Histopathological evaluation of cutaneous malignant melanoma: A retrospective study

DANIELA-ELENA GHEOCA MUTU[1,2], ADELAIDA AVINO[2,3], ANDRA-ELENA BALCANGIU-STROESCU[4,5], MIHAI MEHEDINŢU[2], DANIELA GABRIELA BĂLAN[4], LĂCRĂMIOARA AURELIA BRÎNDUŞE[6], ANA-MARIA POPESCU[7], DORIN IONESCU[8,9], BOGDAN-MIHAI CRISTEA[1], LUMINIŢA FLORENTINA TOMESCU[10], CRISTIAN-RADU JECAN[2,3] and LAURA RĂDUCU[2,3]

[1]Discipline of Anatomy, Faculty of Medicine, 'Carol Davila' University of Medicine and Pharmacy, Bucharest 020021; [2]Department of Plastic and Reconstructive Surgery, 'Professor Dr Agrippa Ionescu' Clinical Emergency Hospital, Bucharest 011356; [3]Discipline of Plastic and Reconstructive Surgery, Faculty of Medicine, 'Carol Davila' University of Medicine and Pharmacy; [4]Discipline of Physiology, Faculty of Dental Medicine, 'Carol Davila' University of Medicine and Pharmacy, Bucharest 020021; [5]Department of Dialysis, Emergency University Hospital, Bucharest 050098; [6]Discipline of Public Health and Management, 'Carol Davila' University of Medicine and Pharmacy, Bucharest 020021; [7]Department of Financial and Economic Analysis and Valuation, Faculty of Accounting and Management Information Systems, Bucharest University of Economic Studies, Bucharest 010731; [8]Department of Medical Semiology, Discipline of Internal Medicine I and Nephrology, Faculty of Medicine, 'Carol Davila' University of Medicine and Pharmacy, Bucharest 020021; [9]Department of Nephrology, Emergency University Hospital, Bucharest 050098; [10]Department of Interventional Radiology, 'Professor Dr Agrippa Ionescu' Clinical Emergency Hospital, Bucharest 011356, Romania

Abstract. Malignant melanoma is a melanocytic neoplasm with a steadily increasing incidence worldwide. In order to define a proper diagnostic protocol and to establish an accurate prognostic method for the disease, specific biomarkers are of notable importance. Their contribution is also significant in the treatment of melanoma for the improvement of newer and more targeted therapeutic approaches. To emphasize the importance of specific immune markers in the diagnosis of melanoma, immunohistochemical analysis was performed on 56 formalin-fixed paraffin-embedded cutaneous melanomas. Besides the traditional prognostic factors, depth of invasion and mitotic rate, the markers tested in the present study were S100 protein family, Melan A, Ki67 and HMB-45. The present results indicated that immunocytochemistry represents a valuable test in the diagnosis and treatment of malignant melanoma and each biomarker had different associations with the progression and prognosis of the disease. Patients with S100 expression were 4.83 times (95% CI=1.2-20.8) more likely to suffer a relapse, whereas patients with a Ki67 expression of >30% had a 5.41-fold higher risk (95% CI=1.3-22.0). The correlation between S100 and the Breslow depth was statistically significant (r-value: 0.43; P=0.027). In addition, the importance of a multidisciplinary team including a plastic surgeon, anatomopathologist and oncologist was highlighted.

Introduction

Malignant melanoma is an aggressive type of cancer that has an increased rate of mortality and morbidity, being responsible for >60% of all deaths from skin cancer. It arises from transformed melanocytes and may occur on cutaneous or non-cutaneous sites, such as the oral mucosa, paranasal sinuses, urinary tract or eye (1). As the incidence of melanoma has increased at a steady rate over the last decades (it is rising by 3-8% per year in the Caucasian population) (2), early detection of malignant melanoma is vital for lowering both mortality and morbidity by identifying patients prone to developing this type of neoplasia, individuals with pre-cancerous lesions or with *de novo* melanoma and adopting an appropriate management of the lesions, with surgical excision frequently being curative for primary cutaneous melanoma (3,4).

Pathological features of the primary melanoma, such as tumor thickness (Breslow index), rate of mitosis and presence of ulceration, are major prognostic factors. These characteristics may be evaluated after localization and biopsy or surgical

resection of the tumor (5). On the other hand, advanced metastatic melanoma requires a more apprehensive approach, as in most of the cases, it cannot be managed only by surgery and requires therapeutic alternatives. In order to attain a proper management of this malignancy, potential metastatic lesions should be detected in a timely manner due to high mortality rates. An improved understanding of the molecular pathogenesis of malignant melanoma proves to be valuable when assessing patients for the requirement of newer therapeutic approaches, such as immunotherapy (6).

Immunohistochemical staining for molecular markers represents an important step not only in the diagnosis of malignant melanoma, but also in staging, evaluating prognosis, establishing treatment management and in predicting recurrence of the disease (7). Actual molecular information suggests that melanoma should be evaluated as a heterogeneous group of lesions with different defects in molecular aspects that involve distinct alterations of cellular processes including cell signaling, cell differentiation, cell adhesion and apoptosis (8). The histological features of melanomas imitate those of lymphomas, sarcomas, neuroendocrine tumors and Merkel cell carcinomas; for instance, both express epithelial cytokeratin 20 and endothelial markers (9). In the present study, the correlations between the specific biomarkers associated with malignant melanoma, including S100 protein family, Ki67, HMB-45 and Melan A, as well as the staging of the malignancy were highlighted, and the important features of each prognostic factor were discussed.

Materials and methods

Patients and treatment. Immunohistochemical analysis was performed on 56 formalin-fixed paraffin-embedded cutaneous melanoma samples. All of the cases covering a period of 2 years (January 2019-December 2020) were retrieved from the archive of 'Prof. Dr. Agrippa Ionescu' Clinical Emergency Hospital (Bucharest, Romania). The cases included the following histological subtypes of melanoma: Lentiginous (n=10), nodular (n=18), superficial spreading melanomas (SSM, n=17), acrallentiginous (n=10) and desmoplastic melanoma (n=1). Out of all the lesions tested, 6 were metastatic malignant melanoma and 11 cases suffered recurrence of the disease after surgical removal of the neoplasia. All 56 patients underwent further investigation by lymphoscintigraphy, with sentinel lymph nodes being positive in 16 cases who were then subjected to lymphadenectomy. A total of 10 patients presented with minor postoperative complications (seroma, wound dehiscence) and special dressings were used.

Patient analysis. The biomarkers tested in tumor samples were protein S100, Ki67, HMB-45 and Melan A, the most important biomarkers used for malignant melanoma (2). Furthermore, each patient's characteristics, including sex, age, smoking status, alcohol consumption, comorbidities and chronic medication, as well as the particular features of the lesions, including topography, lymph node metastasis and histological aspects, were recorded. Regarding the histopathological features of the melanomas, evaluation using the Clark and Breslow scales (1) was performed, the mitotic rate, the subtype of the lesion and the presence of ulceration were determined.

The anatomical sites of the lesions taken into consideration were as follows: The face, trunk and extremities. The entire information was inputted into a database on which statistical analysis was performed.

Statistical analysis. Values are expressed as n (%) for count data and as the mean ± standard deviation for continuous variables. Statistical analysis was performed by using SPSS version 23.0 software (IBM Corp.). Comparison of the averages for the continuous quantitative variables between patients with and without relapse was performed using the nonparametric Mann-Whitney U-test. Furthermore, the frequencies were compared using both Fisher's exact test and the χ^2 test. In order to analyze the relationship between recurrence and immunological or histopathological characteristics, the odds ratios (OR) with a confidence interval (CI) of 95% were determined.

Results

Patients. The patients included in the study had a mean age of 57.9±15.4 years, with no differences regarding the presence of relapses. There was no difference in terms of age. Regarding therapeutic approaches, in all 56 cases, surgical removal of the lesions with oncological safety margins was performed.

The patients who suffered relapses did not exhibit any differences in the prevalence of chronic diseases from those of the patients without recurrences. Among patients with relapses, 90.9% were male patients and the risk of relapse was 8.75 times higher in males (OR=8.75; 95% CI=1.03-74.18; Table I). A total of 3 (27.3%) of the relapsing patients were smokers and 5 (45.5%) of them drank alcohol occasionally. Furthermore, 45.5% (5 patients) of those who suffered recurrences took long-term medication for other pathologies, most commonly type II diabetes associated with chronic renal disease, arterial hypertension or cardiopathies, which was higher than the rate in those who did not relapse (n=21, 46.7%), but there was no difference with this regard.

Among the patients suffering recurrence of the lesions, 90.9% (10 cases) were males. Furthermore, 63.6% of the relapsing patients and 26.7% of the non-relapsing patients presented with melanoma located in the cranial area. The average mitosis rate in patients who suffered recurrences (9.0±3.7) was significantly higher (P=0.023) compared with that in the patients whose melanoma did not recur (5.7±4.3). Similarly, the Breslow depth was significantly higher (P<0.001) in patients who suffered recurrences than that in patients without (9.2±6.1 vs. 3.5±2.3; Table II).

Lesions. Of the lesions tested, the most common subtypes of malignant melanoma investigated at our clinic were nodular melanoma and malignant melanoma with an adjacent component of SSM, followed by the lentiginous, acrallentiginous and desmoplastic melanomas. Nodular melanoma (Fig. 1) was found in 18 patients. superficial spreading melanoma (Fig. 2) in 17 patients and 10 patients were diagnosed with lentiginous melanoma (Fig. 3).

The most frequent location of acrallentiginous melanoma (ALM) was on the lower limbs and it was associated with a higher incidence of recurrence compared to any other subtype. The

Table I. Patient characteristics.

Patient characteristics	RELAPSING (N=11) N (%)	NON-RELAPSING (N=45) N (%)	P value
Age (years) average ± SD	58.2±16.7	57.8±15.2	0.826
Sex			**0.022**
Male	10 (90.9)	24 (53.3)	
Female	1 (9.1)	21 (46.7)	
Comorbidities			
Hypertension	6 (54.5)	18 (40.0)	0.382
Diabetes Mellitus	1 (9.1)	6 (13.3)	0.703
Cardiopathies	3 (27.3)	9 (20.0)	0.335
Venous insufficiency	3 (27.3)	6 (13.3)	0.259
Other cancer types	1 (9.1)	7 (15.6)	0.832
Surgical history	7 (63.6)	19 (42.2)	0.351
Smoking	3 (27.3)	12 (26.7)	0.973
Alcohol use	5 (45.5)	17 (37.8)	0.640
Long-term use of drugs	5 (45.5)	21 (46.7)	0.942

Values are expressed as the mean ± standard deviation or n (%).

Table II. Immunological and histopathological characteristics and their association with relapse.

Patient characteristic	Relapsing (n=11)	Non-relapsing (n=45)	OR (95% CI)	P-value
Localization of melanoma				
Head	7 (63.6)	12 (26.7)	4.81 (1.2-19.4)	0.027
Trunk	0 (0.0)	17 (37.8)	0.08 (0.0-1.3)	0.072
Limbs	4 (36.4)	16 (35.6)	1.04 (0.3-4.1)	0.960
Lymph node metastases	6 (54.5)	10 (22.2)	4.20 (1.1-16.7)	0.041
Capsular invasion	10 (90.0)	24 (53.4)	8.75 (1.1-74.2)	0.047
Immunophenotyping				
HMB-45	1 (9.1)	15 (33.3)	0.20 (0.1-1.7)	0.111
S100	8 (72.8)	16 (35.6)	4.83 (1.2-20.8)	0.034
Melan A	3 (27.3)	14 (31.1)	0.83 (0.2-3.6)	0.804
Ki67			5.41 (1.3-22.0)	0.018
<30%	4 (36.4)	34 (75.6)		
>30%	7 (63.6)	11 (24.4)		
Tumoral foci	1.3±1.7	0.8±2.1	0.32 (-1.9-0.9)	0.467
Histological subtype				
Lentiginous	2 (18.2)	8 (17.8)	1.02 (0.2-5.7)	0.975
Nodular	1 (9.1)	17 (37.8)	0.16 (0.1-1.4)	0.092
Superficial spreading	3 (27.3)	14 (31.1)	0.83 (0.2-3.6)	0.804
Acrallentiginous	5 (45.5)	5 (11.1)	6.67 (1.5-30.1)	0.013
Others	0 (0.0)	1 (2.2)	1.29 (0.1-33.8)	0.878
Mitoses	9.0±3.7	5.7±4.3	(-6.1-0.5)	0.023
Breslow depth	9.2±6.1	3.5±2.3	(-7.9-3.4)	<0.001
Ulcerations	2 (18.2)	16 (35.6)	0.40 (0.1-2.1)	0.279
Clark level	2.8±1.5	3.1±1.3	(-0.6-1.2)	0.508

Values are expressed as n (%) or the mean ± standard deviation. OR, odds ratio.

histological subtype of the melanoma may be an important predictive factor in the evolution of the lesion and patients with ALM had a 6.67-fold increased risk of developing recurrence (P=0.013) compared to those with other histological patterns (Table II).
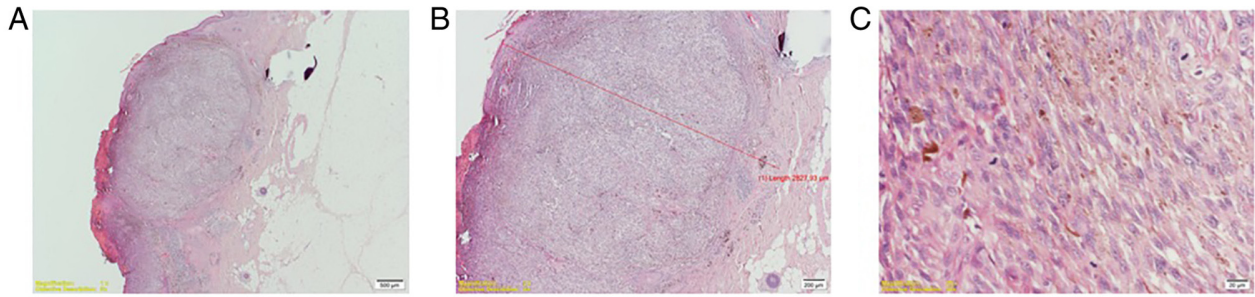
Figure 1. Representative histology images of nodular melanoma. (A) Scale bar, 500 μm; (B) scale bar, 200 μm; (C) scale bar, 20 μm. Asymmetric, well-delimitated lesions with a nodule-like appearance; atypical melanocytes do not extend to the lateral sides related to the dermal component. They exhibit a vertical growth pattern, with an invasive nature. The epidermal component consists of epithelioid melanocytes with abundant clear cytoplasm and large vesicular nuclei with prominent nucleoli and high mitotic rates.
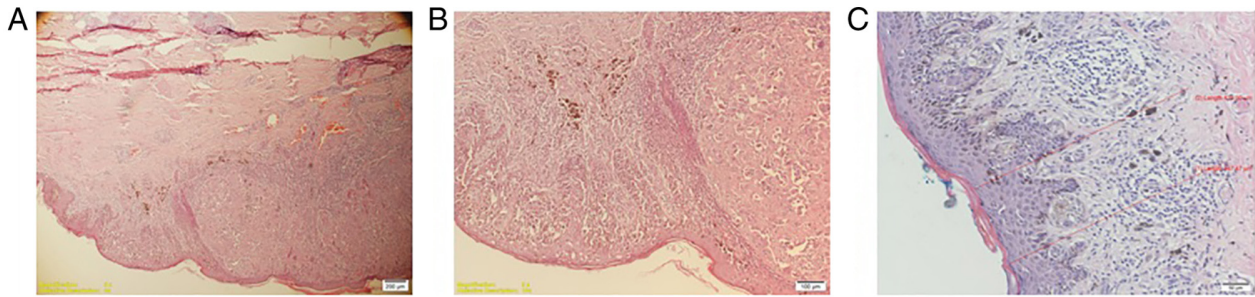

Figure 2. Representative histology images of superficial spreading melanoma. (A) Scale bar, 200 μm; (B) scale bar, 100 μm; (C) scale bar, 50 μm. Proliferation of voluminous atypical epithelioid melanocytes assembled into clusters, having the tendency of nested growth throughout distinct levels of the epidermis. Paget-like pattern.
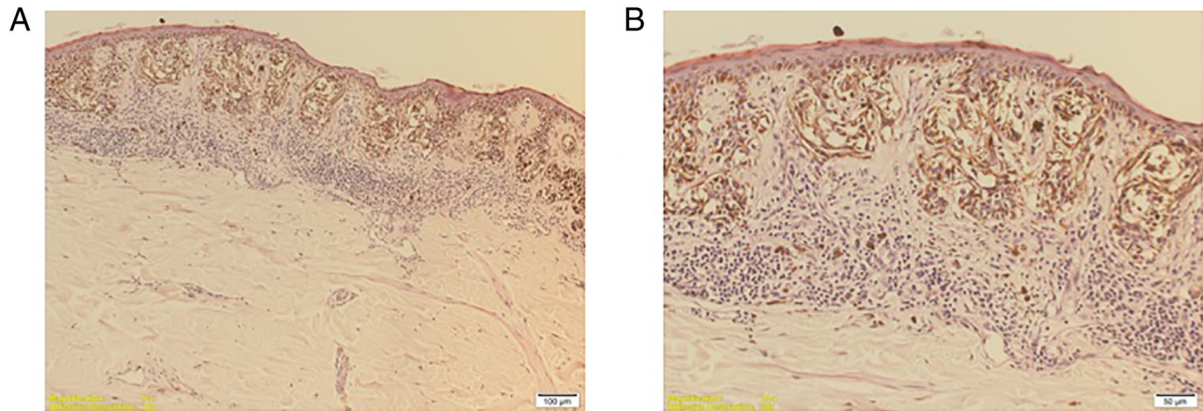

Figure 3. Representative histology images of lentiginous melanoma. (A) Scale bar, 100 μm; (B) scale bar, 50 μm. Radial growth-phase melanoma with lentiginous proliferation of atypical epithelioid melanocytes assembled singularly or into small cellular areas forming nests with focal pagetoid spread. Small fusiform melanocytes with a relatively lower amount of cytoplasm and barely noticeable nuclei; variable degrees of fibrosis and inflammation present (scale bars, 100 and 50 μm in the left and right panel, respectively).

Regarding immunohistochemical analysis, the specificity and sensitivity of S100 protein (Fig. 4A), Ki67 (Fig. 4B), HMB-45 (Fig. 4C) and Melan A (Fig. 4D) biomarkers were assessed. In the present study, the S100 and Ki67 biomarkers were determined to be predictive factors for recurrences. Patients with S100 expression were 4.83 times (95% CI=1.2-20.8) more likely to suffer a relapse, whereas patients with Ki67 expression of >30% had a 5.41-fold higher risk (95% CI=1.3-22.0; Table II). The correlation between S100 and the Breslow depth was statistically significant (r-value: 0.43; P=0.027), the latter being significantly higher in patients with S100 expression.

Discussion

Nodular melanoma accounts for 15-30% of melanoma cases and is the second most common subtype after the superficial spreading lesion (~70%) (10). It consists mostly of lesions >2 mm in thickness, with an increased rate of vertical growth and biologic aggressiveness, evaluated in an advanced stage at
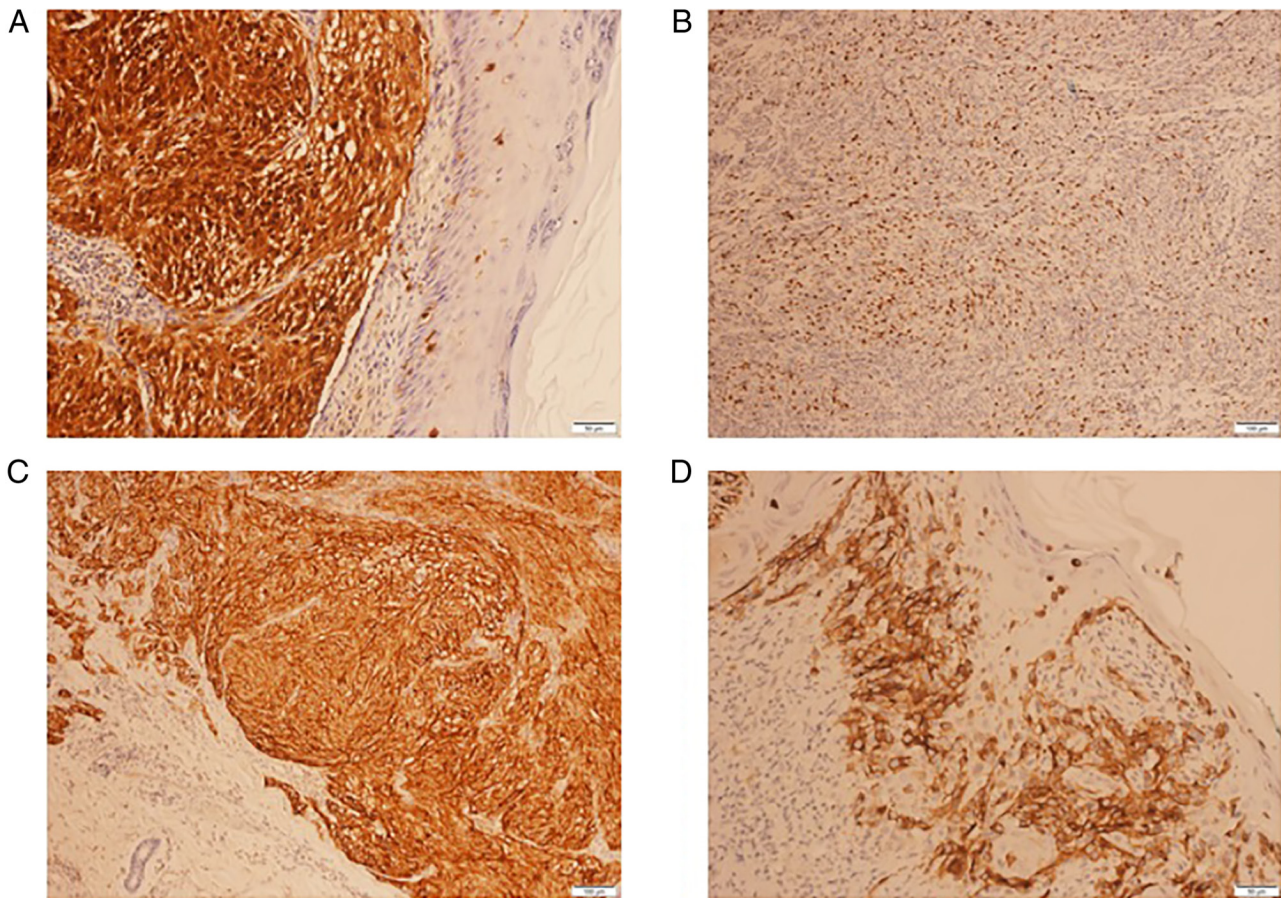
Figure 4. Representative immunohistochemical staining images for the biomarkers. (A) S100 (magnification, x20); (B) Ki67 (magnification, x10); (C) HMB-45 (magnification, x10); and (D) Melan A (magnification, x20).

initial presentation and higher incidence of recurrence. Its cytologic features are epithelioid, resembling the ones of the SSM, with a minimal or no demonstrable macular growth phase (11).

SSM is the most frequent type of melanoma, accounting for >50% and up to 70% of the cases of melanoma diagnosed globally (12). It commonly occurs on the extremities and on the trunk (13). During the last decades, there has been an increase in the number of cases diagnosed during Stage I, while there has also been an increase in the incidence of melanoma (partly due to better diagnostic methods and a higher general awareness), with a possibility for it to double over a period of 1-2 decades (12).

SSM usually consists of intraepidermal spotted (pagetoid) lesions, with voluminous epithelioid melanocytes spread throughout the epidermis, either alone or in packs or nests which vary in size and shape and which may frequently converge (13). The level of atypia of the melanocytes is variable. The lesions are usually flat and barely protuberant, having erratic shapes and edges (12). The SSM is positive for a variety of markers used in the diagnosis of melanoma, which include S100, HBM45 and Melan-A/MART1, which, however, cannot differentiate SSM from benign melanocytomas (14).

ALM is a rare subtype with a higher incidence in people of color (15). It usually implies a worse prognosis than that of all other known malignant skin lesions. Early clinical diagnosis of ALM is essential, but in numerous cases, it is delayed due to atypical location of the lesions, mainly arising on the palms, soles and nail beds. ALM occurring in individuals with dark-colored skin has been demonstrated to have a predilection for lower limb locations, particularly on plantar regions (16). Lentiginous melanoma usually arises on sun-exposed surfaces, such as the face and upper part of the trunk and is a slowly growing entity that may remain *in situ* for a prolonged duration and patients may at times suffer local recurrence after the oncological removal of the tumor. Desmoplastic melanoma is commonly associated with the lentiginous subtype and it consists of bulky tumoral masses that are usually amelanotic. Cells of this type of neoplasia have a storiform pattern and spindle-shaped morphology with high mitotic rates (17).

In the present study, the histopathological findings revealed that 10 lesions were lentiginous melanomas, with the most common topography on the face, and 2 patients had recurrence of the disease. Furthermore, one case was documented as desmoplastic melanoma with facial localization, lymph node involvement, strong positivity for S100 protein and no affinity for Melan A and HMB-45 biomarkers.

The most common chronic disease was hypertension (18), detected in 6 cases (54.5%) of the patients who relapsed and in 18 individuals (40%) of those who did not. Regarding the recurrence of the lesions, 90.9% appeared in male patients, thus making the melanoma 8.75 times more likely to recur in males than in females (OR=8.75; 95% CI=1.03-74.18).

The risk of recurrence was 4.81 times higher in patients with cranial localizations of the melanoma compared to other sites. Those patients with lymph node metastases and those who presented with capsular invasion had a significantly higher risk of recurrence (P=0.041 and P=0.047, respectively).

The ulceration and mitosis rate represented a prognostic factor for melanoma and it provided significant information regarding the aggressiveness of the tumor. It is frequently a characteristic of thick tumors and it is associated with a higher proliferative status of nodular melanomas rather than superficial spreading ones (10). This feature was evaluated by the frequency of mitoses detected for each category. Greater rates of mitosis have been observed to be linked to a fast tumoral size increase, indifferent to the lesion's dimensional characteristics (11). The Breslow thickness is a crucial variable and is the most important prognostic factor in cutaneous melanoma (3).

The level of mitoses was significantly positively correlated with the Breslow depth (r-value: 0.45; P=0.017), meaning that for an increase in the level of mitoses by one unit, the Breslow depth increases by 0.45 mm.

S100 protein is a biomarker used in the evaluation of tumors with a low degree of differentiation, with an almost 100% sensitivity for melanoma (19). It is involved in the process of calcium binding and it is also a regulating component of the microtubules. The protein is involved in the cellular division, in the metabolism of calcium, in protein phosphorylation and secretion, in cellular growth and in the regulation of cellular proliferation (20). S100 has been indicated to be expressed in a variety of poorly-differentiated types of cancer and also in diseases such as neurodegenerative disorders, inflammatory diseases and cardiomyopathies. Recently, it was proven that S100 has a close association with various cancer types, including melanoma (21). This may, in part, be due to the localization of the S100 genes on chromosome 1q21, which is highly susceptible to mutations (22). Among the various subtypes of S100, S100B, S100P, S100A4 (Metastatin), S100A6 and S100A13 are frequently present in melanoma, with S100P being positive in all melanoma subtypes. The association is lesser in oral malignant melanoma than in cutaneous melanoma, the former exhibiting both a lower grade of staining for S100 and a higher biological aggressiveness than the latter (21).

Ki67 is a cell cycle control protein whose specific antibody is used to ascertain the existence of a nuclear antigen only present in tissues with a high cellular proliferation rate, while it is otherwise absent in normal tissue. Ki67 is also involved in the transcription of RNA (23). It is absent during the G0 resting phase but present during the active cellular division phases G1, S, G2 and mitosis (24).

Since the protein's discovery in 1983, Ki67 proved to be a reliable index in both the diagnosis and the prognosis of various types of cancer (23). Specifically, in melanoma, Ki67 is useful for the prevention of false-negative diagnoses of melanoma during the differential diagnosis from benign nevi (24). The level of Ki67 is closely related to the rate of cell proliferation, thus allowing accurate assessment of the presence of the growth fraction of a certain cellular population (25). Furthermore, the expression of Ki67 also corresponds to the evolution of the disease: A higher level of Ki67 is associated with thicker tumors and, consequently, with less favorable prognosis for the patients (25).

HMB-45, which stands for 'human melanoma black', that was discovered in 1986 and recognizes a melanosomal glycoprotein (Pmel17) involved in the synthesis of the melanosomal fibrils and in the process of evolution from stage I pre-melanosomes to stage II. HMB-45 is one of the markers widely used for the positive diagnosis of malignant melanoma and in the assessment of sentinel lymph nodes for ruling out the presence of micrometastases (26). The sensitivity is 95% when using common antigen-retrieval techniques and it increases when using aggressive antigen retrieving techniques (this way, spindle cell melanomas may also be recognized) (27). HMB-45 staining is usually negative in desmoplastic melanoma. HMB-45 has 100% specificity in diagnosing malignant melanoma (26). In malignant melanoma, the level of staining to HMB-45 is proportional to the degree of cellular atypia (27).

Melan A, or MART-1, is a protein occurring in melanocytes, which may be used as a histopathological marker for detecting tumors derived from melanocytic precursors (4). It has been demonstrated that Melan A has the ability to differentiate between melanoma-*in-situ* in its early stages and senile keratosis (8). It is a sensitive and specific marker for the diagnosis of melanoma, but it may also be found in other tumors of melanocytic origin, such as clear cell sarcoma, benign nevi, melanotic neurofibroma or perivascular epithelioid cell tumors (28-30).

Malignant melanoma is considered one of the most virulent diseases, so the importance of a multidisciplinary team including a plastic surgeon, anatomopathologist and oncologist in the treatment patients with malignant melanoma should be highlighted.

Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

DEGM, AA, AEBS, MM, DGB, LAB, AMP, DI, BMC, LFT, CRJ and LR designed the study, analysed and interpreted datasets and wrote the manuscript. DEGM, AA, AEBS and MM collected the data and analysed the datasets. DEGM, AA, AEBS, MM, DGB, LAB, AMP, DI, BMC, LFT, CRJ and LR performed a literature search and selected the studies to be included. CRJ and LR critically revised the manuscript. All authors read and approved the final manuscript. CRJ and

LR checked and approved the authenticity of the raw data of the study.

Ethics approval and consent to participate

The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Committee of Professor Dr 'Agrippa Ionescu' Clinical Emergency Hospital (protocol no. 1736615/05.08.2014). Informed consent was obtained from all subjects involved in the study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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