In Vivo Confocal Microscopy for Diagnosis of Melanoma and Basal Cell Carcinoma Using a Two-Step Method: Analysis of 710 Consecutive Clinically Equivocal Cases

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We describe two algorithms to diagnose basal cell carcinomas (BCCs) and melanomas (MMs) using *in vivo* reflectance confocal microscopy (RCM). A total of 710 consecutive cutaneous lesions excised to exclude malignancy (216 MMs, 266 nevi, 119 BCCs, 67 pigmented facial macules, and 42 other skin tumors) were imaged by RCM. RCM features were correlated with pathology diagnosis to develop diagnostic algorithms. The diagnostic accuracy of the BCC algorithm defined on multivariate analysis of the training set (50%) and tested on the remaining cases was 100% sensitivity, 88.5% specificity. Positive features were polarized elongated features, telangiectasia and convoluted vessels, basaloid nodules, and epidermal shadowing corresponding to horizontal clefting. Negative features were non-visible papillae, disarrangement of the epidermal layer, and cerebriform nests. Multivariate discriminant analysis on the training set (excluding the BCCs) identified seven independently significant features for MM diagnosis. The diagnostic accuracy of the MM algorithm on the test set was 87.6% sensitivity, 70.8% specificity. The four invasive MMs that were misdiagnosed by RCM were all of nevoid subtype. RCM is a highly accurate non-invasive technique for BCC diagnosis. Good diagnostic accuracy was achieved also for MM diagnosis, although rare variants of melanocytic tumors may limit the strict application of the algorithm.

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INTRODUCTION

Cutaneous skin tumors are probably the most common tumors in mankind and represent a major public health problem, particularly in the Western countries. More accurate clinical diagnosis of skin tumors is likely to improve patient management, reduce morbidity and mortality, and generate considerable economic benefits. Recently, dermoscopy has improved the accuracy of the clinical diagnosis of skin tumors and has been shown to reduce the benign/malignant ratio of excised tumors. Nevertheless, misdiagnosis still occurs.

In vivo reflectance confocal microscopy (RCM) allows the visualization of the upper layers of the skin at cellular resolution. Studies investigating the role of RCM in the clinical diagnosis of melanocytic tumors have shown that the identification of specific RCM features can improve the accuracy of diagnosis (Pellacani *et al.* 2005, 2007; Langley *et al.*, 2007; Guitera *et al.*, 2009). A model for the diagnosis of basal cell carcinoma (BCC) with RCM was described in 2004, but was based on the RCM features of an early version of the microscope, which was not in current widespread use (Nori *et al.*, 2004). Another study defined a two-step method for diagnosis, whereby at first, melanocytic lesions were distinguished from non-melanocytic lesions, and second, criteria were then applied to differentiate melanomas (MMs) from nevi. The authors described RCM features of BCCs, based on the analysis of only 26 cases. Moreover, typical clinical cases of BCCs were not excised, and hence, the diagnosis of each case was not confirmed pathologically (Segura *et al.*, 2009).

In this study, we analyzed the RCM characteristics of a large series of melanocytic and non-melanocytic lesions from patients

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Abbreviations: AUC, area under the curve; BCC, basal cell carcinoma; CI, confidence interval; MM, melanoma; OR, odds ratio; RCM, reflectance confocal microscopy; SCC, squamous cell carcinoma

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treated at two specialized skin cancer clinics, to define a model for accurately diagnosing BCCs and MMs, and to compare it with previously published methods of confocal diagnosis. To the best of our knowledge, this represents the largest such series reported to date. Moreover, we analyzed a series of consecutive cases, which were excised because their diagnosis, from their clinical and/or dermoscopic features, was uncertain. First, we characterized the features of BCCs, because, based on our confocal and pathology experience, the diagnosis of BCCs is more straightforward than for melanocytic lesions.

RESULTS

The study population comprised 663 patients (309 females and 354 males, median age of 53 years, interquartile range 39–66, minimum 6 and maximum 90). The RCM features were correlated with the histopathological diagnosis of the lesions (Table 1). The study cohort comprised the following tumors:

- 216 MMs
- 266 nevi
- 119 BCCs
- 67 benign macules of the face, comprising mostly solar lentigines on heavily sun-damaged skin;
- 33 actinic keratoses, Bowen disease, and squamous cell carcinomas (SCC)
- 9 dermatofibroma

The frequencies of 47 features recorded for each diagnostic category are reported in Table 1.

I. BCC features and algorithm

A total of 35 RCM features showed significant correlation with the diagnosis of BCC by histopathology on univariate analysis, compared with all non-BCCs (Table 2). In all, 19 were negative features (odds ratio (OR) <1) and 26 were positive features (OR >1).

Multivariate discriminant analysis was performed for the identification of the independently significant features distinguishing between BCCs and the remaining lesions on the training set, randomly chosen as 50% of the entire series. Eight independently significant features were found (Table 3 and Supplementary Figures S1–4 online). The accuracy of the model having these eight coefficients on the training set was: 97.1% sensitivity (66/68 BCCs), 93.4% specificity (267/269 other lesions); then on the test set: 100% sensitivity (52/52 BCCs), 88.5% specificity, and area under the curve (AUC) $=0.998$ (95% confidence interval (CI): 0.979–0.997).

Interestingly, 37% (3/8) of actinic keratoses and 33% (3/9) of SCCs were deemed to be positive for the BCC algorithm in the test set. Moreover, 11% (12/105) of MMs were also classified as BCCs with this algorithm on the test set. MMs were more frequently diagnosed as BCCs when they were invasive. Positive MMs (10/12) also had obvious MM features and were diagnosed as such with the MM method described below.

II. MM features and algorithm

A total of 35 RCM features showed significant correlation with the MM diagnosis by histopathology on univariate analysis (Table 4). In all, 14 were negative features (OR <1) and 19 were positive features (OR >1). Interestingly, the highest OR was for atypical cells (defined as bright nucleated cells more than twice the keratinocytes around) found at the dermo-epidermal junction (OR $=13.9$) and for pagetoid cells (OR $=11$).

Univariate analysis of MMs of more than 1 mm thickness compared with MMs less than 1 mm showed 14 parameters significantly correlated with thick MMs ($P<0.05$). Cerebriform nests (OR $=8.3$) and convoluted ''glomerular''-like vessels (OR $=7$) were the most important features. Linear telangiectasia-like horizontal vessels, also described in BCCs, were characteristic of thick MMs. A broadened honeycomb pattern was the characteristic of thick MMs.

Multivariate discriminant analysis, based on the training set excluding the BCCs, identified seven independently significant features for the diagnosis of MMs (Table 3). In order of importance, these features were: cerebriform nests, atypical cobblestone pattern with small nucleated cells in the epidermis, marked cytological atypia, and pagetoid cells, and disarranged epidermal layer with no honey comb recognized in some areas were associated with the MM diagnosis. Large inter-papillae spaces filled with honeycomb or cobblestone aspect was negatively associated with MMs.

The accuracy of the MM algorithm (7 features based on multivariate analysis above) on the training set was: 94.5% sensitivity (104/110 MMs), 73.9% specificity (130/176 others without BCCs). On the test set, the accuracy of the algorithm was: 87.6% sensitivity (92/105 MMs), 70.8% specificity (226/319 others without BCCs), AUC $=0.854$ (95% CI: 0.810–0.899). Of the 13 false-negative MMs in the test set, 7 were pigmented, with 4 of these having some dermoscopy specific features of MMs, 1 was partially pigmented and showed dermoscopy-specific features of MMs, 2 were lightly colored with no specific features, and 3 were amelanotic with no specific features. Pathology review showed that three were ''early-stage'' lentigo maligna, three were classic lentigo maligna, two were *in situ* MMs of superficial spreading type associated with nevi, three were thin (<1.0 mm Breslow thickness) superficial spreading MMs (associated with nevi in two cases), and one was a superficial spreading MM associated with nevus (2 mm Breslow thickness). Review of the pathology slides of the four false-negative invasive MMs revealed that each was a subtle ''nevoid'' MM. Nevoid MMs are notoriously difficult to diagnose clinically and pathologically, and the interobserver reproducibility of pathological diagnosis of them is poor.

Unsurprisingly, given their pathological resemblance to MMs, Spitz nevi were a major pitfall for the MM algorithm, with 56% (9/16) classified as MM as previously described for another RCM score (Pellacani *et al.*, 2007).

III. Overall results of the BCC and MM algorithms on a consecutive series of suspicious lesions

The overall analysis (Table 5) on the test set of the two-step model of first diagnosing BCCs and then MMs showed that this two-step method had:

- 89.5% sensitivity for the diagnosis of MMs (94/105) with 92 MMs classified as such, and 2 as BCCs

Table 1. RCM features correlated with the diagnosis by histopathology

RCM features from epidermis to dermis (%)	MIS *N*=113	MM<1 *N*=78	MM>1 *N*=25	Macules of the face[1] *N*=67	Nev Junct *N*=67	Nev Comp *N*=156	Nev Intrad *N*=11	Nev Blue *N*=6	Nev Spitz *N*=26	BCC *N*=119	AK *N*=19	SCC *N*=14	DFibroma *N*=9
Corneal cyst[2]	0.0	6.4	0.0	1.5	3.0	5.8	0.0	0.0	3.8	3.4	5.3	21.4	0.0
Regular HC pattern[3]	66.4	64.1	80.0	86.6	53.7	57.7	90.9	83.3	53.8	90.8	89.5	92.9	100
Atypical HC[3]	17.7	46.2	56.0	32.8	10.4	10.3	9.1	0.0	23.1	37.8	57.9	85.7	44.4
Broadened HC[4]	4.4	20.5	36.0	22.4	4.5	7.1	9.1	0.0	0.0	26.1	26.3	50.0	22.2
Diamond-shape HC[5]	1.8	14.1	16.0	6.0	1.5	2.6	0.0	0.0	3.8	5.9	15.8	64.3	33.3
Polarized in the HC[6] (Supplementary Figures S3 and S4 online)	2.7	9.0	16.0	4.5	4.5	2.6	9.1	0.0	0.0	89.9	5.3	21.4	11.1
Cobblestone pattern[3]	23.9	32.1	20.0	16.4	50.7	58.3	18.2	50.0	57.7	0.0	5.3	0.0	0.0
Atypical cobblestone pattern[3]	19.5	24.4	12.0	1.5	9.0	6.4	0.0	0.0	30.8	0.0	0.0	0.0	0.0
Atypical cobblestone with small nucleated cells[3]	14.2	12.8	8.0	0.0	4.5	2.6	0.0	0.0	19.2	0.0	0.0	0.0	0.0
Epidermal disarray[3]	53.1	47.4	60.0	20.9	16.4	10.3	9.1	0.0	7.7	24.4	31.6	28.6	22.2
HC atypical and disarray[3]	62.8	78.2	84.0	43.3	23.9	19.2	9.1	0.0	30.8	53.8	68.4	92.9	55.6
Pagetoid cells[3]	70.8	79.5	64.0	22.4	22.4	18.6	0.0	0.0	69.2	6.7	42.1	28.6	0.0
Widespread pagetoid infiltration[3]	32.7	57.7	44.0	3.0	14.9	10.9	0.0	16.7	30.8	1.7	15.8	7.1	0.0
Round pagetoid cells[3]	53.1	75.6	60.0	14.9	14.9	14.7	0.0	16.7	53.8	3.4	26.3	21.4	0.0
Dendritic pagetoid cells[3]	50.4	53.8	44.0	11.9	16.4	13.5	0.0	16.7	46.2	5.9	21.1	7.1	0.0
Pagetoid cells around follicular opening[4]	16.8	7.7	8.0	7.5	3.0	0.6	0.0	0.0	3.8	0.0	10.5	0.0	0.0
Edged papillae[3]	46.0	42.3	28.0	55.2	79.1	88.5	72.7	83.3	65.4	3.4	42.1	28.6	77.8
Non-edged papillae[3]	65.5	83.3	80.0	14.9	41.8	32.7	9.1	50.0	61.5	14.3	21.1	7.1	0.0
Edged papillae: absent	54.0	57.7	72.0	44.8	20.9	11.5	27.3	16.7	34.6	96.6	57.9	71.4	22.2
Large interpapillary space[7] (Supplementary Figure S5 online)	0.9	1.3	0.0	22.4	3.0	2.6	0.0	0.0	0.0	0.8	15.8	21.4	0.0
Polycyclic papillae[8]	15.0	2.6	4.0	20.9	0.0	0.6	9.1	0.0	0.0	0.0	5.3	14.3	11.1
Papillae enlarged and polycyclic[7,8]	15.9	3.8	4.0	40.3	3.0	3.2	9.1	0.0	0.0	0.8	21.1	35.7	11.1
Non-visible papillae	11.5	9.0	12.0	31.3	3.0	0.6	18.2	0.0	0.0	79.0	42.1	57.1	22.2
Cell atypia[3]	80.5	87.2	88.0	26.9	28.4	29.5	0.0	33.3	69.2	16.8	42.1	14.3	0.0
Marked atypia[3]	36.3	66.7	76.0	3.0	11.9	10.9	0.0	16.7	23.1	15.1	10.5	7.1	0.0
Sheet of cells[3]	8.8	5.1	20.0	0.0	1.5	2.6	0.0	0.0	7.7	0.8	5.3	0.0	0.0
Junctional nest[3]	30.1	56.4	44.0	3.0	53.7	60.3	18.2	33.3	65.4	0.0	5.3	0.0	11.1
Junctional clusters (three or four cells)[3]	15.0	32.1	20.0	0.0	32.8	41.0	18.2	33.3	46.2	0.0	0.0	0.0	0.0
Junctional thickenings[3]	26.5	48.7	44.0	3.0	47.8	59.0	0.0	33.3	61.5	0.0	5.3	0.0	11.1
Basaloid cord or nodules[9] (Supplementary Figures S1 and S2 online)	1.8	1.3	4.0	3.0	0.0	1.3	0.0	0.0	0.0	61.3	5.3	7.1	0.0
Dendritic-like features within tumor islands[10] (Supplementary Figure S1 online)	1.8	0.0	4.0	0.0	0.0	0.6	0.0	0.0	0.0	21.8	0.0	0.0	0.0

Table 1 continued on following page

Table 1. Continued

RCM features from epidermis to dermis (%)	MIS *N*=113	MM<1 *N*=78	MM>1 *N*=25	Macules of the face[1] *N*=67	Nev Junct *N*=67	Nev Comp *N*=156	Nev Intrad *N*=11	Nev Blue *N*=6	Nev Spitz *N*=26	BCC *N*=119	AK *N*=19	SCC *N*=14	DFibroma *N*=9
Nucleated cell within tumor islands[11] (Supplementary Figure S1 online)	1.8	0.0	4.0	0.0	0.0	1.3	0.0	0.0	0.0	44.5	0.0	0.0	0.0
Fibrillar polarized pattern around tumor[12] (Supplementary Figure S1 online)	1.8	1.3	8.0	1.5	0.0	0.6	0.0	0.0	0.0	41.2	5.3	7.1	0.0
Clefting[13] (Supplementary Figure S1 online)	2.7	0.0	8.0	1.5	0.0	0.6	0.0	0.0	0.0	52.1	5.3	0.0	0.0
Epidermal shadow[14] (Supplementary Figure S4 online)	2.7	5.1	8.0	4.5	3.0	1.9	9.1	0.0	0.0	72.3	15.8	7.1	11.1
Nests[3]	16.8	55.1	60.0	0.0	26.9	57.7	54.5	66.7	42.3	14.3	0.0	7.1	0.0
Dense nest[3]	9.7	17.9	12.0	0.0	25.4	51.9	54.5	66.7	30.8	5.9	0.0	7.1	0.0
Dishomogenous nest[3]	9.7	46.2	48.0	0.0	7.5	17.9	27.3	50.0	26.9	7.6	0.0	0.0	0.0
Sparse nest[3]	0.9	3.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.5	0.0	0.0	0.0
Cerebriform nest[3]	1.8	6.4	24.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Nucleated cells within the papilla[3]	15.9	39.7	36.0	1.5	13.4	10.3	18.2	0.0	15.4	5.9	0.0	0.0	0.0
Plump bright cells[3]	52.2	60.3	56.0	38.8	55.2	67.3	9.1	33.3	76.9	36.1	42.1	21.4	11.1
Plump cells in large aggregation within the papillary dermis[3]	13.3	10.3	4.0	16.4	11.9	3.8	0.0	33.3	11.5	10.1	15.8	7.1	0.0
Vessels: visible[2]	17.7	37.2	48.0	23.9	20.9	19.2	36.4	0.0	15.4	98.3	42.1	64.3	44.4
Vertical vessel within the papillae[2]	8.0	26.9	28.0	10.4	13.4	8.3	9.1	0.0	7.7	16.0	31.6	28.6	11.1
Horizontal vessel[2]	8.8	15.4	36.0	11.9	6.0	4.5	18.2	0.0	0.0	89.9	5.3	35.7	33.3
Linear telangiectasia-like horizontal vessel (Supplementary Figure S3 online)	7.1	9.0	32.0	14.9	6.0	3.2	9.1	0.0	3.8	80.7	15.8	21.4	22.2
Convoluted glomerular-like vessel[15]	0.9	14.1	32.0	6.0	1.5	3.8	9.1	0.0	0.0	37.0	10.5	42.9	11.1
RCM score⩾3	70.8	89.7	88.0	17.9	28.4	25.6	9.1	33.3	65.4	10.1	31.6	7.1	0.0

Abbreviations: AK, actinic keratosis; BCC, basal cell carcinoma; comp, compound; Dfibroma, dermatofibromas; HC, honeycomb; intrad, intradermal; junct, junction; MM, melanoma; MIS, melanoma *in situ*; Nev, nevus; RCM, reflectance confocal microscopy; SCC, squamous cell carcinoma.

The cells highlighted in italics correspond to the features already described in BCCs (Gonzalez and Tannous, 2002; Nori *et al.*, 2004; Agero *et al.*, 2006; Ulrich *et al.*, 2011), and the ones highlighted in bold correspond to the features already described in MMs (Langley *et al.*, 2001, 2007; Gerger *et al.*, 2005; Pellacani *et al.*, 2007).

[1]Benign macules of the face, biopsied in the differential diagnosis of lentigo maligna (mostly solar lentigine, seborrheic keratosis, and lichen planus–like keratosis on heavily sun-damaged skin).

[2]Described in the RCM glossary (Scope *et al.*, 2007).

[3]Described in Pellacani *et al.*, 2007.

[4]Described in Guitera *et al.*, 2009.

[5]HC pattern with irregular diamond-shaped large cells (>20 μm), described in Guitera *et al.*, 2009.

[6]Defined as polarized elongated features in the HC (Nori *et al.*, 2004 and see Supplementary Figures S3 and S4 online).

[7]Defined as large space with HC and/or cobblestone pattern (see Supplementary Figure S5 online).

[8]Described as circumvolution of edged papillae described in solar lentigines (Langley *et al.*, 2006).

[9]Defined as tightly packed cells with palisading, forming cord-like features and nodules with variable brightness (Agero *et al.*, 2006; see Supplementary Figures S1 and S2 online).

[10]Described in Agero *et al.*, 2006 and Segura *et al.*, 2007.

[11]Nucleated cell within tumor island, see Supplementary Figure S2 online.

[12]Defined as fibrillary polarized features drawing a reticulation pattern around tumor island (see Supplementary Figure S1 online).

[13]Defined as reflective tumor islands surrounded by a dark space, well delineated, corresponding to stroma (Ulrich *et al.*, 2011).

[14]Defined as large, dark featureless area with blurred border disrupting the normal epidermis described as horizontal clefting in BCC (see Supplementary Figure S4 online).

[15]Defined as coiled canicular vessels.

Table 2. RCM features showing significant correlations with the BCC diagnosis by histopathology on univariate analysis

BCC features	OR (95% CI)	*P*-value
Junctional nest	0.01 (0.00–0.01)	<0.001
Junctional thickenings	0.01 (0.00–0.10)	<0.001
Edged papillae	0.02 (0.01–0.06)	<0.001
Junctional clusters	0.03 (0.03–0.18)	<0.001
Widespread pagetoid infiltration	0.06 (0.01–0.23)	<0.001
Papillae enlarged and policyclic	0.07 (0.01–0.48)	<0.001
Round pagetoid cells	0.09 (0.03–0.21)	<0.001
Pagetoid cells	0.11 (0.06–0.23)	<0.001
Large interpapillary space	0.16 (0.02–1.21)	0.04
Dendritic pagetoid cells	0.18 (0.09–0.38)	<0.001
Dense nest	0.19 (0.09–0.42)	<0.001
Non-edged papillae	0.21 (0.12–0.35)	<0.001
Cell atypia	0.22 (0.13–0.35)	<0.001
Nests	0.31 (0.18–0.52)	<0.001
Nucleated cells within the papilla	0.34 (0.16–0.76)	0.01
Dishomogenous nest	0.38 (0.18–0.76)	0.01
Plump bright cells	0.48 (0.32–0.72)	<0.001
Atypia marked	0.52 (0.31–0.89)	0.02
Atypical HC	1.8 (1.2–2.7)	0.009
Broadened HC	2.4 (1.5–3.9)	0.001
Regular HC	4.9 (2.6–9.2)	<0.001
Convoluted glomerular-like vessel	7.75 (4.76–12.6)	<0.001
Non-visible papillae	28.2 (17.1–46.7)	<0.001
Dendritic-like features within tumor islands	40.5 (13.8–119)	<0.001
Linear telangiectasia-like horizontal vessels	41.4 (24.4–70.3)	<0.001
Fibrillar polarized pattern around tumor	44.6 (21.0–94.5)	<0.001
Edged papillae: absent	48.4 (17.6–133)	<0.001
Epidermal shadow	62.4 (35.1–111)	<0.001
Horizontal vessel	71.4 (37.9–135)	<0.001
Clefting	77.8 (35.5–170)	<0.001
Basaloid cord or nodules	90.1 (43.6–186)	<0.001
Nucleated cell within tumor islands	92.6 (35.8–240)	<0.001
Vessels: visible	114 (35.8–365)	<0.001
Polarized in the HC	154 (77.6–304)	<0.001

Abbreviations: BCC, basal cell carcinoma; CI, confidence interval; HC, honeycomb; OR, odds ratio; RCM, reflectance confocal microscopy. The cells highlighted in italics correspond to the features already described in BCCs (Gonzalez and Tannous, 2002; Nori *et al.*, 2004; Agero *et al.*, 2006; Ulrich *et al.*, 2011), and the ones highlighted in bold correspond to the features already described in MMs (Langley *et al.*, 2001, 2007; Gerger *et al.*, 2005; Pellacani *et al.*, 2007). For definition and reference of the features see Table 1.

Table 3. Discriminant analysis based on 50% cases (training set): eight independent features distinguishing between BCCs and the remaining lesions, and on MMs compared with all other lesions except BCCs

	Coefficient
BCC features	
Polarized in the HC (Supplementary Figures S3 and S4 online)	1.813
Linear telangiectasia-like horizontal vessels (Supplementary Figure S3 online)	1.339
Basaloid cord or nodule (Supplementary Figure S1 online)	1.288
Epidermal shadow (Supplementary Figure S4 online)	0.754
Convoluted glomerular-like vessels	0.639
Non-visible papillae	0.605
Cerebriform nests	−1.230
Disarray of the epidermal layer	−0.322
Constant	−1.020
MM features	
Cerebriform nests	1.584
Atypical cobblestone with small nucleated cells	1.485
Marked cytologic atypia	1.110
Pageoid Cells	1.091
Epidermal disarray	0.950
Large interpapillary space (Supplementary Figure S5 online)	−1.016
Dense nest	−0.451
Constant	−1.058

Abbreviations: BCC, basal cell carcinoma; HC, honeycomb; MM, melanoma.
Unstandardized canonical discriminant function coefficients.

- 100% sensitivity for the diagnosis of BCCs (52/52), with all 52 BCCs classified as BCCs with the BCC algorithm, and 18 (34.6%) were also classified as MMs by the MM algorithm.
- 68.4% specificity for benign lesions, including actinic keratosis classified as benign in this calculation (130/190).
- Lastly, the overall sensitivity of the method with all epithelial malignant tumors (BCCs and SCCs) and MMs was 91.5% (152/166).

IV. Comparison with other published RCM diagnostic scoring systems

- RCM score–described on melanocytic lesions only (Pellacani *et al.*, 2007):

The RCM score assessed on our test set showed an accuracy for the diagnosis of MMs differentiating from other diagnosis of 77.1% sensitivity, 76.9% specificity (for a

Table 4. RCM features showing significant correlations with the MM diagnosis by histopathology on univariate analysis

RCM features	OR (95% CI)	*P*-value
Basaloid cord or nodule	0.10 (0.04–0.28)	<0.001
Nucleated cell within tumor islands	0.11 (0.04–0.37)	<0.001
Large interpapillary space	0.16 (0.04–0.66)	0.004
Clefting	0.16 (0.06–0.40)	<0.001
Epidermal shadow	0.17 (0.09–0.35)	<0.001
Fibrillar polarized pattern around tumor	0.20 (0.08–0.50)	<0.001
Polarized in the HC	0.21 (0.12–0.38)	<0.001
Dendritic-like features within tumor	0.25 (0.07–0.82)	0.013
Non-visible papillae	0.31 (0.19–0.50)	<0.001
Linear telangiectasia-like horizontal vessel	0.36 (0.22–0.57)	<0.001
Horizontal vessel	0.44 (0.29–0.68)	<0.001
Dense nest	0.45 (0.29–0.70)	<0.001
Vessels: visible	0.56 (0.39–0.79)	0.001
Edged papillae	0.57 (0.41–0.79)	0.001
Atypical HC	1.4 (1.0–2.1)	0.04
Junctional nest	1.5 (1.1–2.1)	0.015
Edged papillae: absent	1.8 (1.3–2.4)	0.001
Polycyclic papillae	2.4 (1.3–4.6)	0.005
Dishomogenous nest	3.0 (2.0–4.6)	<0.001
HC atypical and disarray	4.2 (3.0–6.0)	<0.001
Nucleated cells within the papilla	4.3 (2.8–6.7)	<0.001
Atypical cobblestone pattern	4.8 (2.9–8.1)	<0.001
Epidermal disarray	5.1 (3.6–7.2)	<0.001
Sheet of cells	5.2 (2.3–11.8)	<0.001
Atypical cobblestone with small nucleated cells	6.0 (3.0–12.1)	<0.001
Pagetoid cells localised around follicular opening	6.3 (3.1–13.0)	<0.001
Dendritic pagetoid cells	6.7 (4.6–9.7)	<0.001
Non-edged papillae	7.6 (5.3–11.0)	<0.001
Widespread pagetoid infiltration	7.8 (5.1–11.8)	<0.001
Atypia marked	8.7 (5.9–12.8)	<0.001
Round pagetoid cells	9.7 (6.7–14.1)	<0.001
Pagetoid cells	11.0 (7.5–16.0)	<0.001
Cell atypia	13.9 (9.2–20.9)	<0.001

Abbreviations: BCC, basal cell carcinoma; CI, confidence interval; HC, honeycomb; MM, melanoma; OR, odds ratio; RCM, reflectance confocal microscopy.
The cells highlighted in italics correspond to the features already described in BCCs (Gonzalez and Tannous, 2002; Nori *et al.*, 2004; Agero *et al.*, 2006; Ulrich *et al.*, 2011), and the ones highlighted in bold correspond to the features already described in MMs (Langley *et al.*, 2001, 2007; Gerger *et al.*, 2005; Pellacani *et al.*, 2007). For definition and reference of the features see Table 1.

score >3, determined to be the best threshold for melanocytic lesions).

The RCM score on the test set for a score >2 showed an accuracy for the diagnosis of MMs of 88.6% sensitivity, 62.2% specificity, AUC = 0.828 (95% CI: 0.780–0.876). Only 8% of BCC had an RCM score ⩾3.

- Two-step method of Segura *et al.* (2009):

The two-step method described by Segura *et al.* (2009) used on the test set showed an accuracy for the diagnosis of MMs (calculated with a threshold of zero) and BCCs:

- MMs (differentiating from any other diagnosis): 76.5% sensitivity (80/105, with 72 classified as MMs and 8 as BCCs)
- BCCs (differentiating from any other diagnosis): 88.5% sensitivity (46/52 with 43 classified as BCCs and 3 as MMs)
- A totral of 9 out of 17 actinic keratosis and SCCs were classified as MMs ($n = 6$) and BCCs ($n = 3$)
- The specificity for benign lesions classified as such was 72.5% (132/182)

The AUC calculated only on the subset of melanocytic lesions of the test set (to be comparable between the three methods) was highest for the method reported here, although the differences were not statistically significant; to our knowledge, the previously unreported model described in this article obtained an AUC = 0.848 (0.799–0.897), the algorithm by Segura *et al.* (2009) had an AUC = 0.804 (0.748–0.859) and the RCM score had an AUC = 0.783 (0.726–0.841).

V. Description of benign skin lesions

Table 1 reports the frequency of various RCM features of benign skin lesions. We will comment only on different types of nevi, excluding Spitz nevi, as their features have already been reported (Pellacani *et al.*, 2009).

Most of the RCM features correlate well with the well-known pathological characteristics of melanocytic nevi. For example, compact nests in the dermis are a typical feature of compound and intradermal nevi, and junctional nests are mostly seen in junctional nevi. The two main features of nevi in the epidermis are the regular honeycomb pattern (more than 50% of the nevi, and in particular, more than 80% of dermal and blue nevi) and the regular cobblestone pattern (more than 50% of nevi, with only18% of dermal nevi having this pattern). Disarranged epidermis is more frequently seen in junctional nevi (16%) than in other types of nevi (<10%). Interestingly, pagetoid cells were frequently seen in this suspicious population of junctional and compound nevi (around 20%), but not in blue and dermal nevi. Of note, the pagetoid spread was rarely widespread or composed of small round cells. At the dermo-epidermal junction, edged papillae (corresponding to regular papillae with clearly outlined contours) were seen in more than 73% of the nevi. Atypia of the cells was found at the dermo-epidermal junction in

Table 5. Overall analysis on the test set combining BCC and MM models

	Classified as				
	Neither BCC nor MM (benign), *N* (%)	BCC only, *N* (%)	MM only, *N* (%)	BCC and MM, *N* (%)	Total
In situ MM	8 (14.8)	1 (1.9)	42 (77.8)	3 (5.6)	54
MM <1 mm	2 (5.1)	1 (2.6)	31 (79.5)	5 (12.8)	39
MM>1 mm	1 (8.3)	0 (0.00)	9 (75.0)	2 (16.7)	12
Benign macule of the face	18 (58.1)	4 (12.9)	5 (16.1)	4 (12.9)	31
Junctional nevus	20 (66.7)	1 (3.3)	8 (26.7)	1 (3.3)	30
Compound nevus	69 (75.8)	3 (3.3)	17 (18.7)	2 (2.2)	91
Intradermal nevus	8 (100)	0 (0.00)	0 (0.00)	0 (0.00)	8
Blue nevus	3 (100)	0 (0.00)	0 (0.00)	0 (0.00)	3
Spitz nevus	7 (43.8)	0 (0.00)	8 (50.0)	1 (6.3)	16
BCC	0 (0.00)	34 (65.4)	0 (0.00)	18 (34.6)	52
AK	3 (37.5)	3 (37.5)	2 (25)	0 (0.00)	8
SCC	3 (33.3)	2 (22.2)	3 (33.3)	1 (11.1)	9
Dermatofibroma	2 (66.7)	1 (33.3)	0 (0.00)	0 (0.00)	3
Total	144 (40.4)	50 (14.0)	125 (35.1)	37 (10.4)	356

Abbreviations: AK, actinic keratosis; BCC, basal cell carcinoma; MM, melanoma; SCC, squamous cell carcinoma.

approximately 30% of junctional or compound nevi, but was not observed in dermal nevi (0%). These atypical cells were rarely more than mildly atypical (<10% of nevi). Not surprisingly, nests were a major feature of nevi, but they were never cerebriform. Overall, vessels were visible in only 20% of nevi, but were more common in dermal nevi (36%). They were mostly vertical.

DISCUSSION

The analysis of the cases in this study and the development of the RCM model for clinical diagnosis are based on the real clinical scenario faced by the dermatologist in the clinic where melanocytic and non-melanocytic lesions are mixed and only difficult cases are biopsied. It confirmed our impression that BCC diagnosis is relatively accurate with *in vivo* RCM (similar to pathology assessment). On the contrary, MM diagnosis can be more difficult on a highly selected series of cases chosen for their suspicious criteria by internationally recognized dermoscopy experts. Of note, the vast majority of mildly atypical nevi or in non-atypical nevi with a history of change are usually monitored in our respective institutions with digital dermoscopy or total body photography, and hence not recruited for this study.

Most MMs that are misdiagnosed by RCM are thin tumors, and in some instances, the actual diagnosis may be a question of pathology interpretation. The review of pathology slides of the four invasive MMs that were misdiagnosed by RCM revealed that each of the cases was a subtle nevoid MM; and in one instance, there was some question of whether the lesion was actually an MM or a nevus. Spitz nevi and sun-damaged macules of the face are other good examples of the difficulties in diagnosis commonly faced by both pathologists and RCM experts.

On univariate analysis of BCC features, the majority of positive features have already been described and are highlighted in italics in Table 2 (Gonzalez and Tannous, 2002; Nori *et al.*, 2004; Agero *et al.*, 2006; Ulrich *et al.*, 2011). Not surprisingly, most of the negative features have already been described in MMs and highlighted in bold (Langley *et al.*, 2001, 2007; Gerger *et al.*, 2005; Pellacani *et al.*, 2007). The reverse comment is also true when applying univariate analysis of MM features, excluding BCCs in a second step (see italics and bold in Table 4).

The BCC method that we describe is based on eight independently significant features (Table 3):

- Polarized elongated features in the superficial layer was the most powerful feature (Supplementary Figure S3, 4 online). It has also been reported to be correlated with polarized and elongated nuclei of basal cells (Nori *et al.*, 2004)
- Linear telangiectasia-like horizontal vessels (Supplementary Figure S3 online) were a more important feature than convoluted glomerular-like vessels. The importance of vascularization has been reported in nearly all publications concerning RCM features of BCCs. It is also a well-known dermoscopy criterion (Zalaudek *et al.* 2010)
- Basaloid cord and nodules (Supplementary Figures S1, 2 online) have also been well described in nodular BBCs, as well circumscribed nests of hypo-reflective cells tightly packed in a palisading way (Agero *et al.*, 2006; Segura *et al.*, 2009)

- Epidermal shadow (Supplementary Figure S4 online), defined as large featureless area with blurred border disrupting the normal epidermis and corresponding to the horizontal clefting (due to hyporeflective stroma, Ulrich *et al.*, 2011) is also a useful feature

There were three negative features useful for the diagnosis of BCCs:

- Disarray of the honeycomb epidermal layer is more specific of MMs (Pellacani *et al.*, 2007), and the honeycomb pattern was recognized in more than 90% of the BCCs of this series
- Papillae were ''non-visible,'' meaning that BCC structures altered the normal junction organization. Papillae (corresponding to dark round to oval features surrounded by epidermis in RCM sections) were disrupted in 97% of the 119 BCCs of our series
- Lastly, cerebriform nests are rare, but very specific of nodular MMs (Pellacani *et al.*, 2007; Segura *et al.*, 2008). They were not recorded in our series of 119 BCCs. They were also not recorded in benign melanocytic lesions

MMs (10/12) diagnosed as BCCs by the BCC algorithm also contained obvious MM features, and were diagnosed as such with the MM method described as a second step. It seems that basaloid cords and nodules are highly specific of BCCs. They were found only in few thick MMs, where MM nests simulated BCC islands, but in context, it was often easy to detect specific MM features.

One third of the actinic keratosis and SCC lesions were diagnosed as BCCs, and they were the main pitfall of this BCC method. As actinic keratoses are an important differential diagnosis of BCCs, this RCM finding could lead to unnecessary biopsies of actinic keratoses. It should be noted that actinic keratosis and SCCs have a relatively small sample size in our study; hence, it would be poorly modeled compared with high prevalent lesions. These lesions are often covered by a thick keratin layer that is highly reflective, so the images under this layer are blurred and not readable with RCM. Therefore, the subset of actinic keratoses imaged was pre-selected. Moreover, our consecutive cases included only a limited number of non-melanocytic tumors that would be appropriately considered in the differential diagnosis of BCCs and MMs, such as atypical seborrheic keratoses ($n=8$, pooled in the differential diagnosis of lentigo maligna), atypical dermatofibromas ($n=9$), sebaceous hyperplasia ($n=0$), and pyogenic granulomas ($n=0$). These subsets will need to be studied in a larger series.

In conclusion, to our knowledge, we are reporting a previously unreported two-step RCM method for the diagnosis of skin tumors based on a large series of melanocytic and non-melanocytic lesions, and demonstrate that RCM is a valuable non-invasive tool to diagnose skin tumors. It can be used, in particular, to obtain a non-invasive quasi-histological diagnosis for BCCs and to follow efficiency of treatments. It has also been proposed as a method for determining tumor margins *in vivo*, but its efficacy for this purpose has not been proven. Particular features of different subtypes of BCCs have not been addressed in our series, and more research should be directed to confirm if RCM is a particularly valuable tool on difficult subtypes of BCCs, such as infiltrative ones. RCM is also a very valuable tool for the diagnosis of MMs. RCM is not replacing dermoscopy as it is not a screening tool, because diagnosis of one lesion requires at least 5 minutes. Nevertheless, RCM evaluation can dramatically reduce (by 68% in this series) the unnecessary excisions of benign lesions even in expert institutions. As previously reported (Guitera *et al.*, 2009), we have shown that dermoscopy and RCM are relatively poorly correlated for the diagnosis of atypical melanocytic lesions. In this regard, although RCM and dermoscopy have the same sensitivity for the diagnosis of MMs (91%, 95% CI: 84.6–95.5 for RCM, and 88%, 95% CI: 80.7–92.6 for dermoscopy), the sensitivity increases dramatically to 98% when lesions are excised, because of either the RCM or dermoscopy evidence of MMs.

Improved accuracy of the clinical diagnosis of skin tumors would be of great public health benefit.

MATERIALS AND METHODS

Lesion recruitment was performed in two secondary care settings: the Sydney Melanoma Diagnostic Centre, Australia, and the Department of Dermatology, University of Modena, Italy. Consecutive patients, presenting or found with suspicious lesions, including all macules of the face and neck, suspicious for lentigo maligna, and which would be subjected to biopsy or excision to rule out an epithelial tumor or an MM, following conventional clinical and dermoscopy diagnosis, were entered into the study. The location had to be amenable to examination by RCM, as an adhesive ring of 2 cm must be used, precluding the inclusion of lesions behind the ear, some parts of the edge of the nose or eyes. Because RCM penetration is only 0.2 mm beneath the skin surface, keratotic, sole, and palms lesions were excluded. Therefore, the cases consisted predominantly of BCCs and melanocytic tumors; there were insufficient numbers of SCCs and actinic keratoses to allow development of accurate RCM diagnosis of the latter tumors. Therefore, 19 actinic keratosis, 12 Bowen's disease, and 2 SCCs have been pooled together. The recruitment criteria were the same in the two centers. This study was approved by the Ethics Committees in Sydney (protocol number X05-0218) and in Modena (protocol number 1338/CE), and signed consent was obtained. All clinical investigations were conducted according to the Declaration of Helsinki Principles.

Instruments and acquisition procedures have been similar to ones described in the article (Guitera *et al.*, 2009).

Confocal diagnosis

RCM features were described by two expert observers (GP and PG), blinded from any clinical information, dermoscopy, and clinical aspects, but not for the location and age of the patient. In detail, each observer evaluated the images previously randomized between the two centers, opening codified folders containing all the images acquired for the corresponding case. At the end of the study, the patients' codes were broken and the evaluations were matched with pathology before statistical analysis.

A series of 48 features, corresponding to previous observations (Pellacani *et al.*, 2007; Guitera *et al.*, 2009), and new descriptors were considered at three different depth levels. Description and definitions are summarized in Table 1. All these features were evaluated for their presence/absence (binary non-parametric data).

Statistics

Statistical evaluation was carried out employing the SPSS statistical package (release 12.0. 0, 2003; SPSS, Chicago, Ill., USA).

Absolute and relative frequencies of the observations in benign and malignant lesions were obtained for each RCM feature. By univariate analysis, we compared BCCs with all other lesions, MMs with all other lesions, and thin MMs (<1 mm) with thick MMs (>1 mm). Significant differences were evaluated by means of the χ^2-test of independence (Fisher's exact test was applied if any expected cell value in the 2×2 table was less than 5). For an estimate of risk, a calculation of the OR and 95% CI was carried out.

Multivariate discriminant analysis was performed for the identification of the independently significant features, and for the validation of the efficacy of RCM in distinguishing between BCCs and the rest of the lesions, and then MMs (excluding BCCs) and the rest of the lesions on the training set, randomly chosen as 50% of our series. A coefficient is estimated for each included variable in relation to the likeliness to predict a BCC, and then an MM lesion. The algorithm was then used on the test set constituted by the remaining 50% of the population.

Receiver operating characteristic analysis was performed to investigate sensitivity and specificity of the discriminant analysis equations for the BCC, and then the MM algorithms (McNeil and Hanley, 1984). The AUC, which represents an index of the overall discriminant power, was calculated by the non-parametric trapezoidal method. The model, along with its parameter estimates, was then used for prediction and estimation of the AUC in the test set. Sensitivity, specificity, diagnostic accuracy, OR, and 95% CI, were calculated for each score value. A *P*-value <0.05 was considered significant.

Pathology review

The lesions of the test set found to be false negatives with the MM model, and a subset of false positives (those available and all Spitzoid lesions) were reviewed.

CONFLICT OF INTEREST

The authors state no conflict of interest.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at https://www.sciencedirect.com/journal/journal-of-investigative-dermatology

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