

CDKN2A and MC1R variants influence dermoscopic and confocal features of benign melanocytic lesions in multiple melanoma patients

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Abstract: Non-invasive diagnostic tools are effective in the histomorphological study of melanocytic lesions. The role of melanoma susceptibility genes on melanocytic nevi histopathological features is not clear. The current study aimed to correlate genetic alterations and histomorphological features of melanocytic nevi. Clinical, dermoscopic and confocal features of 34 multiple melanoma patients and 34 controls were compared. Among patients with melanoma, carriers of CDKN2A mutations and/or MC1R variants, and wild-type genes were also compared. In patients with melanoma, a lighter phototype ($P = 0.051$), a higher number of nevi ($P < 0.01$) and clinically atypical nevi ($P < 0.01$) were observed. At dermoscopy, these nevi showed a complex pattern ($P = 0.011$), atypical network ($P = 0.018$) and irregular pigmentation ($P = 0.037$); at confocal, an irregular meshwork pattern ($P = 0.026$) with atypical nests ($P = 0.016$) and an inflammatory infiltrate ($P = 0.048$) were observed. Among patients with melanoma genetically tested, CDKN2A G101W mutation carriers were more frequently younger ($P = 0.023$), with

clinically atypical nevi ($P = 0.050$), with cytological atypia ($P = 0.033$) at confocal. G101W mutation and MC1R variants carriers showed hypopigmented nevi ($P = 0.002$) and, at confocal, roundish cells infiltrating the junction ($P = 0.019$). These data suggest an influence of CDKN2A mutation and MC1R variants in the development of dysplastic melanocytic lesions. Non-invasive histomorphological evaluation, together with genetic studies, improves melanoma risk identification and early diagnosis, for a patient-tailored management.

Abbreviations: CDKN2A, cyclin-dependent kinase inhibitor 2A; CKD4, cyclin-dependent kinase; CTR, control; DMO, Dermatology Dept, Modena; INT, Fondazione IRCCS Istituto Nazionale dei Tumori, Milan; MC1R, melanocortin 1 receptor; MMP, multiple melanoma patients.

Key words: CDKN2A – confocal microscopy – dermoscopy – MC1R – melanocytic nevi – melanoma

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Introduction

The knowledge regarding the biological basis of melanoma and melanocytic lesion development is continually improving. Previous genetic studies have identified high-risk melanoma susceptibility genes, highlighting cyclin-dependent kinase inhibitor 2A (CDKN2A) and cyclin-dependent kinase 4 (CDK4). Casual mutations in these genes cosegregate with melanoma and are inherited in a dominant pattern (1). Additionally, the melanocortin 1 receptor (MC1R) gene (16q24), classified as a low-risk melanoma susceptibility gene, has been proven to be a risk factor for melanoma in families segregating CDKN2A mutations (2–5). Nevertheless, the role of these genes in the development of multiple melanomas and their influence on the histopathological features of melanocytic nevi (e.g. ‘dysplastic’ nevi) are not clear.

By means of instrumental tools commonly used for non-invasive early diagnosis, morphological studies of benign melanocytic lesions can now be performed. Dermoscopy enhances features invisible to the naked eye (6); furthermore, confocal microscopy allows an *in vivo* vision of the cutaneous structures, approaching histological resolution (7). As melanin is a strong source of contrast for confocal, this technology became useful in melanocytic

lesions examination (8–11). The integrated use of dermoscopy and confocal microscopy was demonstrated to be particularly useful in diagnosing equivocal melanocytic lesions lacking frankly malignant features (12–14). An *in vivo*, non-invasive evaluation of melanocytic nevi morphological features in high-risk patients could improve the understanding of the biological behaviour of atypical nevi and identify the risk of melanoma correlated with histomorphological substrates currently detectable by invasive procedures only.

This prospectively designed, hospital-based study aimed to identify histomorphological features of benign melanocytic nevi in multiple melanoma patients (MMP) compared with a control population (CTR) without a history of melanoma. Additionally, genetic studies of MMP were performed to identify mutations and polymorphisms or wild-type gene carriers. The histomorphological features and genetic data were correlated for statistical significance.

Materials and methods

This prospective, hospital-based case–control study was performed from February 2010 to October 2011. Two independent departments were involved: the Melanoma and Sarcoma Unit of General

Oncologic Surgery at the Fondazione IRCCS Istituto Nazionale dei Tumori (INT) in Milan, and the Department of Dermatology at Policlinico of Modena, University of Modena and Reggio Emilia (DMo). The study was approved by the local Ethical Committee, 4/09, Prot n. 1338/CE, 29 April 2009. Written informed consent was obtained from each patient and control. The study was conducted according to the Helsinki Declaration.

Inclusion criteria: 'Cases' included patients undergoing periodical visits at INT with a history of at least two melanomas (MMP), and 'controls' included individuals without a personal or family history of melanoma (CTR) who were age-matched (± 5 years) with the cases.

Following preliminary manual dermoscopy examination, patient consent was acquired for genetic testing, and blood samples were taken at INT. Clinical, digital dermoscopic and confocal examinations were then performed at DMo.

For this analysis, data collected from a total number of 68 subjects were considered, including 34 MMP and 34 CTR. A total of 120 melanocytic nevi were acquired. For the genetic study, 27 of 34 MMP were included, and a comparison of carriers of different germ-line mutations was run as explained below.

Clinical data were collected and analysed for each patient and control. Clinical data included age and sex, age at first melanoma diagnosis, total number of nevi (<50 , $50-100$, ≥ 100), number of clinically atypical nevi (0, 1–4, or ≥ 5) (15) on the body surface, phototype (I–II or III), signs of sun damage (solar lentiginos) and personal and/or familial history of melanoma. The relative risk was evaluated for each patient according to the algorithm by Garbe et al. (1994). Each patient was screened by total body manual dermoscopy at INT, and typical nevi (with a patient-specific 'nevus-type') and, when present, dermoscopically atypical nevi were identified for further examination and/or excision. Manual dermoscopy was performed using the polarized dermatoscope Heine Delta 20[®] (Optotechnik GmbH & Co., Herrsching, Germany). Digital total body dermoscopy was performed in DMo for each patient, recording the lesions previously indicated by INT; a maximum of 5 lesions/patient were selected for confocal study. Frankly, congenital nevi, >10 mm and present from birth, were excluded from the study.

Digital dermoscopy was performed using the polarized dermatoscope DermLite photo 3gen[®] (3gen LLC, San Juan Capistrano, CA, USA), on a photcamera Canon Power Shot G10, 14,7 MegaPixels.

Confocal examinations were performed by Vivascope 1500[®] (Lucid Inc., Henrietta, NY, USA). The collection of images included a minimum of three mosaics on a horizontal plane (VivaBlock[®] modality) covering an area $\geq 6 \times 6$ mm², acquired in the spinous-granular layer, the dermal–epidermal junction and the upper dermis. Furthermore, several sequences of high-resolution images (0.5×0.5 mm²) from the surface up to the dermis with a step increment of 5 μ m in depth (VivaStack[®]) were acquired in the region of interest showing the most important and diagnostic features.

A specific database was developed for clinical, dermoscopic and confocal information. For each recorded nevus, clinical, dermoscopic and confocal data were recorded. Clinical data included size (≤ 5 mm, >5 mm of diameter), anatomical location, palpability and 'Ugly Duckling' sign (16). Dermoscopic evaluation of melanocytic nevi was based on the pattern analysis (17). Each lesion was

classified according to the main dermoscopic pattern (reticular, globular, homogeneous, starburst, complex and multicomponent). Further, according to the 7-point checklist by Argenziano, network (absent, typical, atypical), veil (absent, present), vessels (absent, typical, atypical), pigmentation (absent, regular, irregular), dots/globules (absent, regular, irregular), streaks (absent, regular, irregular) and regression (absent, present) were noted for the calculation of the total 7-point score (18). Additionally, an overall qualitative evaluation of eventual dermoscopic atypia was performed, identifying the presence of an atypical network and/or dishomogeneous pigmentation and/or areas of regression and/or blue-whitish veil.

For confocal features, epidermal pattern (cobblestone, honeycomb, disarranged, aspecific), junctional pattern (architecture: prevalent ringed, meshwork or clod pattern, eventually mixed; papillary contours: edged/non-edged; junctional nests: homogeneous/dishomogeneous) (19), dermal features (nests, inflammatory infiltrate and coarse collagen fibres) (20) and presence, aspect (roundish or dendritic) and number (<5 , $5-10$, >10 per mm²) of atypical cells in any of the examined layers, were evaluated (21). These features were the basis for the calculation of the confocal microscopy total score (10). A cytologic-architectural pattern was considered atypical when the confocal microscopy score was ≥ 3 , based on the presence of major or minor criteria of malignancy.

For excised lesions, the histopathological report was retrieved, and the diagnosis of benign or malignant was inserted in the database. Lesions which were not immediately excised, were controlled during the follow-up at 3–6 months and 8–12 months to confirm lesion stability by dermoscopy and confocal microscopy. Lesions with clinical, dermoscopic or confocal changes were excised and were histologically examined.

Genetic study

The presence of genetic mutations in melanoma susceptibility genes was tested at INT on 27 MMP (approval from the Institutional Ethical Committee in the 'Study of germ line and somatic genetic variants in MMP', January 26 2010); 7 patients did not give their consent for genetic testing. Genomic DNA was isolated from peripheral blood by the QIAamp DNA Blood Mini Kit (QIAGEN, Crawley, West Sussex, UK) and utilized for germ-line analysis of the CDKN2A, CDK4 (exons 2-5-8) and MC1R (exon 1) genes. Gene amplification was performed as described elsewhere (22). The polymerase chain reaction (PCR) products were directly sequenced using the automated 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA), and sequences were analysed with Sequencing Analysis and Vector NTI Suite V.7 (Informax, Bethesda, MD, USA). MC1R variants were categorized as 'R' (red hair variants) or 'r' (non-red hair variants) types according to the reported effect on the phototype of the carrier (23).

Statistics

Statistical evaluation was performed using SPSS 12.0[®] (SPSS, Chicago, IL, USA). Frequencies of the clinical features patients were compared between MMP and CTR. Dermoscopic and confocal parameters of all the recorded melanocytic nevi were considered comparing lesions belonging to MMP versus the ones belonging to CTR. Moreover, within the MMP group that underwent genetical analysis, patient clinical aspects and dermoscopic-confocal lesion features in MMP with genetic alterations were compared with the ones in MMP carriers of wild-type genes. The

statistical analysis was performed only on benign melanocytic lesions, histologically ascertained or stable at a 1-year follow-up, to avoid an evaluation bias of the dermoscopic and histomorphological features due to the malignant nature of the four melanomas found during the study.

Pearson's chi-square test of independence has been used to compare differences between the different subgroups (Fisher's exact test was applied if any expected cell value in the 2×2 table was <5). Statistical significance was considered present with a P value ≤ 0.05 .

Binary logistic regression was calculated to identify dermoscopic and confocal parameters independently significant with respect of total number of nevi and number of atypical nevi.

Results

Patients

This study enrolled 68 patients: 34 MMP and 34 non-melanoma patients (CTR). A total of 124 lesions were examined by dermoscopy and confocal microscopy: 81 lesions in MMP and 43 lesions in CTR. Four melanomas were identified during the study on 3 MMP. Additionally, 120 benign melanocytic nevi were acquired, 15 of which were histologically ascertained; 11 immediate excisions and four within the short-term follow-up (3–6 months). Stability of dermoscopic and confocal features for the remaining nevi ($n = 105$) was confirmed during the long-term follow-up (8–12 months).

Genetic tests

Germ-line mutation research was performed on 27 MMP (79.4%), to identify mutations of CDKN2A and CDK4, and variants of MC1R, which have an established role in the onset of multiple melanomas (23,24). CDKN2A mutations were identified in 11/27 MMP (40.7%). The most frequently observed CDKN2A mutation was the G101W missense mutation (nine cases, 81.8%). Additionally, the R24P and G150V mutations were detected in one case each. CDK4 mutations were not found (Table S1). MC1R variants were detected in 19 patients (70.3%), 9 being carriers of mutant CDKN2A (81%), while wild-type MC1R was shown in two carriers of mutant CDKN2A (18%) and in six non-carriers (37.5%) (Table 1). Genotypes 'R/R', 'R/r', 'r/r', 'R/wt' and 'r/wt' were shown in 1, 5, 0 and 3 patients, respectively, in carriers of mutant CDKN2A, and in 1, 2, 1 and 6 in non-carriers. Carriers of two MC1R variants were six in patients with mutant CDKN2A (55%), while 3 (45%) carried a single variant only. At least one MC1R variant was observed in 7/9 (77.7%)

Table 1. CDKN2A and MC1R genotypes in the study population

Genotype	MC1R	MMP	
		n	%
WT	Total	16	100
	WT	6	37.5
	Any variant	10	62.5
	1 Variant	6	37.5
	2 Variants	4	25
MUT	Total	11	100
	WT	2	18
	Any variant	9	82
	1 Variant	3	27
	2 Variants	6	55

MUT, mutated gene; WT, wild-type gene; MMP, multiple melanoma patients.

G101W mutation carriers. The most commonly identified MC1R variants were V60L (seven cases) and R160W (six cases), which were paired in four cases. The R151C (five cases), D294H, I155T and V92M (two cases each), D84E, R163Q, R160Q and Y152X (one case each) were also identified (Table S1). Clinical, dermoscopic and confocal features concerning 62 melanocytic nevi belonging to the 27 MMP genetically tested were considered. Data were calculated comparing the distribution of these features in nevi of wild-type gene carriers to the ones of G101W mutation carriers, of any MC1R variant, or both G101W mutation and MC1R variant.

Clinical, dermoscopic and histomorphological features in MMP versus CTR

Data calculated on patients

Multiple Melanoma Patients compared with CTR were clinically characterized by a lighter phototype ($P = 0.051$), a higher number of nevi ($P < 0.001$), and a higher number of clinically atypical nevi ($P < 0.001$) (15). No statistically significant differences were noticed for solar lentigines and photodamage. A high relative risk phenotype for melanoma, as outlined by Garbe et al. (25), was present in 62% of MMP ($P < 0.001$).

Data calculated on nevi

Dermoscopic features observed on the melanocytic nevi of MMP more often included a complex/multicomponent pattern compared with a single pattern ($P = 0.011$). An atypical network ($P = 0.018$) and an irregular structureless pigmentation ($P = 0.037$) were prevalent in MMP compared with CTR. Globally, a dermoscopic score suggestive of atypia (new 7-point checklist score ≥ 3) was more often calculated on the lesions of MMP compared with CTR ($P = 0.001$).

Table 2. Main clinical, dermoscopic and confocal findings in benign melanocytic nevi of MMP versus CTR population ($N = 120$)

Data collected on patients	CTR ($N = 34$)	MMP ($N = 34$)	P value ¹
Clinical features			
Phototype I–II (%)	15 (44.1%)	23 (67.6%)	0.051
Phototype III (%)	19 (55.9%)	11 (32.4%)	
>100 nevi	4 (12.1%)	25 (73.5%)	$<0.001^2$
>5 clinically atypical nevi	0	12 (35.3%)	$<0.001^2$
High relative risk ³	1 (3.1%)	21 (61.8%)	$<0.001^2$

Data collected on nevi	Nevi in CTR ($N = 43$)	Nevi in MMP ($N = 77$)	P value ¹
Dermoscopic features			
Single pattern ^{4,5}	32 (74.4)	39 (50.6)	0.011 ²
Complex/multicomponent pattern ^{4,5}	11 (25.6)	38 (49.4)	0.011 ²
Atypical network ^{4,5}	3 (7%)	22 (28.9%)	0.018 ²
Irregular pigmentation ^{4,5}	1 (2.3%)	14 (18.4%)	0.037 ²
Score ≥ 3	0 (0%)	15 (19.5%)	0.001 ²
Confocal features			
Junctional meshwork ^{4,5}	12 (27.9%)	41 (53.2%)	0.026 ²
Papillary contour Non-edged ⁵	11 (25.6%)	40 (52%)	0.018 ²
Dishomogeneous junctional nests ⁴	1 (2.3%)	16 (20.8%)	0.016 ²
Plump bright cells/small bright particles in papillae ^{4,5}	10 (23.3%)	32 (41.6%)	0.048 ²
Coarse collagen fibres ⁴	1 (2.3%)	15 (19.5%)	0.010 ²
RCM atypia ^{4,5}	2 (4.7%)	16 (20.8%)	0.052

CTR, control; MMP, multiple melanoma patients.

¹ P value of Pearson's chi-square/Fisher's exact test.

²Statistical significance ($P < 0.05$).

³Garbe et al., 1994.

⁴Independently significant for '>100 nevi' category.

⁵Independently significant for '>5 clinically atypical nevi' category.

From a histomorphological point of view, as observed at confocal microscopy, MMP melanocytic lesions featured a significantly higher percentage of junctional nests organized into a meshwork pattern ($P = 0.026$), most frequently displaying dishomogeneous junctional nests ($P = 0.016$), and non-edged papillary contour ($P = 0.018$). In the upper dermis, the presence of inflammatory infiltrate ($P = 0.048$) and coarse collagen fibres ($P = 0.010$) was observed in a significantly higher percentage of lesions in MMP compared with CTR (Table 2).

Clinical, dermoscopic and confocal features in benign melanocytic lesions in MMP carriers of genetic alterations compared with wild-type gene carriers

Data calculated on patients

Considering clinical features, MMP with G101W mutation, compared with wild-type gene carriers, were significantly more often male patients ($P = 0.019$), younger than 30 years old at first melanoma onset ($P = 0.023$). MMP with at least 1 MC1R variant were more often younger than 40 years old at the first melanoma diagnosis ($P = 0.025$), with heavy sun damage ($P = 0.027$) compared with wild-type gene carriers. The carriers of both G101W mutation and MC1R variants were younger at the first diagnosis with respect to wild-type gene MMP ($P = 0.050$, borderline). On the other hand, there was no statistical significance in the distribution of the total number of nevi, atypical nevi and previously diagnosed melanomas among the carriers of genetic mutations compared with wild-type MMP. No differences were found among the carriers of one or two MC1R allelic variants, or of 'r' compared with 'R' variants.

Data calculated on nevi

Sixty-two benign nevi were evaluated in MMP undergoing genetic testing. In nevi of G101W mutation carriers, a clinical 'Ugly Duckling sign' was prevalent ($P = 0.050$). In dermoscopy, the presence of vessels was significantly more frequent ($P = 0.027$); under confocal, roundish atypical cells at the dermal–epidermal junction were significantly more frequently observed compared with the wild-type gene population ($P = 0.033$).

In benign nevi of MC1R variants carriers, atypia ($P = 0.023$) and plump bright cells within the upper dermis ($P = 0.041$) were significantly prevalent, as observed by dermoscopy and confocal, respectively. When both G101W mutation and at least 1 MC1R variant were present, vessels were more frequent observed ($P = 0.013$), as was the absence of pigmentation in dermoscopy ($P = 0.002$). Additionally, at confocal examination, roundish cells were prevalent at the dermal–epidermal junction ($P = 0.019$). The infiltration of atypical cells within the upper dermis was also prevalent, although not statistically significant ($P = 0.056$) (Table 3, Fig. 1). Of the three patients with new melanomas, 2 (GM-64 and GM-104) were carriers of both G101W mutation and MC1R variants, and the third was a carrier of a wild-type CDKN2A gene, with 1 MC1R variant (GM-94).

Discussion

The mechanisms by which genetic alterations influence the onset of cutaneous malignancies and the pattern of melanocytic nevi in MMP are not fully understood. The role of CDKN2A and MC1R mutations has been widely studied in different populations (26–28). The correlation between multiple nevi/atypical nevi phenotype and melanoma risk is well known, and in some instances is also correlated with germinal mutations (4,29,30). Recent

Table 3. Dermoscopic and confocal features of benign nevi: the P value expresses statistical significance in the distribution of dermoscopic and confocal features among the carriers of genetic alterations and the wild-type genes carriers (N of patients = 27; N of nevi = 62)

	P value ¹		
	G101W mutation	MC1R variant	CDKN2A mutation +MC1R variant
Data collected on patients ($N = 27$)	$N = 9$	$N = 19$	$N = 9$
Clinical features			
Male sex	0.019 ²	0.398	0.091
Age <30	0.023 ²	1.000	0.050
Age <40	0.406	0.025 ²	0.204
Sign of sun damage (solar lentigines)	0.301	0.027 ²	0.703
Data collected on nevi ($N = 62$)	$N = 19$	$N = 47$	$N = 18$
Clinical features			
Clinical ugly duckling		0.050	0.485
Dermoscopic features			
Vessels		0.027 ²	0.560
Lack of pigmentation		0.060	0.271
Dermoscopic atypia		0.582	0.023 ²
Confocal features			
Roundish cells at the DEJ		0.033 ²	0.445
Plump bright cells/small bright particles in papillae		0.784	0.041 ²
Atypical cells infiltrating dermal papillae		0.90	1.000
		0.056	

¹ P value of Pearson's chi-square/Fisher's exact test.

²Statistical significance ($P < 0.05$).

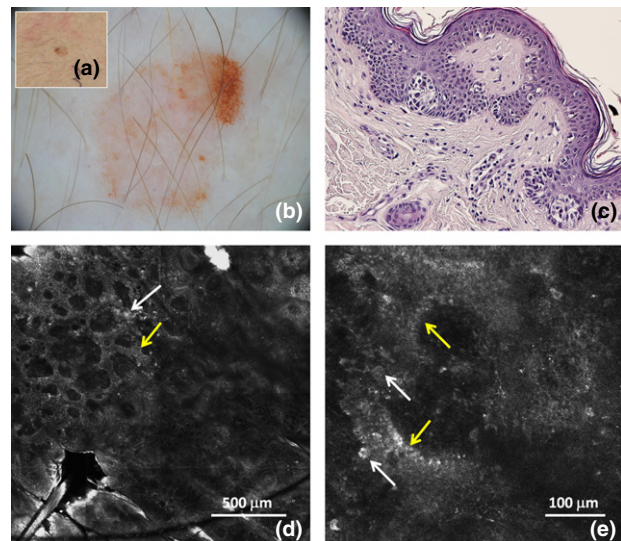


Figure 1. Clinical, dermoscopic and confocal features of a benign melanocytic nevus identified on Multiple Melanoma Patients with G101W mutation and 2 MC1R variants (R151C, D294H—ID: GM75). (a) A flat pigmented lesion on the abdomen of a 36 y.o. man. (b) Dermoscopic features: light diffuse pigmentation and peripheral 'dermoscopic island' with a thickened network. (c) Histopathological detail of the peripheral portion of a lesion (H&E, 40-fold magnified): junctional nests at the peak of the rete ridges, and some cells infiltrating the dermal–epidermal junction. (d) Confocal examination on the peripheral area (2×2 mm) of the lesion shows an irregular meshwork pattern with bright roundish cells infiltrating the dermal–epidermal junction. (e) Confocal detail (0.5×0.5 mm) showing non-edged papillae (yellow arrows) with roundish cells infiltrating the papillary wall (white arrows).

publications which have aimed to describe the correlations between dermoscopic and confocal aspects of melanoma and genetic signature, mainly describe small, hypopigmented lesions,

which are difficult to diagnose in germinal mutation carriers (31,32). Some data exist regarding clinical/dermoscopic aspects of nevi correlated with genetic alterations. Zalaudek highlighted a prevalence of globular pattern (correlating with compound or dermal nevi in histology) in B-RAF-mutated lesions (33). Moreover, MC1R variants carriers tend to develop hypopigmented melanocytic lesions, defined as 'white nevi' and 'red melanomas' (34). In a recent study, authors showed that MC1R variants would play a role in MM development in CDKN2A negative Italian patients, both via pigmentary and non-pigmentary pathways (35).

However, correlation between genetics and histopathological aspects of nevi is limited in clinical practice, because biopsy is only feasible in a few selected lesions, mainly excised for medical reasons (36). Nowadays, confocal microscopy opens a window to *in vivo* examination of living tissue, approaching a histopathological resolution (37). Its diagnostic value is confirmed in this study by the identification of four melanomas in three MMP which were considered clinically negative and difficult to diagnose at dermoscopy. Moreover, confocal microscopy consents the collection of histopathology-correlated information on numerous lesions in the same patient without bioptic procedures, resulting in an improved understanding of the nevus phenotype. Recently, characteristic confocal features have been observed in melanomas in patients with a high Bak serum level, highlighting a possible *in vivo* correlation between biomolecular findings and tumor morphology (38).

The current study aimed to obtain an in-depth characterization analysis of a significant number of benign melanocytic lesions in a population with a proven high susceptibility to melanoma development, compared with the control population.

Previously published dermoscopic findings have outlined a higher frequency of complex pattern (a mix of at least two patterns) in MMP (39). The current study echoes these findings, reporting the prevalence of irregular pigmentation and atypical network in MMP nevi.

Moreover, this study has identified confocal features characteristic of MMP compared with CTR. The meshwork pattern was noted significantly more often in MMP nevi with respect to CTR. This meshwork pattern was characterized by irregular nests and irregular papillary architecture, atypical cells at the dermal-epidermal junction, along with dermal fibrosis and flogosis.

Interestingly, there were no differences between MMP and CTR nevi regarding the ringed and clod pattern distribution, histopathologically corresponding to a junctional/lentiginous proliferation and large dense dermal nests, respectively.

These findings partially support the theory of 'dual origin' of nevi (40,41), affirming that globular nevi (corresponding to the confocal clod pattern and to the histopathological entity of the intradermal nevus) originate from embryogenetic development, whereas reticular nevi (correlated to ringed pattern upon confocal, and junctional lentiginous nevus in histopathology) are correlated to sun exposure. However, this theory does not completely explain the origin and development of junctional/compound nevi, characterized by a predominant melanocytic nests component. In fact, these lesions may appear in the form of globular nevi and then progress to reticular, after an intermediate phase where they show a peripheral rim of globules (40,42,43). Confocal microscopy permitted the identification of different histopathological substrates and the documentation of the globular-network transition in this

subgroup (11). These lesions, regardless of their variable dermoscopic pattern, are characterized by a meshwork pattern at confocal examination, corresponding to a predominant junctional nests proliferation. The data from the current study seems to indicate that MMP develop globular clod mainly intradermal nevi (hypothetically generated during embryogenesis) and reticular-ringed lentiginous nevi (hypothetically related to sun exposure) in the same way as CTR patients (no statistical difference). This finding supports the independence of globular and reticular nevi from germ-line mutation on high-susceptibility melanoma genes. On the other hand, a high number of complex-meshwork-compound/dysplastic nevi has been proven to be related to high melanoma risk (39). The current study not only confirms this data, but suggests that these nevi may also be associated with specific genetic alterations.

In a recent publication regarding the development of neurocutaneous units, the authors suggest that the histological bridging of junctional nests could be the expression of a melanocytic hyperplasia that the epidermal homeostatic feedback failed to block (44). According to the authors, this nevus-type could be considered 'dysplastic' only displaying cytological atypia (44). The *in vivo* results from the current study support these hypotheses. Multiple melanoma patients, and particularly those with G101W and MC1R mutations, displayed a significantly higher frequency of lesions characterized by an irregular meshwork pattern, cytological atypia and stroma reaction, which may delineate the histopathological entity of the 'dysplastic nevus' (45). Thus, the current authors hypothesize that melanoma prone patients, especially those with genetic mutations outlined in this study, may also carry a vulnerable genotype, which may in turn provoke junctional melanocytic hyperplasia and a consequent stroma reaction, responsible for numerous lesions, frequently with clinically atypical and histopathological 'dysplastic' features, according to the original Clark definition (29).

A non-invasive histopathological evaluation of unlimited melanocytic lesions on complex patients without performing unnecessary biopsies together with the identification of genetic alterations represents a step forward in the identification of melanoma risk factors, and early tumor diagnosis, according to a patient-tailored management protocol.

The study was limited by a maximum exploration of ≤ 5 lesions/patient due to the time consuming analytical procedure (approximately 5–10 mins/lesion) required by the commercially available confocal microscope Vivascope[®] 1500, utilized in this study. However, the development of a new hand-held tool (Vivascope[®] 3000) seems to promise a more rapid lesion exploration, which should facilitate the examination of many more patient lesions within the same examination time frame. The ability of confocal microscopy to identify both patients 'at risk' and mutation carriers, based on nevi histomorphological features, from the results of this study, seems promising. However, despite achieving statistical significant data, the study is limited by the small sample size, and further studies on large patient series are needed to confirm these findings.

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Author contributions

Dr. Bassoli, Dr. Maurichi, Dr. Casari, Dr. Pupelli and Dr. Farnetani performed the research on clinical, dermoscopic, confocal aspects on patients and controls. Dr. Rodolfo and Dr. Frigerio performed the research on genetic features of melanoma patients. Prof. Pellacani, Dr. Bassoli and Dr. Maurichi designed the research study. Prof. Santinami, Dr Rodolfo, Dr Frigerio, Prof. Pelosi contributed essential reagents or tools,

histopathological diagnosis and pictures. Dr. Bassoli, Dr. Rodolfo, Dr. Frigerio and Prof. Pellacani analysed the data. Dr. Bassoli and Prof. Pellacani wrote the article.

Conflict of interests

Professor Giovanni Pellacani held confocal training courses, granted by Lucid Inc. and Mavig Inc., in the past years. None of the authors has conflict of interest to declare in the specifically treated topics.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. CDKN2A and MC1R distribution in the study population.