

**UNIVERSIDADE FEDERAL DE CIÊNCIAS DA SAÚDE DE
PORTO ALEGRE – UFCSPA
PROGRAMA DE PÓS-GRADUAÇÃO EM PATOLOGIA**

Thaís Corsetti Grazziotin

**Associação entre a Classificação
Morfológica Confocal e os Fenótipos
Clínicos de Pacientes com Múltiplos
Melanomas Primários e Melanoma
Familiar.**

**Universidade Federal de Ciências da Saúde
de Porto Alegre**

**Porto Alegre
2016**

Thaís Corsetti Grazziotin

Associação entre a Classificação Morfológica Confocal e os Fenótipos Clínicos de Pacientes com Múltiplos Melanomas Primários e Melanoma Familiar.

Tese submetida ao Programa de Pós-Graduação em Patologia da Fundação Universidade Federal de Ciências da Saúde de Porto Alegre como requisito para a obtenção do grau de Doutor

Orientador: Dr. Renan Rangel Bonamigo
Co-orientadora: Dra. Susana Puig

**Porto Alegre
2016**

Catálogo na Publicação

Corsetti Grazziotin, Thaís

Associação entre a classificação morfológica confocal e os fenótipos clínicos de pacientes com múltiplos melanomas primários e melanoma familiar. / Thaís Corsetti Grazziotin. -- 2016.

86 p. : il., graf., tab. ; 30 cm.

Tese (doutorado) -- Universidade Federal de Ciências da Saúde de Porto Alegre, Programa de Pós-Graduação em Patologia, 2016.

Orientador(a): Prof. Dr. Renan Rangel Bonamigo ;
coorientador(a): Profa. Dra. Susana Puig.

1. Melanoma. 2. Dermatoscopia. 3. Genética. 4. Fenótipo. 5. Histologia. I. Título.

Agradecimentos e dedicatórias

À toda a minha família, em especial aos meus pais Verônica Corsetti Grazziotin e Cláudio Antônio Grazziotin e ao meu filho Bernardo Grazziotin Pinto.

Ao meu marido Leonardo Araújo Pinto e minha tia Berenice Corsetti, por compartilharem o entusiasmo pela pesquisa e pela atividade acadêmica.

Ao meu orientador, Prof. Dr. Renan Bonamigo, por sua preciosa orientação e amizade ao longo destes anos e por mais esta oportunidade em minha formação.

À minha co-orientadora Profa. Dra. Susana Puig e ao Prof. Dr. Josep Malvehy, reconhecidos internacionalmente por sua contribuição acadêmica e assistencial para o diagnóstico e tratamento do melanoma. Agradeço a oportunidade de ter realizado o projeto desta tese na Unidade de Melanoma do Hospital Clínic de Barcelona, serviço de excelência acadêmica do qual tenho orgulho de ter participado e onde fui tão bem recebida.

A todos colegas e membros da Unidade de Melanoma do Hospital Clínic de Barcelona e do Serviço de Dermatologia da Universidade Federal de Ciências da Saúde de Porto Alegre.

À Capes e ao Programa Ciências sem Fronteiras por tornar o sonho da pós-graduação no exterior possível.

Aos pacientes que sofrem de melanoma e acreditam no avanço da medicina e da pesquisa.

Sumário

1. Introdução	1
1.1. Histórico do melanoma	1
1.2. Epidemiologia do melanoma no Brasil e no mundo	2
1.3. Fatores econômicos relacionados ao melanoma	8
1.4. Fatores de risco para melanoma	8
1.4.1 Fatores de risco demográficos	10
1.4.2 Fatores de risco ambientais	11
1.4.3 Fatores de risco fenotípicos e constitucionais	12
1.4.4 Fatores de risco genéticos	13
1.5. Estratégias clínicas para a detecção precoce do melanoma	16
1.5.1 Método em duas etapas: dermatoscopia digital e mapeamento corporal total	18
1.5.2 Microscopia confocal de reflectância	20
1.6. Classificação morfológica do melanoma	28
1.7. Referências bibliográficas	30
2. Objetivos	36
3. Artigo científico redigido em inglês	37
4. Considerações finais	75
5. Anexos	77
5.1 Parecer do Comitê de Ética do Hospital Clínic de Barcelona	77
5.2 Normas da Revista	79

Lista de abreviaturas utilizadas

α -MSH: *α melanocyte-stimulating hormone*

CDKN2A: *cyclin-dependent kinase inhibitor 2A*

CM: *cutaneous melanoma*

IC: intervalo de confiança

INCA: Instituto Nacional de Câncer

LM: lentigo maligno

LMM: lentigo maligno melanoma / *lentigo maligna melanoma*

MAU: melanoma do aparelho ungueal

MC1R: *melanocortin 1 receptor*

MCR: microscopia confocal de reflectância

MES: melanoma de espalhamento superficial

MITF: *microphthalmia-associated transcription factor*

MLA: melanoma lentiginoso acral

MN: melanoma nodular

MPM: *multiple primary melanoma*

NM: *nodular melanoma*

NNT - *number needed to treat*

OMS: Organização Mundial de Saúde

OR: *odds ratio*

RCM: *reflectance confocal microscopy*

RHC: *red-hair color variants*

rHV: *non red hair variants*

RR: risco relativo

SD: *standard deviation*

SSM: *superficial spreading melanoma*

TDS: *total dermoscopic score*

Lista de figuras utilizadas

Figura 1: Risco cumulativo de desenvolvimento de melanoma em diferentes países por sexo no período de 2000 a 2002. -----	3
Figura 2: Taxas de incidência de melanoma estimada para 2014 no Rio Grande do Sul em homens e mulheres. -----	5
Figura 3: Relação entre múltiplos fatores de risco para melanoma cutâneo. -----	9
Figura 4: Fatores de risco, em ordem de frequência, são encontrados em mais de 50% dos <i>guidelines</i> por região ou continente.-----	10
Figura 5: Fenótipo de paciente portador de síndrome do nevo atípico. -----	19
Figura 6: Critérios maiores para diagnóstico de melanoma pela microscopia confocal segundo escore proposto por Pellacani e colaboradores (2005). -----	23
Figura 7: Critérios menores para diagnóstico de melanoma pela microscopia confocal segundo escore proposto por Pellacani e colaboradores (2005). -----	23
Figura 8: Critérios morfológicos para diferenciação entre lesão melanocítica e não melanocítica segundo escore proposto por Segura e colaboradores (2009). -----	25
Figura 9: Critérios morfológicos para diferenciação entre lesão melanocítica benigna e maligna segundo escore proposto por Segura e colaboradores (2009). -----	26
Figura 10: Algoritmo proposto por Segura e colaboradores (2009) para diagnóstico por microscopia confocal de lesões equívocas à dermatoscopia.-----	27

Lista de gráficos utilizados

Gráfico 1: Taxas de incidência e mortalidade de melanoma por 100.000 habitantes e taxa de incidência por estágio de melanoma por 100.000 habitantes.-----	7
Gráfico 2: Curva de sobrevida em 5 anos de acordo com estágio do melanoma e período.-----	7

Resumo da Tese

Introdução:

O melanoma se caracteriza como uma doença heterogênea, de diferentes vias causais e manifestações clínicas, histopatológicas e biológicas. Métodos diagnósticos como a dermatoscopia e a microscopia confocal permitem aumentar a acurácia diagnóstica do melanoma e a detectar tumores em estágios iniciais.

Objetivos:

Classificar os melanomas de pacientes de alto risco de acordo com tipos morfológicos na microscopia confocal, a fim de correlacionar com características clínicas, fenotípicas e genéticas dos pacientes e critérios dermatoscópicos e histológicos dos tumores.

Material e Métodos:

Estudo transversal, retrospectivo, hospitalar, selecionou melanomas consecutivamente analisados por dermatoscopia e microscopia confocal e com confirmação de pesquisa de mutação em *CDKN2A* e *MC1R*.

Resultados:

Cinquenta e sete melanomas de cinquenta pacientes foram estudados à microscopia confocal: 23 (40,4%) tipo células dendríticas, 21 (36,8%) células redondas, 2 (3,5%) ninhos dérmicos, 2 (3,5%) combinado e 9 (15,8%) não classificável. O tipo células dendríticas foi caracterizado por idade mais avançada, fototipo cutâneo II e III, exposição solar mais intensa e de lentigos solares moderados a severos; foi mais frequente em lesões faciais e esteve associado ao subtipo histológico lentigo maligno. O tipo de células redondas apresentou tendência a ocorrer mais frequentemente em contexto familiar e em paciente fototipo I; esteve associado a características da microscopia confocal como espessamento da união dermoepidérmica, ninhos dérmicos

densos e células nucleadas dentro da papila dérmica. O melanomas tipo ninhos dérmicos e tipo combinado foram associados à ausência de retículo pigmentado à dermatoscopia e maior espessura tumoral na histologia. O tipo não classificável foi associado a índice dermatoscópico total e escores de microscopia confocal mais baixos. Pacientes com mutação em *CDKN2A* desenvolveram qualquer tipo de melanoma pela classificação de microscopia confocal.

Conclusão:

Características fenotípicas específicas de pacientes de alto risco estiveram associadas com tipos de melanoma classificados pela microscopia confocal. A expressão morfológica dos melanomas na microscopia confocal pode representar diferenças no estágio de crescimento e comportamento biológico do tumor.

Palavras-chave: melanoma, microscopia confocal, dermatoscopia

1. Introdução

1.1 Histórico do melanoma

O melanoma é o mais agressivo entre os cânceres de pele mais comuns, sendo responsável por 75% das mortes por câncer de pele (Siegel e cols, 2014). Apesar de facilmente curável em estágios precoces com tratamento cirúrgico, o melanoma apresenta alto potencial metastático, que aumenta em 10% a cada milímetro de espessura (Balch e cols, 2009).

O tratamento do melanoma tem sofrido mudanças dramáticas desde a primeira descrição da doença há mais de 200 anos. Após a primeira descrição da doença como “la melanose” em 1812 pelo francês René Laennec, foram relatados pelo inglês William Norris oito casos de melanoma na forma de revisão sistemática (Lee e cols., 2013). Características clínicas, epidemiológicas e genéticas, como a possível predisposição hereditária, a associação com múltiplos nevos e o fenótipo de pele clara foram propostas pelo autor na ocasião. Nesta época surgiram os primeiros princípios de manejo cirúrgico da doença, e se tornou evidente os desafios no tratamento de casos avançados.

O período de 1800 a 1970 foi marcado por grandes avanços no diagnóstico e abordagem terapêutica do melanoma. Entretanto, as três décadas seguintes foram marcadas por progresso limitado no campo terapêutico de casos metastáticos, com pouco sucesso ou limitado a grupos selecionados de pacientes.

Avanços recentes no tratamento do melanoma avançado são resultado de mais de 200 anos de investigação do papel do sistema imune e de vias moleculares no desenvolvimento do melanoma. A conclusão do projeto Genoma Humano contribuiu para a produção de DNA em larga escala e a identificação de oncogenes de alta frequência em melanoma, como *b-Raf*, *n-*

Ras e *c-Kit*. Logo estas mutações em kinases possibilitaram o desenvolvimento de terapias-alvo, que com o tempo produziram inibidores cada vez mais seletivos, utilizados atualmente em diferentes estágios de melanoma avançado (Lee e cols., 2013).

As evidências científicas atuais apontam que o melanoma se caracteriza como uma doença heterogênea, de diferentes vias causais e manifestações clínicas, histopatológicas e biológicas, e que requer cada vez mais abordagem e tratamento individualizados para cada caso (Nikolaou e Stratigos, 2013).

1.2 Epidemiologia do melanoma do Brasil e no mundo

A incidência do melanoma tem apresentado taxas crescentes, em especial em populações caucasianas. Pode surgir em qualquer idade, sendo o segundo câncer mais frequente abaixo dos 30 anos (Bleyer e cols., 2006). Por este motivo é um dos cânceres com maior número de anos produtivos de vida perdidos (Hanly e cols., 2015).

A taxa de incidência do melanoma tem aumentado de forma mais rápida do qualquer outro câncer prevenível nos Estados Unidos da América, com um acréscimo médio nos últimos 10 anos de 2,6% ao ano (Mrazek e Chao, 2014; Mayer e cols., 2014). Em indivíduos maiores de 60 anos, a porcentagem média anual estimada de aumento de incidência é de 6,5% (IC 95% 4,31-8,02%) para o período entre 1992 e 2005 (Nikolaou e Stratigos, 2013).

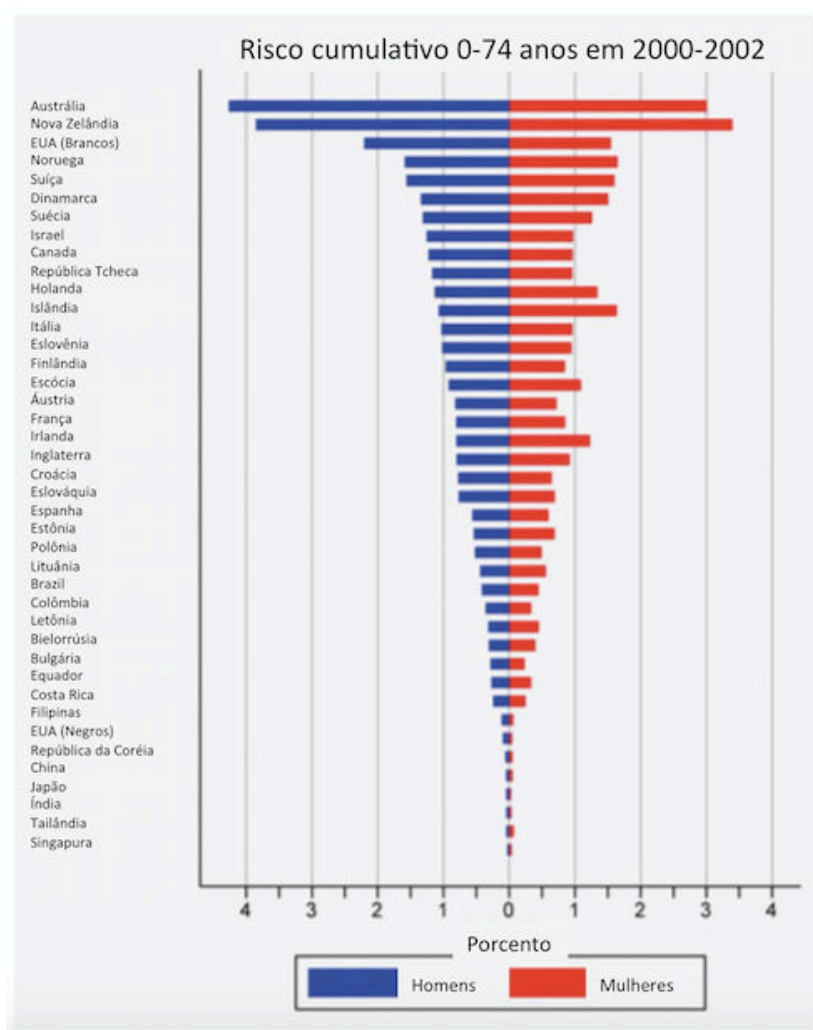
O aumento da detecção de lesões em estágios iniciais e o aumento da exposição recreacional à radiação ultravioleta podem ter contribuído para este aumento na incidência do melanoma (Linos e cols., 2009).

Historicamente é reconhecida inicialmente na Europa uma mudança comportamental e cultural a partir de 1920, que foi seguida por outros países ocidentais (Erdmann, 2013). Esta mudança de atitude representou um movimento de valorização da pele bronzeada como

símbolo de saúde, em detrimento da até então desejada pele clara associada a alto status econômico. No entanto, evidências da literatura sugerem que o acréscimo nas taxas de melanoma é predominantemente explicado pela detecção de melanomas finos, resultado tanto de melhorias em técnicas diagnósticas de detecção precoce, rastreamento e educação da população em relação a câncer de pele (Bataille e de Vries, 2008; de Haas e cols., 2010).

A diferença entre o percentual de risco cumulativo de desenvolvimento de melanoma dos 0 aos 74 anos de 2002 a 2012 em diferentes países pode ser vista na Figura 1, adaptada de Erdmann e cols. (2013).

Figura 1: Risco cumulativo de desenvolvimento de melanoma em diferentes países por sexo no período de 2000 a 2002.



Adaptada de Erdmann e cols., 2013.

O risco ao longo da vida (até os 75 anos) de desenvolver melanoma está claramente relacionado ao tipo de pele predominante e à localização geográfica do país. Na Austrália e Nova Zelândia o risco ao longo da vida chega a 3,6%, comparado a 1,9% nos Estados Unidos, 1,1% no Canadá e de 0,3% a 1,6% em países europeus. Em comparação, o risco ao longo da vida de melanoma em países asiáticos e sul americanos é muito mais baixo (Erdmann e cols., 2013).

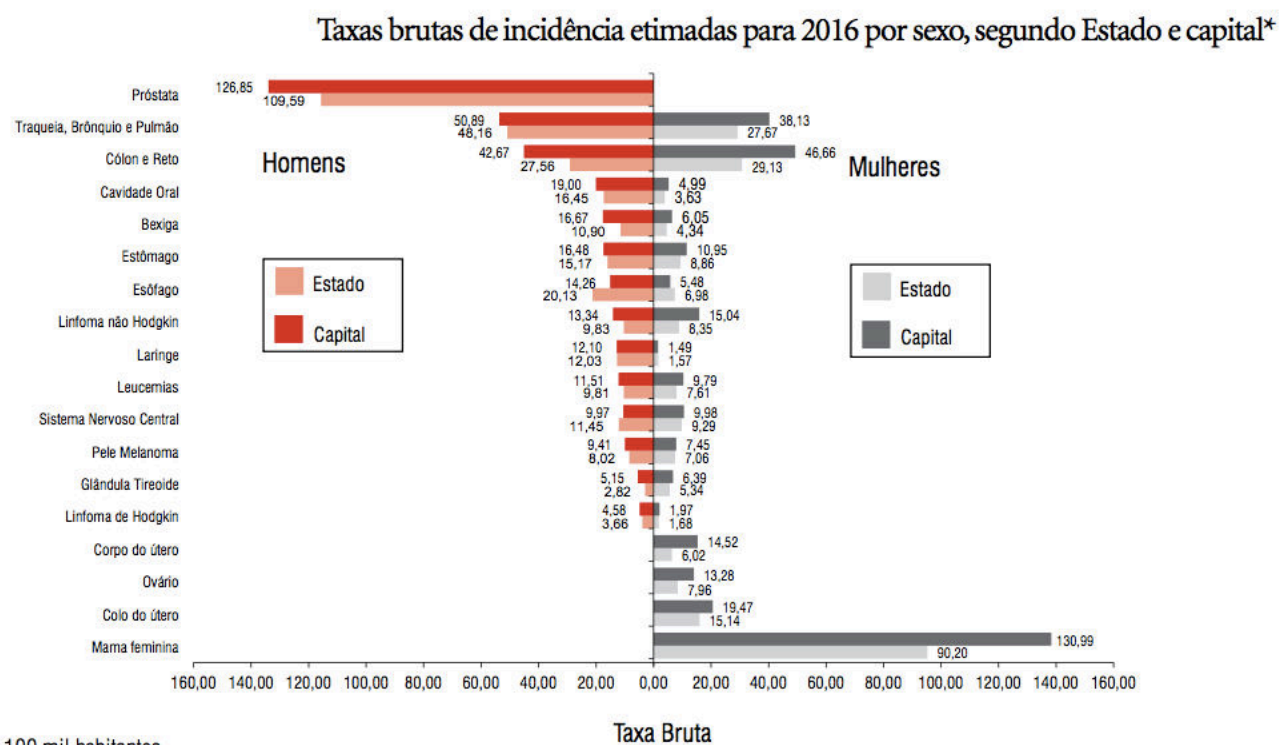
Globalmente, uma tendência de aumento na incidência da doença em populações de pele branca foi demonstrada nos últimos 50 anos, com cerca de 200.000 novos casos diagnosticados anualmente (Ferlay e cols., 2010; Siegel e cols., 2014, Erdman e cols., 2013; Nikolaou e Stratigos, 2013). O aumento dramático nas taxas de incidência a partir de 1990 é observado de forma consistente em indivíduos maiores de 60 anos, enquanto em diversos países tem-se observado estabilização ou mesmo leve queda da incidência em faixas etárias mais jovens (Nikolaou e Stratigos, 2013).

Apesar da detecção precoce de melanomas finos e da melhora de sobrevida observada em muitos países, as taxas de melanomas espessos tem permanecido estáveis ou mesmo apresentado aumento, em especial em grupos de idade avançada (Nikolaou e Stratigos, 2013). Além disso, em regiões de baixa condição socioeconômica, onde os indivíduos são menos propensos a programas de rastreamento, houve aumento nas taxas de incidência de tumores espessos (>4mm) e maior frequência de subtipos nodular e lentiginoso acral (Linos e cols., 2009; Azoury e Lange, 2014; Pollitt e cols., 2009).

Nova Zelândia e Austrália apresentam as maiores taxas de incidência do mundo, de 55,3 a 82,1 novos casos a cada 100.000 habitantes (Azoury e Lange, 2014). Nos Estados Unidos, de 2003 a 2007, as taxas de incidência em indivíduos branco foram em média de 25,4 novos casos a cada 100.000 homens e 16,9 novos casos a cada 100.000 mulheres (Jemal e cols., 2011; Kohler e cols., 2011). No Brasil, segundo dados da estimativa do INCA

(Instituto Nacional de Câncer), são previstos 5670 novos casos de melanoma no ano de 2016 em todo Brasil, 3000 novos casos em homens e 2670 em mulheres. A região sul é a de maior incidência em todo país, com uma taxa bruta de 6,96 novos casos a cada 100.000 habitantes em homens e 6,50 novos casos a cada 100.000 habitantes em mulheres. No Rio Grande do Sul, estima-se 8,02 novos casos para cada 100.000 homens e 7,06 novos casos a cada 100.000 mulheres, as maiores taxas brutas de incidência do país (Figura 2, Estimativa INCA 2016).

Figura 2: Taxas de incidência de melanoma estimada para 2016 no Rio Grande do Sul em homens e mulheres.



Adaptado de Estimativa INCA 2016

No Brasil, a incidência média de melanoma em homens é de 3,03 novos casos a cada 100.000 habitantes e em mulheres de 2,59 novos casos a cada 100.000 habitantes (INCA, 2016).

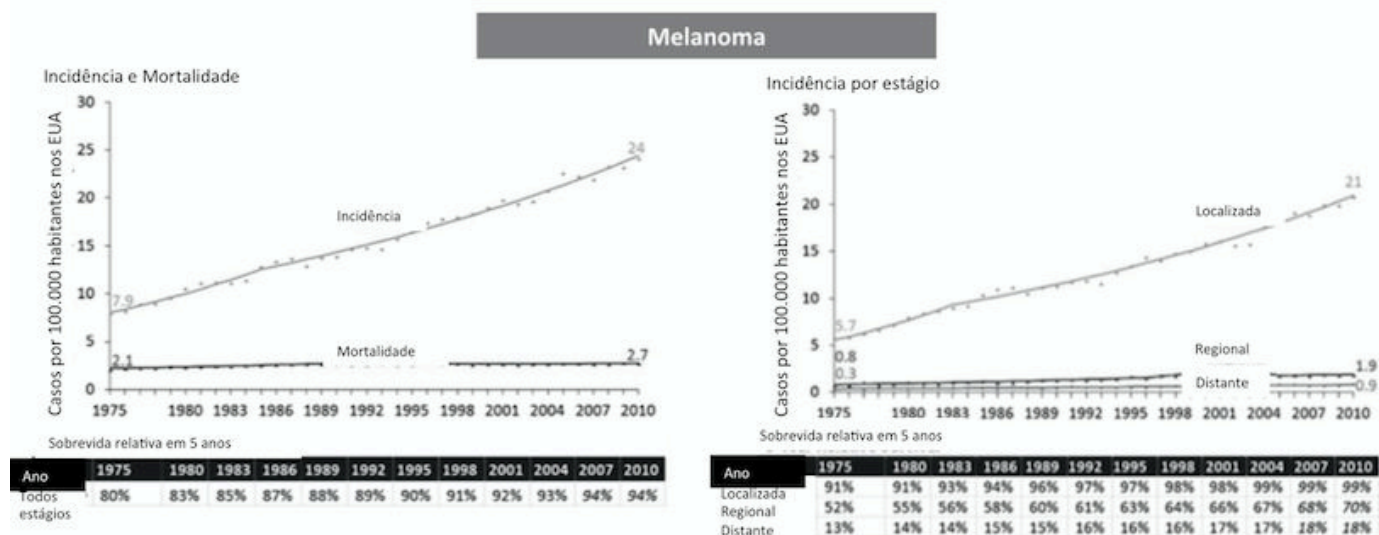
Apesar da maior incidência na região sul, estudos epidemiológicos na população desta região do Brasil tem demonstrado maior frequência de melanomas *in situ* (26,2%) e menores ou iguais a 1mm (36,9%), enquanto apenas 15,9% apresentaram mais de 4mm de espessura (Bonfá e col., 2011).

Ao contrário da crescente incidência do melanoma no mundo, as taxas de mortalidade pela doença permaneceram relativamente estáveis desde 1980, provavelmente refletindo a detecção precoce de tumores em estágios curáveis (Nikolau e Stratigos, 2014). Apesar da melhora na sobrevida de pacientes com melanoma descrita nas últimas décadas, as taxas de mortalidade pela doença continuam a aumentar em vários países da Europa como resultado da crescente incidência, em especial em indivíduos maiores de 60 anos (de Vries e cols., 2003; Erickson e Driscoll, 2007; Giblin e Thomas, 2007; MacKie e cols., 2007). No entanto, as curvas de mortalidade estão estáveis ou já apresentam queda em países como Austrália, Estados Unidos, Reino Unido e região da Escandinávia (Baade e Coory, 2005; Bataille e de Vries, 2008; Erickson e Driscoll, 2010; Tryggvadóttir e cols., 2010; Erdmann e cols., 2013).

A taxa de sobrevida de melanoma entre 1975 e 2010 aumentou de 80% para 94% nos Estados Unidos (EUA). A relação entre taxas de incidência e sobrevida crescentes e mortalidade estável sugere efeito do diagnóstico precoce (*overdiagnosis*), resultado tanto de rastreamento populacional como de achado acidental (Cho e cols., 2014).

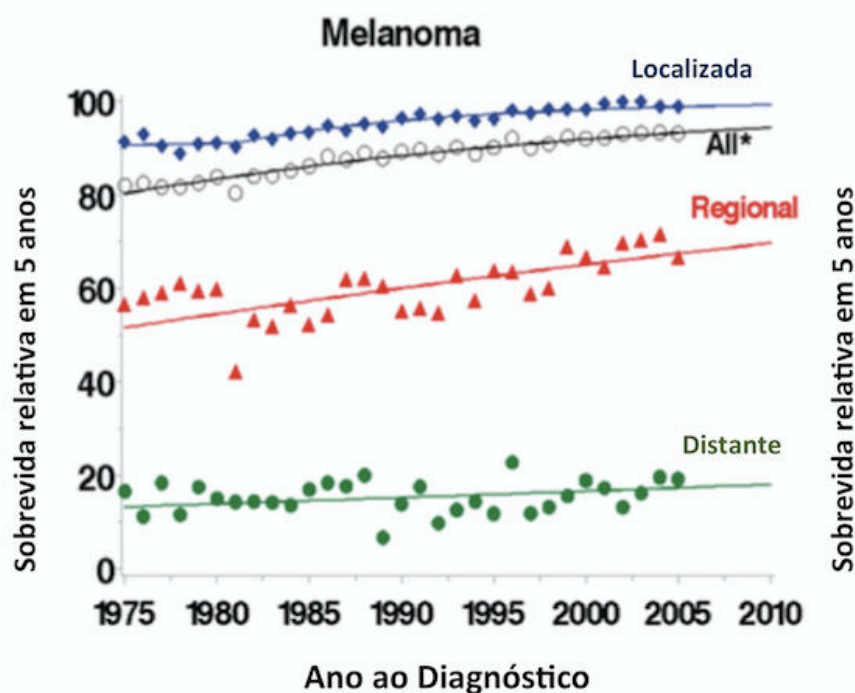
Os gráficos 1 e 2 demonstram a variação na incidência, mortalidade e sobrevida por melanoma nos EUA (Cho e cols., 2014).

Gráfico 1: Taxas de incidência e mortalidade de melanoma por 100.000 habitantes e taxa de incidência por estágio de melanoma por 100.000 habitantes.



Adaptado de Cho e cols., 2014.

Gráfico 2: Curva de sobrevida em 5 anos de acordo com estágio do melanoma e período.



Adaptado de Cho e cols., 2014.

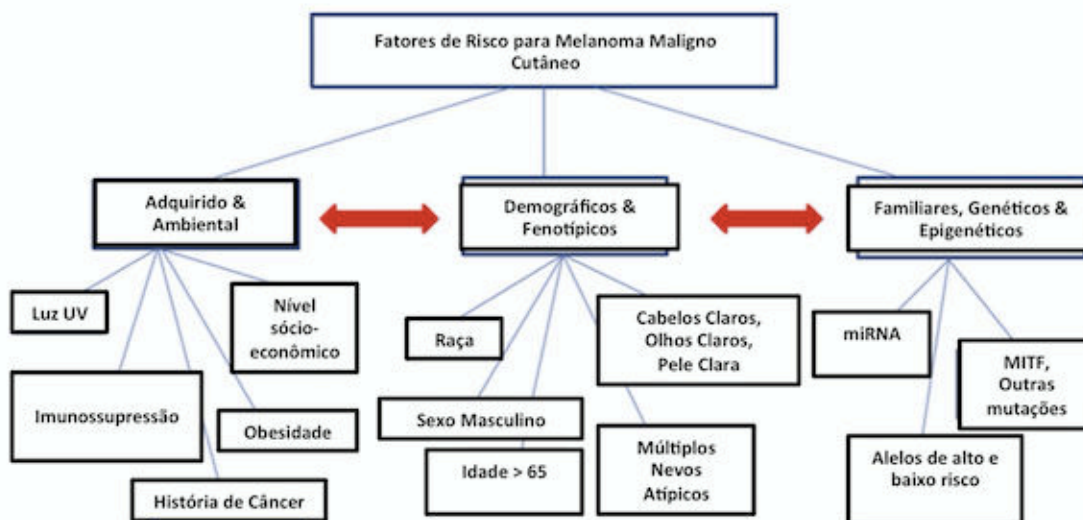
1.3 Fatores econômicos relacionados ao melanoma

A medida que a incidência de melanoma aumenta em todo o mundo, as implicações financeiras referente a medidas de rastreamento, diagnóstico, tratamento, seguimento e morte pela doença devem ser avaliadas. O diagnóstico precoce do melanoma reflete economicamente no sistema de saúde. Estima-se que o custo do tratamento de um paciente Estágio I é cerca de 40 vezes mais baixo do que o de um paciente em Estágio III ou IV. Juntos, os custos de tratamento dos Estágios III ou IV equivalem a 90% de todas despesas diretas anuais com a doença (Tsao e cols., 1998). O custo anual do tratamento do melanoma recentemente diagnosticado é estimado entre 563 milhões a mais de um bilhão de dólares nos Estados Unidos da América (Tsao e cols., 1998). Dado o custo substancial de tratamento do melanoma, as políticas de saúde pública devem incluir estratégias para estimular medidas de prevenção primária (redução da exposição à radiação ultravioleta) e prevenção secundária (detecção precoce) (Guy e cols., 2012). Uma estimativa avaliou que para demonstrar um impacto significativo em mortalidade, cerca de 40 milhões de dólares seriam necessários para rastrear uma população de 350 mil americanos maiores de 45 anos por um período de 4 anos de intervenção e 8 anos de seguimento (Geller, 2009).

1.4 Fatores de risco para melanoma

A etiologia do melanoma é complexa e heterogênea, envolvendo fatores de risco ambientais, fenotípicos e genéticos (Figura 3) (Azoury e cols., 2014; Potrony e cols., 2015).

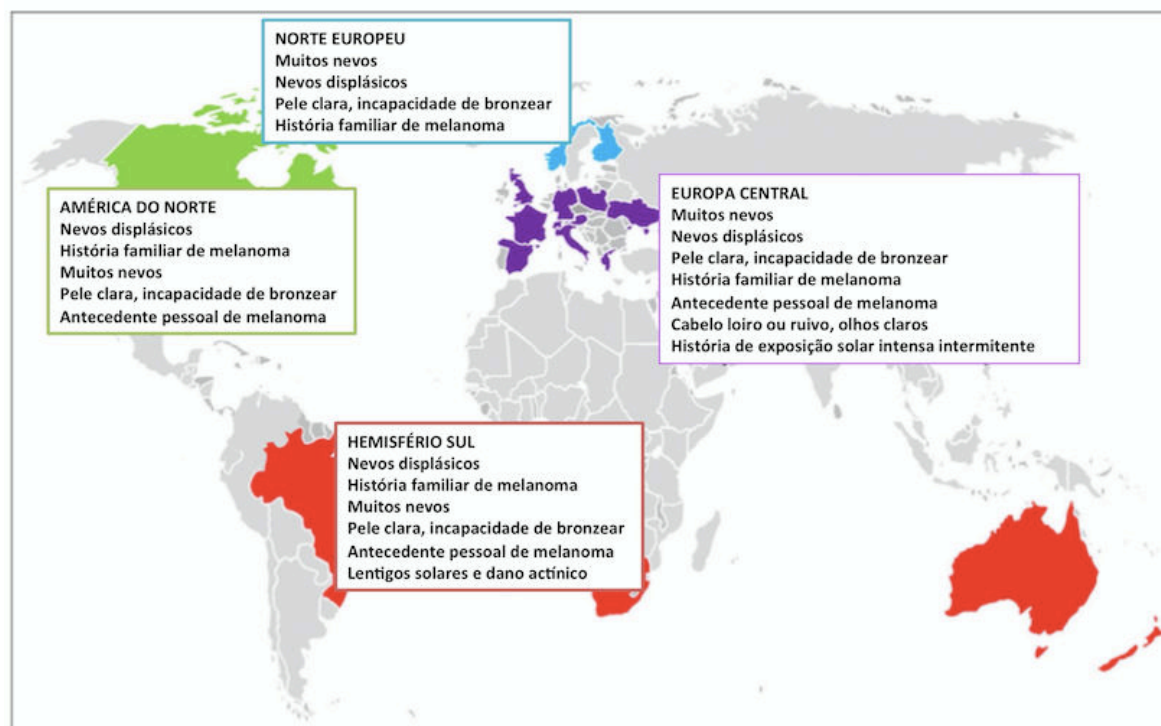
Figura 3: Relação entre múltiplos fatores de risco para melanoma cutâneo.



Adaptado de Azoury e cols., 2014.

A identificação, rastreamento e seguimento de pacientes de alto risco auxilia na otimização de estratégias de prevenção e manejo clínico (Watts e cols., 2015). Em geral, os fatores de risco que conferem o risco mais alto de acordo com uma análise sistemática (Watts e cols., 2015) foram: portador de mutação em *CDKN2A*, mais de 100 nevos, mais de 5 nevos atípicos e história familiar e pessoal de melanoma. Outros fatores como características fenotípicas e exposição à radiação ultravioleta foram citados pela maioria dos *guidelines* como fatores de risco para desenvolvimento de melanoma (Watts e cols., 2015). A figura 4 identifica quais fatores de risco, em ordem de frequência, são encontrados em mais de 50% dos *guidelines* por região ou continente (norte da Europa, Europa central, América do Norte e Canadá e países do hemisfério sul).

Figura 4: Fatores de risco, em ordem de frequência, são encontrados em mais de 50% dos *guidelines* por região ou continente.



Adaptado de Watts e cols., 2015.

Na região sul do Brasil, fatores de risco como fototipos I e II, grande número de nevus melanocíticos adquiridos (mais de 30), nevus displásicos e fotoproteção inadequada demonstraram aumento moderado de risco para melanoma (Bakos e cols., 2002). O uso de filtro solar de fator de proteção igual ou maior a 15 apresentou um fator de proteção ao melanoma (OR 0,2, IC 95% 0,1-0,8), enquanto a história de episódios frequentes de queimadura solar (30 ou mais) foi o fator de risco de maior associação com o desenvolvimento de melanoma na região (OR 11,4, IC 95% 2,6-50,5) (Bakos e cols., 2002).

1.4.1 Fatores de risco demográficos

Fatores demográficos como etnia, sexo e idade têm implicação direta na epidemiologia do melanoma. O melanoma ocorre de forma mais frequente em caucasianos do que qualquer outra etnia. Não caucasianos tendem a apresentar um estágio mais avançado ao diagnóstico e menor taxa de sobrevida em 5 anos comparado a caucasianos (Cormier e cols., 2006). Fatores como menor índice de suspeita de melanoma pelo médico nestas etnias, bem como localização anatômica mais infrequente como em região palmo-plantar e acesso mais limitado à educação e saúde parecem influenciar o prognóstico nestas populações (Azoury e Lange, 2014).

Em geral, o melanoma é mais frequente em homens do que mulheres (Azoury e Lange, 2014). No entanto, muitos estudos relatam uma melhor sobrevida em mulheres comparado a homens (Balch e cols., 2001; Lasithiotakis e cols., 2008).

1.4.2 Fatores de risco ambientais

O principal fator de risco ambiental para o desenvolvimento de melanoma é a exposição à radiação ultravioleta do sol (UV) e de fontes artificiais, como das câmaras de bronzamento (Guy e cols., 2012). A radiação UV causa dano ao DNA através da formação de dímeros de pirimidina, fotoprodutos, mutações genéticas, estresse oxidativo, inflamação e imunossupressão, favorecendo o processo de carcinogênese (Potrony e cols., 2015).

Uma análise sistemática e metanálise demonstrou que o uso de câmaras de bronzamento está associado a um aumento no risco de melanoma com um risco relativo geral de 1,20 (IC 95% 1,08-1,34) e de 1,87 (CI 95% 1,41-2,48) para primeira exposição antes de 35 anos (Boniol e cols., 2012). Cada sessão adicional por ano esteve associada a um aumento de risco de melanoma de 1,8% (IC 95% 0,0 – 3,8%) (Boniol e cols., 2012).

Exposição solar intermitente e queimaduras solares na infância são fatores de risco fortemente relacionados a melanoma no tronco e extremidades, com pouca variação em diferentes latitudes. Exposição solar ocupacional foi mais associada a melanoma de cabeça e pescoço, ou seja, áreas de exposição contínua. Esta associação foi mais pronunciada em populações de baixas latitudes, expostas a maior nível de radiação ultravioleta ambiental (Whiteman e cols., 2006). Um estudo de seguimento pelo período de 10 anos demonstrou que o uso diário de filtro solar reduz a taxa de detecção de melanoma, sugerindo que o uso regular de filtro solar pode prevenir o desenvolvimento da doença (Green e cols., 2011).

Além disso, alguns estudos sugerem associação entre exposição a pesticidas domésticos e ocupacionais e risco aumentado de melanoma (Fortes e cols., 2007). Um estudo de caso controle realizado no sul do Brasil demonstrou que indivíduos expostos a pesticidas (inseticida, rodenticida, herbicida e fungicida) apresentaram duas vezes mais risco de melanoma (OR 2,03, IC 95% 1,03-6,89) (Segatto e cols., 2015). Quando os pesticidas foram usados por mais de 10 anos em ambiente interno, o risco para melanoma aumentou ainda mais (OR 2,84 IC 95% 1,56-5,33). A alta frequência de uso de pesticidas em ambiente interno (4 ou mais vezes ao ano) esteve associada a 44% de aumento de risco para melanoma (OR 1,44 IC 95% 1,11 – 3,49). O uso doméstico de pesticidas em ambiente externo não esteve associado a aumento de risco para melanoma (Segatto e cols., 2015).

1.4.3 Fatores de risco fenotípicos e constitucionais

Características fenotípicas como cabelo loiro ou ruivo, olhos azuis ou verdes, pele clara com pouca capacidade de bronzeamento, sardas, múltiplos nevos melanocíticos (>100) e nevos melanocíticos atípicos (>5) são associados com aumento de risco de desenvolvimento de melanoma (Potrony e cols., 2015). Indivíduos com mais de 100 nevos

apresentam um risco quase 7 vezes maior de melanoma (RR 6,89, IC 95% 4,63-10,25) do que pessoas com poucos nevos (<15). O número de nevos comuns indica também um risco de melanoma significativo entre indivíduos com número intermediário de nevos (<40), comparado aos com poucos nevos (<15) (RR 1,45 IC 95% 1,36-1,59) (Gandini e cols., 2005). A presença de nevos atípicos também foi associada a aumento de risco de melanoma em uma série de estudos, formando um gradiente de risco de acordo com o número de lesões atípicas. O risco relativo de pacientes portadores de um nevo displásico é de 1,45 (IC 95% 1,31-1,60), 3,03 (2,23-4,06) para pacientes com 3 displásicos e 6,36 (6,80-10,33) para aqueles com 5 nevos displásicos (Gandini e cols., 2005).

Além disso, indivíduos com antecedente pessoal de câncer de pele do tipo melanoma ou não melanoma apresentam um risco relativo de melanoma de cerca de 3 vezes de desenvolver melanoma (Azoury e Lange, 2014). História pessoal de melanoma aumenta em 5 a 8% o risco de um segundo melanoma (Levi e cols., 2005).

A ancestralidade também parece ser um fator associado a risco em muitas populações, em especial no sul do Brasil (Bakos e cols., 2009). Pacientes com ancestralidade italiana ou alemã apresentaram maior risco relativo para melanoma na região sul (3,5 IC 95% 1,8-6,7; 9,7 IC 95% 3,9-24,2, respectivamente), enquanto a ancestralidade indígena foi associada a efeito protetor (RR 0,16 IC 95% 0,04-0,7) (Bakos e cols., 2009).

1.4.4 Fatores de risco genéticos

História familiar de melanoma é amplamente associada a um aumento de risco de melanoma (Gandini e cols., 2005). Aproximadamente 5 a 10% dos melanomas ocorre em um contexto familiar (Florell e cols., 2005), seguindo um padrão de transmissão autossômica dominante com penetrância incompleta (Anderson e cols., 1991). Indivíduos com múltiplos

melanomas primários também podem apresentar susceptibilidade hereditária a melanoma (Puig e cols., 2005).

Em pacientes com melanoma classificados na categoria de melanoma familiar, o risco de desenvolvimento da doença é de 30 a 70 vezes maior do que na população em geral (Kefford e cols., 1999). O critério de inclusão para aconselhamento genético de melanoma na América Latina foi recentemente considerado semelhante ao de países de incidência média-baixa: ao menos dois melanomas em pacientes com múltiplos melanomas primários esporádicos e dois ou mais casos em familiares em primeiro ou segundo grau (Puig e cols., 2015).

Genes de alto risco para melanoma são genes que apresentam mutações que conferem um alto risco de desenvolvimento da doença, e são usualmente associados a múltiplos casos de melanoma em uma família. O inibidor da quinase 2A dependente de ciclina (*CDKN2A*) foi o primeiro gene associado a suscetibilidade a melanoma. Mutações neste gene são encontradas em 20% das famílias de alto risco, porém a frequência pode variar de 5 a 72%, dependendo do critério utilizado e da área geográfica. Já a frequência de mutação neste gene em pacientes com múltiplos melanomas primários esporádicos é de cerca de 9% (Potrony e cols., 2015).

A penetrância para o desenvolvimento de melanoma em portadores de mutação no *CDKN2A* varia conforme localização geográfica, níveis de exposição solar e presença de mutações em outros genes modificadores, aumentando com a idade. Aos 50 anos, a penetrância para melanoma em portadores de mutação em *CDKN2A* foi de 13% na Europa, 50% nos Estados Unidos e 32% na Austrália; aos 80 anos a penetrância foi de 58% na Europa, 76% nos Estados Unidos e 91% na Austrália (Potrony e cols., 2015).

Na população da região sul do Brasil, a variante genética p.A148T do gene *CDKN2A* foi significativamente mais frequente em pacientes com melanoma do que em controles

(12,6% versus 3,9%, $p=0,009$) (Bakos e cols., 2011), sendo considerada um alelo de susceptibilidade nesta região. Todos pacientes do estudo portadores da variante genética p.A148T referiram ancestralidade europeia, especialmente germânica, o que foi confirmado por painéis de marcadores de ancestralidade (Bakos e cols., 2011).

Outro gene de susceptibilidade a melanoma é o receptor da melanocortina 1 (*MC1R*), regulador chave da pigmentação cutânea e codificador do receptor 1 do hormônio estimulador de melanócito α (α -MSH). As variantes mais comuns do *MC1R*, classificadas como r (p.V60L, p.V92M, p.R163Q) são fracamente associadas ao fenótipo cabelo ruivo, enquanto as classificadas como R (p.D84E, p.R142H, p.R151C, p.I155T, p.R160W, p.D294H) são altamente associadas ao fenótipo cabelo ruivo, e são as mais implicadas em suscetibilidade a melanoma. O risco conferido a portadores é de 2 vezes por alelo R na população em geral e de 3 vezes no contexto familiar. O risco é aditivo, sendo que portadores de 2 alelos R apresentam risco de desenvolvimento de melanoma 4 a 6 vezes maior do que a população em geral (Potrony e cols., 2015). A variante r p.R163Q é associada a aumento de risco para melanoma em áreas geográficas de alta incidência de exposição solar e ao subtipo de melanoma associado a fotodano crônico, lentigo maligno melanoma (Puig-Butille e cols., 2013).

Na região sul do Brasil, a frequência de mutações em genes de alta susceptibilidade, em especial no *CDKN2A*, é baixa por estudos em populações de alto risco (Ashton-Prolla e cols., 2008, Grazziotin e cols., 2012). As duas mutações funcionais encontradas no estudo de 29 famílias do sul do Brasil em um estudo de 2008, G-34T e M53I, são provavelmente originadas na Europa. (Ashton-Prolla e cols., 2008). Mais recentemente a frequência de variantes infrequentes no *CDKN2A* e no *MC1R* foi estudada em uma amostra de indivíduos de alto risco (história pessoal ou familiar de melanoma) da região sul do Brasil (Grazziotin e cols., 2012). A presença dos polimorfismos C540U, G442A e C500G foi demonstrada em

24,2% dos participantes, sendo que ao menos 70% apresentava alguma variante infrequente no *MCIR* (Grazziotin e cols., 2012).

1.5 Estratégias clínicas para a identificação precoce do melanoma

O melanoma é uma neoplasia maligna de linhagem melanocítica que mais frequentemente se origina de melanócitos da pele, mas também pode surgir de melanócitos de órgãos internos, como o sistema nervoso central. Apesar de poder surgir a partir de um nevo preexistente, a maioria dos melanomas primários não se encontra associado a um nevo precursor (Bastian e cols., 2014).

O reconhecimento precoce do melanoma é a intervenção mais efetiva para melhorar o prognóstico da doença (Salerni e cols., 2011). No entanto, nenhuma técnica diagnóstica não invasiva isoladamente permite a detecção correta em todos os casos, devido à heterogeneidade de apresentação clínica da doença (Stanganelli e cols., 2015).

Na maioria dos casos o melanoma apresenta um componente intrapidérmico, quando as células malignas proliferam ao longo da camada basal (melanoma *in situ*). Após um período de meses a anos, os melanócitos malignos não proliferam somente radialmente, mas também adquirem a capacidade de invadir verticalmente para a derme (melanoma invasivo) (Zalaudek e cols., 2008). No entanto, em alguns casos o melanoma pode apresentar ausência de comprometimento epitelial significativo, como nos melanomas uveais ou nodulares (Bastian e cols., 2014).

Segundo a Organização Mundial de Saúde (OMS) o melanoma pode ser classificado em quatro subtipos histológicos principais: espalhamento superficial, nodular, lentigo maligno e lentiginoso acral.

O melanoma de espalhamento superficial (MES) é o subtipo histológico mais frequente (65-70%), caracterizado por uma fase inicial macular de pigmento geralmente marrom a preto com modificação gradual de cor, tamanho e formato (Bologna e cols., 2012; Dummer e cols., 2016). Posteriormente o MES desenvolve uma área papular ou nodular de crescimento vertical. Este subtipo pode por vezes apresentar coloração avermelhada, azul-acinzentada ou hipopigmentada (Bologna e cols., 2012).

O melanoma nodular (MN) representa cerca de 15-20% dos melanomas e geralmente não apresenta critérios clínicos da regra do ABCD para lesões clinicamente suspeitas (assimetria, bordas irregulares, cores variadas, diâmetro > 6mm). Tempo mais curto de evolução e espessura mais avançada no momento do diagnóstico reforçam a importância do acréscimo do E (evolução, elevação) para a regra do ABCD (Greenwald e cols., 2012). Geralmente o MN se apresenta como um nódulo enegrecido ou azulado, às vezes de cor vermelha ou rosa (amelanótico), que pode sangrar ou ulcerar (Bologna e cols., 2012). O acrônimo “EFG” (E=elevado, F=firme, G=*growing*, crescimento progressivo por mais de um mês) indica critérios clínicos para o diagnóstico do MN (Giacomel e cols., 2008).

O lentigo maligno (LM) e o lentigo maligno melanoma (LMM, forma invasiva do LM) é responsável por cerca de 10-15% dos melanomas e surge em média em torno da sétima década de vida, em áreas cronicamente fotoexpostas como face e pescoço, mas também na região superior do tronco e extremidades (Pralong e col., 2012). O reconhecimento do LMM em estágios precoces pode ser difícil pela semelhança com lentigos solares e ceratoses actínicas pigmentadas (Alarcon e cols., 2014).

O melanoma lentiginoso acral (MLA) acomete a região palmo-plantar e o aparelho ungueal, representando 5% de todos os melanomas. Em negros, no entanto, este subtipo histológico pode chegar a 70% dos melanomas diagnosticados. O MLA tipicamente se apresenta como uma mácula marrom ou preta com variação de cor e bordas irregulares.

Quando amelanótico pode ser confundido com verruga ou outras condições benignas (Bologna e cols., 2012). O melanoma do aparelho ungueal (MAU) é pouco frequente (0,1-2,8%), com uma incidência relativa mais alta entre indivíduos de etnia afriacana e asiática (Richert e cols., 2013). Quando originado na matriz ungueal, o melanoma se apresenta como uma pigmentação longitudinal (melanoníquia estriada) caracterizada por fundo acastanhado com linhas irregulares em cor, espessura e paralelismo. O MAU pode também surgir no leito ungueal, formando um nódulo que pode ulcerar ou sangrar, com destruição da lâmina ungueal. O Sinal de Hutchinson descreve a presença de pigmento na prega ungueal proximal, lateral ou distal, e representa a fase de crescimento radial do melanoma subungueal (Levit e cols., 2000).

Variantes raras de melanoma como o melanoma nevíde e de mucosa representam não mais do que 5% dos melanomas (Dummer e cols., 2016).

Apesar da classificação em subtipos histológicos facilitar o reconhecimento e diagnóstico das variações de apresentação clínica e histopatológica do melanoma, apresenta limitações em prever prognóstico e resposta terapêutica dos tumores (Viros e cols., 2008).

1.5.1 Método em duas etapas: dermatoscopia digital e mapeamento corporal total

A dermatoscopia surgiu como um método diagnóstico de primeira linha, comprovadamente aumentando a sensibilidade e especificidade do diagnóstico de melanoma comparado ao olho nú (Menzies, 2013). Esta técnica diagnóstica utiliza um equipamento de magnificação seguido da aplicação de um líquido na superfície cutânea ou lentes de polarização cruzada. A dermatoscopia permite a visualização de estruturas morfológicas submacroscópicas da epiderme até a derme, não visíveis a olho nú (Menzies, 2013). Estudos de metanálise demonstraram que a dermatoscopia aumenta a acurácia diagnóstica para

melanoma comparado ao olho nú, atribuindo um grau de evidência A de recomendação (conjunto de evidências confiáveis para guiar a prática clínica) (Menzies, 2013).

Entretanto, em pacientes com múltiplos nevos atípicos (Figura 5), pode ser difícil distinguir melanoma iniciais de nevos, tanto na apresentação clínica como dermatoscópica (Argenziano e cols., 2011; Puig e Malvehy, 2013).

Figura 5: Fenótipo de paciente portador de síndrome do nevo atípico.



Arquivo pessoal

No contexto de pacientes de alto risco com síndrome do nevo atípico, o seguimento por dermatoscopia digital e mapeamento corporal total é uma das estratégias mais eficientes em detectar melanomas iniciais, reduzindo a necessidade de exéreses desnecessárias (Kittler e Binder, 2001). A dermatoscopia digital permite a identificação de melanomas que não apresentam critérios específicos de malignidade em um primeiro momento (Salerni e cols.,

2012). Diversos estudos demonstram que lesões melanocíticas benignas de pacientes com síndrome do nevo atípico tendem a ficar estáveis ao longo do tempo, enquanto o melanoma apresenta modificações graduais (Puig e Malvehy, 2013).

Enquanto o mapeamento corporal total permite a detecção de lesões novas ou modificações macroscópicas em lesões preexistentes, a dermatoscopia digital permite a identificação precisa de modificações nas cores e estruturas das lesões mais atípicas (Puig e Malvehy, 2013). Em um estudo recente de 10 anos de seguimento, a combinação de mapeamento corporal e dermatoscopia digital foi superior ao uso de qualquer uma das técnicas isoladas (Salerni e cols., 2011).

Em estudos observacionais prospectivos comparando o uso de dermatoscopia versus dermatoscopia digital, houve uma significativa melhora na taxa de exérese de lesões benignas por melanoma no grupo usando dermatoscopia digital (8,1 versus 2,4). Além disso, 55% dos melanomas foram detectados exclusivamente por dermatoscopia digital no último grupo (Tromme e cols., 2012).

A proporção de melanomas *in situ* ou finos detectados por seguimento digital e mapeamento corporal total é maior do que o esperado na população geral (Salerni e cols., 2013).

1.5.2 Microscopia confocal de reflectância (MCR)

A MCR é utilizada como um método diagnóstico não invasivo complementar, combinada à dermatoscopia, para aumentar a especificidade diagnóstica de tumores cutâneos, incluindo melanoma (Pellacani e cols., 2014). Há mais de 10 anos é utilizada para estudo de tumores melanocíticos e não melanocíticos *in vivo* como uma técnica diagnóstica complementar para aumentar a acurácia diagnóstica de lesões equívocas à dermatoscopia

(Pellacani e cols., 2007; Segura e cols., 2009). A microscopia confocal é uma técnica não invasiva que permite o exame da epiderme e derme papilar com resolução a nível celular.

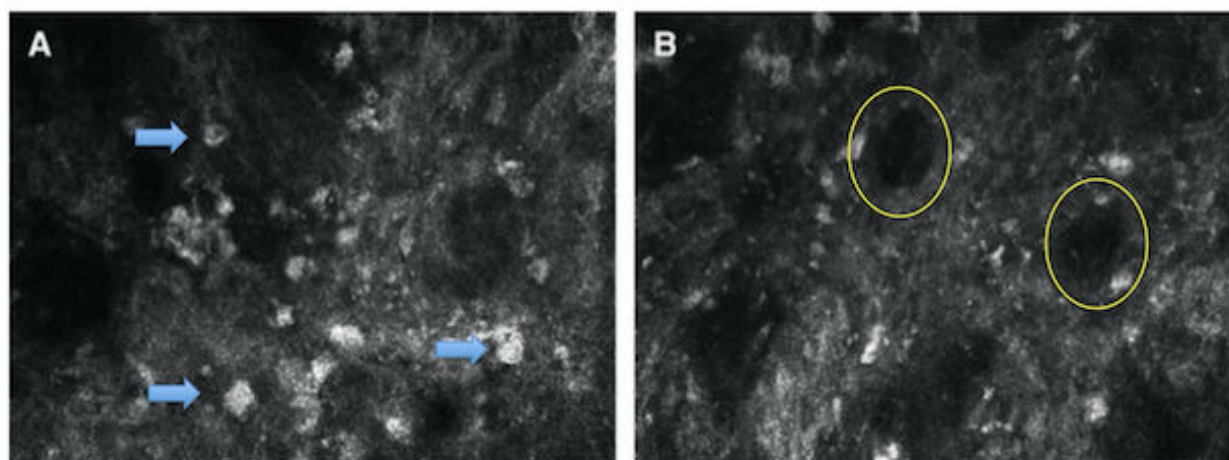
A MCR comercialmente disponível (Vivascope ®) utiliza um laser diodo de 830 nm com um comprimento de luz próximo ao infravermelho. O laser de 830 nanomilímetros não causa danos ao tecido cutâneo, mas limita a profundidade da imagem a 200-300µm, que corresponde à derme papilar, dependendo da localização. O laser diodo é uma fonte de luz monocromática e coerente que penetra na pele e ilumina um pequeno ponto dentro do tecido. Esta luz é refletida, vai para um pinhole e forma uma imagem no detector. O pequeno pinhole não permite que a luz refletida (reflectância) de outros pontos do tecidos atinja o detector. Desta forma, apenas a luz refletida da região focal (confocal) é detectada. Através de movimentos consecutivos do laser sobre a pele, uma imagem bi-dimensional representando secções horizontais da pele em mosaico é obtida. Cada unidade individual registrada é formada por um campo de 500µm x 500µm, e a área máxima de visualização por lesão é de até 8x8mm. O contraste das imagens na MCR resulta da diferença de reflectividade dos tecidos, que depende de estruturas moleculares e químicas. Estruturas com maior índice refrativo aparecem brilhantes na MCR. Melanina e melanossomos são as fontes naturais de contraste mais intenso, formando um tom esbranquiçado na MCR (Kolm e Braun, 2012).

Diversos estudos tem demonstrado aumento de acurácia com o uso da MCR para lesões equívocas, com melhora significativa na detecção de melanomas incipientes, iniciais ou amelanóticos e redução no número necessário de exéreses desnecessárias (Alarcon e cols., 2014). Segundo estudo de Alarcon e cols. (2014), o número necessário de lesões a serem excisadas (*number needed to treat ratio* – NNT) para diagnóstico de um melanoma obtido com o uso da dermatoscopia foi de 3,73, enquanto ao associar-se o uso da MCR, o NNT resulta em 2,87, e reduz ainda mais, para 1,12, com o uso isolado da MCR ($P < 0,0001$). Estes dados favorecem o uso da MCR para aumentar a acurácia diagnóstica e reduzir a necessidade

de exéreses desnecessárias, bem como o impacto econômico associado ao manejo do câncer cutâneo (Alarcon e cols., 2014).

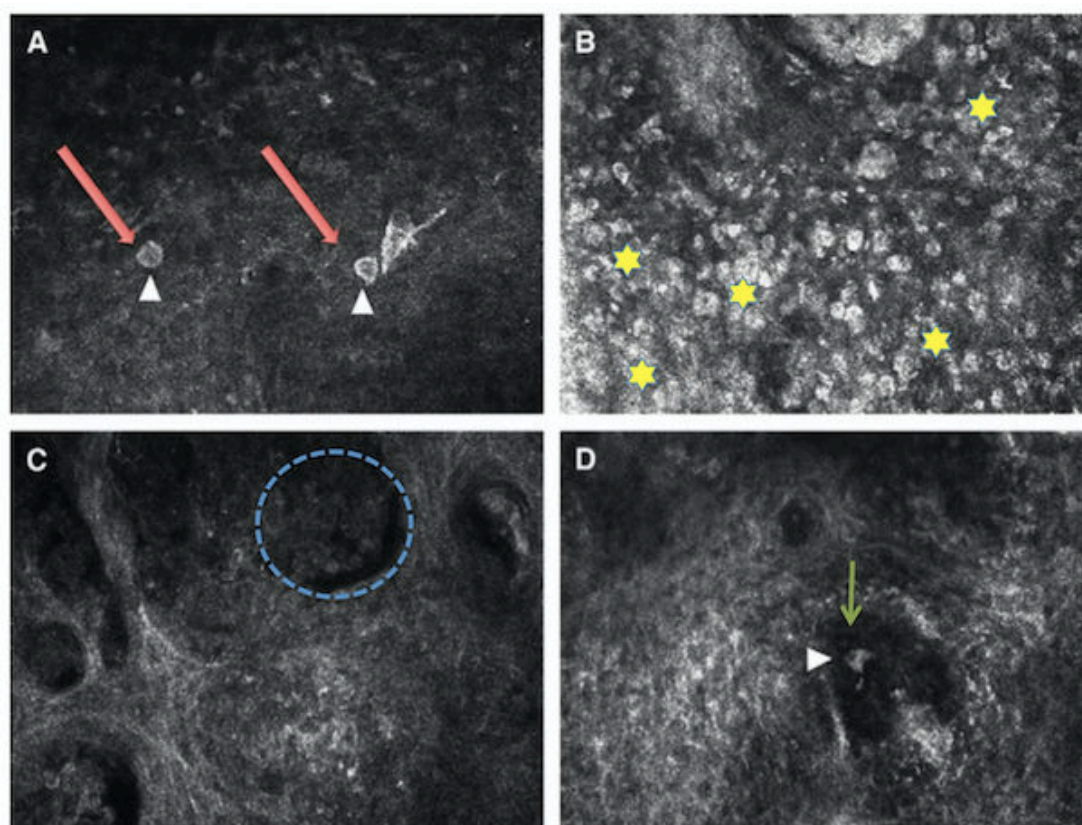
Os critérios para diferenciação de lesões melanocíticas benignas e malignas por MCR foram descritos em estudos europeus que resultaram na elaboração de escores para diagnóstico de tumores cutâneos pela técnica de microscopia confocal (Pellacani e cols., 2005; Segura e cols., 2009). Pellacani e cols. (2005) descreveram inicialmente os seis critérios associados independentemente por análise multivariada ao diagnóstico de melanoma: presença de papilas dérmicas mal delimitadas, células atípicas na camada basal, células isoladas nucleadas dentro da papila dérmica, células pagetóides, infiltração disseminada de células pagetóides e ninhos cerebriformes. O escore total maior ou igual a 3 pontos é sugestivo de melanoma (97,3% de sensibilidade e 72,3% de especificidade), sendo os dois primeiros critérios descritos (atipia citológica marcada nas células da camada basal e papilas mal delimitadas na união dermoepidérmica (Figura 6) considerados critérios maiores (2 pontos) e os demais (células pagetóides redondas em camadas superficiais, células pagetóides difusas na lesão, ninhos cerebriformes na derme papilar e células nucleadas dentro da papila dérmica (Figura 7), critérios menores (1 ponto) (Pellacani e cols., 2005).

Figura 6: Critérios maiores para diagnóstico de melanoma pela microscopia confocal segundo escore proposto por Pellacani e cols. (2005).



A. Atipia citológica marcada na camada basal (setas azuis). B. Papilas dérmicas mal delimitadas na união dermoepidérmica (círculos amarelos). Adaptado de Pellacani e cols., 2005.

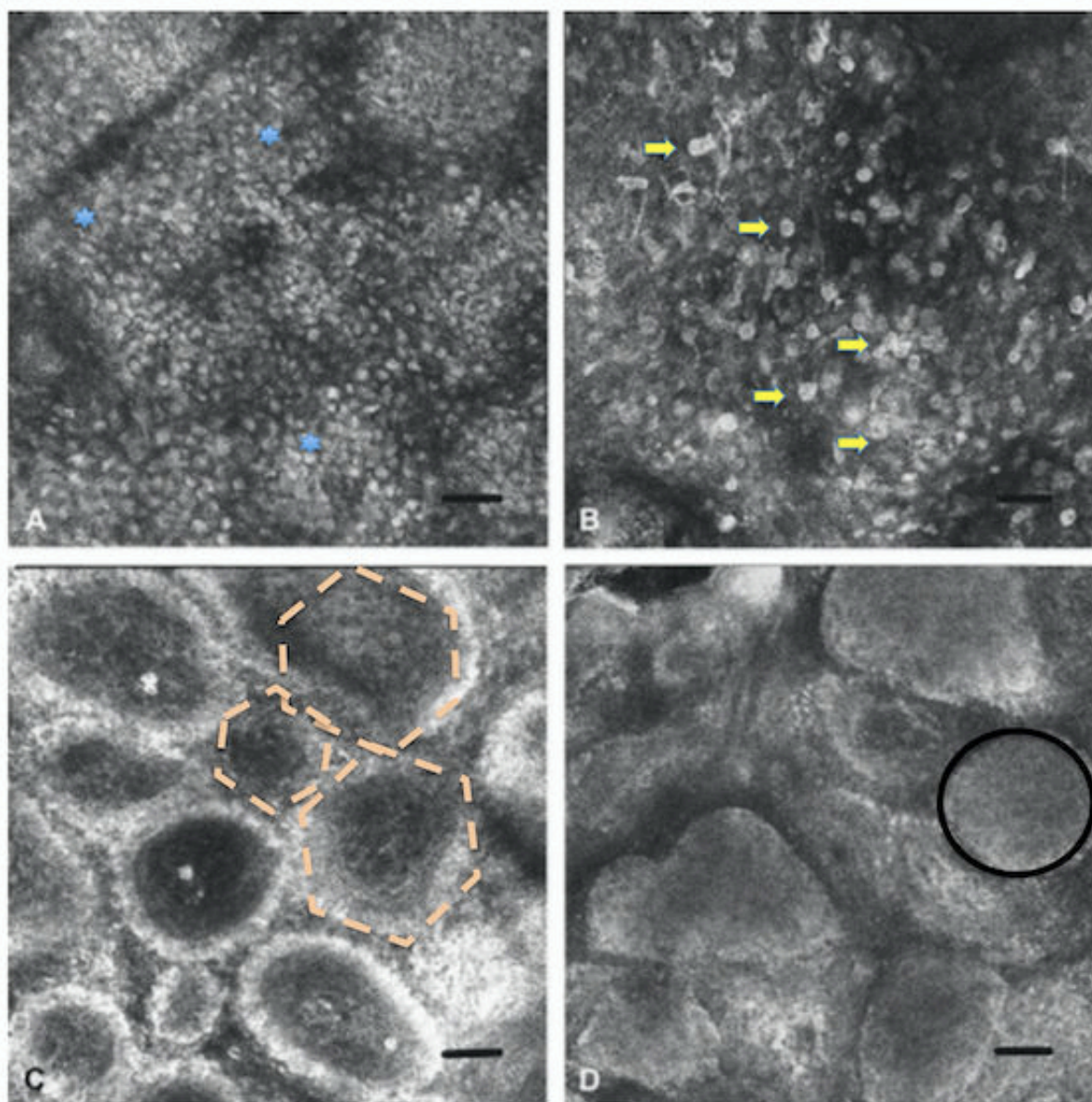
Figura 7: Critérios menores para diagnóstico de melanoma pela microscopia confocal segundo escore proposto por Pellacani e cols. (2005).



A. Células redondas em camadas superficiais em disseminação pagetóide (setas vermelhas). B. Células pagetóides disseminadas ao longo da lesão (asteriscos amarelos). C. Ninhos cerebriformes na derme papilar (círculo azul). D. Células nucleadas dentro da papila dérmica (seta verde). Adaptado de Pellacani e cols., 2005.

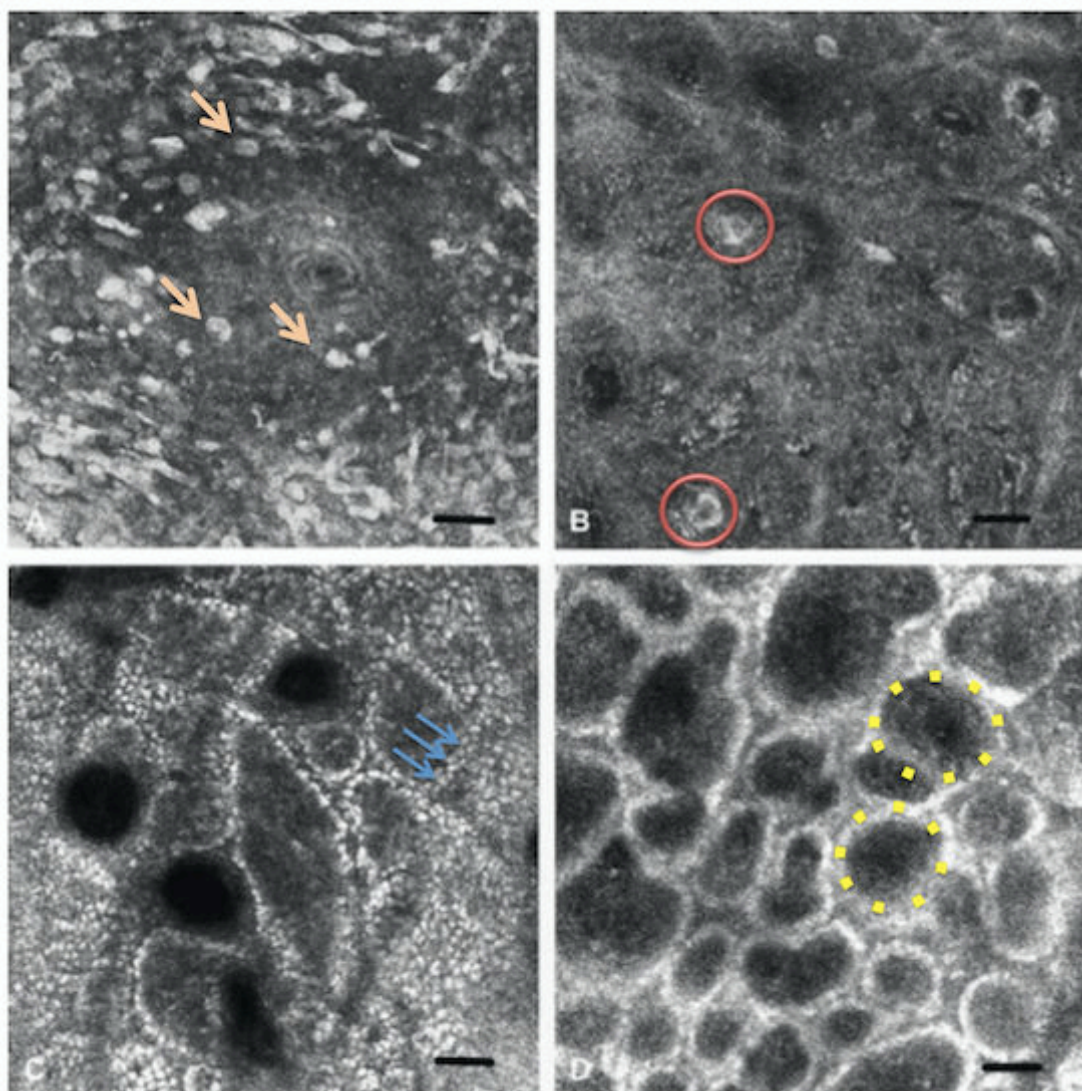
Posteriormente Segura e cols. (2009) descrevem um algoritmo em duas etapas, sendo na primeira etapa utilizados critérios para diferenciação de lesão melanocítica de não melanocítica. Na primeira etapa, quatro critérios na microscopia confocal foram associados estatisticamente com o diagnóstico de lesão melanocítica: padrão em pedra de calçamento (*cobblestone pattern*), células em disseminação pagetóide, padrão de rede (*mesh appearance*) das papilas dérmicas disseminado na união dermoepidérmica e ninhos dérmicos (Figura 8) (Segura e cols., 2009).

Figura 8: Critérios morfológicos para diferenciação entre lesão melanocítica e não melanocítica segundo escore proposto por Segura e cols. (2009).



- A. Padrão epidérmico em pedra de calçamento (*cobblestone pattern*, asteriscos azuis).
B. Disseminação pagetóide (setas amarelas). C. Padrão em rede (*mesh appearance*) disseminado das papilas dérmicas na união dermoepidérmica (tracejado laranja). D. Ninhos dérmicos na derme papilar (círculo preto). Adaptado de Segura e cols., 2009.

Figura 9: Critérios morfológicos para diferenciação entre lesão melanocítica benigna e maligna segundo escore proposto por Segura e cols. (2009).

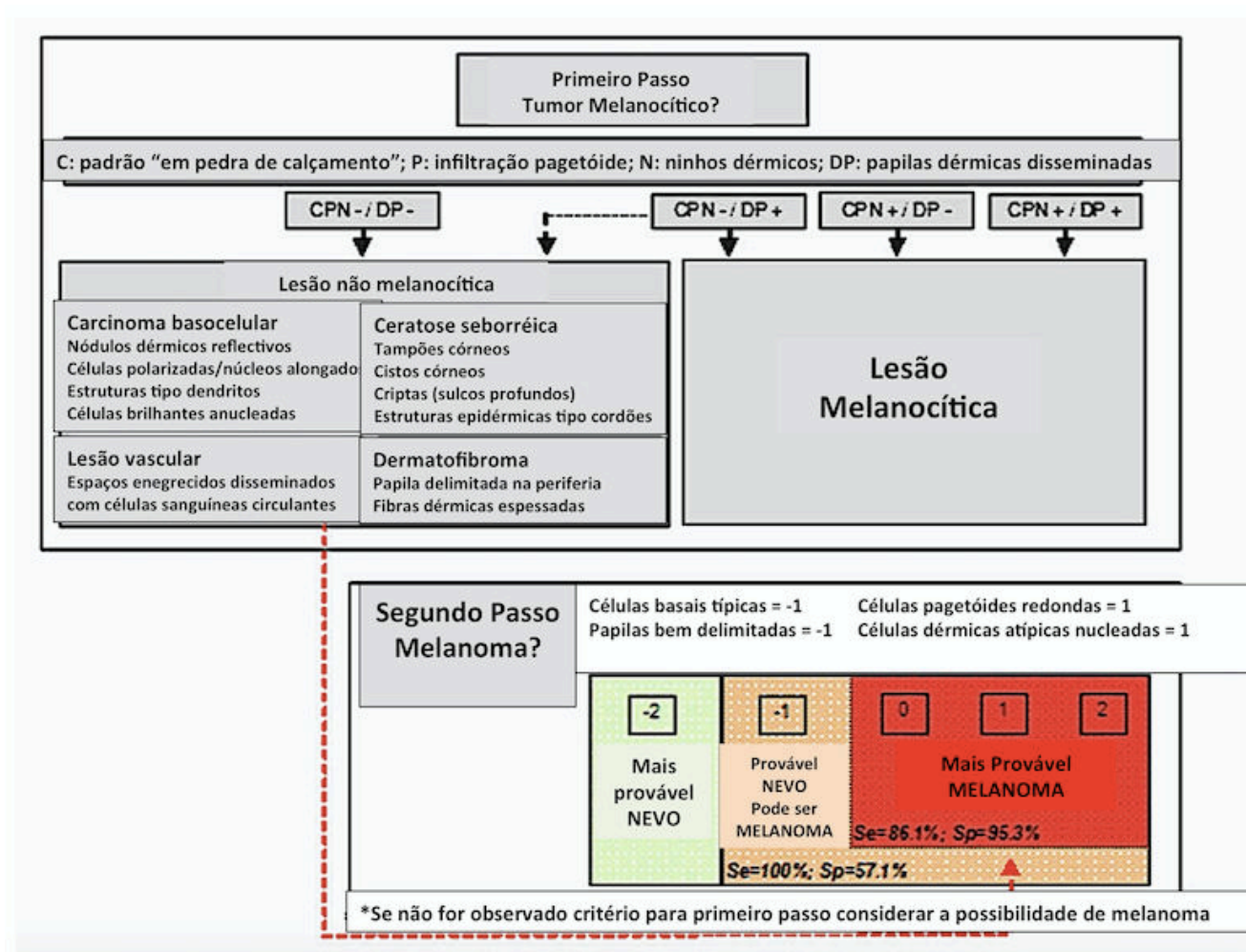


Critérios associados com melanoma (A e B) e nevo (C e D). A. Células pagetóides redondas (setas laranja). B. Células nucleadas atípicas na derme (círculos vermelhos). C. Células basais típicas (setas azuis). D. Papilas bem delimitadas (pontos amarelos). Adaptado de Segura e cols., 2009.

Na segunda etapa são utilizados 4 critérios maiores para diferenciar nevo de melanoma, dois critérios protetivos e dois fatores de risco para melanoma. A presença de papilas dérmicas bem delimitadas e células típicas na camada basal foi associada ao

diagnóstico de nevo, e a cada um dos critérios foi atribuído o valor de -1. A presença de células pagetóides redondas suprabasais e células nucleadas atípicas na derme foi associada ao diagnóstico de melanoma, sendo que cada um dos critérios de risco pontua o valor de 1 (Figura 9). Neste estudo, lesões sugestivas de melanoma por dermatoscopia que apresentaram escore maior ou igual a 0 (um fator de risco associado a um protetivo, ou dois fatores de risco independentemente dos protetivos) foram diagnosticadas como melanoma com uma especificidade de 95,3% e valor preditivo positivo de 91,2% (Figura 10) (Segura e cols., 2009).

Figura 10: Algoritmo proposto por Segura e colaboradores (2009) para diagnóstico por microscopia confocal de lesões equívocas à dermatoscopia.



Adaptado de Segura e cols., 2009.

1.6 Classificação morfológica do melanoma

A classificação do melanoma é baseada em características anatômicas, epidemiológicas e padrão de progressão (Segura e cols., 2008). Tradicionalmente os melanomas são classificados histopatologicamente de acordo com o comportamento biológico e padrão de crescimento em lentigo maligno melanoma, melanoma de espalhamento superficial, melanoma nodular e melanoma lentiginoso acral. (Pellacani e cols., 2014; Viros e cols., 2008). Apesar da categorização proposta ter validade para representação clínica e histológica, seu impacto no manejo clínico é limitado. O principal motivo é o fato de não haver diferença em sobrevida total ou resposta terapêutica entre as categorias quando tumores de espessura equivalente são comparados ou após a ocorrência de metástases.

Estudos recentes têm demonstrado diferenças moleculares e genéticas entre os subtipos de melanoma, que podem determinar variação na evolução natural da doença (Segura e cols., 2008). No exame histopatológico a arquitetura tumoral (como disseminação pagetóide e formação de ninhos) parece estar associada a diferentes variações genéticas e padrões de crescimento (Viros e cols., 2008). A reclassificação morfológica dos melanomas primários pode ser usada para formar subgrupos que são geneticamente e/ou biologicamente mais homogêneos e que provavelmente diferem em desfechos clínicos e padrão de metástases, facilitando a estratificação para terapia (Viros e cols., 2008).

Uma nova classificação morfológica baseada em subtipos de melanoma por critérios de MCR foi proposta recentemente por Pellacani e cols. (2014). Os autores descrevem quatro tipos de melanoma à microscopia confocal: melanoma de células dendríticas, melanoma de células redondas, melanoma de ninhos dérmicos e melanoma de tipos combinados. O melanoma de células dendríticas é caracterizado por predomínio de população de células dendríticas e padrão anular ou reticular fino na união dermoepidérmica. O melanoma de

células redondas é formado principalmente por grandes células redondas formando agregados ou ninhos intraepidérmicos e padrão reticular alargado na união dermoepidérmica. O melanoma tipo ninhos dérmicos é caracterizado por grandes agregados celulares (ninhos cerebriformes) invadindo a derme. A combinação dos três padrões é considerado melanoma de tipo combinado e a ausência de padrão definido como melanoma não classificável (Pellacani e cols., 2014).

Neste estudo os melanomas de células dendríticas se apresentaram tipicamente como lesões planas e grandes, caracterizadas por retículo pigmentado na dermatoscopia, com baixo índice de Breslow na histopatologia, o que falaria a favor de melanomas de crescimento lento. Já os de células redonda apresentaram menor diâmetro clinicamente e maior espessura histologicamente do que os de células dendríticas e ocorreram principalmente em pacientes com número alto de nevus. Histologicamente os melanomas de células redondas tipicamente demonstraram um padrão de crescimento predominantemente horizontal, com invasão da derme em alguns casos. Melanomas de ninhos dérmicos apresentaram maior espessura no momento do diagnóstico e menor diâmetro clinicamente, sugerindo padrão de crescimento rápido, invasão dérmica e melanócitos pobremente diferenciados (Pellacani e cols., 2014).

Em resumo, a microscopia confocal permite distinguir diferentes tipos celulares em uma lesão. A conexão entre a morfologia e o comportamento biológico dos tumores pode ser útil para o desenvolvimento de estratégias *in vivo* orientadas morfolologicamente para diagnosticar e tratar diferentes tipos de melanoma (Pellacani e cols., 2014).

O objetivo desta tese é avaliar o potencial da MCR como ferramenta confiável para classificar melanomas em subtipos morfológicos e correlaciona-los com fenótipos clínicos e características genéticas dos pacientes com melanoma familiar e múltiplo.

1.7 Referências bibliográficas

Alarcon I, Carrera C, Palou J, Alos L, Malveyh J, Puig S. Impact of in vivo reflectance confocal microscopy on the number needed to treat melanoma in doubtful lesions. *Br J Dermatol*. 2014 Apr;170(4):802-8.

Anderson DE, Badzioch MD: Hereditary cutaneous malignant melanoma: a 20-year family update. *Anticancer Res*. 1991;11:433-7.

Argenziano G1, Catricalà C, Ardigo M, Buccini P, De Simone P, Eibenschutz L, Ferrari A, Mariani G, Silipo V, Zalaudek I. Dermoscopy of patients with multiple nevi: Improved management recommendations using a comparative diagnostic approach. *Arch Dermatol*. 2011 Jan;147(1):46-9.

Ashton-Prolla P, Bakos L, Junqueira G Jr, Giugliani R, Azevedo SJ, Hogg D. Clinical and molecular characterization of patients at risk for hereditary melanoma in southern Brazil. *J Invest Dermatol*. 2008 Feb;128(2):421-5.

Azoury SC, Lange JR. Epidemiology, risk factors, prevention, and early detection of melanoma. *Surg Clin North Am*. 2014;94:945-62.

Baade P, Coory M. Trends in melanoma mortality in Australia: 1950-2002 and their implications for melanoma control. *Aust N Z Public Health*. 2005;29:383-386.

Bakos L, Masiero NC, Bakos RM, Burtet RM, Wagner MB, Benzano D. European ancestry and cutaneous melanoma in Southern Brazil. *J Eur Acad Dermatol Venereol*. 2009 Mar;23(3):304-7.

Bakos L, Wagner M, Bakos RM, Leite CS, Sperhacke CL, Dzekaniak KS et al. Sunburn, sunscreens, and phenotypes: some risk factors for cutaneous melanoma in southern Brazil. *Int J Dermatol*. 2002 Sep;41(9):557-62.

Bakos RM, Besch R, Zoratto GG, Godinho JM, Mazzotti NG, Ruzicka T, et al. The CDKN2A p.A148T variant is associated with cutaneous melanoma in Southern Brazil. *Exp Dermatol*. 2011 Nov;20(11):890-3.

Balch BCM, Soong S, Gershenwald JE, Thompson JF, Reintgen DS, Cascinelli N et al. Prognostic factors analysis of 17,600 melanoma patients: melanoma staging system. *J Clin Oncol*. 2001;19:3622-34.

Balch CM, Gershenwald JE, Soong SJ, Thompson JF, Atkins MB, Byrd DR et al. Final version of 2009 AJCC melanoma staging and classification. *J Clin Oncol*. 2009;27:6199-206.

Bastian B. The molecular pathology of melanoma: an integrated taxonomy of melanocytic neoplasia. *Annu Rev Pathol Mech Dis*. 2014;9:239-71.

Bataille V, de Vries E. Melanoma--Part 1: epidemiology, risk factors, and prevention. *BJM* 2008; 337: a2249.

Bleyer A, Viny A, Barr R. Cancer in 15- to 29-year-olds by primary site. *Oncologist* 2006;11:590-601.

Bolognia JL, Jorizzo JL, Schaffer JV. Chapter 113, Melanoma, from *Dermatology*, 3rd Edition, 2012.

Bonfá R, Bonamigo RR, Bonfá R, Duro KM, Furian RD, Zelmanowicz Ade M. Early diagnosis of cutaneous melanoma: an observation in southern Brazil. *An Bras Dermatol*. 2011;86(2):215-21.

Boniol M, Autier P, Boyle P, Gandini S. Cutaneous melanoma attributed to sunbed use: systematic review and meta-analysis. *BJM* 2012;345:e4757.

Cho H, Mariotto AB, Schwartz LM, Luo J, Woloshin H. When do changes in cancer survival mean progress? The insight from population incidence and mortality. *J Natl Cancer Inst Monogr*. 2014;49:187-97.

Cormier JN, Xing Y, Ding M, Lee JE, Mansfield PF, Gershenwald JE et al. Ethnic Differences Among Patients With Cutaneous Melanoma. *Arch Intern Med*. 2006;166:1907-1914.

de Haas E, Nijsten T, de Vries E. Population education in preventing skin cancer: from childhood to adulthood. *J Drugs Dermatol*. 2010;2: 112–6.

de Vries E, Bray FI, Coebergh JWW, Parkin DM. Changing epidemiology of malignant cutaneous melanoma in Europe 1953-1997: rising trends in incidence and mortality but recent stabilizations in western Europe and decreases in Scandinavia. *Int J Cancer*. 2003;107:119–26.

Dummer R, Siano M, Hunger RE, Lindenblatt N, Braun R, Michielin O, et al. The updated Swiss guidelines 2016 for the treatment and follow-up of cutaneous melanoma. *Swiss Med Wkly*. 2016 Feb 22;146:w14279.

Erdmann F, Lortet-Tieulent J, Schüz J, Zeeb H, Greinert R, Breitbart EW *et al*. International trends in the incidence of malignant melanoma 1953-2008--are recent generations at higher or lower risk? *Int J Cancer*. 2013;132:385–400.

Erickson C, Driscoll MS. Melanoma epidemic: Facts and controversies. *Clin Dermatol*. 2010;28:281–6.

Ferlay Jacques, Shin Hai-Rim, BrayFreddie, Forman David MC and PDM. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *Int. J. Cancer*. 2010;127:2893–2917.

Florell SR, Boucher KM, Garibotti G, et al. Population- based analysis of prognostic factors and survival in familial melanoma. *J Clin Oncol*. 2005;23:7168-77.

Fortes C, Mastroeni S, Melchi F, Pilla MA, Alotto M, Antonelli G, et al. The association between residential pesticide use and cutaneous melanoma. *Eur J Cancer*. 2007

Apr;43(6):1066-75.

Gandini S, Sera F, Cattaruzza MS, et al. Meta-analysis of risk factors for cutaneous melanoma: I. Common and atypical naevi. *Eur J Cancer*. 2005;41(1):28–44.

Geller AC. Educational and screening campaigns to reduce deaths from melanoma. *Hematol Oncol Clin North Am* 2009;23:515–27.

Giacomel J, Zalaudek I, Mordente I, Nicolino R, Argenziano G. Never perform laser treatment of skin tumors with clinical "EFG" criteria. *J Dtsch Dermatol Ges*. 2008;6(5):386-8.

Giblin a-V, Thomas JM. Incidence, mortality and survival in cutaneous melanoma. *J Plast Reconstr Aesthet Surg*. 2007;60:32–40.

Grazziotin TC, Rey MC, Bica CG, Pinto LA, Bonamigo RR, Puig-Butille JA, et al. Genetic variations of patients with familial or multiple melanoma in Southern Brazil. *J Eur Acad Dermatol Venereol*. 2013 Feb;27(2):e179-85.

Green AC, Williams GM, Logan V, et al. Reduced melanoma after regular sunscreen use: randomized trial follow-up. *J Clin Oncol*. 2011;29:257-63.

Greenwald HS, Friedman EB, Osman I. Superficial spreading and nodular melanoma are distinct biological entities: a challenge to the linear progression model. *Melanoma Res*. 2012;22(1):1-8.

Guy GP, Ekwueme DU, Tangka FK, Richardson LC. Melanoma treatment costs: a systematic review of the literature, 1990-2011. *Am J Prev Med*. 2012;43:537–45.

Hanly P, Soerjomataram I, Sharp L. Measuring the societal burden of cancer: the cost of lost productivity due to premature cancer-related mortality in Europe. *Int J Cancer*. 2015;136:E136-45.

Instituto Nacional do Câncer José Alencar Gomes da Silva (INCA), Ministério da Saúde. Estimativa 2016. Incidência de Câncer no Brasil.

Jemal A, Saraiya M, Patel P, Cherala SS, Barnholtz-Sloan J, Kim J et al. Recent trends in cutaneous melanoma incidence and death rates in the United States, 1992-2006. *J Am Acad Dermatol*. 2011;65:S17–25.e1–3.

Kefford RF, Newton Bishop JA, Bergman W, Tucker MA. Counseling and DNA testing for individuals perceived to be genetically predisposed to melanoma: a consensus statement of the Melanoma Genetic Consortium. *J Clin Oncol*. 1999;17:3245–51.

Kittler H, Binder M. Risks and benefits of sequential imaging of melanocytic skin lesions in patients with multiple atypical nevi. *Arch Dermatol*. 2001;137(12): 1590–5.

Kohler B a, Ward E, McCarthy BJ, Schymura MJ, Ries L a G, Ehemann C et al. Annual report to the nation on the status of cancer, 1975-2007, featuring tumors of the brain and other nervous system. *J Natl Cancer Inst*. 2011;103:714–36.

Kolm I and Braun RP. How reflectance confocal microscopy works. Chapter 2. From Reflectance Confocal Microscopy for Skin Diseases. Hofmann-Wellenhof R, Pellacani G, Malvehy J, Soyer HP. 1st Ed. 2012

Lasithiotakis K, Leiter U, Meier F, Eigentler T, Metzler G, Moehrle M et al. Age and gender are significant independent predictors of survival in primary cutaneous melanoma. *Cancer*. 2008;112:1795–804.

Lee C, Collichio F, Ollila D, Moschos S. Historical review of melanoma treatment and outcomes. *Clin Dermatol*. 2013;31:141–7.

Levi F, Randimbison L, Te VC, et al. High constant incidence rates of second cutaneous melanomas. *Int J Cancer*. 2005;117:877-9.

Levit EK, Kagen MH, Scher RK, Grossman M, Altman E. The ABC rule for clinical detection of subungual melanoma. *J Am Acad Dermatol*. 2000;42(2 Pt 1):269-274.

Linos E, Swetter SM, Cockburn MG, Colditz G a, Clarke C a. Increasing burden of melanoma in the United States. *J Invest Dermatol*. 2009;129:1666–74.

MacKie RM, Bray C, Vestey J, Doherty V, Evans a, Thomson D et al. Melanoma incidence and mortality in Scotland 1979-2003. *Br J Cancer*. 2007;96:1772–7.

Mayer JE, Swetter SM, Fu T, Geller AC. Screening, early detection, education, and trends for melanoma: Current status (2007-2013) and future directions. *J Am Acad Dermatol*. 2014;71:599.e1–599.e12.

Menzies SW. Evidence-based dermoscopy. *Dermatol Clin*. 2013;31:521-24.

Mrazek A a, Chao C. Surviving cutaneous melanoma: a clinical review of follow-up practices, surveillance, and management of recurrence. *Surg Clin North Am*. 2014;94:989–1002.

Nikolaou V, Stratigos a J. Emerging trends in the epidemiology of melanoma. *Br J Dermatol*. 2014;170:11–9.

Pellacani G, Cesinaro AM, Seidenari S. Reflectance-mode confocal microscopy of pigmented skin lesions--improvement in melanoma diagnostic specificity. *J Am Acad Dermatol*. 2005 Dec;53(6):979-85.

Pellacani G, De Pace B, Reggiani C, Cesinaro AM, Argenziano G, Zalaudek I, Soyer HP, Longo C. Distinct melanoma types based on reflectance confocal microscopy. *Exp Dermatol*. 2014 Jun;23(6):414-8.

Pellacani G, Guitera P, Longo C, Avramidis M, Seidenari S, Menzies S. The impact of in vivo reflectance confocal microscopy for the diagnostic accuracy of melanoma and equivocal melanocytic lesions. *J Invest Dermatol*. 2007;127: 2759-65.

Pellacani G, Pepe P, Casari A, Longo C. Reflectance confocal microscopy as a second-level

examination in skin oncology improves diagnostic accuracy and saves unnecessary excisions: a longitudinal prospective study. *Br J Dermatol*. 2014 Nov;171(5):1044-51.

Pollitt R a, Clarke C a, Swetter SM, Peng DH, Zadnick J, Cockburn M. The expanding melanoma burden in California hispanics: Importance of socioeconomic distribution, histologic subtype, and anatomic location. *Cancer*. 2011;117:152–61.

Potrony M, Badenas C, Aguilera P, Puig-Butille JA, Carrera C, Malvehy J, et al. Update in genetic susceptibility in melanoma. *Ann Transl Med*. 2015 Sep;3(15):210.

Pralong P, Bathelier E, Dalle S, Poulalhonn N, Debarbieux S, Thomas L. Dermoscopy of lentigo maligna melanoma: report of 125 cases. *Br J Dermatol*. 2012 Aug;167(2):280-7.

Puig-Butille JA, Carrera C, Kumar R, Garcia-Casado Z, Badenas C, Aguilera P, et al. Distribution of MC1R variants among melanoma subtypes: p.R163Q is associated with lentigo maligna melanoma in a Mediterranean population. *Br J Dermatol*. 2013;169:804-11.

Puig S, Malvehy J, Badenas C, Ruiz A, Jimenez D, Cuellar F, et al. Role of the CDKN2A locus in patients with multiple primary melanomas. *J Clin Oncol*. 2005;23:3043-51.

Puig S, Malvehy J. Monitoring patients with multiple nevi. *Dermatol Clin*. 2013;31:565-77.

Puig S, Potrony M, Cuellar F, Puig-Butille JA, Carrera C, Aguilera P et al. Characterization of individuals at high risk of developing melanoma in Latin America: bases for genetic counseling in melanoma. *Genet Med*. 2015;Dec17(Ahead of Print).

Richert B, Lecerf P, Caucanas M, André J. Nail tumors. *Clin Dermatol*. 2013;31(5):602-17.

Salerni G, Carrera C, Lovatto L, Martí-Laborda RM, Isern G, Palou J, et al. Characterization of 1152 lesions excised over 10 years using total-body photography and digital dermatoscopy in the surveillance of patients at high risk for melanoma. *J Am Acad Dermatol*. 2012;67(5):836-45.

Salerni G, Lovatto L, Carrera C, Puig S, Malvehy J. Melanomas detected in a follow-up program compared with melanomas referred to a melanoma unit. *Arch Dermatol*. 2011;147(5):549–55.

Salerni G, Terán T, Puig S, Malvehy J, Zalaudek I, Argenziano G, et al. Meta-analysis of digital dermoscopy follow-up of melanocytic skin lesions: a study on behalf of the International Dermoscopy Society. *J Eur Acad Dermatol Venereol*. 2013;27(7):805-14.

Segatto MM, Bonamigo RR, Hohmann CB, Müller KR, Bakos L, Mastroeni S, et al. Residential and occupational exposure to pesticides may increase risk for cutaneous melanoma: a case-control study conducted in the south of Brazil. *Int J Dermatol*. 2015 Dec;54(12):e527-38.

Segura S, Pellacani G, Puig S, Longo C, Bassoli S, Guitera P, Palou J, Menzies S, Seidenari S, Malvehy J. In vivo microscopic features of nodular melanomas: dermoscopy, confocal microscopy, and histopathologic correlates. *Arch Dermatol*. 2008. Oct;144(10):1311-20.

Segura S, Puig S, Carrera C, Palou J, Malvehy J. Development of a two-step method for the diagnosis of melanoma by reflectance confocal microscopy. *J Am Acad Dermatol*. 2009 Aug;61(2):216-29.

Siegel R, Ma J, Zou Z, et al. Cancer statistics, 2014. *CA Cancer J Clin*. 2014;64:9-29.

Siegel R, Naishadham D, Jemal A. Cancer statistics, 2013. *CA Cancer J Clin*. 2013;63:11–30.

Stanganelli I, Longo C, Mazzoni L, Magi S, Medri M, Lanza G, Farnetani F, Pellacani G. Integration of reflectance confocal microscopy in sequential dermoscopy follow-up improves melanoma detection accuracy. *Br J Dermatol*. 2015 Feb;172(2):365-71.

Tromme I, Sacré L, Hammouch F, Legrand C, Marot L, Vereecken P, et al. Availability of digital dermoscopy in daily practice dramatically reduces the number of excised melanocytic lesions: results from an observational study. *Br J Dermatol*. 2012;167(4):778-86.

Tryggvadóttir L, Gislum M, Hakulinen T, Klint A, Engholm G, Storm HH et al. Trends in the survival of patients diagnosed with malignant melanoma of the skin in the Nordic countries 1964-2003 followed up to the end of 2006. *Acta Oncol*. 2010;49:665–72.

Tsao H, Rogers GS, Sober AJ. An estimate of the annual direct cost of treating cutaneous melanoma. *J Am Acad Dermatol* 1998;38:669-80.

Viros A, Fridlyand J, Bauer J, Lasithiotakis K, Garbe C, Pinkel D, Bastian BC. Improving melanoma classification by integrating genetic and morphologic features. *PLoS Med*. 2008 Jun 3;5(6):e120.

Watts CG, Dieng M, Morton RL, Mann GJ, Menzies SW, Cust AE. Clinical practice guidelines for identification, screening and follow-up of individuals at high risk of primary cutaneous melanoma: a systematic review. *Br J Dermatol*. 2015 Jan;172(1):33-47.

Whiteman DC, Stickley M, Watt P et al. Anatomic site, sun exposure, and risk of cutaneous melanoma. *J Clin Oncol*. 2006; 24:3172– 7.

Zalaudek I, Marghoob AA, Scope A, Leinweber B, Ferrara G, Hofmann-Wellenhof R, et al. Three roots of melanoma. *Arch Dermatol*. 2008;144(10):1375-1379.

2. Objetivos

O objetivo geral deste projeto foi classificar os melanomas cutâneos de pacientes de alto risco com múltiplos melanomas primários e/ou melanoma familiar da Unidade de Melanoma do Hospital Clínic de Barcelona de acordo com tipos morfológicos na MCR e determinar a associação com características clínico-epidemiológicas e genéticas dos pacientes.

2.1 Objetivos específicos

- a) Classificar os melanomas em cinco tipos morfológicos pela MCR: tipo células dendríticas, células redondas, ninhos dérmicos, combinado e não classificável.
- b) Estudar a associação dos tipos classificados pela MCR com características fenotípicas e genéticas dos pacientes e morfológicas (achados de dermatoscopia, confocal e histologia) dos tumores.

Artigo aceito para publicação no periódico JAMA Dermatology em 29/03/2016

De: jamaderm@msubmit.net
 Assunto: DER15-2267R Decision Letter
 Data: 29 de março de 2016 12:52
 Para: tcgrazziotin@gmail.com
 Cc: tcgrazziotin@yahoo.de



March 29, 2016

Mrs Thais Corsetti Grazziotin
 UFCSPA
 Programa de Pós-Graduação em Patologia
 Rua Sarmento Leite, 245
 Porto Alegre, RS 90050-170
 Brazil

RE: Association between confocal morphological classification and clinical phenotypes of multiple primary and familial melanoma patients

Dear Mrs Grazziotin:

We have completed our review of your manuscript and are pleased to accept it for publication in JAMA Dermatology. Your manuscript is accepted with the understanding that its contents, all or in part, have not been published elsewhere and will not be published elsewhere in print or electronic format without the consent of the editor.

Any information you may receive about the proposed publication date of your article is tentative and should be kept confidential. Shortly before publication, the publication date can be confirmed by contacting the manuscript editor or the journal office. Prepublication distribution of the accepted manuscript in hard copy or electronic format without permission is prohibited. Do not use social media to post information or "tweet" about your article before publication.

Many authors work with their institution's media relations department to send out press releases about their publications. However, please note that the news media should not release any information about your article until 10 AM Central Time on the day of its publication. There is a strict embargo until then. If you or your media relations department need help in coordinating press conferences or releases, please contact the JAMA Network Media Relations Department at mediarelations@jamanetwork.org or 312-464-JAMA (312-464-5262).

First authors (ie, those listed first in the article byline) may claim 10 CME credits (AMA PRA Category 1 Credit) after publication of articles in JAMA Network Journals. For additional information on eligibility, see

<http://www.ama-assn.org/resources/doc/cme/direct-credit-application.pdf>

An edited version of your manuscript will be sent to you for final approval. It is likely that you will also receive questions about the text, tables, or figures. Your manuscript is accepted, therefore, with the understanding that it may later be necessary to consider some changes in text or ancillary material.

NOTE: Please verify and confirm that all financial disclosure information for you and all coauthors is accurate, complete, and up-to-date. Our policy requires that "potential conflicts of interest, including relevant financial interests, activities, relationships, and affiliations (e.g., employment, affiliation, grants or funding, consultancies, honoraria or payment, speakers' bureaus, stock ownership or options, expert testimony, royalties, donation of medical equipment, or patents planned, pending, or issued) with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript are completely disclosed" and that "all financial and material support for the research and the work should be clearly and completely identified on title page of the manuscript." Please contact me immediately if you have any questions about this policy.

Potential conflicts of interest include but are not limited to employment, affiliation, grants or funding, consultancies, honoraria or payment, speakers' bureaus, stock ownership or options, expert testimony, royalties, donation of medical equipment, or patents planned, pending, or issued. For example, authors of a manuscript about hypertension should report all financial relationships they have with all manufacturers of products used in the management of hypertension, not only those relationships with companies whose specific products are mentioned in the manuscript. Authors without potential conflicts of interest, including relevant financial interests, activities, relationships, and affiliations, should indicate no such interests. Please contact me immediately if you have any questions about this policy.

All research articles published in JAMA Dermatology are freely available on the journal web site 12 months after publication (<http://jamaderm.com>) and on the day of publication for a limited time on The JAMA Reader (<http://mobile.jamanetwork.com>). If your accepted manuscript reports research that was funded by an organization that requires the article to be posted in an institutional repository, you may deposit the submitted manuscript or a copy of the published PDF as per our policy at:

<http://archderm.jamanetwork.com/public/instructionsForAuthors.aspx>

We appreciate your submitting the manuscript for our consideration and look forward to seeing it in JAMA Dermatology in print and online.

Sincerely,

June Robinson, MD
 Editor
 JAMA Dermatology

COVER LETTER

Dear Editor,

Please find enclosed our manuscript entitled “**Association between confocal morphological classification and clinical phenotypes of multiple primary and familial melanoma patients**”, which we submit for consideration for publication as an original article in the *JAMA Dermatology*.

Melanoma is known to have a heterogeneous presentation as regards morphological and molecular expression and the association with patient characteristics. *In vivo* diagnostic tools such as dermoscopy and confocal microscopy provide a deeper understanding of tumor morphology and behavior. The categorization of the disease into types according to a confocal classification may help to identify distinct development pathways and stages of tumors and highlight phenotypic features that predispose to melanoma.

The results suggest that this classification has a role in the evaluation of melanoma patients and we therefore think that our findings are of interest to the readership of the *JAMA Dermatology*.

All authors declare that they have no competing financial or personal interests. This research has been funded by Grants from Fondo de Investigaciones Sanitarias, Spain; by the *CIBER de Enfermedades Raras* of the Instituto de Salud Carlos III, Spain, co-funded by “Fondo Europeo de Desarrollo Regional (FEDER)”; by GenoMEL and Diagnostics; by the National Cancer Institute (NCI) of the US National Institute of Health (NIH) and by the Brazilian Post-Graduation Agency Capes. T.C. Grazzotin wrote the first draft of the manuscript and no

specific payment was given to anyone to produce the manuscript. Neither the manuscript nor any parts of its essential substance have been submitted for publication elsewhere.

Yours sincerely,

Thais Corsetti Grazziotin, MD

CORRESPONDING AUTHOR

Thais Corsetti Grazziotin: tcgrazziotin@gmail.com

Department of Dermatology, Universidade Federal de Ciências da Saúde de Porto Alegre
(UFCSPA)

Rua Sarmiento Leite, 245 - CEP 90050-170 - Porto Alegre, Rio Grande do Sul, Brazil

tel +55 513303-9000 Fax: +55 51 3303-8810

Email: tcgrazziotin@gmail.com

Key Points

Question

Is reflectance confocal microscopy a reliable tool to classify melanomas into subtypes and correlate them with phenotypic and genetic background?

Findings

This cross-sectional, retrospective study evaluated 57 melanomas of high-risk patients.

Dendritic-cell type melanoma was characterized by older age at diagnosis, phototype II and III, more intense solar exposure and solar lentigines. Round cell melanomas were identified more often in familial context and phototype I individuals. No melanoma confocal type presented association with the genetic variations studied.

Meaning

Specific phenotypic features of high-risk patients were associated with some types of melanomas upon confocal microscopy classification.

Association between confocal morphological classification and clinical phenotypes of multiple primary and familial melanoma patients

Thaís Corsetti Grazziotin, MD^{1,2,3}, Ivette Alarcon, MD¹, Renan Rangel Bonamigo, MD, PhD², Cristina Carrera, MD, PhD^{1,4}, Miriam Potrony, Ph¹, Paula Aguilera, MD, PhD^{1,4}, Joan-Anton Puig-Butillé, PhD^{1,4}, Johanna Brito, MD¹, Celia Badenas, PhD^{1,4}, Llúcia Alós, MD¹, Josep Malvehy, MD, PhD^{1,4}, Susana Puig, MD, PhD^{1,4}

1 Hospital Clinic I Provincial de Barcelona, Universitat de Barcelona & IDIBAPS (Institut d'Investigacions Biomèdiques August Pi i Sunyer), Barcelona, Spain.

2 UFCSPA Universidade Federal de Ciências da Saúde de Porto Alegre, Department of Dermatology, Post-Graduation Program in Pathology, Porto Alegre, Brazil.

3 PUCRS Pontifícia Universidade Católica do Rio Grande do Sul, School of Medicine, Porto Alegre, Brazil

4 Centro de Investigación Biomédica en Red en Enfermedades Raras (CIBERER), Barcelona, Spain

CORRESPONDING AUTHOR

Thais Corsetti Grazziotin: tcgrazziotin@gmail.com

Department of Dermatology, Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA)

Rua Sarmento Leite, 245 - CEP 90050-170 - Porto Alegre, Rio Grande do Sul, Brazil

tel +55 513303-9000 Fax: +55 51 3303-8810

Date of revision: February 14th, 2016.

Word count: 2558

Number of figures: 3

Number of tables: 2

Abstract 348 words

Importance: The improved knowledge of clinical, morphologic and epidemiologic heterogeneity of melanoma in the context of multiple primary and familial melanoma patients (MP&FM) may improve prevention, diagnosis and prognosis of melanoma.

Objective: To characterize reflectance confocal microscopy (RCM) morphological patterns of melanomas in MP&FM.

Design: Cross-sectional, retrospective study. Patients were recruited between March 2010 and August 2013; data analysis was conducted from September 2013 to May 2014.

Setting: Hospital-based, referral center.

Participants: Consecutive primary melanomas, documented by dermoscopic and confocal examination, from MP&FM with known *CDKN2A* mutational status.

Main outcomes and measures: Epidemiological, genetic, dermoscopic and histological data were evaluated according to a RCM morphological classification: dendritic-cell, round-cell, dermal-nest, combined and non-classifiable type.

Results: Fifty-seven melanomas from 50 patients were included: 23 dendritic-cell (40.4%), 21 round-cell (36.8%), 2 dermal nests (3.5%), 2 combined (3.5%) and 9 non-classifiable (15.8%). Dendritic-cell type melanoma was characterized by older age at diagnosis, phototype II and III, more intense solar exposure and moderate to severe solar lentigines; it was the most prevalent confocal type in facial lesions and was associated with the lentigo

maligna histological subtype. Round cell melanomas were identified more often in familial context and in phototype I individuals; RCM features such as junctional thickening, dense dermal nests and nucleated cells within papillary dermis were more frequently found in this subtype. Dermal nest type and combined type melanoma were associated with absence of pigmented network on dermoscopy and thicker tumors on histology. Non-classifiable type was associated, by RCM, with absence of pagetoid cells on confocal examination and lower frequency of marked atypia on melanocytes in the basal cell layer; it presented lower TDS and RCM scores compared to other types. *CDKN2A* mutation carriers may develop any RCM type of melanoma.

Conclusions and relevance: Different routes to develop melanoma can be identified according to RCM morphological classification, dendritic cell melanomas being associated with chronic sun damage, and round cell melanoma with early age of onset and phototype 1, in the context of MP&FM. The morphological expression of melanomas via dermoscopy and confocal varies according to differences in tumor stage and biological behavior.

Introduction

At the present moment, morphology-based melanoma classification is insufficient to provide enough information about causative factors or to select patients for specific treatments.¹ In fact, refining classification of melanomas into morphological subtypes that correlate with phenotypical characteristics or prognostic factors may enhance knowledge about the disease and create a model for future investigations with molecular analysis and/or therapy stratification.

In vivo reflectance confocal microscopy (RCM) is a non-invasive imaging technique that allows for the *en face* visualization of microscopic structures and cellular detail in the epidermis and superficial dermis at histopathological resolution.^{2,3} Furthermore, integrating dermoscopy and confocal techniques is useful for distinguishing early-stage melanoma lacking frankly malignant features, especially in high-risk patients during follow-up,^{4,5} adding to morphological information about the biology of the lesions. The use of *in vivo* RCM added a new dimension in melanoma knowledge as all the surface of melanoma can be analyzed at histological resolution. It is already known that not only the tumor but also patient characteristics may influence the morphology. In fact, cyclin- dependent kinase inhibitor 2A (*CDKN2A*) germline mutations and genetic variants in the melanocortin-1 receptor gene (*MC1R*) may influence dermoscopic features. Among Spanish carriers of *CDKN2A* mutations the presence of two *MC1R* red-hair color variants (RHC) was associated with melanomas with a less suspicious clinical and dermoscopic appearance.^{2,6-10} Recently, four distinct melanoma phenotypes upon RCM were described as follows: dendritic-cell, round-cell, dermal-nest and combined-type melanomas.¹¹ The authors showed that melanomas with a predominant population of dendritic cells were thinner by Breslow index and melanomas typified by roundish melanocytes or dermal nests were smaller in size but thicker by Breslow index.¹¹

The purpose of this study was to correlate morphological RCM patterns with clinical data, genetic variants, dermoscopic features and histologic criteria in the context of multiple primary and familial melanoma patients (MP&FM)

Materials and methods

Patient and melanoma selection

A cross-sectional, retrospective, hospital-based study was performed including patients treated and followed-up at the Melanoma Unit of the Hospital Clínic, Barcelona, Spain, from March 2010 to August 2013; data analysis was conducted from September 2013 to May 2014.

The inclusion criteria were patients undergoing periodic follow-up at the Melanoma Unit with a history of at least two melanomas (multiple primary melanoma, MPM) or hereditary familial melanoma (two or more patients affected by melanoma in first or second degree relatives or at least one primary melanoma and at least one first or second degree relative with pancreatic cancer). Primary melanomas proven by histopathological examination (any histological subtype, Breslow Index ≤ 3 mm) and documented by photographic dermoscopic and confocal examination and known *CDKN2A* status (wild-type or mutated) were included. Tumors thicker than 3mm were excluded to avoid outliers that could modify the interpretation of results. As this study is focused on morphological classification based on confocal microscopy of primary melanomas we wanted to avoid that more advanced tumors, losing primary morphology and acquiring more heterogeneity, could obscure the results of our study. Patients with genetic conditions such as Li-Fraumeni, Xeroderma Pigmentosum and Albinism, as well as patients undergoing systemic treatment for advanced melanoma, were excluded. Clinical data were collected for each patient and tumor characteristics were also registered.

The study was approved by the ethical committee of Hospital Clinic of Barcelona. Written informed consent was obtained from each patient.

Dermoscopic analysis

Dermoscopic images were captured using digital cameras (Olympus Camedia, Canon G7 and/or Nikon Coolpix 4500) equipped with a polarised dermatoscope (DermlitePhoto®; 3 GEN, LLC, Dana Point, CA, USA) and with a epiluminescence microscope system (Mole Max II; Derma Medical Systems, Vienna, Austria). Dermoscopic evaluation was based on the pattern analysis and ABCD Total Dermoscopic Score (TDS) as follow: (A) asymmetry, (B) borders, (C) colors and (D) different structural components (structureless areas, pigment network, branched streaks, dots and globules).^{12,13} A TDS < 4.75 is indicative of a benign melanocytic lesion, a TDS of 4.75–5.45 is suggestive but not diagnostic of melanoma and a TDS > 5.45 should be considered as melanoma.

Confocal analysis

Confocal examinations were performed by Vivascope 1500" (Lucid Inc., Henrietta, NY, USA). Distinct melanoma types based on reflectance confocal microscopy were classified as recently proposed by Pellacani et al: i) Dendritic-cell melanomas, with a predominantly dendritic-cell population and rings and/or thin meshwork pattern; ii) Round-cell melanomas with predominantly large roundish cells with tendency to aggregate into nests forming a meshwork pattern; iii) Dermal nest melanomas with predominantly large aggregates of cells in the papillary dermis; iv) Combined type melanomas with combination of the three previous confocal patterns and v) Non-classifiable melanoma type without any of the previous patterns.¹¹

Based on the presence of major or minor criteria of malignancy, the lesion was classified according to the Pellacani and Barcelona confocal microscopy total score.^{14,15} According to Pellacani's score, the cutoff for malignancy was a score equal to or greater than 3. By Barcelona's algorithm, the cutoff for malignancy was a score equal to or greater than -1..

Genetic study

The study only included patients where *CDKN2A* and *MC1R* sequencing results were available. Peripheral blood sample was collect and the DNA extracted from lymphocytes of all patients by standard methods. Molecular analysis was conducted at the Molecular Genetics Department of the Hospital Clínic, Barcelona, Spain. The technique of analysis of single strand conformational polymorphism (SSCP) was used to detect specific mutations in *CDKN2A*. Polymorphisms in *MC1R* were studied by entire sequencing of the gene as previously described.^{16,17,18,19,20} *MC1R* polymorphisms were classified as red hair variants (RHV) or non-red hair variants (rHV). Variants that produce no change in amino acid sequence (synonymous changes) were considered as wild type for statistical analysis.

Statistical analysis

Continuous variables were analyzed to mean, standard deviation (SD), median and interquartile values. The analysis of the association between categorical variables and outcomes used Pearson's chi-square test. Fischer's exact test was applied when the expected frequency in the 2x2 table is below 5 and Student's t-test in the comparison of quantitative variables. A κ (kappa) value for the evaluation of the interobserver reproducibility was calculated for the percentage of concordant ratings. Interobserver reproducibility was calculated based on the independent evaluation of two blinded readers that analyzed

dermoscopy and confocal images of 10 pre-selected cases. The differences are considered significant if $p < 0.05$. The calculations were made using Haploview and SPSS 16.0.

Results

Fifty-seven melanomas from 50 patients were included in the study; 28 individuals were female (56%). The median age of the participants was 53.0 years (SD = 16.9 years; interquartile range 41.75 – 71.25) and the median age at the first melanoma was 46.0 years (SD = 17.1 years; interquartile range 35.75 – 61.50). The most prevalent histological subtype was superficial spreading melanoma (SSM) (81.1%), followed by lentigo maligna melanoma (LMM) (11.3%), nodular melanoma (NM) (5.7%) and nevoid melanoma (1.9%). According to AJCC classification, 31 lesions were *in situ* melanomas (54.4%), 23 stage I (40.3%) and 3 stage II (5.3%). Forty patients had MPM (80%) and the 10 individuals with single melanoma (20%) were members of families with hereditary cancer syndrome. Ten patients (20%) were *CDKN2A* mutation carriers.

The mean Breslow thickness was 0.79 mm (SD = 0.75 mm) and the median thickness was 0.52 mm. TDS general score was 6.2 (SD = 0.7), Barcelona general RCM score was 1.0 (SD = 1.0) and Pellacani general RCM score was 5.0 (SD = 1.7). Melanomas arising in MPM patients had a significantly lower mean TDS than single melanomas developed in a familial context (6.0 SD = 0.6 versus 7.0 SD = 0.4, $p < 0.001$).

The classification of melanomas according to morphological subtypes via confocal analysis resulted in 23 dendritic-cell type (40.4%), 21 round-cell type (36.8%), 2 dermal nest type (3.5%), 2 combined type (3.5%) and 9 non-classifiable type (15.8%) (Table 1 and Figure 1). The description of the data of the three less representative types (dermal nest, combined and non-classifiable type) is shown in Tables 1 and eTable2.

The interobserver kappa index was moderate to very good for single confocal features included in RCM melanoma scores, ranging from 0.458 to 1.000 (eTable 1).

Description of melanomas by RCM pattern

Individuals with dendritic-cell melanoma were on average 10.5 years older than round-cell melanoma patients (60.4 years, SD = 17.5 years x 49.9 years, SD = 15.6 years; $p = 0.043$). There was a trend of older age at first melanoma diagnosis in the dendritic cell group, compared to round-cell type (53.6 years SD = 17.5 versus 43.8 years SD = 14.8 years, $p = 0.053$). All patients with dendritic-cell type were of phototype II or III. Compared with round-cell type, patients had a more intense history of solar exposure after 18 years of age (43.5% versus 10%, $p = 0.019$) and moderate to severe solar lentigines (38.9% versus 6.7%, $p = 0.046$). The majority of dendritic-cell type patients had less than 100 nevi (63.6%) and had no family history of melanoma or pancreatic cancer (60.9%). The frequency of *CDKN2A* mutations in this group was 5/23 (21.7%) and at least one *MC1R* variant allele was present in 69.6% of patients (with or without the red-hair phenotype). The comparative epidemiological and clinical data between the two most prevalent types of melanoma on RCM, dendritic- and round-cell types, are described in the Table 2.

Dendritic-cell melanomas were located mainly on the trunk (43.5%) and extremities (41.2%). Although not the most frequent location, the dendritic-cell type was the most prevalent confocal type in facial lesions (5/6 lesions, 83.3%). Most of the dendritic-cell melanomas were sized between 5 and 10 mm. The most common histological subtype found in dendritic-cell tumors was superficial spreading melanoma (78.3%). However, the confocal pattern of dendritic-cell melanoma was significantly associated with LM histological subtype compared with other RCM types (83.3% versus 16.7%, $p = 0.034$). The mean tumor thickness in the dendritic-cell type was 0.5 mm (SD = 0.1 mm). More than half (13/23,

56.6%) of dendritic-cell lesions were in *situ* melanomas (Figure 3). Regarding dermoscopic features, dendritic-cell melanomas demonstrated an atypical pigmented network (78.3%) and atypical dots and globules (95.7%) (Figure 2). A multicomponent pattern was seen in 21.7% of lesions. The mean TDS of the dendritic-cell type was 6.3 (SD = 0.8), which was not statistically different from the general score (6.1, SD = 0.6, $p = 0.575$). The Barcelona confocal score achieved a mean value of 1.0 (SD = 1.0) and Pellacani score of 4.9 (SD = 1.7).

With confocal microscopy, dendritic-cell melanomas frequently exhibited pagetoid cells larger than 20 μ m (100% versus 61.8%, $p = 0.001$) and pleomorphic cellular populations (87% versus 47.1%, $p = 0.002$). Dendritic-cell melanomas also presented a smaller nucleus, less than 10 μ m (82.6% versus 32.4%, $p < 0.001$) and a central ringed pattern more often than the other types (30.4% versus 8.8%, $p = 0.035$) (eTable 2).

Round-cell melanomas were identified more often in a familial context (66.7%) than dendritic-cell melanomas (39.1%, $p = 0.068$). Phototype I was more prevalent than in the dendritic cell type (23.8% versus 0.0%, $p = 0.019$) and 50% of the patients in this group had more than 100 nevi (Table 2). The most common location of round-cell melanomas was on the trunk (61.9%). The majority (61.9%) of round-cell type melanomas were between 5 and 10 mm in diameter and were of the superficial spreading histological subtype (88.2%) (Table 1). Most round-cell melanomas were *in situ* (13/21, 61.9%) and the mean tumor thickness was 0.6 mm (SD = 0.5 mm) (Figure 3). Only three of the round-cell melanomas occurred in a *CDKN2A* mutation carrier (14.3%). The frequency of any *MC1R* variant allele in this melanoma type was 80%. The most prevalent dermoscopic findings in round-cell melanomas were atypical pigment network (81.0%) and atypical dots and globules (100%) (Figure 2). The global dermoscopic pattern was classified as multicomponent in 23.8% of the lesions. TDS presented a mean value of 6.4 (SD = 0.6). Mean RCM Barcelona Score of 1.4 (SD = 0.7) was statistically higher than other groups (0.7 SD = 1.1, $p = 0.006$). Mean round-cell

type Pellacani Score was 5.4 (SD = 1.1). Single confocal features such as junctional thickening, dense dermal nests and nucleated cells within papillary dermis were more frequently found in round-cell melanomas (81.0% versus 44.4%, $p = 0.007$, 38.1% versus 11.1%, $p = 0.022$ and 66.7% versus 36.1%, $p = 0.026$) (eTable2).

There was no significant association between the presence of *CDKN2A* mutations and a specific confocal melanoma type being any RCM melanoma type present in *CDKN2A* carriers. The presence of at least one *MC1R* variant was associated with widespread pagetoid cells within the epidermis compared to wild type status (28.6% vs. 0.0%, $p=0.026$).

Discussion

In the present study performed on MP&FM, we stratified melanomas into the five confocal types previously described; it was possible to associate specific phenotypic features with some types of melanomas. Dendritic-cell melanomas appeared in older patients with a history of a more intense solar exposure and moderate to severe solar lentigines as previously described by Bassoli et al.⁴ Round-cell melanomas had a predisposition for fair skinned individuals and tended to occur with a family history of melanoma. However, in this study, the association with a high nevus count or presence of atypical nevi was not observed in the round-cell melanoma type, as previously reported.¹¹

Dendritic-cell and round-cell melanomas were not different regarding tumor thickness or tumor size, differing from that previously described.¹¹ These findings reinforce the hypothesis that at least two different routes of melanoma development exist. One associated with multiple nevi, predominance on the trunk and younger age of onset, characterized via RCM by round-cell pattern with a second one associated with chronic sun damage, older age of onset, characterized via RCM as dendritic-cell type. A third possible, less frequent route may exist, characterized by thick and fast growing tumors with a nested pattern via confocal

microscopy. More advanced lesions showed a multicomponent pattern probably reflecting lesion evolution with one of the three previous patterns. Finally, lesions with unspecific pattern are probably very early melanomas in which the pattern is as yet undefined.²¹

Mean Barcelona score was higher in round-cell type probably due to the higher frequency of atypical nucleated dermal cells. These findings support the behavior of round-cell melanomas to aggregate into clusters at the dermal-epidermal junction and papillary dermis, and also to spread as isolated cells into the dermis. The dendritic-cell melanomas, adjusting for tumors of similar stage and thickness, may take a longer time for single cells to aggregate into nests and to disseminate into the dermis, possibly representing slow-growing melanomas as previously proposed.¹¹

Considering TDS, RCM scores and tumor thickness, it is possible that combined-type and non-classifiable type melanomas represent opposite ends of a morphological spectrum. In fact, milder morphological expression in dermoscopy and confocal examination of non-classifiable type is characteristic of early-stage tumors that are frequently difficult to diagnose by *in vivo* techniques.

CDKN2A mutation carriers may develop any RCM type of melanoma, even those less represented as dermal nests. It is possible that the low sample size was not able to demonstrate any differences in the distribution between different types, if they actually exist.

Conclusion

Specific phenotypic features of MP&FM patients were associated with some types of melanomas based upon confocal microscopy classification. Morphological expression of melanomas under dermoscopy and confocal examination may be associated with differences in tumor stage and biological behavior. Future studies are necessary to enhance our

knowledge of the interaction of multiple coexisting causal factors that drive melanoma development.

Acknowledgment Section

Author Contributions: Dr(s) Grazziotin and Puig had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. *Study concept and design:* Puig, Grazziotin. *Acquisition, analysis, and interpretation of data:* Grazziotin, Puig, Alarcon, Carrera, Aguilera, Badenas, Potrony, Puig-Butillé, Alós, Brito. *Drafting of the manuscript:* Grazziotin, Puig, Bonamigo. *Critical revision of the manuscript for important intellectual content:* Puig, Bonamigo, Carrera, Malvey. *Statistical analysis:* Grazziotin, Puig. *Obtained funding:* Grazziotin, Potrony. *Administrative, technical, or material support:* Badenas, Potrony, Puig-Butillé, Alós, Brito. *Study supervision:* Puig, Bonamigo.

Funding/Support: This study at the Melanoma Unit in Barcelona was supported in part by Grants PI03/0019, PI05/0302, PI06/0265, PI09/01393, PI12/00840, PI15/00716 and PI15/00956 from Fondo de Investigaciones Sanitarias, Spain; by the *CIBER de Enfermedades Raras* of the Instituto de Salud Carlos III, Spain, co-funded by “Fondo Europeo de Desarrollo Regional (FEDER). Unión Europea. Una manera de hacer Europa”; by the AGAUR 2014_SGR_603 of the Catalan Government, Spain; by the European Commission under the 6th Framework Programme, Contract nr: LSHC-CT-2006-018702 (GenoMEL) and under the 7th Framework Programme (Diagnoptics); by a grant from “Fundació La Marató de TV3, 201331-30”, Catalonia, Spain; and by the National Cancer Institute (NCI) of the US National Institute of Health (NIH) (CA83115). The work was carried out at the Esther Koplowitz Centre, Barcelona (Spain). Grazziotin was funded by the Brazilian Post-Graduation Agency Capes (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior). Potrony is the recipient of a PhD Fellowship (PFIS) from Instituto de Salud Carlos III, Spain.

Funding/Sponsor was involved?

Design and conduct of the study	No
---------------------------------	----

Collection, management, analysis and interpretation of data	No
---	----

Preparation, review, or approval of the manuscript	No
--	----

Decision to submit the manuscript for publication	No
---	----

Financial Disclosure: None reported

The specific role of the funding organization or sponsor is: design and conduct of the study: 0 (none); collection, management, analysis, and interpretation of the data: 0 (none); preparation, review, or approval of the manuscript: 0 (none); decision to submit the manuscript for publication: 0 (none).

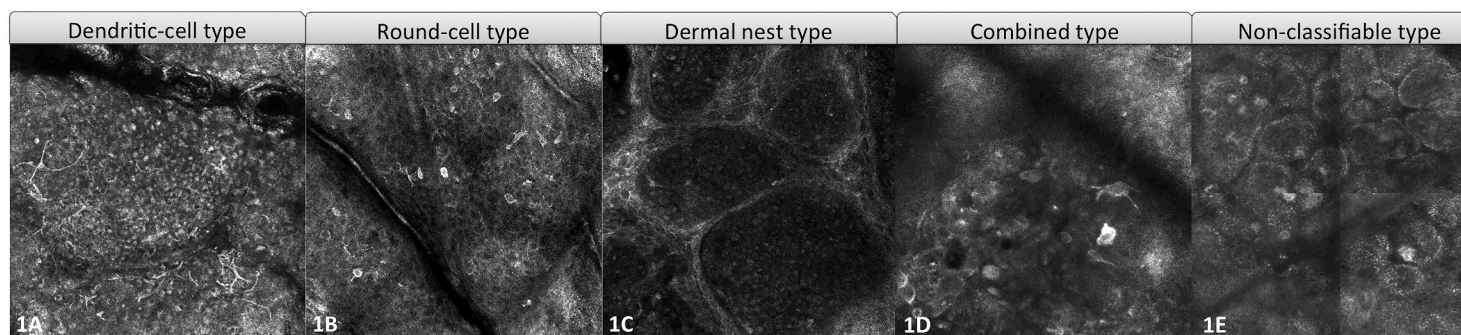
References

1. Viros A, Fridlyand J, Bauer J, Lasithiotakis K, Garbe C, Pinkel D, Bastian BC. Improving melanoma classification by integrating genetic and morphologic features. *PLoS Med*. 2008;5(6):e120.
2. Carrera C, Palou J, Malvehy J, et al. Early stages of melanoma on the limbs of high-risk patients: clinical, dermoscopic, reflectance confocal microscopy and histopathological characterization for improved recognition. *Acta Derm Venereol*. 2011;91(2):137–146.
3. Alarcon I, Carrera C, Palou J, Alos L, Malvehy J, Puig S. Impact of in vivo reflectance confocal microscopy on the number needed to treat melanoma in doubtful lesions. *Br J Dermatol*. 2014;170(4):802-808.
4. Bassoli S, Maurichi A, Rodolfo M, et al. CDKN2A and MC1R variants influence dermoscopic and confocal features of benign melanocytic lesions in multiple melanoma patients. *Exp Dermatol*. 2013;22(6):411–416.
5. Lovatto L, Carrera C, Salerni G, Alós L, Malvehy J, Puig S. In vivo reflectance confocal microscopy of equivocal melanocytic lesions detected by digital dermoscopy follow-up. *J Eur Acad Dermatol Venereol*. 2015;29(10):1918-1925.
6. Goldstein AM, Chan M, Harland M, et al. Features associated with germline CDKN2A mutations: a GenoMEL study of melanoma-prone families from three continents. *J Med Genet*. 2007;44(2):99–106.
7. Badenas C, Aguilera A, Puig-Butille JA, Carrera C, Malvehy J, Puig S. Genetic counselling in melanoma. *Dermatol Ther*. 2012;25(5):397-402.
8. Nelson AA, Tsao H. Melanoma and genetics. *Clin Dermatol*. 2009;27(1):46–52.

9. Potrony M, Badenas C, Aguilera P, et al. Update in genetic susceptibility in melanoma. *Ann Transl Med.* 2015;3(15):210.
10. Cuéllar F, Puig S, Kolm I, et al. Dermoscopic features of melanomas associated with MC1R variants in Spanish CDKN2A mutation carriers. *Br J Dermatol.* 2009;160(1):48–53.
11. Pellacani G, De Pace B, Reggiani C, et al. Distinct melanoma types based on reflectance confocal microscopy. *Exp Dermatol.* 2014;23(6):414–418.
12. Argenziano G, Soyer HP, Chimenti S, et al. Dermoscopy of pigmented skin lesions: results of a consensus meeting via the Internet. *J Am Acad Dermatol.* 2003;48(5):679–693.
13. Nachbar F, Stolz W, Merkle T, et al. The ABCD rule of dermatoscopy. High prospective value in the diagnosis of doubtful melanocytic skin lesions. *J Am Acad Dermatol.* 1994;30(4):551–559.
14. Pellacani G, Cesinaro AM, Seidenari S. Reflectance-mode confocal microscopy of pigmented skin lesions--improvement in melanoma diagnostic specificity. *J Am Acad Dermatol.* 2005;53(6):979–985.
15. Segura S, Puig S, Carrera C, Palou J, Malvehy J. Development of a two-step method for the diagnosis of melanoma by reflectance confocal microscopy. *J Am Acad Dermatol.* 2009;61(2):216–229.
16. Puig S, Ruiz A, Castel T, et al. Inherited susceptibility to several cancers but absence of linkage between dysplastic nevus syndrome and CDKN2A in a melanoma family with a mutation in the CDKN2A (P16INK4A) gene. *Hum Genet.* 1997;101(3):359–364.
17. Puig S, Malvehy J, Badenas C, et al. Role of the CDKN2A locus in patients with multiple primary melanomas. *J Clin Oncol.* 2005;23(13):3043–3051.

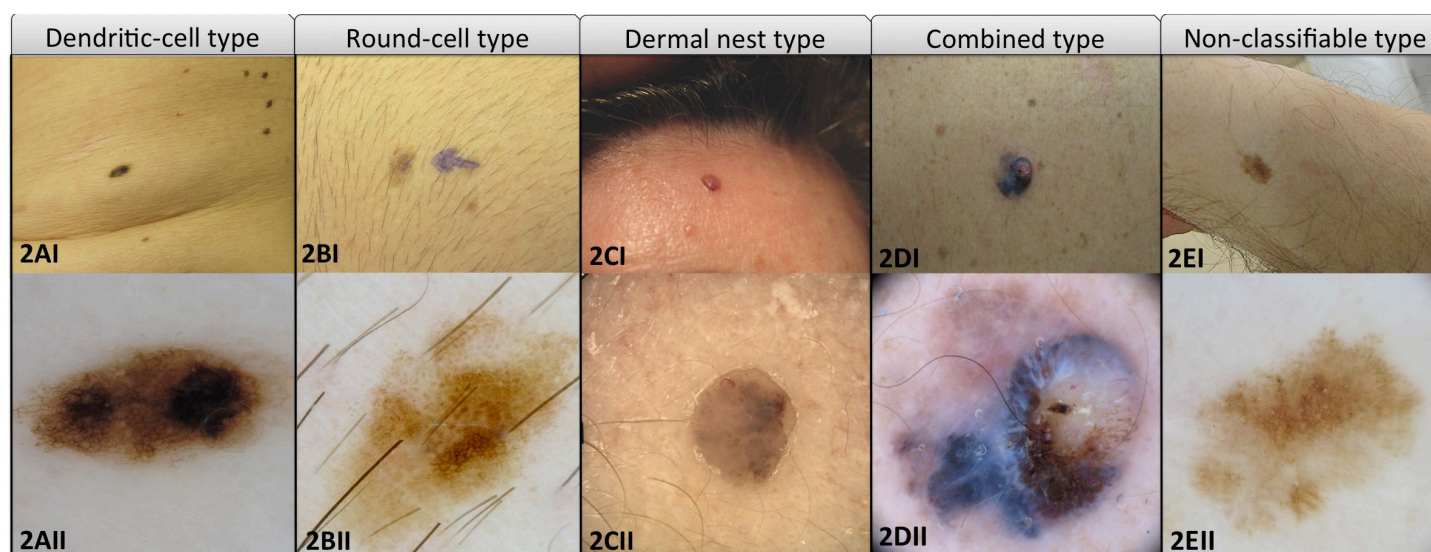
18. Badenas C, Torra R, San Millán JL, et al. Mutational analysis within the 3' region of the PKD1 gene. *Kidney Int.* 1999;55(4):1225–1233.
19. Puig-Butillé JA, Carrera C, Kumar R, et al. Distribution of MC1R variants among melanoma subtypes: p.R163Q is associated with lentigo maligna melanoma in a Mediterranean population. *Br J Dermatol.* 2013;169(4):804-811.
20. Potrony M, Puig-Butillé JA, Aguilera P, et al. Increased prevalence of lung, breast, and pancreatic cancers in addition to melanoma risk in families bearing the cyclin-dependent kinase inhibitor 2A mutation: implications for genetic counseling. *J Am Acad Dermatol.* 2014;71(5):888-895.
21. Zalaudek I, Marghoob AA, Scope A, et al. Three roots of melanoma. *Arch Dermatol.* 2008;144(10):1375-1379.

Figure 1: RCM morphological presentation according to confocal microscopy classification



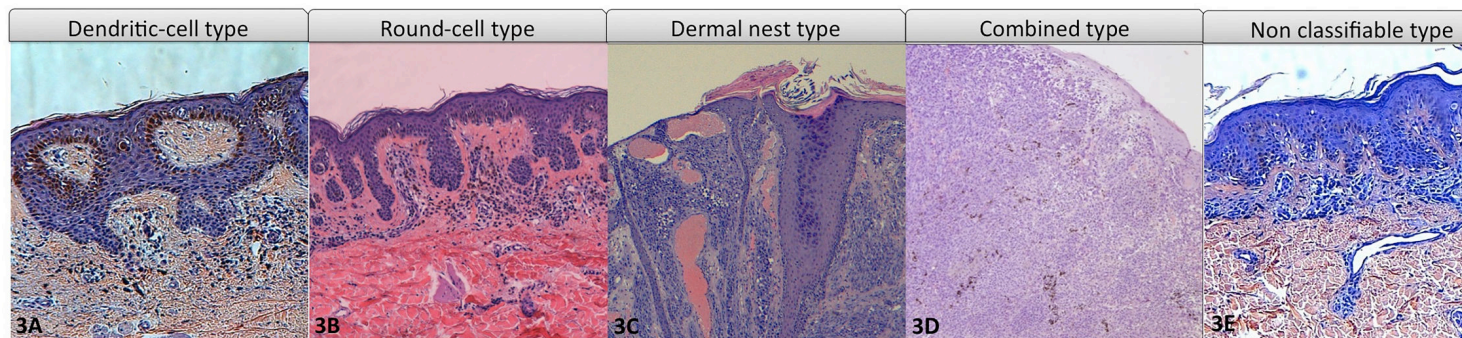
1A. Dendritic-cell melanoma. RCM shows dendritic cells with large projections and nucleus within upper epidermis layers seen as atypical cobblestone pattern. 1B. Round-cell melanoma. RCM shows small round nucleated cells with refractive cytoplasm and dark nucleus in an atypical honeycomb epidermal pattern. 1C. Dermal nest melanoma. RCM shows large cerebriform nests formed by confluent aggregates of low reflecting cells, brain-like in appearance. 1D. Combined type melanoma. RCM shows pleomorphic cellular population with dendritic and round cells within a disarranged epidermis. 1E Non-classifiable type. RCM shows dermoepidermal junction with ringed pattern, edged and non-edged papillae with irregular junctional nests and mild cytological atypia in basal layer.

Figure 2: Clinical and dermoscopic presentation according to confocal microscopy classification



2A. Dendritic-cell melanoma. 2AI. Clinical image of a melanocytic macular lesion on the left lateral trunk. 2AII. Dermoscopy shows reticular global pattern with atypical pigment network. 2B. Round-cell melanoma. 2BI. Clinical image of a melanoma on the right arm. 2BII. Dermoscopy shows a globular pattern with irregular dots and globules and focal atypical network. 2C. Dermal nest melanoma. 2CI. Clinical image of slightly erythematous papular lesion on the frontal region. 2CII. Dermoscopy shows unspecific pattern with sparse atypical globules and vessels. 2D. Combined type melanoma. 2DI. Clinical image of a palpable tumor on the infraescapular region. 2DII. Dermoscopy shows multicomponent pattern with atypical globules, blue whitish veil, blue regression and short white streaks. 2E. Non-classifiable type. 2EI. Clinical image of a flat melanocytic lesion on the forearm. 2EII. Dermoscopy shows reticular pattern with atypical pigment network, structureless areas and segmental streaks.

Figure 3: Histopathological presentation according to confocal microscopy classification



3A. Dendritic-cell melanoma. Histopathology of an *in situ* superficial spreading melanoma.

3B. Round-cell melanoma. Histopathology of an *in situ* nevus-associated superficial

spreading melanoma. 3C. Dermal nest melanoma. Histopathology shows an 1.1 mm thick

nevoid melanoma. 3D. Combined type. Histopathology shows an ulcerated 3.0 mm thick

superficial spreading melanoma. 3E. Non-classifiable type. Histopathology shows a 0.2 mm

thick superficial spreading melanoma with more than 50% regression.

Table 1: Description of tumor characteristics and genetic findings according to RCM pattern

Tumor feature	Dendritic-cell	Round-cell	Dermal-nest	Combined	Non-classifiable
Location - trunk	10/23 (43.5%)	13/21 (61.9%)	0/2 (0%)	1/2 (50%)	7/9 (77.8%)
Location - extremity	7/23 (41.2%)	7/21 (33.3%)	1/2 (50%)	1/2 (50%)	1/9 (11.1%)
Location - acral	1/23 (4.3%)	1/21 (4.8%)	0/2 (0%)	0/2 (0%)	1/9 (11.1%)
Location - facial	5/23 (21.7%)*	0/21 (0%)	1/2 (50%)	0/2 (0%)	0/9 (0%)
Size of tumor <5mm	6/23 (26.1%)	4/21 (19%)	1/2 (50%)	1/2 (50%)	3/9 (33.3%)
Size of tumor 5-10 mm	13/23 (56.5%)	13/21 (61.9%)	0/2 (0%)	1/2 (50%)	5/9 (55.6%)
Size of tumor >10 mm	4/23 (17.4%)	4/21 (19%)	1/2 (50%)	0/2 (0%)	1/9 (11.1%)
Tumor thickness (mm)	0.5 (SD=0.1)	0.6 (SD=0.5)	2.0 (SD=1.3)*	2.1 (SD=1.2)*	0.3 (SD=0.2)*
Subtype SSM	18/23 (78.3%)	15/17 (88.2%)	0/2 (0%)	1/2 (50%)	9/9 (100%)
Subtype NM	0/23 (0%)	1/17 (5.9%)	1/2 (50%)	1/2 (50%)	0/9 (0%)
Subtype LM	5/23 (21.7%)*	1/17 (5.9%)	0/2 (0%)	0/2 (0%)	0/9 (0%)
Subtype nevoid	0/23 (0%)	0/17 (0%)	1/2 (50%)	0/2 (0%)	0/9 (0%)
<i>CDKN2A</i> mutation	5/23 (21.7%)	3/21 (14.3%)	1/2 (50%)	2/2 (100%)	2/9 (22.2%)
Any <i>MC1R</i> variant	16/23 (69.6%)	16/20 (80%)	1/2 (50%)	2/2 (100%)	8/9 (88.9%)
RHC variant**	5/23 (21.7%)	7/20 (35%)	0/2 (0%)	2/2 (100%)	4/9 (44.4%)
ABCD TDS	6.3 (SD=0.8)	6.4 (SD=0.6)	6.3 (SD=0.6)	6.1 (SD=0.7)	5.6 (SD=0.5)*

*Significance= $p < 0.05$ compared to other types. ** Red Hair Color (RHC) variants in

patients: R151C, R160W, D294H, I155T

Table 2: Association between RCM dendritic-cell and round-cell melanoma types and clinical and epidemiological findings

Epidemiological data	Dendritic-cell	Round-cell	Significance (p)
Gender (female)	12/23 (52.2%)	15/21 (71.4%)	0.190
Age (years)	60.4 (SD=17.5)	49.9 (SD=15.6)	0.043*
Age at first CM	53.6 (SD=17.5)	43.8 (SD=14.8)	0.053
Multiple CM	19/23 (82.6%)	16/21 (76.2%)	0.716
Familial CM	8/23 (34.8%)	13/21 (61.9%)	0.072
Phototype I (%)	0/23 (0%)	5/21 (23.8%)	0.019*
Phototype II (%)	13/23 (56.5%)	9/21 (42.9%)	0.365
Phototype III (%)	10/23 (43.5%)	6/21 (28.6%)	0.305
Phototype IV (%)	0/23 (0%)	0/21 (0%)	<i>NA</i>
Phototype V (%)	0/23 (0%)	1/21 (4.8%)	0.477
Nevus number >100	8/22 (36.4%)	8/16 (50%)	0.401
Solar exp severe <10y	9/23 (39.1%)	10/21 (47.6%)	0.570
Solar exp severe 10-18y	11/23 (47.8%)	7/21 (33.3%)	0.329
Solar exp severe >18y	10/23 (43.5%)	2/20 (10%)	0.015*
Solar lentigos mod/sev	7/18 (38.9%)	1/15 (6.7%)	0.046*

*Significance= $p < 0.05$. Statistic tests: Pearson Chi-Square, Fisher's Exact, T Student.

eTable 1: Kappa correlation between observers for confocal features

Confocal criteria or score	Observer 1 (%)	Observer 2 (%)	Kappa value	Significance (p)
Barcelona Score	<i>NA</i>	<i>NA</i>	0.690	0.000
Pellacani score	<i>NA</i>	<i>NA</i>	0.837	0.000
Atypical cobblestone	56.3	81.3	0.458	0.029
Pagetoid cells yes/no	75.0	81.5	0.818	0.001
Pagetoid cell 1-5/500μ	68.8	81.3	0.673	0.004
Pagetoid cell 5-10/500μ	37.5	62.5	0.529	0.016
Pagetoid cell >10/500μ	50.0	43.8	0.875	0.000
Pagetoid cell small (<20μ)	81.3	81.3	1.000	0.000
Pagetoid cell large (>20μ)	75.0	75.0	1.000	0.000
Shape round	81.3	81.3	1.000	0.000
Shape dendritic	62.5	62.5	1.000	0.000
Pleomorphic cells	62.5	62.5	1.000	0.000
Widespread pagetoid cells	31.3	18.8	0.673	0.004
Typical basal cells	6.3	6.3	1.000	0.000
Atypical basal cells	87.5	93.8	0.636	0.006
Mild atypia DEJ	50.0	50.0	1.000	0.000
Marked atypia DEJ	43.8	43.8	0.746	0.003
Meshwork pattern DEJ	43.8	43.8	0.746	0.003
Ringed pattern DEJ	37.5	31.3	0.586	0.018
Junctional thickening	56.3	37.5	0.636	0.006
Irregular junctional nests	37.5	43.8	0.871	0.000
Dermal nests	31.3	25.0	0.846	0.001
Nucleated cell within dermis	56.3	62.5	0.613	0.013

*Significance= $p < 0.05$. Statistic tests: Pearson Chi-Square, Fisher's Exact.

eTable2: Association between RCM types and RCM findings

RCM feature	Dendritic-cell	Round-cell	Dermal-nest	Combined	Non-classifiable
Epidermis					
Pagetoid cell <20µm y/n	20/23 (87.0%)	20/21 (95.2%)*	2/2 (100.0%)	2/2 (100.0%)	0/9 (0.0%)*
Pagetoid cell >20µm y/n	23/23 (100%)*	17/21 (81%)	2/2 (100.0%)	2/2 (100.0%)	0/9 (0.0%)*
Pleomorphic pagetoid y/n	20/23 (87.0%)*	12/21 (57.1)	2/2 (100.0%)	2/2 (100.0%)	0/9 (0.0%)*
Nucleus small (<10µm) y/n	19/23 (82.6%)*	10/21 (47.6%)	1/2 (50.0%)	0/2 (0.0%)	0/9 (0.0%)*
Nucleus large (>10µm) y/n	12/23 (52.2%)	15/21 (71.4%)*	2/2 (100.0%)	2/2 (100.0%)	0/9 (0.0%)*
DEJ and upper dermis					
Central ringed pattern y/n	7/23 (30.4%)*	1/21 (4.8%)	0/2 (0.0%)	0/2 (0.0%)	2/9 (22.2)
Junctional thickening y/n	12/23 (52.2%)	17/21 (81.0%)*	0/2 (0.0%)	0/2 (0.0%)	4/9 (44.4%)
Dense dermal nests y/n	3/23 (13.0%)	8/21 (38.1%)*	0/2 (0.0%)	0/2 (0.0%)	1/9 (11.1%)
Cerebriform nests y/n	0/23 (0.0%)	0/21 (0.0%)	2/2 (100.0%)*	2/2 (100.0%)*	0/9 (0.0%)
Nucleated cells dermis y/n	8/23 (34.8%)	14/21 (66.7%)*	0/2 (0.0%)	2/2 (100.0%)	3/9 (33.3%)
RCM Scores					
Barcelona Score	1.0 (SD=1.0)	1.4 (SD=0.7)*	1.0 (SD=0.0)	2.0 (SD=0.0)*	-0.2 (SD=0.9)*
Pellacani Score	4.9 (SD=1.7)	5.4 (SD=1.1)	6.5 (SD=0.7)	7.5 (SD=0.7)*	3.2 (SD=1.7)*

*Significance= $p < 0.05$ compared to other types.

REVISION LETTER

Dear Editor,

Please find enclosed the revised version of manuscript DER15-2267 entitled **“Association between confocal morphological classification and clinical phenotypes of multiple primary and familial melanoma patients”**. The modifications afforded a meaningful improvement of the manuscript, and we appreciate and thank the reviewers for all their comments and suggestions.

Below we answer the editor's and the reviewer's comments:

Editor's comments:

1. It seems that the sample of 57 lesions is inadequate to provide statistically significant results. Did you have a biostatistician do a power calculation? Without an adequate sample size, this is an observational descriptive study.

Authors (A). The sample size depends on the strength of the association. We performed a sample size estimation using a 2-sided 5% significance level and a power of 80%. In the present study, a sample size adequate to provide statistically significant results depends on the variable tested between the two main melanoma subtypes by confocal microscopy (dendritic-cells and round-cells). For the association with severe solar exposition, for example, 27 patients per group would be required (<http://www.stat.ubc.ca/~rollin/stats/ssize>) and for *CDKN2A* mutations a total sample size of 48. It may be considered a descriptive study, but in fact it was adequate to test the more prevalent variables with strong or moderate association.

2. The manuscript preparation needs to be improved by reducing extraneous thoughts.

A. The first paragraph was corrected and all the suggestions of reviewer 1 were accepted and included in the new version. Indeed the manuscript acquired significant improvement in the scientific writing and translation.

3. Please include nodular melanomas in the analysis or state that you excluded them and why.

A. Nodular melanomas were included in the analysis (line 164), as long as the tumor was a primary melanoma and not a metastasis. Tumors thicker than 3mm were excluded to avoid outliers that could modify the interpretation of results. This study is focused on morphological classification based on confocal microscopy of primary melanomas and our impression is that more advanced tumors lose primary morphology acquiring more heterogeneity and mixing of the primary subtypes as was finally also shown in our study, with thicker and larger combined types.

4. If you have photographs of the lesions shown with dermoscopy in Fig 2 A-D please add them to this figure.

A. Figure 2 now includes the clinical photographs of the lesions seen with dermoscopy.

Include the clinical, dermoscopic and confocal images of a non-classifiable lesion.

A. Non-classifiable type was represented in figures 1, 2 and 3.

Reviewer 1

Thank you for submitting your article to Dermatology. I have a few suggestions re style and English that I hope you will agree will improve your manuscript.

- 1) Lines 51-51: "... on dermoscopy and thicker tumors on histology."
- 2) Line 53: please clarify "atypia on basal cells". Do you mean basal cell atypia or atypia of melanocytes in the basal cell layer or at the DEJ?
- 3) Lines 5859: "...confocal examination may present with differences in tumor stage and biologic behavior."
- 4) Line 69: "...with a confirming confocal and histopathological correlation in clinically featureless early stage melanomas."
- 5) Line 163: "... lentigo maligna melanoma."
- 6) Lines 187-190: "Compared withpatients had a more intense history of solar exposure after 18 years of age.... to severe solar lentigines (38.9%....).
- 7) Line 193: "... of patients (with or without the redhair phenotype).
- 8) Line 198: "Although not the most frequent anatomic location, the dendritic..."
- 9) Line 200: "The most common histologic subtype..."
- 10) Line 206: "...melanomas demonstrated an atypical pigmented..."
- 11) Line 211: "...melanomas frequently exhibited..."
- 12) Line 216: "Roundcell melanomas were identified more often..."
- 13) Lines 220-221: "... were between 5 and 10 mm in diameter and were ..."
- 14) Line 235: "One lesion occurred in a patient who was a carrier..."

- 15) Line 236: "Dermal nest type melanoma was associated with thicker tumors (2.00 mm...
- 16) Lines 249-250: "... (Figure 3). Dermoscopically they presented ...patterns with a mean TDS of..."
- 17) Lines 2612: "... TDS was 5.6, lower than recorded in other groups and demonstrated less atypical..."
- 18) Lines 264-265: same issue as #2 above
- 19) Lines 275-276: "...performed on high risk patients, we stratified melanomas into the five confocal types previously described; it was possible..."
- 20) Line 278: "...older patients with a history of more intense solar exposure and moderate to severe solar lentigines as..."
- 21) Line 279: "... had a predisposition for fair..."
- 22) Lines 280-282: "... with a family history of melanoma. However, in this study, the association with a high nevus count or presence of atypical nevi was not..."
- 23) Lines 288-289: "... with a second one associated with chronic sun damage, older age of onset,"
- 24) Line 292: "... probably reflecting lesion evolution with one of the..."
- 25) Line 296: "These findings support the behavior..."
- 26) Line 297: "... clusters at the dermalepidermal junction..."
- 27) Lines 298-299: "...dermis. The dendriticcell melanomas, adjusting for tumors of similar stage and thickness, may take a longer time..."
- 28) Line 302: "...thickness, it is possible that combinedtype..."
- 29) Lines 307-308: "...RCM types with the exception that the presence of round pagetoid cells was associated with the presence of..."
- 30) Line 314: "... melanomas based upon ..."

31) Line 315: "... examination may be associated with differences in tumor ..."

32) Line 316: "... to enhance our knowledge of the ..."

A. All remarks to the author (1 to 32) were extremely pertinent and included in the new version.

Reviewer 2

R2. 1. Introduction - The introduction does not sufficiently specify the reason / motivation for the study but rather lists in sequence what is dermoscopy, confocal, genetic mutations in melanoma, and the study aims to correlate all of these. Why did you decide to study this topic? What was your hypothesis going into the study? What can be gained if this hypothesis will prove correct? What is known about such correlations from prior literature? Why did you focus the study only on multiple primary / familial melanoma cases?

A. At the present moment, morphology-based melanoma classification is insufficient to provide enough information about causative factors or to select patients for specific treatments. In fact, refining classification of melanomas into morphological subtypes that correlate with phenotypical characteristics or prognostic factors may enhance our knowledge of the disease and create a model for future investigations with molecular analysis and/or therapy stratification.

The main hypothesis in this study was that differences in morphological criteria of melanoma by reflectance confocal microscopy could be associated with different phenotypic and genetic background of patients. If this hypothesis proves to be correct, confocal microscopy could be able to give not only

information about primary tumor cellular and architectural features, but also tell us about etiological agents such as environmental, genetic and phenotypic factors. Besides, the association of specific subtypes of melanoma with tumor thickness and other prognostic factors could tell us about tumor stage even before biopsy.

A previous study of Pellacani et al (2014) was the first to propose classification of melanomas into subtypes according to cellular and architectural morphology by confocal microscopy and the correlation with patient and tumor characteristics. However, the genetic background of the patient was not accessed in this previous study and sporadic melanomas were also included. The aim of the inclusion of high-risk patients only (multiple primary / familial cases) was to analyze the RCM morphology of early melanomas in a specific context where we can know the genetic background. Then, we select individuals with confirmation of *CDKN2A* status, as they are part of routine genetic counseling in the investigation center where the study was conducted. Sporadic melanoma cases have a very low frequency of germinative mutations in *CDKN2A* (1-2%). Genetic testing of these patients is not routinely performed and would take extra time and costs with low benefit to detect genetic variations.

R2. 2.Methods - There is a conflict in the paper about the inclusion criteria. In the methods lines 106-108 it states that the study included patients with multiple primary melanomas or patients with multiple cases of melanoma in the family. Results lines 167-168 & 191 it states that members of family with melanoma and pancreatic cancer could also be included. Please be consistent on who is included in the study.

A. Currently, the definition of familial susceptibility to melanoma includes families with multiple cases of melanoma or melanoma associated with pancreatic cancer or multiple primary melanomas. In this study we included patients with at least two melanomas (multiple melanoma) or familial melanoma (two or more patients affected by melanoma in first or second degree relatives or at least one primary melanoma and at least one- first or second -degree relative with pancreatic cancer). It is now explained in text (patient and melanoma selection section). Thank you for the correction.

R2. 3. Methods line 109 - why was Breslow index limited to <3mm? Or is this just part of the results?

A. As previously answered to the editor, nodular melanomas were included in the analysis (line 164), as long as the tumor was a primary melanoma and not a metastasis. Tumors thicker than 3mm were excluded to avoid outliers that could modify the interpretation of results. This study is focused on morphological classification based on confocal microscopy of primary melanomas and our impression is that more advance tumors loose primary morphology acquiring more heterogeneity and mixing the primary subtypes as finally also showed in our study, being combined types thicker and larger.

R2. 4. Methods lines 122-125 and 136-137 - readers do not necessarily know what are the scales of TDS, confocal-Pellacani and confocal-Barcelona. Please remind readers what are the cutoffs for malignant scores.

A. The cutoffs of TDS, confocal-Pellacani and confocal-Barcelona scores were included in the text.

R2. 5. Methods - you give later in the results information about sun-exposure history, nevus counts and lentigines density. Give details of the procedure of how you collected these data. Did you administer questionnaires? Total body nevus counts?

A. All the phenotyping of the patients was performed by dermatologist during the screening visits. Concerning sun exposure habits and sunburns, a questionnaire is administered by the dermatologist to the patients in the first visit or at inclusion to the study.

R2. 6. Methods - line 135 - why is a subset of melanomas non-classifiable? What kind of cells do they show? Is this a uniform group or a potpourri of unrelated melanomas?

A. Melanomas without any of the previous confocal patterns (dendritic cell, round cell, dermal nest type or combined type) were characterized as non-classifiable type. These lesions presented lower confocal scores and were thinner in Breslow index than the other melanoma confocal types. They were all superficial spreading melanomas that represent early-stage tumors without full morphologic expression in dermoscopy and confocal examination. A brief explanation was added to non-classifiable type in the new version.

R2. 7. Results - in general, the results section is very long and hard to follow. It could be reduced by more than half. It may be better to leave all the details of the in the comparative tables and give the highlights of only the major differences between the dendritic and round cell melanomas in the results text.

A. We resumed the information in the text and think it now provides a better reading of the results section.

R2. 8. Results line 169 and 245 - were all patients or only a few tested for MITF? If not all, then you could consider deleting the information about the few MITF mutation cases. If all were tested, then state that in the methods.

A. Not all the patients were tested for MITF. We have now deleted the information.

R2. 9. Results lines 179-180 - give details in the methods of how the interobserver agreement study was done. E.g., 2 readers blinded to the diagnosis independently reviewed all (or pre-selected) dermoscopy and confocal imagers from all (or x) cases.

Interobserver reproducibility was calculated based on the independent evaluation of two blinded readers that analyzed dermoscopy and confocal images of 10 pre-selected cases.

R2. 10. Results and methods - as for the groups of dermal nest (n=2) and combined (n=2), these groups are too small for any descriptive statistics or conclusions. It is, in my opinion, best to remove these small groups from the study altogether. At minimum, I would only give the details in the table and avoid any statistical comparisons and details in the text of the results.

A. Data concerning these two less representative subtypes was excluded from the text and is shown only in the tables.

R2. 11. Figures - there is no depiction of the non-specific group in any of the figures (even though this is the 3rd group in term of size).

Non-classifiable type is now represented in all figures.

R2. 12. Tables - I suggest to put Table 2 (which does not give central information for this paper) as an e-table, and to make eTable 2 (which summarized the key findings) as Table 2.

A. We have incorporated this suggestion and we agree that it improved the reading of the text.

R2. Is there a representative figure of non specific melanoma?

A. Non-classifiable type is now represented in all figures.

Reviewer 3

R3. I suggest the authors conclude including that the confocal microscopy is not suitable for predicting neither the genetic mutations nor the histological subtype.

A. The suggestion has been added to the text (discussion section).

Yours sincerely,

Thais Corsetti Grazziotin, MD

CORRESPONDING AUTHOR

Thais Corsetti Grazziotin: tcgrazziotin@gmail.com

Department of Dermatology, Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA)

Rua Sarmiento Leite, 245 - CEP 90050-170 - Porto Alegre, Rio Grande do Sul, Brazil

tel +55 513303-9000 Fax: +55 51 3303-8810

Email: tcgrazziotin@gmail.com

4. Considerações finais

O projeto de pesquisa desenvolvido durante o período de doutorado sanduíche na Espanha é resultado de uma colaboração internacional com os professores Susana Puig e Josep Malvehy da Universidade de Barcelona. O vínculo acadêmico se iniciou em 2009 com o estudo multicêntrico **Genomel**, Consórcio de Genética em Melanoma, no qual a UFCSPA foi incluída como centro participante, gerando conhecimento científico acerca da situação genética de pacientes com melanoma no sul do Brasil.

Posteriormente foi possível consolidar a colaboração com o grupo espanhol através de um novo projeto, tema da tese atual, no qual os pesquisadores brasileiros puderam presenciar a excelência em ensino, pesquisa e assistência da Unidade de Melanoma do Hospital Clínic de Barcelona. O projeto intitulado “**Associação entre a Classificação Morfológica Confocal e os Fenótipos Clínicos de Pacientes com Múltiplos Melanomas Primários e Melanoma Familiar**” foi aprovado por Comitê de Ética em Pesquisa do Hospital Clínic em 21/02/14 (Parecer n. HCB/2014/0023) e executado integralmente na cidade espanhola. A análise estatística, interpretação e redação do artigo e tese foram finalizados no Brasil, sob supervisão do orientador brasileiro e co-orientadora espanhola.

O artigo científico resultante deste trabalho conjunto traz informações originais e de grande interesse para o estudo e aplicação das novas técnicas diagnósticas em melanoma. Além disso, reforça a experiência positiva da colaboração estabelecida e possibilita estreitar o vínculo com um dos maiores centros de referência internacional na área de oncologia cutânea para futuros projetos.

O presente estudo demonstra a associação entre características clínico-epidemiológicas e fenotípicas como idade, história familiar de melanoma, sinais de

fotodano, exposição solar e fototipo com tipos específicos de melanoma em uma classificação morfológica de microscopia confocal, permitindo uma maior compreensão sobre as rotas de desenvolvimento e evolução dos melanomas. Conclui-se também que a correlação de achados específicos na microscopia confocal com a classificação de subtipo histológico e os critérios prognósticos de microestadiamento está possivelmente associada a diferenças no estágio tumoral e de comportamento biológico das lesões. A influência de fatores genéticos nos subtipos morfológicos dos melanomas não pôde ser estabelecida neste estudo, sendo necessárias investigações adicionais em amostras maiores de pacientes de alto risco.

5. Anexo

5.1 Parecer do Comitê de Ética do Hospital Clínic de Barcelona



DICTAMEN DEL COMITÉ ÉTICO DE INVESTIGACIÓN CLÍNICA

NEUS RIBA GARCIA, Secretaria del **Comité Ético de Investigación Clínica del Hospital Clínic de Barcelona**

Certifica:

Que este Comité ha evaluado la propuesta del promotor, para que se realice el estudio:

CÓDIGO:

DOCUMENTOS CON VERSIONES:

Tipo	Subtipo	Versión

TÍTULO: Dermoscopic and confocal features of melanomas associated with CDKN2A and MC1R variants in multiple and familial melanoma patients.

PROMOTOR:

INVESTIGADOR PRINCIPAL: SUSANA PUIG SARDÁ

y considera que, teniendo en cuenta la respuesta a las aclaraciones solicitadas (si las hubiera), y que:

- Se cumplen los requisitos necesarios de idoneidad del protocolo en relación con los objetivos del estudio y están justificados los riesgos y molestias previsibles para el sujeto, teniendo en cuenta los beneficios esperados.
- La capacidad del investigador y los medios disponibles son apropiados para llevar a cabo el estudio.
- Que se han evaluado la compensaciones económicas previstas (cuando las haya) y su posible interferencia con el respeto a los postulados éticos y se consideran adecuadas.
- Que se cumplen los preceptos éticos formulados en la Orden SAS 3470/2009 y la Declaración de Helsinki de la Asociación Médica mundial sobre principios éticos para las investigaciones médicas en seres humanos y en sus posteriores revisiones, así como aquellos exigidos por la normativa aplicable en función de las características del estudio.
- Que dicho estudio se incluye en una de las líneas de investigación biomédica acreditadas en este centro, cumpliendo los requisitos necesarios, y que es viable en todos sus términos.

Este CEIC acepta que dicho estudio sea realizado, debiendo ser comunicado a dicho Comité Ético todo cambio en el protocolo o acontecimiento adverso grave.

CIF – G-08431173

y hace constar que

1º En la reunión celebrada el día 23/01/2014, acta 2/2014 se decidió emitir el informe correspondiente al estudio de referencia.

2º El CEIC del Hospital Clínic i Provincial, tanto en su composición como en sus PNTs, cumple con las normas de BPC (CPMP/ICH/135/95)

3º Listado de miembros:

Presidente:

- FRANCISCO JAVIER CARNE CLADELLAS (Médico Farmacólogo Clínico, HCB)

Vicepresidente:

- BEGOÑA GOMEZ PEREZ (Farmacéutica Hospitalaria, HCB)

Secretario:

- NEUS RIBA GARCIA (Médico Farmacólogo Clínico, HCB)

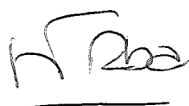
Vocales:

- ITZIAR DE LECUONA (Jurista, Observatorio de Bioética y Derecho, UB)
- MONTSERRAT GONZALEZ CREUS (Trabajadora Social, Servicio de Atención al Usuario, HCB)
- MIRIAM MENDEZ GARCÍA (Abogada, HCB)
- MONTSERRAT NUÑEZ JUÁREZ (Enfermera, HCB)
- JOSE RIOS GUILLERMO (Estadístico, Farmacología Clínica, USEM, UASP, HCB)
- JOSE MIGUEL SOTOCA (Farmacéutico Atención Primera, CAP Les Corts)
- ANTONI TRILLA GARCIA (Médico Epidemiólogo, HCB - Director UAPS)
- OCTAVI SANCHEZ LOPEZ (Representante de los pacientes)
- MARIA JESÚS BERTRAN LUENGO (Médico Epidemiólogo, HCB)
- MARTA AYMERICH GREGORIO (Médico Hematólogo, HCB)

En el caso de que se evalúe algún proyecto del que un miembro sea investigador/colaborador, este se ausentará de la reunión durante la discusión del proyecto.

Para que conste donde proceda, y a petición del promotor,

Barcelona, a 21 de febrero de 2014



CLÍNICA
BARCELONA
Hospital Universitari
COMITÈ ÈTIC
INVESTIGACIÓ CLÍNICA

Reg. HCB/2014/0023

Mod_03 (V2 de 11/10/2013)

EPA

5.2 Normas da Revista

***JAMA Dermatology* Instructions For Authors**

General Information

JAMA Dermatology is an international, peer-reviewed, dermatologic journal, with distribution to readers in more than 95 countries. Manuscripts submitted to *JAMA Dermatology* should meet the following criteria: the material is original; the writing is clear; the study methods are appropriate; the data are valid; the conclusions are reasonable and supported by the data; the information is important; and the topic has general clinical dermatology relevance. From these basic criteria, we assess a paper's eligibility for publication. We receive approximately 1600 papers each year, but publish only about 21% of unsolicited manuscripts. Because of this competition for space in *JAMA Dermatology*, we advise authors to follow these instructions and to keep papers as brief as possible while still meeting the quality criteria described herein.

Editorial Office Contact Information

June K. Robinson, MD, Editor, *JAMA Dermatology*, 132 E Delaware Pl, #5806, Chicago, IL 60611 USA; email: jamaderm@jamanetwork.org. Send inquiries about submitting manuscripts by email. Manuscripts may be submitted online at <http://manuscripts.jamaderm.com>.

Categories of Articles

JAMA Dermatology publishes Original Investigations, Evidence-Based Reviews, Special Communications, Viewpoints, Letters, and other categories of articles. Topics of interest include all subjects that are related to the practice of dermatology and the betterment of public dermatologic health worldwide. The most frequently published types of articles are described herein.

Original Investigation

These reports typically include randomized trials (see Clinical Trial), intervention studies, cohort studies, case-control studies, epidemiologic assessments, other observational studies, surveys with high response rates (see Survey Research), cost-effectiveness analyses and decision analyses (see Reports of Cost-effectiveness Analyses and Decision Analyses), and studies of screening and diagnostic tests (see Reports of Diagnostic Tests). Each manuscript should clearly state an objective or hypothesis; the design and methods (including the study setting and dates, patients or participants with inclusion and exclusion criteria and/or participation or response rates, or data sources, and how these were selected for the study); the essential features of any interventions; the main outcome measures; the main results of the study; a discussion section placing the results in context with the published literature and addressing study limitations; and the conclusions and relevant implications for clinical practice or health policy. Data included in research reports must be original and should be as timely and current as possible (see Timeliness of Data). A structured

abstract is required; for more information, see instructions for preparing structured Abstracts for Reports of Original Data. The text should include the full name, academic degrees, and a single institutional affiliation for each author and the email address for the corresponding author. Other persons who have contributed to the study may be indicated in an Acknowledgment, with their permission, including their academic degrees, affiliation, contribution to the study, and an indication if compensation was received for their role. Please provide a word count on the title page. Maximum length: 3000 words of text (not including acknowledgment, abstract, tables, figures, references, and online-only material) with no more than a total of 5 tables and/or figures.

Manuscript Preparation and Submission Requirements

Manuscript Submission

All manuscripts must be submitted online via the *JAMA Dermatology* online manuscript submission and review system. At the time of submission, complete contact information (affiliation, postal/mail address, email address, and telephone number) for the corresponding author is required. First and last names, email addresses, and institutional affiliations of all coauthors are also required. After the manuscript is submitted, the corresponding author will receive an acknowledgment confirming receipt and a manuscript number. Authors will be able to track the status of their manuscripts via the online system. After manuscript submission, all authors of papers under consideration for publication will be sent an Authorship Form to complete and submit (see sample Authorship Form). See Manuscript Checklist, Manuscript Preparation and Submission Requirements, and other details in these instructions for additional requirements.

Cover Letter

Include a cover letter and complete contact information for the corresponding author (affiliation, postal/mail address, email address, and telephone numbers) and whether the authors have published or submitted any related papers from the same study (see Duplicate/Previous Publication or Submission).

Manuscript Style

Manuscripts should be prepared in accordance with the *AMA Manual of Style*, 10th edition,¹ and/or the ICMJE Recommendations for the Conduct, Reporting, Editing, and Publication of Scholarly Work in Medical Journals.²

Manuscript Components

Include in the manuscript file, in the following order, the Title Pages (including Acknowledgment section), abstract, text, references, and appropriate figure legends and tables. Start each of these sections on a new page, numbered consecutively, beginning with the title pages. Include figures as separate files.

Recommended File Sizes

We recommend individual file sizes of no more than 500 kB and not exceeding 1 MB, with the total size for all files not exceeding 5 MB (not including any video files).

Manuscript File Formats

For submission and review, acceptable manuscript file formats include Word. Do not submit your manuscript in .pdf format. Microsoft Vista users should save the text as a Microsoft Word 97-2003 file (.doc NOT .docx).

Manuscripts are double-spaced throughout, including abstract, text, references, legends for illustrations, and tables. Use only Times New Roman 12-point font size.

Ample margins of at least 2.5 cm (1 in) should be used all around. Do not use proportional spacing; use unjustified (ragged) right margins and spell-check the manuscript prior to submission.

Figure File Formats

For digital images, submit initially as a single JPG file for each figure (see Figures).

Title Pages

The title page should be the first pages of your main manuscript file. It should include a manuscript title; the full names, highest academic degrees, and affiliations of all authors (if an author's affiliation has changed since the work was done, the new affiliation also should be listed); name and complete contact information for corresponding author; authors' contributions and conflict of interest disclosures (see the Template for Author Attribution and Conflict of Interest Disclosure); and word count (not including title, abstract, acknowledgments, or references).

Title

Titles should be concise, specific, and informative and should contain the key points of the work.^{1(p8)} Please limit the length of titles to 150 characters for reports of research and other major articles and 100 characters for Editorials, Viewpoints, Commentaries, and Letters. For scientific manuscripts, overly general titles are not desirable and questions and declarative sentences should be avoided. For reports of clinical trials, meta-analyses, and systematic reviews, include the type of study as a subtitle (eg, A Randomized Clinical Trial, A Meta-analysis, A Systematic Review). For reports of other types of research, do not include study type or design in the title or subtitle.

Abstracts

Include a *structured abstract* of no more than 350 words for reports of original data, reviews (including meta-analyses), and consensus statements. Abstracts should be prepared in JAMA Network style—see instructions for preparing structured abstracts for specific article types below. Abstracts are not required for Editorials, Viewpoints, and some special features.

Abstracts for Reports of Original Data:

Reports of original data should include an abstract of no more than 350 words using the headings listed below. For brevity, parts of the abstract may be written as phrases rather than complete sentences. Each section should include the following content:

Importance: The abstract should begin with a sentence or two explaining the clinical (or other) importance of the study question.

Objective: State the precise objective or study question addressed in the report (eg, “To determine whether...”). If more than 1 objective is addressed, the main objective should be indicated and only key secondary objectives stated. If an a priori hypothesis was tested, it should be stated.

Design: Describe the basic design of the study. State the years of the study and the duration of follow-up. If applicable, include the name of the study (eg, the Framingham Heart Study). As relevant, indicate whether observers were blinded to patient groupings, particularly for subjective measurements.

Setting: Describe the study setting to assist readers to determine the applicability of the report to other circumstances, for example, general community, a primary care or referral center, private or institutional practice, or ambulatory or hospitalized care.

Participants: State the clinical disorders, important eligibility criteria, and key sociodemographic features of patients. The numbers of participants and how they were selected should be provided (see below), including the number of otherwise eligible individuals who were approached but refused. If matching is used for

comparison groups, characteristics that are matched should be specified. In follow-up studies, the proportion of participants who completed the study must be indicated. In intervention studies, the number of patients withdrawn because of adverse effects should be given. For selection procedures, these terms should be used, if appropriate: random sample (where random refers to a formal, randomized selection in which all eligible individuals have a fixed and usually equal chance of selection); population-based sample; referred sample; consecutive sample; volunteer sample; convenience sample.

Note: For accepted manuscripts, the above 3 sections are usually combined during the editing process (as "Design, Setting, and Participants"), but for manuscript submission these sections should be kept separate.

Intervention(s) for Clinical Trials or Exposure(s) for observational studies: The essential features of any interventions, or exposures, should be described, including their method and duration. The intervention, or exposure, should be named by its most common clinical name, and nonproprietary drug names should be used.

Main Outcome(s) and Measure(s): Indicate the primary study outcome(s) and measurement(s) as planned before data collection began. If the manuscript does not report the main planned outcomes of a study, this fact should be stated and the reason indicated. State clearly if the hypothesis being tested was formulated during or after data collection. Explain outcomes or measurements unfamiliar to a general medical readership.

Results: The main outcomes of the study should be reported and quantified, including baseline characteristics and final included/analyzed sample. Include absolute numbers and measures of absolute risks (such as increase/decrease or absolute differences between groups), along with confidence intervals (for example, 95%) or P values. Approaches such as number needed to treat to achieve a unit of benefit may be included when appropriate. Measures of relative risk also may be reported (eg, relative risk, hazard ratios) and should include confidence intervals. Studies of screening and diagnostic tests should report sensitivity, specificity, and likelihood ratio. If predictive value or accuracy is reported, prevalence or pretest likelihood should be given as well. All randomized clinical trials should include the results of intention-to-treat analysis, and all surveys should include response rates.

Conclusions and Relevance: Provide only conclusions of the study that are directly supported by the results. Give equal emphasis to positive and negative findings of equal scientific merit. Also, provide a statement of relevance indicating implications for clinical practice or health policy, avoiding speculation and overgeneralization. The relevance statement may also indicate whether additional study is required before the information should be used in clinical settings.

Trial Registration: For clinical trials, the name of the trial registry, registration number, and URL of the registry must be included.

Key Points

With the manuscript, a separate Supplemental file called "Key Points," should be submitted.

This feature provides a quick structured synopsis of the findings of your manuscript (required only for research and review manuscripts), following 3 key points: Question, Findings, and Meaning. Limit this section to 75-100 words or less.

Question: Focused question based on the study hypothesis or goal/purpose. Limit to 1 sentence.

Findings: Results of the study/review. Include the design (eg, clinical trial, cohort study, case-control study, meta-analysis, systematic review). Focus on primary

outcome(s) and finding(s). Do not emphasize secondary outcomes. Report basic numbers only but state if results are statistically significant or not significant; do not include results of statistical tests or measures of variance (see example below). Can include 1-2 sentences.

Meaning: Key conclusion and implication based on the primary finding(s). Limit to 1 sentence.

Abbreviations

Do not use abbreviations in the title and limit their use in the text. Expand all abbreviations at first mention in the text.

Methods and Statistics

Authors are encouraged to consult “Reporting Statistical Information in Medical Journal Articles.”¹³ Describe statistical methods with enough detail to enable a knowledgeable reader with access to the original data to reproduce the reported results. When possible, quantify findings and present them with appropriate indicators of measurement error or uncertainty (such as confidence intervals). Avoid relying solely on statistical hypothesis testing, such as the use of P values, which fails to convey important quantitative information. Give details about randomization. Describe the methods for and success of any blinding of observations. Report complications of treatment. Give numbers of observations. Report losses to observation (such as dropouts from a clinical trial). For multivariate models, report all variables included in models, and report model diagnostics and proportion of variance explained by both individual variables and the complete model.

Put a general description of methods in the Methods section. Restrict tables and figures to those needed to explain the argument of the article and to assess its support. Use graphs as an alternative to tables with many entries; do not duplicate data in graphs and tables. Avoid nontechnical uses of technical terms in statistics, such as random (which implies a randomizing device), normal, significant, correlations, and sample. Define statistical terms, abbreviations, and most symbols.

Units of Measure

Laboratory values are expressed using conventional units of measure, with relevant Système International (SI) conversion factors expressed secondarily (in parentheses) only at first mention. Articles that contain numerous conversion factors may list them together in a paragraph at the end of the Methods section. In tables and figures, a conversion factor to SI units should be provided in a footnote or legend. The metric system is preferred for the expression of length, area, mass, and volume. For more details, see the Units of Measure conversion table on the website for the *AMA Manual of Style*.¹

Names of Drugs, Devices, and Other Products

Use nonproprietary names of drugs, devices, and other products, unless the specific trade name of a drug used is essential to the discussion.^{1(pp567-569)}

Gene Names, Symbols, and Accession Numbers

Authors describing genes or related structures in a manuscript should include the names and official symbols provided by the US National Center for Biotechnology Information (NCBI) or the HUGO Gene Nomenclature Committee. Before submission of a research manuscript reporting on large genomic data sets (eg, protein or DNA sequences), the data sets should be deposited in a publicly available database, such as NCBI’s GenBank, and a complete accession number (and version number, if appropriate) must be provided in the Methods section of the manuscript.

Reproduced Material

Please verify that all information and materials in the manuscript are original. *JAMA*

Dermatology generally does not republish text, tables, figures, or other material from other publishers, except in rare circumstances. If you believe that you must include content that is owned by a third party, please let us know and provide information about all material that has been previously published and, when applicable, include author(s), title of article, title of journal or book or other publication, and complete citation, doi, and/or URL. The publisher or other third party's permission to reproduce in print and online and in licensed versions of *JAMA Dermatology* should be submitted to *JAMA Dermatology* when the manuscript is submitted. (See Permission to Reproduce Copyright-Protected Material Guidelines.)

References

Authors are responsible for the accuracy and completeness of their references and for correct text citation. Number references in the order they appear in the text; do not alphabetize. In text, tables, and legends, identify references with superscript arabic numerals. When listing references, follow AMA style¹(pp39-79) and abbreviated names of journals according to the journals list in PubMed. List all authors and/or editors up to 6; if more than 6, list the first 3 followed by "et al." Note: Journal references should include the issue number in parentheses after the volume number.

Examples of reference style:

1. Tempark T, Shwayder T. Chinese fortune-telling based on face and body mole positions: a hidden agenda regarding mole removal. *Arch Dermatol*. 2012;148(6):772-773.
2. Centers for Medicare & Medicaid Services. CMS proposals to implement certain disclosure provisions of the Affordable Care Act. <http://www.cms.gov/apps/media/press/factsheet.asp?Counter=4221>. Accessed January 30, 2012.
3. McPhee SJ, Winker MA, Rabow MW, Pantilat SZ, Markowitz AJ, eds. *Care at the Close of Life: Evidence and Experience*. New York, NY: McGraw Hill Medical; 2011.

Tables

Number all tables in the order of their citation in the text. Include a title for each table (a brief phrase, preferably no longer than 10-15 words). Include all tables at the end of the manuscript file. Refer to Categories of Articles because there may be a limit on the number of tables for the type of manuscript. If a table must be continued, repeat the title on the second page, followed by "(continued)." Make certain that each item in the table sits in its own table cell. Do not use paragraph returns (to start new rows) or tabs (to start new columns) to format the table. Tables must be modifiable within the manuscript file. Do not insert images of tables. Online-only tables will not be edited (see Online-Only Supplements and Multimedia).

Figures

Number all figures (graphs, charts, photographs, and illustrations) in the order of their citation in the text. Include a title for each figure (a brief phrase, preferably no longer than 10 to 15 words). For initial manuscript submission, figures must be submitted as high-resolution, uncompressed TIF or JPG files. Generally, each figure exceeds 200 kB. Please submit photographic images at 5 inches (13 cm) wide at 300 pixels per inch (120 pixels per centimeter), minimum. This applies to each part for multipart figures. Save files as TIF (with LZW compression), JPG (with "maximum quality" setting). Please do not add arrowheads, "a," "b," asterisks, etc, directly to the file that contains the single figure. A separate, composite figure in PowerPoint may be submitted to indicate the location of arrows, asterisks, etc. Submit photographs and

illustrations as separate JPG files for each figure. Do not embed figures in the Word file of the manuscript. If the manuscript is accepted, authors will be asked to provide figures that meet the Guidelines for Figures. Graphs, charts, and legends in accepted manuscripts will be recreated or edited according to JAMA Network style and standards prior to publication. Online-only figures will not be edited or recreated (see Online-Only Supplements and Multimedia).

Acceptable Figure File Size

To reduce the time that it takes to upload files to the *JAMA Dermatology* submission site and for reviewers to download files from the site, we recommend that the file size of figures be compressed before uploading them. This can be done by using compression software or by decreasing the resolution of individual files.

Acceptable Figure File Formats

At submission, the following file formats are acceptable: ai, bmp, doc, emf, eps, jpg, pdf, ppt, psd, tif, wmf, or xls. Figures should be submitted as separate files (1 file per figure or composite figure) and not included in the manuscript text. PPT files can be used to show where arrows may be applied to the image.

Figure Titles and Legends

At the end of the manuscript, include a title and legend for each figure. The figure title should be a brief descriptive phrase, preferably no longer than 10 to 15 words. The figure legend can be used for a brief explanation of the figure or markers if needed (preferred maximum length, 40 words) and expansion of abbreviations. For photomicrographs, include the type of specimen, original magnification or a scale bar, and stain in the legend. For gross pathology specimens, label any rulers with unit of measure. Digitally enhanced images must be clearly identified in the figure legends as enhanced or manipulated, eg, computed tomographic scans, magnetic resonance images, photographs, photomicrographs, x-ray films.

Figures With Labels, Arrows, or Other Markers

Photographs, clinical images, photomicrographs, gel electrophoresis, and the like that include labels, arrows, or other markers must be submitted in 2 versions: one version with the markers and one without. Provide an explanation for all labels, arrows, or other markers in the figure legend. The Figure field in the File Description tab of the manuscript submission form allows for uploading of 2 versions of the same figure.

Number of Figures

Refer to Categories of Articles as there may be a limit on the number of figures for the type of manuscript.

Online-Only Supplements and Multimedia

Authors may submit supporting material to accompany their article for online-only publication when there is insufficient space to include the material in the print article. This material should be important to the understanding and interpretation of the report and should not repeat material in the print article. The amount of online-only material should be limited and justified. Online-only material should be original and not previously published.

Online-only material will undergo editorial and peer review with the main manuscript. If the manuscript is accepted for publication and if the online-only material is deemed appropriate for publication by the editors, it will be posted online at the time of publication of the article as additional material provided by the authors. This material will not be edited or formatted; thus, authors are responsible for the accuracy and presentation of all such material.

Online-only material should be submitted in a single Word document (Supplement)

with pages numbered consecutively. Each element included in the online-only material should be cited in the text of the main manuscript (eg, “see eTable in the Supplement”) and numbered in order of citation in the text (eg, eTable 1, eTable 2, eFigure 1, eFigure 2, eMethods). The first page of the online-only document should list the number and title of each element included in the Supplement.

Online-Only Text

Online-only text should be set in Arial font, 10 point in size, and single-spaced. The main heading of the online-only text should be in 12 point and boldface; subheadings should be in 10 point and boldface.

Online-Only References

All references cited within the online-only document must be included in a separate reference section, including those that also were cited in the main manuscript. They should be formatted just as in the main manuscript and numbered and cited consecutively in the online-only material.

Online-Only Tables

Online-only tables should be inserted in the document and numbered consecutively according to the order of citation as eTable 1, eTable 2, etc. The text and data in online tables should be Arial font, 10 point in size, and single-spaced. The table title should be set in Arial font, 12 point, and bold. Headings within tables should be set in 10 point and bold. Table footnotes should be set in 8 point and single-spaced. See also instructions for Tables. If a table runs on to subsequent pages, repeat the column headers at the top of each page. Wide tables may be presented using a landscape orientation.

Online-Only Figures

Online-only figures should be inserted in the document and numbered consecutively according to the order of citation as eFigure 1, eFigure 2, etc. Figure titles should be set in Arial font, 12 point, bold, and single-spaced. Text within figures should be set as Arial font, 10 point. Figure legends should be set in 8 point and single-spaced. Graphs and diagrams should be exported directly out of the software application used to create them in a vector file format, such as WMF, and then inserted into the Word document. Image file formats such as JPG, TIF, and GIF are generally not suitable for graphs. Photographs, including all radiological images, should be prepared as JPG (highest option) or TIF (uncompressed) files at a resolution of 300 dpi and width of 3 to 5 inches, but the resolution of photographic files with an original resolution less than 300 dpi should not be increased digitally to achieve a 300-dpi resolution. Photographs should be inserted in the document with the “Link to File” button turned off. Wide figures may be presented using a landscape orientation.