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EXPRESSION OF CYCLIN D1, BCL2, p53 AND OTHER MELANOCYTE MARKERS IN MALIGNANT SKIN MELANOMAS AND MELANOCYTE NEVI -

COMPARATIVE ANALYSIS OF IMMUNOHISTOCHEMICAL EXPRESSION, MORPHOLOGICAL PROFILE, AND RELEVANCE TO DIAGNOSIS AND TUMOUR PROGRESSION

Abstract

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Abbreviations used in the dissertation work
Introduction
Purpose and tasks
Purpose 10
Tasks 10
Materials and methods
Material 12
Methods 12
Macroscopic analysis of biopsy material researched 12
Section of the material under examination 12
Histological processing of materials 13
Histological methods 13
Immunohistochemical test 17
Reporting of immunohistochemical test results 19
Statistical methods for the analysis of results: 21
Descriptive methods 21
Non-parametric analysis 21
Correlation analysis 22
T-test analysis 22
Variation analysis 22
Spearman correlation analysis (rho) 23

Results

Demographic indicators of the material under examination 26

Topographical distribution (localization) of benign **nevi, atypical nevi, and melanomas:** 29

Basic morphological characteristics of the test material 31

Results of immunohistochemically tested expression of Cyclin D1, BCL2, and p53 35

Cyclin D1 study results 36

Results of the p53 study 46

BCL2 Expression Results 53

S100 protein expression results 60

Melanosome clone HMB45 results 64

Ki 67 study results 74

Statistical analysis for pre-statistical value of Cyclin D1, p53, and Melanosome 76

clone HMB45 76

Discussion

Key points

Contributions

Original scientific and applied contributions 118

Confirmatory contributions 118

Abbreviations used in the dissertation work

BCL2 protein - B-Cell Lymphoma apoptotic regulator

P53 protein – tumor protein p53

Cyclin D1 -B cell Lymphoma 1 protein

MM - malignant melanoma

WCRF- World Cancer Research Fund International

AICR – American Institute for Cancer Research

HE/HE - HematoxylinEosin stain

Melan A (MART) - Melanoma Antigen Recognized By T-Cells

S100 protein - S100 Calcium Binding Protein

SOX10 - SRY-Box Transcription Factor 10

HMB45 – Human Melanoma Black 45 (Melanosome clone HMB45)

Ki 67 (MIB1) – Ki 67 antigen clone MIB1

PAS - Periodic Acid-Schiff (PAS) Staining

CK - CytoKeratin

EMA – Epithelial Membrane Antigen

CD45 – Leucocyte Common Antigen

MYOD1 - Myogenic Differentiation 1

HLA - Major Histocompatibility Complex

DOPA - 3,4-dihydroxyphenylalanine

PEComa family - Perivascular Epithelioid Cell family of tumor**s**

NKI/C3 - CD63 Antibody (NKI/C3**)**

RB gen - RB Transcriptional Corepressor 1

IAP family - endogenous caspase inhibitors with multiple biological activities

PCNA - Proliferating Cell Nuclear Antigen

TNM - T category describes the primary tumor site and size, N category describes the regional lymph node involvement, M category describes the presence or otherwise of distant metastatic spread

TILs (TIL) – Tumor-Infiltrating Lymphocytes in the stroma

Introduction

Melanoma is a malignant tumor of melanocyte origin, unfavorable prognosis, and increasing frequency worldwide, the most significant type and with the highest frequency is skin melanoma. It is a rare malignancy but is the most common cause of death from malignant skin tumors. The epidemiological characteristics of cutaneous melanoma in Bulgaria are low morbidity, moderate growth rate, relatively low mortality in men, and moderately high in women, but relatively rapid growth rate in both sexes. Bulgaria has the lowest patient survival among Eastern European countries (Dimitrova N, 2015).

Malignant melanoma occurs de novo or by malignant transformation of a previous benign melanocyte lesion. The main risk factor for its occurrence is ultraviolet radiation, as well as the family burden. The multistage mechanism of tumorigenesis in melanoma includes accumulating consecutive genetic defects, with a significant modifying risk factor - overexposure to ultraviolet radiation, especially periodically acting, whether from direct sunlight or an artificial source (Weir H, 2011). The identified genetic mutations in malignant

melanoma play a vital role in carcinogenesis, programmed cell death, and cell cycle control. Today, the study and knowledge of the resulting mutations are a necessary and targeted component for diagnosis for pathogenetically determined therapy with inhibitors, specified by determining the patient's molecular-pathological tumor profile.

The morphological diagnosis of melanoma is complex and requires knowledge and evaluation of many histological parameters and often immunohistochemical analysis proving the origin of the tumor and its differentiation from atypical pigmented lesions. The use of markers, which are usually used in tumors to confirm their malignant nature and possibly interpret their role as predictors of tumor progression, is already widely used in practice. The present study aims to, by comparative analysis of the expression of markers such as BCL2, p53 protein, Cyclin D1, and others, determine the pigmented nature of tumors S100 protein, Melanosome HMB45, against the background of morphological features for each type of lesion, to determine their significance for morphological diagnosis, early diagnosis, and prevention, stage-oriented therapy, and prediction of cutaneous malignant melanoma.

Purpose and tasks

Purpose

On a selected, morphologically diagnosed, and analyzed cohort of pigmented (benign and malignant) skin tumors, to perform immunohistochemical analysis of the expression of certain melanocytes (S100 protein, Melanosome clone HMB45) and non-melanocytes (Cyclin D53, protein BioL2) in order to look for significant differences that would allow the development of criteria to support the morphological diagnosis, especially for the differentiation of borderline lesions and MM, and for a possible prediction of the biological behavior of these tumors.

Tasks

Achieving this goal includes performing the following tasks:

1. In separate groups of benign, atypical, and malignant pigment lesions to conduct a synchronous immunohistochemical study of the expression of melanocyte markers S100 protein and HMB45 and non-melanocyte BCL2, p53 protein, and Cyclin D1, as well as proliferative activity Ki 67

2. To determine whether or not there are features and significant differences in the expression of the respective markers in and between the different groups.
3. To determine the significance of the manifested differences and look for prognostic characteristics for malignant potential.

Materials and methods

Material

A total of 91 pigmented neoplasms were identified and selected as suitable for the present study, including 57 benign melanocyte nevi, ten atypical nevi, and 24 malignant melanomas. The biopsy sheet accompanying the material and in some cases, the patient or his relatives provided data on the patient's age and sex, location, primary or secondary lesion, time of onset, rate/rate of development, including the degree of increase and change in the type of lesion, as well as suspected provoking factors.

Methods

Macroscopic analysis of biopsy material researched

Including macroscopic assessment and description of the lesion in terms of size, (contours) outlines of its boundaries, the presence of symmetry, color and size, the presence of ulcerations, crusts, satellite, resection boundaries.

Section of the material under examination

By making sections for subsequent histological processing. Transversal sequential topographic sections 2 to 3 mm thick were made from the entire biopsy material, including the lesion with its peripheral and deep edges.

Histological processing of materials

Each material was fixed in 10% neutral formalin, buffered with 4% methanol, with a fixation duration of 8 to 48 hours. It was processed in a tissue processor, then prepared a paraffin block from each slice embedded in paraffin with a low melting point of 52 to 54 degrees. Histological sections were prepared from each paraffin block on a paraffin microtome, followed by routine histological staining and immunohistochemical procedures.

Histological methods

Sections of the paraffin blocks were dewaxed in xylene and subsequent dehydration with an ascending row of ethanol, stained with hematoxylin-eosin, and incorporated into a cover glass. The microscopic examination was performed with an Olympus CX31 light microscope. The microscopic evaluation

of each lesion according to the parameters necessary for the morphological diagnosis of the pigmented lesions, studied morphologically, morphometrically, and by semi-quantitative methods, namely:

Evaluation of the basis (positioning) of melanocyte proliferation

Evaluation of microscopic symmetry of the lesion profile

Type of cellularity, cellular atypia with the determination of the degree of expression, localization, and distribution in the tumor

Lesion thickness (tumor mass thickness) determined on a four-point Breslow scale, measured with an ocular micrometer in mm, from the level of the granular epidermal layer or the edge of the defect in ulcerated tumors, the deepest infiltrating group of melanocytes in the dermis/subcutis. The measurement is performed on histological sections perpendicular to the epidermal surface. The thickness measured in this way qualifies as follows: Breslow I - thickness up to 0.75 mm, Breslow II - from 0.76 to 1.50 mm, Breslow III - from 1.51 to 4.00 mm, Breslow IV - thickness over 4mm.

Mitotic activity - detection of mitoses by the "hot spot" method (mitotic figures per square millimeter, calculated as the sum of mitoses in the field with the largest number of mitoses / hot

spot / and four adjacent fields, at a magnification of 400x, according to the protocol of 1982, at the International Pathology Workshop, included in the seventh revision of the TNM classification) (Amin M, 2017). Mitotic activity is reported as moderate mitotic activity (1 to 5 mitoses), high mitotic activity (6 to 10 mitoses), very high mitotic activity (over 11 mitoses).

Clark micro invasion - five degrees of micro invasion to the individual anatomical layers of the skin: Clark 1 - malignant proliferation in the epidermis, Clark 2 - an invasion of malignant proliferation in the papillary dermis, Clark 3 - crossing the border with the reticular dermal dermis, C, Clark 5 - engagement of the hypodermis (Clark W, 1967).

Ulceration in melanomas - assessment of the type of ulceration: infiltrative ulceration - invasion and erosion of the epithelium, most common in superficial melanomas; tumorigenic ulceration, in nodular melanomas without epidermal invasion, which thin and tear the epidermis and traumatic ulceration, which has no prognostic meaning. The size of the ulceration (below or above 70% of the total tumor length and up to 5 mm in diameter or above 5 mm in diameter) is also determined,

which has prognostic significance (Eggermont A, 2012-2; Haydu L, 2012; Bønnelykke-Behrndtz M, 2014).

Tumor-infiltrating lymphocytes (TILs), a manifestation of an immune response to melanoma cells. The reaction is categorized into severe, moderate, and minimal to absent (Plaza J, 2017). It is a high-intensity TIL in continuous lymphoid infiltration in the tumor periphery and between tumor cell aggregates. Moderate TIL in the presence of focal but relatively dense cellular distribution lymphoid infiltrates in the tumor periphery and focal between tumor cell aggregates. It is reported as weak to absent TIL in cases where separate, low-density lymphoid infiltrates are found in the tumor periphery and/or in the tumor stroma.

Regression in melanomas - regression is defined as replacing melanoma cells by fibrosis, melanophages, lymphocyte infiltrate, and telangiectatic vessels, with or without a residual intradermal component. Regression can be focal or extensive.

Satellite melanoma - microscopically, satellites are skin or subcutaneous tumor cell groups with a diameter greater than or equal to 0.05 mm, located around the primary melanoma but separated by normal collagen or adipose tissue (Day C, 1981; Barnhill R, 2004).

Immunohistochemical test

Dewaxed and dehydrated sections of the same paraffin blocks were treated with a universal DAKO system (En vision flex TRS high pH / Link /), including buffers for antigenic recovery, dewaxing, rehydration, washing, and subsequent staining with HRP Magenta Substrate Chromo visualization system Omnis GV925, in which the target antigenic objects and antibodies are colored red. Antibody exposure was performed according to a standardized antibody protocol. The preparations were carried out in one stage on an automated (robotic) closed system of DAKO autostainer Link 48. The following antibodies were used:

DAKO IR504 anti S100 protein, ready-to-use polyclonal rabbit antibody, pH nine antigen repair buffer, to demonstrate the melanocyte character of the lesion. Cells labeled with the antibody-positive for nuclear and cytoplasmic expression.

Anti Melanosome, clone HMB 45 (Human Melanoma Black), DAKO IR052 monoclonal murine antibody, ready to use, pH nine antigen-repair buffer, and demonstrate the melanocyte character of the lesion. Cells labeled with the antibody positioned cytoplasmic expression.

Ki 67 antigen clone MIB-1, a ready-to-use DAKO IR626 monoclonal mouse antibody, monitors the proliferative activity of melanocyte cells. Labeled with the antibody, they positively express nuclear expression. Expression for study purposes was refined by dividing into five groups: Ki 67 0 to 2%, 3 to 5%, 6 to 10%, 11 to 20%, over 20%. The expression was determined at low magnification in the most proliferative zone, without a severe inflammatory infiltrate. At this hot spot, the percentage of stained nuclear cells is estimated at 200 tumor cells, allowing thin and thick lesions (Uguen A, 2015).

Antibody p53 protein, monoclonal mouse, clone D07, DAKO IR616, ready to use. Cells labeled with the antibody show nuclear staining, but in some cases, cytoplasm may be detected. BCL2 oncoprotein antibody, mouse monoclonal, clone 124, DAKO IR614, ready to use. Cells labeled with the antibody show cytoplasmic staining.

Cyclin D1 antibody, rabbit monoclonal, clone EP12, ready to use, DAKO IR083. Cells labeled with the antibody show predominantly nuclear staining.

Reporting of immunohistochemical test results

Evaluation of the expression of the various antibodies used was performed light microscopically, based on semi-quantitative analysis. Ten visual fields were examined, at a magnification of x400 times, from each case. The following were reported: 1. intensity of expression on a four-point scale, from 0 (negative) to 4 degrees (highly positive); 2. the nature of the distribution of the expression as focal/spotted or diffuse/continuous; 3. topographic characteristics of the expression: superficially in the dermo-epidermal border (upper portion), in the deeply located parts of the lesion or diffusely in its entire thickness; 4. presence or absence of differences in the degree of intensity of expression in the same lesion. For positive expression, we considered the staining of the cells in red with three degrees of intensity: bright red staining (+), corresponding to low / low-intensity expression; red color (++) corresponding to moderately intense expression and dark red color (+++) corresponding to high intensity expression. Based on these basic requirements for the assessment of immunohistochemical reactions and based on the accumulated experience, we have

developed a detailed report of the expression by intensity and distribution in eleven groups (models), as follows:

1. Lack of expression - complete lack of expression or expression in single cells

2. Low intensity expression in the upper portion of the lesion

3. Low intensity spot expression, i. e., low-intensity focal expression in individual sections of the entire lesion thickness

4. Low intensity diffuse - low-intensity expression throughout the thickness of the lesion

5. Moderate intensity in the upper portion of the lesion

6. Moderate intense spotted expression - a focal expression of moderate intensity, in separate sections of the entire thickness of the lesion

7. Moderate intensity diffuse expression - an expression of moderate-intensity throughout the thickness of the lesion

8. High intensity expression in the upper portion of the lesion

9. High intensity spot expression - high-intensity focal expression in individual sections of the entire lesion thickness

10. High intensity diffuse expression – high intensity expression throughout the thickness of the lesion

11. Heterogeneous expression - expression throughout the thickness of the lesion, but varying in intensity

Digital photos of the cases were taken using a Leica Aperio Scan Scope AT2 device (Aperio Technologies, Vista, CA) Image Scope V12.1.0.5029 (Aperio).

Statistical methods for the analysis of results:

A statistical analysis program was used - IBM, SPSS v. 23. The results are presented in tables using Microsoft Excel v.2010.

Types of statistical methods:

Descriptive methods

Calculation of the arithmetic means and standard deviation of quantitative characteristics.

Non-parametric analysis

Analysis of categorical features by crosstabulation and Pearson's $\chi 2$ (chi-square) criterion. Crosstabulation forms bidirectional and multidirectional tables. The table's structure is arranged by categories, which help determine the type of

statistical test used. The obtained results were evaluated as statistically significant when the p-value <0.05.

Correlation analysis

Correlations measure the degree of association between two or more variables. Correlation coefficient - accepts values between -1 and 1, as the sign depends on the direction of the association, and values above 0.5 are considered astrong correlation. The obtained results were evaluated as statistically significant when the p-value <0.05.

T-test analysis

Calculates the differences between the values of the variables for each patient case and tests whether the mean values differ from 0, i.e., whether there is a difference in the comparison or not, and whether this difference is statistically significant.

Variation analysis

ANOVA parametric data test and Kruskal-Wallis test for nonparametrically distributed data were used to compare the different indicators examined between benign nevi, atypical

nevi, and melanomas. The graphical visualization of the compared values of the indicators is presented with Error Bar Graphs. Differences between values were considered significant at $p < 0.05$, the accepted statistically significant difference for biological experiments. Post-Hoc test by Tukey and Dunn - used to show which groups these statistically significant differences are found.

Spearman correlation analysis (rho)

Used to determine the relationships between variables. The degree of associative dependence is: significant at 0.5 <rho = 0.7; large at 0.7 <rho = 0.9; extremely large at rho> 0.9. Statistical significance is assumed at $p \leq 0.05$.

Results

The diagnosed pigment tumors, for the period 2014 - 2019 in the laboratory in DCC2 EOOD Dobrich, were selected as suitable for this study, based for the most part on immunohistochemical analysis - a total of 91 pigment neoplasms, including 57 benign melanocyte nevi, ten atypical nevi and 24 malignant melanomas (Table 4.1 - Table 5.3).

Table4.1. Distribution of tested benign nevus by histological type

	Compound	intradermal	Border	Reed pigment nevus	Combined	congenital	Total
Number (n=)	30	22	1	1	1	2	57

Table4.2. Distribution of atypical nevi tested according to their histological appearance

Number (n=)	atypia in the border component		atypism in the dermal component	Total
	low-grade	high-level		
8		1	1	10

Table4.3. Distribution of melanomas tested according to their histological appearance

Number (n=)	nodular		superficially advancing	Spitz melanoma	malignant son nevus	childhood melanoma	Total
	primary dermal	vertical growth phase					
2		16	3	1	1	1	24

In studies for the present study material, the selected lesions, strictly meeting the clinical and morphological criteria for the respective group, are presented compared to Table 4.4. Benign melanocyte lesions account for 62.63% (n=57), atypical nevi are 11% (n=10), and cases of malignant melanoma - 26.37% (n=24).

Table4.4. Distribution of the tested pigment neoplasias in the three target groups

	Benign nevi	Atypical nevi	Melanomas	Total
Number (n=)	57	10	24	91
%	62,63%	11%	26,37%	100%

Demographic indicators of the material under examination

They include data on the sex distribution of the lesions, age characteristics, and location of the formations:

The gender distribution by groups is as follows:

Sex distribution of benign nevi: in cases of benign nevi, the female sex prevails - 42 cases, men are - 15 cases, respectively:

- 46.15% women and 16. 48% men

Sex distribution of atypical nevi: in patients with atypical nevi, women are 5 cases; men are also 5 cases, respectively:

- 5.50% men and 5.50% women

Gender distribution in malignant melanomas in the studied material: the distribution shows equality of the two sexes - 12 cases in men and 12 in women: 13.2% men and 13.2% women.

The relative share of the distribution of lesions by sex in the individual groups of pigment lesions is relative as follows: (Table 4.5) In all the material studied by us, the female sex dominates, almost 2: 1 predominance of women with pigmented tumors compared to men.

Table4.5. Comparative percentage distribution of lesions studied according to patient sex

gender	Benign nevi % (n=)	Atypical nevi	Melanomas	**Total**
Men	16,48 % (n=15)	5,50 % (n=5)	13.20 % (n=12)	35,17 % (n=32)
Women	46.15 % (n=42)	5,50 % (n=5)	13.20 % (n=12)	64,83 % (n=32)
Total	62,63 % (n=57)	11 % (n=10)	26.37 % (n=24)	100 % (n=91)

The distribution by age:

The mean age of melanoma patients in the study material was 59.82 years (range 8 - 89 years).

In all age groups, except for the group 11 to 20 years, cases of melanoma were found, with the highest frequency in the age group 61 to 70 years, followed by 71 to 80 and 51 to 60 (Fig. 4.1).

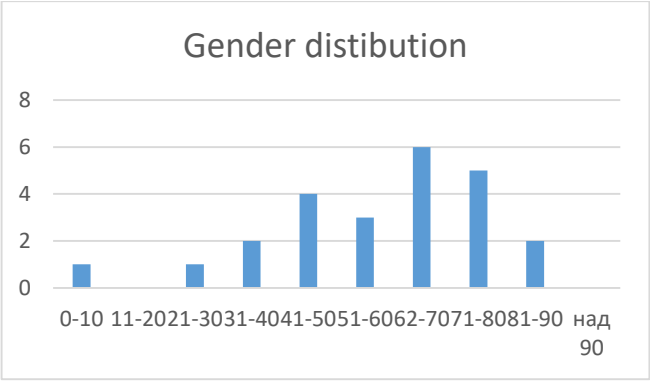

Figure4.1: Distribution of malignant melanoma cases by age group

From these data, it follows that as a percentage, the majority of cases of melanoma were diagnosed in patients over 40 years of age, and the difference compared to the other age groups was statistically significant ($X^2 = 38.13$, $p = 0.001$). The number of possible melanomas detected increases with increasing age, i. e., there is a positive correlation (rho = 0.463, p = 0.001).

Topographical distribution (localization) of benign **nevi, atypical nevi, and melanomas:**

These data show that most benign nevi are in the head and neck area, followed by those in the back, chest, and abdomen, less commonly in the upper and least in the lower extremities.

Atypical nevi are often localized on the skin of the back and abdomen, followed by those in the head and neck (atypical is the only nevus found in the face).

The cases of malignant melanoma diagnosed in the area of the skin of the lower extremities prevail, followed by those of the back and chest (the last two localizations have equal values). (Fig. 4.2)

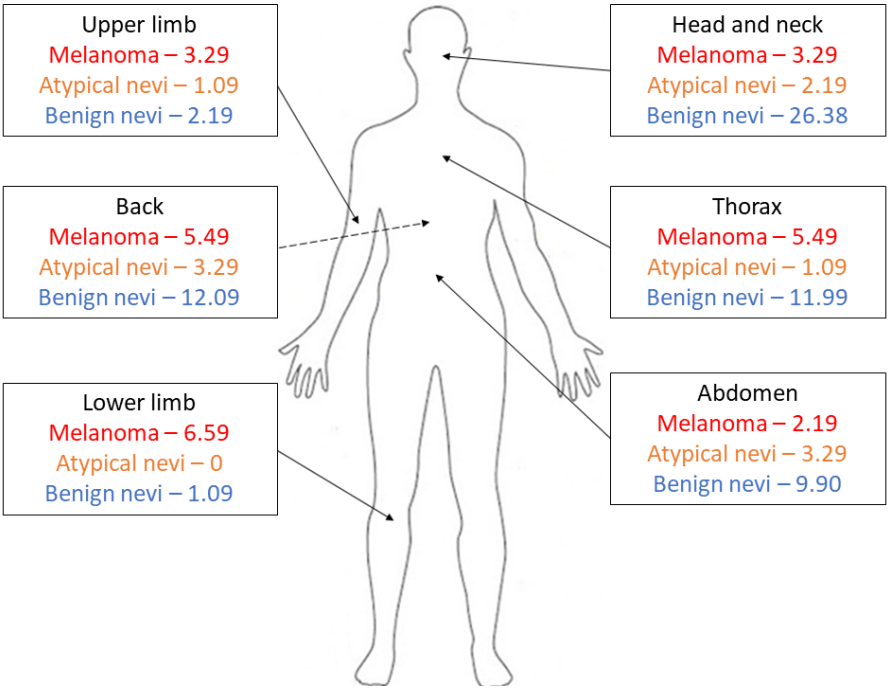

Figure4.2: Topographical distribution (localization) of pigment lesions from the test material.

Basic morphological characteristics of the test material

The diagnostic cohort included a total of fifty-seven nevi - 30 (thirty) are compound, 22 (twenty-two) intradermal, 1 (one) borderline, 1 (one) combined (Spitz with conventional compound), 1 (one) pigmented Reed nevus, 2 (two) congenital small composite type. Atypical nevi are 10 (ten): 1 (one) composed of low-grade atypia in the dermal component, four composed of low-grade dysplasia in the borderline component, and 5 (five) of borderline type with low-grade atypia in four of them and one with severe dysplasia. Melanomas are 18 (eighteen) nodular, 3 (three) superficially advanced, 1 (one) malignant blue nevus, 1 (one) Spitz melanoma, and 1 (one) pediatric primary dermal nodular melanoma:

Tumor thickness of melanomas in research material:

In the study material of twenty-four melanomas, the relative proportion of cases of MM according to the thickness of the lesion is as follows: one (4.17%) - Breslow 1, one (4.17%) - Breslow 2, eight (33.33 %) - Breslow 3, fourteen (58.33%) - Breslow 4

(Fig. 4.3). The predominant cases are with Breslow 4, followed by those with Breslow 3.

Figure 4. 3: Distribution of melanomas according to their thickness, with a horizontal showing the thickness by Breslow and the vertical showing the number of melanomas of the corresponding thickness

Presence of ulcerations in melanomas from the studied material:

In the study material, seventeen melanomas had ulceration; all were nodular, ten with ulceration sizes below 5 mm and seven from 5 to 12 mm. The other melanomas, including one nodular - primary dermal, the three superficially advanced, spitzoid melanoma, pediatric, and malignant blue nevus are without ulceration.

Satellites in melanoma:

Three of the melanomas in the study material (malignant blue nevus and two nodular) have microsatellites in the hypodermis and peripheral dermis

Mitotic activity of melanomas (quantitative measurement of melanoma proliferation) - i. e., the determination of mitoses in 1 sq. mm area in the studied cases with MM shows the following distribution:

1. The mitotic activity in thin melanomas (up to 1 mm thick) is low. i.e., less than one mitosis / sq.mm; in the second, with a thickness of 1 mm, two mitoses / sq.mm are established.

2. In two of the superficially advancing melanomas, which are thin - the mitotic activity is low; in the third, with a thickness of 2 mm - the mitotic activity is high (17 mitoses / 1 sq. Mm).

3. In nodular melanomas, the mitotic activity is high, on average 22.90 mitoses, / one sq.mm, with an average melanoma thickness of 14.42 mm.

4. The average mitotic activity of non-ulcerated nodular melanomas is 17 mitoses / 1 sq. Mm, with an average lesion thickness of 8 mm.

5. The average mitotic activity of ulcerated melanomas in the selected material is 24.62 mitoses / 1 sq. Mm, at an average thickness of 11.29 mm, ie.

6. The average mitotic activity of ulcerated melanomas is higher than that of non-ulcerated mitoses with 7.62 / 1 sq. Mm at a greater thickness, on average 3.29 mm, i. e., 2.32 mitoses / 1 sq. Mm. more for each mm thick more.

Clark level of melanoma invasion in the study material:

In the studied material, the defined micro invasion by Clark is as follows) - the most significant number is melanomas with micro invasion by Clark 3 - nine cases (37.50%), followed by Clark 5 - seven cases (29.16%), and an equal number of melanomas with micro invasion Clark 4 - four (16.67%) and Clark 2 - four (16.67%) (Fig. 4.4).

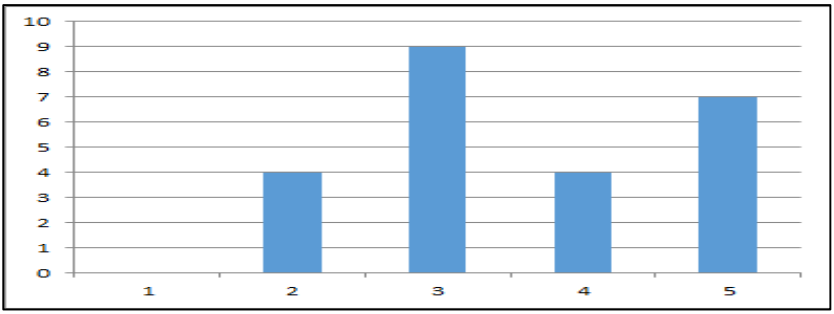

Figure 4. 4. Graphical visualisation of Clark micro invasion in research material

Regression of melanomas: In the studied material, no signs of regression in thin melanomas were found. It is found only in one of the nodular melanomas, where the regression is partial (focal), in the central part of the lesion with fibrosis and melanophages, without melanoma cells in the dermal regression area Tumor-infiltrating lymphocytes in melanomas (TILs), which are a manifestation of an immune response to tumor cells, in this case, melanoma, in the studied material show the following characteristic: in three of the melanomas an intense, represented by dense infiltrates in the stroma between (complete) in the periphery, lymphocytic infiltration; in four cases it is moderate in intensity, represented by sparse lymphocyte groups focal in the tumor stroma and focal one at the border of invasion; the largest is the group of melanomas with minimal and mostly absent steep lymphocytic infiltration - seventeen. In our material, only three of the melanomas have high-grade (high intensity) TIL**.**

Results of immunohistochemically tested expression of Cyclin D1, BCL2, and p53

Based on our experience in the diagnosis of pigment tumors showing marked diversity in the immunohistochemical

expression of the same antibody in the same diagnostic group, in order to present this diversity in a systematic way useful for practice, we reported the expression in eleven groups. These groups or models refine it in detail, both in intensity and in topographic distribution (see chapter methods - reporting the results of immunohistochemical examination):

Cyclin D1 study results

Cyclin D1 expression in benign pigment lesions (Fig. 4. 5):

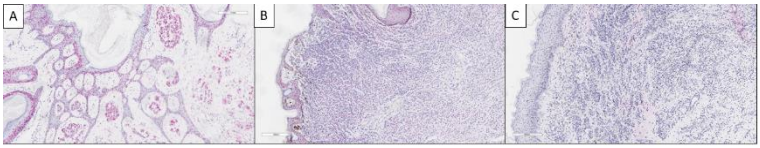

Figure 4. 5: IHC reaction Cyclin D1 in nevi: A – high-intense excretion in the upper portion of the lesion, original magnification 100x; B – low-intense spotted expression, original magnification 100x; C – lack of expression in the lesion, original magnification 100x

- In summary, the results show the following:
- In benign pigmented lesions, in a tiny percentage of cases - one (1.75%), there is no expression of the antibody.
- Spotted expression was found more often - in forty-two (73.68%) of the total number of benign lesions in the material, with varying intensity, at most with moderate intensity - thirty (71.43%) cases, followed by these with low intensity - nine (21.43%) and the lowest number of cases, with high intensity - three (7.14%);
- Next in frequency is the expression in the upper portion of the lesion - thirteen (22.80%) of the total number of benign lesions, also varying in intensity, from low-intensity in six (46.15%), moderately intense in six (46.15). %) and high intensity in one (7.69%) case.
- Diffuse expression in the entire thickness of the lesion is found in one (1.75%) of the nevi and with low intensity.
- There are no nevi with expression throughout the lesion that is moderately intense, highly intense, or heterogeneous.

Cyclin D1 expression in atypical nevi (Fig. 4.6):

(The cases were reduced due to insufficient IHC examination material after the incisions made for the histological examination in 4 of the lesions, which were very small in size).

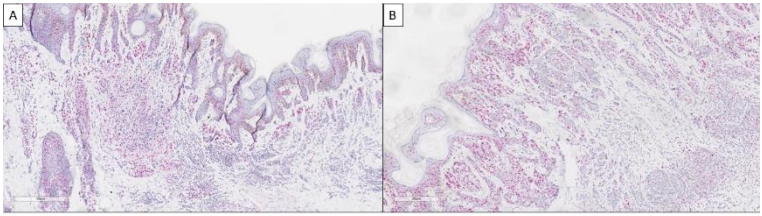

Fig. 4.6: IHH reaction Cyclin D1 in dysplastic nevi A – moderately intense spotted expression original magnification 100x; B – high intense expression in the upper portion original magnification 80x

- All atypical lesions express Cyclin D1.
- There are no cases that show low-intensity diffuse expression, moderately intense diffuse or heterogeneous in intensity throughout the thickness of the lesion expression.
- In equal percentage, the lesions show low intensity in the upper portion and spotted, low-intensity expression (respectively 16.67%), moderately intense in the upper

portion or moderately intense spotted (respectively 16.67%), high-intensity in the upper portion, high-intensity spotted, and high-intensity diffuse (respectively 16.67%);

- No expression of Cyclin D1 expression heterogeneous in intensity throughout the lesion.

Expression of Cyclin D1 in melanomas (Fig. 4. 7):

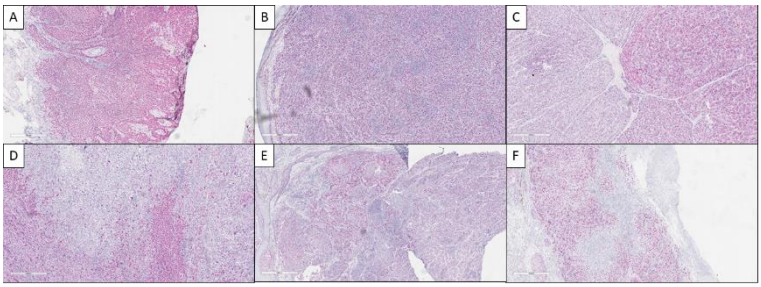

Figure 4. 7: Cyclin D1expression in melanomas: A -high-intensity diffuse expression; B moderately intense diffuse expression; C – heterogeneous (different in intensity throughout the thickness of the lesion; D – heterogeneous (different in intensity throughout the thickness of the lesion); E – high-intensity spotted expression; F – high-intensity spotted expression; original magnification 40x,

The results of the expression of Cyclin D1 in malignant melanomas show:

- All melanomas express Cyclin D1.
- There are no melanomas with expression only in the upper portion of the lesion; the expression is distributed throughout the thickness of the lesion - spotted or diffuse.
- The intensity of the expression is moderate, high, low intensity, or heterogeneous.

The comparative analysis of the expression of Cyclin D1 antibody in benign nevi, atypical nevi, and melanomas presented as a percentage of the total number of lesions is as follows (Table 4.6):

Table 4.6- Comparative expression of the antibody Cyclin D1 in benign nevi, atypical nevi, and melanomas

	diagnostic group			
Cyclin D1	benign nevi % (n=)	Atypical nevi % (n=)	Melanomas % (n=)	Total % (n=)
no expression to single cells with weak expression	1,15% (n=1)	0% (n=0)	0% (n=0)	1,15% (n=1)
low-intensity expression in the upper portion of the	6,9% (n=6)	0% (n=0)	0% (n=0)	6,9% (n=6)

lesion				
Low intensity spotted expression	10,34% (n=9)	1,15% (n=1)	0% (n=0)	11,49% (n=10)
Low intensity diffuse expression	1,15% (n=1)	0% (n=0)	0% (n=0)	1,15% (n=1)
Moderate intensity expression in the upper portion of the lesion	6,9% (n=6)	1,15% (n=1)	0% (n=0)	8,05% (n=7)
Moderate intensity spotted expression	34,48% (n=30)	1,15% (n=1)	1,15% (n=1)	36,78% (n=32)
Moderate intensity diffuse expression	0% (n=0)	0% (n=0)	1,15% (n=1)	1,15% (n=1)
High intensity expression in the upper portion of the lesion	1,15% (n=1)	1,15% (n=1)	0% (n=0)	2,3% (n=2)
high intensity spotted expression	3,45% (n=3)	1,15% (n=1)	10,34% (n=9)	14,94% (n=13)
High intensity diffuse expression	0% (n=0)	1,15% (n=1)	13,74% (n=12)	14,94% (n=13)
heterogeneous expression	0% (n=0)	0% (n=0)	1,15% (n=1)	1,15% (n=1)
Total	65,52% (n=57)	6,9% (n=6)	27,59% (n=24)	100% (n=87)

The percentage differences in cyclin D1 expression between the groups was statistically significant ($X2 = 73.7$, $p = 0.001$)

To determine whether or not there was a significant difference in the expression of Cyclin D1 in the studied groups, an additional variation analysis, ANOVA rank test, and Kruskal-Wallis test for nonparametrically distributed data with graphical visualization of the compared values presented with Error Bar Graphs.

The differences between the values in this analysis are significant, with the value $p < 0.05$ accepted for biological experiments. These additional analyses assessed the relationship between Cyclin D1 expression and the type of melanocyte lesion.

The table (Table 4.7) shows the mean values of Cyclin D1 expression in the different diagnostic groups. It is evident that they are highest in melanomas (9.375 ± 1.056), lowest in benign nevi (5,070 +/- 1,860), and intermediate in atypical nevi (6,833 +/- 2,639).

Table 4.7. Mean Cyclin D1 expression values in the target groups studied

diagnostic group	arithmetic average	standard deviation	count
Benign nevi	5.070	1.860	57
Atypical nevi	6.833	2.639	6
Melanomas	9.375	1.056	24

The graphical visualization of the compared values of the indicators is presented with Error Bar Graphs (Fig.4.7). The range of values is the widest in atypical nevi, marginally high in melanomas, and marginally low in benign nevi, i. e., the largest and most significant difference in expression is between benign and malignant lesions.

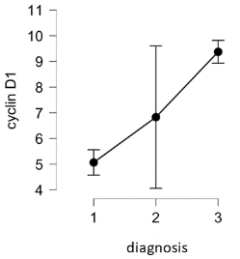

Figure 4. 7: Graphical range of Cyclin D1 expression in the target groups studied: 1 - benign nevi, 2 - atypical nevi; 3 - melanomas

The analysis of the results on whether there are significant statistical differences between the three diagnostic groups (benign nevi, atypical nevi, and melanoma) compared to the values of Cyclin D1 performed with ANOVA test shows a statistically significant difference ($F = 51.935$, $p < 0.001$) (Table 4.8):

Table 4. 8. ANOVA test for the expression of Cyclin D1

Cases	**Amount**	**df**	**Square**	**F**	**p**
Diagnosis	314.305	2	157.153	51.935	< 0.001
Remainder	254.178	84	3.026		

Specific statistical differences were also found in the Post Hoc analysis by Tukey and Dunn (Table 4.9) between benign nevi and melanomas, where the differences compared to the values of the indicator are the largest, $t = 10.170$, $p < 0.001$. There is also a statistical difference between atypical nevi and melanomas ($t = 3.201$, $p = 0.005$).

Table 4. 9. Post Hoc test using the Tukey and Dunn methods to identify the differences between The Cyclin D1expression in the three target groups:

	Average difference	**Upper**	**Lower**	**95% CI**	**SE**	**t**	**p**
Benign - atypical	-1.763	-3.545	0.018	0.747	-2.362	0.053	
Benign nevi - melanomas	- 4.305	-5.315	- 3.295	0.423	-10.170	< .001	***
Atypical nevi - melanomas	- 2.542	-4.436	- 0.647	0.794	-3.201	0.005	**

A high average difference is reported between benign and atypical nevi, between atypical nevi and melanoma, between benign nevi and melanoma, as statistically very high. The most

significant is that between benign nevi and melanoma - average difference 4,305. Therefore, the highly statistically significant difference in Cyclin D1 expression between benign and malignant lesions in the study material reflects the possibility of using Cyclin D1 expression to predict malignant potential in pigment tumors.

Results of the p53 study

The exact number of lesions was studied - 57 benign, six atypical, and 24 melanomas presented in the results as absolute value and relative share.

Expression of p53 in benign nevi (Fig. 4.8):

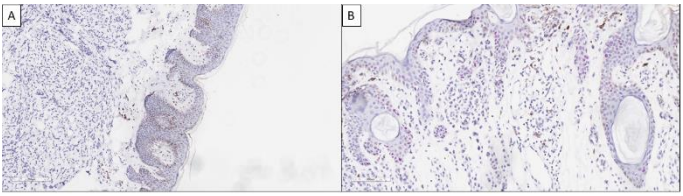

Figure 4. 8: expression of p53 in benign nevi: A – without expression original magnification 100x; B – low intense expression in the upper portion of the lesion original magnification 200x

Fifty-six (98.25%) of benign nevi lacked p53 expression, except for one (1.75%), which showed low-intensity expression in the upper portion of the lesion.

Expression of p53 in atypical nevi (Fig. 4.9):

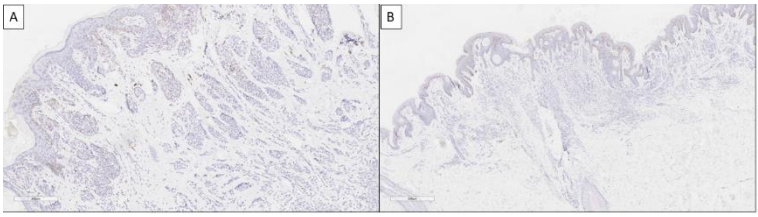

Figure 4.9: IHC p53 expression in atypical nevi: A – moderately intense spotted expression original magnification 100x; B – lack of expression original magnification 80x.

In the studied material - one (16.67%) of the atypical nevi shows moderately intense spotted expression; the rest lacks such, i. e. the majority of 83.33% of atypical nevi do not show p53 protein expression.

Expression of p53 in malignant melanomas (Fig. 4.10):

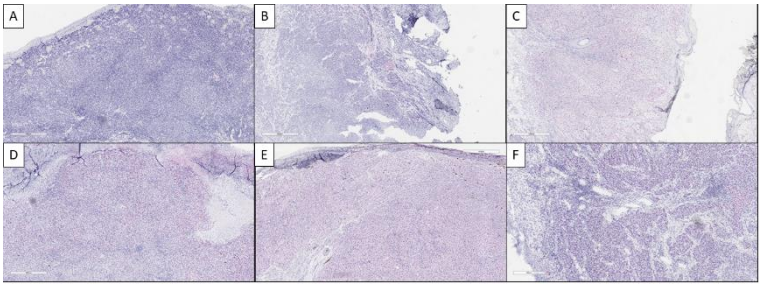

Figure 4.10: immunohistochemical p53 protein expression in melanomas: A -negative; B slightly intense diffuse; C – slightly intense diffuse; D – moderately intense diffuse; E – moderately intense diffuse; F – heterogeneous expression; original magnification 40x

The expression of p53 in the studied 24 melanomas summarizes the following results:

- Nineteen (79.17%) of malignant melanomas in the study material express p53, with the highest percentage of 8 (33.33%) of them showing moderately intense macular expression, followed by a low-intensity macular expression in 4 (16.67%). From high-intensity spotted in 3 (12.50%), the moderately intense diffuse expression has in 2 (8.33%), and similar, low-intensity expression in the upper portion and heterogeneous intensity varying in intensity throughout the

lesion thickness was reported in 1 (respectively 4.17%) of melanomas.

- Five (20.73%) of the studied melanomas do not show expression.

The comparative analysis of p53 antibody expression in benign nevi, atypical nevi, and melanomas is presented as a percentage of the total number of lesions in the following table (Table 4.10)**:**

Table 4.10with a comparative analysis of p53 expression in the three target groups

	diagnostic group			
p53	Benign nevi % (n=)	Atypical nevi % (n=)	Melanomas % (n=)	**Total** % (n=)
no expression to single cells with weak expression	64.37 % (n=56)	5.75% (n=5)	5.75 % (n=5)	75.86 % (n=66)
Low intensity expression in the upper portion of the lesion	1.15 % (n=1)	0 % (n=0)	1.15 % (n=1)	2.30 % (n=2)
Low intensity spotted expression	0 % (n=0)	0 % (n=0)	4.60 % (n=4)	4.60 % (n=4)
Moderate intensity spotted expression	0 % (n=0)	1.15 % (n=1)	9.20% (n=8)	10.35 % (n=9)
Moderate intensity diffuse expression	0 % (n=0)	0 % (n=0)	2.30 % (n=2)	2.30% (n=2)

High intensity spotted expression	0 % (n=0)	0 % (n=0)	3.45 % (n=3)	3.45 % (n=3)
heterogeneous expression	0 % (n=0)	0 % (n=0)	1.15 % (n=1)	1.15 %
Total	65.52 % (n=57)	6.90 % (n=6)	27.59 % (n=24)	100 % (n=87)

The percentage differences in p53 expression between the groups were statistically significant (X2 = 58.6, p = 0.001).

An ANOVA test was additionally performed to highlight differences in expression between the study groups.

The result of the additional variation analysis, ANOVA test for parametric data and Kruskal-Wallis test for nonparametrically distributed data, to compare the differences in p53 protein expression between benign nevi, atypical nevi, and malignant melanomas show the following: (Table 4.11**)**

Table 4.11. Variational analysis for p53 expression in melanocyte lesions

Diagnostic group	**Arithmetic mean**	**Standard deviation**	**Total**
Benign nevi	1.018	0.132	57
Atypical nevi	1.833	2.041	6
Melanomas	4.958	2.985	24

The differences between the values in this analysis are significant, with the value p <0.05 accepted for biological experiments. At p53, mean values and standard deviations confirmed, as expected, the highest mean value in the group of melanomas (M = 4.958 ± 2.985), and the lowest mean value - in benign nevi (M = 1.018 ± 0.132); in atypical nevi the values occupy an intermediate position (M = 1,833 +/- 2,041). The graphical visualization of the compared values of p53 is presented with Error Bar Graphs, which shows the latitude in the range of the indicator in the individual groups (Fig. 4.11)**.**

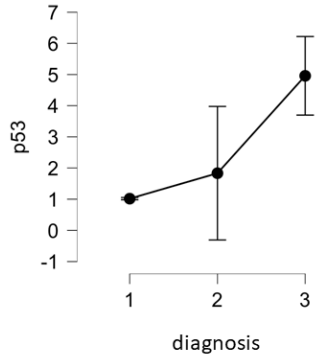

Figure 4.11: Graphical range for p53 expression in melanocyte lesions:1- benign nevi, 2-atypical nevi; 3-melanomas

Analysis of the results for whether there were significant statistical differences between the three diagnostic groups (benign nevi, atypical nevi, and melanoma) compared to p53 values performed with the Kruskal-Wallis test showed a statistically significant difference ($H = 55.381$, $p < 0.001$) (Table 4.12).

Table 4.12. Kruskal-Wallis Test

Factor	**Amount**	**df**	**p**
Diagnosis	55.381	2	< .001

In particular, statistically significant differences in Dunn's Post-Hoc analysis were found between benign nevi and melanomas (here the differences compared to the values of the indicator are the largest, ($z = 7.429$, $p < 0.001$) and atypical nevi and melanomas ($z = 3.191$, $p = 0.001$) (Table 4.13).

Table 4.13.: Confidence interval of p53 expression in the test groups of *melanocyte lesions Dunn's Post Hoc Comparisons – diagnosis*

Comparative analysis	**z**	**W $_{i}$**	**W $_{j}$**	**p**	**p $_{bonf}$**	**p $_{holm}$**
Benign nevi – atypical nevi	-0.819	34.096	40.750	0.207	0.620	0.207
Benign nevi - melanomas	-7.429	34.096	68.333	< .001	< .001	< .001
Atypical nevi - melanomas	-3.191	40.750	68.333	< .001	0.002	0.001

Therefore, the highly statistically significant difference in p53 expression between benign and malignant lesions in the study material reflects the possibility of using p53 expression to predict malignant potential in pigment tumors.

BCL2 Expression Results

BCL2 expression results in the benign nevi studied is shown in Fig. 4.12:

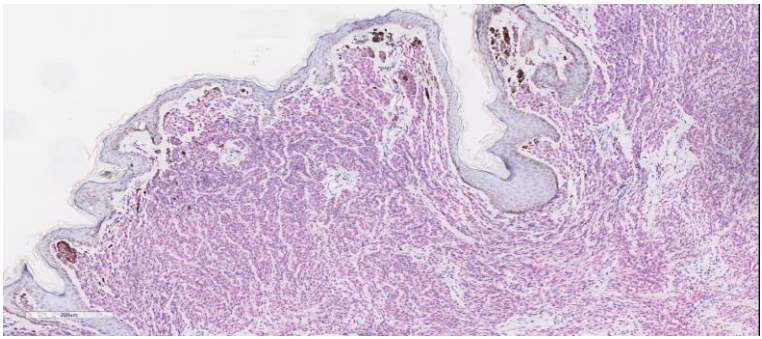

Figure 4.12: IHC expression BCL2 in melanotic nevus – high intense diffuse, original magnification 100x.

All benign nevi in the studied material - 57 cases, i. e., 100% express uniform, high intense, and diffuse BCL2.

BCL2 expression results in atypical nevi (Fig. 4.13):

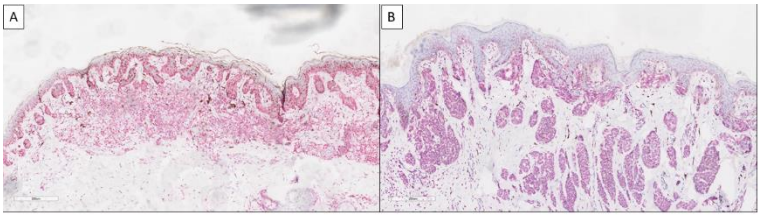

Figure 4.13: BCL2 expression in atypical nevi: A – moderately intense diffuse original magnification 100x; B – high intense diffuse original magnification 100x

Atypical nevi show moderately intense spotted expression in 1 (16.67%) cases, moderately intense diffuse in 1 (16.67%) cases, high-intensity diffuse expression in 4 (66.67%) cases. I.e., the largest number of lesions - 4 (66.67%), shows high-intensity diffuse expression, identical to benign lesions.

Results of BCL2 expression in the studied malignant melanomas (Fig. 4.14):

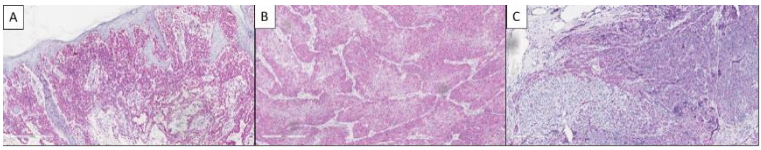

Figure 4.14: IHC reaction BCL2 in melanomas: A – high intense diffuse *expression, original magnification 100x; B – heterogeneous expression, original magnification 100x; C – high intense spotted expression, original magnification 100*

All melanomas express BCL2, with high-intensity diffuse expression (similar to that in benign lesions) present in 9 (41.67%) of melanomas, followed by moderately intense diffuse in 6 (25%) cases, varying in intensity - heterogeneous in 5 (20.83%), followed by high-intensity spotting in 2 (8.33%) of them and moderately intense spotting in 1 (4.17%) of

melanomas. I.e., high intensity BCL2 expression in malignant melanomas decreased below 50% of the studied and was distributed between expressions with decreasing intensity, spotting expression, and heterogeneous expression, in total in 58.33% of melanomas in the studied material.

The comparative analysis of BCL2 antibody expression in benign nevi, atypical nevi, and melanoma, presented in tabular form (Table 4.14), is as follows:

Table 4.14: *BCL2 expression in the three target groups represented as % of the total number of pigmented neoplasias studied:*

	Diagnostic group			
BCL 2	Benign nevi % (n=)	Atypical nevi % (n=)	Melanomas % (n=)	**total** % (n=)
Moderate intensity spotted expression	0 % (n=0)	0 % (n=0)	1.15 % (n=1)	1.15 % (n=1)
Moderate intensity diffuse expression	0 % (n=0)	1.15 % (n=1)	6.90 % (n=6)	8.05% (n=7)

High intensity spotted expression	0 % (n=0)	1.15 % (n=0)	2.30% (n=2)	3.45 % (n=3)
High intensity diffuse expression	65.52 % (n=57)	4.60 % (n=4)	11.49 % (n=10)	81.61 % (n=71)
Heterogeneous expression	0 % (n=0)	0 % (n=0)	5.75 % (n=5)	5.75 % (n=5)
Total	65.52 % (n=57)	6.90 % (n=6)	27.59 % (n=24)	100 % (n=87)

The difference in BCL2 expression between the groups was statistically significant ($X2 = 43.3$, $p = 0.001$).

The summary results of BCL2 expression in the study groups showed that 71 of 87 lesions studied represented high-intensity diffuse expression - 57 of 57 benign lesions, 4 of 6 atypical nevi and 10 of 24 melanoma. This required further analysis. For the BCL2 expression, the Anova test cannot be used as an additional analysis because the data are clustered around one type of expression - high-intensity diffuse in 71 of the 87 pigment lesions examined, making variation analysis

impossible. Therefore, we used X-square test (X2) analysis (Table 4.15).

Table 4.15: Results of chi-square analysis of *BCL2 expression in the three targeted groups of melanocyte lesions*. *In the table of digits 6, 7, 9, 10, and 11, respectively, the models of BCL2 expression manifested in the targeted groups were noted: 6 – moderately intense spotted 7 – moderately intense diffuse, 9 – highly intense spotted, 10 – highly intense diffuse, 11 – heterogenic expression (according to the model proposed by us and used in the study to assess the degree and localization of antibody expression).*

Diagnostic group	**6 % (n=)**	**7 % (n=)**	**9 % (n=)**	**10 % (n=)**	**11 % (n=)**	**total % (n=)**
Benign nevi	0 % (n=0)	0 % (n=0)	0 % (n=0)	65.52 % (n=57)	0 % (n=0)	65.52 % (n=57)
Atypical nevi	0 % (n=0)	1.15 % (n=1)	1.15 % (n=1)	4.60 % (n=4)	0 % (n=0)	6.90 % (n=6)

Melanomas	1.15 % (n=1)	6.90 % (n=6)	2.30 % (n=2)	11.49 % (n=10)	5.75 % (n=5)	27.59 % (n=24)
Total	1.15 % (n=1)	8.05 % (n=7)	3.45 % (n=3)	81.61 % (n=71)	5.75 % (n=5)	100 (n=87)

The results of the X-square test show an existing difference between the values of BCL2 expression in the different diagnostic groups (X2 = 43.349, p <0.001). However, they appear to be concentrated mainly around high-intensity diffuse BCL2 expression (10) in benign nevi. The indicator distributions are similar, but percentage less, in the other two groups - atypical nevi and malignant melanomas (Table 4.16**).**

Table 4.16: *Chi-Squared Tests showing the statistical significance of a non-parametric test at p ≤0.05*

	Amount	**df**	**p**
X^2	43.349	8	< 0.001

N	87

S100 protein expression results

The study of S100 protein expression in benign pigment lesions shows the following distribution (Fig. 4.15):

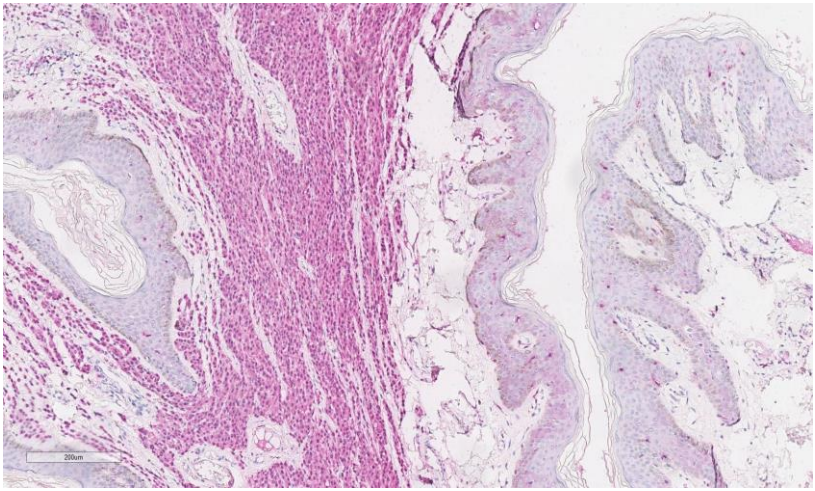

Figure 4.15: S100 protein expression in benign melanocytic nevus, high intense diffuse original magnification 100x.

All benign nevi show diffuse and highly intense S100 protein expression.

Results of S100 protein expression in the atypical nevi studied are shown in Fig. 4.16:

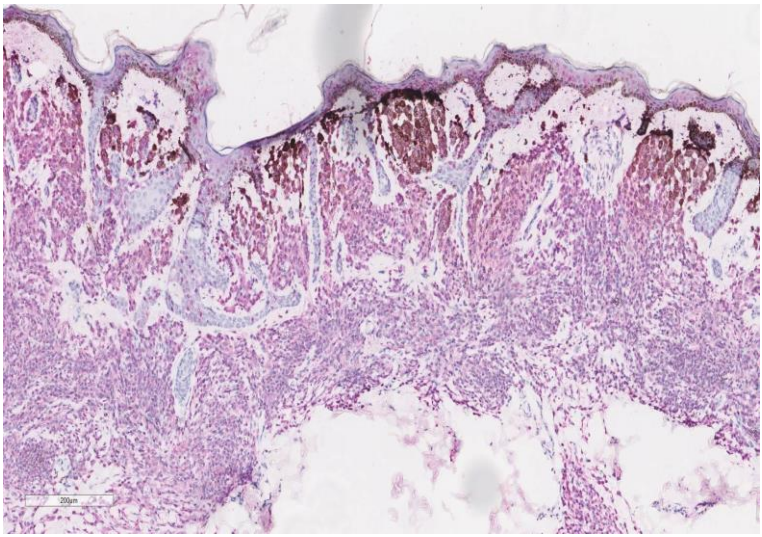

Figure 4.16: IHC expression S100 protein in atypical melanocytic nevus: moderately intense diffuse expression original magnification 100x.

All atypical nevi in the research material expressed S100 protein, diffuse, mostly high intense – 83.33% of them, with only 16.67% expressing the antibody of moderate intensity.

Results of the expression of S100 protein in the malignant melanomas studied is shown in Fig. 4.17:

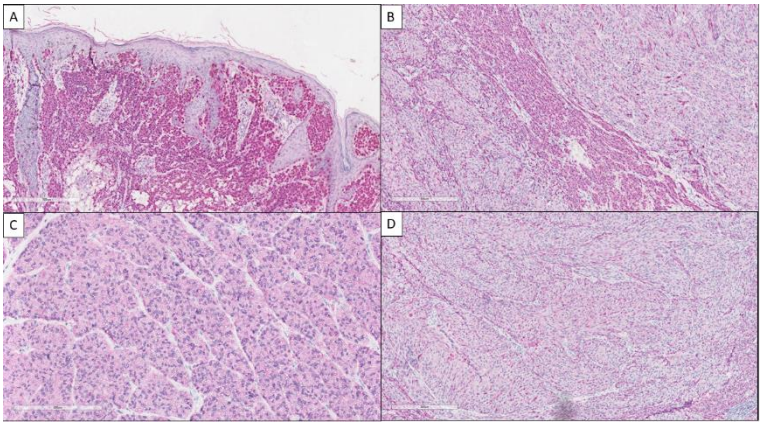

Figure 4.17: IHC reaction S100 protein in melanomas: A – high-intensity diffuse expression original magnification 100x; B – heterogeneous expression (in the entire thickness of the lesion of different intensity) original magnification 100x; C - moderately intense diffuse expression original magnification 100x; D – low intense expression original magnification 100x

The comparative analysis of the expression of antibody S100 protein in benign nevi, atypical nevi, and melanomas show (Table 4.17) that:

All melanomas in the studied material express S100 protein, the most significant part - nine (37.50%) of them express the antibody diffusely with moderate intensity, followed, in equal proportions - seven (respectively 29.17%) with high intense diffuse expression and heterogeneous with varying intensity throughout the lesion thickness; a minor part - one (4.17%) of melanomas shows low-intensity diffuse expression.

Table 4.17: S100 protein expression in the three targeted groups with the presentation and as % of the total number of pigmented *neoplasias studied:*

	diagnostic group			
S100 protein	Benign nevi % (n=)	Atypical nevi % (n=)	Melanomas % (n=)	**Total** % (n=)
Low intensity diffuse expression	0 % (n=0)	0 % (n=0)	1.15 % (n=1)	1.15 %(n=1)
Moderate intensity diffuse expression	0 % (n=0)	1.15 % (n=1)	10.35 % (n=9)	11.50 % (n=10)
High intensity diffuse expression	65.52 % (n=57)	5.75 % (n=5)	8.05 % (n=7)	79.31 % (n=69)

heterogeneous expression	0 % (n=0)	0 % (n=0)	8.046 % (n=7)	8.05% (n=7)
Total	65.52 % (n=57)	6.90% (n=6)	27.59 % (n=24)	100 % (n=87)

All studied pigment neoplasms 57 (65.52%) benign, 6 (6.90%) atypical, and 24 (27.59%) melanomas express S100 protein. Expression of S100 protein in all lesions studied is an opportunity for the antibody to be used to demonstrate melanocyte origin.

Melanosome clone HMB45 results

Expression of HMB45 in benign nevi (Fig. 4.18):

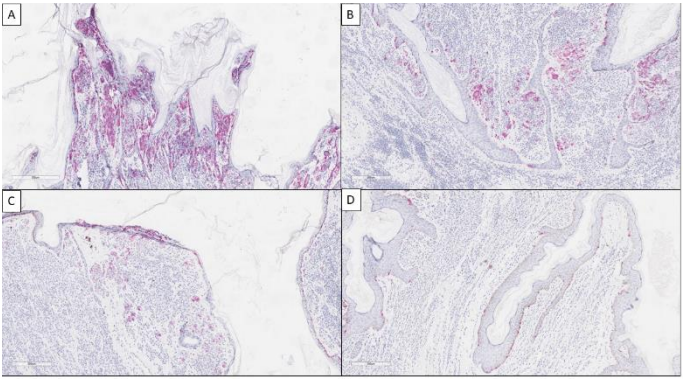

Figure 4.18: IHC expression of HMB45 in benign nevi: A – high-intense expression in the upper portion of the lesion magnification 100x; B – moderately intense in the upper

portion of the lesion, original magnification 100x; C – low intense expression in upper lesion portion, original magnification 100x; D – the absence of HMB 45 expression in the lesion, original magnification 100x

The majority - 53 (92.98%) cases of the total examined nevi express HMB45, and only 4 (7.02%) of them remain without expression.

The expression is in the upper portion - 94.34% of the expressing lesions and only 5.26% show moderately intense macular expression.

The expression in the upper portion varies in intensity from low to high; in most cases, the expression is of moderate and high intensities in equal proportions - resp. 40% and twice less or one-fifth of the total number for this group have a low-intensity expression - 20.00%.

To summarize the results, it can be said that nevi predominantly express HMB45 in the upper portion of the

lesion with varying intensity, from low to high, i.e., there is a gradient in expression in benign lesions.

Expression of HMB45 in atypical nevi is shown in Fig. 4.19

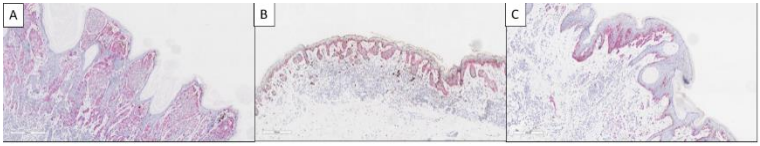

Figure 4.19: HMB45 expression in atypical nevi: A – high intense diffuse original magnification 80x; B – measuredly intense in upper portion original magnification 100x; C – high intense in upper portion original magnification 100x.

All atypical nevi in the test material express Melanosome clone HMB45.

In atypical nevi, low intensity expression is not recorded.

The majority of atypical nevi - 4 (66.66%) of them show expression in the upper portion of the lesion, i. e., show a gradient in expression, with 50% of those with a gradient exhibiting an expression gradient with moderate expression intensity and 50% with high expression intensity

One-third (33.33%) of atypical nevi did not show a gradient of HMB45 expression, with 1 (16.67%) of atypical nevi showing moderately intense spotting and 1 (16.67%) of high-intensity diffuse expression.

Expression of Melanosome clone HMB 45 in the studied malignant melanomas (Fig.4.20)

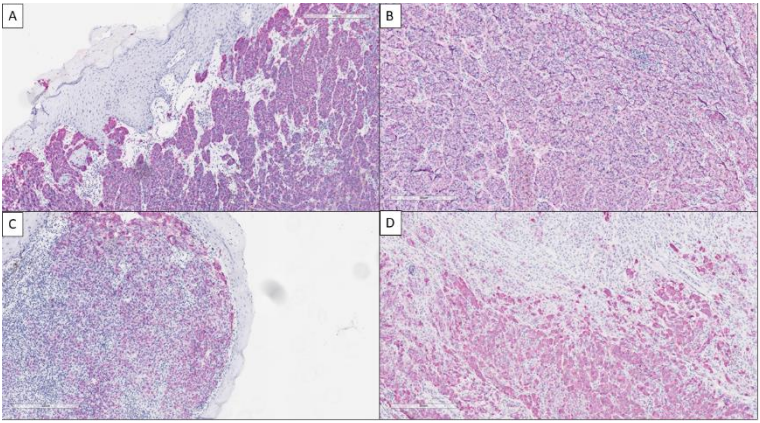

Figure 4.20: IHC reaction HMB45 in melanomas: A – highly intensive diffuse expression original magnification 100x; B – moderately intense diffuse expression original magnification 100x; C – high intensity spotted original expression

magnification 100x; D – heterogeneous expression original magnification 100x

The comparative analysis of the expression of Melanosome clone HMB45 antibody in benign nevi, atypical nevi, and melanomas (Table 4.18) is as follows:

All melanomas in the test material express Melanosome clone HMB45, with no gradient in expression, i. show expression throughout the thickness of the lesion.

In melanomas, low-intensity expression is not reported.

The majority of melanomas - 18 (75%), show high - intensity expression: 12 (50%) show high - intensity spotted expression and 6 (25%) show high - intensity diffuse expression.

The most frequently observed expression in melanomas after the high intensity, diffuse or mottled distribution is the heterogeneous (varying in intensity expression throughout the thickness of the lesion), found in 5 (20.83%) of the melanomas in the studied material.

A minor proportion of melanomas - 1 (4.17%) showed moderately intense diffuse expression.

Table 4.18: Comparative expression of*HMB45 in the three target groups presented as % of the total number of pigmented neoplasias studied:*

	diagnostic group			
HMB 45	Benign nevi % (n=)	Atypical nevi % (n=)	Melanomas % (n=)	**Total** % (n=)
no expression to single cells with weak expression	4.60 % (n=4)	0 % (n=0)	0 % (n=0)	4.60 % (n=4)
Low intensity expression in the upper portion of the lesion	11.49 % (n=10)	0 % (n=0)	0 % (n=0)	11.49 % (n=10)
Moderate intensity expression in the upper portion of the lesion	22.99 % (n=20)	2.30 % (n=2)	0 % (n=0)	25.29 % (n=22)
Moderate intensity spotted expression	3.45 % (n=3)	1.15 % (n=1)	0 % (n=0)	4.60 % (n=4)

Moderate intensity diffuse expression	0 % (n=0)	0 % (n=0)	1.15 % (n=1)	1.15 % (n=1)
High intensity expression in the upper portion of the lesion	22.99 % (n=20)	2.30 % (n=2)	0 % (n=0)	25.29 % (n=22)
High intensity spotted expression	0 % (n=0)	0 % (n=0)	13.79 % (n=12)	13.79 % (n=22)
High intensity diffuse expression	0 % (n=0)	1,15% (n=1)	6.90 % (n=6)	8.05 % (n=7)
heterogeneous expression	0 % (n=0)	0 % (n=0)	5.75 % (n=5)	5.75 % (n=7)
General	65.52 % (n=57)	6.90 % (n=6)	27.59 % (n=24)	100 % (n=87)

Almost all studied pigment lesions 83 (95.40%) express HMB45, and only 4 (4.60%) of them are absent. The comparative analysis (cross-tabulation and chi-square test) found that the percentage differences in HMB45 expression between the groups were statistically significant ($X2 = 88.16$, $p = 0.001$). A difference in the type of expression was found between the groups. In benign nevi, it is typically localized in the upper portion of the nevus in 50 (57.47%). There is a

gradient expression varying in intensity from low to high, primarily high intensity. Only 3 (3.45%) benign lesions show moderately intense macular expression, i. e., missing gradient. All atypical nevi in the test material express HMB45, two-thirds of them show a gradient of high or moderate-intensity in equal proportions. In the MM group, no expression gradient of HMB45 was detected. The lack of a gradient in malignant tumors (MM and some atypical nevi is a characteristic feature.

Comparative analysis of HMB45 expression against the presence or absence of a gradient (presence or absence of expression in the upper portion of the lesion): A comparative analysis (cross-tabulation and chi-square test) showed that the percentage differences between the different categories were statistically significant (X2 = 58.179, p = 0.001) (Table 4.20) (Table 4.19)

Table 4.19: Comparative analysis of gradient in HMB45 expression in the three target groups:

	diagnostic group			
HMB 45	**Benign nevi %**	**Atypical nevi %**	**Melano mas**	**Total % (n=)**

	(n=)	(n=)	% (n=)	
Presence of gradient in expression	51	4	0	55
	58.62 % (n=51)	4.60% (n=4)	0 % (n=0)	63.21 % (n=55)
Lack of gradient in expression	6	2	24	32
	6.90 % (n=6)	2.30 % (n=2)	27.59 % (n=24)	36.79 % (n=32)
Total	57	6	24	87
	65.52 % (n=57)	6.90 % (n=6)	27.59 % (n=24)	100 % (n=87)

According to the reported results, it is evident that the gradient in HMB45 expression was found in 51 of 57 benign nevi and absent in 24 of 24 melanomas.

This significant difference was further tested by correlation analysis by the Spearman method to establish statistical significance to show whether the expression gradient can be

used to predict malignant potential in pigment tumors. (Table 4.21)

Table 4.20: Chi-Squared Tests

	Amount	df	p
X^2	58.179	2	< .001
N	87		

The table shows that this method presents a high level of association between the expression gradient of HMB45 and benign nevi, a tendency to decrease the expression gradient in atypical nevi, and a lack of gradient in melanomas (rho = 0.794, p = 0.001).

Table 4.21: Spearman correlation analysis for the HMB45 gradient in the three target groups:

Factor		**HMB 45**	**Diagnosis**
HMB 45	Spearman's rho	—	
	p-value	—	

Diagnosis	Spearman's rho	0.794	—
	p-value	< .001	—

Ki 67 study results

Expression of Ki 67 in benign nevi from the test material:

The majority of benign nevi have low proliferative activity of 0 to 2% - 53 (92.98%) of them, 3 (5.26%) of them express 3 to 5%, and only 1 (1), 15%) is in the group of 6 to 10% proliferative activity. There are no nevi with proliferative activity of 11 to 20% and over 20%. The mean proliferative activity for benign pigmented tumors (nevi) is Ki 67 = 0.90%.

Expression of Ki 67 in the studied atypical nevi

In atypical nevi, the reported proliferative activity is close to that of benign lesions: most of them show proliferative activity from 0 to 2%, as reported in 3 (50%) of dysplastic nevi, followed by 2 (33.33%), with a proliferative index in the group of 6 to 10% and 1 (16.67%) of atypical lesions with a proliferative index in the group of 3 to 5%. Mean proliferative activity for the studied atypical nevi is Ki 67 = 2.75%

Expression of Ki 67 in the studied malignant melanomas

The largest share of melanomas in the target group, 15 (62.50%), show high proliferative activity, as 8 (33.33%) of them show a proliferative index of the group over 20% and seven (29.17%) with a proliferative index of the group 11 to 20%. Moderate proliferative activity, i. e., with an index of 6 to 10%, shows 2 (8.33) of melanomas. Low proliferative activity was reported in 7 (29.17%) melanomas, with 4 (16.67%) having an index of 0 to 2% and 3 (12.50%) with an index in the group of 3 to 5%. Proliferative activity in the studied melanomas is Ki 67 = 14.96%.

The comparative analysis of the proliferative activity in the three target groups shows the following: (Table 4.22).

Table 4.22: Comparative analysis of Ki67 immunohistochemical expression in the three target groups

	diagnostic group			
Ki 67	Benign nevi % (n=)	Atypical nevi % (n=)	Melanomas % (n=)	**Total** % (n=)
0-2%	60.92 % (n=53)	3.45 % (n=3)	4.60 % (n=4)	68.97 % (n=60)
2-5%	3.45% (n=3)	1.15 % (n=1)	3.45 % (n=3)	8.05 % (n=7)
6-10%	1.15 % (n=1)	2.30 % (n=2)	2.30 % (n=2)	5.75 % (n=5)
11-20%	0 % (n=0)	0 % (n=0)	8.05 % (n=7)	8.05 % (n=7)

Over 20%	0 % (n=0)	0 % (n=0)	9.20 % (n=8)	9.20 % (n=8)
Total	65.52 % (n=57)	6.90 % (n=6)	27.59% (n=8)	100 % (n=87)

It is highest in melanomas, lowest in benign nevi, and intermediate in atypical lesions. The percentage differences in Ki 67 expression between groups was statistically significant ($X2 = 65.4$, $p = 0.001$).

Statistical analysis for prestatistical value of Cyclin D1, p53, and Melanosome clone HMB45

The study on the immunoreactivity of all markers in the three groups of pigment tumors showed the most significant differences in expression between the groups in Cyclin D1, p53 protein, and Melanosome clone HMB45, with marginal results in the expression between benign and malignant pigment tumors.

Therefore, these markers were further examined for statistical significance as predictors of malignant potential in two designs: The first study design was based on a combination of the expression of Cyclin D1, p53 protein, and HMB45

established by the eleven model scheme we introduced to account for immunoreactivity.

The second design uses the immunoreactivity of Cyclin D1 and p53 protein established by the eleven - model scheme for measuring expression in combination with a gradient in the expression of HMB45. The three markers were additionally tested with X - square test and Spearman correlation analysis, graphically visualized with ROC curve analysis:

Results of the study of the three markers Cyclin D1, p53, Melanosome clone HMB45, as predictors of malignant potential, according to the first design (based on their expression, evaluated according to the eleven-model antibody expression scheme):

Combined analysis with X - square test and Spearman correlation analysis shows the high predictive value of each of the three criteria and in combination. ROC Curve Analysis best visualizes this high predictive value.

Graphically, a ROC curve with a curve area close to 1.0 means high predictability (almost 100%). In this case, the three indicators have high predictability, the highest being HBM45 (area = 0.975), the lowest at p53 (area = 0.886) (Fig. 4.21**).**

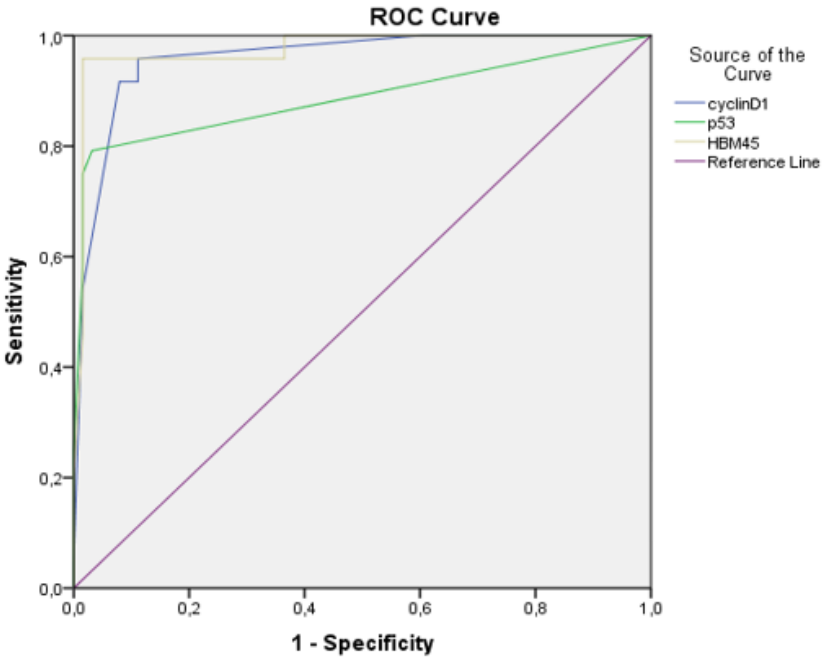

Figure 4.21: Graphical representation of the ROC curve analysis, presenting the three curves in a different color *follows: Cyclin D1 in blue (AUC=0.959, 95% Cl: 0.917-1.000, p<0.0001). P53 in*

green (AUC=0.886, 95% Cl: 0.786-0.986, p<0.0001). Melanosome clone HMB45 yellow (AUC=0.975, 95% Cl: 0.939-1.000, p<0.0001)

The specified values of the parameters in the graph (AUC) are reflected in the following table: **(Table 4.23)**.

Table 4.23: *ROC curve analysis (AUC) parameters for the three criteria examined (indicator)*

	Area	Standard Error	Level of significance	95% confidence interval	
				Lower Spacebar	Top Spacebar
cyclinD1	0,959	0,021	0,0001	0,917	1,000
p53	0,886	0,051	0,0001	0,786	0,986
HMB45	0,975	0,018	0,0001	0,939	1,000

In the Cyclin D1 indicator, the moderately intense expression - spotted or diffuse is a cut-off point, has the best sensitivity (95.8%) and specificity as a prognostic indicator that the pigment lesion is malignant (AUC = 0.959, 95% Cl: 0.917-1.000, p <0.0001).

In the p53 protein index, even low-intensity expression in the upper portion of the lesion can serve as a cut-off point or an indicator of possible malignant potential, i. as a predictor of melanoma (AUC = 0.886, 95% Cl: 0.786-0.986, p <0.0001).

HBM45 has the highest sensitivity in high intensity diffuse or heterogeneous expression.

With this type of expression, the probability of predicting a malignant potential of the pigment lesion or melanoma is 97.5% (AUC = 0.975, 95% Cl: 0.939-1.000, p <0.0001). Results of examination of the three markers Cyclin D1, p53, Melanosome clone HMB45, as predictors of malignancy, according to the second design (based on the expression evaluated according to the eleven-model scheme for Cyclin D1 and p53 and the two-stage gradient scheme in HMB45 expression).

Again, a ROC curve analysis was applied to represent the predictive precision of the three indicators graphically.

Again, the three studied indicators have a high value as predictors of malignant potential (Fig. 4.22**)**

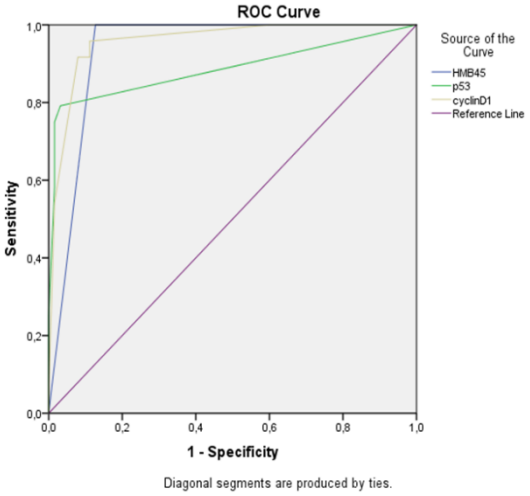

Figure 4.22: Graphical image of roc curve analysis with presentation of the three curves for the individual indicators in different color as follows: Cyclin D1 curve in yellow (AUC=0.959, 95% Cl: 0.917-1.000, p<0.0001). P53 green curve (AUC=0.886, 95% Cl: 0.786-0.986, p<0.0001). Melanosome clone HMB45 curve in blue (AUC = 0.937.95% CI:o,886 – 0.988, p <0.0001) (table 5.32-33)

The specified values of the parameters in the graph (AUC) are reflected in the following table **(Table 4.24).**

Table 4. 24: ROC curve analysis parameters (AUC) for the three criteria examined (indicator)

	Area	Standard Error	Level of significance	95% confidence interval	
				Lower Spacebar	Top Spacebar
CyclinD1	0,959	0,021	0,0001	0,917	1,000
p53	0,886	0,051	0,0001	0,786	0,986
HMB45	0,937	0,026	0,0001	0,886	0,988

Here the indicator HMB45 has the highest sensitivity. In the absence of a gradient, the sensitivity as a predictor of malignant potential is 100%.

Discussion

The results of a comparative morphological and immunohistochemical study of 91 cases of pigmented skin tumors, divided into groups according to the criteria set by the WHO (Elder D, 2018) and based on our own experience, namely - 57 benign melanocyte nevi, ten atypical nevi, and 24 malignant melanomas show that the demographic and purely morphological characteristics of pigmented neoplasms are not sufficient for a definitive assessment of their malignant potential and biological behavior. Gender distribution, for example, which in our material includes twice the number of benign nevi in women and equal distribution in both sexes of atypical nevi and melanoma, is not a sign of particular value for diagnosis and prognostic guidelines. Although according to separate clinical studies (Chernev G, 2015), the female sex is associated with a better prognosis than the male, even in patients with metastatic lymph nodes, and the 10-year survival in women with melanoma is 86% without the presence of metastases, compared with 68% in men. It follows that sex can only be considered as a clinical prognostic sign in malignant melanoma.

Regarding the age distribution of cases in our material, no significant differences in the groups with benign nevi and atypical lesions were found. Their age range is between 11 and 80 years, respectively. 11 - 70 years, with the highest frequency at a young age (31 - 40 years, resp. 21 - 30 years). Malignant melanoma is found in all age groups (except for the group 11-20 years). The difference in the percentage of cases diagnosed in our material over 40 years, compared to other age groups, is statistically significant ($X^2 = 38.13$, $p = 0.001$), i.e., with age, the number of possible melanomas detected increases (rho = 0.463, p = 0.001). It follows that age in patients with melanoma can be considered as an independent prognostic factor. The latter is also confirmed by the literature data that with increasing age at the time of diagnosis, the 5 - and 10 - year prognosis for survival deteriorates (Chernev G, 2015). In cases diagnosed with malignant melanoma before the age of 30, the 5 - year survival rate is 87%, compared to 78%, 71%, and 60%, respectively, in 60, 70, and 80-year-old patients (Chernev G, 2015).

According to the topographic distribution of the lesions in the target groups, most of the benign nevi are in the head and neck area; most atypical nevi are diagnosed in the skin of the

abdomen, followed by those of the head and neck. Cases of malignant melanoma have been found, mainly in the skin of the lower limbs, back and chest. The literature assumes that the localization of MM in the limbs is associated with a better prognosis than that of the trunk, head, and neck. In the absence of distant metastases, regardless of tumor thickness, the 10-year survival for patients with recent localizations is 70%, compared with 90% survival for melanomas localized on the skin of the limbs (Chernev G, 2015). Therefore, the localization of the primary tumor in melanomas should be considered a clinical prognostic factor, while the localization of benign and atypical lesions is of little importance.

However, in the morphological characteristics of pigment tumors, several parameters have a high value for diagnosis, for reporting the malignant potential, and prognostic conclusions. According to the requirements for a diagnosis of pigmented lesions (Elder D, 2018), these are defining diagnostic and prognostic features, mainly related to malignant melanoma of the skin and include: tumor lesion thickness, degree of micro invasion, mitotic activity, ulcerations, tumor-infiltrating lymphocytes, the manifestations of regression and satellite. The study of Breslow tumor thickness in our material shows that

the largest relative share has tumors with thickness - Breslow 4 (58.33%), followed by Breslow 3 (33.33%). In 1970, A. Breslow compared the prognostic value of tumor diameter, Clark micro invasion, and tumor thickness measured with a micronodular in mm, determining five prognostic cut-off points of 0.75 mm, 1.5 mm, 2, 25 mm, 3.00 mm, and over 3.00 mm (Breslow, 1970). Subsequently, the Breslow staging was changed to four levels, and together with the Clark levels, it determined the T category in the TNM classification up to the sixth and 2002 versions (Kirov K, 2017). Tumor thickness is considered a specific independent prognostic factor of high importance, a significant criterion for pT-staging of cutaneous melanoma according to the 8th edition of AJCC (Amin M, 2017). In the eighth edition of the TNM classification, 2017, the T-category is determined by Breslow thickness and the absence or presence of ulcerations (Amin M, 2017). Microscopic measurement of tumor volume in the direction of underlying structures (so-called vertical growth) remains the primary criterion for pT - the staging of cutaneous melanoma (Amin M, 2017). In addition, a direct relationship between survival and Breslow assessment has been demonstrated in

retrospective clinical and morphological studies (Marghoob A, 2000; Balch C, 2001).

The determined Clark micro invasion in our study showed the highest number of melanomas such as Clark 3 (37.50%), followed by Clark 5 (29.16%), and almost twice as many cases of Clark 4 micro invasion and Clark 2 (respectively 16.67%). According to Scolier, Clark micro invasion has prognostic significance only for 5-year survival and tumors up to 1 mm thick (T1) (Elder D, 2018).

Mitotic activity is a critical component of diagnosis. Our material's mitotic activity in thin (up to 1 mm thick) melanomas is low, less than 1 to 2 mitoses / 1 sq. Mm. In nodular MM, the mitotic activity is high, on average 22.90 mitoses / 1 sq. Mm, at an average tumor thickness of 14.42 mm, as in non-ulcerated lesions, it is 17 mitoses / 1 sq. Mm (average thickness 8 mm), and in ulcerated - 24.62 mitoses /1 sq. mm (average thickness 11.29 mm), i.e., the average mitotic activity of ulcerated melanomas is higher than that of non-ulcerated forms by 7.62 mitoses / 1 sq. mm and increases by 2.32 mitoses / 1 sq. mm. for each mm greater thickness. The relationship between the two parameters (number of mitoses/thickness of the lesion) is apparent in the graphical representation of the relationships

between the two quantities. This is confirmed by research by other authors. According to K. Kirov, the average number of mitoses increases with tumor thickness, from 1 to 9.6 mitoses / 1 sq. Mm with increasing lesion thickness from 1 mm or less than 1 mm to over 8 mm (Kirov K, 2017). The author points out that the mitotic activity increases with tumor ulceration, namely more than five mitoses / 1 sq. Mm in 59% of cases with ulcerated melanoma, compared to 16% without ulceration. To Azzola, the number of mitoses in an area of 1 sq. m. Mm. has an independent prognostic value, as at mitotic sum over six mitoses / sq.mm, the 8 - year survival is up to 38%, against 95% in the absence of mitoses (Azzola M, 2003). Attis M. also defines mitotic activity as a significant prognostic factor (Attis M, 2007). In the seventh revision of the TNM classification (2010), the number of mitoses determines the division of T1 stage - T1a at less than one mitosis per sq. mm and T1b at one and more than one mitosis per sq. mm in thin melanomas (Amin M, 2017). It follows that mitotic activity is a significant factor for tumor progression and overall survival of patients with MM.

From the above results, it is clear that mitotic activity is directly related to tumor ulceration. Ulceration of MM is

another sign of significant value for the diagnosis. It is accepted as a specific independent prognostic factor with high statistical significance (Kirov K, 2017). It determines the specification of the T-category in the TNM classification as T1a - T4a without ulceration and T1b - T4b with ulceration. In our material, ulcerated forms of MM are over 50% of cases (17 patients). Literature data suggest that ulcer size correlates with patient survival. At minimal or moderate size (less than 70% of total tumor length or diameter less than 5 mm), survival is better compared to patients with significant ulceration (more than 70% or more than 5 mm), i. e., the increase in ulceration is a prognostic sign for poorer survival of patients (Haydu L, 2012; Bønnelykke-Behrndtz M, 2014; Bønnelykke-Behrndtz M, 2017). It follows that in our material, we should expect significantly less favorable prognostic survival in 29.17% (7 cases) of ulcerated cases, as the ulceration is 5 mm and over 5 mm (from 5 mm to 12 mm). Analyzes of EORTC studies 18952 and 18991 found that ulceration of primary melanoma was statistically significant ($p < 0.001$) and the only predictive factor for the effect of therapy. These patients' survival is significantly improved with interferon-alpha adjuvant immunotherapy (Eggermont A, 2005; Eggermont A, 2012-2).

The effect of adjuvant therapy with interferon-alpha is most likely due to restoring the JAK-STAT function (Jewell R, 2015). According to research by Rakosy and Jewell, patients with ulcerated MM have a different genotype than those without ulceration (Rakosy Z, 2013; Jewell R, 2015). The genes FGFR2, FGFR3, HLF, DSP, EGFR, CEBRA, PTGS1, KCNK6, are associated with ulceration (Rakosy Z, 2013; Jewell R2015). These features in the biological behavior of ulcerative melanomas and their different genetic profile give grounds to authors such as Eggermont to raise the question of their separation into a separate nosological subgroup (Eggermont A, 2012-1). Therefore, accurate assessment and reflection of ulceration is an essential component in the morphological diagnosis of MM, especially given the prediction of survival and therapeutic response.

Evaluation of tumor-infiltrating lymphocytes is another important point in the diagnosis of pigmented neoplasms. Many authors accept lymphoid infiltration as a protective mechanism of the immune system against tumors and a cause of their regression (Dudley M, 2002; Besser M, 2009; Goff S, 2010; Joseph R, 2011; Robbins P, 2011). This motivates the application of immunotherapy, which is widely used today in

oncology practice. In the material we studied, we found a correlation between the thickness of melanomas and TIL. In thick melanomas, steep lymphocytic infiltration is minimal to absent. According to Plaza results, there is a clear correlation between pronounced inflammatory infiltrate (brisk TILs) and better survival (Plaza J, 2017). The weak steep lymphocytic reaction, or lack thereof, correlates with worsening prognosis. Five-year survival decreased from 55% to 27% with a decrease in the severity of TIL according to Barnhill, and according to Mihm, 5- and 10-year survival decreased from 77%, respectively. 55%, up to 37%, respectively 27%. It is clear that the reporting of TIL is a mandatory part of the diagnosis and has a decisive role as a prognostic factor (Mihm M, 1996; Barnhill R, 2004).

Regarding the regression of MM, such in the material we studied, we observed only in one of the cases - nodular type MM and partial (focal) regression. In some studies (Morris K, 2008), the occurrence of regression is reported as a poor prognostic sign for thin melanomas (less than 0.76 mm thick). According to others (Plaza J, 2017), the correlation of the regression phenomenon with the prognosis is contradictory.

One of the reasons for this is the lack of consensus regarding the precise definition of the process and its measurement.

In the literature, the presence of microsatellites in MM was described in 4.6% of tumors with a thickness of 1.5 mm and in 65% of those with a thickness of more than 4 mm; their presence is associated with a high risk of regional lymph nodular metastases at a thickness above 1.5 mm (Plaza J, 2017). In our material, satellites were found in three melanomas - in malignant blue nevus and two nodular melanomas, with respective thicknesses of 7 mm, 14 mm, and 25 mm. However, as we do not know the lymphonodular status of the patients at the time of primary diagnosis, we could not be relevant to this morphological criterion.

From the analysis of these results, comparable to the literature data, it is clear that in the characteristics of pigmented tumors, the main demographic criteria such as gender, age, location do not play a significant role and place in the process of morphological diagnosis. They can be taken into account only and mainly as clinical prognostic features for the survival of patients with malignant pigmented lesions. However, in the assessments of biological behavior and prognosis for survival in malignant melanoma, the value of the characteristics for

malignant lesion thickness, depth of invasion, mitotic activity, and lymphocyte infiltration is significantly high. They illustrate the degree of malignant potential and prediction of patient survival, particularly for the expected therapeutic response. Therefore, their accurate assessment and reflection in the morphological diagnosis are mandatory components in its construction. In principle, as evidenced by the results of our study, research and diagnosis of pigmented tumors is not an easy process. The complexity and difficulties in morphological diagnosis and prediction of their behavior stem from several factors determined by their diversity, from challenging to detect, sometimes, signs of malignancy, in many cases due to blurring of histological parameters for benign and malignant lesions, and more especially between benign and atypical nevi on the one hand, and atypical nevi and MM on the other, from the difficulty of proving their melanocyte nature in their differentiation from other tumors. The search for methods other than histological to help the diagnosis is justified to solve these diagnostic problems. Immunohistochemical examination of pigment lesions in our material with melanocyte and non-melanocyte markers aims to detect marginal (radically opposite) traits showing the most significant statistical

differences between different types, which to be defined as predictors of malignant potential. The clinical and morphological meaning is that they should be used in the diagnosis of morphologically complex pigmented lesions, such as, most often, atypical nevi, especially those with severe dysplasia.

The comparative immunohistochemical analysis was performed based on evaluation of the expression of the respective biomarkers in 57 cases with benign, 6 cases with atypical, and 24 cases with malignant pigment tumors, i. e., the standard asymmetric profile of study design (2: 1 benign: malignant tumors), with a predominance of benign lesions as more common in the human population. To assess immunoreactivity, based on our own experience, we developed eleven model schemes for the distribution of expression. This reporting scheme was necessary because, in the study, we observed a great variety, both in the intensity of expression and in the topographic distribution of the marker within the individual tumor lesions. Moreover, knowledge of the nuances of immunoreactivity in pigmented tumors is essential to avoid misinterpretations, especially in assessing malignant potential. For all tested markers in our material, this scheme's detailed

study of immunoreactivity shows gravity around one of the most common, market-specific expression patterns in the respective group (benign, atypical, and malignant tumors), which can be called characteristic or typical. At the same time, we found a wide range of other expression models, defined as atypical, with a narrower or broader overlap of the expressed expression in the benign and malignant tumor groups. Furthermore, the diversity of expression suggests that the assessment in some cases cannot and should not be based solely on the expression of one antibody but on a combination of several.

Using the two melanocyte markers (S100 protein and Melanosome clone HMB45), which essentially confirm the affiliation of the studied tumors to the pigment tissue, we found certain features in the expression that are important for diagnosis. All pigmented neoplasms in the studied groups express S100 protein. The expression in benign nevi is diffuse and highly intense in 100% and with the same intensity, but in 83.33% of cases with atypical lesions. In cases of MM, the antibody is expressed mainly diffusely with moderate intensity in 37.50%, high-intensity diffuse expression and heterogeneous with varying intensity throughout the thickness of the lesion is

detected in equal proportions - in 29.17% (respectively 7 cases) and only in 1 case (4.17%) is low-intensity diffuse. Therefore, the pronounced immunoreactivity in all studied pigment neoplasms in the three targeted groups confirms the high sensitivity of S100 protein to prove their melanocyte nature and makes it suitable for differentiation of the pigment lesion from other, non-pigmented tumors. This antibody characteristic is also widely supported in the literature (Gaynor R, 1981; Cochran A, 1982; Gatter K, 1985; McNutt N, 1998). However, the difference in the intensity of expression found between the target groups in our material, namely that it is consistently high in benign nevi and moderate in intensity mainly in MM and some atypical nevi, is a character whose knowledge may help the diagnostic process.

Several features are found in the expression of HMB45. Benign lesions in 92.98% express the antibody, and only in 4 cases (7.02%), such is absent. However, the expression is typically localized in the upper portion of the nevus (in 87.72%). There is an expression gradient and only a tiny part; 5.26% (3 cases) show moderately intense spotted expression, i. e., there is no such thing. The gradient expression varies in intensity from low to high, as in most nevi, it is high intensity (in 40%) and

twice less (in one-fifth of the total number for this group) it is low intensity. All atypical nevi in the test material express HMB45, with the majority (66.66%) showing a high or moderate intensity gradient in equal proportions. There is no expression gradient; the expression is the high intensity diffuse or moderately intense spot in them. In the MM group, no HMB45 expression gradient was detected in any case. Immunoreactivity is highly intense, spotted (in 50%) or diffuse (in 25%), and heterogeneous (varying in intensity throughout the thickness of the lesion) (in 20.83%). It follows that the lack of a gradient in malignant tumors (MM) and some atypical nevi are characteristic features. This is also confirmed by the X - square test and Spearman correlation analysis, which confirmed, with high statistical reliability, that the lack of gradient in HMB45 expression should be considered as a predictor of malignant potential. A similar conclusion is made by Uguen, namely that the lack of a gradient in HMB 45 expression is more common in malignant pigmented tumors (Uguen A, 2015). Nat Pernik summarizes that HMB45 is a marker for MM in cases where it is manifested, at least with deep focal reactivity of the antibody, in contrast to nevi, in which there is no expression in the deep areas of the lesion

(Pernik N, 2021). Our study has proven HMB45 expression in all three groups of pigmented tumors is another convincing evidence of their melanocyte origin, which provides certainty in its use in diagnosing tumors with unclear histogenesis. The absence of a gradient in HMB45 expression in only a tiny proportion of atypical nevi requires a careful approach to addressing the biological potential of the lesion. The latter should be formulated based on a combined multifactorial (histological, immunohistochemical) analysis to avoid overdiagnosis of malignancy.

Therefore, the diversity of expression in different tumors in different groups, the degree of intensity, and its distribution in different parts of the lesion are signs that must be known and adequately considered. Knowing the nuances of expression will protect the morphologist from misinterpretation, especially in reporting malignant potential. All this once again emphasizes the importance and practical value of the model of immunoreactivity developed by us according to the eleven-model scheme. Its application ensures that all features of the expression of each antibody are taken into account, both in terms of intensity and topographic distribution, and distribution gradient.

In the characteristics of the pigment lesions, it is clear that the melanocyte markers used have a particular place, primarily to prove their melanocyte nature, but to some extent as predictors of malignancy, especially the expression of HMB45. However, the search for other non-melanocytic markers, with even greater certainty characterizing and reflecting the tumor process and the directions of tumor development, prompted us to study the role of Cyclin D1, p53 protein, and BCL2 in the diagnosis of pigmented tumors. With the sole purpose to increase the possibility for the more reliable establishment of malignant potential of the pigment lesion and more accurate prediction of its biological behavior.

In the study of Cyclin D1, we found statistically significant differences in expression in different groups of pigment tumors. All but one benign nevus expressed the antibody, with the most common expression pattern in benign lesions being spotted expression (73.68%) of moderate intensity (more than 50%); expression, mainly in their upper portion, is observed in 22.81% and only in one nevus (1.75%) it is diffuse (in the entire thickness), but with low intensity. The highest expression is observed in malignant melanomas, in which it is diffuse or mottled throughout the thickness of the tumor.

Atypical nevi, in equal proportions (respectively 16.67%), show a different type of expression of Cyclin D1, namely, with low and moderate intensity in the upper or spotted, high intensity in the upper portion, spotted or diffuse, i. e., there is no characteristic model. Thus, the expression of Cyclin D in atypical lesions is intermediate, with characteristics close to benign and malignant lesions.

The additional variation analysis revealed the most significant statistical difference in the expression of Cyclin D1 in benign nevi and melanomas. The significantly higher expression of Cyclin D1 in melanomas compared to that in benign lesions reveals and proves the connection and role of Cyclin D1 in the development of malignant phenotype, as it is known that Cyclin D1 activation plays a significant role in the transition from benign to malignant immunophenotype. Therefore, the use of Cyclin D1 for the prediction of malignant potential in pigmented tumors is justified. In their research, Alekseenko et al. also emphasize the statistical significance of differences in Cyclin D1 expression between melanomas and common nevi and between dysplastic and common nevi (Alekseenko A, 2010). The antibody's unusual (intermediate) expression in atypical nevi reported in our material should be considered

difficult to determine their biological potential. In addition, the topographic distribution of Cyclin D1 expressing cells is impressive in terms of antibody expression. In the results of Alekseenko et al. Immunoreactivity is described mainly in the upper portion (in melanocytes located near the dermo-epidermal border) of the nevi (Alekseenko A, 2010), while our results show mainly spotted, i. e., focally distributed throughout the thickness of the lesion expression of moderate-intensity (in 73.68%) and only in 22.81% of the studied nevi expression in the upper portion. Also, according to this team, dysplastic nevi and melanomas show an even distribution of Cyclin D1 - positive cells. Our results report diffuse and mottled in distribution and heterogeneous in intensity expression within the same lesion. These results are close to those reported by other authors (Ewanowich C, 2001), according to which benign nevi show zonal expression, dysplastic nevi overexpress Cyclin D1, and expression correlates with the degree of cytological atypia. The nuances in the characteristics of expression in our results and those of the cited authors are probably due to the more detailed description according to the eleven - model scheme for distribution of expression used by us. In conclusion, the expression of Cyclin D1 reveals the

development of a malignant phenotype. Therefore it is a suitable marker for the prediction of malignant potential.

A comparative study of p53 protein expression in the three groups of pigment tumors showed that expression was absent in 98.25% of benign lesions and 83.33% of atypical nevi, in contrast to melanoma, which is its central part (79.17%) typically express p53. An interesting fact in atypical nevi is that those with low-grade dysplasia do not express the antibody. Only one with severe dysplasia exhibits immunoreactivity and moderately intense spotting throughout the thickness of the lesion. The differences in p53 expression between the three groups were statistically significant (X sq. = 58, p = 0.001). These results are confirmed in several other studies in which Pierard states that expression is absent in melanocyte nevi. 25% to 60% is present in its mutated form in cutaneous malignant melanomas (Piérard G, 2012). According to Kanoko, all nevi studied, including Spitz nevi, dysplastic and ordinary nevi, are negative (do not express p53) (Kanoko M, 1996). According to Bergman, when studying p53 expression in primary melanomas, organ metastases from melanoma, in Spitz and benign composite nevi, there is a high degree of expression in 31% of primary cutaneous melanomas,

and there is no such expression in the studied nevi (Bergman R et al., 1995). Indeed, according to our results and those in the literature where it is generally reported that a large percentage of melanomas are p53 positive. Simultaneously, nevi are negative, p53 expression should be considered a characteristic of a malignant immunophenotype (Cristofolini M., 1993). However, the established, albeit in a small proportion of benign lesions (in 1.75%) and atypical nevi in our material immunoreactivity, does not allow us to accept p53 as an absolute (autonomous) criterion for determining biological potential. On the contrary, there is a danger of overdiagnosis of malignancy. This is emphasized by other authors who even find significantly higher values of p53 expression (in 15%) in nevi and significantly lower expression in MM (in 30%) compared to our results (Cristofolini M., 1993).

In the study of BCL2 expression, we found that 100% of the benign nevi in the present material express BCL2 uniformly, highly intensely, and diffusely. All atypical lesions also expressed BCL2, with the most significant number of lesions (66.67%) showing high-intensity diffuse expression identical to benign lesions. In other cases, it was of lower intensity. All 24

malignant melanomas tested expressed BCL2. High-intensity diffuse expression, similar to that of benign lesions, occurs in 41.67% of melanomas, and in the remaining 58.33%, it is of reduced intensity or partial. Therefore, BCL2 immunoreactivity is present in all three study groups, highly intense and diffuse in benign lesions and most atypic nevi. Although the small relative proportion of atypical nevi with low intensity of expression similar to that prevalent in melanomas confirms the position, they occupy intermediate lesions between benign and malignant pigmented neoplasms. According to Santamaria studies, BCL2 immunoreactivity is widespread in normal melanocytes and their derived neoplasms, from intradermal nevi to cutaneous melanoma and its metastases (Carmen Saenz-Santamaría M, 1994). According to the authors, the positivity of BCL2 in normal melanocytes and nevus cells is equally intense. In contrast, melanoma cells show variable but mostly weak reactivity and suggest that decreased expression is associated with tumor progression and correlates with increased proliferative activity. Based on their relatively high expression levels in MM and benign nevi (respectively 93.1% for melanomas and 94.3% for benign melanocyte lesions), other authors found that BCL2 expression in cutaneous

melanoma had no prognostic significance. 1995). It follows that these data reflected in the literature and our results, respectively, in which there is a significant partial overlap between expression in the three groups, lead to the conclusion that the use of BCL2 immunoreactivity in pigmented neoplasms is not an appropriate criterion for assessing their biological potential.

The study of proliferative activity by expression of the Ki 67 proliferative index in different tumor groups is another important indicator of the characteristics of the tumor process. In summary, in the studied material, benign nevi have a low average proliferative activity (0.9%), with an index range of 0 to 5%, and very rarely (in isolated cases) up to 10%. Atypical nevi occupy an intermediate site, with a moderate proliferative activity of 2.75% and in the range of 0 to 10%. Melanomas have a high mean proliferative activity (14.96%), mainly in 11 to 20% and over 20% Ki 67 expressing cells. The indicated differences between the different groups are statistically significant (X sq. = 65, p = 0.001). A significantly high Ki 67 proliferation index in malignant melanocyte tumors, compared to nevi, was reported by other authors, respectively 29.9% for

melanomas and 1.7% for nevi (Uguen A, 2015). Compared to our results, the indicated proliferative activity in MM is much higher. According to Lebe, comparative results between the different groups of pigmented tumors show that the Ki 67 index is significantly higher in dysplastic nevi than in ordinary melanocyte nevi and in melanoma compared to dysplastic nevi (Lebe B, 2007). Based on these results, the authors believe that dysplastic nevi are biologically separated from benign pigmented lesions by this criterion (Ki 67 proliferative index). Despite our statistically significant differences in the Ki 67 proliferative index, we should note some data overlap between groups. Despite the predominant low proliferative activity in nevi and atypical lesions, isolated cases have a 6 - 10% proliferative index. In MM, against the background of the markedly high index characteristic of them, also isolated cases show moderate (6 - 10%), low (3 - 5%), or deficient (0 - 2%) Ki 67 proliferative index. This makes it an uncertain independent differential diagnostic criterion for determining malignant potential. It follows that its use should be concurrent with other markers (e.g., Cyclin D1) or other indicators that have been shown to have statistically significant differences

between benign and malignant pigmented tumors. The latter is also recommended by other authors (Lebe B, 2007).

From the comparative analysis of the present study results, it is clear that the different immunohistochemical markers show diversity, both in intensity and in the topographic distribution of expression, characteristic or not for the different groups of pigment tumors. Despite the variability, some of them have high statistical significance when comparing the groups in the direction of benign - malignant tumors, which allows them to be defined as predictors of malignancy. In other cases, the results show close or overlapping expression characteristics, which blurs the boundaries between the different groups and makes it impossible to use the respective marker as a separate, autonomous criterion for malignancy. Therefore, for higher reliability of malignant prediction, it is correct to base the assessment on a study of a combination of immunohistochemical markers that show the statistically most significant difference in expression in malignant and benign pigmented tumors. According to our results, the most significant ones are the expression of Cyclin D1, p53, and HMB45. The expression of Cyclin D1 was found to have the

most significant statistical differences in the groups with benign nevi and melanoma.

In contrast to MM, expression of p53 was shown to be absent in benign pigmented lesions, for which it is, and with high intensity, typical. The inclusion of HMB45 in the combination was accepted based on the established, with high statistical reliability lack of gradient in the distribution of expression in malignant melanoma and the presence of such in nevi. The analysis performed with X - square test and correlation analysis by Spearman confirms the high predictive value of each of the three criteria alone and even higher in their combination. The performed ROC curve analysis particularly well visualizes their high predictive value (see Fig. 4.21 and Fig. 4.22), first, based on an evaluation of the eleven-model scheme for determining expression and second, of the expression evaluated on the eleven - model scheme in combination with HMB45 expression gradient. The graphical expression of the Rock curve analysis proves that at an area of the curve close to 1, there is a predictive possibility of almost 100%.

According to the first analysis, all three indicators have high predictability, the highest being HBM45 (area = 0.975), the lowest at p53 (area = 0.886).

According to the second comparative analysis, the indicator - lack of gradient in HBM45 expression has 100% (area = 0.937) sensitivity as a predictor of malignant potential. Therefore, these actual results are evidence of the high potential of the combination of Cyclin D1, P53, and HMB45 to establish malignant potential in pigmented tumors. This emphasizes once again that a reasonable approach requires a combination of markers. In this case, our chosen triple combination, Cyclin D1, p53, and HMB45, whose maximum statistical reliability provides sufficient certainty in predicting malignancy and contributes to the maximum accuracy of diagnosis.

Of course, the construction of morphological diagnosis is a multi-stage and challenging process, based first and foremost on the accurate morphological assessment of the lesion, subject to the basic requirements for a diagnosis of pigmented tumors (Elder D, 2018), reflecting all the specifics and features in each case. The inclusion and use of immunohistochemical markers in the diagnosis of pigmented tumors are now recognized as necessary. The correct assessment and interpretation of their expression is an important indicator to prove the origin of the tumor and confirm malignant potential, respectively, for the differentiation of malignant melanoma, from imitating border

lesions. Aggression in the biological behavior of malignant melanoma requires a correct and early diagnosis, as only it would ensure the success of treatment and the possibility of a reasonable prognosis of survival. In this aspect, the results of our research, as well as any scientific research on the problems of diagnosis of pigmented tumors, represent a scientific and practical contribution to the fight against this aggressive malignant neoplasia - cutaneous malignant melanoma.

Conclusion

The wide variety of pigmented neoplasms, including the very discrete morphological differences between malignant pigmented tumors and atypical melanocyte lesions mimicking malignant melanoma, make morphological diagnosis difficult. A reliable diagnostic approach requires knowledge and evaluation of many histological parameters and often immunohistochemical analysis to prove the origin of the tumor and differentiate malignant melanoma from its imitating borderline lesions. In addition to histological data on tumor thickness, mitotic activity, the presence or absence of ulceration, which are significant prognostic factors, the modern diagnostic practice includes the use of markers, which are usually used in tumor diagnosis to confirm the malignant potential of the lesion and as predictors of tumor progression. In the present study, we investigated the expression of Cyclin D1, p53, BCL2, and Ki 67 markers, which are essential in the division cycle and tumor progression. We also confirmed the fundamental role of S100 protein as a marker for melanocyte differentiation and the importance of Melanosome clone HMB45 in proving the tumor origin of pigment cells. The detailed study of the expression according to an eleven-model

scheme for distribution and evaluation demonstrates the great diversity in the immunoreactivity of the studied markers. Knowing the nuances in this immunoreactivity prevents hasty and inaccurate decisions regarding the biological potential of pigment lesions, especially in cases we try to build an interpretation based on one researched criterion. I.e., brings to mind the conclusion that the diagnostic assessment should be based on a combined study on the expression of several markers, carefully selected, depending on the difficulty of the diagnostic task we are facing. The present study was focused on the search for markers predicting malignant potential in pigmented neoplasms to the greatest extent. Based on the processing of the obtained results by different statistical methods, we selected the most statistically significant immunohistochemical markers - predictors of the malignant potential in pigmented neoplasms. In this regard, we found it impossible to use the BCL2 protein and showed the indisputably high potential of Cyclin D1, P53, and HMB45 to establish the malignant potential in melanocyte tumors. The study showed that a reasonable approach required a combination of markers, in our selection triple one, based on Cyclin D1, p53, and HMB45 expression, to provide a

predictive value with maximum statistical reliability. This contributes to the most accurate diagnosis in the study of pigmented tumors. Aggression in the biological behavior of malignant melanoma requires a correct and early diagnosis, as it alone determines the success of treatment and survival. The essential components in the fight against this aggressive malignant neoplasia are timely diagnosis and stage-oriented therapy, for which there is a specific practical contribution and the current study. The study conducted in this way gives us reason to formulate the following conclusions:

Key points

1. Based on the characteristics of pigmented skin tumors, with an analysis of their morphological features and immunoreactivity to search for criteria for accurate diagnosis and prediction of their biological behavior, the following conclusions can be made:
2. Knowledge and accurate assessment of the morphological characteristics of pigmented tumors is an absolute necessity for the morphological diagnosis and determination of their type, respectively, as benign lesions (nevi), atypical nevi, and malignant melanoma.
3. The reflection of all the features and characteristics that characterize each pigmented lesion and are significantly related to malignant melanoma lesion thickness, depth of invasion, lymphocyte infiltration, ulceration, and mitotic activity determine morphological diagnostic and prognostic criteria.
4. The demographic characteristics of pigment tumors, namely gender, age, location, can be considered only as clinical prognostic features.

5. The immunohistochemically studied S100 protein and Melanosome clone HMB45, expressed in all studied cases in the three groups, confirmed their importance as markers proving the melanocyte nature of the tumors.
6. The established peculiarity in the expression of HMB45, referring to the absence of a gradient in the distribution of expression in malignant melanoma and the presence of such in nevi, is a significant prognostic criterion for malignancy.
7. The proven statistically significant difference in the expression of Cyclin D1 in the studied groups allows the use of this marker as a predictor of malignant potential with cut-off point - moderately intense expression, spotted or diffuse.
8. Despite the high positive expression of p53 in malignant melanomas and harmful in nevi, a characteristic of malignant immunophenotype, however, established, although in a small proportion of benign lesions and atypical nevi immunoreactivity, does not allow to accept p53 as an absolute (autonomous) criterion to determine biological potential.

9. The expression of BCL2 in the target groups, characterized by overlap (uniformity) in the degree of expression in benign and malignant pigmented tumors, makes it unsuitable for predicting malignant potential in these neoplasms.
10. Proliferative activity (Ki 67 expression), due to the presence of an extensive range in the degree of expression in the individual groups, cannot be a reliable, independent indicator of possible malignant potential; its application must be in parallel with other markers or other indicators that have a proven statistically significant character.
11. A reasonable approach in applying immunohistochemical markers predicting malignant potential in pigmented tumors, for more excellent reliability, requires their use in combination. According to our results, the best combination is a study of the three markers - Cyclin D1, p53 protein and HMB 45, in which it presents the highest statistical reliability in the direction of malignancy.
12. The 11-model scheme for a detailed accounting of the expression intensity and topographic distribution of the

expressing cells in the tumor introduced by us provides accuracy in the interpretation and determination of the immunoreactivity of each marker, an essential detail for increasing the certainty of morphological diagnosis and differential diagnosis, especially in borderline lesions.

Contributions

Original scientific and applied contributions

1. A detailed study of cyclin D1, p53 protein, BCL2, S100 protein, Melanosome clone HMB45 has been carried out with the application of an 11- model evaluation scheme made by us, both of the degree (intensity) and the topographical distribution of expression; its use is advisable, since it reflects the significant variability and nuances in the immunoreactivity of the test markers in pigment neoplasia, thus avoiding the danger of inaccurate conclusions and errors in the assessment of their biological potential.
2. A high prognostic value of our proposed triple combination of markers has been established - Cyclin D1, p53 protein expression, and HMB45 as a prequel of malignant potential in pigment lesions.

Confirmatory contributions

1. The high value of S100 protein and HMB 45 as markers of melanocytic origin is confirmed with their expression in all the pigment tumors of the three groups studied.

2. Of a confirmatory nature is the established high intensity of expression of S100 protein in benign lesions and in the majority of atypical nevi studied, as well as the wide range and peculiarities in the expression of HMB 45.

3. Confirmatory character is the established absence of statistically significant differences in expression of BCL2 in all pigment lesions, which makes it impossible to use the marker as a pre-marker of malignant.

4. Variability in the expression of Ki 67 in the three groups has been confirmed, a feature in benign and malignant pigment tumors, the knowledge of which is necessary for assessing their malignant potential.