VILNIUS UNIVERSITY

Ingrida Vaišnorienė

IN VIVO REFLECTANCE CONFOCAL MICROSCOPY STUDY TO IDENTIFY DYSPLASIA OF MELANOCYTIC NEVUS

Summary of Doctoral Dissertation

Biomedical Sciences, Medicine (06 B)

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The doctoral dissertation was prepared and study was carried out during 2008–2013 academic years at the doctoral studies at the National Cancer Institute (previous Institute of Oncology, Vilnius University). The doctoral dissertation to be defended externally.

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The doctoral dissertation is available at Vilnius University Library and Vilnius University website: *http://www.vu.lt/lt/naujienos/ivykiu-kalendorius.*

VILNIAUS UNIVERSITETAS

Ingrida Vaišnorienė

IN VIVO KONFOKALIOS ATSPINDŽIO MIKROSKOPIJOS TYRIMAS NUSTATANT MELANOCITŲ KILMĖS APGAMO DISPLAZIJĄ

Daktaro disertacijos santrauka

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ABBREVIATIONS

ABCD(E)	signs of melanoma
AUC	area under the curve
CI	confidence interval
EDJ	epidermal-dermal junction
EUROCARE	European Cancer Registry-Based Study of Cancer Patients' Survival and Care
H&E	hematoxylin and eosin
HMB 45	human melanoma black 45
IHC	immunohistochemistry
Melan-A	melanoma antigen recognized by T cells
NCI	National Cancer Institute
NPV	negative prognostic value
p	level of significance
PPV	positive prognostic value
OR	odds ratio
RGB	red, green, blue
ROC	receiver operator characteristic
SD	standard deviation
TNM	Tumor, Nodes, Metastasis
WHO	World Health Organization
χ^2	chi square criterion

1. INTRODUCTION

The incidence of cutaneous melanoma is increasing in Lithuania as well as worldwide [Stang A. et al., 2006]. Despite being the rarest malignant skin condition, melanoma is the commonest cause of skin cancer associated death. According to National Cancer Institute (NCI) data, 200-300, and even more, new cases of cutaneous melanoma is diagnosed every year in our country. The yearly increase in incidence of the disease is about 6%. The incidence of cutaneous melanoma was 10.5 cases per 100000 inhabitants in Lithuania in 2012. According to the European Cancer Registry and EUROCARE-5 (European Cancer Registry-Based Study of Cancer Patients' Survival and Care) research data, the five-year survival rate for cutaneous melanoma in Lithuania is 69,19%, and the overall survival rate in European countries is 83,22% [De Angelis R. et al., 2014]. After analysing survival rates of cutaneous melanoma in Lithuania, the better survival rates were observed for women, younger patients and townspeople [Smailyte G. et al., 2011]. Differences in survival rates motivate improvement in the early detection of cutaneous melanoma and prophylaxis in Lithuania.

According to World Health Organization (WHO) data, people with pale, sun sensitive skin and with many atypical nevi are prone to develop cutaneous melanoma the most. Atypical nevus is the major risk factor for melanoma, and its diagnosis and treatment is one of the most relevant purposes for increasing cutaneous melanoma survival rates.

In 1978, when investigating nevi of patients diagnosed with melanoma and their family members, W.Clark et al. observed and described atypical or dysplastic nevus [Clark W. et al., 1978]. Dysplastic nevus is characterized as a nevus with signs of architectural and cytological atypia as well as signs of host reaction to nevus cells that is observed in histopathological examination. Referring to the data of clinical records, dysplastic nevus is associated with increased risk of melanoma in three aspects: due to the

resemblance to melanoma; as risk factor of melanoma; as possible precursor of melanoma [Elder D. et al., 1993; Goldstein A. et al., 2013; Farber M. et al., 2012; Gandini S. et al., 2005; Elder D. et al., 2010].

Based on the histological classification of a cutaneous melanocytic tumor, proposed by W.Clark, dysplastic nevus is an intermediate stage between non-malignant nevi and cutaneous melanoma when grading morphologically progressive atypia. It is thought, that melanocytic tumors go through a sequential progression: common nevus → dysplastic nevus → *in situ* melanoma → invasive melanoma [Clark W. et al., 1984]. Different authors claim, 25 – 30% of melanomas arise from common nevi [Marks R., et al., 1990; Togawa Y. et al., 2010; Marghoob A. et al., 2002]; therefore, it is essential to recognise signs of atypia in order to evaluate the probability of concrete nevus to become malignant.

Diagnostic challenges of atypical nevus are caused by its resemblance to cutaneous melanoma. Most frequently cited definitions of atypical nevus characterise it as larger than 6mm in diameter, with varying shades of colour and irregular borders. It corresponds to signs of the ABCD rule used for identification of cutaneous melanoma, where A is asymmetry, B – border irregularity, C - various shades of colour, D – greater than 6mm diameter. The accuracy of the diagnosis of melanocytic origin skin tumors evaluated by the naked eye is about 60% [Duffy K. et al., 2012].

Non-invasive optical techniques used in clinical practice are applied to suspect nevus dysplasia and cutaneous melanoma depending on observed signs of atypia. Dermoscopy, or epiluminescence skin microscopy, is currently the main non-invasive diagnostic tool for skin cancer that enables to suspect melanoma and to identify dysplastic changes of nevi more accurately. When compared to a naked eye examination, dermoscopy increases diagnostic accuracy of the examiner from 5 to 30% [Bafounta M. et al., 2001; Kittler H. et al., 2002, Vestergaard M. et al., 2008]. Dysplastic nevus is diagnosed after an histological examination is performed. Therefore, an invasive skin biopsy is needed to diagnose dysplastic nevus. Besides, histopathological diagnosis can be delayed.

The accuracy of the non-invasive diagnostic method is very important for patients with dysplastic nevi syndrome when it is impossible to remove all atypical nevi at once. Accurate non–invasive diagnosis helps to select nevi with highest grade of atypia (risk for melanoma) and remove them earlier.

Accuracy of the non-invasive diagnosis is important for choosing an optimal treatment as well. Guidelines for prophylaxis of melanoma or treatment of nevi suggest a follow-up or excise nevi when signs of atypia are suspicious for melanoma. In case signs of atypia progress, nevi have to be removed surgically with incisions made through the healthy tissue. In cases of severe dysplasia, it is recommended to keep 0.5 cm margins of normal skin around the removed nevus. Surgical treatment tactics for melanoma differ according to the stage of the disease: I stage of melanoma, 1 cm; II stage of melanoma, 2 cm; III-IV stage of melanoma, 2.5 cm or more margins of normal surrounding skin. [NCCI Clinical Practice Guidelines in Oncology, Melanoma. V.3. 2011]. Hence, differences in the treatment of atypical nevi and cutaneous melanoma are another inducement for accurate non-invasive diagnosis.

New non-invasive diagnostic possibilities emerge as a result of developing technologies of medical research. The diagnosis of atypical skin nevi might be made more accurate by high frequency ultrasound, optical coherentive tomography, optical spectroscopy, surface magnetic resonance imaging and *in vivo* reflectance confocal microscopy.

In vivo reflectance confocal microscopy enables the examination of morphologic changes of skin for skin disease diagnostics in real time on the cellular level [Rajadhyaksha M. et al., 1995; Ahlgrimm-Siess V. et al., 2009]. This way the skin is examined in real time, without any invasion or alteration of the tissue examined, and can be repeated with no limitations in time or space. Melanocytic cutaneous lesions have become an important object of this test due to major interest from the doctors who seek for more accurate diagnostics of cutaneous melanoma. *In vivo* reflectance confocal microscopy is currently at the stage of clinical research not only for melanocytic skin tumors, but also for other skin disease diagnostics [Cinotti E. et al.,

2015; Ulrich M. et al., 2015]. Based on preliminary research data, *in vivo* reflectance confocal microscopy corresponds to the results of histological examinations [Rajadhyaksha M. et al. 1999], and diagnostic specificity for melanocytic skin tumors up to 95% [Pellacani G. et al., 2005].

It would be possible to safely follow-up nevi with signs of atypia as well as accurately diagnose melanoma in its early stage, if diagnostic accuracy of *in vivo* reflectance confocal microscopy for diagnosing dysplastic nevi was close to "gold standard" – histological examination. Nevertheless, no reliable research was yet performed that would confirm the accuracy of this method when diagnosing dysplastic nevi.

In comparative clinical trials of dermoscopy and *in vivo* reflectance confocal microscopy, the latter is shown to increase the diagnostic accuracy for melanocytic skin tumors. Nonetheless, the ability of the test to differentiate between dysplastic nevus and melanoma is not explored sufficiently; what is more, diagnostic criteria of dysplastic nevi and cutaneous melanomas are the same. Therefore, there is a need for further research to estimate the importance of separate signs of atypia for differential non-invasive diagnosis of benign, dysplastic and malignant cutaneous melanocytic tumors, comparing results of *in vivo* reflectance confocal microscopy and histological examination.

1.1. Aim and objectives of the study

The aim of this study is to assess the accuracy of *in vivo* reflectance confocal microscopy in the diagnosis of dysplastic melanocytic nevus.

Objectives of the study

1. To estimate the correspondence of features of melanocytic dysplastic nevus and cutaneous melanoma (epidermal disarray, pagetoid cells, discohesive or sparse cells clusters, non-homogeneous clusters, and dense cells clusters) found using *in vivo* reflectance confocal microscopy to features of cytological atypia of histological examination.

2. To assess the sensitivity and specificity of *in vivo* reflectance confocal microscopy for diagnosing melanocytic dysplastic nevi.
3. To assess the sensitivity and specificity of *in vivo* reflectance confocal microscopy for diagnosing cutaneous melanoma.

1.2. Scientific novelty and practical value of the study

Collaboration among scientists, medical physics and doctors of the National Cancer Institute enabled the realization of a research project and the purchase of the *in vivo* reflectance confocal microscopy equipment.

There is a small amount of data regarding use of *in vivo* reflectance confocal microscopy for the diagnosis of dysplastic nevi of melanocytic origin. According to data from individual researchers, atypic changes of dysplastic nevi in histological examinations correlate with ones obtained from *in vivo* confocal reflectance microscopy. Non-homogeneous clusters are used to distinguish dysplastic nevi from common ones. Results from our study did not confirm the diagnostic value of non-homogeneous clusters. Other evaluated factors (epidermal disarray, padgetoid cells, discohesive or spare cells clusters, dense cells clusters) also were not significant to diagnose melanocytic dysplastic nevi.

To find new possible criteria for melanocytic dysplastic nevi, we performed reflectance confocal microscopy of histologic skin and skin tumor sections together with applied immunohistochemical melanocyte markers. No publications were found about tests of melanocytes reflection on histological skin sections using reflectance confocal microscopy to be previously performed; therefore, we patented this method of the test (national (No 2014-100) and international (No PCT/IB2014/067441) requests "Melanocyte imaging by using reflectance confocal microscopy in histological skin sections" were requisitioned).

2. OBJECT AND METHODS OF THE STUDY

2.1. Selection and characteristics of participants, and research process

A prospective observational study was carried out at the National Cancer Institute (NCI) from 2010 to 2013. The approval for the study from the Vilnius Regional Biomedical Research Ethics Committee was received before the beginning of the research, in 5 November, 2010 (Protocol ADT-01, No 158200-11-256-59).

138 patients were included into the study based on these criteria:

- Older than 18 years;
- Atypical nevus or cutaneous melanoma suspected during dermoscopy.

Exclusion criteria:

- Patient younger than 18 years;
- Allergy for local anaesthetics.

All patients included in the study underwent surgical treatment or excisional biopsy of the skin tumor, and an histological examination was performed to identify cutaneous melanocytic tumor.

All patients were informed about the aim and the course of the study. All of the participants signed the Subject Information and Consent Form.

The selection of patients, dermoscopy and *in vivo* reflectance confocal microscopy of skin cancer, tumor excision, reflectance confocal microscopy of histological sections of skin tumor, and analysis of *in vivo* reflectance confocal microscopy and histological findings were performed at the NCI. An histological examination of the cutaneous tumor, the establishment of the final pathologic diagnosis, scanning of the histological samples as well as the preparation of histological sections for reflectance confocal microscopy and their immunohistochemical examination were performed

in the National Center of Pathology, Affiliate of Vilnius University hospital Santariskiu Klinikos. Statistical analysis of the data was performed in the Faculty of Mathematics and Informatics at Vilnius University.

2.2. Baseline characteristics of participants

138 patients were included in the study: 90 women and 48 men. The mean age of the patients was 42 years, standard deviation (SD) – 16, the youngest patient was 18 years old, the oldest – 78 years.

Following surgical treatment / excisional biopsy and histological examination of the skin cancer, participants were divided into three groups based on final the pathologic diagnosis: I group – patients diagnosed with common nevus with no signs of cytological atypia (n=25), II group – patients diagnosed with dysplastic nevus (n=69), and III group – patients diagnosed with cutaneous melanoma (n=44). Based on TNM (Tumor, Nodes, Metastasis) Classification of Malignant Tumours, 9 out of 44 cases were stage 0 melanoma (melanoma *in situ*), 35 – invasive, i.e. stage I-IV; mean *Breslow's* thickness of cutaneous melanoma was 1,35 mm.

2.3. Methods

2.3.1. Dermoscopy

Features of atypical nevus or cutaneous melanoma of 138 patients were identified during consultation by an NCI doctor after examination and dermoscopy in year 2010-2013. Dermoscopic examination was performed with a manual *Heine Delta 20* (*Heine Optotechnik*, Germany) contact dermoscope. Dermoscopic signs of atypia were assessed according to structural analysis algorithm, and dermoscopic criteria of face, soles and palms proposed by *T.Saida*. Digital dermoscopic pictures of the cutaneous tumor with signs of atypia, which were selected for the study, were saved using a *VivaCam* digital camera.

2.3.2. In vivo *reflectance confocal microscopy*

In vivo reflectance confocal miscroscopy was performed using the skin visualisation system *VivaScope 1500* (*Lucid Inc*., USA).

In vivo reflectance confocal microscopy is an optical method of examination, which allows the imaging of the skin in cellular resolution. Confocal microscopy is the test, which enables imaging of cells *in vivo* in real time, and evaluating shape, size and nuclear contours in the superficial layers of the skin. This test is best suited to diagnosing pigmented skin lesions, as melanin is the best contrast agent when examining skin with reflectance confocal microscopy. During the examination, the skin is illuminated using a diagnostic (830 nm wavelength with illuminating power up to 10 mW) safe laser, and reflectance of the light returning from skin microstructures is registered in the chosen depth of the skin in 500μm^2 field using a complex optical system.

The software creates a mosaic skin macro image from the micro images scanned, therefore, enabling visualization of the structure of up to 8 mm^2 of horizontal skin surface. By using the confocal skin imaging system, skin can be visualized to 250 μm in depth from the skin surface. The depth of imaging is limited because the light is scattered and absorbed by skin tissues. A skin-contact device consisting of a metal ring and a coverglass window is fixed to the clean, dry skin using special glue after centrating the skin tumour to be examined. The device is fixed to the skin using a drop of immersion oil (*Crodamol STS oil*; USA). A x30 objective lens is then attached to the skin-contact device (transmission medium – *Aquasonic gel 100*; USA).

The reflectance confocal skin imaging system captures horizontal images of tissue in these parameters: 500 x 500 μm, 1024 x 1024 pixels. An automatic scanner scans up to 8 x 8 mm field-of-view, and afterwards these images are composed to mosaic image of horizontal tissue section. During the examination, images of reflectance confocal microscopy were scanned in skin depth of 30, 60 and 90 μm.

Structural changes of the skin that are characteristic of dysplastic nevus, which were identified using *in vivo* reflectance confocal microscopy in the mentioned depth, were analysed and evaluated in digital mosaic (*Vivablock*) images. The typical "honeycomb" pattern of a normal epidermis was assessed in 30 μm skin depth (below *stratum corneum*). The absence of it, i.e. irregular arrangement of cells with no clearly visible borders of cells, was considered as "epidermal disarray." Large (bigger than 20 μm), bright or branched atypical cells found in the same depth, i.e. 30 μm below *stratum corneum*, were considered as "pagetoid infiltration." Cell clusters, their homogeneity and borders were assessed in the level of dermoepidermal junction – 60 and 90 μm below *stratum corneum*. Cell clusters with no clearly visible borders or loss of integrity were assessed as "discohesive" or atypical sparse cells clusters. Common bright monomorphic cell clusters with clearly visible borders were called "dense cells clusters."

2.3.3. Histological examination of skin tumor, establishment of final pathologic diagnosis and scanning of histological samples

After surgical treatment/excisional biopsy, tissue samples were fixed in a 10 percent buffered formalin solution. Regular histological and immunohistochemical tests were performed to establish histopathological diagnosis.

20 times magnified histological images were scanned using *Aperio ScanScope GL* cover slip scanner (*Asperio Technologies, Vista*, Canada, USA).

Additional histological tests were performed in case of melanocytic tumors with signs of atypical nevocytes observed in *in vivo* reflectance confocal microscopy images. Adjacent sections of histological samples of those cutaneous tumors were stained with H&E, Melan-A and HMB 45 in order to estimate the relation between reflection of melanocytes under *in vivo* reflectance confocal microscopy and histological/immunohistochemical features.

2.3.4. Analysis of in vivo *reflectance confocal microscopy images and histological microsamples*

Signs of cutaneous melanocytic tumors that are characteristic of cytological atypia – epidermal dissaray, pagetoid infiltration, dense cells clusters, nonhomogeneous clusters, irregular discohesive and sparse cells clusters – observed under *in vivo* reflectance confocal microscope were evaluated comparing *in vivo* reflectance confocal microscopy images and images of the same tumors' microsamples stained with H&E. Depending on the differences in features characteristic of particular localisation (e.g. tumors with atypical clusters observed locally in the periphery of the tumour), their correspondence to the results of histological/immunohistocheminal tests was searched according to the known depth of *in vivo* reflectance confocal microscopy images from the skin surface.

2.3.5. Reflectance confocal microscopy examination of histological sections (ex vivo)

A reflectance confocal microscopy examination of histological skin sections was performed to assess the reflection of skin melanocytes visualized by reflectance confocal microscopy in a healthy human skin and cutaneous melanocytic tumors (nevi and melanomas). The examination was performed with tumors that were previously examined using *in vivo* reflectance confocal microscopy. After excising the tumour, samples were fixed in 10 percent buffered formalin solution. Routine histological tests were made to establish histopathological diagnosis. Subsequently, tumors were additionally sectioned; their reflectance confocal microscopy and immunohistochemical tests were performed to mark melanocytes.

Preparation of histological skin sections. A formalin-fixed paraffin embedded tissue column was sectioned into 5 μm thick slices using microtome. Afterwards, sections were floated on a warm water bath, transferred to clean glass microscopic slides and dried at 80-90°C temperature.

Such sections of melanocytic tumors were examined using reflectance confocal microscopy.

Reflectance confocal microscopy of histological sections (*ex vivo*). As it was already mentioned, reflectance confocal microscopy was performed on prepared histological skin sections that were examined under a *Nikon "Eclipse TE2000"* (Japan) microscope with *C1si* confocal scanning system (lens – *x60 Plan Apo VC oil*, 1.4 numerical aperture). A 515 nm wavelength argon ion laser was used for the illumination, and a standard 12-bits three colors RGB detector and a 500-530 nm bandpass filter were used to detect the reflection.

Immunohistochemical tests. After receiving reflectance confocal microscopy images of histological sections, immunohistochemical (IHC) tests of paraffin embedded sections were performed using specific melanocyte markers – Melan-A and HMB 45. *Ventana BenchMark Ultra* automated staining instrument (*Ventana Medical Systems, Tucson, Arizona*, USA) was used for protein expression analysis of biological markers. Antigen epitope retrieval was made using Ventana Cell Conditioning Solution CC1 (pH 8,5) - 95°C (Melan-A – 36 min., HBM 45 – 64 min.). Later, sections were incubated with monoclonal antibodies - 37°C 32min., with 1:100 diluted solution, using the *Ventana Ultraview DAB* detection system. Monoclonal mouse antibodies (Ab) used for the study were these: HMB 45 melanosome (clone HMB 45; *DAKO Diagnostics, Glostrup*, Denmark) and Melan-A (clone A103; *DAKO Diagnostics, Glostrup*, Denmark).

Analysis of reflectance confocal microscopy and immunohistochemical images of histological samples. After performing reflectance confocal microscopy of histological sections of healthy skin, Spitz nevus and cutaneous melanoma, and after receiving the images, immunohistochemical tests of these histological samples were performed. Images of immunohistochemical tests of histological samples were magnified 20 times and scanned using an *Aperio ScanScope GL* cover slip scanner (*Asperio Technologies, Vista*,

Canada, USA). Reflectance confocal microscopy images of histological sections were compared to immunohistochemical test images. The contrast of melanocyte and melanocyte nucleus and cytoplasm was assessed in comparison with the contrast of surrounding cells and structures. The reflection of melanocytes was evaluated in regard to the maturation of melanosomes identified by immunohistochemical tests.

2.3.6. Statistical data analysis

The accuracy, sensitivity and specificity of the test, and positive and negative prognostic values of separate features were calculated to assess the reliability of *in vivo* reflectance confocal microscopy for diagnosis of dysplastic nevi and cutaneous melanoma.

True/false and positive/negative results were established depending on the histological examination as a diagnostic gold standard.

Binary logistic regression was used to measure the diagnostic value of separate criteria of *in vivo* reflectance confocal microscopy depending on the diagnostic likelihood ratios and their confidence intervals, ROC (receiver operator characteristic) curves, and area under the curve (AUC).

The diagnostic value of the test, depending on the area under the curve, was assessed as follows: when area under the curve is 0,9-1 – the test is great, 0,8-0,9 – very good, 0,6-0,7 – satisfactory, 0,5-0,6 – insufficient.

The inter-relation of qualitative criteria was assessed adjusting chi square (χ^2) criterion. The significance level of 0,05 was used to test statistical hypotheses. Models in tables were set under statistical values of probability (χ^2). Statistical analysis of the data was performed with SAS 9.3 statistical package.

3. RESULTS

Skin tumors that had dermoscopic features of atypia were examined using *in vivo* reflectance confocal microscopy. Later, the examined tumor was surgically removed, and an histological examination was performed. One hundred thirty-eight cases of cutaneous melanocytic tumor were examined and divided into three groups according to the final histopathological diagnosis:

- Common melanocytic nevi with no signs of atypia (n=25);
- Dysplastic nevi (n=69);
- Cutaneous melanomas (n=44).

Dyplastic nevi were further divided according to the level of cytological atypia assessed during the histological examination:

- Mild cytological atypia (n=);
- Moderate cytological atypia (n=42);
- Severe cytological atypia of melanocytes (n=20).

Nine cases in the cutaneous melanoma group were stage 0 (melanoma *in situ*), remaining – stage I-IV. The mean *Breslow's* thickness of cutaneous melanoma was 1,35 mm, the thickest – 9 mm.

The results were analysed according to the stages of the research:

1. Features of cutaneous melanocytic tumor that were assessed using *in vivo* reflectance confocal microscopy and their correspondence to the results of histological examination were estimated. These features were analysed:
   - Dense cells clusters;
   - Nonhomogeneous clusters;
   - Irregular discohesive and sparse cells clusters;
   - Pagetoid cells;
   - Epidermal disarray.

2. Sensitivity and specificity of *in vivo* reflectance confocal microscopy was evaluated for diagnosing dysplastic nevi and cutaneous melanoma according to signs mentioned.
3. Diagnosis of cutaneous melanoma was assessed after two criteria of *in vivo* reflectance confocal microscopy.
4. Clinical case analysis of cutaneous melanoma was performed and a working hypothesis proposed, stating that melanocytes could have light-transmitting cytoplasm and refractile nucleus when examined by *in vivo* reflectance confocal microscopy.
5. Visualization of melanocytes with reflectance confocal microscopy of histological sections (*ex vivo*) was performed.

Figure 3.1 represents characteristics of melanocytic tumors according to the features identified by *in vivo* reflectance confocal microscopy.

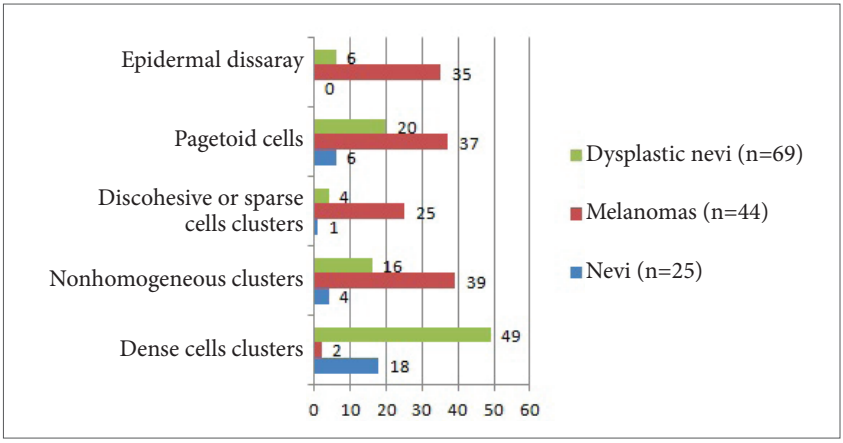

Figure 3.1. Characteristics of melanocytic tumors according to the features identified by *in vivo* reflectance confocal microscopy (n – number of tumors with positive feature).

After examining the images of *in vivo* reflectance confocal microscopy of 138 cutaneous melanocytic tumors, epidermal disarray was not found in a single common nevus. This sign was positive in 35 melanomas out of 44. Pagetoid cells were frequently observed in melanomas as well: this sign was positive in 37 cases out of 44. Discohesive and sparse cells clusters are rarely found in common nevi and in dysplastic nevi; nevertheless, this sign was positive in 25 melanomas out of 44. Nonhomogeneous clusters were observed in 16 dysplastic nevi out of 69, in 39 melanomas out of 44, and in 4 common nevi out of 25. Dense cells clusters were more common for nevi, i.e. 18 out of 25 common nevi were composed of dense cells clusters.

3.1. Correspondence of features of *in vivo* reflectance confocal microscopy to histological features

Further, images of cutaneous melanocytic tumors – dysplastic nevi and cutaneous melanoma – obtained using *in vivo* reflectance confocal microscopy are presented, describing them according to the signs mentioned above. Images obtained using *in vivo* reflectance confocal microscopy and images of micro samples stained with H&E (hematoxylin and eosin) of the identical tumor are also presented, analysing correspondence of signs observed by *in vivo* reflectance confocal microscopy to histological signs of atypia of cutaneous melanocytic tumor.

Dense cells clusters

When analysing images of *in vivo* reflectance confocal microscopy, dense cells clusters were defined as compact aggregates of well demarcated reflective cells. This feature was identified in images, made 30-60 μm below *stratum corneum*, i.e. at the level of dermoepidermal junction. Dense cells clusters observed using *in vivo* reflectance confocal microscopy corresponded to the histological evaluation of atypical melanocytes in 51 out of 69 cutaneous melanocytic tumors examined.

By analysing *in vivo* reflectance confocal microscopy of different cutaneous melanocytic tumors and histological images of the same tumors' micro samples stained with H+E, it was found that dense cells clusters correspond to clusters and aggregates of melanin-rich cells. Cells having melanin pigment in their cytoplasm are highly refractile when examined by *in vivo* reflectance confocal microscopy (Fig. 3.1.1).

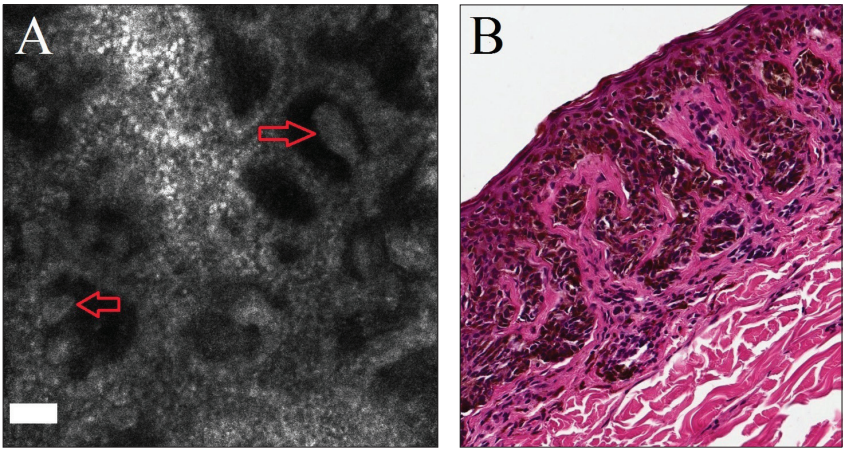

Figure 3.1.1. *In vivo* reflectance confocal microscopy image of dysplastic nevus with moderate cytological atypia (A), where the tumor is observed 30 μm below *stratum corneum*, i.e. in the superficial level of epidermal-dermal junction (EDJ) (0,5 x 0,5 mm, scale = 50μm). Dense cells clusters that deform skin architecture characteristic of EDJ (red arrows) are visible in entire area. Histological image (H&E stain, x20) (B) of the identical tumor's micro sample.

Figure 3.1.1 displays clusters of atypical melanocytes, which under the *in vivo* reflectance confocal microscope seem like aggregates of monomorphic light-reflecting cells that deform typical architecture of the skin. The same clusters, which are seen in an histological examination, are composed of melanocytes containing melanin granules in cytoplasm.

Next represented is a mosaic image of dense cells clusters of dysplastic nevus with severe cytological nevomelanocytic atypia obtained with *in vivo* reflectance confocal microscopy (Fig. 3.1.2). Differently arranged clusters are visible in the presented image of dyplastic nevus.

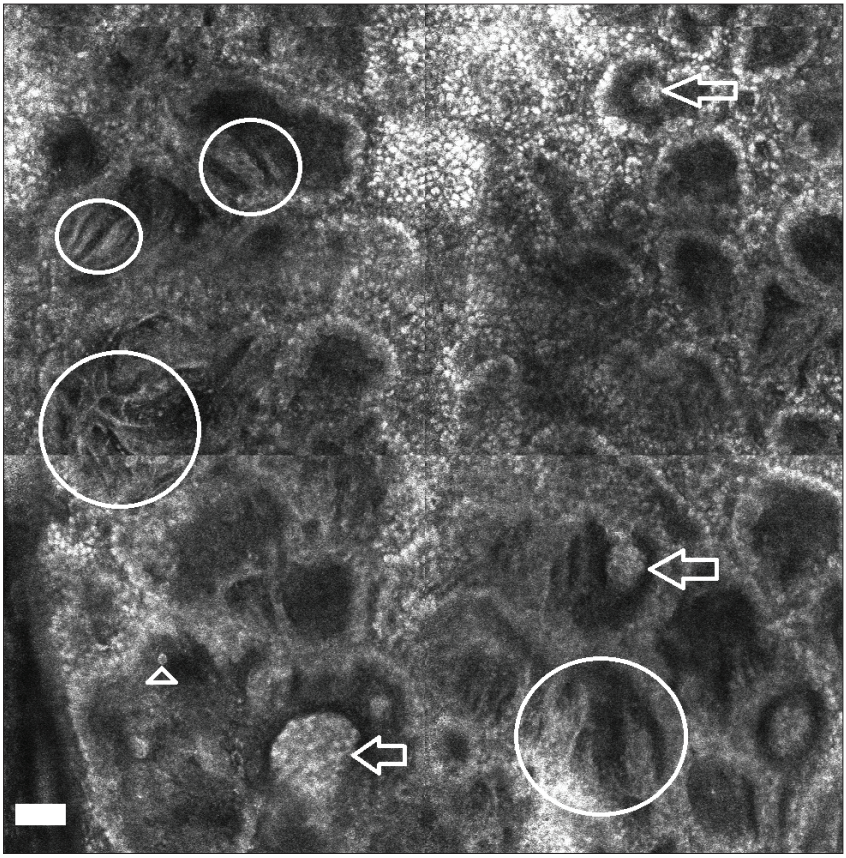

Figure 3.1.2. Mosaic image of dyplastic nevus with severe cytological atypia obtained using *in vivo* reflectance confocal microscopy, where the tumor is identified 30 μm below *stratum corneum* (1 x 1 mm, scale = 50 μm).

It can be seen in Figure 3.1.2 that dense cells clusters found in the level of the epidermal-dermal junction are irregularly scattered, deforming typical architecture of the skin (arrows). Dense cells clusters (encircled) are bridging between adjacent rete ridges. This kind of clusters' arrangement is characteristic histological feature of dysplastic nevus. Aberrant cell with large hipo-refractile nucleus and refractile cytoplasm, marked by a triangle, is likely to be an atypical melanocyte.

Non homogeneous clusters

The term "non homogeneous clusters" defines a collection of cells with varying reflection and irregular, hardy distinguishable borders. This feature is identified 30-60 μm below the skin surface, i.e. at the dermoepidermal junction. The sign of nonhomogeneous clusters found by *in vivo* reflectance confocal microscopy corresponds to histological evaluation of atypical melanocytes in 55 out of 59 cutaneous melanocytic tumors examined.

By analysing *in vivo* reflectance confocal microscopy of different cutaneous melanocytic tumors and histological images of the same tumors' micro samples stained with H+E, it was found that non homogeneous clusters correspond to coalescent, irregularly shaped clusters with sparse melanin-rich cells in the epidermal-dermal junction (Fig. 3.1.3).

Figure 3.1.1 of *in vivo* reflectance confocal microscopy displays nonhomogeneous clusters connecting adjacent rete ridges, thus forming "bridges" that deform typical skin architecture. Additional signs characteristic of dysplastic nevus – fibrosis and inflammatory infiltration in papillary dermis – are seen as well. Compared to an histological image of the same tumor's micro sample (B), cells (nevocytes) composing clusters are polymorphic: several contain melanin granules, others – epithelioid pigment-free cells.

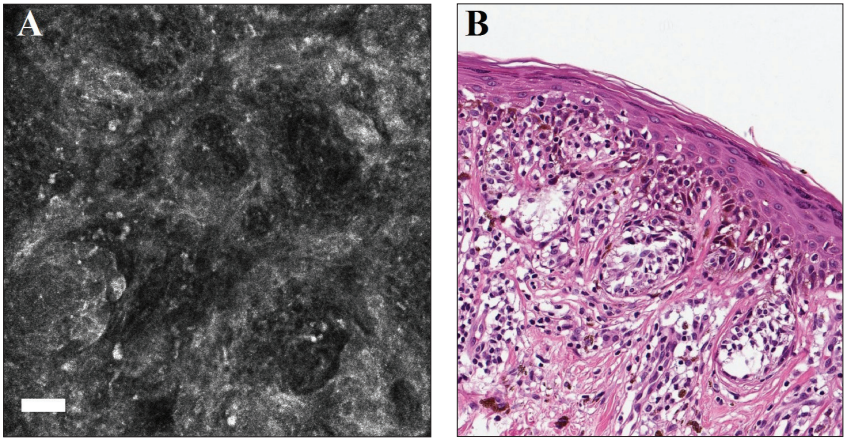

Figure 3.1.3. *In vivo* reflectance confocal microscopy images of dysplastic nevus with severe cytological atypia (A), with tumour seen 60 μm below *stratum corneum* at the level of epidermal-dermal junction (0,5 mm x 0,5 mm, scale = 50 μm); histological image of the same tumor's micro sample (H&E stain, x20) (B).

Irregular discohesive or sparse cells clusters

All collections were called irregular discohesive or sparse cells clusters when discohesive cells clusters with irregular cell borders (Fig. 3.1.4) or dark (non-reflecting light) structures with sparse light reflecting cells and clear margins of clusters (Fig. 3.1.5) were seen in epidermal-dermal junction 30-60 μm below *stratum corneum*. The sign of irregular discohesive or sparse cells clusters identified under *in vivo* reflectance confocal microscope corresponds to the histological evaluation of atypical melanocytes in 29 cutaneous melanocytic tumor cases out of 30.

The images in figure 3.1.4 display that irregular discohesive (in red circle) clusters are located in the epidermal-dermal junction: the majority of cluster composing cells have refractile cytoplasm and hipo-refractile nucleus, dark areas up to 30-100 μm are seen between refractile cells, and tiny up to 10 μm refractile structures are found within these areas. Histologically. irregular discohesive clusters correspond to atypical epithelioid cells clusters, the majority of which have pigment melanin.

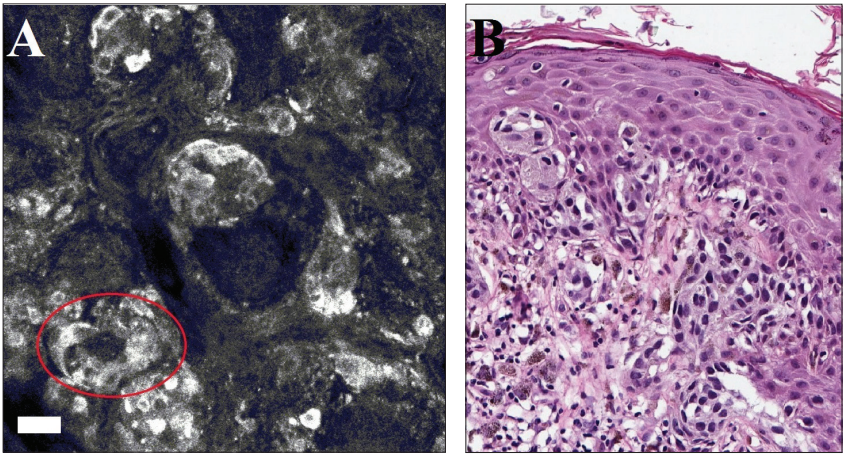

Figure 3.1.4. *In vivo* reflectance confocal microscopy image of superficial spreading cutaneous melanoma (Breslow thickness – 0,9 mm) (A), tumor is found 50 μm below *stratum corneum* at the level of epidermal-dermal junction (0,5 mm x 0,5 mm, scale = 50 μm); histological image of the same tumor's micro sample (H&E stain, x20) (B).

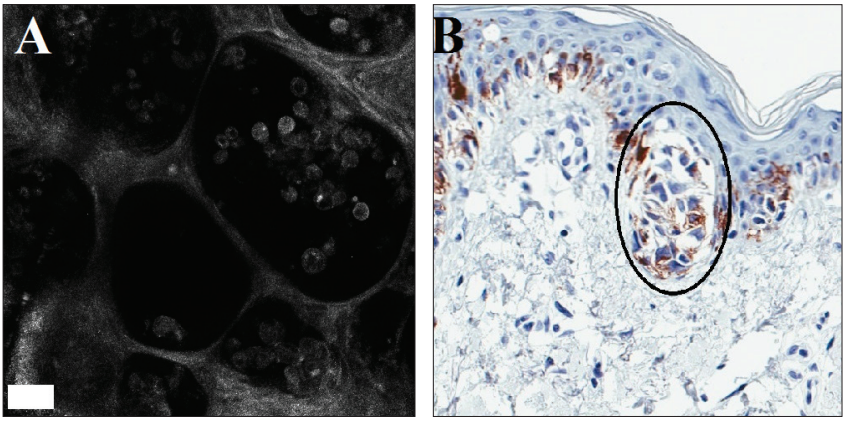

Figure 3.1.5. *In vivo* reflectance confocal microscopy image of superficial spreading cutaneous melanoma (Breslow thickness – 0,9 mm) (A), tumor is found 50 μm below *stratum corneum* at the level of epidermal-dermal junction (0,5 mm x 0,5 mm, scale = 50 μm); an histological image of the same tumor's micro sample (immunohistochemical examination with HMB 45 markers, x20) (B).

The *in vivo* reflectance confocal microscopy image (Fig. 3.1.5) displays the entire cancer area filled with sparse cells clusters, i.e. dark structures with clear margins up to 300 μm, and easily distinguishable sparse cells with refractile cytoplasm and hipo-refractile nucleus. In an histological examination, these clusters are equivalent to irregular discohesive (B; black circle) intraepidermal clusters composed of cells with immature melanosomes (HMB 45 positive cells). These clusters spread from 20 to 75 μm below *stratum corneum* when examined by *in vivo* reflectance confocal microscopy (see chapter 3.3 "Clinical case analysis of cutaneous melanoma").

Pagetoid cells

Bright branching or round cells with hipo-refractile nuclei, identified under reflectance confocal microscopy *in vivo*, were estimated as atypical melanocytes (Fig. 3.1.6 – 3.1.7). Pagetoid cells observed under reflectance confocal microscope *in vivo* correspond to histological evaluation of atypical melanocytes in 57 out of 63 cutaneous melanocytic tumors examined.

Images in Fig. 3.1.6 display that atypical bright branching or round cells (encircled) are clearly seen in superficial skin layers (A – D). These cells match the definition of atypical melanocyte in superficial epidermal layers (or pagetoid cell).

Analysing the *in vivo* reflectance confocal microscopy image of dysplastic nevus displayed in Fig. 3.1.7, it can be seen that atypical, highly refractile round or branching cells distinguish among pigmented monomorphic epidermal keratinocytes. The comparison of pagetoid cells in images of *in vivo* reflectance confocal microscopy and histological examination is presented in Fig. 3.1.8.

Fig. 3.1.8 reveals that atypical melanocytes marked by arrows and located in the intermediate epidermal layers (so called pagetoid cells) are identified by both *in vivo* reflectance confocal microscopy and histological examination.

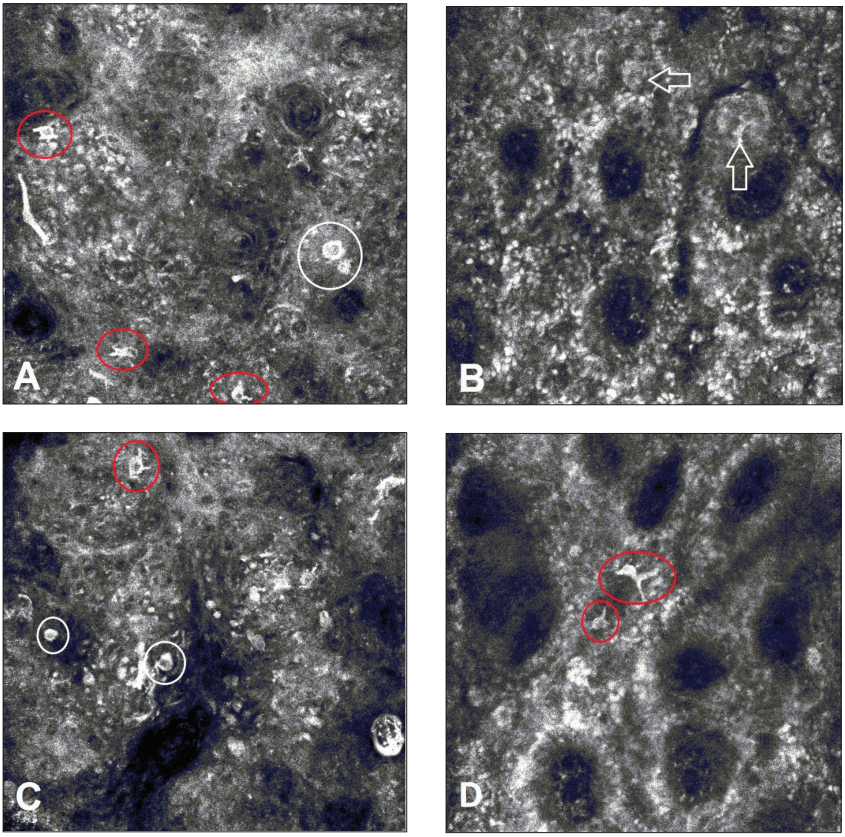

Figure 3.1.6. *In vivo* reflectance confocal microscopy images (0,5 x 0,5 mm) of cutaneous melanoma observed in granular layer of the epidermis (A) and at various depths of epidermal-dermal junction (B – D).

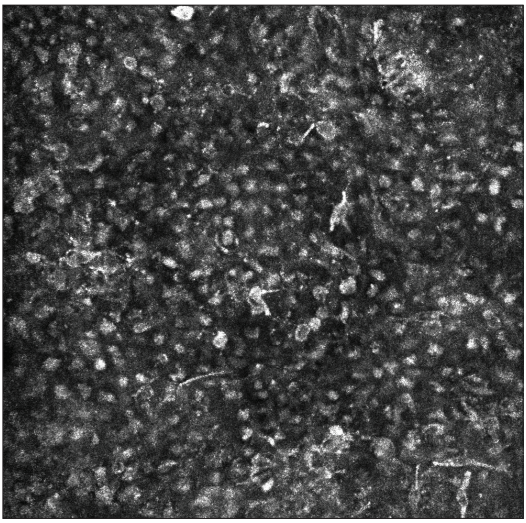

Figure 3.1.7. *In vivo* reflectance confocal microscopy image of dysplastic nevus with severe cytological atypia, the tumor is observed in intermediate epidermal layers (0,5 x 0,5 mm).

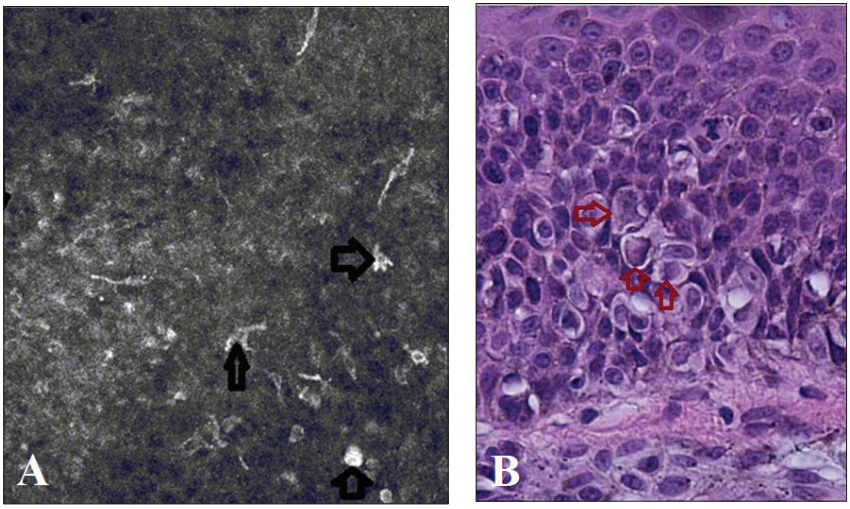

Figure 3.1.8. The *In vivo* reflectance confocal microscopy image of a cutaneous melanoma (A), tumor is identified in intermediate epidermal layers (0,5 x 0,5 mm), and the histological image of the same tumor's micro sample (B).

Epidermal disarray

When examining the skin by *in vivo* reflectance confocal microscopy, the typical honeycomb pattern is observed in the granular layer of the skin epidermis, i.e. 10-20 μm below *stratum corneum* (Fig. 3.1.9). The sign of epidermal disarray under *in vivo* reflectance confocal microscope corresponds to the histological evaluation of atypical melanocytes in 41 out of 41 cutaneous melanocytic tumors examined.

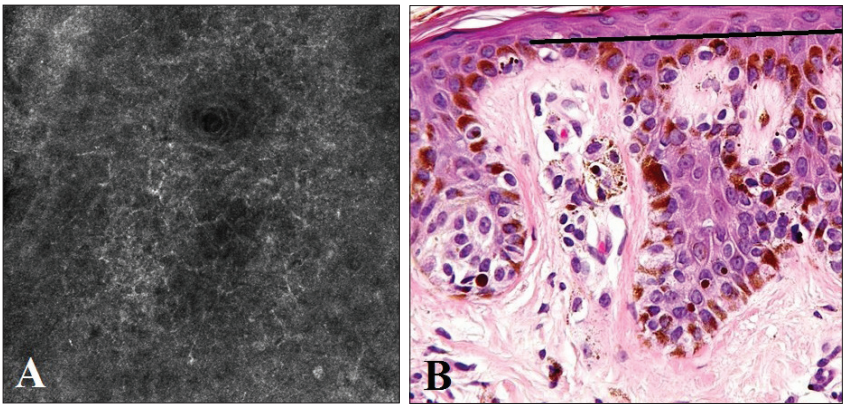

Figure 3.1.9. The *in vivo* reflectance confocal microscopy image of dysplastic nevus (A), where the tumor is identified 30 μm below *stratum corneum* in intermediate epidermal layers (0,5 x 0,5 mm); histological image of the same tumor's histopathological micro sample (H&E stain, x20) (B).

As it is displayed in Figure 3.1.9, the honeycomb pattern, characteristic of an epidermis with regular arrangement of unchanged keratinocytes, is observed in intermediate epidermal layers, i.e. granular layer, when examined by *in vivo* reflectance confocal microscopy. The granular layer (*stratum granulosum*) is composed of flattened keratinocytes (diameter – 25-35 μm) with large oval nuclei. Nuclei of the cells are dark central cellular formations. The cytoplasmic edge is brighter and granulated by keratohyalin granules. The regular arrangement of keratinocytes seen in the granular layer of the epidermis resembles a honeycomb.

If the regular honeycomb pattern is absent in the granular epidermal layer when examining by *in vivo* reflectance confocal microscopy, it is called epidermal disarray (Fig. 3.1.10, B).

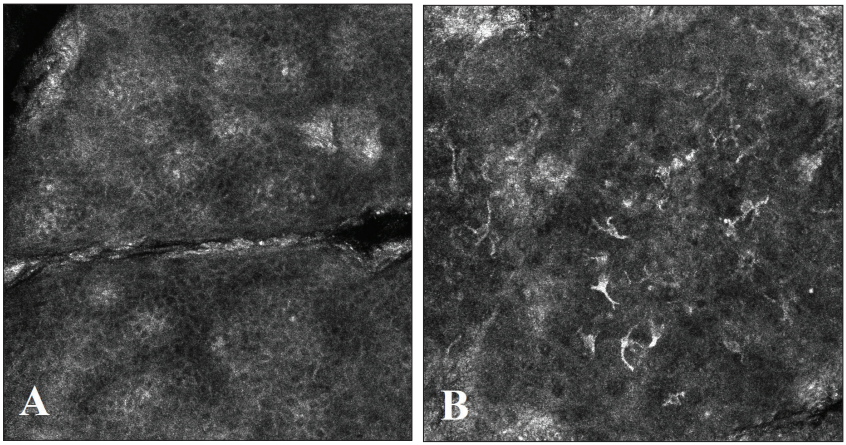

Figure 3.1.10. *In vivo* reflectance confocal microscopy images of healthy skin (A) and cutaneous melanoma (B), observed in granular layer of epidermis (0,5 x 0,5 mm; depth – 20-30 μm below *stratum corneum*).

As it can be seen in Figure 3.1.10, when examining with an *in vivo* reflectance confocal microscope, the regular epidermis of healthy skin differs from epidermal disarray with pagetoid infiltration (atypical melanocytes – large bright branching cells) in case of cutaneous melanoma. The sign of epidermal disarray observed in cutaneous melanocytic tumors is equivalent to the same signs identified in histological images.

Depending on *in vivo* reflectance confocal microscopy images in case of dense cells clusters observed, it could be stated that adding this diagnostic test to non-invasive diagnosis helps to forecast histological evaluation and final pathological diagnosis more accurately.

3.2. Diagnostic value of features of cutaneous melanocytic tumor observed by *in vivo* reflectance confocal microscopy

Further, diagnostic accuracy of *in vivo* reflectance confocal microscopy is presented, according to different features, in order to differentiate between common, dysplastic nevi and cutaneous melanoma. Accuracy, sensitivity and specificity of *in vivo* reflectance confocal microscopy, its positive and negative prognostic values for different features were evaluated for diagnosis of dysplastic nevi and cutaneous melanoma, and are presented in Tables 3.2.1 and 3.2.2 respectively. Data presented in these tables show that the accuracy of *in vivo* reflectance confocal microscopy is better when diagnosing cutaneous melanoma compared to dysplastic nevi.

Table 3.2.1. Accuracy, sensitivity, specificity, positive and negative prognostic values of *in vivo* reflectance confocal microscopy for diagnosing dysplastic nevi (when analysing benign cutaneous melanocytic tumors – common nevi and dysplastic nevi – according to signs listed, n=94).

Feature	Accuracy (%)	95 % CI	Sensitivity (%)	95% CI	Specificity (%)	95% CI	PPV	NPV
Dense cells clusters	59,58	49,46–68,94	71,02	59,37–80,44	28,00	14,06–47,80	73,13	25,93
Non-homogeneous clusters	39,36	30,08–49,48	23,19	14,71–34,49	84,00	64,73–94,21	80,00	28,38
Discohesive or sparse cells cluster	29,79	21,45–39,71	5,8	1,85–14,40	96,00	78,86–99,99	80,00	26,97
Pagetoid cells	41,49	32,05–51,60	28,99	19,56–40,63	76,00	56,25–88,83	76,92	27,94
Epidermal disarray	32,98	24,28–43,01	8,7	3,72–18,02	100,00	84,24–100,00	100,00	22,12

CI- confidence interval; PPV- positive prognostic value, NPV- negative prognostic value

Table 3.2.2. Accuracy, sensitivity, specificity, positive and negative prognostic values of *in vivo* reflectance confocal microscopy for diagnosing cutaneous melanoma (when analysing all cutaneous melanocytic tumors according to features listed, n=138).

Feature	Accuracy (%)	95 % CI	Sensitivity (%)	95% CI	Specificity (%)	95% CI	PPV	NPV
Dense cells clusters	21,01	15,00–28,59	4,55	0,42–15,97	28,72	20,52–38,60	2,9	39,13
Non-homogeneous clusters	81,88	74,57–87,47	88,64	75,57–95,50	78,72	69,33–85,86	66,10	93,67
Discohesive or sparse cells cluster	82,61	75,37–88,09	56,82	42,21–70,33	94,68	87,85–98,01	83,33	82,41
Pagetoid cells	76,09	68,29–82,47	84,09	70,32–92,39	72,34	62,52–80,41	58,73	90,67
Epidermal disarray	89,13	82,74–93,43	79,55	65,28–89,07	93,62	86,50–97,31	85,37	90,72

CI- confidence interval; PPV- positive prognostic value, NPV- negative prognostic value

The specificity of *in vivo* reflectance confocal microscopy can be estimated for common nevi (all nevi, including dysplastic ones) or only for dysplastic nevi. The specificity of *in vivo* reflectance confocal microscopy according to different features is compared in Fig. 3.2.1, and sensitivity for diagnosing dysplastic nevi and cutaneous melanoma – in Fig. 3.2.2. Data in these charts demonstrate that the specificity of *in vivo* reflectance confocal microscopy differs little and is high enough when diagnosing both, all nevi and dysplastic nevi, according to all features listed, except in case of dense cells clusters. Therefore, dense cells clusters are little specific as feature for dysplastic nevus diagnosis by *in vivo* reflectance confocal microscopy. However, this feature is sensitive enough when diagnosing dysplastic nevi. Other features are less sensitive for dysplastic nevi, but more sensitive for cutaneous melanoma.

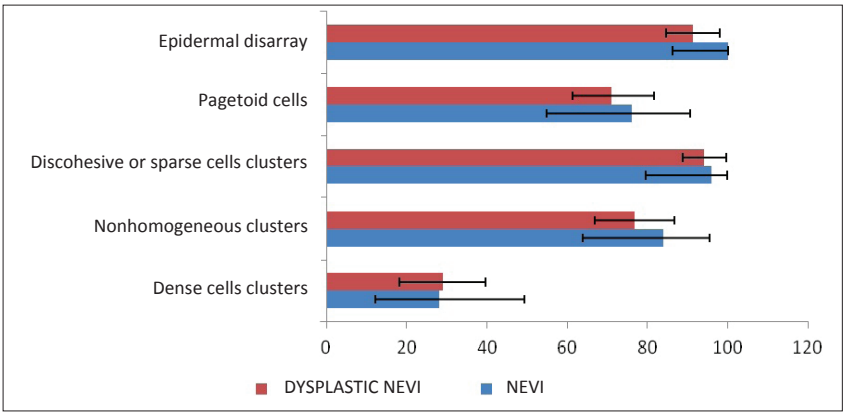

Figure 3.2.1. Specificity (%) of *in vivo* reflectance confocal microscopy

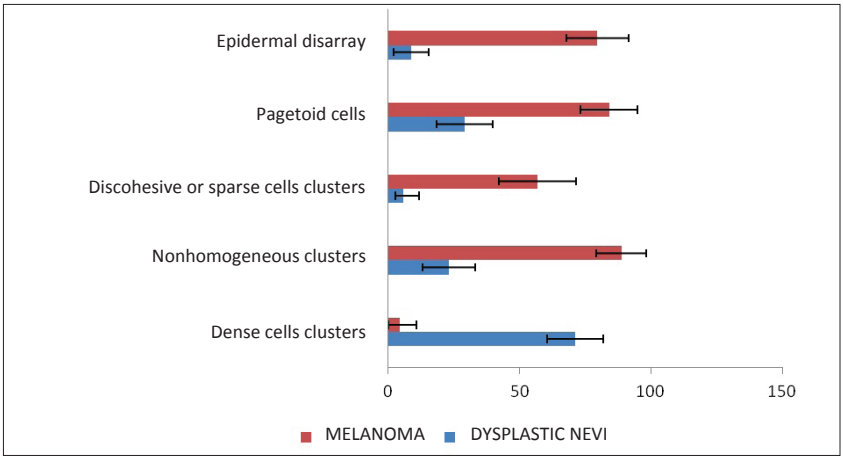

Figure 3.2.2. Sensitivity (%) of *in vivo* reflectance confocal microscopy

After performing logistic regression analysis of *in vivo* reflectance confocal microscopy, neither of the features evaluated were statistically significant for diagnosing dysplastic nevi. However, all together and each separately, they were significant for diagnosing cutaneous melanoma. The results of statistical analysis, i.e. odds ratio and *p* value of *in vivo* reflectance

confocal microscopy, for diagnosing cutaneous melanoma, is presented in Table 3.2.3. Data in this table show that epidermal disarray is the most significant cutaneous melanoma feature for *in vivo* reflectance confocal microscopy.

Table 3.2.3. The employment of features of *in vivo* reflectance confocal microscopy for diagnosing cutaneous melanoma

Feature	OR (95 % CI)	*p* value
Epidermal disarray	57,037 (18,896–172,165)	<0,0001
Non-homogeneous clusters	28,857 (10,059–82,787)	<0,0001
Dense cells clusters	0,019 (0,004–0,085)	<0,0001
Irregular discohesive or sparse cells clusters	23,421 (7,95–68,995)	<0,0001
Pagetoid cells	13,824 (5,478–34,886)	<0,0001

OR – odds ratio; CI – confidence interval

Further presented is the analysis of features of cutaneous melanocytic tumors observed by *in vivo* reflectance confocal microscopy.

Dense cells clusters

By evaluating the significance of feature of dense cells clusters in *in vivo* reflectance confocal microscopy for diagnosing cutaneous melanocytic tumors, it was found that the accuracy of *in vivo* reflectance confocal microscopy for diagnosing dysplastic nevus according to the criterion of dense cells clusters is 59,58 %, sensitivity – 71,02 %, specificity – 28,00 %, PPV – 73,13 %, NPV – 25,93 %. Therefore, despite the fact that this feature is the most accurate of all features examined for diagnosis of dysplastic nevi, it is not informative enough to differentiate between dysplastic nevi and common nevi due to low specificity.

The accuracy of dense cells clusters for differential diagnosis of cutaneous melanoma and dysplastic nevus is 21,01 %, sensitivity – 4,55 %, specificity –28,72 %, PPV – 2,90 %, NPV – 39,13%. The Odds Ratio (OR) of feature of dense cells clusters is 0,019, *p* value <0,0001. The presented

data show that dense cells clusters in tests of *in vivo* reflectance confocal microscopy are a statistically significant feature, characteristic of benign cutaneous melanocytic tumor.

Thus, dense cells clusters are an informative feature characteristic of common nevi observed by in vivo reflectance confocal microscopy of cutaneous melanocytic tumor, and is little informative when diagnosing dysplastic nevi.

Non-homogeneous clusters

The accuracy of *in vivo* reflectance confocal microscopy for diagnosing dysplastic nevus according to the criterion of non-homogeneous clusters is 39,36 %, sensitivity – 23,19 %, specificity – 84,00 %, PPV – 80,00 %, NPV – 28,38 %. Therefore, this feature is of little accuracy when differentiating between dysplastic nevi and common nevi.

The accuracy of criterion of non-homogeneous clusters for differential diagnosis of cutaneous melanoma and dysplastic nevus is 81,88 %, sensitivity – 88,64 %, specificity – 78,72 %, PPV – 66,10 %, NPV – 93,67 %. OR of criterion of non-homogeneous clusters is 28,857, p value <0,0001. The resented data show that non-homogeneous clusters are a statistically significant feature of cutaneous melanoma in *in vivo* reflectance confocal microscopy.

Thus, non-homogeneous clusters are an important feature of cutaneous melanoma in *in vivo* reflectance confocal microscopy, and is little informative when diagnosing dysplastic nevi.

Irregular discohesive or sparse cells clusters

The accuracy of *in vivo* reflectance confocal microscopy for diagnosing dysplastic nevus according to the criterion of irregular discohesive or sparse cells clusters is 29,79 %, sensitivity – 5,80 %, specificity – 96,00 %, PPV – 80,00 %, NPV – 26,97 %. Therefore, this feature gives little information when differentiating between dysplastic nevi and common nevi.

The accuracy of the criterion of irregular discohesive or sparse cells clusters for differential diagnosis of cutaneous melanoma and dysplastic nevus is 82,61 %, sensitivity – 56,82 %, specificity – 94,68 %, PPV – 83,33 %, NPV – 82,41%. OR of criterion of irregular discohesive or sparse cells clusters is 23,421, p value <0,0001. The presented data show that irregular discohesive or sparse cells clusters are a specific feature of melanoma for *in vivo* reflectance confocal microscopy.

Thus, irregular discohesive or sparse cells clusters are a specific feature of melanoma in *in vivo* reflectance confocal microscopy. But they are rarely met and thus give little information when diagnosing dysplastic nevi.

Pagetoid cells

The accuracy of *in vivo* reflectance confocal microscopy for diagnosing dysplastic nevus according to the criterion of pagetoid cells is 41,49 %, sensitivity – 28,99 %, specificity – 76,00 %, PPV – 76,92 %, NPV – 27,94 %. Therefore, this feature gives little information when differentiating between dysplastic nevi and common nevi.

The accuracy of the criterion of pagetoid cells for the differential diagnosis of cutaneous melanoma and dysplastic nevus is 76,09 %, sensitivity – 84,09 %, specificity –72,34 %, PPV – 58,73 %, NPV – 90,67%. OR of criterion of pagetoid cells is 13,824, p value <0,0001. The presented data show that pagetoid cells are a reliable feature of cutaneous melanoma when observed by *in vivo* reflectance confocal microscopy.

Thus, pagetoid cells are an important feature of melanoma in *in vivo* reflectance confocal microscopy, and is little informative when diagnosing dysplastic nevi.

Epidermal disarray

The accuracy of *in vivo* reflectance confocal microscopy for diagnosing dysplastic nevus according to epidermal disarray is 32,98 %, sensitivity – 8,70 %, specificity – 100,00 %, PPV – 100,00 %, NPV – 22,12 %. Therefore, because of low sensitivity, this feature gives little information when differentiating between dysplastic nevi and common nevi.

The accuracy of the criterion of epidermal disarray for the differential diagnosis of cutaneous melanoma and dysplastic nevus is 89,13 %, sensitivity – 79,55 %, specificity –93,62 %, PPV – 85,37 %, NPV – 90,72%. OR of criterion of epidermal disarray is 57,037, 95%, p value <0,0001. The presented data show that epidermal disarray is a statistically significant feature of cutaneous melanoma when observed by *in vivo* reflectance confocal microscopy.

Thus, epidermal disarray is an important feature of cutaneous melanoma in *in vivo* reflectance confocal microscopy, and is the most accurate of all features assessed; however it is rarely met and thus gives little information when diagnosing dysplastic nevi.

To sum up the results of the research, it is worth mentioning that neither of the features of cutaneous melanocytic tumors observed by *in vivo* reflectance confocal microscopy are not accurate, nor statistically significant for differential diagnosis of dysplastic nevi and common nevi. However, all together and each separate feature of *in vivo* reflectance confocal microscopy are significant for diagnosing cutaneous melanoma.

In order to assess in detail the accuracy of *in vivo* reflectance confocal microscopy for the diagnosis of cutaneous melanoma, the analysis of prognostic value upon two and more features of *in vivo* reflectance confocal microscopy was performed.

3.3. Analysis of cutaneous melanoma diagnosis based upon two features of *in vivo* reflectance confocal microscopy

The data of the analysis of cutaneous melanoma diagnosis based upon two features of *in vivo* reflectance confocal microscopy are presented in table 3.3.1. Models in the table are ranged based on chi square (χ^2) criterion. Epidermal disarray, being the most accurate feature characteristic of cutaneous melanoma, is being analyzed matching it to other sings of *in vivo* reflectance confocal microscopy.

Table 3.3.1. Diagnostic accuracy of melanoma when matching two features of *in vivo* reflectance confocal microscopy

Feature	Area under the curve (AUC)	OR (95 % CI)	*p* value
Non-homogeneous clusters and epidermal disarray	0,9125	8,761 (2,553–30,064)	0,0006
		22,534 (6,824–74,407)	<0,0001
Dense cells clusters and epidermal disarray	0,9206	0,075 (0,015–0,376)	0,0017
		19,254 (5,84–63,48)	<0,0001
Irregular discohesive or sparse cells clusters and epidermal disarray	0,8920	5,446 (1,351–22,118)	0,0172
		30,349 (9,41–97,876)	<0,0001
Pagetoid cells and epidermal disarray	0,8713	0,699 (0,190–2,567)	0,5893
		62,484 (19,364–201,619)	<0,0001

OR- odds ratio; CI- confidence interval

Based on the data presented in Table 3.1.1, epidermal disarray together with non-homogeneous clusters is the most significant pair of diagnostic criteria for diagnosing cutaneous melanoma; whereas at least significant – in pair with pagetoid cells.

Statistical models according to three or four diagnostic features of *in vivo* reflectance confocal microscopy had at least one statistically insignificant feature. Therefore, such statistical models were not analyzed. The significant correlation among all four diagnostic criteria of *in vivo* reflectance confocal microscopy (p<0,0001) was found when analyzing the dependence among these criteria.

To summarize the performed analysis of features of *in vivo* reflectance confocal microscopy, it can be stated that adding *in vivo* reflectance confocal microscopy to non-invasive diagnostics of cutaneous melanocytic tumors allows the detection of the features, which enable to reach 89% of accuracy in diagnosis of cutaneous melanoma. By assessing the diagnostic value of the test, depending on the area under the curve, *in vivo* reflectance confocal microscopy is a very good or excellent test, in case not less than two examined features are detected. However, this test is not significant for diagnosing dysplastic nevi according to the features mentioned.

3.4. Formulation of working hypothesis and case analysis of cutaneous melanoma

After identifying that features of atypia of *in vivo* reflectance confocal microscopy correlate to histological features of atypia of pigment-rich melanocytes, and the equivalent of discohesive or sparse cell clusters found in epidermis to histological ones is not completely clear, a hypothesis was formulated, stating that not all melanocytes are able to reflect light when examined by this diagnostic method. In addition to this, clinical cases of superficial spreading melanoma, where all examined features of melanocytic atypia were observed using *in vivo* reflectance confocal microscopy, were analyzed. Sparse cells clusters identified in superficial layers of the skin were analyzed in images of *in vivo* reflectance confocal microscopy using *VivaStack* by 5μm step deeper. The analysis of cells composing sparse cells clusters is presented in Fig. 3.4.1.

The mosaic fragment marked by the red rectangle is enlarged in picture C and is analyzed in images of *in vivo* reflectance confocal microscopy by 5 μm step deeper in C-L images. The view corresponds to the feature of sparse cells clusters. Based on the joint agreement of definitions by researchers analyzing *in vivo* reflectance confocal microscopy, sparse cells clusters are defined as round, bright, non-reflecting light structures with clear borders, having separate round or oval cells with dark nuclei and light reflecting cytoplasm. A standard defined cell, marked by the horizontal arrow, is visualized in five consecutive images (G-K) of *in vivo* reflectance confocal microscopy (*VivaStack*) going deeper by every 5 μm. An atypical target resembling cell (vertical arrow) has a refractile nucleus and plasma membrane, is 25-30 μm in size and is observed in five images (C-F) of *in vivo* reflectance confocal microscopy (*VivaStack*).

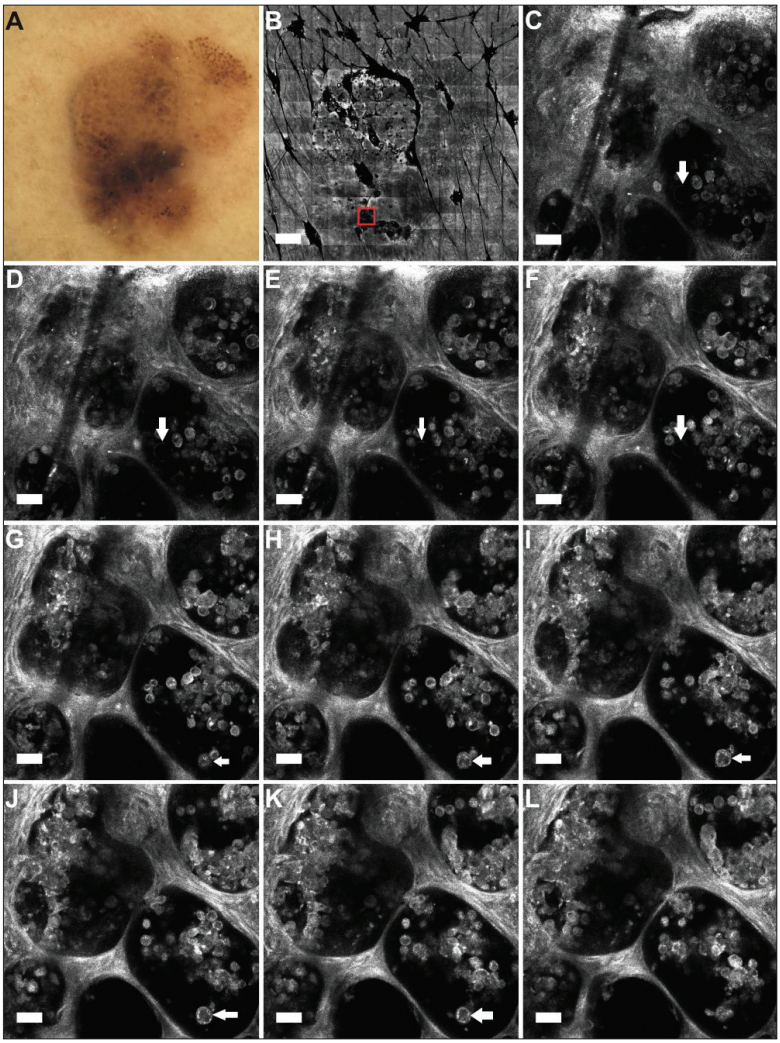

Figure 3.4.1. Dermoscopic image of cutaneous melanoma (A), mosaic of *in vivo* reflectance confocal microscopy images (8 x 8 mm cancer area) made at the level of epidermal-dermal junction 30 μm below *stratum corneum* (B), and red rectangle in image B displays images of *in vivo* reflectance confocal microscopy by 5 μm step deeper (C-L) to 75 μm below *stratum corneum* (L). Scale = 50 μm. Histopathological diagnosis: superficial spreading melanoma pT1A (level III of invasion according to *Clark* classification, *Breslow* thickness – 0,9mm).

Based on case analysis of melanoma presented in Fig.3.4.1, a working hypothesis was formulated, stating that melanocytes, when examined by reflectance confocal system *in vivo*, can have light-transmitting cytoplasm and refractile nucleus. It was decided to perform reflectance confocal microscopy of histological sections of the same tumor. After comparing reflectance confocal microscopy of histological sections and *in vivo*, it was found that sparse cells clusters observed in tumor periphery by *in vivo* reflectance confocal microscopy (Fig. 3.4.1) correspond to intraepidermal discohesive clusters in the histological sample (Fig. 3.4.2).

Figure 3.4.2. Image of histological micro sample of superficial spreading melanoma (immunohistochemical test with HMB 45 markers, x20)

Further delivered are the results of reflectance confocal microscopy of histological sections and results of immunohistochemical test of the same cutaneous melanoma case.

3.5. Reflectance confocal microscopy of histological sections

When examining the skin with *in vivo* reflectance confocal microscope, it is possible to say which cell is keratinocyte and which one is melanocyte. In order to measure the reflection of the melanocyte when examining with the reflectance confocal microscope, reflectance confocal microscopy tests and immunohistochemical tests with melanocyte specific markers were performed for identical histological sections of skin cancer. The image below depicts identical cells visualized by reflectance confocal microscopy (Fig. 3.5.1 – 3.5.3, A images) compared to histological examination (Fig. 3.5.1 – 3.5.3, B images).

Reflectance confocal microscopy and immunohistochemical tests of histological sections (*ex vivo*) of healthy skin (Fig. 3.5.1), *Spitz* nevus (Fig. 3.5.2) and cutaneous melanoma (Fig. 3.5.3) were performed as well. Immunohistochemical tests were performed using melanocyte specific markers: Melan-A or HMB 45. Arrows in histological images point to melanocytes of epidermal layer. The reflection of their cytoplasm and nucleus in images of *ex vivo* reflectance confocal microscopy was assessed comparing reflections of melanocytes and surrounding cells – keratinocytes.

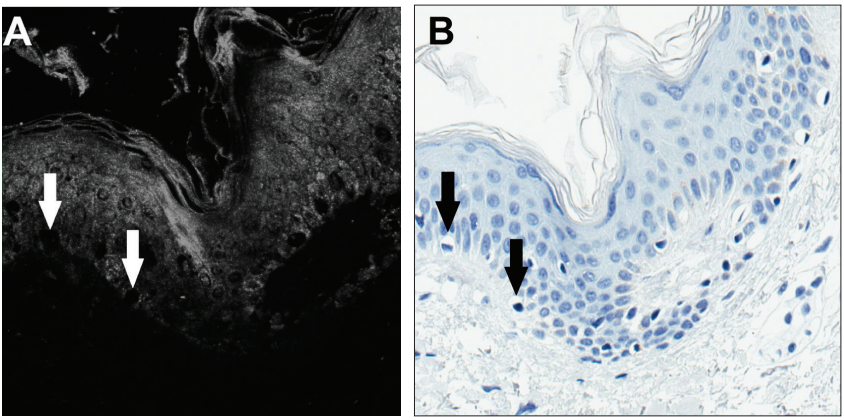

Figure 3.5.1. Histological section of healthy skin (53-year-old patient; localisation – inner surface of the wrist; skin phototype I-II): (A) image of *ex vivo* reflectance confocal microscopy; (B) image of immunohistochemical HMB 45 test of the same section

Images in Fig.3.5.1 display that cytoplasm of melanocytes in basal epidermal layer (marked by arrows) is not refractile (i.e. is dark), whereas weekly refractile are their nuclei.

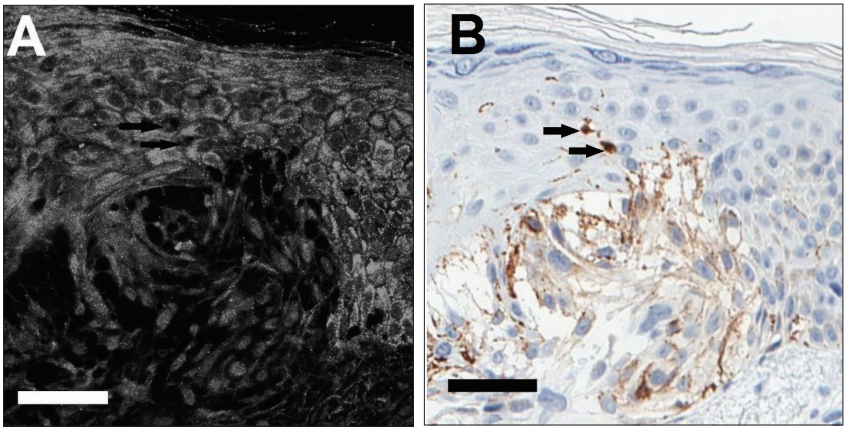

Figure 3.5.2. *Spitz* nevus – 3 mm diameter amelanotic papule with clear borders (56-year-old patient; nevus of inner wrist surface; skin phototype II): (A) image of *ex vivo* reflectance confocal microscopy; (B) image of immunohistochemical HMB 45 test

As it can be seen in Fig.3.5.2, nevus cells melanocytes contain refractile nuclei, and the contrast of cytoplasm is blurred when examined with a reflectance confocal microscope and is directly correlated to a more intense reaction of HMB 45 (image B displays HMB 45 positive melanocytes with their cytoplasm coloured in brown). Sparse epidermal melanocytes marked by arrows are hipo-refractile when examined by reflectance confocal microscopy, compared to surrounding keratinocytes with refractile cytoplasm.

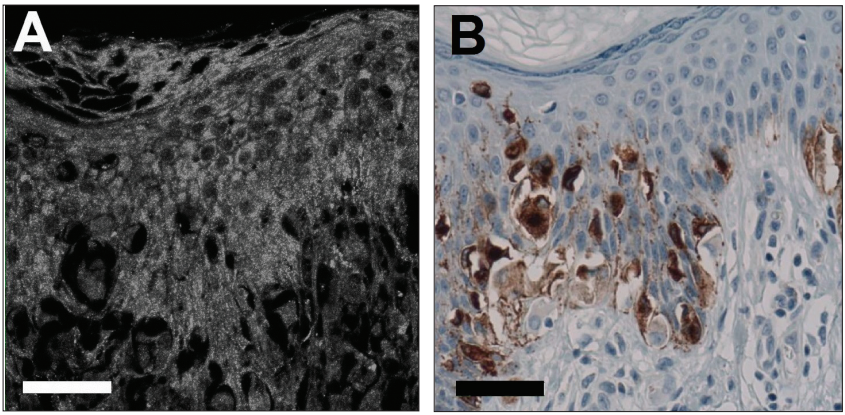

Figure 3.5.3. Melanoma *in situ* (53-year-old patient; pigmented skin cancer of the thigh; skin phototype II): (A) image of *ex vivo* reflectance confocal microscopy; (B) image of immunohistochemical Melan-A test (melanocytes are Melan-A positive, their cytoplasms are brown)

As it can be seen in images of Fig.3.5.3, the majority of melanocytes (melanoma cells) are hipo- or iso-refractile compared to surrounding keratinocytes.

Thus, *ex vivo* reflectance confocal microscopy visualizes both skin keratinocytes and melanocytes, and when it is combined with immunohistochemical tests, it is possible to evaluate reflection of skin melanocytes and their maturity both in healthy skin and cutaneous melanocytic tumor.

Based on primary results of tests performed, when melanocytes located between keratinocytes of epidermal layer were more hipo-refractile than hiper-refractile, refractile nucleus was observed in both healthy skin melanocytes and nevus nevocytes, and melanoma cells (atypical melanocytes) were hipo-, iso- hiper-refractile in regard to keratinocytes, it can be concluded that characteristics of reflection of melanocyte cytoplasm differs depending on maturity stage of melanosomes. Melanocytes containing immature melanosomes might have hipo-refractile, i.e. light-transmitting, cytoplasm and refractile nucleus when examined under reflectance confocal microscopy.

Formulated hypothesis needs to be specified by continuing research of reflectance confocal microscopy of skin cancer sections (*ex vivo*).

4. CONCLUSIONS

1. *In vivo* reflectance confocal microscopy features of melanocytic dysplastic nevi and cutaneous melanoma – epidermal disarray, pagetoid cells, discohesive or sparse cells clusters, non-homogeneous clusters, dense cells clusters – identified using *in vivo* reflectance confocal microscopy, correspond to features of cytological atypia identified by histological examination from 73,91 to 100,00%.
2. The sensitivity of *in vivo* reflectance confocal microscopy for diagnosing dysplastic nevi is 71,02%, and the specificity is 28,00%. The most accurate feature of melanocytic dysplastic nevi, identified by *in vivo* reflectance confocal microscopy, is dense cells clusters.
3. The sensitivity of *in vivo* reflectance confocal microscopy for diagnosing cutaneous melanoma is 79,55%, and the specificity is 93,62%. The most accurate feature of cutaneous melanoma, identified by *in vivo* reflectance confocal microscopy, is epidermal disarray.
4. *In vivo* reflectance confocal microscopy is significant for diagnosing cutaneous melanoma. However, it is not sufficiently accurate for diagnosing melanocytic dysplastic nevi and does not enable to distinguish melanocytic dysplastic nevi from common nevi.

5. PRACTICAL RECOMMENDATIONS

Referring to research results, their interpretation and comparison to data from other authors, we formulated such practical recommendations:

1. *In vivo* reflectance confocal microscopy can help to avoid unnecessary biopsies when no features of melanocytic dysplastic nevi or cutaneous melanoma are identified by *in vivo* reflectance confocal microscopy.
2. If features of cutaneous melanoma are identified by *in vivo* reflectance confocal microscopy, the tumor can be removed with wide, i. e. 5 mm, healthy skin borders. This way the primary diagnostic tumor excision can be avoided in case of severe nevus dysplasia and melanoma *in situ*.
3. Though *in vivo* reflectance confocal microscopy is a very promising method, it does not replace tumor biopsy, histological and immunohistochemical examination due to its little specificity in diagnosing melanocytic dysplastic nevi.
4. In order to identify more accurate features of melanocytic dysplastic nevi, identified by *in vivo* reflectance confocal microscopy, it is purposeful to perform reflectance confocal microscopy (*ex vivo*) and immunohistochemical analysis of histological tumor sections in addition.

6. PUBLICATIONS AND PRESENTATIONS UNDER THE TOPIC OF DISERTATION

Scientific articles

1. Ingrida Vaišnorienė, Janina Didžiapetrienė, Konstantinas Povilas Valuckas, Ričardas Rotomskis. Optical biopsy for diagnosis of cutaneous melanocytic lesions. Sveikatos mokslai, 2011 (21): 140-147.
2. Ingrida Vaišnorienė, Janina Didžiapetrienė, Giedrė Smailytė, Konstantinas Povilas Valuckas. Early diagnosis of cutaneous melanoma. Medicinos teorija ir praktika, 2013 (4): 362-368.
3. Ingrida Vaišnorienė, Ričardas Rotomskis, Vytautas Kulvietis, Rimantas Eidukevičius, Violeta Žalgevičienė, Aida Laurinavičienė, Jonas Venius, Janina Didžiapetrienė. Nevomelanocytic atypia detection by *in vivo* reflectance confocal microscopy. Medicina, 2014 (50): 209-215.

Scientific presentations

1. Theses and poster. I. Vaisnoriene, J. Venius, J. Didziapetriene, R. Rotomskis, K. P. Valuckas. Atypia grading in nevi by reflectance confocal microscopy. Pigment cell Melanoma Res, 2011 (24), Abstract Nr. P138. Blackwell. ISSN 1755-1471. XXIst International Pigment Cell Conference, 21-24 Sept 2011, Bordeaux (France).
2. Oral presentation. I. Vaišnorienė, J. Didžiapetrienė, G. Pellacani. Diagnosis of melanoma using reflectance confocal skin imaging system. Doctoral internship in foreign scientific centers 2012-2013, Research Council of Lithuania Project „*Promotion of Students' Scientific Activities*", 2013-11-11, Vilnius (Lithuania).
3. Oral presentation. I. Vaišnorienė. Malignant melanoma: case report. Salzburg Weill Cornell Seminar in Dermatology, 2014-11-13, Salzburg (Austria).

7. SUMMARY IN LITHUANIAN

Darbo aktualumas

Lietuvoje, kaip ir visame pasaulyje, didėja sergamumas odos melanoma. Nors melanoma yra rečiausia iš piktybinių odos ligų, tačiau būtent ji yra dažniausia mirties nuo odos vėžio priežastis [*Stang A.* ir bendraaut., 2006]. Nacionalinio vėžio instituto (NVI) duomenimis, mūsų šalyje kasmet diagnozuojama 200–300 ir daugiau naujų odos melanomos atvejų. Kasmetis susirgimų prieaugis yra apie 6 procentai. 2012 metais sergamumas odos melanoma Lietuvoje sudarė 10,5 atvejo 100 000 gyventojų. Remiantis Europos šalių vėžio registrų tyrimo EUROCARE-5 (angl. *European cancer registry-based study of cancer patients' survival and care)* duomenimis, susirgusiųjų odos melanoma penkerių metų reliatyvusis išgyvenamumas Lietuvoje sudaro 69,19 proc., o vidutinis išgyvenamumas Europos šalyse – 83,22 proc. [*De Angelis R.* ir bendraaut., 2014]. Išanalizavus Lietuvos žmonių, susirgusių odos melanoma, išgyvenamumo rodiklius paaiškėjo, kad geresnis išgyvenamumas yra moterų, jaunesnių pacientų ir miesto gyventojų [Smailyte G. ir bendraaut. 2011]. Nustatyta sergamumo odos melanoma pasaulyje ir Lietuvoje didėjimo tendencija ir minėti išgyvenamumo skirtumai skatina gerinti ankstyvąją odos melanomos diagnostiką ir profilaktiką Lietuvoje.

Pasaulio sveikatos organizacijos (PSO) duomenimis, dažniausiai odos melanoma serga asmenys, turintys šviesią, saulei jautrią odą ir daug atipinių apgamų. Atipinis apgamas yra svarbiausias melanomos rizikos veiksnys, o jo diagnostika ir gydymas yra vienas aktualiausių uždavinių gerinant išgyvenamumą susirgus odos melanoma.

1978 metais dermatopatologas *W. Clark* ir bendraautoriai pastebėjo ir aprašė atipinį apgamą tyrinėdami melanoma sergančių pacientų šeimos narių apgamus [*Clark W.* ir bendraaut., 1978]. Remiantis histologiniu

apibrėžimu, nevocitinis navikas, pasižymintis ląstelių ir architektonikos atipijos požymiais bei kartu pasireiškiančiais organizmo reakcijos požymiais, vadinamas displaziniu apgamu. Klinikinių tyrimų duomenimis, displazinis apgamas yra susijęs su padidėjusia melanomos tikimybe trimis aspektais: dėl panašumo į melanomą, kaip melanomos rizikos žymuo, kaip galimas melanomos pirmtakas [*Elder D.* ir bendraaut., 1993; *Goldstein A.* ir bendraaut., 2013; *Farber M.* ir bendraaut., 2012; *Gandini S.* ir bendraaut., 2005; *Elder D.* ir bendraaut., 2010].

Remiantis *W. Clark* pasiūlyta melanocitų kilmės odos navikų histologine klasifikacija, morfologiškai progresuojanti atipija laipsniuojama išskiriant displazinį apgamą kaip tarpinę stadiją tarp įprastinių apgamų ir odos melanomos. Manoma, kad melanocitų kilmės navikams būdinga nuosekli progresija: įprastinis apgamas → displazinis apgamas → melanoma *in situ* → invazinė melanoma [*Clark W*. ir bendraaut., 1984]. Įvairių tyrimų duomenimis, 20–30 proc. melanomų atsiranda iš apgamų [*Marks R.,* ir bendraaut., 1990; *Togawa Y.* ir bendraaut., 2010; *Marghoob A.* ir bendraaut., 2002], todėl siekiant įvertinti apgamo tikimybę supiktybėti tikslinga atpažinti displazinio apgamo požymius.

Melanocitų kilmės odos navikų – įprastinio apgamo, atipinio apgamo ir odos melanomos – diferencinės diagnostikos iššūkius lemia atipinių apgamų ir odos melanomos panašumas bei didelė odos melanomos požymių įvairovė. Dažniausiai minimuose atipinio apgamo apibrėžimuose šio tipo apgamas apibūdinamas kaip didesnis nei 6 mm skersmens, įvairių spalvų ir nelygių kraštų navikas. Tai atitinka odos melanomos ABCD požymius, kai A – asimetrija (angl. *asymetry*), B – nelygūs kraštai (angl. *borders*), C – įvairios spalvos navike (angl. *colour*) bei D – didesnis kaip 6 mm skersmuo (angl. *diameter*). Apžiūros plika akimi tikslumas melanocitų kilmės odos navikų diagnostikai yra apie 60 proc. [*Duffy K.* ir bendraaut., 2012].

Klinikinėje praktikoje naudojami neinvaziniai optiniai tyrimai padeda tiksliau nustatyti apgamo atipiją ir odos melanomą. Svarbiausias iš

jų – dermoskopija arba epiliuminescencinė odos mikroskopija. Lyginant su apžiūra plika akimi, dermoskopija pagerina tyrėjo diagnostinį tikslumą nuo 5 iki 30 proc [*Bafounta M*. ir bendraaut., 2001; *Kittler H*. ir bendraaut., 2002, *Vestergaard M*. ir bendraaut., 2008]. Nustačius atipijos požymius rekomenduojama atlikti odos naviko biopsiją ir displazinio apgamo ar odos melanomos diagnozė patvirtinama morfologiškai.

Neinvazinės diagnostikos tikslumas ypač svarbus displazinių apgamų sindromo atveju, kai visų atipinių apgamų iš karto pašalinti nėra galimybių. Tiksli neinvazinė diagnostika padeda atrinkti apgamus, pasižyminčius didžiausia atipija (melanomos rizika) ir juos pašalinti anksčiausiai.

Neinvazinės diagnostikos tikslumas svarbus ir parenkant optimalų gydymą. Atipiniai apgamai šalinami tik chirurginiu būdu pjūvį atliekant per sveikus audinius. Ryškios displazijos apgamus rekomenduojama pašalinti kartu su 0,5 cm sveikos odos kraštais. Chirurginio odos melanomos gydymo rekomendacijos skiriasi priklausomai nuo ligos stadijos: I stadijos odos melanomos atveju rekomenduojama pašalinti 1,0 cm, II – 2,0 cm, III-IV - 2,5 cm ir daugiau sveikos aplinkinės odos [*NCCI Clinical Practice Guidelines in Oncology, Melanoma*. V.3.2011]. Taigi, atipinių apgamų ir odos melanomos gydymo skirtumai yra dar viena tikslios neinvazinės diagnostikos būtinybės priežastis.

Tobulėjant medicininių tyrimų technologijoms atsiranda naujos neinvazinės diagnostikos galimybės. Atipinių apgamų diagnostika gali būti tikslinama naudojant aukšto dažnio ultragarsą, optinę koherencinę tomografiją, optinę spektroskopiją, paviršiaus magnetinį rezonansinį vaizdinimą, *in vivo* konfokalią atspindžio mikroskopiją.

Taikant konfokalią atspindžio mikroskopiją yra įmanoma stebėti odos morfologinius pokyčius *in vivo* ląsteliniame lygyje [*Rajadhyaksha M*. ir bendraaut., 1995; *Ahlgrimm-Siess V.* ir bendraaut., 2009]. Šio tyrimo metu oda vaizdinama be invazijos į organizmą ar pažeidimo tiriamiems audiniams ir gali būti neribotai kartojama. *In vivo* konfokalios atspindžio

mikroskopijos pritaikymas šiuo metu yra tiriamas ne tik melanocitų kilmės odos navikų, bet ir kitų odos ligų diagnostikai [*Cinotti E.* ir bendraaut., 2015; *Ulrich M.* ir bendraaut., 2015]. Preliminarių tyrimų duomenimis, *in vivo* konfokalios atspindžio mikroskopijos tyrimų rezultatai atitinka histologinių tyrimų rezultatus [*Rajadhyaksha M.* ir bendraaut. 1999], o šio tyrumo specifiškumas melanocitinių odos navikų diagnostikai siekia 95 proc. [*Pellacani G.* ir bendraaut., 2005].

Tiksliai nustatyti apgamo displaziją ir odos melanomą ankstyvojoje ligos stadijoje būtų galima, jei *in vivo* konfokalios atspindžio mikroskopijos diagnostinis tikslumas būtų artimas „auksiniam" diagnostikos standartui – histologiniam tyrimui.

Atsižvelgiant į tai, kad lyginamuosiuose dermoskopijos ir *in vivo* konfokalios atspindžio mikroskopijos klinikiniuose tyrimuose konfokali atspindžio mikroskopija pagerina nepiktybinių melanocitų kilmės odos navikų diagnostiką, tačiau jos galimybės nustatyti apgamo displaziją nėra pakankamai ištirtos, todėl yra aktualūs tyrimai siekiant išsiaiškinti *in vivo* konfokalios atspindžio mikroskopijos tyrimo vertę diferencinėje įprastinių apgamų, displazinių apgamų bei odos melanomos diagnostikoje, lyginant rezultatus su histologinių tyrimų rezultatais.

Tyrimo tikslas

Įverinti *in vivo* konfokalios atspindžio mikroskopijos tyrimo tikslumą diagnozuojant melanocitų kilmės displazinius apgamus.

Tyrimo uždaviniai

1. Ištirti, ar *in vivo* konfokalios atspindžio mikroskopijos tyrimu nustatyti melanocitų kilmės displazinių apgamų ir odos melanomos požymiai – epidermio netaisyklingumas, pedžetoidinės ląstelės, diskoheziniai arba pavienių ląstelių lizdai, nehomogeniški lizdai, tankių ląstelių lizdai – atitinka ląstelių atipijos požymius, nustatytus histologiniais tyrimais.

2. Nustatyti *in vivo* konfokalios atspindžio mikroskopijos tyrimo jautrumą ir specifiškumą diagnozuojant melanocitų kilmės displazinius apgamus.
3. Nustatyti *in vivo* konfokalios atspindžio mikroskopijos tyrimo jautrumą ir specifiškumą diagnozuojant odos melanomą.

Darbo mokslinis naujumas ir praktinė reikšmė

Bendradarbiaujant Nacionalinio vėžio instituto (NVI) mokslininkams, medicinos fizikams ir gydytojams buvo įgyvendintas mokslinis projektas, kuriam vykdyti buvo įsigyta *in vivo* konfokalios atspindžio mikroskopijos aparatūra.

Atlikus melanocitų kilmės odos navikų *in vivo* konfokalią atspindžio mikroskopiją nustatyta, kad melanocitų atipijos požymiai atitinka pigmento melanino turinčių melanocitų atipijos požymius histologiniuose tyrimuose. Šie požymiai leidžia diferencijuoti nepiktybinius melanocitų kilmės odos navikus nuo piktybinių, tačiau nepadeda nustatyti displazinio apgamo.

Darbe atlikta histologinių odos bei jos navikų pjūvių konfokali atspindžio mikroskopija kartu su imunohistocheminiais melanocitų žymenų tyrimais padeda nustatyti melanocitų atspindį odoje bei melanocitų kilmės odos navikuose, todėl šių tyrimų duomenų analizė gali patikslinti *in vivo* konfokalios atspindžio mikroskopijos melanocitų kilmės odos navikų charakteristikas ir pagerinti šio tyrimo tikslumą.

Melanocitų vaizdinimas histologiniuose odos navikų pjūviuose *ex vivo* konfokalios atspindžio mikroskopijos būdu buvo patentuotas neradus šių tyrimų ankstesnių publikacijų.

Tiriamieji ir tyrimo metodai

Į perspektyvųjį žvalgomąjį tyrimą įtraukti 138 pacientai, kuriems Nacionaliniame vėžio institute (NVI) 2010–2013 metais atlikta atipinių melanocitų kilmės odos navikų ekscizinė biopsija. Remiantis galutine patologine diagnoze, tiriamieji suskirstyti į tris grupes: I grupė – pacientai, kuriems diagnozuotas melanocitų kilmės apgamas, neturintis citologinės atipijos požymių (n=25), II grupė – pacientai, kuriems diagnozuotas displazinis apgamas (n=69), ir III grupė – pacientai, kuriems diagnozuota odos melanoma (n=44). Naudoti tyrimo metodai:

- dermoskopijos tyrimas;
- *in vivo* konfokali atspindžio mikroskopija;
- histologiniai tyrimai;
- *in vivo* konfokalios atspindžio mikroskopijos požymių ir histologinių mikropreparatų analizė;
- histologinių pjūvių *ex vivo* konfokali atspindžio mikroskopija ir imunohistocheminiai tyrimai;
- statistinė duomenų analizė atlikta naudojant SAS 9.3 statistinį paketą.

Išvados

1. *In vivo* konfokalios atspindžio mikroskopijos tyrimu nustatomi melanocitų kilmės displazininių apgamų ir odos melanomos požymiai – epidermio netaisyklingumas, pedžetoidinės ląstelės, diskoheziniai arba pavienių ląstelių lizdai, nehomogeniški lizdai, tankių ląstelių lizdai – 73,91–100,00 proc. atitinka ląstelių atipijos požymius, nustatomus histologiniais tyrimais.
2. *In vivo* konfokalios atspindžio mikroskopijos tyrimo jautrumas diagnozuojant melanocitų displazinius apgamus yra 71,02 proc., specifiškumas – 28,00 proc. Tiksliausias melanocitų kilmės displazinių

apgamų požymis, nustatomas atliekant *in vivo* konfokalią atspindžio mikroskopiją, yra tankių ląstelių lizdai.

3. *In vivo* konfokalios atspindžio mikroskopijos tyrimo jautrumas diagnozuojant odos melanomą yra 79,55 proc., specifiškumas – 93,62 proc. Tiksliausias odos melanomos požymis, nustatomas atliekant *in vivo* konfokalią atspindžio mikroskopiją, yra epidermio netaisyklingumas.
4. *In vivo* konfokalios atspindžio mikroskopijos tyrimas yra reikšmingas odos melanomos diagnostikoje, tačiau šis tyrimas yra nepakankamai tikslus melanocitų kilmės displazinių apgamų diagnostikoje ir nepadeda atskirti melanocitų kilmės displazinių apgamų nuo įprastinių apgamų.

9. CURRICULUM VITAE

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Education:

Vilnius University, Faculty of Medicine:

1995–2001	medical doctor
2001–2002	resident of medical doctor
2002–2006	resident of dermatovenerology

Employment:

Since 2006	National Cancer Institute (previous Institute of Oncology, Vilnius University), dermatovenerologist.
2006–2007	Centre of Dermatovenerology, Vilnius University Hospital Santariskiu Klinikos, dermatovenerologist.

Training courses:

2014	*Weill Cornell* Dermatology seminars, Salzburg, Austria.
2012	Learning Course in Dermoscopy, Congress of Melanoma 2012, Barcelona, Spain.
2012	Traineeship at the Department of Dermatology of University Hospital – Policlinico of Modena and at the Arcispedale S. Maria Nuova, Hospital of Reggio Emilia, Italy. Head – prof. G. Pellacani.

2011 *In Vivo* Confocal Microscopy Introductory Course for the Evaluation of Melanocytic Lesions, Skin Tumors, Inflamatory Skin Diseases and Cosmetology Applications. Department of Dermatology, University of Modena and Reggio Emilia, Modena, Italy.

2005 Dermatovenereology Learning Course in London, EADV (*European Academy of Dermatology and Venereology)*.

Overall publications: 3

Memberships:
Member of Lithuanian Association of Dermatovenereologists