‘‘CAROL DAVILA” UNIVERSITY OF MEDICINE AND PHARMACY, BUCHAREST
DOCTORAL SCHOOL
FIELD OF MEDICINE

DOCTORAL THESIS

SUMMARY

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2024

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Evaluation of the Aggressiveness of Cutaneous Melanomas Staged pT3 and pT4 through Histopathological and Immunohistochemical Methods

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Introduction

Cutaneous melanomas represent approximately 4% of all skin cancer types. However, the morbidity and mortality associated with cutaneous melanomas, especially in locally advanced cases, exceed all other skin tumors [1]. In this context, the highest mortality rate is recorded among patients with deeply invasive melanomas, diagnosed in stages pT3 and pT4 according to the guidelines issued by the American Joint Committee on Cancer (AJCC) [2, 3]. However, even these subgroups of patients often present unpredictable outcomes. For these reasons, thorough research into prognostic factors is justified to improve the identification of patients at high risk of disease progression, so that they can benefit from the best monitoring and treatment options.

Prognostic factors for cutaneous melanomas can be divided into clinical, histopathological, and immunohistochemical, but their predictive value is sometimes uncertain, especially in the case of melanomas pT3 or pT4 melanomas. Among clinical factors, we mention the gender and age of patients, as well as the primary tumor location. Regarding the first two characteristics, certain studies have shown that male gender and older age at the time of diagnosis are unfavorable prognostic factors [4, 5, 6]. However, there are also studies that refute these associations [7, 8]. Similarly, the primary tumor location is a parameter with debatable prognostic value, in the sense that, depending on the author, the location on acral skin or on the head and neck have been associated with a negative prognosis [6, 9], while other studies refute the predictive value of the primary tumor location [10].

Histopathological factors considered to have prognostic value according to AJCC include: the histopathologic type of melanoma, epidermal ulceration, invasion depth measured in millimeters (Breslow index), Clark's level of invasion, the number of mitoses/mm^2, the presence of tumor microsatellites, lympho-vascular and perineural invasion, the type of inflammatory infiltrate associated with the tumor, and the status of resection margins [2]. However, deeply invasive melanomas often exhibit unpredictable outcomes, hence the prognostic value of the aforementioned factors may be significantly lower. In this regard, it is worth noting firstly that for tumors diagnosed at stage pT4, Breslow index values over 4 mm continue to retain prognostic significance up to a certain threshold beyond which some authors have demonstrated that invasion depth no longer influences patient outcomes [11, 12, 13].

Regarding this threshold value, reports vary; depending on the study, the Breslow index loses its prognostic significance when it exceeds 10 mm or 15 mm [14, 15].

In addition to the Breslow index, the presence or absence of epidermal ulceration is the second characteristic used for the staging of the primary tumor according to AJCC guidelines [2]. However, numerous studies have shown that the majority of deeply invasive cutaneous melanomas are also associated with epidermal ulceration, so the prognostic value of this parameter is limited in such cases [11, 16-23].

In the context of deeply invasive cutaneous melanomas, frequently contradictory results have been reported for the other histopathological parameters considered as highly valuable for estimating the prognosis of cutaneous melanomas overall [11, 18, 24].

Due to the low predictive values of conventional histopathological parameters, it is necessary to identify new prognostic factors for deeply invasive melanomas. In this regard, we mention perineural invasion and tumor necrosis, phenomena that are scarcely studied in such tumors. However, the results obtained so far are significant, with perineural invasion and tumor necrosis being associated with decreased survival [25, 26].

A new parameter with promising prognostic potential for cutaneous melanomas is the maximal width of the tumor. This parameter can be evaluated both microscopically according to the method described by Saldamha G. et al [27] and macroscopically [28]. However, studies on the width of invasion are still quite limited, necessitating further research to validate their prognostic value.

Last but not least, the prognosis estimation of patients with cutaneous melanomas could be enhanced through immunohistochemical tests. Currently, these analyses primarily hold diagnostic value, especially in the case of undifferentiated melanomas [29]. Dedifferentiation, defined as the loss of immunohistochemical expression of usual melanocytic markers, coupled with immunoreactivity for markers specific to other cell lines, is a rare phenomenon in primary cutaneous tumors but relatively common in metastatic lesions. In such situations, a thorough immunohistochemical analysis is necessary to establish a diagnosis [30].

However, certain immunohistochemical markers such as the proliferation index Ki67 could also be useful for evaluating the prognosis of patients with cutaneous melanomas. Although the predictive value of the Ki67 index has been demonstrated for numerous different malignancies, its role in estimating the prognosis of cutaneous melanomas remains uncertain

[31, 32]. Additionally, a standardized method for evaluating the percentage of Ki67-positive tumor cells is required in order to reduce inter-observer variability, a possible cause of the lack of agreement regarding the prognostic value of the Ki67 index in cutaneous melanomas. Lastly, for a better estimation of the relationship between the expression of this proliferation marker and patient outcomes, establishing a standardized threshold value is necessary, as to date, numerous different threshold values of the Ki67 index have been used in studies analyzing the prognosis of cutaneous melanomas [31, 33].

Study Hypothesis and Objectives

Starting from the hypothesis that locally advanced cutaneous melanomas, despite significant morbidity and mortality, often exhibit unpredictable progressions that cannot be solely explained by conventional prognostic factors, new studies are necessary to enhance the identification of patients at high risk of disease progression, enabling them to receive the most appropriate monitoring and treatment measures.

The main goal of this scientific endeavor was to establish new prognostic factors that can be quantified through classical histopathological examination or widely used immunohistochemical tests, making their analysis accessible without significantly increasing the cost associated with diagnosis.

Starting from the aforementioned goals, this doctoral research comprised three retrospective studies that analyzed patients diagnosed with cutaneous melanomas at the Emergency University Hospital in Bucharest between 2012 and 2018. In total, 143 patients were included, who were followed for at least five years from the time of the initial diagnosis or until death, if this event occurred earlier. Overall, various clinical and histopathological prognostic factors were evaluated, along with the expression of the Ki67 proliferation index concerning the progression of patients diagnosed with locally advanced cutaneous melanomas (stages pT3 and pT4) as follows:

1. The first study analyzed 94 patients diagnosed with cutaneous melanomas with a depth of invasion exceeding 4 mm (stage pT4), and evaluated the prognostic value of various demographic, clinical, and histopathological factors, focusing in particular on

establishing the predictive significance of the Breslow depth of invasion when it exceeds 4 mm;

2. The second study analyzed 49 patients diagnosed with cutaneous melanomas with invasion depth between 2 and 4 mm (stage pT3). This analysis also included various demographic, clinical, and histopathological prognostic factors, focusing particularly on the predictive value of a new parameter - invasion width, measured both macroscopically and microscopically;
3. The third study included 33 patients diagnosed with cutaneous melanomas in stages pT3 and pT4 and analyzed the prognostic value of the proliferative activity through the assessment of both the mitotic index and of the expression of the Ki67 proliferation index.

Research Methodology

The scientific research methodology included imaging evaluations to detect the presence of metastases, macroscopic analysis of surgically excised specimens, preparation of microscopic slides, histopathological and immunohistochemical analyses of tissue fragments collected according to standard protocols, database compilation, and statistical analysis of various variables in relation to disease progression and patient overall survival using the GraphPad Prism 10.0 software (GraphPad Inc.; San Diego, CA, USA). The statistical analysis involved calculating means, medians, standard deviations, minimum and maximum values, as well as 95% confidence intervals (95%CI) for continuous variables. Univariate and multivariate Cox regression analyses were performed, calculating hazard ratios (HR) and 95% confidence intervals, Kaplan-Meier survival curves, Harrell's concordance statistic test to demonstrate the prognostic value of multivariate regression, and Pearson correlation coefficient. Results were considered statistically significant for p-values < 0.05. The level of agreement between the Ki67 index values reported by two pathologists was evaluated using Bland-Altman analysis.

First Study: The Study of the Prognostic Value of Clinico-pathological Factors in pT4 Cutaneous Melanomas

In the study presented in the fifth chapter, the influence of various clinicopathological factors on disease-free interval and overall survival of patients with cutaneous melanomas with an invasion depth exceeding 4 mm was analyzed. Evaluated parameters included demographic factors such as age and gender of patients, clinical factors such as the primary tumor location and ulceration, as well as histopathological parameters including melanoma subtype, Breslow thickness, Clark's level of invasion, the number of mitoses/mm^2, lympho-vascular invasion, perineural invasion, regression, tumor microsatellites, tumor necrosis, intra-tumoral lymphocyte distribution, and the minimum distance between resection margins and the tumor.

Starting with the analysis of demographic factors, it was demonstrated that patients who developed metastases and those who died during the follow-up had, on average, older ages compared to those without disease progression, but these differences were not statistically significant. On the other hand, within this research, it was shown that the female gender is significantly associated with increased disease-free interval (HR=0.49; 95% CI=0.28-0.83; p=0.0103) and survival (HR=0.42; 95% CI=0.23-0.74; p=0.0036) in the univariate Cox analysis.

Following the analysis of the clinical factors, no statistically significant association could be established between the primary tumor location and disease-free interval or patient survival using univariate analysis models. Regarding epidermal ulceration, it was demonstrated that this characteristic was present in the majority of the cases included in the study and was not associated with disease-free interval or patient survival.

In terms of histopathological parameters, higher disease-free interval was significantly associated with the superficial spreading subtype of melanoma in univariate Cox analyses. The same analysis showed that a decrease in disease-free interval was significantly influenced by higher Breslow thickness and higher mitotic index values, as well as the presence of tumor necrosis, microsatellites, and perineural invasion. In the same context, Clark's level of invasion, the type of inflammatory infiltrate associated with the tumor, lympho-vascular invasion, regression, or the minimum distance between resection margins and the tumor formation were not significantly associated with disease-free interval. Regarding patient survival, this was

positively associated with the superficial spreading subtype of melanoma and negatively associated with Breslow thickness, necrosis, microsatellites, and perineural invasion.

Further, through multivariate Cox analyses, the independent predictive value of factors significantly associated with disease-free interval and patient survival in the univariate models was evaluated. Thus, the only factors independently associated with disease-free interval were Breslow thickness, necrosis, perineural invasion, and tumor microsatellites (Table V.12).

Tabel V.12. Cox multivariate anyalysis on factors associated with disease free interval

	HR	CI 95%	p
Necrosis	2.27	1.21-4.42	0.0126
Microsatellites	2.59	1.33-5.8	0.0069
Perineural invasion	2.45	1.21-4.93	0.0117
Breslow index	1.04	1.005-1.07	0.0205
Gender (female)	0.76	0.34-1.42	0.3943
Superficial spreading melanoma	0.63	0.23-1.49	0.3249
Mitotic index	1.02	0.96-1.08	0.4288

Similarly, the only factors independently associated with survival were Breslow thickness, necrosis, microsatellites, and perineural invasion (Table V.13).

Tabel V.13. Cox multivariate analysis on factors associated with patient survival.

	HR	CI 95%	p
Necrosis	2.19	1.14-4.34	0.0203
Microsatellites	2.84	1.42-5.5	0.0024
Perineural invasion	2.19	1.09-4.32	0.0249
Breslow index	1.038	1.005-1.07	0.0177
Gender (female)	0.61	0.32-1.16	0.1346
Superficial spreading melanoma	0.56	0.19-1.33	0.2339

In conclusion of this study, considering that the initial premise that Breslow thickness remains an important prognostic factor even in cases of melanomas with invasion depth exceeding 4 mm was validated, a Kaplan-Meier analysis was conducted to highlight the relationship between Breslow thickness values and survival rate. The Breslow thickness threshold values in this analysis were as follows: Breslow under 6 mm; Breslow between 6-8 mm, Breslow between 8-10 mm; Breslow between 10-15 mm, and Breslow over 15 mm (Figure 5.23).

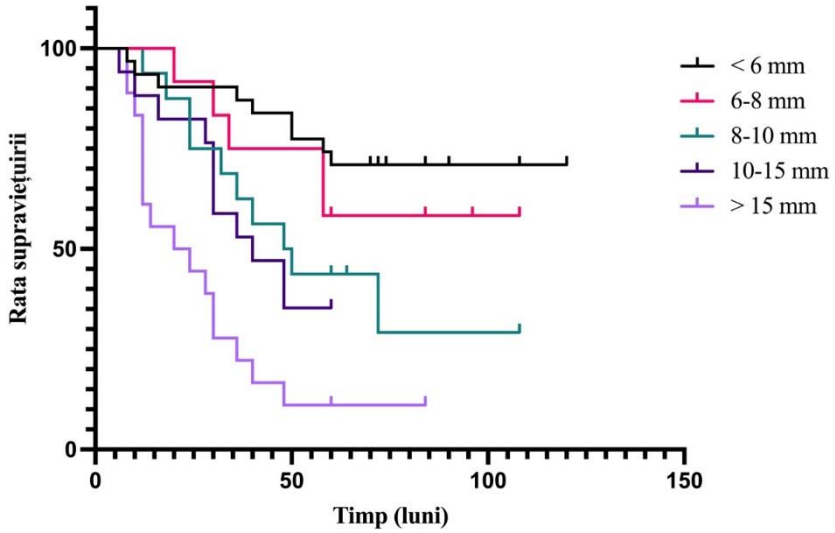

Fig 5.23. Kaplan-Maier survival proportions based on Breslow depth.

Based on these data, a significant decrease in the percentage of survivors can be noted as the Breslow thickness value continues to increase. The death rate progressively increased from 29.03% in the group with Breslow under 6 mm, to 41.66% in the group with Breslow between 6-8 mm, to 62.5% in the group with Breslow between 8-10 mm, to 64.7% in the group with Breslow between 10-15 mm, and reached 88.88% in patients with Breslow over 15 mm. These differences were statistically significant ($p<0.0001$, log-rank test).

For the last part of this study, we analyzed the survival rate using the Kaplan-Meier curve in relation to other parameters independently associated with patient survival. We demonstrated that patients with perineural invasion have a significantly lower survival rate ($p<0.0001$) than those without perineural invasion (HR=5.323; 95% CI=2.55-11.1). Similarly, the presence of tumor necrosis is strongly associated with a decrease in the survival rate (HR=3.218; 95% CI=1.572-6.588; $p<0.0001$). The last parameter we evaluated through Kaplan-Meier analysis

was the presence of tumor microsatellites. Also, the presence of tumor microsatellites is strongly associated (HR=3.538; 95% CI=2.023-6.19; p=0.0001).

Second Study: The Study of the Prognostic Value of the Width of Invasion in pT3 Cutaneous Melanomas

The study presented in the sixth chapter analyzes the prognosis of cutaneous melanomas diagnosed at stage pT3, emphasizing the importance of understanding negative prognostic factors for guiding appropriate treatment options.

Factors such as age at diagnosis, gender, and primary tumor location were analyzed through univariate Cox analysis in relation to disease-free interval and patient survival. In this context, it is worth noting that patient age was the only variable significantly associated with both disease-free interval (HR=1.06; 95%CI=1.02-1.1; p=0.0053) and overall survival (HR=1.07; 95%CI=1.02-1.13; p=0.0045).

Univariate Cox analyses were used to evaluate the prognostic significance of histopathological characteristics. In this regard, variables significantly associated with a decreased disease-free interval included nodular melanoma subtype, Breslow thickness, microsatellites, lympho-vascular invasion, and perineural invasion. Additionally, variables significantly associated with decreased patient survival were Breslow thickness, microsatellites, and perineural invasion.

The primary objective of this study was to evaluate the prognostic value of the maximum macroscopic tumor diameter and microscopic invasion width. Invasion width was defined as the maximum distance between the two outmost invasive tumor cells, measured in a plane approximately parallel to the skin surface and perpendicular to the Breslow thickness measurement plane (Figure 6.1), following the method developed by Saldanha G. et al. [27].

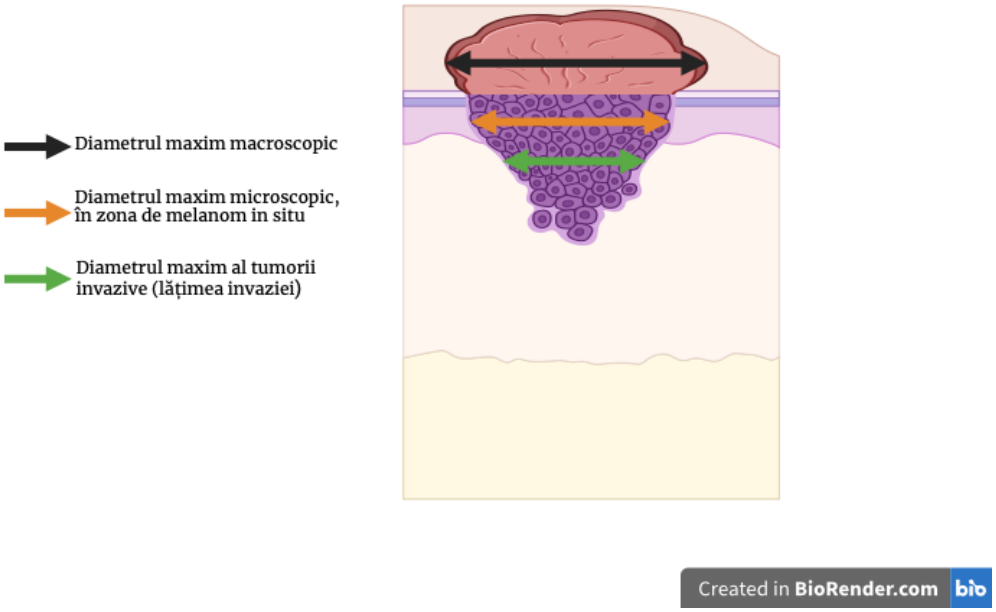

Fig. 6.1. Measurement of tumor width (biorender.com)

Firstly, these two parameters were found to be significantly correlated (Pearson correlation coefficient r=0.6422; 95% CI=0.4405-0.7821; p<0.0001). However, microscopic invasion width was significantly associated with both disease interval and patient survival, while no such associations were established for maximum macroscopic diameter in the univariate Cox analysis. In the same context, it was shown that invasion depth is a significant prognostic factor for disease-free interval and survival (Table VI.8).

Tabel VI.8. Cox univariate analysis on the prognostic value of the maximum microscopic diameter, the width of invasion and depth of invasion

	Disease free interval			Overall survival		
	HR	95%CI	p	HR	95%CI	p
Breslow depth	6,8	2,4-24,8	0,0011	8,81	2,48-48,92	0,0034
Invasion width	1,27	1,16-1,4	<0,0001	1,35	1,32-1,5	<0,0001
Macroscopic tumor diameter	1,05	0,98-1,11	0,1207	1,05	0,97-1,13	0,1260

Subsequently, in the multivariate Cox analyses, microscopic invasion width was shown to be the only independent factor associated with the disease interval and patient survival (Table VI.12).

Tabel VI.12. Cox multivariate analyses on clinical and histopathological prognostic factors.

	Disease free interval			Overall survival		
	HR	95%CI	p	HR	95%CI	p
Age	1.04	0.96-1.15	0.3373	1.2	1.02-1.57	0.0787
Gender (Male)	2.04	0.5-14.68	0.3794	14,22	0.03-11769	0.3134
Tumor location						
Members	Ref					
Head&neck	1.6	0.07-19.5	0.7345	2,03	0.0005-76544	0.8447
Trunk	0.58	0.07-4.33	0.5761	30,82	0.21-10036	0.1668
Acral skin	0.57	0.04-7.04	0.6637	6,17	0.0004-5719	0.5872
Breslow depth	2.06	0.45-12.00	0.3746	28,09	0.14-8252	0.1891
Invasion width	1.3	1.09-1.6	0.0057	2,08	1.28-10.96	0.0280
Mitotic index	0.85	0.62-1.1	0.2753	1,35	0.63-3.375	0.4135
Ulceration	3.63	0.57-30.76	0.1947	0,01	0.81-20607	0.1362
Regression	2.28	0.32-19.94	0.4038	25,01	8.328e-006 – 4.1	0.0911
Microsatellites	6.88	0.62-75.9	0.1099	7,56	0.0008-89652594	0.9967
Lympho-vascular invasion	6.85	1.81-101.1	0.0153	0,06	8.463e-009 – 4.22	0.3341
Perineural invasion	1.19	0.19-12.41	0.8620	1,35	0.15-18375	0.4005

To go on, we conducted a Kaplan-Meier survival analysis to highlight how microscopic invasion width influences survival over time. The chosen threshold values for invasion width were: width <5 mm; width between 5-10 mm; and width >10 mm (Figure 6.18).

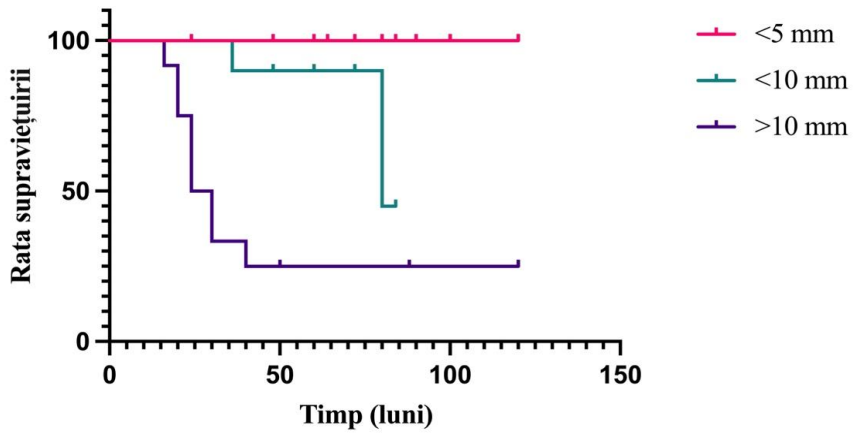

Fig. 6.18. Kaplan-Meier survival analysis based on the width of invasion.

Thus, we demonstrated that there is a significant decrease in the percentage of survivors as the invasion width increases. Survival decreased from 100% in the group with an invasion width of less than 5 mm to 80% for cases with an invasion width between 5-10 mm, and was only 18.18% among patients with an invasion width greater than 10 mm ($p < 0.0001$, log-rank test).

Third study: The Study of the Prognostic Value of Clinico-pathological Factors in pT4 Cutaneous Melanomas

The research presented in chapter seven aimed to evaluate the relationship between Ki67 index expression and disease-free interval and patient survival in cutaneous melanomas diagnosed at stages pT3 and pT4. According to the guidelines established by AJCC, the proliferative activity of melanomas should be assessed by reporting the mitotic index, considered to have prognostic value. For this reason, the correlation between Ki67 index values and the number of mitoses/mm², as well as the correlation between these parameters and invasion depth, were analyzed. Additionally, the predictive value for the number of mitoses and Ki67 index was investigated through Cox analysis and Kaplan-Meier survival curves.

Before conducting these analyses, we established a standardized and easily replicable method to estimate Ki67 index values. This method involved the following steps:

- Evaluating the entire section at a 10x objective magnification to identify areas with maximum proliferative activity;
- Capturing 10 photographs of these areas at a 40x objective magnification;
- Printing the photographs and counting 100 cells within each;
- Determining the number of Ki67-positive cells out of each 100 cells counted;
- A cell had to exhibit intense nuclear expression of Ki67 to be considered positive;
- Calculating the average Ki67 index by summing the values obtained for each photograph and dividing by 10.

This method demonstrated a high level of inter-observer agreement. The Bland-Altman analysis showed a small mean difference between the two measurements, suggesting that there is no significant systematic difference between observers (bias=0.6061; standard deviations=5.695; with 95% agreement limits between -10.56 and 11.77) (Figure 7.3).

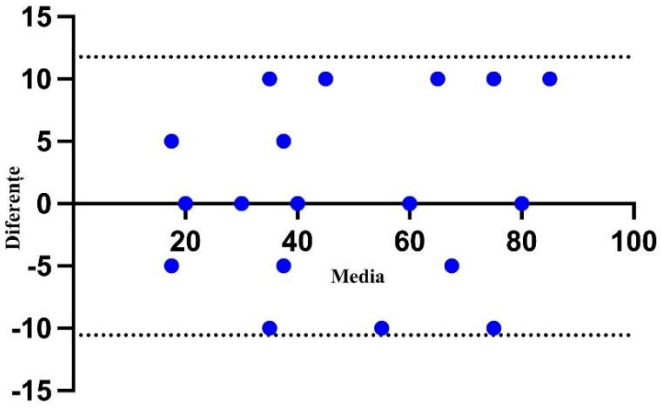

Fig.7.3. Bland-Altman plot for inter-observer agreement.

To go on, we evaluated the level of correlation between the mitotic index and the Ki67 index, demonstrating that these parameters are statistically significantly correlated (Pearson correlation coefficient r=0.5439; 95% CI=0.2366-0.7522; p=0.0011).

Subsequently, we analyzed the prognostic value of these two variables through univariate Cox regression. It was shown that the mitotic index is significantly associated with patient survival, but not with the disease-free interval (Table VII.3).

Tabel VII.3. Cox univariate analysis on the predictive value of the mitotic count

	Disease free interval			Overall survival		
	HR	95%CI	p	HR	95%CI	p
Univariate analysis	1.239	0.9538-1.653	0.1049	1.1	1.299-1.664	0.0126

Similarly, the Ki67 proliferation index was significantly associated with patient survival, but not with the disease-free interval (Table VII.5).

Tabel VII.5. Cox univariate analysis on the predictive value of the Ki67 index

	Disease free interval			Overall survival		
	HR	95%CI	p	HR	95%CI	p
Univariate analysis	1.144	1.011-1.368	0.0696	1.1	1.033-1.237	0.0302

However, in the multivariate Cox analyses, the mitotic index was associated with both the disease-free interval and patient survival, while the Ki67 index values no longer had statistical significance (Table VII.6).

Tabel VII.6. Cox multivariate analysis on the clinical and histopathological prognostic factors.

	Disease free interval			Overall survival		
	HR	95%CI	p	HR	95%CI	p
Age	1.038	0.99-1.09	0.0998	1.23	1.08-1.51	0.0147
Gender (Male)	5.79	1.405-26.59	0.0171	84.46	5.31-3744	0.0076
Melanoma subtype						
Superficial spreading	Ref					
Acral lentiginous	9.79	0.86-132.2	0.0686	2.48	0.13-75.44	0.5652
Nodular	1.08	0.14-9.71	0.9405	0.08	0.003-1.41	0.0989
Breslow depth	1.08	1.01-1.15	0.0144	1.31	1.13-1.61	0.0018

Ki67 index	0.99	0.96-1.02	0.6727	0.98	0.94-1.02	0.4263
Mitotic index	1.16	1.01-1.33	0.0276	1.31	1.05-1.79	0.0428
Ulceration	0.41	0.05-2.29	0.3421	0.4	0.008-8.07	0.5804
Regression	0.88	0.04-7.079	0.9228	0.61	0.008-18.64	0.7985
Microsatellites	0.55	0.09-2.68	0.9228	0.01	0.0001-0.61	0.058
Necrosis	0.26	0.05-1.13	0.084	0.62	0.08-3.67	0.6032
Lympho-vascular invasion	1.08	0.26-4.56	0.9091	0.09	0.005-1,26	0.075
Perineural invasion	6.45	1.48-30.09	0.0131	35.68	2.8-1065	0.0167

Furthermore, the correlation between the Breslow index and the Ki67 marker expression, as well as the number of mitoses/mm², were examined in order to determine if the proliferative activity is correlated with invasion depth. In this context, the Ki67 index was found to be significantly correlated with the Breslow index (Pearson correlation coefficient r=0.4749, 95% CI=0.1468-0.7088; p=0.0052), while the mitotic index was not correlated with invasion depth (Pearson correlation coefficient r=0.01687; 95% CI=-0.3386-0.3664; p=0.9301).

The last method used to estimate the predictive value of the number of mitoses and the Ki67 proliferation index was represented by survival analysis using Kaplan-Meier curves. A cutoff value of 8 mitoses/mm^2 was used for the mitotic index. In this context, the survival rate for patients with <8 mitoses/mm^2 was 68.42%, while for those with ≥8 mitoses/mm^2, it was 21.42% (p=0.0003, log-rank test).

Regarding the Ki67 index, a threshold value of 50% was chosen. The survival rate in the group with Ki67 <50% was 63.63%, and it was 18.18% in the group of patients with Ki67 ≥50% (p=0.0072, log-rank test).

Lastly, we draw attention to a particular situation where despite relatively low values of the mitotic index (5 mitoses/mm^2) and Ki67 proliferation index (15%), the patient's outcome was unfavorable. This was the case of a 65-year-old male patient diagnosed with an ulcerated nodular melanoma, with an invasion depth of 3.51 mm, without other negative prognostic histopathological factors. However, the patient developed multiple metastases and passed away after 30 months. The uniqueness of the case lies in the fact that in the metastatic lesions, the tumor proliferation exhibited a sarcomatoid appearance, composed of spindle cells with marked

atypia and the formation of vascular spaces, resembling angiosarcoma. Angiosarcomatoid dedifferentiation was confirmed by the loss of immunohistochemical expression of melanocytic markers, except for SOX10, and by the positivity of vascular markers such as ERG or CD31.

Conclusions and Personal Contributions

This doctoral research focused on estimating the prognosis of patients with cutaneous melanoma in stages pT3 and pT4, considering the unpredictable nature of these cases and the challenges they pose for treatment decisions. The research was centered on evaluating the predictive value of various clinical and histopathological factors. Additionally, the importance of immunohistochemical analysis of the Ki67 proliferation marker in relation to the prognostic value of the mitotic index was explored.

Chapter 5 highlighted the clinical and histopathological prognostic factors for cutaneous melanoma with an invasion depth greater than 4 mm. Female gender was statistically significant in predicting a longer disease-free survival and overall survival in the univariate analysis. In the same context, the superficial spreading melanoma subtype was significantly associated with an increase in disease-free survival and overall survival. On the other hand, the mitotic index was significantly associated only with disease-free survival in the univariate analysis.

The Breslow index, necrosis, microsatellites, and perineural invasion were identified as independent prognostic factors for a decrease in disease-free interval and overall survival. Additionally, Kaplan-Meier analyses illustrated statistically significant associations between reduced survival and the Breslow index, perineural invasion, and tumor necrosis. For these reasons, a significant personal contribution lies in establishing the prognostic value for two histopathological factors (tumor necrosis and perineural invasion) that have been previously analyzed superficially in relation to the progression of cutaneous melanomas. Furthermore, by demonstrating that the patient survival rate continues to decrease as the Breslow index exceeds the 4 mm threshold, a significant contribution was made to improving the identification of patients at high risk of disease progression.

A significant personal contribution in Chapter 6 was the identification of prognostic values for the macroscopically measured maximum tumor diameter and the microscopically

measured invasion width. The correlation between these two parameters was determined, showing a significant correlation between the macroscopically determined maximum tumor diameter and the microscopically measured invasion width. However, the prognostic values of these two variables differed significantly. The maximum tumor diameter was not significantly associated with disease-free interval (HR=1.05; 95% CI=0.98-1.11; p=0.1207) or patient survival (HR=1.05; 95% CI=0.97-1.13; p=0.126), while the invasion width was an independent prognostic factor for both disease-free interval (HR=1.3; 95% CI=1.09-1.6; p=0.0057) and patient survival (HR=2.08; 95% CI=1.28-10.96; p=0.028). Furthermore, Kaplan-Meier analysis demonstrated a significant decrease in patient survival with increasing invasion width ($p<0.0001$).

Finally, the third study contributed to understanding the utility of evaluating the proliferative activity of cutaneous melanomas through histopathological and immunohistochemical methods. In Chapter 7, a reproducible method, with a good degree of inter-observer concordance, was proposed and standardized to accurately measure the percentage of Ki67-positive cells. Additionally, a significant correlation between the mitotic index and the Ki67 index was demonstrated. However, the mitotic index was independently associated with disease-free interval and patient survival, unlike the Ki67 index, which was not an independent prognostic factor for either disease-free interval or survival. Furthermore, through Kaplan-Meier survival curves, it was shown that both increased values of the number of mitoses/mm^2 and the Ki67 proliferation index were associated with decreased survival, but the statistical significance was stronger for the analysis based on the number of mitoses. These results emphasize the essential contribution of the study in demonstrating that the analysis of Ki67 expression, when performed correctly, is inferior in prognostic value compared to evaluating the number of mitoses/mm^2, highlighting the limited utility of additional immunohistochemical analysis of Ki67 in predicting the prognosis of cutaneous melanomas.

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2. **Țăpoi D.A.**, Dumitru A, Ciongariu A, Chetroiu D, Grădinaru S, Aliuș C, Costache M. Primary cutaneous melanoma with rhabdomyoblastic differentiation – histopathological and immunohistochemical analysis of an extraordinarily rare malignancy – poster presentation at the XXXV European Congress of Pathology, Dublin, 2023