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PhD THESIS

Clinical, morphological and immunophenotypic correlations in cutaneous and mucous melanoma with CDKN2A mutational status

Thesis abstract

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- 49 figures, of which 8 in the General Part
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- 287 bibliographic sources

Note: in this summary, the table of contents has been kept in the same form as in the doctoral thesis.

CURRENT STATE OF KNOWLEDGE

1. Melanoma. General considerations

Melanoma is a form of malignancy that derives from melanocytes, cells specialized in the synthesis and release of melanic pigment, and that is localized in the skin, uvea and mucosal epithelium. Cutaneous melanoma represents the most common type and is characterized by various risk, prognostic and predictive factors, compared to melanoma originating from other tissues [1]. From the epidemiological point of view, incidence varies according to sex: 16.8/100000 women and 27.4/100000 men, but the data regarding mortality does not follow the same ratio: 1.6/100000 in the feminine population, in contrast with 3.9/100000 among male patients [2].

CDKN2A represents a susceptibility gene investigated especially in the cases of familial melanoma (FM); systematic reviews and metanalyses have not been identified at the moment; some studies examined the potential associations between gender and the germinal mutations of this gene, took into account the genetic penetration and the clinical and pathological characteristics of the melanoma cases diagnosed among carriers, but the findings were consistent with the absence of any proven relationship.

Ultraviolet radiation captured by the tegument following exposure to the sun and to artificial tanning devices were clearly and definitively associated with the development of melanoma, according to the International Agency for Cancer Research. Various patterns of solar exposure have been described, the major type being represented by the intermittent model, approached during the weekend by people that work indoors, generally leading to skin burns. The metanalyses available up to this point show that chronic, persistent exposure to the solar light (i.e. the daily occupational pattern) does not increase the risk of melanoma [3].

Regular intake of coffee proved, due to the antioxidant value of its intrinsic polyphenols, a 38% lower risk in women compared to the male population (where no statistically significant difference was recorded) and to the group of female patients that drink coffee occasionally or do not consume it [4].

Synthetically, the major forms of clinical presentation are, according to Fitzpatrick et al: in situ melanoma, lentigo maligna melanoma, superficial spreading melanoma, nodular melanoma, desmoplastic melanoma, acral lentiginous melanoma, amelanotic melanoma, mucosal melanoma and the particular type of metastatic melanoma [5].

The tumoral growth is currently measured according to the last published edition of the American Joint Committee on Cancer (AJCC) [6]. The local and regional lymph nodes that present metastases are classified, based on their number, under the umbrella of the “N” indicator. The descriptors previously defined empirically as “microscopic” and “macroscopic” are now subject to a new nomenclature: “clinically occult” (e.g. clinical stages I-II with lymph node metastases identified by the sentinel lymph node biopsy) and “clinically apparent” regional nodal disease (e.g. clinical stage III). The lesional volume present in the sentinel lymph node, identified after the sentinel lymph node biopsy (SLNB), is considered a prognostic factor for regional disease, that must be established for every patient with a positive result, but that is not used to establish the “N” category. “Microsatellites” refer to cutaneous or subcutaneous microscopical metastases, adjacent to or underlying the primary tumor; microsatellite cells are not contiguous to those originating in the primary melanoma; moreover, the interposition of dermal inflammation, fibrosis or scar tissue is absent. The “satellite” metastasis defines the intralymphatic metastasis identified at a maximum

of 2 cm from the primary melanoma. Contrastively, in-transit metastases are located at more than 2 cm from the primary site of malignancy, but not distal to the regional lymph nodes [7].

Concerning the melanoma staging, the definitions of stage IA and IB have been revised; another amendment consists of the fact that stage IV is no longer subdivided consecutively (i.e. M1c represents stage IV, not stage IV C, like in the past). It should be noted that defining lymph nodes as "clinically detectable" is required when they can be objectified by palpation during the clinical examination and when they can be confirmed as locoregional metastases of a melanoma at the histopathological evaluation that follows the excision or biopsy. Moreover, the clinical staging should be conventionally used after the complete excision of the primary tumor, associated with the complete clinical evaluation of regional and distant metastases [8].

The Breslow index, that refers to the tumoral growth measured, with the aid of a calibrated ocular micrometer, from the most superficial area (the epidermic stratum granulosum) to the deepest part of the malignant melanocytic population, represents the most important prognostic factor in melanoma: a greater value is correlated with an increased risk of metastases [9]. Other negative prognostic and predictive factors are the particularly high levels of lactate dehydrogenase [10], the increased number of mitoses per mm^2 (histological element), the advanced Clark level of invasion and the anatomic distribution (i.e. melanomas localized at the cervical and cephalic level are recognized for their distinct aggressivity).

The therapeutic approach of melanoma varies depending on the degree of extension, thus the wide local excision of the primary tumor, with safety margins personalized according to the Breslow index, suffices in case of local disease. SLNB is recommended for all patients with at least pT1b, according to the TNM staging system provided by the AJCC, and complete lymph node dissection (CLND) is indicated in case of isolated locoregional lymph node metastases that are clinically detectable. Furthermore, patients with resected stage III disease must be evaluated for adjuvant therapy, as anti-PD1 agents, Nivolumab, Pembrolizumab, or Dabrafenib/Trametinib represent the preferred options. Patients with metastatic melanoma must be evaluated for the detection of the BRAF V600 mutation (using the specimen obtained from the primary tumor or, preferably, from the identified metastasis), because a combination of BRAF inhibitors and MEK inhibitors is applicable in those cases with BRAF positivity.

Given the fact that 8% of patients with melanoma develop a second primary melanoma during the first 2 years after the initial diagnosis [11], patients are clinically monitored in the course of post-therapeutic screening in order to precociously identify potential relapses or other cutaneous tumors, especially new primary melanomas [12]. At the moment, there is no consensus regarding the frequency of post-treatment examinations or the use of imagistic and laboratory techniques in patients with excised melanomas; however, the intervals between clinical appointments and imagistic evaluations can be personalized depending on the individual risk and the personal characteristics of the patients [13].

2. Current concepts and methods of melanoma investigation

Suspicious pigmented lesions are usually analyzed clinically following the ugly duckling sign, that represents the macroscopic evidence of a large melanocytic nevus among a series of small-sized nevi or of a single hypochromic nevus observed among multiple dark nevi. This semiologic element is fundamentally described after a comparative clinical evaluation of the aspect of all the pigmented lesions recorded in a patient, in case they present a nevus with clinical

characteristics different from the others [14]. Additionally, the “ABCDE” algorithm represents an instrument for the clinical morphological evaluation of pigmented lesions and is defined as an acronym for: asymmetry, irregular borders, uneven color, diameter greater than 6 mm and specific evolution pattern (debuting with a radial extension, followed by the vertical growth phase) [15].

Even though these criteria used for the naked eye cutaneous evaluation improved the identification of superficial spreading and nodular melanomas, a lot of other clinical subtypes remained undiagnosed, therefore the technological methods were refined, with the introduction of the portable device called dermatoscope, that insures a 10x magnification, associated with tegument illumination, so that it minimizes the light reflection and facilitates the chromatic and structural visualization of the layers located under stratum corneum, untraceable with a naked eye [16]. Synthetically, the dermoscopic criteria that plead for the diagnosis of melanoma comprise: the atypical pigment network [17], the angulated lines [18], the negative pigment network [19], the atypical dots and globules [20,21], the blue white veil [22], the atypical spots [23], the regression structures [24], the atypical vascular patterns [25] and the irregular striae [26].

The histopathological description includes asymmetric and poorly circumscribed lesions, with cytoarchitectural alterations and significant cellular atypia. The specific elements comprise epidermal consumption, pagetoid distribution of nested melanocytes, with dimensional and morphological polymorphism (that may be confluent or lacking maturation), melanocytes in the lymphovascular spaces and atypical mitoses associated with intense apoptotic processes. If ulceration is detected, it constitutes a negative prognostic factor. The main identified cells can be classified in two major types: epithelioid and spindle cells. The first ones represent round, large cells, with abundant eosinophilic cytoplasm, prominent vesicular nuclei and conspicuous nucleoli and usually appear in the context of superficial spreading and nodular melanomas. Spindle cells present a greater capacity of cellular cohesion, compared to epithelioid cells, and imply smaller, oval nuclei with mild-moderate pleomorphism, that contain small, hardly visible nucleoli. Marked cellular atypia and giant tumoral cells are rarely identified [27].

The histopathological differential diagnosis, in the absence of clinical lesional pigmentation, must be made with other entities that associate spindle cells, such as leiomyosarcoma, spindle-cell squamous carcinoma, atypical fibroxanthoma and dermatofibrosarcoma protuberans; in this context, special stains and immunohistochemical techniques are useful to evaluate the melanocytic origin of the tumoral cells. Amelanotic neoplastic lesions may be confounded with Langerhans histiocytosis, anaplastic carcinoma or lymphoma [28].

The molecular biology testing that targets actionable mutations is mandatory in patients with stage III or IV, resectable or non-resectable melanoma at diagnosis, and have a high degree of recommendation for stage IIC, associated with the resection of the primary tumor and high risk, thus the BRAF molecular identification is requisite [29]. Furthermore, approximately 10% of patients with cutaneous melanoma present positive familial history of this malignancy, and the CDKN2A mutation constitutes the most frequent cause of familial melanoma. Carriers of this genetic mutation harbor an increased risk of developing melanoma during their lifetime, of approximately 70%, with a younger age at diagnosis [30]. Taking into account the uncertainty of the direction and degree of implication of the CDKN2A mutation (CDKN2A-mut) in the evolution and survival of its carriers, Ipenburg *et al.* noted that CDKN2A-mut patients develop multiple primary melanomas (MPM) more frequently and that the age at diagnosis is 15 years lower than the category of patients with sporadic melanoma [31]; moreover, the CDKN2A mutation was

associated with thinner malignant melanocytic lesions, with an average Breslow index of 0.6 mm (compared to 0.9 mm in the latter group) [32].

PERSONAL CONTRIBUTIONS

1. General research hypotheses and motivation of the study

Recent molecular biology studies indicated the particular role of CDKN2A mutations in the etiopathogenesis of cutaneous and mucosal melanoma, characterized by cases identified in two members of the same family and by individuals with multiple primary melanomas. The financial burden of the exhaustive investigation of melanoma cases becomes clear when implementing standard personalized diagnostic and therapeutic protocols, therefore clarification of the potential prognostic and diagnostic value of certain auxiliary immunohistochemical markers – that could work as surrogate or additional techniques – is needed, in order to thoroughly characterize this pathological entity. Supplementarily, the possible correlations between the studied parameters will influence early detection, with direct impact on prognosis, by raising the life expectancy and quality of life of patients in this category.

2. General objectives

The present paper proposes the dermoscopic, histopathological, immunohistochemical and genetic investigation of the various clinical forms of cutaneous and mucosal melanomas, examining the presence of the most eloquent correlations between the immunohistochemical parameters (p14, p16, CD8, Ki-67) and the CDKN2A mutational status investigated by molecular biology techniques, as well as their prognostic role. Under the umbrella of the limitations of the derived correlations, the thesis aims to elaborate a tailored algorithm for facilitating the differential diagnosis between CDKN2A-positive melanomas and wild type cases, useful for dermatologists and pathologists.

Furthermore, the integration of the dermato-oncological theme in the current context of technological advancements is also proved by the research that explored the capacity of the AI-mediated software QuPath to quantify the CD8 positivity of the intra- and peritumoral lymphocytic infiltrate, as well as by the study that investigated the role of social media in the promotion of good sun protection practices and in the prophylaxis of UV-induced damage.

3. Line of research 1. Cutaneous and mucosal melanomas: Epidemiological, clinical, dermoscopic and histopathological characterization of patients with familial/multiple primary melanoma versus sporadic melanoma

The first part of the research evaluated the epidemiological, clinical, histopathological and dermoscopic features of the cutaneous and mucosal melanoma cases selected from the two reference centers where the study was conducted. Through the noted observations, the paper defined the correlations between the aforementioned parameters and the clinical, histopathological and dermoscopic characteristics most frequently registered among patients diagnosed with this dermato-oncological disease.

Study 1.1 comprised the analysis of the demographic, epidemiological, clinical and microscopical indicators of the selected cases. Therefore, a group of patients admitted in the Clinical Emergency County Hospital “Sf. Apostol Andrei” of Constanta and in the Emergency

University Hospital "Elias" of Bucharest for the suspected or confirmed diagnosis of cutaneous or mucosal melanoma was evaluated. After the analysis of the clinical evolution files, dermoscopic reports and histopathological features that certified the diagnosis of cutaneous or mucosal melanoma, the research was completed, in a personalized manner, by immunohistochemical techniques – consisting of individual panels of specific and non-specific biomarkers and percentual indicators of tumoral proliferation – and molecular biology testing used for establishing the mutational status of the pathological entities. The study of clinical cases from a 5-year period (January 2018-December 2022) identified a total number of 169 patients with cutaneous or mucosal melanomas diagnosed histopathologically and confirmed by immunohistochemistry.

The demographic profile of the examined cohort revealed the preponderance of cases originating from the county of Constanta, melanomas developed on cutaneous sites (95.26%), diagnosed in 2018 (29.58%), in male patients from urban residential areas. The main histopathological subtype was represented by superficial spreading melanomas (67.45%), the average value of the Breslow index was 3.65 mm, while 41.42% of the microscopically evaluated surgical specimens presented ulceration. Regression areas were objectified in a minority of cases, lympho-vascular invasion was absent in 53.84% of cases, and the absence of perineural invasion was predominant (76.33%). Concerning the mitotic rate, the average recorded a value of 1.35/mm^2. The most common localization of secondary determinations was the tegument and almost a fifth of the investigated patients were diagnosed in the metastatic stage. The dermatological exam, associated with the dermoscopic/videodermoscopic evaluation, detected an average number of pigmented lesions of 45 per patient.

In *Study 1.2*, the iconographic evaluation was performed via FotoFinder for the clinical profile and dermoscopic pattern of the nevi present in patients with a confirmed diagnosis of multiple primary melanoma or familial melanoma in the skin or mucosa. The information obtained after the examination of patients with the aforementioned diagnosis from the Dermatology Clinic of the "Elias" Emergency University Hospital in Bucharest comprised: the epidemiological and demographic data (age, sex, area of residence), the clinical features (topography of benign melanocytic lesions and primary melanomas, familial and/or personal history of melanoma), the histopathological details (histopathological subtype, Breslow index, presence/absence of ulceration) and dermoscopic characteristics (diameter, border regularity, symmetry, color).

The final group included in the study involved 10 patients with exclusive MPM, 4 patients with exclusive FM and 2 cases with an overlap of MPM and familial history of melanoma in at least one first-degree relative. Using the FotoFinder bodystudio ATBM master system (FotoFinder Systems GmbH, Bad Birnbach, Germany), total body images of the examined patients were taken. The identified melanocytic lesions were selected for regular follow-up after the histopathological diagnosis of melanoma, following five clinical criteria: a) asymmetry, b) irregular borders, c) diameter $\geq$ 6 mm, d) presence of erythematous or variable brownish areas, e) simultaneous identification of macular and papular components. The dermoscopic images corresponding to each significant pigmented lesion were subsequently analyzed in order to define the global dermoscopic pattern of every nevus (reticular, globular, starburst, multicomponent and atypical). The secondary phase included the classification of the melanocytic lesions as benign or malignant, based on criteria such as uniformity or heterogeneity of the structures on the lesional surface, border regularity, pigmentation model, symmetry of the pigment network, size, regularity and distribution of brownish globules, localization of atypical dots in relation to the periphery of the lesion and the presence of irregular and/or peripherical depigmentation. Taking into account the aforementioned elements, lesions with regular borders, centrifugal attenuation of the pigment network, lacking

pseudopods or radial disposition of globules were considered benign melanocytic nevi, while melanocytic macules or papules with irregular borders, pigment network abruptly discontinued at the periphery, peripherical aggregation of brownish globules, with or without pseudopods, were suggestive for the clinical suspicion of atypical nevi.

The average age at diagnosis in the sub-group of patients with exclusive MF was 35.5 years, while the one identified in the MPM cohort was higher (52.58 years). Furthermore, male patients with Fitzpatrick phototype II and superficial spreading melanomas were predominant. Most of the malignant lesions were diagnosed on the trunk and the average value of the Breslow index calculated microscopically in every first primary melanoma was 2.37 mm and 0.71 mm in the MPM sub-group and MF cohort, respectively. Concerning the MPM patients, the average interval between the diagnosis of the first and second primary melanomas was 33.33 months. The total number of nevi monitored with the aid of FotoFinder was 890, with an average value of 53.33 and 62.5 melanocytic lesions per individual in subjects with MPM and MF, respectively. Afterwards, the representative lesions recorded in every patient were evaluated, therefore 158 nevi with various characteristics were classified as follows: 81 with reticular pattern, 26 with globular pattern, 41 with multicomponent aspects, 9 with atypical dermoscopic elements and one starburst nevus. 73.41% of lesions presented homogeneous structures and 74.05% of the total number of the analyzed nevi were characterized by regular borders. Among the nevi with a reticular pattern, 67 of them indicated morphological and chromatic symmetry of the melanic network (42.40%) and other 65 (41.13%) were asymmetric, while 26 lesions did not expose a reticular model (16.45%). Depigmentation was revealed in 70.88% of the melanocytic lesions either at the periphery, in the center, or focally. Thus, the present study identified a predominant pattern of the nevi monitored in the analyzed population diagnosed with MPM and/or FM, defined by lesions with regular borders, homogeneous structures on the lesional surface, most frequently reticular, with symmetric pigment network and depigmentation.

The distribution of the cohort included in *Study 1.1* during the 5 calendar years indicated a gradual decrease in the number of diagnosed cases, from 2018 to 2022, a fact that may be correlated with the impact of SARS-CoV-2 pandemic on the patients' perceptions on the priority of health matters, particularly regarding the lesions developed on the skin. The results of *Study 1.2* indicated lower average Breslow index values in the second primary melanomas, compared to the first ones, which proves the utility of regular dermatological and videodermoscopic screenings (that also offer perspective over the evolutive, dynamic character of the lesions) in the attainment of the supreme purpose: the early diagnosis. Moreover, the sub-group of patients with familial melanoma present, on average, 1.17 times more nevi than the group diagnosed with MPM and 1.38 times more benign pigmented lesions than individuals with sporadic melanoma, findings that support the theory according to which the number and distribution of cutaneous nevi represent inherited genetic characteristics.

4. Line of research 2. Immunohistochemical examination and the investigation of the CDKN2A mutational status in patients with cutaneous and mucosal familial and multiple primary melanoma

Scientific gaps are still recognized regarding the existence of clear correlations between the CDKN2A mutations and specific phenotypes or dermoscopic characteristics in the field of MPM and FM; their formulation is essential for the particular oncological management of this patient category, by introducing them in special screening programs and post-diagnosis follow-up procedures for the early detection of the first primary melanoma or of the primary tumors developed

subsequently. Given the recommendation for genetic testing in cases where the probability of detecting a mutation surpasses 10%, corroborated with the personal history of MPM or familial history of melanoma, this part of the research aims to detect the frequency of CDKN2A mutations in a selected population diagnosed with MPM or FM and to identify the correlations between the genetic analysis and the immunohistochemical evaluation of the associated proteins (p14 and p16).

Study 2.1 comprises the analysis of tissue specimens by employing immunohistochemical techniques in order to detect the expression of p14 and p16 proteins in cutaneous and mucosal MPM and/or FM. The retrospective cross-sectional study was conducted over a period of 5 years (2018-2022) in a group of patients diagnosed with cutaneous and/or mucosal melanoma, with a positive personal or family history of melanoma. Clinical and epidemiological information, such as sex, age at diagnosis, area of residence, anatomic localization of melanoma, total number of pigmented lesions, were extracted from the archives of the Clinical Service of Pathology from the Clinical Emergency County Hospital of Constanta, considered a point of reference for the casuistry from the South-Eastern region of Romania. The tissue fragments obtained after the elliptical surgical excision and re-excision with safety margins adapted to the individual characteristics of every case were evaluated in order to describe the histopathological subtype, the Breslow index, mitotic rate, presence/absence of lympho-vascular and perineural invasion, and the type of lymphocytic infiltrate. Furthermore, the p14 and p16 immunohistochemical techniques were applied, according to the protocol from the manufacturer: for p14, the nuclear reaction was evaluated in a minimum of 1000 nuclei comprised in the tumoral proliferation, while p16 was considered to have present expression when it revealed intense nuclear and cytoplasmic reactions.

In the final cohort, that involved 23 subjects (7 patients originating from families with at least one first-degree relative diagnosed with melanoma and 16 individuals with MPM), the average age at diagnosis was 62.3 years, with an almost equal sex distribution, the majority of cases being identified in the eighth decade of life, and the most frequently affected anatomical sites were represented by the anterior/posterior thorax. Among the 23 cases that met the eligibility criteria, 13 exhibited p14 and/or p16 immunohistochemical absence. Exclusive p16 absent reaction was more commonly associated with the presence of distant metastases (85.71%), compared to the 50% of the 4 specimens that revealed exclusive loss of p14 expression. The average value of the Breslow index correlated with the loss of p16 and p14 expressions was 6.79 mm and 5.37 mm, respectively. Cases with p16 absent reaction presented an average mitotic rate of 5.28/mm^2, while those with exclusive loss of p14 immunoexpression recorded an average value of 2/mm^2.

Study 2.2 refers to the detection of the CDKN2A mutational status via fluorescent in situ hybridization (FISH) in the 23 melanoma samples described in Study 2.1, an investigation that was performed at the Center for Research and Development of the Morphological and Genetic Studies of Malignant Pathology (CEDMOG), Constanta. The evaluation via the epifluorescent microscope (Zeiss – Axio Imager.M2, Zeiss, Oberkochen, Baden-Württemberg, Germany), equipped with optimal filters and an image analysis system (MetaSystem Isis, MetaSystems Probes, Heidelberg, Germany), followed the genetic diagnostic criteria. In a cell with CDKN2A deletion, a reduced number of green signals was identified. The specimens showing ≥15 nuclei with the absence of orange signals afferent to the chromosome 9 centromere and the presence of at least two green signals suggestive for CDKN2A were considered positive for homozygous deletion. Cases of heterozygous CDKN2A deletion were objectified by the pattern that included an orange signal and two green signals of reduced dimensions.

The results obtained after the CDKN2A molecular FISH-mediated evaluation recognized 3 cases of heterozygous deletion (13.04%) and 7 cases of homozygous deletion (30.43%). Disomy was identified in 7 patients (30.43%), while CDKN2A monosomy was diagnosed in 6 cases (26.08%).

Corroborating the immunohistochemical information (p14 and p16) and the molecular biology data (CDKN2A) derived from the research conducted in Study 2, the association of all 7 cases of CDKN2A homozygous deletion and the immunohistochemical loss of p16 was observed. Moreover, 6 out of the 7 cases of CDKN2A homozygous deletion were correlated with absent p14 expression. Among the patients included in the personal study, 8 out of 10 cases with immunohistochemical absence of the p16 marker presented metastases at the time of diagnosis, and the other 2 patients with loss of p16 expression showed no secondary distant tumoral foci. Considering the epidemiological data, associated with the focus of the personal analysis on the casuistry from the South-Eastern region of Romania, the correlation between the p14-p16 immunoprofile and the CDKN2A genetic status revealed statistical significance.

5. Line of research 3. Evaluation of the CD8 immunoreaction through the manual technique versus via artificial intelligence

CD8 may be used as a prognostic marker, being associated with superior survival rates, and understanding the relationship between the CD8 positivity percentage and disease evolution facilitates patient classification based on their prognosis. Furthermore, the percentage of CD8-positive tumor-infiltrating lymphocytes (TILs) and the screening of the aforementioned values before and after treatment may offer information regarding the dynamic impact of different therapeutic strategies on the tumoral microclimate.

The retrospective observational study was conducted over a 5-year period (2018-2022) and included patients diagnosed with cutaneous and/or mucosal melanoma, with positive familial or personal history of primary melanomas, from the Clinical Service of Pathology, Clinical Emergency County Hospital of Constanta.

The 23 most representative microscopic specimens were selected for the application of CD8 immunohistochemical techniques, and a specific focus region was chosen inside the tumoral invasion area of each sample and marked as “0” or “1”, depending on the absence or presence of CD8-positive cells. Lymphocytes in direct contact with the tumor margins or comprised in the malignant cell population were labeled as “intratumoral”, while those located externally were considered ”peritumoral”. The reactions were defined as positive when >5% of TILs presented different degrees of staining intensity. Afterwards, all the slides were scanned with the Huron LE120TM 4000XT scanner (Huron Technologies International Inc., St. Jacobs, ON, Canada), at the Center for Research and Development of the Morphological and Genetic Studies of Malignant Pathology (CEDMOG), Constanta. A whole slide image (WSI) was obtained for every histological specimen, that was subsequently visualized via the HuronViewer™ software, while the image analysis was performed with the aid of QuPath platform (version 0.4.3), that utilized proprietary artificial intelligence based on machine learning.

The final cohort, that met the inclusion criteria, comprised 23 patients: 7 originating from families with at least a first-degree relative diagnosed with melanoma and 16 individuals with MPM; for the latter, a single primary tumor was chosen for further immunohistochemical evaluation.

Concerning the manual examinations, the average percentage of CD8-positive peritumoral nuclei in the analyzed population was 13.47% ± 22.81, while the average percentual index of intratumoral nuclei that presented CD8 immunohistochemical positivity was 20.86% ± 24.65. Additionally, the average percentage of CD8-positive intra- and peritumoral TILs was 14.23% and 16.53%, respectively, in the category of superficial spreading melanomas. On the other hand, nodular melanomas presented an average percentage of 26.11% in the context of CD8-positive intratumoral TILs and 10.55% in the context of peritumoral T cells. The single case of acral lentiginous melanoma revealed CD8 positivity in 60% of the intratumoral population and absent CD8 reaction in the peritumoral environment.

Among the 23 cases processed using the CD8 marker, only 56.52% could be supplementarily and optimally examined via QuPath. The other 10 tissue samples did not generate quantifiable results because of the artifacts generated in the preanalytical and analytical processing phases. The automatic examination determined a percentage of peritumoral CD8-positive nuclei of 45.49%, while the naked-eye evaluation identified an average of 24.23%. The interelement covariance matrix revealed a positive linear relation between "QuPath" and "Human Examiner", given that the covariance index (99.236) was substantial, exposing the fact that – when one of the variables tends to deviate from the average – the other follows the same tendency. These findings suggested a significant connection between the two indicators and highlighted their individual variability.

The number of analyzed cases will be extended in future studies, thus targeting the evaluation of changes in the statistical significance. There was no significant inverse correlation between the high value of CD8-positive TILs and melanoma progression in the personal study, an idea supported by the cases with multiple visceral metastases and elevated percentage of CD8-positive cells in the peritumoral infiltrate, as well as by those without distant metastases presenting a low number of CD8-positive lymphocytes. The potential molecular role of the CDKN2A mutation on the survival rates of CDKN2A-mut patients should be thoroughly investigated to confirm the possible mechanisms of action that could be targeted by specific novel therapies.

6. Line of research 4. The digital means of information and their contribution to the prevention and early diagnosis of melanoma

Accelerated digitalization was precipitated by the massive use generated by patients, as well as by the high percentage of health care professionals (65%) that created social media accounts.

The objectives of the current study were to identify the role of social media in augmenting the awareness on cutaneous and mucosal melanoma. Therefore, the most frequent risk factors and malpractices of primary/secondary prophylaxis were extracted, and we aimed to present the correlation between different social media usage patterns and various degrees of personal responsibility in the avoidance of specific high-risk behaviors.

This cross-sectional study was based on e-questionnaires that included 20 questions, divided into 4 categories: (1) general patient characteristics, (2) types of social media usage and people's opinion on its modifying role in melanoma prevention attitudes, (3) the personal applicability of correct sun protective behaviors and (4) information about the personal and familial history of melanoma and Clark nevi (dysplastic nevi). These surveys were disseminated to the general public during four consecutive weeks, between January and February 2023, with the aid of online channels, aiming to extract qualitative data in regard to the relationship between social media, epidemiological variables and the behaviors associated with melanoma development, as well as

with solar protection practices. SurveyPlanet (PollDeep, Inc., Hamilton HM, Bermuda) was the platform that hosted the questionnaire during the deployment of the study and the obtained information was exported and stored as Excel documents (Microsoft Office, USA) for analysis and processing.

The extraction strategy followed the principles of chain sampling, represented by the process that implies the collection of the first sample group which, if accepted after the preliminary analysis, permits the approbation of the entire cohort of respondents. Thus, the final database included 221 persons, with an average age between 26-35 years, with a female:male ratio of 3.5:1 and predominant residency in urban areas (71.94%). Among the evaluated social media users, 44.79% declared that they actively follow medical educational content on virtual channels, most of them (80.99%) reported that they use products with sun protection factor (SPF), 54.29% wear sunglasses with special UV filter, while 53.84% avoid UV exposure between 10 AM and 4 PM.

When asked to evaluate, on a scale from 1 (inapplicable) to 10 (applicable, superposable), the level on which a certain solar prevention measure was included in their routine, the average value attributed to the use of creams/lotions/sprays with SPF and their reapplication every 2 hours or after perspiration/entering the pool or sea was 6.77, and the use of clothing that covers a large cutaneous surface and/or of textiles with UV filters registered an average of 5.86. In this section, the avoidance of solar exposure between 10 AM and 4 PM reached the highest score (7.3). Concerning the dermatological advice transmitted on social media that was incorporated into their regime, participants were provided with different options that define the most frequent suggestions elaborated by dermatologists online. In total, 50.67% of the individuals included in the study affirmed that they no longer go to the beach without using SPF products, 46.15% quit sunbathing at improper hours and 45.70% started avoiding any type of solar exposure between 10 AM and 4 PM. Moreover, 47.51% of the respondents integrated the application of SPF in their daily routine, while 38.00% of them learned about the correct dosage and application of SPF products in order for them to cover the entire cutaneous surface. Contrastively, only 12.21% reported that they did not actively access any dermatological practice disseminated online.

In this analysis, the hypothesis according to which a higher applicability of dermatological recommendations transmitted via social media would be corroborated with certain specific epidemiological characteristics was tested. First of all, the association between the younger age (under 35) and the prominent adoption of appropriate skincare practice was confirmed, due to the positive values of the covariance determinant (0.02-0.05), but could not be considered significative according to the correlation coefficients that varied between 0.09 and 0.23 and that highlighted a weak correlation. Secondly, the link between the urban area of residence (taking into consideration the elevated accessibility to technology) and the superior acquisition of photoprotective practices was negligible, as indicated by the covariance analysis (with results comprised between 0.005 and 0.008), corroborated to the correlation coefficients (0.05-0.08).

In the third section, dedicated to the personal applicability of the correct sun protection behaviors, it was found that only 21.26% of participants benefited from dermatological visits once a year or more frequently (2-4 times annually), depending on their cutaneous particularities and history, but the vast majority stated that they never underwent dermoscopic or videodermoscopic evaluations (63.34%).

Furthermore, regarding the personal and familial history of dysplastic nevi, 2.26% of the participants were diagnosed with Clark nevi, and 1.35% of the respondents originated in families

with at least one first-degree relative with the aforementioned diagnosis. As far as the melanoma history is concerned, only 3 respondents (1.35%) had one family member affected by this oncological disease, while none of them presented a personal history of melanoma.

To comprehensively evaluate the role of virtual channels in the propagation of this type of information, Instagram and Facebook were the chosen platforms in the present study, representing the main sites for disseminating the link that guided the interested population to the e-questionnaire. During the 60 days of active status, the form was majorly accessed via Instagram, proving the key attribution of this platform in the digital industry and its potential to become a financially efficient and almost bias-free tool, convenient for the propagation of scientific data and dermatological advice. The short amount of time needed to obtain 221 valid responses additionally pleads to the use of virtual channels, to the detriment of conventional forms or standard medical archives used to collect data. After performing the analysis, this particular type of electronic survey does not only generate statistical information about dermato-oncological screening behaviors but can also act as a tool for raising awareness about the adoption of optimal sun protective measures and about the dermatological visits scheduled at correct and personalized intervals of time, especially when the questionnaire encompasses scientifically based concepts and is elaborated by a health care professional.

Additional studies that investigate the characteristics of the target audience are essential in order to attain a higher level of addressability of dermatological recommendations. The criteria that can be further explored as potential influencing factors for the design of awareness campaigns on cutaneous cancers are the social economic status, the marital status and the profession of the participants.

7. Line of research 5. Development of a personalised immunophenotypical screening algorithm for patients with familial and multiple primary melanoma, with variable CDKN2A mutational status

Alterations of the CDKN2A tumoral suppressing gene (p16INK4a) were demonstrated to play an important role in certain tumoral types and the corresponding p16 protein acts by blocking the progression of the cellular cycle by its inhibiting effect on the cyclin D/CDK2-pRb pathway. Recent studies in the field of dermatopathology showed the major utility of immunohistochemistry in the correct and exhaustive diagnosis of malignant melanocytic tumors.

Taking into account the significant prevalence of melanoma, the costs associated with its diagnosis and treatment are exponentially augmented. Therefore, new approaches in patient selection for different types of well-known or novel treatments are necessary to obtain optimal therapy response rates as well as the cost efficiency of the diagnostic investigations.

In the present study, 23 patients were evaluated, among which 7 diagnosed with familial melanoma and 13 with multiple primary melanomas, identified over a period of 5 years (2018-2022); hence, the total number of primary tumors was 50, of which the most illustrative malignant melanocytic lesions were selected for every case, favoring those with optimal tissue preservation and histopathological slides. Immunohistochemical techniques were performed on one representative tumor per patient, so that, in the study, the number of lesions examined via immunohistochemistry was equal to the number of included patients. Furthermore, epidemiological parameters, such as age at diagnosis and sex, clinical indicators (anatomical localization of the

primary tumor(s) and histopathological characteristics (histopathological melanoma subtype, Breslow index, mitotic rate) were investigated and retained.

The immunohistochemical techniques comprised the p16 protein (clone MX007, Master Diagnostica), the Ki-67 antibody (clone SP6, Biocare) and the CD8 marker (clone SP16, Master Diagnostica). The Ki-67 index was determined in the area with the most intense proliferation and the absence of major inflammatory infiltrate, criteria evaluated at low magnification (x10), in hot spot. The percentage of nuclei stained with diaminobenzidine (DAB) was estimated in approximately 200 tumoral cells. The percentual analysis of Ki-67-positive cells was, therefore, utilized, as in the case of the p16 protein, for which the percentage of immunomarked cells was classified at the cytoplasmic, nuclear or cytoplasmic and nuclear level.

Regarding the genetic analysis of CDKN2A mutations, it was performed with the aid of fluorescent in situ hybridization (FISH), according to the specifications described in Study 2.2. The initial evaluation was made individually for every immunohistochemical marker (p16, CD8, Ki-67), after the characterization of the tumors included in the study, as CDKN2A-mutated (CDKN2A-mut, with homo- or heterozygous deletions) or CDKN2A-wild type (CDKN2A-wt, comprising cases with disomy or monosomy).

Based on the data obtained after the use of p16 protein, CD8 marker and Ki-67 index, a comparative analysis of the results of the 3 parameters was firstly conducted separately among the MPM and FM category with CDKN2A mutation or with the absence of this mutation. With the aid of the correlations obtained between various values of the 3 aforementioned indicators and the data available in the specialty literature, individual classification systems for each parameter were established, with different limits established depending on their clinical, diagnostic or evolutive significance. The final aim was to create a semi-quantitative evaluation algorithm of the two mutational contexts. The two sub-groups (synthetically named CDKN2A-wt and CDKN2A-mut) were then randomly divided into two almost equal parts: the first part of each (the test set) was used to obtain the total values of the triple p16-CD8-Ki-67 score, while the second part (the validation set) included the cases analyzed for the confirmation of the accuracy of the method and the performance of the proposed score.

Cases with CDKN2A-mutated expression totaled 10 patients, while 13 tissue specimens revealed the absence of this genetic mutation. The individual evaluation of p16 registered an average value of 42.30% in MPM and FM without CDKN2A mutations and 15.00% in the category of patients with homo- or heterozygous deletions of CDKN2A, respectively. The average value of CD8-positive peritumoral lymphocytes in the sub-group of CDKN2A-mutated patients was 5.00%, while cases without CDKN2A genetic alterations identified via molecular biology were associated with an average percentage of CD8-positive peritumoral TILs of 19.61%. Moreover, the Ki-67 expression in individuals with MPM and FM that revealed CDKN2A mutations after FISH was more prominent (42.50%) compared to the group of CDKN2A-wt patients, where the average value was 35.38%.

Corroborating the correlation data between the mutational status-anatomical localization with those regarding sex-anatomical localization, the logical hypothesis that was extracted defined the predominance of CDKN2A-wt tumors in male patients and of CDKN2A deletions in women. This supposition was supported by the results obtained previously, that indicated a percentage of 61.53% CDKN2A-wt tumors among male patients and 50.00% of CDKN2A-mut melanomas in female

subjects. Furthermore, the absence of statistical differences of MPM-FM distribution was remarked, indifferent of the mutational characteristics associated with CDKN2A.

Based on the results obtained via the individual analysis of the parameters included in the present study, the objective consisted of creating a score whose progressively superior values could be superposed to those cases with unfavorable prognosis (tumors with CDKN2A mutations), compared to the total inferior values applicable to CDKN2A-wt tumors. Therefore, the percentage of p16-positive cells was stratified according to a digressive scale with 4 classes: expression present in >50% of cells (score 0), in 11-50% of cells (score 1), in 1-10% of cells (score 2) and complete absence of the p16 immunoreaction (score 3). The significance of the CD8 antibody in the peritumoral lymphocytic population was interpreted in conjunction with the evolutive and therapeutic responsivity patterns observed in the research cohort and in previous studies; thus, the limit values considered for classification were: intense CD8 staining of peritumoral TILs >60% (Score 0), moderate intensity, with 20-60% CD8-positive TILs (score 1), reduced intensity <20% (score 2) and the absence of CD8-positive peritumoral TILs (score 3). The quantification of the Ki-67 proliferation index was based on the use of a progressively descendant scale with 5 classes, that determined the neoplastic characterization from less proliferative tumors (Score 0) to the most intense proliferation (score 4): value <2% (score 0), 2-5% (score 1), 6-10% (score 2), 11-20% (score 3) and over 20% (score 4). In this global context, the total value after applying the proposed score per tumor varied between 0 and 10.

The limit of 9 points of the triple score was proved to differentiate melanomas with CDKN2A homozygous deletion from CDKN2A-wt tumors and those with heterozygous deletion. All the 8 cases of cutaneous/mucosal MPM and FM with CDKN2A homozygous deletion corresponded to a total score of ≥9. Moreover, setting the detection limit of melanomas with CDKN2A homozygous deletion to a total minimal value of 9 generates a test sensitivity of 100%, with a specificity of 94.11%.

Thus, the association of the 3 parameters included in the algorithm was performed through the proved utility of p16, CD8 and Ki-67 in cases of cutaneous and mucosal melanoma. The limitations of the personal study involve the reduced number of patients that met the inclusion criteria in the examined time interval. Furthermore, the cut-off values for each of the 3 immunohistochemical markers (p16, CD8, Ki-67) were established based on previous research that aimed to indicate correlations between the immunohistochemical characteristics of cutaneous and mucosal melanomas at diagnosis and their identified patterns of evolution in time; however, the potential confirmation bias should be recognized due to the heterogeneity of the data currently available in the specialty literature.

In order to standardize and augment the utilization of the triple p16-CD8-Ki-67 score in the prediction of the CDKN2A mutational status in individuals with clinical proof of MPM and/or FM, future studies with comprehensive designs, performed on extended groups of patients or the integration of the proposed algorithm in clinical trials are necessary.

8. General conclusions

In conclusion, the distribution of the analyzed group over the 5 calendar years indicated a progressive decline in the number of diagnosed cases (from 2018 to 2022), a fact that can be correlated with the impact of the SARS-CoV-2 pandemic on patient awareness and their perspective on the degree of priority that cutaneous lesions receive. The superficial spreading melanoma

constituted the most common histopathological subtype in the population with sporadic melanoma, as well as in patients diagnosed with MPM and/or FM.

Individuals with multiple primary melanomas presented lower average values in the second primary melanomas compared to those registered in the first primary melanomas. Concerning the predominant dermoscopic pattern of the nevi monitored in patients with MPM and/or FM, the most frequent characteristics included lesions with regular borders, homogeneous structures on the nevus surface, most commonly reticular, with symmetric pigment network and depigmentation.

The doctoral study revealed that the investigation of the p14-p16 immunophenotype in patients with MPM and FM represents a useful and cost-effective method for the detection of the CDKN2A genetic profile in this group. The immunohistochemical absence of p16 has predictive value for the aggressive biological behavior and unfavorable prognosis of MPM and/or FM, indifferent of the histopathological subtype. Melanomas with double mutational load (BRAF and CDKN2A) are more frequently associated with distant metastases, therefore are correlated with elevated aggressivity and fulminant evolution.

Taking into account the current era where digital pathology benefits from rapid advancements, the use of automatized platforms for image scanning becomes more popular in the detriment of naked eye microscopical evaluations. The accuracy and replicability of the results obtained through artificial intelligence techniques are superior to those reported after evaluations performed by human examinators. A higher percentage of intratumoral versus stromal CD8-positive lymphocytes was positively associated with melanomas with multiple metastatic determinations.

Similarly, the ubiquitous access to technology nowadays contributes to the metamorphosis of social media in the ideal environment for the dissemination and organization of future awareness raising campaigns about dermato-oncological diseases. Taking into consideration the reduced proportion of subjects that undergo regular dermatological visits and dermoscopic evaluations, the implementation of widespread educational programs on this topic, with the aim of correctly informing the general population, is necessary.

Eventually, the algorithm composed from immunohistochemical parameters (p16-CD8-Ki-67), selected based on their prognostic and evolutive importance, represents a valuable auxiliary diagnostic instrument in the field of CDKN2A-mutated MPM and FM, as a cost-effective way of predicting the mutational status. By corroborating the significance of the three indicators included in the proposed immunohistochemical score, the results derived from the personal study and the data collected from the specialty literature, exploring the utility of the aforementioned algorithm for the distinction between atypical nevus and melanoma represents a valid future line of research.

9. Elements of thesis originality

- The first study that investigates the presence of CDKN2A mutation in a group of patients from the South-Eastern region of Romania diagnosed with familial and/or multiple primary melanoma via immunohistochemical techniques (p14 and p16 proteins), as well as fluorescent in situ hybridization (FISH).
- The first analysis that evaluates the CD8 reaction in the intra- and peritumoral lymphocytic population, with the aid of artificial intelligence (the QuPath software) in a concomitant and comparative manner with the classical evaluation performed by pathologists with a “naked eye”, in the context of familial and/or multiple primary melanoma cases diagnosed in the Dobruja region.

- The first research that proposes the use of a standardized classification system that exclusively employs common immunohistochemical antibodies (p16, CD8 and Ki-67) for the distinction between MPM and FM with homozygous CDKN2A deletion and those without CDKN2A mutations or with heterozygous deletion of this gene.
- The additional data, centered on the cutaneous and mucosal melanoma identified in the Romanian population, derived from the personal doctoral research, completes the existent knowledge presented in the specialty literature and gains particular utility in the epidemiological, clinical, histopathological and immunophenotypic characterization of this nosological entity.
- The results generated by the analysis conducted in the present paper may constitute the starting point for ulterior lines of research in the field of biomarkers associated with familial and multiple primary melanomas or for the development of CDKN2A targeted therapies.

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