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PHYSIOPATHOLOGY DISCIPLINE II

THE STUDY OF THE PHYSIOPATHOLOGICAL MECHANISMS OF THE MOLECULES INVOLVED IN THE PROGRESSION OF MELANOMA

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SUMMARY

I. GENERAL PART – CURRENT STATE OF KNOWLEDGE

Melanoma is the most severe type of skin cancer. It is the fifth most common cancer in the United States, among both men and women, and its incidence increases with age. [1] The survival of melanoma patients depends on the time of diagnosis of the disease and early detection is essential for a good prognosis and an excellent survival rate. The incidence of melanoma has increased greatly in recent years worldwide and mortality has begun to decline, probably due to sustained efforts at early detection and important advances in the treatment of advanced melanomas.

Melanoma is considered a multifactorial disease and occurs as a result of the interaction between genetic predisposition and exposure to environmental factors. Superficial spreading melanoma is the most common type of melanoma and accounts for 70% of all cases, causing 25% of all deaths from this disease [5]. 5% of patients with this subtype of melanoma are at risk of metastasis and even death within 10 years [6]. The overall 10-year survival rate is approximately 4.5-8%. [7]–[9] Fortunately, in most cases of thin cutaneous melanomas, surgical excision is curative and patients have a generally favorable prognosis. Thus, for melanomas in the T1a stage, survival at 10 years is 98%. [10] Therefore, identification of strong predictive factors for metastasis and survival is mandatory, especially for patients with thin melanomas.

Currently, the diagnosis, prognosis and treatment of patients with melanoma are based on the staging system of malignant tumors TNM (T – *tumor*, N – *node,* M – *distant metastasis*) which takes into account clinico-pathological risk factors: tumor thickness, ulceration, mitotic rate, sentinel node status and the presence of metastases [11]. But the American Joint Committee on Cancer (AJCC) classification cannot predict the distinct course and different responses to treatment of melanomas classified at the same stage. Thus, it is necessary to identify new parameters as quickly as possible for a better stratification and to establish the optimal treatment for each patient, according to his particular risk.

Melanoma has been considered an immunogenic tumor for many years and this particularity is used in the development of new therapeutic strategies. The immunogenicity of a tumor is the ability to induce responses of the adaptive immune system that can counteract the growth and progression of malignant cells. [12], [13] Melanoma is one of the most immunogenic tumors and its interaction with the immune system is still under investigation. It appears that the immunogenicity of melanoma depends on the interaction between tumor cells and immune cells,

as well as on the released factors by cancer cells into the tumor microenvironment. [14] An important step in understanding tumor immunobiology is the analysis of the populations and subsets of immune cells that form the tumor-associated lymphocytic infiltrate (TILs). TILs constitute a polymorphic group that is mainly composed of effector T lymphocytes, regulatory T lymphocytes, natural killer (NK) cells, dendritic cells and macrophages [15]. The distribution, density, profile and activation state of the cells that constitute TILs can vary and alter the clinical progress.

More recently, histological regression is considered an immune phenomenon with positive prognostic value in patients with primary cutaneous melanoma. For many years, however, it was interpreted as a negative prognostic factor. This represents, together with the lymphocytic tumor infiltrate, an early sign of the activation of the host's immune system against the tumor cells. [16] No clear conclusion has yet been reached regarding the prognostic significance of the regression phenomenon in melanoma. Immunotherapy in the case of melanomas diagnosed in advanced stages has shown promising results in recent years. Thus, a better understanding of the interaction between tumor cells and cells of the immune system could have beneficial results in the treatment of these patients. [16] In most of the studies that showed a good prognosis in the case of melanomas with regression, thin melanomas (with Breslow index below 1 mm) were prevalent. [17]–[19] In studies that included melanomas with Breslow index greater than 1 mm, in most cases no association was found between the presence of regression and patient survival. [20], [21]

I. PERSONAL CONTRIBUTION

3. HYPOTHESIS AND GENERAL OBJECTIVES OF THE RESEARCH

The main aim of the present study is the complete and detailed evaluation of the pathophysiological mechanisms of tumor progression and the identification of new prognostic factors in primary cutaneous melanoma. Considering that the incidence of melanoma is constantly increasing and the survival rate is dependent on the time of diagnosis of the disease, early detection is essential for a good prognosis and an excellent survival rate. Identifying and understanding the pathophysiological mechanisms that take place at the molecular level and the interactions between them can provide us valuable information regarding tumor initiation and progression, but also about the prognosis of melanoma patients.

I have beed analyzed cutaneous melanomas and common nevi with conventional histopathological methods but also with modern methods, such as immunohistochemistry. I compared the results obtained with the most relevant data from the specialized literature. I have been analyzed demographic and clinical data, histopathological parameters, immunohistochemical markers for the detection of new prognostic and therapeutic targets.

In order to achieve the aim of this study, I have considered the fulfillment of several objectives:

- Description of the most relevant aspects related to the demographic and clinical data of the analyzed groups with the aim of identifying the risk factors involved in the pathogenesis of melanoma;
- Expanding the area of knowledge regarding the histopathological and immunohistochemical aspects of cutaneous melanoma in order to identify the most important markers of evolution and prognosis;
- Achievement of comparisons and correlations between the established prognostic markers and the immunohistochemical studied ones in order to identify new correlations that could help to understand the evolution of melanoma lesions and to identify possible therapeutic targets.

4. GENERAL RESEARCH METHODOLOGY

4.1. MATERIALS AND METHODS

I performed a retrospective case-control study in which I have been analyzed clinical, histopathological and immunohistochemical prognostic factors for primary cutaneous melanomas and nevi. The study group finally included 70 samples, divided as follows: 56 spreading cutaneous melanomas, of which 30 regressed melanomas and 26 melanomas without regression and 14 common nevi. The lesions were from patients with no history of malignant skin tumors. The cases included in the study are part of the archive of the Pathological Anatomy Department of Colentina Clinical Hospital. These have been diagnosed between 2012-2016 and were selected within the CNCS-UEFISCDI research project, PN-III-P4-ID-PCE-2016-0641, crt no 183/2017, within PNCDI III, Project financed by the Ministry of Research and Innovation, with the title "*Characterization of the mechanisms involving CEACAM1 in melanoma based on the study of tumor regression, an innovative approach to optimize the management of patients with thin melanoma*" - CEACAMMEL. The study was approved by the Scientific Research Ethics Committee of Colentina Clinical Hospital from Bucharest. (Appendix 2)

The inclusion criteria used for case selection were:

- the histopathologically confirmed presence of spreading cutaneous melanoma lesions;
- the histopathologically confirmed presence of common nevi lesions;
- full resection sample taken for all cases.

The exclusion criteria used for case selection were:

- lesions of mucous membranes and of the hands and feet;
- cases confirmed by punch biopsy;
- lesions from the same patient;
- nodular melanoma lesions.

To perform immunohistochemical staining, it was necessary to make multi-tissue blocks. After selecting the cases, I took out the corresponding slides from the histotheque of the Pathological Anatomy Department of the Colentina Clinical Hospital. Thus, the formalin-fixed and paraffin-embedded tissues were subsequently analyzed under the optical microscope. After encircling and cutting tissue fragments with histopathological aspects characteristic of the analyzed tumors, multi-tissue blocks were made. Although we initially selected 68 cutaneous

melanoma lesions and 22 common nevi lesions, we subsequently excluded cases with thin tissue fragments from the study. I kept only the cases for which the cutting of tissue fragments will not impede the subsequent rediagnosis. The tissues that remained after cutting and were not included in the multi-tissue blocks are kept in the histotheque of the hospital according to the norms in force. Multitissue blocks were subsequently sectioned and each section was placed on a pretreated slide for immunohistochemical assays.

After completing these steps, the final study group comprises a total of 70 samples, of which 30 regressed cutaneous melanoma lesions and 26 non-regressed cutaneous melanoma lesions and 14 common nevi lesions. The obtained multitissue blocks were subsequently stained with immunohistochemical markers for:

- Langerhans cell expression analysis: CD1a;
- Analysis of the inflammatory tumor microenvironment cells: CD3, CD4, CD8, CD20, CD11c, FoxP3;

  CD3, CD4 and CD8 immunohistochemical markers were used for conventional T lymphocytes, CD20 marker for B lymphocytes, and FoxP3 marker for regulatory T lymphocytes (Tregs).
- Analysis of the adhesion and invasiveness molecule CEACAM1.

I have been studied their expression within each group by making correlations with classic histopathological prognostic factors. In addition, I performed a comparative analysis between the three types of samples taken.

I selected and analyzed data from histopathology reports as follows:

- For melanoma group:
  1. General parameters: sex, age, location of the lesion;
  2. Parameters related to the macroscopic appearance of the lesion: the presence or absence of macroscopic ulceration
  3. Histological parameters of the lesion: the level of invasion of the tumor, the assessment of atypical mitoses, the presence of perivascular and/or perineural invasion
  4. Parameters that characterize inflammatory infiltrate
- For nevi group:
  1. General parameters: sex, age, location of the lesion;
  2. Histological parameters of the lesion

The histopathological analysis of the samples was perfomed in the laboratory of the Pathological Anatomy Department of the Colentina Clinical Hospital from Bucharest. The pieces were oriented macroscopically according to the current protocols of the Pathological Anatomy specialty, issued by the Ministry of Health of Romania and published in the Official Gazette no. 723 of October 29, 2010.

4.2.5. STATISTICAL TESTS USED

The statistical analysis was performed by using the IBM SPSS Statistics 21 program. The statistical tests used in the analysis were:

- ***Chi-Square Test*** - is used to check if two nominal / dichotomous variables are associated. The *Phi* correlation coefficient is used to identify associations between two dichotomous variables. It takes values in the range of -1 to 1. The direction of the association is given by the sign. Cramer's correlation coefficient V measures the strength of association between variables and takes values between 0 and +1.
- ***Mann–Whitney U test*** - non-parametric test is used to determine whether there are differences between two independent groups when the dependent variable is either ordinal or continuous but not normally distributed.
- ***Kruskal–Wallis H test*** - nonparametric test is used to determine whether there are statistically significant differences between two or more independent groups when the dependent variable is continuous or ordinal.

5. ANALYSIS OF CLINICAL-PATHOLOGICAL CORRELATIONS IN THE GROUP OF CUTANEOUS MELANOMAS – RESULTS AND DISCUSSIONS

Patients from the melanoma group were distributed in a balanced way regarding of sex. It was made of 51.8% women and 48.2% men. The patients' ages ranged from 31 to 87 years, the average age being 57.29 years. The mean age of female patients was slightly higher (58 years) than male patients (56.52 years). The distribution of patients according to the decade of age was uniform, only 7.1% (4) presented ages over 80 years. More than half (51.72%) of female melanoma patients were between 41 and 60 years of age. On the other hand, in the case of men, the decades of age 31 – 40 years (33.3%), respectively 61 – 70 years (22.2%) recorded the most cutaneous melanomas. Only after the age of 70 the distribution of melanomas by sex was similar.

Regarding the location of the tumors, most of the them were located on the trunk, 32% of the tumors being registered on the posterior part and 30% on the anterior part of the trunk. These were followed by the lower limbs, the upper limbs and the head and neck region. Among men, most tumors were located on the posterior aspect of the trunk (48%) and respectively on the anterior aspect of the trunk (24%). In the case of women, most of the tumors were located on the anterior part of the trunk (36%) and the lower limbs (32%). These observations agree with the specialized literature. Thus, in an extensive study carried out in Sweden on 12,533 patients, it showed that women had melanoms in 53.5% of cases located on the extremities and 30.2% on the trunk, while men had melanomas in 25.2% of cases on the extremities and 56.7% on the trunk [198].

The distribution of cases in the group of melanomas studied according to Clark level of invasion was relatively uniform, with 48.2% of melanoma cases having Clark level between I and III and 51.8% level IV-V. Clark indices IV-V predominated among men (62.96%, 17 patients) and in the case of women the situation was relatively balanced, recording Clark indices between I and III in 58.62% of patients and IV-V in 41.38%. The average age of patients with Clark index IV-V (62.93 years) was higher than that of patients with Clark level between I and III (51.21 years). Except for the decade 41-50 years, we observed a sustained increase in the incidence of levels IV-V of Clark invasion in favor of levels I-III, with the increase in the age of the patients from one decade to another. The Mann-Whitney U test revealed statistically significant differences between the age of patients with Clark index I-III (mean rank 22.24) and the age of patients with Clark

index IV-V (mean rank 34.33, U=222.500, Z= -2.773, p=0.006). Advanced age is known to be a poor prognostic factor for melanoma patients. Many studies have indicated an inverse correlation between the depth of tumor invasion and patient survival, and the correlation of the Clark level with this parameter is in agreement with the data in the literature. [195], [212], [213].

Regarding Breslow tumor thickness, tumors with Breslow index between 0.25 – 0.49mm (28.6%) predominated, followed by those with values between 1 and 1.9 mm (21.4%). In addition, there is a higher proportion of Breslow index values between 1 and 1.9 mm among men (29.63%) and among women between 0.25 and 0.49 mm (27.59%). If we talk about the average age of the patients according to the Breslow tumor thickness, we noticed that it is lower in the case of patients with Breslow values of 0.25 – 0.49 mm, which is 48.50 years (standard deviation 13.13; CI: [41.5, 55.5]) and higher in patients with Breslow index ≥ 2mm, namely 66.2 years (standard deviation 13.73; CI: [56.38, 76.02]). These data agree with those in the specialized literature, knowing that advanced age and increased tumor thickness are negative prognostic factors.

The ulceration was found in 21.4% of patients, with a uniform distribution according to gender, thus 22.22% of male patients and 20.69% of female patients have ulceration. Patients whose tumors showed ulceration ranged in age from 37 to 84 years, with a mean age of 64 years, higher than that of patients without ulceration, who had a mean age of 55.45 years.

All melanomas with a mitotic index higer than 5 mitoses/mm^2 associated a Clark index of IV or V, while in the case of tumors with a mitotic index of 0-5 mitoses/mm2, 57.78% of them had a Clark index between I and III and 42.22 % Clark index of IV-V. These differences between the distributions were confirmed as statistically significant by applying the Chi-square test ($\chi 2$=9.073, p=0.011) and Cramer's Phi and V coefficients (0.414) reveal a link between the two variables (p=0.011). Numerous studies have shown the prognostic value of mitotic rate in patients with cutaneous melanoma. Several studies have demonstrated that mitotic rate has an independent prognostic value and is also the most important prognostic factor for survival according to the Breslow index [5], [191], [220]. Barnhill *et al* showed that a mitotic index of 1-6 mitoses/mm2 increases the risk of 5-year mortality 8-fold, while a mitotic rate greater than 6 mitoses/mm2 increases 5-year mortality 11-fold [221].

6. IMMUNOHISTOCHEMICAL CHARACTERIZATION OF THE MELANOMA GROUP – RESULTS AND DISCUSSION

The distribution of cases according to CD1a expression was as follows: 16.7% of cases had negative CD1a expression, 50% of them had rare CD1a expression, 33.3% of cases had frequent CD1a.

Without marking a statistically significant difference, it is observed that tumors with a lower Breslow index tend to have negative CD1a expression more often, while those with a higher Breslow index tend to have frequent CD1a more often. At the same time, an increase in the incidence of the Clark IV-V index is noted as the intensity of CD1a expression increases. In addition, we obtained a statistically significant association between CD1a expression and Clark level (Chi-square test, $\chi 2=6.964$, $p=0.031$), and Cramer's Phi and V coefficients (0.482) reveal a strong relationship between the two variables ($p=0.031$).

Regarding the ulceration, there is a significant increase in its incidence in tumors that showed frequent CD1a (60%) compared to those that had negative (20%) or rare (13.3%) CD1a expression. Moreover, we obtained a statistically significant association between CD1a expression and the ulceration (Chi-square test, $\chi 2=6.508$, $p=0.039$). Cramer's Phi and V coefficients (0.466) showed a strong relationship between the two variables ($p=0.039$). Thus, tumors with frequent CD1a had ulceration significantly more often compared to tumors that had another expression of CD1a (negative, rare).

If we refer to the mitotic index, there is no statistically significant association between it and CD1a expression, but a slight increase in the mitotic index is observed as the level of CD1a expression increases. In the group of melanomas studied, the incidence of tumor regression was 80% in the tumors with negative CD1a and in the group with frequent CD1a, respectively 60% in the group of tumors with rare CD1a.

FoxP3 expression was more frequent in tumors with Breslow index between 0.75-0.99 mm (33.33%) and over 2 mm (33.33%), without a statistically significant association between the two parameters. ($p=0.401$) Regarding ulceration, it is noted that its presence follows a similar trend in patients with negative or rare FoxP3 expression, having an incidence of approximately 16%, while in the case of patients with frequent FoxP3 expressions, the incidence of ulceration is much higher (57.14%). The Chi-square test revealed the existence of a statistically significant association

between FoxP3 expression and the presence of ulceration, $\chi 2=6.070$, $p=0.048$. Cramer's Phi and V coefficients (0.345) reveal that the link between the two variables is moderate ($p= 0.048$). Thus, tumors with frequent FoxP3 ulcerate significantly more often than tumors that have a negative or rare value of FoxP3.

In the group of cutaneous melanomas, cases with increased expression of CD4 predominated (52.94%). 29.42% had moderate density of CD4 expression and only 5.88% had negative expression of this parameter. Regarding the distribution of cases according to CD4 expression and Breslow index, we observed that, regardless of Breslow index, 42% - 66% of cases showed increased density of CD4 expression. By applying the Chi-square test, no statistically significant association was observed between CD4 expression and the Breslow index determined in patients with cutaneous melanoma. ($p=0.796$)

60.78% of patients showed an increased density of CD8 expression, 35.29% showed moderate density and only 3.92% low density. No CD8 negative cases were recorded. Kruskal-Wallis test did not reveal statistically significant differences in the age of patients in the groups with low, moderate or high CD4 expression. ($p=0.685$) Depending on the gender of the patient, a higher incidence of cases with increased density of CD8 is observed among men (73%) than women (48%). Female patients had a balanced ratio of cases with increased density and moderate compared to men. However, the differences are not statistically significant ($p=0.0168$).

Regarding the distribution of cases according to CD8 expression and Breslow index, an increased incidence of CD8 expression is observed with percentages varying between 37.5% and 75%. After applying the Chi-square test, no statistically significant association was observed between these parameters. ($p=0.604$) No statistically significant association between Clark invasion level and CD8 expression was find ($p=0.310$). In the group of patients with cutaneous melanoma, the absence of ulceration was noted in cases with low density of CD8 expression, and for those with moderate or increased density, the incidence of ulceration was similar (in about a quarter of cases). There was no statistically significant evidence to indicate an association between CD8 expression density and the presence of ulceration. ($p=0.697$)

Regarding the mitotic index, it is observed that patients with low density of CD8 expression exclusively had mitotic rate between 0 and 5 mitoses/mm^2, and in the case of those with moderate or increased densities, mitotic rate of 6-10 mitoses/mm^2 and $\geq$ 11 mitoses/mm^2 had similar

incidences. However, there is no statistically significant evidence to indicate an association between CD8 expression density and tumor-associated mitotic index. (p=0.977)

Although the trend is not statistically significant (p=0.162), an increase in the incidence of tumor regression is observed with the increase in CD8 expression density. Thus, none of the cases with low density of CD8 expression showed tumor regression. 44.44% of cases with moderate density of CD8 expression and 61.29% of cases with increased density of expression showed tumor regression.

In the group of cutaneous melanomas, CEACAM1 expression was positive in 80.39% of patients. Of these, 78% showed positive expression at the cytoplasmic level, 36% at the membrane level and 33.33% had positive CEACAM1 expression in both sites. We also noticed that the vast majority of cases that presented membranar CEACAM1 also associated cytoplasmic CEACAM1 (94.44%). Among the cases with cytoplasmic CEACAM1, only 43.58% also showed membranar CEACAM1. It is observed that the tumor ulceration is present exclusively in patients who showed positive CEACAM1, more precisely in 26.83% of them. We can think that patients with ulceration are characterized by a positive CEACAM1 expression, but the association between the presence of ulceration and CEACAM1 expression is not statistically significant (p=0.064). We also noted that the incidence of ulceration increases in patients with positive cytoplasmic CEACAM1 expression (28.21%). We obtained a statistically significant association ($\chi 2$ =3.978, p=0.046) between cytoplasmic CEACAM1 and tumor ulceration. Cramer's Phi and V coefficients (0.282) indicate a direct but weak relationship between them (p=0.046).

7. ANALYSIS OF CLINICAL-PATHOLOGICAL CORRELATIONS IN THE CONTROL GROUP (NEVI) AND COMPARATIVE ANALYSIS BETWEEN THE TWO GROUPS STUDIED - RESULTS AND DISCUSSIONS

The studied control group includes 14 patients with nevi, the majority of whom are women (78.57% of patients). The average age of the analyzed patients was 36.14 years (standard deviation 13.50; CI: [28.35; 43.94]), without registering major differences between the sexes. The average age of female patients was 34.73 years and that of men 41.33 years.

In the control group, FoxP3 expression showed negative values in most cases. Thus, 71.4% of patients recorded negative values of FoxP3 expression and 28.57% of patients recorded rare values. In contrast to them, patients with cutaneous melanomas recorded negative expression of FoxP3 in 37.25% of cases, rare in 49.02% of cases and frequent in 13.73% of cases. However, applying the Chi-square test did not indicate statistically significant differences between the two groups in terms of the distribution of FoxP3 expression values. ($p=0.054$)

Patients from the control group tend to have negative values of CD4 expression, while patients in the group of cutaneous melanomas predominantly have increased densities of CD4 expression. Thus, more than half of the patients in the control group (57.1%) presented negative values of CD4 expression and the rest equally presented either low densities or moderate densities (21.4% for each), without registering increased densities of this one. Compared to them, more than half of the patients with cutaneous melanomas showed increased density of CD4 expression (52.94%), 29.42% showed moderate density (15), 11.76% low density (6) and only 5.88% had negative values of CD4 expression (3). There is a statistically significant association between the value of CD4 expression and the patient's group ($\chi2=25.461$, $p\leq0.001$) and Cramer's Phi and V coefficients (0.626) reveal that the connection between the two variables is strong ($p\leq0.001$).

CD8 expression was negative in approximately two-thirds of patients from the control group (64.29%) and no increased CD8 densities were found. In the melanoma group, CD8 expression did not register negative values but had increased density in 60.78% of cases, moderate density for 35.29% and low density for only 3.92% of patients. We obtained a statistically significant association between CD8 expression and the patient's group ($\chi2=43.867$, $p\leq0.001$) and Cramer's Phi and V coefficients (0.822) reveal that the connection between the two variables is

very strong ($p \leq 0.001$). We can state that the patients in the control group tend to have negative values of CD8 expression, while the patients in the group of cutaneous melanomas mainly have increased densities of CD8 expression.

All patients in the nevi group showed a negative value of CD3 expression. Melanoma patients frequently had moderate or increased CD3 expression (35.29% and 41.18%, respectively). We obtained a statistically significant association between the CD3 expression value and the patient's group ($\chi 2=27.231$, $p \leq 0.001$) and Cramer's Phi and V coefficients (0.937) revealed a very strong link between the two variables.

CD20 expression recorded negative values in all patients in the group of nevi and predominantly positive in the case of melanomas. The Chi-square test revealed a statistically significant association between the CD20 expression value and the patient's group ($\chi 2=17.029$, $p=0.001$) and Cramer's Phi and V coefficients (0.729) showed that the connection between the two variables is strong ($p=0.001$).

Patients from the group of nevi tend to have negative values (64.29%) or low expression (35.71%) of CD11c, while patients from the group of cutaneous melanomas more frequently show increased or moderate expression of it. The association between CD11c expression and the patient's group was statistically significant ($\chi 2=11.005$, $p=0.004$) and Cramer's Phi and V coefficients (0.596) revealed a strong link between the two variables.

In the control group, only 21.43% of the cases presented a positive CEACAM1 expression, this being expressed exclusively at the cytoplasmic level. In the group of melanomas, however, CEACAM1 was expressed in a proportion of 80.39%, predominantly at the cytoplasmic level (95.12%). Unlike the group of nevi, in the group of cutaneous melanomas CEACAM1 expression was also found at the membrane level, representing 43.9% of the cases with a positive CEACAM1 expression value. The Chi-square test revealed a statistically significant association between CEACAM1 expression and the patient's group ($\chi 2=17.463$, $p \leq 0.001$) and Cramer's Phi and V coefficients (0.518) indicated a strong link between the two variables. These tests show us that melanoma patients have a positive CEACAM1 expression value more frequently, unlike patients with nevi, who in most cases show negative CEACAM1 expression values. Patients from the melanoma group more frequently associated a positive value of cytoplasmic CEACAM1 expression, unlike patients from the control group, who, in most cases, present negative values of CEACAM1 expression. This association was statistically significant ($\chi 2=15.517$, $p \leq 0.001$) and

Cramer's Phi and V coefficients (0.492) revealed a moderate-strong connection between the two variables. Regarding the expression of CEACAM1 at the membrane level, it showed that patients from the melanoma group have more frequently associated a positive value of this expression compared to patients with nevi, who exclusively present negative values. There is a statistically significant association between membrane CEACAM1 expression and the patient's group ($\chi 2$=7.012, p=0.008) and Cramer's Phi and V coefficients (0.331) revealed a moderate link between the two variables (p = 0.008).

8. CONCLUSIONS AND PERSONAL CONTRIBUTION

CONCLUSIONS

- ✓ I obtained a statistically significant correlation between the Clark index and the age of the patients, thus, the increase in the level of invasion occurs with the increase in the age of the patients. Increased Clark level also correlates with the presence of ulceration, this being more frequent in tumors with invasion level IV or V. ($p=0.002$) Advanced age and ulceration are known to be poor prognostic factors for melanoma patients. Many studies have indicated an inverse correlation between the depth of tumor invasion and patient survival, and the correlation of the Clark level with these parameters is in agreement with the data in the literature.
- ✓ I noted a statistically significant strong association between the Breslow index and Clark level of invasion. Thus, a lower Breslow index is associated with a lower Clark level and an increased Breslow index is associated with a higher Clark. Increased Breslow index also strongly correlates with the presence of ulceration ($p<0.001$). Data from the literature indicate that the presence and prognostic role of ulceration is influenced by tumor thickness and that its incidence increases with increasing Breslow index. [219]
- ✓ There is an association between the mitotic index and the age of melanoma patients, meaning that patients with a mitotic index greater than 5 tend to be much older compared to those with a mitotic index ≤ 5. ($p=0.021$) The mitotic index was also associated with Clark level. All melanomas with a mitotic index above 5 mitoses/mm^2 were associated with Clark index of IV or V. In the case of tumors with a mitotic index of 0-5 mitoses/mm^2, Clark indices between I and III predominated. ($p=0.011$). In a recent retrospective study, researchers revealed that mitotic rate is the strongest predictor of survival after positive sentinel lymph node biopsy [185]. Another large study concluded that mitotic rate is an independent predictor for survival [222].

STUDY OF TUMOR REGRESSION

- ✓ The prognostic role of histological regression in primary cutaneous melanoma is controversial. Traditionally, histological regression has been considered a negative prognostic factor because it is thought to affect the correct assessment of Breslow thickness [20], [178]–[180] Other authors believe that tumor regression has a positive role in that the immune system of the patient because

is intact and able to generate an immune response [18], [19], [181] and decrease the risk of developing metastases. [182] I obtained statistically significant differences between the age of patients with tumor regression and the age of patients without tumor regression. Patients with tumor regression tend to be older compared to those without tumor regression.

- ✓ Tumor regression was also associated with the presence of ulceration. I obtained a higher frequency of ulceration in melanomas with tumor regression compared to those without tumor regression. (p=0.02)
- ✓ I also obtained a statistically significant association between tumor regression and the mitotic index (p=0.015). It can be said that, in the case of the group of melanomas studied, tumor regression tends to be specific to all tumors that have associated a mitotic index above 5 mitoses/mm^2.
- ✓ In the group of melanomas, there was an increase in the incidence of cases with tumor regression with the increase in Breslow tumor thickness and a higher frequency in the case of Clark IV-V index tumors. However, no statistically significant associations were obtained for these parameters. (p=0.157)
- ✓ I noted the tendency for melanomas with regression to have associated increased CD4 densities, while those without regression are more often associated with moderate densities of CD4 expression. (p=0.018)
- ✓ Although the trend is not statistically significant (p=0.162), an increase in the incidence of tumor regression is observed with the increase in the density of CD8 expression. Thus, none of the cases with low density of CD8 expression showed tumor regression.
- ✓ I observed that the incidence of tumor regression is higher in cases where CD20 expression is negative or has low density. With the increase in the density of CD20 expression, tumor regression no longer occurs. However, an association between tumor regression and CD20 expression is not statistically significant (p=0.561)
- ✓ I noted that the incidence of tumor regression increases with the increase in CD11c expression, but without showing a statistically significant association between the two parameters. (p=0.334) Tumor regression was absent in cases with negative CD11c.
- ✓ There was an increase in the incidence of tumor regression in the case of patients who showed positive CEACAM1 compared to those who had negative expression. Thus, although it does not mark a significant difference (p=0.360), tumor regression is found in more than half of the cases

with positive CEACAM1 (56.1%), while, in patients with negative CEACAM1, it is found less frequently (40 %). The incidence of tumor regression remains high also among patients with positive expression of cytoplasmic CEACAM1, the highest level being recorded in patients with moderately positive cytoplasmic CEACAM1 (70%) without marking, however, an association between cases of tumor regression and the intensity of cytoplasmic CEACAM1 expression (p=0.637). In the case of patients with membrane CEACAM1, an increased presence of tumor regression is noted, but I did not obtain correlations between the intensity of expression of membrane CEACAM1 and the presence of tumor regression (p=0.325).

THE STUDY OF THE INFLAMMATORY INFILTRATE

- ✓ Within the studied group, no statistically significant associations were revealed between the intensity of the inflammatory infiltrate and age, sex, tumor regression or the Clark and Breslow indexes.
- ✓ No statistically significant associations were observed between the age or gender of the patients and the presence of inflammatory infiltrate and, respectively, the presence of intratumoral inflammatory infiltrate and the Breslow index.
- ✓ Without a statistically significant difference, a higher incidence of intratumoral inflammatory infiltrate was found in those with Clark index I-III (54.11%), compared to those with Clark index IV-V (45.83%).
- ✓ Tumor regression was found more frequently in patients with intratumoral inflammatory infiltrate compared to those without intratumoral inflammatory infiltrate, without obtaining a statistically significant association between the two parameters. (p=0.394)
- ✓ Both in the case of tumors with Clark index I-III, and in those with level IV-V, peritumoral inflammatory infiltrate was found in a proportion of 81.48%.
- ✓ Regarding CD1a expression, an increase in its density can be noted with the increase in the incidence of the Clark IV-V index. This association is statistically significant and the link between the two variables is strong. (p=0.031) CD1a expression also correlates with the presence of ulceration. Thus, tumors with frequent CD1a present ulceration significantly more often compared to tumors that have another expression of CD1a (negative, rare). (p=0.039) Several studies have shown that infiltration of tumor cells with CD-1a+ dendritic cells at the tumoral and peritumoral level correlates with low tumor thickness and the presence of the radial growth phase

of the tumor. [289], [290] Another study, however, observed a significant increase in CD-CD1a+ in primary cutaneous melanomas compared to normal skin and melanocytic nevi.

- ✓ Tumors with frequent FoxP3 present ulceration significantly more often compared to tumors that have a negative or rare value of FoxP3. (p=0.048) FoxP3 reflects the totality of T cells at the epithelial level and thus is considered in most cases an unfavorable prognostic factor.
- ✓ We noticed that patients with low density of CD3 expression tend to present ulceration more frequently compared to patients who have other values of CD3 expression. (p=0.014)

ANALYSIS OF CEACAM1 EXPRESSION

- ✓ I noted that the incidence of ulceration increases in patients with positive cytoplasmic CEACAM1 expression (28.21%). I obtained a statistically significant association between the two parameters (p=0.046).
- ✓ In the case of melanomas with positive CEACAM1 at the cytoplasmic level, the low expression of CD20 predominated (66.67%) and for membrane CEACAM1 the negative one (62.5%). These differences were statistically significant and the connection between the two variables is strong (p=0.04).

COMPARATIVE IMMUNOHISTOCHEMICAL ANALYSIS BETWEEN THE TWO GROUPS STUDIED

- ✓ Patients from the control group tend to have negative expression of CD4 while patients with cutaneous melanomas predominantly have increased densities of CD4 expression. The association between the CD4 expression and the patient's group is statistically significant ($p \leq 0.001$) and the link between the two variables is strong. There is little data in the literature on the prognostic role of CD4+ lymphocytes in melanoma. A study in metastatic melanoma identified a significant correlation of increased infiltration with CD4+ TILs and increased overall survival. [303] Other studies, however, could not identify a link between the presence of these cells in the inflammatory tumor infiltrate and the prognosis of melanoma patients. [304] As with CD8+ T lymphocytes, CD4+ T helper lymphocytes possess a variety of functions and phenotypes [305], making interpretation of their prognostic role in cancer difficult.
- ✓ Patients from the control group tend to have negative values of CD8 expression, while patients from the group of cutaneous melanomas predominantly have increased densities of CD8

expression. ($p \leq 0.001$) A recent cohort study of primary cutaneous melanomas observed an association between increased tumor-level CD8+ T lymphocytes and increased survival. Thus, the 5-year survival of patients was 78% for cases of melanomas with increased CD8+ lymphocytic tumor infiltrate, 44% for moderate infiltrate and 25% for low infiltrate. [293], [309] Another large study published in 2011, however, did not identify a significant correlation between CD8+ TILs and patient survival. [310] These studies suggest the complexity of demonstrating the prognostic role of cytotoxic T lymphocytes.

- ✓ All patients from the nevi group showed a negative value of CD3 expression and melanoma patients frequently had moderate or increased CD3 densities. ($p \leq 0.001$)
- ✓ CD20 expression recorded negative values in all patients from the group of nevi and predominantly positive in the case of melanomas. The Chi-square test revealed a statistically significant association between the CD20 expression value and the patient's group ($p=0.001$) and the link between the two variables is strong ($p=0.001$). For primary cutaneous melanoma, most studies have shown a positive association with survival. [316], [317]
- ✓ Patients from the group of nevi tend to have negative values or low expressions of CD11c expression, while patients from the group of cutaneous melanomas more frequently show increased or moderate expression of it. The association between CD11c expression and the patient's group was statistically significant and the link between the two variables is strong. ($p=0.004$)
- ✓ Patients from the melanoma group more frequently presented a positive value of CEACAM1 expression, in contrast to patients with nevi, who in most cases present negative values of CEACAM1 expression. ($p \leq 0.001$)
- ✓ Patients from the melanoma group more frequently associated a positive value of cytoplasmic CEACAM1 expression, unlike patients from the control group, who, in most cases, present negative values of CEACAM1 expression. This association was statistically significant and the link between the two variables was moderate-strong ($p \leq 0.001$). Regarding the expression of CEACAM1 at the membrane level, I noticed that patients from the melanoma group have more frequently associated a positive value of this expression compared to patients with nevi, who exclusively present negative values. There is a statistically significant association between membrane CEACAM1 expression and the patient's group ($p=0.008$). In a study published by Thies et al. in 2002 [242], [254] a highly statistically significant correlation between CEACAM1

expression and the occurrence of metastases was identified by Kaplan-Meier analysis ($p < 0.0001$). In addition, multivariate Cox regression analysis confirmed that CEACAM1 is an independent factor for the risk of metastasis, having a higher predictive value than tumor thickness. [242] The results of another study published in 2009 by Gamblicher et al. supported previous studies in that CEACAM1 may have an important role in tumor progression in cutaneous melanomas. [242], [262] However, there are few studies that systematically analyze CEACAM1 expression in melanoma.

PERSONAL CONTRIBUTION

In the present study, I performed an analysis of the specialized literature regarding the current guidelines and classifications in melanoma and highlighted the lack of firm criteria for risk stratification of patients with this pathology. Taking as a starting point the need to identify new prognostic factors and new possible therapeutic options, I performed clinicopathological correlations in melanoma with and without regression and common nevi. I used classic histopathological stainings but also modern immunohistochemistry techniques, the latter constituting the basis of numerous current researches regarding the understanding of the complex mechanisms of carcinogenesis.

The results obtained from this study were based on the expansion of knowledge regarding the histopathological and immunohistochemical aspects that characterize melanoma and the identification of specific markers of evolution and prognosis for it. At the same time, I described and analyzed the main mechanisms underlying immunogenicity in melanoma, namely the phenomenon of tumor regression and the presence and composition of the tumoral lymphocytic infiltrate. Additional studies are needed to exhaustively analyze the described mechanisms.

This complex analysis of cutaneous melanoma highlights the need for further research that correlates the immunological profile of the lesions obtained with data on the evolution, response to treatment, and local or distant recurrence rates of the tumors. These would lead to optimized management in cutaneous melanoma. I therefore consider this study to be an important contribution to the field and provide new research insights into progression in melanoma.

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