTICS WITH HISTOLOGY IN THE DETECTION OF CERVICAL NEOPLASIA. Medical Physics in the Baltic States: proceedings of the 11th international conference on medical physics. 2013; 11:12-4.

2. **Vansevičiūtė R,** Letautienė SR, Venius J. 5-AMINOLEVULINIC ACID BASED FLUORESCENCE DIAGNOSTICS OF CERVICAL PREINVASIVE CHANGES. Medicina 2014; 50(3):137-43.

3. **Vansevičiūtė R,** Letautienė SR, Venius J, Žukovskaja O, Kanopienė D, Rotomskis R. 5-AMINOLEVULINIC-ACID-BASED FLUORESCENCE SPECTROSCOPY AND CONVENTIONAL COLPOSCOPY FOR *IN VIVO* DETECTION OF CERVICAL PRE-MALIGNANCY. BMC Women's health 2015; 15:35.

Presetations:

1. **Vansevičiūtė R,** Venius J, Žukovskaja O. COMPARISON OF 5-ALA BASED FLUORESCENCE DIAGNOSTICS WITH HISTOLOGY IN THE DETECTION OF CERVICAL NEOPLASIA. Annual Meeting "Medical physics in the Baltic states 11". Kaunas (Lithuania), Oct. 10-12, 2013.

2. **Vansevičiūtė R.** „Gimdos kaklelio ikivėžinių būklių tyrimas sensibilizacinės fluorescencijos spektroskopijos metodu *in vivo*" 2014-09-10; Nacionalinio vėžio instituto mokslinė konferencija.

3. **Vansevičiūtė R.** „Gimdos kaklelio ikivėžinių būklių tyrimas sensibilizacinės fluorescencijos spektroskopijos metodu *in vivo*" 2015-04-14; Mokslinių tyrimų centras Biomedicininės fizikos laboratorijos darbuotojų posėdis.

8. SUMMARY IN LITHUANIAN

Darbo aktualumas

Lietuvoje sergamumas gimdos kaklelio vėžiu didžiausias Baltijos šalių regione ir Rytų Europoje antroje vietoje po Rumunijos [Arbyn M. et al., 2011]. 2004 m. Lietuvoje buvo pradėta ir toliau tęsiama atrankinės patikros dėl gimdos kaklelio patologijos programa, kurioje naudojamas profilaktinis testas - citologinis gimdos kaklelio tyrimas. Pagal gimdos kaklelio citologinio tyrimo rezultatą atrenkamos moterys, kurioms reikalingas detalesnis ištyrimas: kolposkopija, histologinis tyrimas ar aktyvus stebėjimas. Diferencijuojant gimdos kaklelio patologijas kolposkopijos jautrumas yra didelis, tačiau specifiškumas mažas. Žinoma, kad kolposkopijos tikslumas tiesiogiai priklauso nuo tiriančiojo klinikinės patirties [Palma PD. et al., 2008; Cremer ML. et al., 2010]. Todėl diagnozei patvirtinti tenka atlikti gimdos kaklelio intervencinius diagnostinius tyrimus ne visada pagrįstai.

Pastaruoju metu atliekami klinikiniai tyrimai ieškant metodų, padedančių objektyviau diagnozuoti gimdos kaklelio ikivėžines būkles neinvaziniu būdu [Adelman MR. 2014]. Atliekant tokį tyrimą pakitimus turėtų būti galima atpažinti be histologinio tyrimo (t.y. neatliekant biopsijos) ir tyrėjo klinikiniai įgūdžiai turėtų kuo mažiau lemti tyrimo rezultatus. Daugėja tyrimų, kurių tikslas analizuoti fluorescencijos spektroskopijos diagnostinę vertę tiriant gimdos kaklelio ikivėžines būkles. Nustatyta, kad sensibilizacinės fluorescencijos spektroskopijos metodas yra tinkamas tirti biocheminius ir struktūrinius pakitimus audiniuose, kurie atsiranda vystantis ikivėžinėms būklėms ir vėžiui. Taigi šis metodas tinka „optinei biopsijai“ atlikti.

Darbo tikslas

Įvertinti sensibilizacinės fluorescencijos spektroskopijos metodo tikslumą diagnozuojant ikivėžines gimdos kaklelio būkles *in vivo*.

Darbo uždaviniai

1. Išmatuoti savitosios fluorescencijos intensyvumą tiriamuosiuose gimdos kaklelio audiniuose *in vivo* ir apskaičiuoti skiriamąją vertę didelės rizikos ikivėžiniams gimdos kaklelio pakitimams diferencijuoti.

2. Išmatuoti sensibilizacinės fluorescencijos intensyvumą gimdos kaklelio audiniuose *in vivo* ir apskaičiuoti skaitinę skiriamąją vertę didelės rizikos ikivėžiniams gimdos kaklelio pakitimams nustatyti.

3. Palyginti sensibilizacinės fluorescencijos spektroskopijos, kolposkopijos ir šių metodų derinio tikslumą bei įvertinti sensibilizacinės fluorescencijos spektroskopijos metodo tinkamumą diagnozuojant didelės rizikos ikivėžinius gimdos kaklelio pakitimus.

Ginamieji teiginiai

1. Savitosios fluorescencijos spektroskopija yra tinkamas metodas didelės rizikos ikivėžiniams gimdos kaklelio pakitimams diagnozuoti, tačiau metodo tikslumą sąlygoja matavimo sąlygos (šviesolaidžio zondo priglaudimo kampas, prispaudimo stiprumas, organizmo skysčiai esantys matavimo vietoje, patalpos apšvietimas ir kt.).

2. Sensibilizacinės fluorescencijos spektroskopijos verčių normavimas į savitosios fluorescencijos vertes leidžia sumažinti gautų fluorescencijos intensyvumų santykių išsibarstymą pacientei, o gauto santykio papildomas normavimas į sveiko audinio fluorescencijos vertę leidžia sumažinti diagnostinių koeficientų išsibarstymą tarp pacienčių.

3. Sensibilizacinės fluorescencijos spektroskopija yra objektyvus ir didelės klinikinės patirties nereikalaujantis tyrimo metodas, kurio tikslumas nustatant didelės rizikos pakitimus gimdos kaklelyje yra didesnis nei kolposkopijos.

Darbo mokslinis naujumas ir praktinė reikšmė

1. Mokslinių – klinikinių duomenų apie sensibilizacinės fluorescencijos matavimus pakitusiame gimdos kaklelio audinyje *in vivo* dar nepakanka, šio perspektyvaus tyrimo metodikos visuotinai nėra patvirtintos, buvo atlikti tokių (gimdos kaklelio audinio) pakitimų sensibilizacinės fluorescencijos spektroskopijos tyrimai bei įvertintas šio metodo tikslumas atskiriant didelės rizikos ikivėžines būkles. Dėl to atlikti gimdos kaklelio audinių fluorescencijos spektrų matavimai prieš ir po sensibilizuojančios medžiagos naudojimo, nustatyta sensibilizacinės fluorescencijos spektroskopijos skaitinė skiriamoji vertė (rizikos lygio riba), leidžianti atskirti didelės rizikos ikivėžines gimdos kaklelio būkles tiriant juos sensibilizacinės fluorescencijos spektroskopijos metodu.

2. Mokslinio darbo metu Lietuvoje gimdos kaklelio ikivėžinių būklių diagnostikai pradėta taikyti sensibilizacinės fluorescencijos spektroskopija naudojant sensibilizuojantį preparatą 5-ALA ir nustatyta, kad sensibilizacinės fluorescencijos spektroskopija yra objektyvus tyrimas, tinkamas taikyti klinikinėje praktikoje, gimdos kaklelio didelės rizikos pakitimams identifikuoti, t.y. atlikti optinę biopsiją.

Tiriamieji ir tyrimo metodai

Į tyrimą įtrauktos 48 pacientės, kurioms Vilniaus universiteto Onkologijos institute (dabar Nacionalinis vėžio institutas) 2012-2014 metų laikotarpiu kreipėsi konsultacijai dėl citologiniame gimdos kaklelio tyrime įtariamų žymių intraepitelinių pokyčių (angl. *HSIL*).

Naudoti tyrimo metodai:

- anketinė apklausa;
- kolposkopija;
- savitosios fluorescencijos spektrų registravimas ir analizė;
- sensibilizacinės fluorescencijos spektrų registravimas ir analizė;
- histologinis gimdos kaklelio audinių tyrimas (atlikus biopsija ar konizaciją);
- statistinė duomenų analizė atlikta naudojant "Origin Pro 8" ir "MedCalc" programinės įrangos paketus.

Išvados

1. Nustatyta savitosios fluorescencijos spektroskopijos skiriamoji vertė didelės ir mažos rizikos ikivėžiniams pakitimams gimdos kaklelyje diferencijuoti (skiriamoji vertė yra 108). Taikant savitosios fluorescencijos spektroskopijos metodą apskaičiuotas tyrimo tikslumas nėra pakankamas didelės ir mažos rizikos ikivėžiniams pakitimams gimdos kaklelyje diferencijuoti.

2. Sensibilizacinės fluorescencijos spektroskopijos metodo skaitinė skiriamoji vertė (rizikos lygio riba), pagal kurią diagnozuoti didelės rizikos ikivėžiniai pakitimai gimdos kaklelyje, yra apskaičiuota atliekant dvigubą normavimą. Šis normavimas leidžia lyginti tos pačios pacientės ir skirtingų pacienčių matavimų duomenis. Nustatyta sensibilizacinės fluorescencijos spektroskopijos skaitinė skiriamoji vertė yra 3,87.

3. Palyginus sensibilizacinės fluorescencijos spektroskopijos, kolposkopijos bei šių metodų derinio tikslumo rodiklius (jautrumą, specifiškumą) nustatant didelės rizikos

pakitimus gimdos kaklelyje, statistiškai reikšmingo skirtumo nenustatyta, šių metodų tikslumas yra panašus. Taigi sensibilizacinės fluorescencijos spektroskopija yra objektyvus ir didelės klinikinės patirties nereikalaujantis metodas tinkamas didelės rizikos ikivėžiniams pakitimams gimdos kaklelyje diagnozuoti.

PADĖKA

Dėkoju Nacionalinio vėžio instituto administracijai ir vadovams prof. Eduardui Aleknavičiui, prof. Narimantui Evaldui Samalavičiui, prof. Konstantinui Povilui Valuckui už suteiktą galimybę ir paramą vykdant šį mokslinį darbą. Dėkoju disertacinio darbo recenzentams ir disertacijos gynimo komisijos nariams už šio darbo įvertinimą.

Esu nuoširdžiai dėkinga mokslinio darbo vadovei doc. Simonai Rūtai Letautienei už palaikymą, pagalbą organizuojant mokslinio darbo rengimą, supratimą ir gerą nuotaiką visose situacijose. Dr. Jonui Veniui dėkoju už mokslines diskusijas, idėjas ir dosniai skirtą laiką rašant straipsnius ir disertacijos rezultatus bei jų aptarimą. Dėkoju gerbiamai prof. Janinai Didžiapetrienei už kantrybę, supratimą, svarbias mokslines pastabas ir skatinimą tobulėti, prof. Ričardui Rotomskiui ir dr. Vydmantui Atkočiui už vertingus patarimus darbo eigoje. Taip pat dėkoju medicinos fizikos studentei magistrei Olgai Žukovskajai už pagalbą registruojant ir analizuojant fluorescencijos spektrų duomenis, bei statistinius skaičiavimus. Dėkoju kalbos redaktorei Gražinai Pruskuvienei už disertacijos teksto redagavimą.

Dėkoju visoms tyrime dalyvavusioms pacientėms už sutikimą dalyvauti tyrime. Taip pat esu dėkinga kolegėms gydytojoms, nukreipusioms pacientes konsultavimui. Labai ačiū kolegoms Konsultacinės poliklinikos vedėjai dr. Daivai Kanopienei už dalyvavimą tiriant pacientes, bei palaikymą ir supratimą, onkoginekologijos skyriaus gydytojams ir slaugos personalui už pagalbą atliekant intervencines procedūras – kaip mokslinio darbo etapą, dr. Jolitai Rimienei už histologinių tyrimų atlikimą. Nuoširdžiai dėkoju savo kolegei slaugytojai Onutei Stankevičienei už kasdienį palaikymą, supratimą, profesionalų darbą ir gebėjimą sudaryti pozityvų psichologinį klimatą nepaisant gausaus sudėtingų pacientų srauto.

Taip pat esu labai dėkinga savo šeimai Mamai, Tėčiui ir savo artimiausiam žmogui Vaidui už visapusišką palaikymą, supratimą, kantrybę ir rūpestį mokslinio darbo rengimo metu.

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