



**UNIVERSIDADE ESTADUAL PAULISTA “JÚLIO DE
MESQUITA FILHO”
FACULDADE DE MEDICINA**

Carolina Nunhez da Silva

Expressão de endotelina-1, receptores de endotelina A e B,
bem como receptor de hidrocarboneto arílico (AhR) no
melasma facial em comparação com a pele sã adjacente
fotoexposta

Tese apresentada à
Faculdade de Medicina,
Universidade Estadual
Paulista “Júlio de Mesquita
Filho”, Campus de
Botucatu, para obtenção do
título de Doutora em
Patologia.

Orientadora: Prof^a. Dr^a. Ana Cláudia Cavalcante Espósito
Co-orientador: Prof. Dr. Hélio Amante Miot

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da Silva, Carolina Nunhez

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Orientador: Ana Cláudia Cavalcante Espósito Lemos

Coorientador: Hélio Amante Miot

1. Melanose. 2. Endotelina. 3. Ar poluição. 4. Imunofluorescência. 5. Imunohistoquímica. I. Título.

Impacto potencial desta pesquisa

Impacto científico: Contribuição com a expansão do conhecimento sobre as bases fisiopatológicas do melasma, dermatose multifatorial de etiologia não completamente elucidada, com achados que podem fundamentar novas abordagens terapêuticas para esta dermatose.

Impacto social: A partir da fundamentação de possíveis novas abordagens terapêuticas, melhorar o impacto psicossocial do melasma, o qual afeta a percepção da autoimagem, implica em sofrimento psíquico nos pacientes e está associado a ansiedade e uso de psicotrópicos.

Impacto econômico: A partir de novas abordagens terapêuticas, contribuir para a relação custo-efetividade na escolha do tratamento e com isso, uso racional dos recursos destinados à saúde.

Potential impact of this research

Scientific impact: Contribution to expanding knowledge about the pathophysiological bases of melasma, a multifactorial dermatosis whose etiology is not fully understood. Findings presented in this thesis may support new therapeutic approaches for this dermatosis.

Social impact: Based on the foundation of possible new therapeutic approaches, improve the psychosocial impact of melasma, which affects self-image perception, implies psychological suffering in patients and is associated with anxiety and the use of psychotropic drugs.

Economic impact: Based on new therapeutic approaches, contribute to the cost-effectiveness of treatment selection and, therefore, rational use of resources allocated to health.

ATA DA DEFESA PÚBLICA DA TESE DE DOUTORADO DE CAROLINA NUNHEZ DA SILVA, DISCENTE DO PROGRAMA DE PÓS-GRADUAÇÃO EM PATOLOGIA, DA FACULDADE DE MEDICINA - CÂMPUS DE BOTUCATU.

Aos 26 dias do mês de fevereiro do ano de 2025, às 15h, por meio de Videoconferência, realizou-se a defesa de TESE DE DOUTORADO de CAROLINA NUNHEZ DA SILVA, intitulada **Expressão de endotelina-1, receptores de endotelina A e B, bem como receptor de hidrocarboneto arílico (AhR) no melasma facial em comparação com a pele sã perilesional**. A Comissão Examinadora foi constituída pelos seguintes membros: Profa. Dra ANA CLÁUDIA CAVALCANTE ESPÓSITO LEMOS (Orientador(a) - Participação Virtual) do(a) Universidade do Oeste Paulista (Unoeste), Profa. Dra. LUCIANE DONIDA BARTOLI MIOT (Participação Virtual) do(a) Fundação para o Desenvolvimento Médico e Hospitalar (Famesp), Prof. Dr. DANIEL PINHO CASSIANO (Participação Virtual) do(a) Depto. de Dermatologia / EPM/São Paulo - Unifesp. Após a exposição pela doutoranda e arguição pelos membros da Comissão Examinadora que participaram do ato, de forma presencial e/ou virtual, a discente recebeu o conceito final: 10,00. Nada mais havendo, foi lavrada a presente ata, que após lida e aprovada, foi assinada pelo(a) Presidente(a) da Comissão Examinadora.

Ana Cláudia Espósito Lemos
Profa. Dra ANA CLÁUDIA CAVALCANTE ESPÓSITO LEMOS

Dedicatória

Dedico à minha família, composta por tantos professores, a grande conquista que este trabalho representa.

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Resumo

Fundamentos: O melasma é uma hipermelanose focal adquirida crônica, de etiologia multifatorial, que acomete principalmente a face de mulheres em idade fértil. Embora seja altamente prevalente, sua patogênese ainda não é totalmente compreendida. A radiação ultravioleta (RUV) é seu principal gatilho ambiental, agindo direta e indiretamente na melanogênese. Uma alta prevalência de melasma é observada em países com maior exposição solar e população miscigenada, mas também com altas taxas de poluição. Endotelina-1 (ET-1) é um peptídeo sintetizado por diferentes tipos de célula e tem ação vasoconstritora. Na pele, ET-1 é produzida pelos queratinócitos em resposta à exposição RUV do tipo B, e desempenha um papel na melanogênese ao se ligar a receptores específicos na superfície dos melanócitos - receptores de endotelina A e B (ERA e ERB). O receptor de hidrocarboneto arílico (AhR) é uma proteína citoplasmática que sofre translocação nuclear quando ativada, e atua como um fator de transcrição regulador para a resposta a estímulos ambientais e manutenção da homeostase celular. O AhR pode ser ativado por bifenilos policlorados, dioxinas ou outros hidrocarbonetos aromáticos policíclicos (HAP) presentes na poluição ambiental, além de metabólitos endógenos induzidos pela RUV. Quando ativado, AhR regula positivamente enzimas envolvidas na depuração de xenobióticos e aumenta espécies reativas de oxigênio (ERO), induzindo a expressão de p38. Por sua vez, p38 aumenta a atividade do fator de transcrição MITF, regulando diretamente a expressão da tirosinase nos melanócitos. Até o momento, a expressão de ET-1, ERA, ERB e AhR no melasma não foi sistematicamente investigada.

Objetivos: Avaliar a expressão de ET-1, ERA, ERB e AhR na pele de mulheres com melasma facial em comparação com a pele sã adjacente fotoexposta.

Métodos:

Estudo transversal, com 40 amostras de pele (20: melasma facial; 20: pele sã adjacente) obtidas de biópsias por *punch* (3mm), realizadas em mulheres com melasma facial moderado a grave, sem tratamento há ao menos 30 dias, exceto por uso de protetor

solar, obtidas por conveniência entre as pacientes atendidas em hospital terciário. Coletaram-se dados clínicos e demográficos das pacientes. Foram excluídos extremos fenotípicos, gestantes, lactantes e portadoras de outras dermatoses. As amostras foram emblocadas em parafina e, após, realizou-se técnica de imunofluorescência de tripla marcação para anti-vimentina, DAPI, mais um dos seguintes anticorpos testes: (a) anti-ET1, (b) anti-ERA; (c) anti-ERB. Para análise de Ki67 (anticorpo monoclonal clone MIB1, Dako, M7240, Glostrup, Denmark – 1/50), e para AhR (ab190797, Abcam, Cambridge - MA – 1/100), 40 lâminas foram previamente coradas para Hematoxilina & Eosina, e submetidos à imuno-histoquímica (DAB). De maneira cega para topografia (melasma; pele sã), as áreas interfoliculares de maior intensidade nas lâminas foram fotografadas em triplicata. Para as técnicas de imunofluorescência, foi utilizado microscopia confocal a laser (LEICA TCS SP8) para diferentes filtros de cor. Já para imuno-histoquímica, as lâminas foram fotografadas em microscópio óptico convencional. Usando o software ImageJ v1.51, realizou-se análise semiquantitativa das imagens obtidas, sendo a análise cega quanto à topografia.

Resultados: A média (DP) de idade dos participantes foi de 44,9 (9,2). A expressão de ET-1 mostrou-se aumentada em toda a epiderme com melasma quando comparada à pele sã adjacente, sendo 32,8% (IC95% 14,7%—52,6%) maior na camada espinhosa ($p=0,013$), 30,4% (IC95% 13,7 % —47,9%) maior na camada basal ($p=0,014$) e 29,7% (IC95% 11,4%—49,7%) maior nos melanócitos ($p=0,006$). Houve correlação positiva entre a expressão de ET-1 na epiderme com melasma e a espessura da epiderme ($r=0,51$; $p=0,02$) e com a idade ($r=0,58$; $p=0,01$). Não houve expressão perceptível de ET-1 nas células da derme superior. Nem o ERA nem o ERB resultaram em expressão epidérmica diferencial entre melasma e a pele sã adjacente ($p\geq 0,1$). A imuno-histoquímica revelou maior ativação do AhR na epiderme do melasma em comparação com o epitélio adjacente (1,3 vs. 1,1 núcleo por campo; $p=0,03$). Não houve marcação citoplasmática do AhR na epiderme. Nos folículos pilosebáceos, houve marcação tanto nuclear quanto citoplasmática, sendo mais frequente no melasma que na pele sã adjacente (70% vs. 30%; $p=0,03$). A epiderme com melasma apresentou

menor espessura do epitélio (187 vs. 207 μm ; $p<0,01$), entretanto, maior taxa de proliferação (HSCORE 36 vs. 27; $p=0.02$). Não houve, porém, correlação entre o HSCORE, a espessura da epiderme e a expressão epitelial do AhR ($p>0,3$).

Limitações: Foram incluídas apenas mulheres (embora isso garanta a homogeneidade da amostra) oriundas de apenas um centro de pesquisa e não houve um estudo funcional das vias ativadas pelo AhR.

Conclusão: A ET-1 é expressa mais intensamente na pele de mulheres com melasma facial em comparação com a pele sã adjacente fotoexposta e este aumento se dá às custas de ET-1 epidérmica em detrimento da expressão dérmica. Evidenciou-se ativação do AhR epidérmica e maior expressão do AhR nas unidades pilossebáceas da pele com melasma em comparação com a adjacente.

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Palavras-chave: melasma, técnica de imunofluorescência, imuno-histoquímica, endotelina-1, receptor de endotelina-A, receptor de endotelina-B, Ki-67, poluição, receptor de hidrocarboneto arílico.

Abstract

Fundamentals: Melasma is a chronic acquired focal hypermelanosis of multifactorial etiology, primarily affecting the faces of women of childbearing age. Although it is highly prevalent, its pathogenesis is not yet fully understood. Ultraviolet radiation (UVR) is its main environmental trigger, acting directly and indirectly on melanogenesis. A high prevalence of melasma is observed in countries with greater sun exposure and mixed populations, but also with high rates of pollution. Endothelin-1 (ET-1) is a peptide synthesized by different types of cells and has vasoconstrictive action. In the skin, ET-1 is produced by keratinocytes in response to UVB exposure, mediated by an increase in IL-1 α or reactive oxygen species. ET-1 plays a role in melanogenesis by binding to specific receptors on the surface of melanocytes - endothelin A and B receptors (ERA and ERB) - and is involved in the hyperpigmentation of skin lesions. The aryl hydrocarbon receptor (AhR) is a cytoplasmic protein that undergoes nuclear translocation when activated and acts as a regulatory transcription factor for the response to environmental stimuli and maintenance of cellular homeostasis. AhR can be activated by polychlorinated biphenyls, dioxins, or other polycyclic aromatic hydrocarbons (PAHs) present in environmental pollution, as well as endogenous metabolites induced by UVR. When activated, AhR positively regulates enzymes involved in the clearance of xenobiotics and increases reactive oxygen species (ROS), inducing the expression of p38, which enhances the activity of the transcription factor MITF, directly regulating the expression of tyrosinase in melanocytes. To date, the expression of ET-1, ERA, ERB, and AhR in melasma has not been systematically investigated.

Objectives: To evaluate the expression of ET-1, ERA, ERB, and AhR in the skin of women with facial melasma compared to adjacent sun-exposed healthy skin.

Methods: Cross-sectional study with 40 skin samples (20: facial melasma; 20: adjacent healthy skin) obtained from punch biopsies (3mm) performed on women with moderate to severe facial melasma, without treatment for at least 30 days, except for the use of sunscreen, collected by convenience among patients attending a tertiary hospital.

Clinical and demographic data from the patients were collected. Phenotypic extremes, pregnant and lactating women, and those with other dermatoses were excluded. The samples were embedded in paraffin and then subjected to triple-label immunofluorescence for anti-vimentin, DAPI, plus one of the following test antibodies: (a) anti-ET1, (b) anti-ERA; (c) anti-ERB. In a blinded manner regarding topography (melasma; healthy skin), the interfollicular areas of greatest intensity on the slides were photographed in triplicate under laser confocal microscopy (LEICA TCS SP8) for different color filters. Using ImageJ v1.51 software, a semi-quantitative analysis of the obtained images was conducted. The median values of the mean intensities of the staining from the image histograms (intensity from 0 to 255 pixels) were estimated for different cell types (suprabasal keratinocytes, basal layer, and upper dermis), correcting the obtained value for the background staining of each slide. For Ki67 analysis (monoclonal antibody clone MIB1, Dako, M7240, Glostrup, Denmark – 1/50), and for AhR (ab190797, Abcam, Cambridge - MA – 1/100), 40 slides were previously stained for Hematoxylin & Eosin and subjected to immunohistochemistry (DAB). The values were compared blindly between topographies and correlated with clinical and demographic data.

Results: The mean (SD) age of the participants was 44.9 (9.2). The expression of ET-1 was found to be increased across the epidermis with melasma compared to adjacent healthy skin, being 32.8% (95% CI 14.7%-52.6%) higher in the spinous layer ($p=0.013$), 30.4% (95% CI 13.7%-47.9%) higher in the basal layer ($p=0.014$), and 29.7% (95% CI 11.4%-49.7%) higher in melanocytes ($p=0.006$). There was a positive correlation between ET-1 expression in the melasma epidermis and epidermal thickness ($\rho=0.51$; $p=0.02$) and with age ($\rho=0.58$; $p=0.01$). No perceptible expression of ET-1 was found in the upper dermal cells. Neither ERA nor ERB resulted in differential epidermal expression between melasma and adjacent healthy skin ($p\geq 0.1$). Immunohistochemistry revealed greater activation of AhR in the epidermis of melasma compared to the adjacent epithelium (1.3 vs. 1.1 nuclei per field; $p=0.03$). No cytoplasmic labeling of AhR was observed in the epidermis. In the pilosebaceous follicles, both nuclear and cytoplasmic labeling was observed, being more frequent in melasma than in adjacent healthy skin

(70% vs. 30%; $p=0.03$). The epidermis with melasma (Figure 1) showed a thinner epithelium (187 vs. 207 μm ; $p<0.01$), but a higher proliferation rate (HSCORE 36 vs. 27; $p=0.02$). However, there was no correlation between HSCORE, epidermal thickness, and epithelial expression of AhR ($p>0.3$).

Limitations: Only women were included (although this ensures sample homogeneity) from only one research center, and there was no functional study of the pathways activated by the AhR.

Conclusion: ET-1 is expressed more intensely in the skin of women with facial melasma compared to adjacent sun-exposed healthy skin, and this increase is due to epidermal ET-1 at the expense of dermal expression. There was evidence of epidermal AhR activation and greater AhR expression in the pilosebaceous units of melasma skin compared to the adjacent skin.

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Keywords: melasma, immunofluorescence technique, immunohistochemistry, endothelin-1, endothelin-A receptor, endothelin-B receptor, Ki-67, pollution, aryl hydrocarbon receptor.

Lista de abreviaturas / Siglas

ACTH - hormônio adrenocorticotrófico
 AhR - receptor de hidrocarboneto arílico
 ARNT - Aryl Hydrocarbon Receptor Nuclear Translocator
 CGRP - peptídeo relacionado ao gene da calcitonina
 CTGF - fator de crescimento de tecido conjuntivo
 DLQI - Dermatology Life Quality Index
 ERA - receptor de endotelina A
 ERB - receptor de endotelina B
 ET-1 - endotelina 1
 EROs - espécies reativas de oxigênio
 FICZ - formilindolo- (3,2-b)-carbazol
 FMB - Faculdade de Medicina de Botucatu
 HAPs - hidrocarbonetos aromáticos policíclicos
 HGF - fator de crescimento de hepatócitos
 IFD - imunofluorescência direta
 iNOS - óxido nítrico sintetase induzida
 LGR5 - Leucine-rich repeat-containing G-protein coupled receptor 5 (receptor acoplado à proteína G 5 rico em repetições contendo leucina)
 MASI - Melasma Area and Severity Index
 MC1-R - receptor de melanocortina 1
 MITF - fator de transcrição da microftalmia
 mMASI - MASI modificado
 MMP - metaloproteinase
 MSH - fator estimulador de melanócitos
 NGFβ - fator de crescimento de nervo beta
 OPN3 - receptor opsin 3
 RUV - radiação ultravioleta
 SCF - fator estimulador de *stem cell*
 SFRP2 - secreted Frizzled-Related Protein 2 (Proteína 2 secretada relacionadas a “frizzled”)
 TCLE - Termo de Consentimento Livre e Esclarecido
 UNESP - Universidade Estadual Paulista “Júlio de Mesquita Filho”.
 UVA - radiação ultravioleta A
 UVB - radiação ultravioleta B
 VEGF - fator de crescimento do endotélio vascular
 WIF-1 - fator 1 inibidor de Wnt
 Wnt5a - wingless-type MMTV integration site family, member 5A (família de sítios de integração do MMTV do tipo wingless, membro 5A)
 XRE - elemento de resposta a xenobióticos
 ZMB - zona de membrana basal

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TÍTULO

Expressão de endotelina-1, receptores de endotelina A e B, bem como receptor de hidrocarboneto arílico (AhR) no melasma facial em comparação com a pele sã perilesional.

REVISÃO DE LITERATURA

Introdução

O melasma é uma dermatose pigmentar crônica, adquirida, recidivante, focal que acomete regiões fotoexpostas de adultos, especialmente mulheres em idade fértil e de fototipo III a IV de Fitzpatrick. Tem distribuição geográfica ampla, sendo mais prevalente em populações de países intertropicais: Oriente Médio, Ásia Leste, Índia e América. (1,2) Segundo estudo conduzido em amostra multirracial no Brasil, a prevalência do melasma foi de 36,3%. O relato de acometimento por melasma entre parentes de primeiro grau em 49,1% das pacientes avaliadas, favorece a hipótese de seu caráter genético hereditário. (1) A proporção entre mulheres e homens acometidos varia conforme o país: no Brasil, há 32 mulheres acometidas a cada um homem, enquanto na Índia essa relação é 6:1. (3) Em uma população miscigenada como a brasileira, o melasma facial está relacionado à maior ancestralidade africana. (4–7)

Uma alta prevalência de melasma é observada em países com maior exposição solar e população miscigenada, mas também com altas taxas de poluição, como Índia, sudeste asiático e China. (8)

Um aumento significativo na incidência de melasma está bem documentado na última década. Estudo analisando o volume relativo de pesquisas online sobre o assunto na plataforma Google encontrou um aumento de 54,4% de interesse por melasma em 29 países, entre 2010 e 2019, o que poderia refletir a epidemiologia desta dermatose. No mesmo estudo, em análise multivariada, este dado mostrou associação com a insolação do país, sua taxa de uso de contraceptivo oral e de emissão de gás carbônico. (9)

Clínica e diagnóstico

Clinicamente, caracteriza-se por máculas acastanhadas de limites precisos e irregulares, bilaterais e simétricas, acometendo preferencialmente a face: região malar, fronte, glabella, nariz, mento e região supralabial. O melasma extra-facial é menos comum e pode ocorrer nos membros superiores e tórax, sempre restringindo-se a áreas fotoexpostas. [Figura 1]



Figura 1. Paciente do sexo feminino com melasma facial: máculas hiperocrômicas simétricas distribuídas em área fotoexposta. [Acervo do pesquisador]

Em geral, o melasma não infringe sintomas físicos. No entanto, devido a seu grande potencial de gerar alteração da percepção da autoimagem, culmina, em grande parte dos pacientes, em sofrimento psíquico relevante e impacto negativo psicossocial, tendo associação de risco independente com ansiedade e uso de psicotrópicos. (7). Estudo de

2021, conduzido com mulheres brasileiras portadoras de melasma, identificou, por meio de aplicação de questionário *online*, comprometimento da autoestima em mais de 28% das participantes e forte correlação com ansiedade e depressão. (10)

O diagnóstico do melasma, em geral, não representa um desafio ao dermatologista e é eminentemente clínico, tendo como base as características das lesões, localização e epidemiologia. A dermatoscopia pode ser ferramenta de grande utilidade em casos de difícil diagnóstico e, principalmente, para o estabelecimento de diagnóstico diferencial com outras dermatoses. Na dermatoscopia de lesões faciais de melasma, é descrita a ocorrência de pseudorrede difusa acastanhada, com múltiplos pontos acastanhados a enegrecidos, grânulos e glóbulos esparsos, além de estruturas arciformes ou anulares acastanhadas perifoliculares. (11) A epiluminescência pela lâmpada de Wood (365nm) é útil especialmente por realçar lesões de melasma ainda subclínicas. À microscopia confocal, as lesões de melasma apresentam elastose solar e aumento dos vasos sanguíneos dérmicos, bem como distribuição heterogênea dos melanóforos entre diferentes regiões; apesar de não ser uma ferramenta disponível na prática clínica diária, pode permitir prever a resposta clínica às abordagens terapêuticas. (11–14)

Os principais diagnósticos diferenciais do melasma são: efélides, lentigo solar, melanoderma tóxica, hiperpigmentação pós-inflamatória, melanose friccional, ocronose, líquen plano pigmentoso, lúpus eritematoso cutâneo, fitofotodermatose, nevus de Ota ou Hori, pseudoacantose nigricante, manchas café com leite, ceratose seborreica, poiquilodermia de Civatte, hiperpigmentação periocular e pigmentação induzida por droga. (13)

Classificação

Como meio de objetivar a avaliação clínica do paciente acometido por melasma, elaboraram-se escores, como o MASI (*"Melasma Area and Severity Index"*), descrito em 1994 por Kimbrough-Green e colaboradores. Esta escala é utilizada para avaliação da

gravidade e da resposta terapêutica em pacientes com melasma facial. (14,15) Através da inspeção visual, a face é dividida em quatro regiões (frontal, malar direita e esquerda, mento) e, em cada uma delas, avalia-se a área de acometimento, intensidade de pigmentação e homogeneidade.

As regiões frontal, malar direita, malar esquerda e mento representam, respectivamente, 30%, 30%, 30% e 10% da área total da face. Intensidade de pigmentação e homogeneidade são avaliados em uma escala de 0 a 4 (0, ausente; 1, leve; 2, moderado; 3, importante; 4, máximo) - Figura 2. (13)

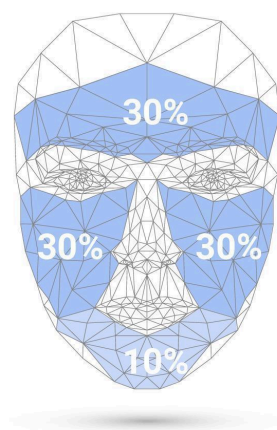


Figura 2: Diagrama representativo das regiões faciais para cálculo do MASI. [Imagem dos autores]

O MASI foi submetido a um processo de validação, tendo sido eliminado o critério de homogeneidade - MASI modificado (mMASI) -, o que não só manteve sua validade, confiabilidade e responsabilidade a mudanças na intensidade do quadro clínico, como também o tornou mais reprodutível. (16–18) Para o cálculo de mMASI, utiliza-se apenas a avaliação da intensidade da pigmentação e a área de envolvimento nas quatro regiões da face, o que tornou o escore mais fácil de ser calculado. (16). O escore total do mMASI varia de 0 a 24 e consiste da somatória ($\sum e$) dos escores (e) de cada área facial conforme tabela abaixo:

Tabela 1: mMASI. Esquema de cálculo do índice de gravidade do melasma simplificado. O escore varia de 0 a 24, sendo mais grave quanto maior a pontuação.

	Área afetada *	Intensidade de pigmentação **	Fator de multiplicação	Valor
Frontal		x	x 0,3	
Malar Direita		x	x 0,3	
Malar Esquerda		x	x 0,3	
Mento		x	x 0,1	
mMASI			SOMA	

* Área afetada (0-6) : 0 pele normal; 1: < 10%; 2: 10 - 30 %; 3: 30-49%; 4: 50-69%; 5: 70-89%; 6: 90-100%

** Intensidade de pigmentação (0-4) : 0: nenhuma; 1: leve ; 2: média; 3: marcante e 4: máxima.

Fisiopatologia do melasma

Daremos ênfase à fisiopatologia desta dermatose, que é o objeto de estudo desta tese. A pele com melasma apresenta alterações morfológicas e funcionais em relação à pele são adjacente, as quais lhes conferem características únicas e que não se limitam apenas ao fotoenvelhecimento. Apesar das diversas pesquisas recentes na área e o aprofundamento em seu conhecimento, a fisiopatologia do melasma é complexa e ainda não completamente conhecida.

Alterações morfológicas e funcionais

O melasma envolve alteração fenotípica localizada na pele, com comprometimento funcional e morfológico da epiderme, zona de membrana basal (ZMB) e derme superior, o que reforça sua complexidade. (19)

Há compactação da camada córnea, atraso da restauração da função barreira cutânea e atrofia da camada granulosa, com achatamento dos cones epidérmicos [Figura 3]. (19,20)

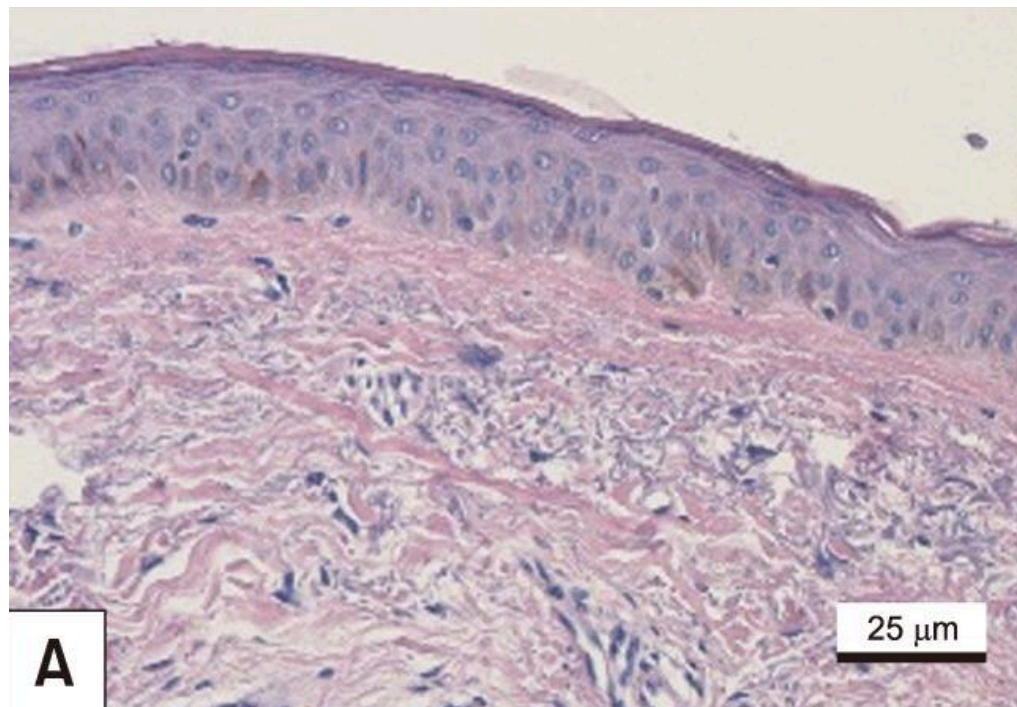


Figura 3: Compactação da camada córnea, atrofia epidérmica e elastose solar proeminente visível na derme de amostra cutânea de melasma facial (Hematoxilina e eosina; x100). (19)

O aumento na densidade de eumelanina dispersa nos queratinócitos em todas as camadas da epiderme é a alteração mais preponderante no melasma, com melanócitos em número normal, porém hipertróficos e hiperfuncionantes. Quando comparada à pele são adjacente, os dendritos dos melanócitos da pele com melasma são mais proeminentes, os melanossomos são mais maduros e há maior taxa de transferência de melanossomos aos queratinócitos. (21)

É mais frequente na pele com melasma a ocorrência de melanócitos projetados da camada basal à derme papilar (“melanócitos em pêndulo”), cuja função na fisiopatogênese da dermatose não é bem estabelecida, mas são considerados

marcadores do fotoenvelhecimento e sua frequência parece ter correlação com a resposta terapêutica. [Figura 4] (22,23)

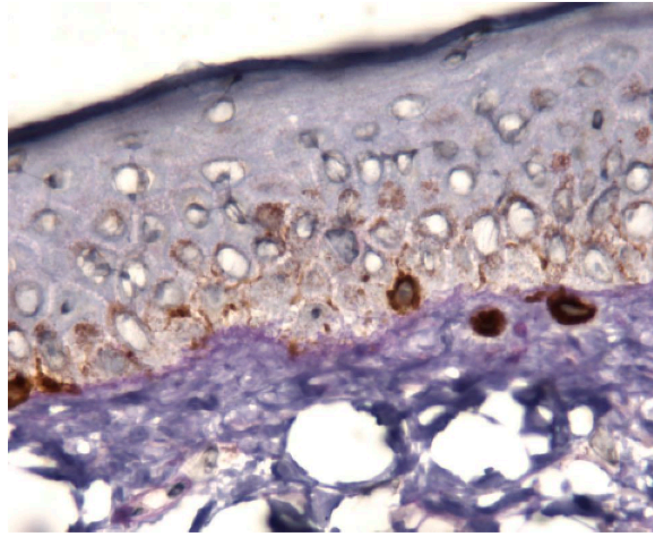


Figura 4: Melanócitos “em pêndulo” e descontinuidade da membrana basal, imunomarcação pelo Melan- A e contracoloração pelo ácido periódico de Schiff (PAS). (19)

Há perda de continuidade estrutural da zona de membrana basal [Figura 4], a qual apresenta-se fragmentada, com lâmina densa mais fina e perda das fibrilas de ancoragem na lâmina lúcida, provavelmente resultantes da degradação dos colágenos IV e VII pelas metaloproteinases (MMPs) 2 e 9. (24) Essas alterações facilitam o trânsito de citocinas na comunicação parácrina entre a derme e a epiderme, a qual é considerada responsável pelo estímulo sustentado à melanogênese do melasma, considerando seu caráter crônico e recidivante. (25,26)

A derme da pele com melasma apresenta elastose solar mais proeminente se comparada à pele sã adjacente [Figura 5], resultante da ativação de metaloproteinases pela radiação ultravioleta (RUV), ativação mastocitária e perfil citocinas pró-inflamatórias e pró-melanogênicas (ex.: SCF, HGF, NGF β) secretadas pelos fibroblastos

precocemente senescentes. (19,22,27) Tais fibroblastos senescentes resultam da exposição à RUV e estão aumentados na pele com melasma. Caracterizam-se por serem menos fusiformes, com menor taxa mitótica e terem alteração no perfil secretório, conforme mencionado. (28,29)

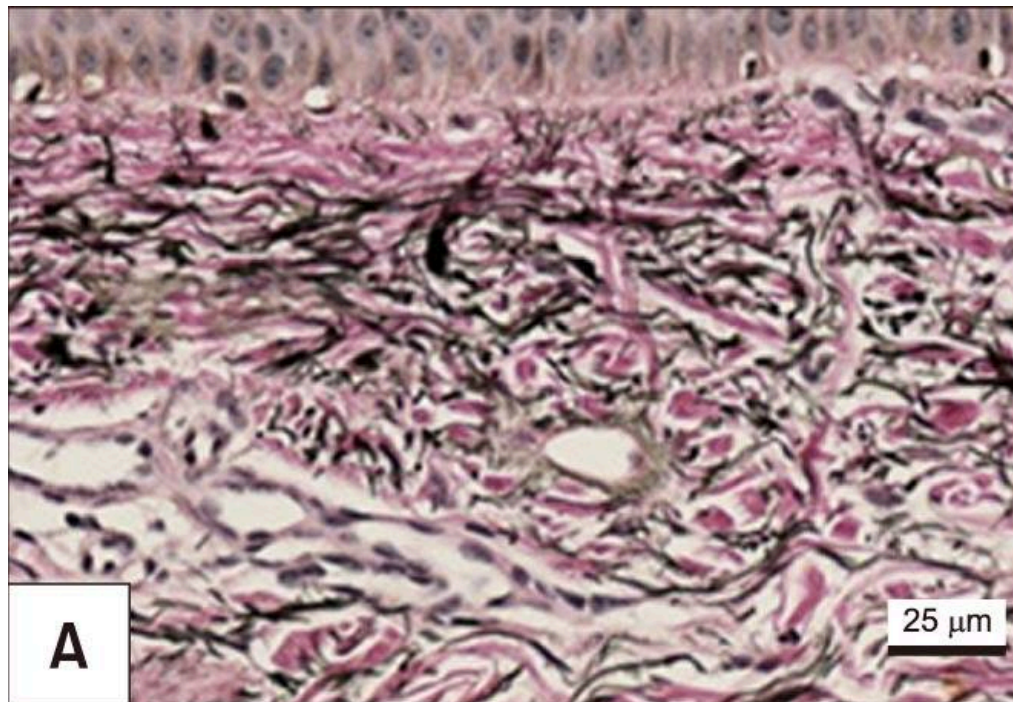


Figura 5: Elastólise proeminente de fibras elásticas em amostra de pele com melasma facial. (Verhoeff; x 200). (19)

O incremento do componente vascular dérmico no melasma também contribui com a hiperpigmentação, em decorrência do aumento de fatores melanogênicos como o fator de crescimento do endotélio vascular (VEGF) e fator estimulador de *stem cell* (SCF). (29–32) Mastócitos estão anormalmente aumentados em número na pele com melasma, preferencialmente ao redor dos vasos, e esta migração à derme parece estar relacionada à secreção de SCF pelos fibroblastos senescentes. (33)

Os mastócitos, em resposta a diversos estímulos ambientais - como calor, pressão e RUV -, degranulam e a histamina liberada tem ação direta sobre os melanócitos, via receptor H2, promovendo síntese de melanina. A triptase também liberada, por sua vez,

ativa MMPs, que contribuem para a degradação de colágeno. Os mastócitos também são capazes de promover a indução da proliferação de vasos na derme superior. (20)

O mecanismo de autofagia - processo catabólico de defesa celular que elimina macromoléculas anormais – está comprometido nos melanócitos da pele com melasma, o que pode favorecer o acúmulo de melanossomas alterados e repletos de melanina. (34–36)

No melasma há expressão aumentada de genes relacionados à melanogênese (TYR, TYRP1 e MLNA), relacionados ao aumento de transferência de melanossomos entre melanócitos e queratinócitos (Myosin 5a e GDA), genes moduladores da via melanogênica Wnt/ β -catenina (Wnt5a, SFRP2, LGR5 e WIF1), genes relacionados às prostaglandinas (PTGIS e PTG1S), além de genes ligados às vias de reparo (SER-PINB3) e angiogênese (angiopoitina-like 1 e 2, heparanase e MMP2). Também há incremento na expressão de genes relacionados à dendricidade melanocítica, à supressão da degradação da enzima tirosinase, ao aumento de fatores melanogênicos pró-inflamatórios, aos defeitos de reparo, à autofagia e genes relacionados ao aumento da degradação de estruturas da zona de membrana basal (ZMB). (28,37–39)

Fatores Etiopatogênicos

Em indivíduos geneticamente predispostos, a exposição solar é o fator de risco mais relevante para a patogênese do melasma. Diversos comprimentos de onda do espectro solar agem sobre a pele de modo a estimular, isolada e conjuntamente, a pigmentação cutânea. A radiação ultravioleta (RUV) - especialmente nas faixas de comprimento UVA (320 - 400nm) e UVB (280 - 320 nm) - tem ação direta sobre os melanócitos e também indireta, ao promover secreção de citocinas pró-melanogênicas pelos queratinócitos, fibroblastos e mastócitos, que induzem a síntese de melanina de forma parácrina. (40)

A exposição solar crônica acarreta fotoenvelhecimento, estresse oxidativo, inflamação e melanogênese sustentada, os quais ocorrem no melasma. A UVB está mais relacionada ao eritema RUV-induzido e, UVA, mais relacionada à pigmentação imediata e persistente. (40) A ação da UVA na pele e sua participação na melanogênese envolve a

ocorrência de estresse oxidativo a partir de espécies reativas de oxigênio formadas à fotoestimulação. (41)

Nos queratinócitos, a RUV induz expressão de p53, promovendo a síntese de proopiomelanocortinas, como hormônio adenocorticotrópico (ACTH), fator estimulador de melanócitos (MSH), β -endorfina e laminina-332, os quais estimulam a melanogênese e a transferência de melanossomos, paracrinamente. (40) Há também produção epidérmica de endotelina, óxido nítrico, leucotrienos e prostaglandinas, que atuam sobre os melanócitos, promovendo aumento dos dendritos e aumento de expressão do gene TYR. (33,42)

A UVB contribui também para a degradação das pontes de heparan-sulfato na ZMB, o que favorece o trânsito de estímulos melanogênicos dérmicos à epiderme. (43) Por fim, a UVB também aumenta a produção de mediadores pró-inflamatórios, como prostaglandinas e fator de crescimento endotelial vascular (VEGF), induzindo proliferação vascular. Neuropeptídeos envolvidos na melanogênese, como peptídeo relacionado ao gene da calcitonina (CGRP), também são estimulados pela RUV. (30)

A luz visível é capaz de induzir pigmentação, entretanto apenas em indivíduos de fototipo mais alto (III-VI), a altas dosagens e em comprimentos de onda menores (420 - 470 nm, luz azul-violeta), por meio da ativação do receptor opsina 3 (OPN3) dos melanócitos. (44) No entanto, não há diferença na expressão de OPN3 na superfície de diferentes tipos celulares da pele com melasma em comparação com a pele sã adjacente, sugerindo não ser a superexpressão de OPN-3 o mecanismo fisiopatológico que explique a hiperpigmentação pela luz visível na pele com melasma. (45)

Metade do espectro da luz solar é composto pela radiação infra-vermelha (IV), percebida como calor. Sabe-se que a IV ativa vias inflamatórias, de degradação de matriz extracelular, de vasodilatação e de aumento do estresse oxidativo, todos componentes presentes na fisiopatologia do melasma. Ainda assim, o papel da IV no melasma é

incerto, apesar de haver piora da dermatose em situações de exposição crônica ao calor. (40,46,47) Há, inclusive, maior prevalência de melasma entre mulheres que trabalham expostas a fontes de calor, como as cozinheiras. (47)

A preponderância do melasma em mulheres, seu surgimento em idade fértil e a melhora da condição após a menopausa são fatos que apontam a favor de seu componente etiológico hormonal. Além disso, a gestação e o uso de anticoncepcionais hormonais também são conhecidos fatores desencadeantes da dermatose. (4,6,7,48,49)

O estradiol age sobre receptores nucleares específicos nos queratinócitos: receptor de estrogênio α (ER1) e β (ER2). Através deles, o estradiol é capaz de ativar vias melanogênicas, além de modular funções de proliferação epitelial e síntese de colágeno em fibroblastos. (50,51) O papel da progesterona sobre a pele ainda é incerto. (52,53)

Outro provável componente etiológico do melasma é a alteração do equilíbrio oxidativo do microambiente cutâneo. A exposição à UVR, poluição atmosférica, excesso de exercícios físicos e a privação de sono são estímulos que levam à formação de espécies reativas de oxigênio (EROs) na pele, que promovem dano oxidativo celular, principalmente ao interagirem com membranas lipídicas. Em condições normais, o dano oxidativo de EROs é inibido por substâncias antioxidantes endógenas. (54,55)

A pele com melasma apresenta sinais de estresse oxidativo quando comparada à pele sã adjacente, como superexpressão de p38 e, cabe ressaltar, a própria melanogênese é um processo oxidativo. (56,57) Vários dentre os tratamentos eficazes para melasma envolvem substâncias com ação antioxidante, como vitamina C, niacinamida, cisteamina, ácido kójico, ácido fítico e picnogenol, o que encoraja o estudo dos mecanismos que alterem o equilíbrio oxidativo e que possam promover a manutenção desta dermatose. (58)

Poluentes atmosféricos parecem ser um importante fator contributivo para deflagrar o melasma, já que são capazes de ligar-se ao receptor de hidrocarboneto arílico (AhR) que, ao ser ativado, promove aumento de tirosinase nos melanócitos e síntese de

melanina. (59,60) Além disso, o número de pacientes com melasma mostra-se maior em países com maior poluição atmosférica e sua incidência crescente parece acompanhar o ritmo da urbanização e industrialização da sociedade. (9,61)

Endotelina

Inicialmente descritas como potentes vasoconstritores produzidos por células endoteliais e células musculares lisas vasculares, as endotelinas são polipeptídeos compostos por 21 aminoácidos [Figura 6] e são importantes no equilíbrio hidro-pressórico dos sistemas cardiovascular, pulmonar e renal (62) pela modulação de óxido nítrico, principalmente, estando envolvidas na patogênese de diversas doenças vasculares. (63) Das 3 endotelinas descritas (endotelina-1, endotelina-2 e endotelina-3), a endotelina-1 (ET-1) é a mais abundante e é codificada pelo gene EDN1 expresso por vários tipos celulares de vertebrados como células imunes, endoteliais, neurônios e queratinócitos. (64)

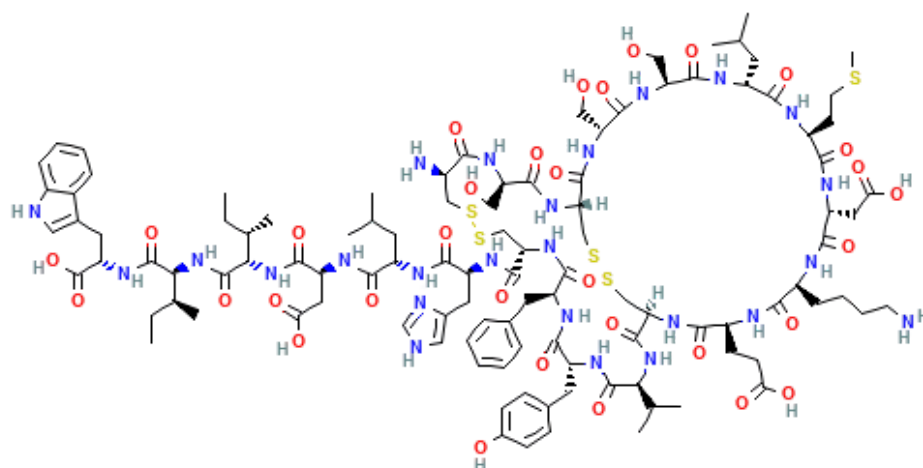


Figura 6 : Representação da estrutura química molecular da endotelina. (65)

As endotelinas possuem receptores específicos na superfície dos melanócitos (receptor de endotelina A (ERA) e receptor de endotelina B (ERB)) e, atuam sobre essas e outras células da crista neural desde o processo de embriogênese, regulando sua proliferação, migração, diferenciação e melanogênese. (25,66–68)

ET-1 é estudada na fisiopatogênese de outras afecções cutâneas. Mostrou-se aumentada em condições como dermatite atópica e psoríase, nas quais parece estar relacionada ao prurido por vias histamina-independentes. (64,69,70) Fibroblastos estimulados com ET-1 mostraram aumento da produção de colágeno tipo 1 e fator de crescimento de tecido conjuntivo, e seu potencial papel regulatório na fibrose dérmica de condições como a esclerose sistêmica direciona esforços na busca de alvos terapêuticos. (71)

O receptor de endotelina B (ERB), é uma proteína transmembrana de 7 domínios ligada à proteína-G, e é igualmente estimulado por todas as formas de endotelina e altamente expresso na superfície dos melanócitos, tendo papel central na pigmentação UVB induzida. (25)

À mínima exposição à radiação UVB, queratinócitos produzem, entre outros fatores, a ET-1 que age paracrinamente sobre os melanócitos através do ERB, ativando vias melanogênicas nestas células capazes de manterem-se ativas por até 10 dias após a exposição solar. (25,72,73) A expressão de fatores melanogênicos como o fator de transcrição de microftalmia (MITF), receptor de melanocortina 1 (MC1R), tirosinase (TYR) e a expressão do ERB são aumentadas pela ação da ET-1, causando aumento da tirosinase e dopacromotautomerase por sinalizações intracelulares via MAPKs ERK1/2 e p38. (31,74)

ET-1 é capaz de promover alterações estruturais nos melanócitos, como aumento no número de dendritos celulares, prolongamento de dendritos e aumento na taxa de transferência de melanossomos. (29) Está envolvida no aumento de componentes necessários à formação de melanossomos em lentigos solares, o que mostra possível relação da ET-1 com mecanismos de autofagia responsáveis pela degradação de melanossomos, deficitários na pele com melasma. (75)

A expressão de ET-1 e ERB está aumentada em lesões de lentigo solar em humanos, e ET-1 derivada de queratinócitos está aumentada também em outras lesões pigmentares como queratoses seborreicas e carcinomas basocelulares pigmentados (76,77) bem como na epiderme sobrejacente à derme hipervascularizada de lesões vasculares benignas. (31)

Apesar de já estabelecida a relação entre endotelina e melanogênese, esta relação ainda não foi sistematicamente avaliada na pele acometida por melasma.

Receptor de hidrocarboneto arílico

O receptor de hidrocarboneto arílico (AhR) [Figura 7] é um receptor citoplasmático que libera suas proteínas acessórias quando ativado e inicia um sinal de translocação nuclear, intermediando diversos processos celulares. Está presente em vários tipos de células da pele, incluindo melanócitos, queratinócitos, células de Langerhans, mastócitos e fibroblastos e, participa da proliferação, diferenciação, apoptose e hiperpigmentação celular.

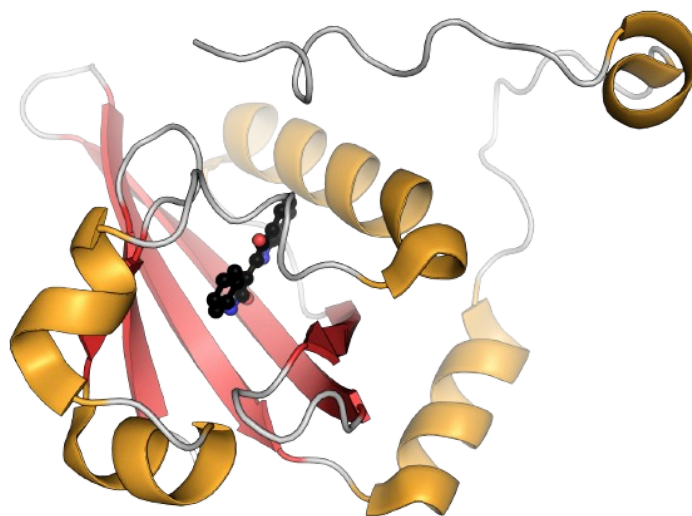


Figura 7: Representação do receptor de hidrocarboneto arílico ligado a indirubin, um composto químico.

(Fonte: https://commons.wikimedia.org/wiki/File:Aryl_hydrocarbon_receptor_PDB_7ZUB.png)

O AhR é um conhecido sensor pleiotrópico de xenobióticos, termo usado para descrever os poluentes ambientais sintéticos produzidos em grandes volumes nas atividades industriais, agrícolas, domésticas e urbanas, como ozônio, bifenilos policlorados, dioxinas e outros hidrocarbonetos aromáticos policíclicos (HAPs), substâncias estas altamente lipofílicas. A dioxina, por exemplo, está amplamente presente na fumaça dos escapamentos veiculares. Esses agentes podem ligar-se ao AhR e torná-lo ativado. (78) Quando submetida à intensa poluição atmosférica, a pele apresenta déficit de barreira, com menor produção de esqualeno, desequilíbrio oxidativo e alteração da microbiota local, podendo contribuir com o desencadeamento de doenças inflamatórias e neoplásicas, além do envelhecimento extrínseco da pele. (78–80)

Na derme, material particulado proveniente da poluição atmosférica, pode induzir fenótipos senescentes nos fibroblastos, com aumento de metaloproteinases e citocinas inflamatórias, comprometendo a colagênese. (81)

Estudo *in vitro* com fibroblastos dérmicos expostos a material particulado mostrou aumento significativo da expressão de metaloproteinase-1 (MMP-1), metaloproteinase-3 (MMP-3) e citocinas inflamatórias e queda de fator de crescimento transformador beta (TGF-beta), colágeno tipo 1 e elastina, apresentando alterações associadas ao envelhecimento cutâneo. (81)

A luz UV, principalmente UVB, e a exposição à luz visível geram formilindolo-(3,2-b)-carbazol (FICZ), um derivado do triptofano. FICZ é um ligante de alta afinidade e ativador endógeno do fator de transcrição do receptor de hidrocarboneto arílico (AhR), o que indica uma ação sinérgica da radiação solar e poluentes atmosféricos.

Quando a via AhR é ativada, promove a regulação positiva da tirosinase nos melanócitos. A produção de melanina também é altamente regulada por atividade parácrina dos queratinócitos circundantes. (59,82)

Observações clínicas após exposição a tóxicos ambientais sugeriram uma ligação entre a ativação do AhR e a melanogênese. No Japão, em 1968 e em Taiwan em 1979, eventos acidentais de envenenamento em massa ocorreram por óleo de cozinha, entre os quais estão fortes ligantes AhR. As pessoas expostas apresentaram aumento da pigmentação da pele e da mucosa gengival. Os mecanismos moleculares subjacentes à hiperpigmentação induzida por xenobióticos não foram estabelecidos. (59,82)

Há acúmulo de evidências científicas de que a poluição do ar desempenha um papel importante no envelhecimento extrínseco e na pigmentação. De maneira sinérgica à exposição solar e a fatores genéticos, a poluição pode ser um fator de risco para o melasma por meio da penetração cutânea de material particulado e HAPs, promovendo ativação da melanogênese e processos de senescência celular via AhR.

Até o momento, a expressão de AhR não foi avaliada em melanócitos e queratinócitos da pele com melasma. (8)

Tratamento

Considerando a RUV como o principal fator etiopatogênico do melasma, sua primeira medida terapêutica inclui o uso de protetor solar com cor, de amplo espectro de proteção, o qual é capaz de diminuir a hiperpigmentação, prevenir recidivas e evitar o desencadeamento em indivíduos de alto risco para desenvolvimento do melasma. (30,43,83). O êxito do tratamento está vinculado à adequada utilização do protetor solar, bem como adesão a outras medidas fotoprotetoras, como uso de barreiras físicas (óculos de sol, chapéu, sombrinhas, etc).

As medicações tópicas despigmentantes podem ter diferentes mecanismos de ação, sendo o principal descrito a inibição da tirosinase (forma como atua o ácido azelaico,

arbutin, hidroquinona e tiamidol). (84–86) Outros agentes tem ação antioxidantes e/ou no transporte da melanina, assim como a niacinamida e a vitamina C. (67,68) A fórmula tríplice (hidroquinona 4% + fluocinolona acetonida 0,01% + tretinoína 0,1%) ainda é o padrão-ouro do tratamento, em associação ao uso do fotoprotetor, e tem taxas de resposta superiores ao uso isolado de hidroquinona 4%. (87)

Medicações sistêmicas incluem o uso de antioxidantes orais, como pycnogenol (extrato de *Pinus pinaster*) e *Polypodium leucotomos*. No entanto, a medicação sistêmica mais promissora e estudada é o ácido tranexâmico, antifibrinolítico inibidor da plasmina e endotelina-1, capaz de intervir na comunicação parácrina entre queratinócitos e melanócitos, além de diminuir atividade mastocitária, e fatores secretados por fibroblastos. (88–90) O ácido tranexâmico oral é eficaz no tratamento do melasma e é considerado um adjuvante à terapêutica com tríplice associação e protetor solar. (91) Formulações tópicas e intradérmicas com ácido tranexâmico mostram resultados mais limitados de clareamento do que a administração por via oral. (22,90,92)

Medidas comportamentais, medicações tópicas e sistêmicas têm resultados otimizados pela associação de procedimentos realizados em consultório, tendo como destaque o microagulhamento (IPCA ® protocolo de injúria moderada), que já pode ser considerado primeira linha do tratamento do melasma, quando associado aos demais tratamentos. (93,94)

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OBJETIVOS

OBJETIVO PRINCIPAL

Avaliar a expressão de endotelina-1 (ET-1), receptor de endotelina-A (ERA) e receptor de endotelina- B (ERB) na epiderme e derme de mulheres com melasma facial em comparação com a pele sã adjacente.

Avaliar a expressão do receptor de hidrocarboneto arílico na epiderme e derme da pele com melasma em comparação com a pele sã adjacente.

OBJETIVO SECUNDÁRIO

Avaliar a expressão do receptor de hidrocarboneto arílico nas glândulas sebáceas da pele com melasma em comparação com a pele sã adjacente.

ARTIGO PUBLICADO

Aponte uma câmera de celular ao QR code abaixo para ser direcionado à página do artigo na íntegra intitulado “Expression of Endothelin-1, Endothelin Receptor-A, and Endothelin Receptor-B in facial melasma compared to adjacent skin”, anexado nas páginas a seguir.

**ACESSO AO ARTIGO**

Expression of Endothelin-1, Endothelin Receptor-A, and Endothelin Receptor-B in facial melasma compared to adjacent skin

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Background/Objectives: Although melasma is highly prevalent, its pathogenesis is not yet fully understood. In the skin, endothelin-1 (ET-1) is primarily produced by keratinocytes in response to UVB exposure, which is mediated by an increase in IL-1 α or reactive oxygen species. ET-1 plays a role in melanogenesis by binding to specific receptor B (ERB) or receptor A (ERA). However, the expression of ET-1, ERA, and ERB in melasma has not been systematically investigated. The objective of this study was to evaluate the expression of ET-1, ERA, and ERB in facial melasma compared to the adjacent unaffected skin.

Methods: Cross-sectional study, with 40 skin samples (20: facial melasma; 20: adjacent unaffected skin) from women with facial melasma without treatment for 30 days except for sunscreen. A triple staining immunofluorescence technique was performed for anti-vimentin, DAPI, plus one of the following antibodies: (a) anti-ET-1, (b) anti-ERA; (c) anti-ERB. Interfollicular areas on the slides of each topography (melasma; unaffected skin) were photographed in triplicate under confocal laser microscopy. The mean staining intensities of the image histograms (0–255 pixels intensity) were estimated for different types of cells (suprabasal keratinocytes, basal layer, and upper dermis) and were blindly compared between topographies.

Results: The mean (SD) age of the participants was 44.9 (9.2). The expression of ET-1 was increased in the whole epidermis with melasma when compared to the adjacent skin, being 32.8% (CI95% 14.7%–52.6%) higher in the spinous layer ($p=0.013$), 30.4% (CI95% 13.7%–47.9%) higher in the basal layer ($p=0.014$), and 29.7% (CI95% 11.4%–49.7%) higher in the melanocytes ($p=0.006$). There was no noticeable expression of ET-1 within the cells on the upper dermis. Neither ERA nor ERB resulted in differential epidermal expression between melasma and unaffected skin ($p\geq 0.1$).

Conclusion: ET-1 is expressed more intensely on the epidermis from the skin with facial melasma compared to the unaffected adjacent skin.

Keywords: melasma, immunofluorescence technique, endothelin-1, endothelin receptor-A, endothelin receptor-B

Introduction

Melasma is a chronic, acquired hypermelanosis with a multifactorial aetiology that mainly affects the faces of women during their childbearing years. Although melasma is highly prevalent, its pathogenesis is not yet fully understood.¹ Sunlight exposure is the most relevant environmental trigger in its pathogenesis.² However, the exact role of different wavelengths of solar radiation and the combined effects of these wavelengths are not acknowledged.³

Ultraviolet radiation (UVR) plays both a direct and indirect role in the melanogenesis process. It specifically stimulates melanocytes to produce melanin and also activates keratinocytes, mast cells, and fibroblasts, which paracrinally regulate melanogenesis.⁴ Exposure to UVR upregulates the production of basic fibroblast growth factor, endothelin-1

(ET-1), and GM-CSF (Granulocyte-Macrophage Colony Stimulating Factor) by keratinocytes, which in turn also stimulate the growth of melanocytes.⁵⁻⁸

ET-1 is a 21-amino acid peptide encoded by the *EDN1* gene and expressed by various cell types, including immune cells, endothelial cells, neurons, and keratinocytes.⁹ Among the three endothelins described, ET-1 is the most abundant. When synthesized by endothelial cells, it acts as a potent vasoconstrictor and has been associated with the pathophysiology of several vascular diseases.¹⁰ In addition to being enhanced in the skin of patients with atopic dermatitis and psoriasis, ET-1 has been shown to induce pruritus in mice and humans via a histamine-independent mechanism.^{9,11,12} ET-1 is also a keratinocyte-derived factor that stimulates nearby melanocytes by binding to its receptor, either endothelin receptor A (ERA) or endothelin receptor B (ERB). This binding activates intracellular signaling cascades, which regulate melanocyte proliferation and melanogenesis, throughout ERB activation.¹³

In vitro studies have shown that treatment with ET-1 in a culture system of sheep skin melanocytes increases the number of melanocytes and melanin content. Furthermore, the expression of microphthalmia-associated transcription factor (MITF), melanocortin 1 receptor (MC1R), tyrosinase (TYR), and endothelin receptor B (ERB) in melanocytes was upregulated by ET-1.¹⁴

In humans, the expression of ET-1 and its receptor ERB are increased in senile lentigos, and ET-1 has been shown to induce melanogenesis and increase melanosome transport from melanocytes to keratinocytes. Inhibition of ERB function substantially reduces melanogenic ability in tissue-cultured senile lentigos. ET-1 also upregulates the expression of components necessary for early melanosome formation in these lesions, indicating its counteraction against autophagy-targeting melanosome degradation in melanocytes.¹⁵ Enhanced expression of keratinocyte-derived ET-1 has also been associated with hyperpigmentation observed in other diseases, such as pigmented basal cell carcinoma and seborrheic keratosis.¹⁶⁻¹⁸

The expression of ET-1, ERA, and ERB in melasma has not been systematically explored to date. Therefore, the objective of this study was to evaluate the expression of ET-1, ERA, and ERB in the epidermis and upper dermis from facial melasma and compare it with the unaffected adjacent skin.

Methods

The protocol was approved by the Institution's Ethics Committee from Unesp Medical School, and all the participants signed the consent form. The study complies with the Declaration of Helsinki.

Twenty women with facial melasma (moderate to severe mMASI - modified Melasma Area and Severity Index), clinically diagnosed by a qualified dermatologist, without treatment for at least 30 days, except for the sunscreen use, were enrolled in the study. They were selected among patients treated at the Hospital das Clínicas da Faculdade de Medicina de Botucatu - FMB/Unesp.

Patients with concomitant facial dermatoses, photosensitive dermatoses, collagenosis, blood dyscrasias, or those using anticoagulant medication, immunosuppressive drugs, pregnant, or lactating women were excluded from the study. Phenotypic extremes, such as individuals with red hair or intensely dark skin, were also excluded since melasma is rare in these groups.

Clinical and demographic information were required from these participants. Two skin samples were obtained from each participant using a 3mm punch biopsy under local anesthesia (lidocaine 2%). One sample was taken from the facial melasma lesion, and the other was taken from adjacent unaffected skin that was exposed to UV radiation (with a maximum distance of 2cm from the lesion, having been evaluated clinically and with the aid of dermoscopy to define the limits of pigmentation). The samples were fixed in 10% buffered formalin and embedded in paraffin for histochemical staining (H&E) and immunofluorescence.

A triple immunofluorescence staining technique was performed on each skin sample using anti-vimentin (ab8978 / 1:100) and DAPI (ab104139/ 1:1), in addition to one of the following antibodies: (a) anti-ET1 (ab113697/ 1:100), (b) anti-ERA (ab76259: 1/500), or (c) anti-ERB (ab117529: 1/100). Vimentin is an intermediate filament of 52–58 kD expressed in both benign and malignant melanocytes, as well as being the primary intermediate filament of fibroblasts. Standardization of immunofluorescence protocols involved titrating the lowest concentration of antibodies that could be detected on slides of positive controls.

The areas with the highest fluorescence intensity on the slides from each location (melasma and unaffected skin) were captured using confocal microscopy equipment, specifically the LEICA TCS SP8, which was equipped with a laser with emission at 405, 488, 552, and 638 nm, as well as two photomultiplier-type detectors and a hybrid-type detector that allowed for the simultaneous acquisition of numerous fluorescence channels. TIFF-format images with a resolution of 813×813 pixels were saved for each marker channel.

In regular interfollicular areas, a quantitative analysis was performed in a blinded manner, without knowledge of the location of the sample (melasma or adjacent healthy skin), in suprabasal keratinocytes, basal layer melanocytes, and upper dermal fibroblasts. Melanocytes and dendritic cells of the upper dermis were stained with vimentin (red fluorescence) while cell nuclei were stained with DAPI (blue fluorescence), enabling topographic orientation of markings by ET-1, ERA, and ERB (green fluorescence).

To evaluate the pixel intensity associated with the fluorescence of the primary antibody, standardized photos (regarding laser intensity) were taken. The average color histogram intensity (ranging from 0 to 255 shades of gray) within the cytoplasm of the target cells was computed across various sites. These intensities were adjusted by dividing the cytoplasmic intensity by the background intensity (represented by the black area) for each photograph, yielding the relative pixel intensity, which served as the primary outcome of this study. Therefore, a relative intensity of 30 represents a semi-quantitative measurement indicating that the cytoplasmic intensity of the antibody is 30 times greater than that of the dark background.

The thickness of the epidermis was calculated by digital histomorphometry. ImageJ 1.51 was the software used for the semiquantitative digital analysis.

Statistical Analysis

The normality of the data obtained was assessed using the Shapiro–Wilk test.¹⁹ Data were compared between topographies using a linear model generalized mixed effects, with robust covariance structure, distribution of probability adjusted to each sample (normal or gamma) and post hoc analysis by the sequential Šidák algorithm.²⁰

The correlation between the fluorescence of ET-1 and histomorphometric parameters was assessed by the Spearman rank correlation coefficient.²¹

The analysis was performed using the IBM SPSS 22v software, and significance was defined for $p < 0.05$.²² The sample size was based on the expectation of difference between the topographies of more than 20% of the expressions among the sampled skins, with alpha 0.05 and beta 0.2.

Results

The main clinical and demographic information from the 20 participants are presented in the Table 1.

The relative fluorescence of ET-1 in whole epidermis was higher in melasma, than in the adjacent skin (Figures 1 and 2, [Supplementary Table 1](#)), being 32.8% (CI95% 14.7%–52.6%) higher in the spinous layer ($p = 0.013$), 30.4% (CI95% 13.7%–47.9%) higher in the basal layer ($p = 0.014$), and 29.7% (CI95% 11.4%–49.7%) higher in the melanocytes ($p = 0.006$). Neither ERA nor ERB resulted in differential epidermal expression between melasma and unaffected skin ($p > 0.1$).

There was no relevant fluorescence in upper dermis for ET-1, ERA, and ERB.

Table 1 Main Clinical and Demographic Data from the Participants ($n = 20$ Women)

Variables	Values
Age (in years), mean (SD)	44.9 (9.2)
Skin phototype, n (%)	
II	8 (40%)
III	5 (25%)
IV–V	7 (35%)
Age of disease onset, mean (SD)	33.3 (10.8)
mMASI ^a , mean (SD)	4.0 (2.4)

Abbreviation: ^amMASI, Modified Melasma Area and Severity Index.

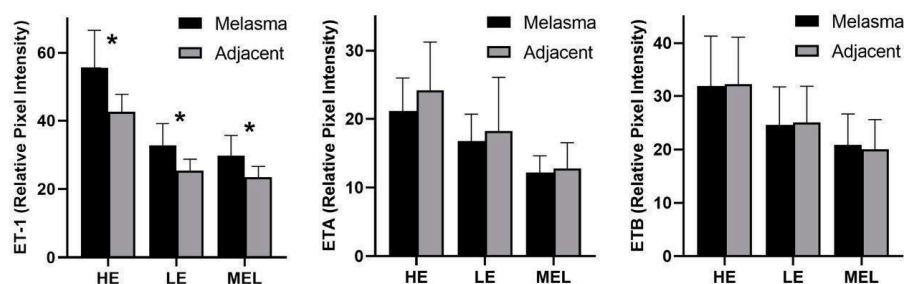


Figure 1 Relative fluorescence (ET-1, ERA, and ERB) in the cytoplasm of cells from the epidermis in facial melasma and in the adjacent photoexposed nonlesional skin ($n = 20$ women). * $p < 0.05$.

Abbreviations: HE, high epidermis (spinous and granular layers); LE, lower epidermis (suprabasal); MEL, melanocyte.

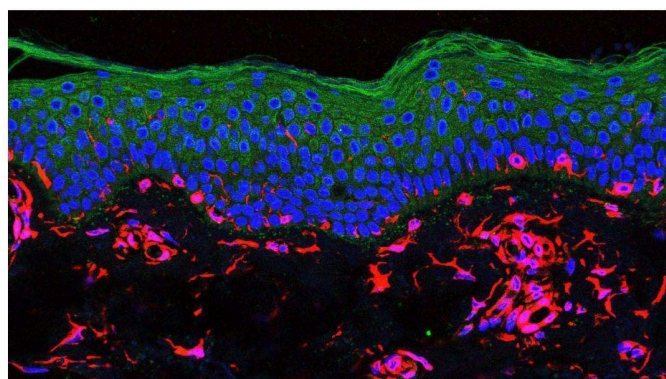


Figure 2 Overlapping direct immunofluorescence images in melasma: cytoplasmic labeling of melanocytes and keratinocytes ($\times 100$). Anti-endothelin-1—green; vimentin—red; 4',6-diamidino-2-phenylindole—blue.

There was a positive correlation between the expression of ET-1 in the epidermis of melasma with the thickness of the epidermis ($\rho = 0.51$; $p = 0.02$), and with age ($\rho = 0.58$; $p = 0.01$), but not with mMASI scores ($\rho = 0.18$; $p = 0.45$), nor with the receptors ERA and ERB ($p > 0.3$).

Discussion

Our study demonstrated that ET-1, derived from keratinocytes and melanocytes in the epidermis, is overexpressed in skin with melasma compared to adjacent non-lesional skin exposed to sunlight. This suggests that epidermal ET-1, but not from upper dermis, may play a role in the augmented melanogenesis observed in melasma.^{13,23}

Upon exposure to UV radiation, endothelin is produced in the epidermis, which in turn stimulates the ET1-ERB pathway in melanocytes, which is prolonged up to 10 days after UVB exposition.²⁴ This pathway modulates MITF phosphorylation, leading to pigmentation. However, the inhibition of ERA does not result in melanogenesis.^{25,26} Our results suggest that there is an increase in ET1 expression in the epidermis of skin with melasma, but without a compensatory increase in the expression of ERB or ERA receptors.

Endothelin receptors are G-protein coupled receptors expressed on the surface of melanocytes and play a critical role in their survival and homeostasis. During prenatal life, ERB is the receptor for ET-3 and is expressed on melanoblasts. In

postnatal life, ERB is the main receptor for ET-1, which has the same binding affinity and effects as ET-3. Activation of ERB by ET-1 leads to increased intracellular Ca^{2+} mobilization and PKC activity.²⁷

Extensive research on ET-1 has primarily focused on endothelial secretion, given that it is a potent vasoconstrictor agent. In the epidermis, melanogenesis, keratinocyte proliferation, pruritus and pain can be mediated by ET-1.^{28,29} It is a relevant cytokine in the pathogenesis of pigmented seborrheic keratosis, lentigo senilis and pigmented basal cell carcinoma.^{18,30,31} Our study confirmed the higher expression of ET-1 in the epidermis in sites with increased melanogenesis (melasma) and it was correlated with greater epidermal thickness.

UV irradiation induces a dose-dependent increase in melanocyte dendritic percentage and dendrite length, which is promoted by keratinocyte-derived ET-1.³² However, in vitro irradiation of endothelial cells with UVB or UVA leads to a more intense synthesis of SCF (stem cell factor), which is also a pro-melanogenic factor. In another study, the use of 590 nm LED light, which targets vascular components, led to an improvement in skin pigmentation and erythema in melasma patients by inhibiting the expression of vascular growth factors and SCF in dermal endothelial cells.³³

Despite the presumable role of dermal ET-1 produced by endothelial cells from the prominent vascular component in the upper dermis in melasma, epidermal ET-1 is more relevant for melanogenesis in healthy and melasma skin. The present study reinforces this finding, as it did not show noticeable ET-1 expression in the upper dermis from photoexposed skin samples, neither in melasma nor in the unaffected adjacent skin. However, fibroblasts from melasma display a senescent phenotype and overexpress ET-3, which also activates ERB, contributing to the sustained melanogenesis in melasma.³⁴

ET-1, as well as keratinocyte plasmin, has been identified as a target of tranexamic acid (TA) in topical formulations that have shown promising results in clearing melasma.³⁵ Given the limited skin penetration of topical formulations, targeting the epidermis rather than the dermis may be the key to the positive results observed in this therapy approach, which is consistent with our findings. Other agents which acts as direct ET-1 inhibitors are being prospected as potential treatments for pigmentary disorders.^{36,37} To date, no specific topical ERB blockers are available, while potent ERA antagonist (eg, bosentana) have no role in melanogenesis.

Recently, sebocytes have been demonstrated to be responsible for some epidermal ET-1 production, as well as the secretion of other inflammatory mediators, melanogenic factors, and senescence markers observed in melasma. This discovery makes sebocytes a potential target for treatment.³⁸

As melasma is more prevalent among women at childbearing age, some researchers suggest that estrogen can regulate the production of endothelin-1 in skin cells by modulating the activity of enzymes involved in the synthesis and degradation of ET-1. In addition, estrogen can also modulate the sensitivity of skin cells to the effects of ET-1. Nevertheless, the interaction between estrogen receptors (through the noncanonical pathway) and endothelin-1 in the skin is complex and still not fully understood.^{39,40}

Melasma skin present a reduced autophagy in basal layer melanocytes compared to the adjacent unaffected skin.⁴¹ Furthermore, ET-1 also induces a decrease in autophagy, which is well documented in cardiac hypertrophy and contractile dysfunction and can be another action in melasma skin.⁴²

Other changes found in melasma skin are damage to the basement membrane zone, including disruptions, gaps, lower density and thinning in the lamina densa and damage in anchoring fibrils from *lamina lucida*, in addition to an increase in oxidative stress in upper dermis.⁴³⁻⁴⁵ There is a proinflammatory and cellular repair environment in melasma skin, which may contribute to maintaining the increase in ET-1 in the epidermis, as it is also released in repair situations.⁴⁶

The leading hypothesis behind the overexpression of ET-1 in the epidermis of melasma is associated with the local production of nitric oxide (NO). The oxidative and proinflammatory conditions in the upper dermis stimulate NO release, resulting in vasodilation.⁴⁷ ET-1 is produced as a countermeasure to the vasodilatory effects induced by NO and other inflammatory mediators. Given the increased expression of iNOS in melasma-afflicted skin, which leads to heightened local NO production, there is a potential for sustained production of ET-1 to counteract the effects of NO, leading to a sustained hypermelanogenesis.^{48,49} A study that concurrently examines the skin expression of ET-1 and NO is warranted.

This study is limited in that only women were included, although this does ensure homogeneity of the sample. Furthermore, the overall severity of melasma, as assessed by the mMASI score, is not particularly high. However, this factor does not undermine the likelihood of detecting a significant difference among the examined topographies.

Additional research is required to explore the underlying mechanisms responsible for the overexpression of ET-1 in melasma. Moreover, it is crucial to develop therapeutic approaches that specifically target epidermal ET-1 to achieve efficient depigmentation and long-term resolution of melasma.

Conclusion

The expression of ET-1 is more pronounced in the epidermis of facial skin affected by melasma compared to the adjacent unaffected skin.

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Disclosure

The authors report no conflicts of interest in this work.

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SUPPLEMENTARY TABLE

Relative fluorescence (ET-1, ERA and ERB) in the cytoplasm of cells from the epidermis in facial melasma and in the adjacent photoexposed nonlesional skin (n = 20 women). HE: high epidermis (spinous and granulous layers); LE: lower epidermis (suprabasal); MEL: melanocyte.

ET- 1		Melasma - Mean (SD)	Values
	HE	55.7 (23.2)	42.7 (10.8)
	LE	32.8 (13.6)	25.4 (7.2)
	MEL	29.8 (12.6)	23.5 (6.6)
ERA	HE	21.2 (10.3)	30.2 (25.5)
	LE	16.8 (8.3)	24.6 (21.9)
	MEL	12.2 (5.2)	18.6 (18.0)
ERB	HE	31.9 (20.2)	32.2 (19.0)
	LE	24.6 (15.2)	25.1 (14.5)
	MEL	20.8 (12.4)	20.0 (11.9)

ARTIGO SUBMETIDO

Página de Título

Seção: Research Letter

Título: Expressão do receptor de hidrocarboneto arílico (AhR) na pele com melasma facial em comparação com a pele sã perilesional.

Title: Expression of the aryl hydrocarbon receptor (AhR) in facial melasma skin compared to healthy perilesional skin.

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Conflito de interesses: Nenhum.

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Participação no trabalho:

CNS: concepção e planejamento do estudo; análise e interpretação dos dados; redação do artigo; revisão crítica de literatura; revisão crítica do manuscrito; aprovação da versão final do manuscrito.

TFG: análise e interpretação dos dados; redação do artigo; objeção dos dados; aprovação da versão final do manuscrito.

ACCE: concepção e planejamento do estudo; análise e interpretação dos dados; redação do artigo; revisão crítica de literatura; revisão crítica do manuscrito; aprovação da versão final do manuscrito.

HAM: concepção e planejamento do estudo; análise e interpretação dos dados; análise estatística; redação do artigo; revisão crítica de literatura; revisão crítica do manuscrito; aprovação da versão final do manuscrito.

Prezado Editor,

Melasma é hipermelanose adquirida com elevada prevalência em mulheres adultas e de fototipos intermediários (III a V), especialmente em países intertropicais, como do Oriente Médio, Índia e América Latina.^{1,2}

Na última década, evidenciou-se aumento da prevalência do melasma no Brasil.² Um estudo que avaliou o volume de pesquisas *online* na plataforma Google demonstrou aumento de 54,4% do interesse pelo tema, e a análise multivariada dessa tendência mostrou associação com a insolação do país, sua taxa de uso de contraceptivo oral e de emissão de gás carbônico.³

O receptor de hidrocarboneto arílico (AhR) é uma proteína citoplasmática que sofre translocação nuclear quando ativada, e atua como um fator de transcrição regulador para a resposta a estímulos ambientais e manutenção da homeostase celular. Está expresso em vários tipos de células da pele, incluindo queratinócitos, melanócitos, fibroblastos e folículo piloso, participando de processos como proliferação celular, diferenciação, apoptose, inflamação e pigmentação.^{4,5}

AhR pode ser ativado por bifenilos policlorados, dioxinas ou outros hidrocarbonetos aromáticos policíclicos (HAP) presentes na poluição ambiental, além de metabólitos endógenos induzidos pela radiação ultravioleta. Quando ativado, AhR regula positivamente enzimas envolvidas na depuração de xenobióticos (como CYP1A1, CYP1A2, CYP1B1, UGT1A6). A atividade dessas enzimas resulta na produção de espécies reativas de oxigênio (ERO), que induzem a expressão de p38 que aumenta a atividade do fator de transcrição MITF, regulando diretamente a expressão da tirosinase nos melanócitos.⁴⁻⁷

A poluição do ar desempenha um papel importante no envelhecimento extrínseco. De maneira sinérgica com a exposição solar e fatores genéticos, a exposição à poluição pode ser um fator de risco para o desencadeamento e manutenção da pigmentação do melasma, em decorrência da penetração cutânea de HAPs e ativação do AhR.⁸

Como, até o momento, nenhum estudo avaliou o papel do AhR no melasma, conduzimos uma investigação para comparar sua expressão diferencial no melasma e na pele sã adjacente.

Após aprovação do comitê de ética institucional (#4.642.565), 20 mulheres adultas com melasma facial sem tratamento há mais de 30 dias, exceto pelo uso de protetor solar, foram submetidas à retirada de dois fragmentos de pele (*punch* de 3 mm): um da pele com melasma facial e outro da pele sã adjacente, distante até 2 cm do limite visível da lesão.

Após fixação em parafina, os 40 espécimes foram corados para Hematoxilina & Eosina, e submetidos à imunohistoquímica (DAB) para Ki67 (anticorpo monoclonal clone MIB1, Dako, M7240, Glostrup, Denmark – 1/50), e para AhR (ab190797, Abcam, Cambridge - MA – 1/100).

O desfecho principal foi o número de núcleos epidérmicos marcados por campo de 400x, analisados cegamente em relação à topografia (melasma ou pele sã). Foram ainda avaliadas as marcações das unidades pilossebáceas. A taxa de proliferação epidérmica foi calculada pelo escore (HSCORE) da marcação nuclear do Ki67. A espessura da epiderme viável foi avaliada a partir de três medidas em áreas interfoliculares.

Os dados foram comparados entre as topografias utilizando modelo linear de efeitos mistos, e a significância definida como $p < 0,05$. O tamanho da amostra foi baseado na expectativa de diferença entre as topografias de mais de 10% das expressões entre as peles amostradas (alfa 0,05 e beta 0,2).

Os dados clínicos e demográficos da amostra estão apresentados na tabela 1. Nenhuma das participantes era fumante, e apenas uma era usuária de contraceptivo oral.

A marcação imuno-histoquímica (Figuras 1 e 2) revelou maior ativação do AhR na epiderme do melasma em comparação com o epitélio adjacente (1,3 vs. 1,1 núcleo por campo; $p = 0,03$). Não houve marcação citoplasmática do AhR na epiderme.

Nos folículos pilossebáceos, houve marcação tanto nuclear quanto citoplasmática (Figura 3) sendo mais frequente no melasma que na pele sã adjacente (70% vs. 30%; $p = 0,03$).

A epiderme com melasma (Figura 1) apresentou menor espessura do epitélio (187 vs. 207 μm ; $p < 0,01$), entretanto, maior taxa de proliferação (HSCORE 36 vs. 27; $p = 0,02$). Não houve, porém, correlação entre o HSCORE, a espessura da epiderme e a expressão epitelial do AhR ($p > 0,3$).

Poluentes microparticulados penetram na pele por vias intracelulares, intercelulares e pelos folículos pilosos. No melasma, há prejuízo da barreira cutânea e redução da espessura do epitélio, em comparação com a pele adjacente, o que pode favorecer a permeação de poluentes.² E as ERO geradas pela ativação do AhR contribuem para danos oxidativos da epiderme e derme superior. Esse microambiente inflamatório acelera a degradação de colágeno e secreção de mediadores inflamatórios que induzem a melanogênese persistente.^{2, 4, 6, 7}

A maior expressão de AhR na epiderme e nas unidades pilossebáceas no melasma reforça a participação de estímulos ambientais (especialmente poluição) na sua patogênese e subsidia, inclusive, a maior prevalência de melasma em grandes centros urbanos. Além disso, a luz ultravioleta, principalmente UVB, e a exposição à luz visível geram formilindolo-(3,2-b)-carbazol (FICZ), um derivado do triptofano. FICZ é um ligante de alta afinidade e ativador endógeno da transcrição do AhR, o que reforça a ação sinérgica da radiação solar aos poluentes atmosféricos.⁹

A ativação do AhR pode mediar a melanogênese por via canônica ou não-canônica. Na via canônica, após a ativação, AhR transloca-se para o núcleo, onde interage com o parceiro de heterodimerização ARNT (*Aryl Hydrocarbon Receptor Nuclear Translocator*), formando um complexo que se liga a elementos de resposta específicos no DNA, como o elemento de resposta a xenobióticos (XRE). Isso resulta na transcrição de genes como *CYP1A1* e *CYP1B1*, que influenciam a síntese de melanina e podem exacerbar hiperpigmentação do melasma. Já a via não canônica envolve interações diretas ou indiretas do AhR com outras proteínas sinalizadoras, independentemente de ARNT, incluindo a ativação de quinases intracelulares e vias como a MAPK (*Mitogen-Activated Protein Kinase*). Estas vias podem promover a expressão de fatores pró-melanogênicos, como o MITF (*Microphthalmia-Associated Transcription Factor*), que também promovem aumento da síntese de melanina.^{4, 10}

As principais limitações do estudo residem na amostra modesta, incluir apenas mulheres e não haver um estudo funcional das vias ativadas pelo AhR. Estudos posteriores devem explorar o efeito do bloqueio do AhR na supressão da melanogênese e do envelhecimento cutâneo.

Concluindo, evidenciou-se ativação do AhR epidérmica e maior expressão do AhR nas unidades pilossebáceas da pele com melasma em comparação com a adjacente.

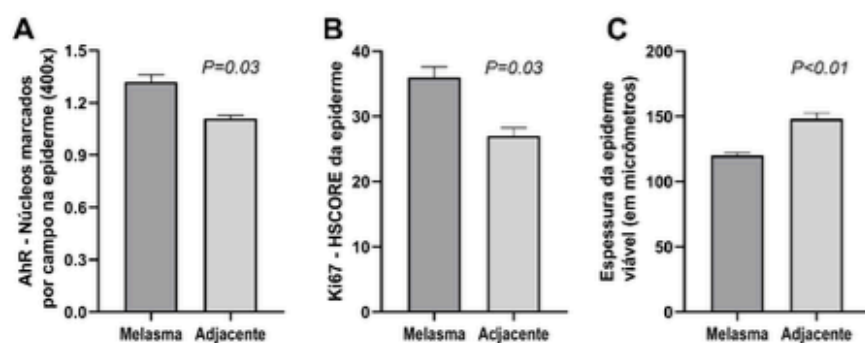
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Tabela 1. Principais informações clínicas e demográficas das participantes ($n = 20$ mulheres).

Variáveis	Valores
Idade (anos), média (DP)	44,9 (9,2)
Fototipo, n (%)	
II	8 (40%)
III	5 (25%)
IV-V	7 (35%)
Histórico de gestação, n (%)	
Nunca	3 (15%)
1-2	11 (55%)
3 ou mais	6 (30%)
Exposição solar diária, n (%)	7 (35%)
Antecedente familiar de melasma, n (%)	15 (75%)
Idade de início, média (DP)	33,3 (10,8)
mMASI, média (DP)	4,0 (2,4)

mMASI: *Modified Melasma Area and Severity Index*.

**Figura 1.** Gráficos comparativo da expressão nuclear do AhR na epiderme (A), do escore de Ki67 (B) e da espessura do epitélio (C) com melasma e da pele sã adjacente ($n = 20$).

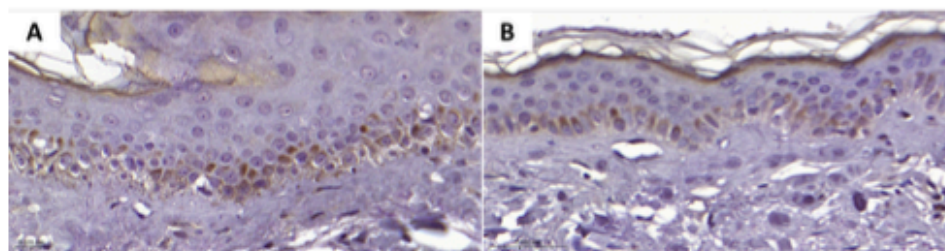


Figura 2. Expressão nuclear epidérmica do AhR (400x). A. Melasma. B. Pele adjacente.

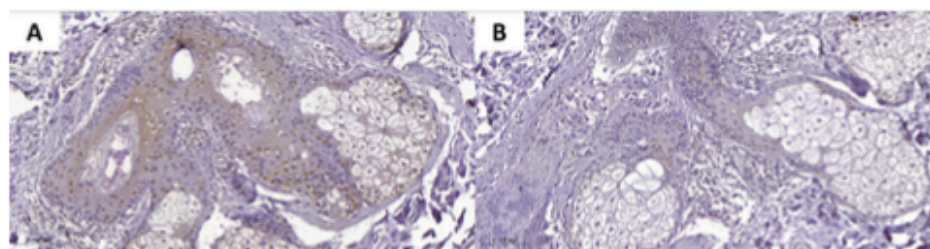
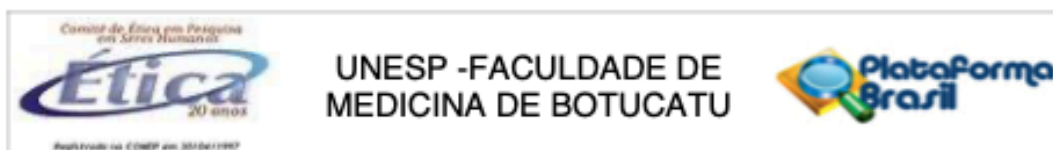


Figura 3. Expressão do AhR no folículo pilossebáceo (200x). A. Melasma. B. Pele adjacente.

CONCLUSÕES

1. Há aumento da expressão de ET-1 na epiderme da pele com melasma em comparação com a pele adjacente sã.
2. O aumento da expressão de ET-1 na epiderme da pele com melasma em comparação com pele sã adjacente ocorre tanto na camada espinhosa, quanto na camada basal.
3. Não há expressão diferencial de ERA e ERB na epiderme da pele com melasma em comparação com a pele adjacente sã.
4. Não há fluorescência relevante para ET-1, ERA e ERB na derme superior da pele com melasma ou pele sã adjacente.
5. Há maior ativação epidérmica do AhR na pele com melasma em comparação com a pele adjacente sã.
6. Há maior expressão do AhR nas unidades pilossebáceas da pele com melasma em comparação com a adjacente.

ANEXO 1: Aprovação no Comitê de Ética em Pesquisa



PARECER CONSUBSTANCIADO DO CEP

DADOS DA EMENDA

Título da Pesquisa: Avaliação da autofagia e da senescência na epiderme e derme superior na pele com melasma em comparação com a pele sã adjacente.

Pesquisador: Ana Cláudia Cavalcante Espósito

Área Temática:

Versão: 3

CAAE: 88076618.9.0000.5411

Instituição Proponente: Faculdade de Medicina de Botucatu/UNESP

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 4.642.565

Apresentação do Projeto:

Previamente avaliado.

Objetivo da Pesquisa:

A emenda tem por objetivo solicitar a inclusão da avaliação da expressão de quatro antígenos adicionais, endotelina-1, endotelina-3, e receptores da endotelina-A e B, na pele com melasma facial, em comparação com a pele sã adjacente. Serão utilizados os mesmos blocos de parafina do projeto previamente submetido, assim como a mesma técnica de imunofluorescência direta descrita previamente. As alterações propostas não envolvem outras mudanças na metodologia ou inclusão de novos participantes.

Há solicitação para alteração do título do projeto para: Avaliação da autofagia, da senescência e papel da endotelina na epiderme e derme superior na pele com melasma em comparação com a pele sã adjacente".

Avaliação dos Riscos e Benefícios:

Previamente avaliados.

Comentários e Considerações sobre a Pesquisa:

Trata-se de emenda com a finalidade de:

1. A inclusão da avaliação da expressão de quatro antígenos adicionais, endotelina-1, endotelina-3, e receptores da endotelina-A e B, na pele com melasma facial, em comparação com a pele sã

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Continuação do Parecer: 4.842.565

adjacente. Serão utilizados os mesmos blocos de parafina do projeto previamente submetido, assim como a mesma técnica de imunofluorescência direta descrita previamente. As alterações propostas não envolvem outras mudanças na metodologia ou inclusão de novos participantes.

2.Há solicitação para alteração do título do projeto para: Avaliação da autofagia, da senescência e papel da endotelina na epiderme e derme superior na pele com melasma em comparação com a pele sã adjacente".

Pesquisa de interesse na área em que se insere. A inclusão de novas proteínas a serem avaliadas e a alteração de título não comprometem a estrutura do projeto, previamente analisada.

Considerações sobre os Termos de apresentação obrigatória:

Foram apresentados:

- 1.Folha de Rosto atualizada devidamente assinada;
- 2.Carta de Anuência atualizada;
- 3.Projeto com as emendas destacadas em amarelo;

Recomendações:

Não há.

Conclusões ou Pendências e Lista de Inadequações:

Após análise em REUNIÃO ORDINÁRIA, o Colegiado deliberou APROVAÇÃO da Emenda.

Considerações Finais a critério do CEP:

Conforme deliberação do Colegiado, em REUNIÃO ORDINÁRIA do Comitê de Ética em Pesquisa FMB/UNESP, realizada em 05/04/2021, a Emenda encontra-se APROVADA. O Pesquisador deverá enviar Relatório Final de Atividades ao final da pesquisa.

Atenciosamente,

Comitê de Ética em Pesquisa FMB/UNESP

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

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APÊNDICE 1: Protocolos de imunofluorescência direta de tripla marcação para os anticorpos teste anti-endotelina-1, anti-receptor de endotelina A e anti-receptor de endotelina B.

Foram recuperados 40 blocos de parafina (20 de pele sã e 20 de pele adjacente) de pacientes com melasma facial oriundos do projeto previamente desenvolvido pelo grupo (2018/10827-3).

Foram catalogados os dados clínicos, colorimétricos (*L de cada região biopsiada), histomorfométricos da epiderme, e demográficos da amostra de pacientes com melasma facial. (Tabela Complementar 1). Não houve perdas de dados ou de casos, todos foram elegíveis para o experimento.

Tabela Complementar 1 : Principais dados clínicos, colorimétricos, histomorfométricos e demográficos da amostra com melasma facial.

Topografia	Lâmina	Idade	Fototipo	mMASI	*L	Espessura total	Espessura total	Compactação da córnea
melasma	TB 6/18 1	45	III	4,4	50,2	236,9	149,5	1
melasma	TB 7/18 1	53	IV	1,8	55,9	130,0	104,2	2
melasma	TB 8/18 1	45	IV	5,7	47,1	173,7	143,7	2
melasma	TB 9/18 1	51	II	5,1	59,3	226,4	154,9	1
melasma	TB 10/18 1	42	II	1,5	58,7	291,2	160,0	1
melasma	TB 11/18 1	68	IV	10,1	50,0	202,6	122,4	1
melasma	TB 12/18 1	34	II	2,4	53,7	175,6	111,8	1
melasma	TB 13/18 1	37	II	4,0	54,5	179,2	145,6	1
melasma	TB 14/18 1	45	II	4,8	54,4	178,2	118,0	1
melasma	TB 15/18 1	37	II	3,6	51,4	185,9	96,4	1
melasma	TB 16/18 1	38	III	7,8	55,5	234,4	90,8	0
melasma	TB 17/18 1	44	V	0,6	55,9	187,8	118,2	1
melasma	TB 18/18 1	53	IV	3,5	56,1	242,0	138,7	1
melasma	TB 19/18 1	45	III	2,4	50,9	217,7	111,1	1
melasma	TB 25/18 1	48	IV	2,4	61,6	142,1	107,6	2
melasma	TB 26/18 1	39	III	3,4	48,9	137,7	91,9	2
melasma	TB 27/18 1	40	III	6,3	50,5	131,1	103,3	2
melasma	TB 28/18 1	37	II	1,7	54,7	157,5	108,3	1
melasma	TB 29/18 1	63	IV	2,2	55,4	127,0	103,2	2
melasma	TB 31/18 1	33	II	6,0	50,5	186,6	126,8	1
adjacente	TB 6/18 2				73,8	228,2	184,0	1
adjacente	TB 7/18 2				79,3	271,6	232,1	2
adjacente	TB 8/18 2				88,7	264,1	197,0	1
adjacente	TB 9/18 2				89,9	230,5	183,2	1
adjacente	TB 10/18 2				81,8	273,3	234,4	2
adjacente	TB 11/18 2				76,0	238,5	160,3	1
adjacente	TB 12/18 2				75,3	200,6	144,4	2
adjacente	TB 13/18 2				80,6	192,6	104,4	0
adjacente	TB 14/18 2				84,6	266,1	176,0	1
adjacente	TB 15/18 2				73,8	240,1	176,3	2
adjacente	TB 16/18 2				78,0	303,9	150,1	0
adjacente	TB 17/18 2				77,1	172,6	136,0	2
adjacente	TB 18/18 2				52,9	127,5	79,0	2
adjacente	TB 19/18 2				75,2	158,5	105,4	1
adjacente	TB 25/18 2				84,6	163,3	76,3	1
adjacente	TB 26/18 2				71,7	160,4	140,4	2
adjacente	TB 27/18 2				76,9	143,5	95,3	1
adjacente	TB 28/18 2				76,7	130,9	103,1	2
adjacente	TB 29/18 2				81,1	174,9	144,3	2
adjacente	TB 31/18 2				78,1	194,0	145,3	2

1. Recuperação antigênica e montagem das lâminas

O processo de recuperação antigênica seguiu o seguinte protocolo:

- a. Microtomia dos blocos 4 micra e posicionamento dos cortes em lâmina silanizada;
- b. Desparafinização, através de banho sem xilol (3 vezes) 5 minutos cada;
- c. Hidratação, realizada através da passagem do material por banhos sucessivos em álcool absoluto (3 vezes), álcool 100%, álcool 95%, álcool 70% e água destilada;
- d. Etapa de recuperação antigênica: mergulhar as lâminas em solução de Tampão Citrato 10mM pH 6,0 e utilizar a Câmara de Pressão a 117° C por 3 minutos;
- e. Após o procedimento, deixar as lâminas esfriarem em temperatura ambiente por 20 minutos e lavar com PBS (3 vezes de 5 minutos);
- f. Etapa de bloqueio de ligações inespecíficas: realizada mergulhando-se as lâminas em solução de leite desnatado a 5% por no mínimo 1 hora;
- g. Circular o corte com caneta hidrofóbica e sem lavar a lâmina, incubar com anticorpo primário. Transferir as lâminas para bandeja e aplicar o anticorpo primário (ver diluição). Fechar a bandeja e deixar incubando em câmara úmida na geladeira à 4°C “overnight”;
- h. No dia seguinte, lavar as lâminas com PBS e aplicar o anticorpo secundário e incubar por 60 minutos em ambiente escuro;
- i. Após a lavagem das lâminas com PBS, aplicar o anticorpo Anti-Vimentin antibody (Abcam® 8978/ 1:100) e incubar por 120 minutos em ambiente escuro;
- j. Depois de lavagem das lâminas com PBS, aplicar o anticorpo Goat Anti- Mouse IgG H&L (Alexa Fluor® 647) e incubar por 30 minutos em ambiente escuro;
- k. Após, lavar as lâminas com PBS e
- l. Aplicar Mounting Medium With DAPI - Aqueous (Abcam® 104139 / 1:1), Fluoroshield em ambiente escuro, aguardar 5 minutos e, colocar lamínula;
- m. Seguir para leitura sob microscopia.

2. Validação dos anticorpos para imunofluorescência de tripla marcação

Com os recursos de custeio do projeto, foram adquiridos os insumos para a imunofluorescência de tripla marcação, e os anticorpos anti-Endotelina-1, anti-vimentina (Abcam® 8978) , DAPI (Abcam® 104139), anti- Receptor de Endotelina-A (Abcam® 76259) , e anti-Receptor de Endotelina-B (Abcam® 117529).

Após subseqüentes testes com as concentrações dos anticorpos em pele sã (teste), os anticorpos foram validados nos seus devidos controles positivos, segundo as informações da tabela complementar 2 e das figuras complementares 1 a 3.

Tabela Complementar 2. Validação dos anticorpos para a imunofluorescência direta.

Anticorpo	Código	Diluição
Anti-endotelina 1	Abcam® 113697	1/100
Anti-receptor de endotelina A	Abcam® 76259	1/500
Anti-receptor de endotelina B	Abcam® 117529	1/100
Anti-vimentina	Abcam® 8978	1/100
DAPI	Abcam® 104139	1/1
Anticorpo secundário verde	Abcam® 150081	1/500
Anticorpo secundário vermelho	Abcam® 150119	1/200

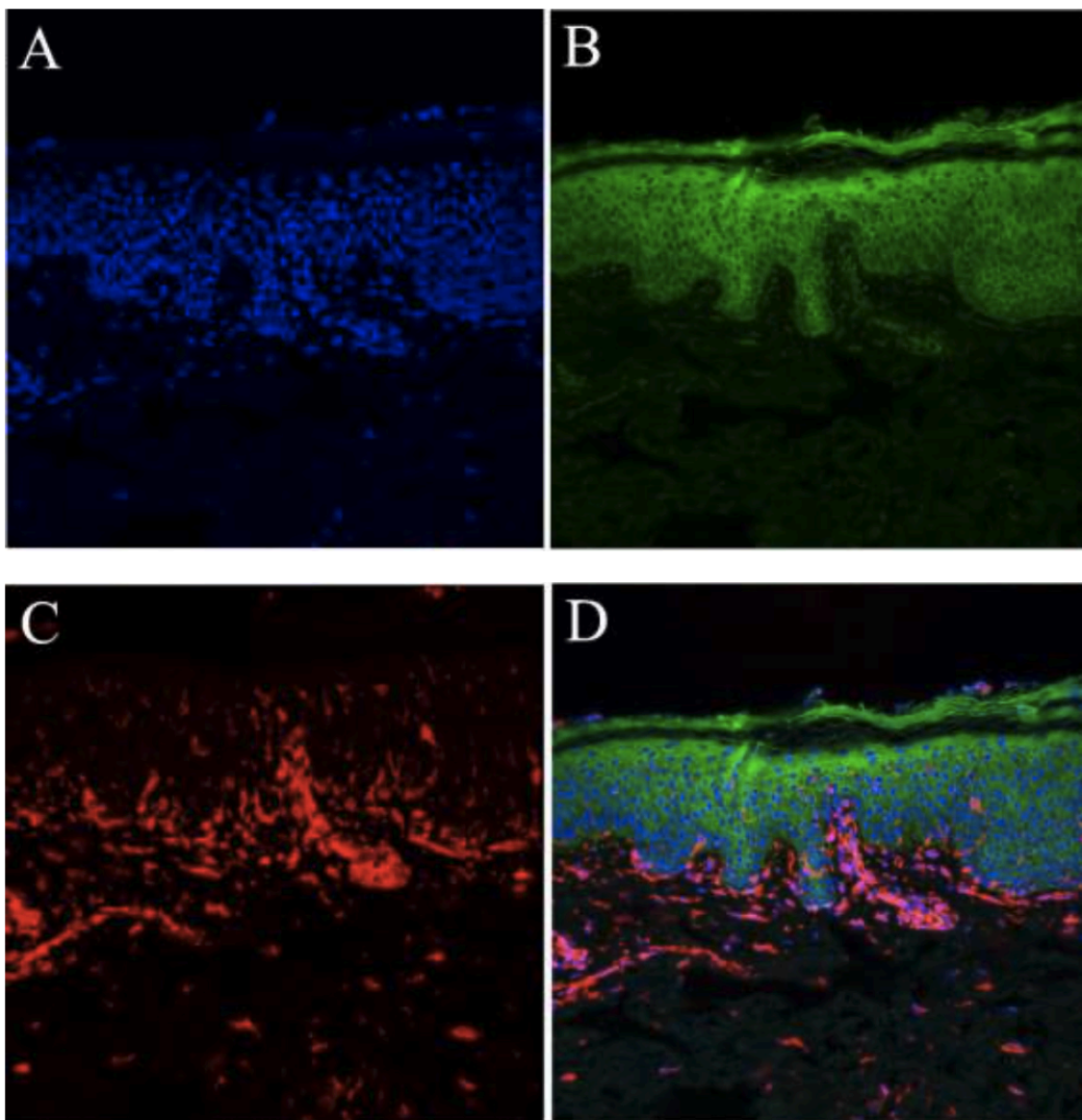


Figura Complementar 1. Validação do anticorpo anti-endotelina 1 (1/100) para imunofluorescência de tripla marcação. (Fig. Compl. 1: A) DAPI; B) Anti-endothelin; C) Anti-vimentin; D) Overlap)

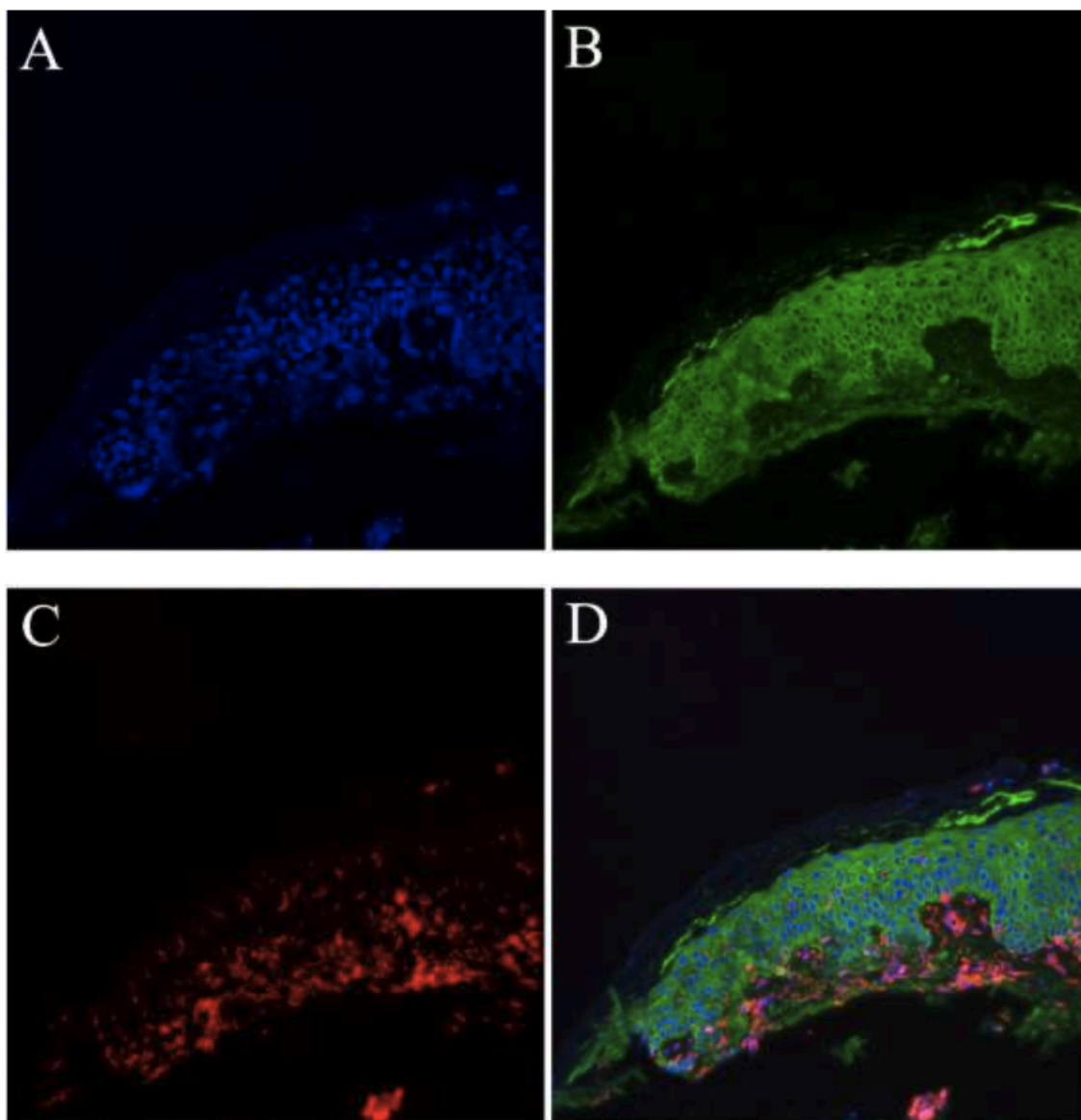


Figura Complementar 2: Validação do anticorpo anti-receptor de endotelina A (1/500) para imunofluorescência de tripla marcação. (Fig. Compl. 2: A) DAPI; B) Anti-Endothelin A Receptor/ ERA; C) Anti-vimentin e D) Overlap)

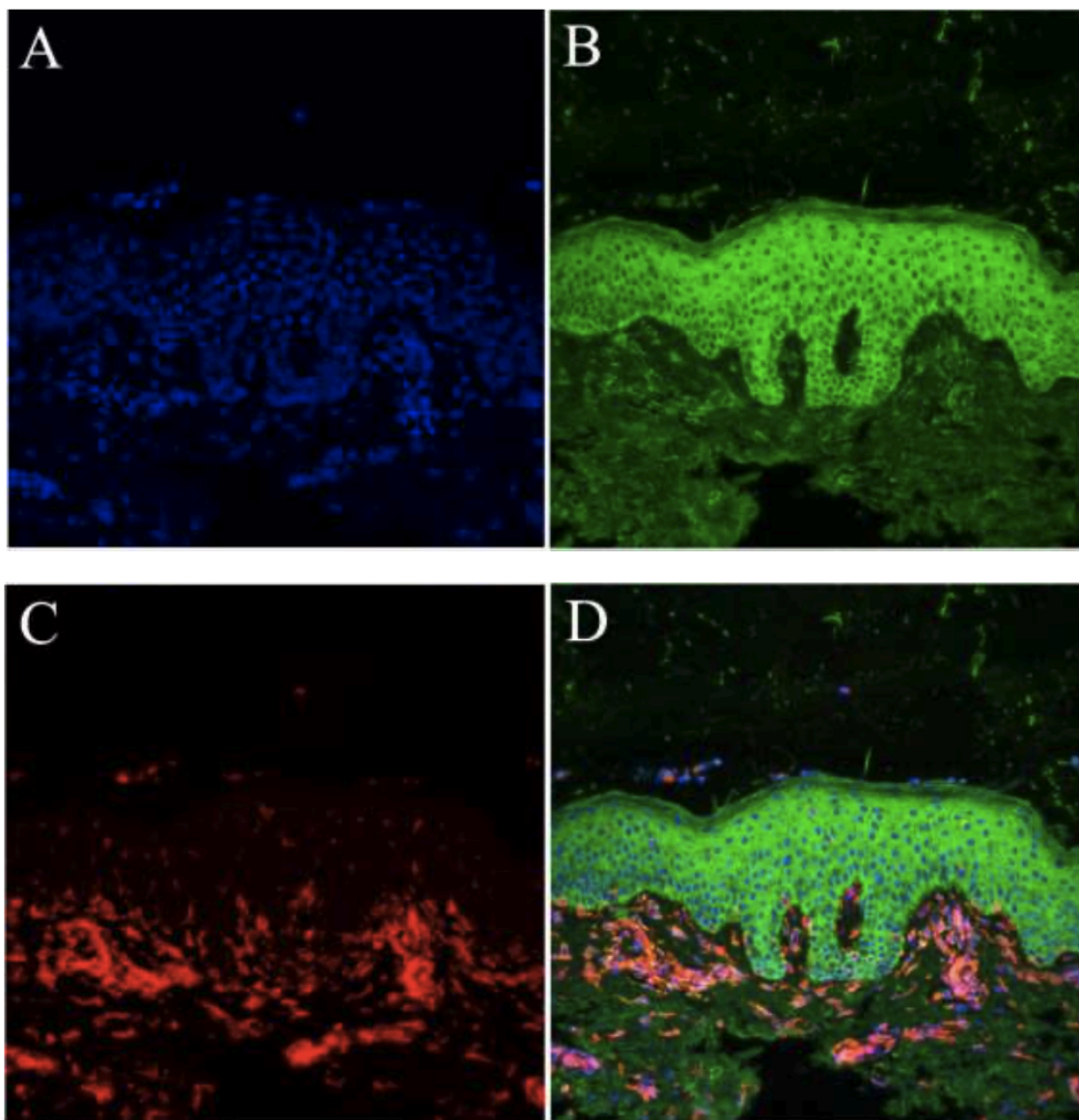


Figura Complementar 3. Validação do anticorpo anti-receptor de endotelina B (1/100) para imunofluorescência de tripla marcação. (Fig. Compl. 3: A) DAPI; B) Anti-Endothelin B Receptor/ ERB; C) Anti-vimentin; D) Overlap)

3. Obtenção de imagens

De maneira cega para topografia (melasma e pele sã), foram obtidas fotografias padronizadas em triplicata de áreas interfoliculares de maior intensidade de marcação através de microscópio confocal LEICA TCS SP8, equipado com emissão de laser nos

comprimentos 405, 488, 552 e 638 nm, bem dois detectores do tipo fotomultiplicador e um detector do tipo híbrido que permitiu a aquisição simultânea de vários canais de fluorescência. Para cada canal de fluorescência (marcadores), foram geradas imagens em formato TIFF com resolução de 813 x 813 pixel.

4. Análise de imagens

Por meio do software ImageJ v1.51 e de maneira cega para topografia (melasma e pele sã), foram analisadas as intensidades (medianas das intensidades) dos histogramas (variação de 0 a 255 tons de cinza) para os diferentes canais de cor.

Os valores de intensidade de histograma foram obtidos para queratinócitos suprabasais, melanócitos basais e derme papilar e, ajustados para a intensidade do histograma de fundo (representado por fundo preto) de cada fotografia, obtendo-se a intensidade relativa de pixels de cada área analisada. (Figura Complementar 4).

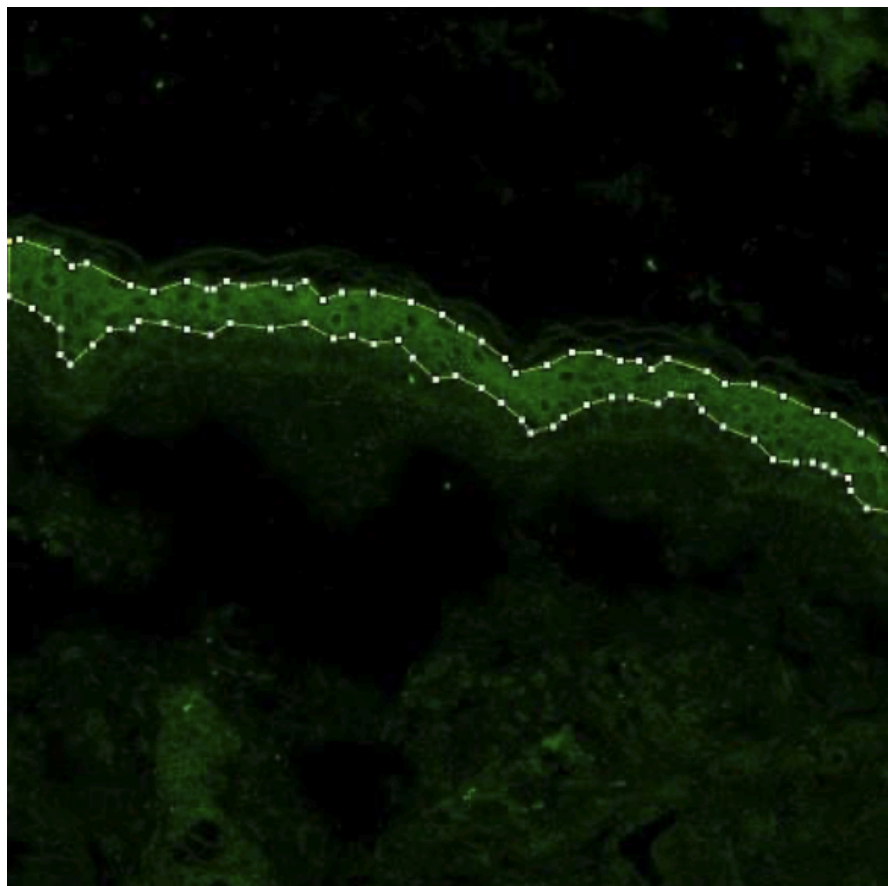


Figura Complementar 4. Cada imagem foi selecionada manualmente, de forma cega quanto ao grupo. Sendo selecionado o valor mediano da intensidade do histograma dos pixels. No exemplo acima, a área de epiderme alta (HE).

5. Análise Estatística

A normalidade dos dados obtidos foi avaliada por meio do Shapiro-Wilk. Os dados foram comparados entre topografias utilizando um modelo linear de efeitos mistos generalizados, com estrutura de covariância robusta, distribuição de probabilidade ajustada para cada amostra (normal ou gama) e análise *post hoc* pelo algoritmo Sidak sequencial.

A correlação entre a fluorescência da ET-1 e os parâmetros histomorfométricos foi avaliada pelo coeficiente de correlação de Spearman.

A análise foi realizada no software IBM SPSS 22v e a significância foi definida para $p < 0,05$. O tamanho da amostra baseou-se na expectativa de diferença entre as topografias de mais de 20% das expressões entre as peles amostradas, com alfa 0,05 e beta 0,2.

APÊNDICE 2: Protocolos de imuno-histoquímica para análise de anti-receptor de hidrocarboneto arílico

O protocolo de reação imuno-histoquímica foi realizado conforme descrito a seguir.

Foram recuperados 40 blocos de parafina (20 de pele sã e 20 de pele adjacente) de pacientes com melasma facial oriundos do projeto previamente desenvolvido pelo grupo (2018/10827-3).

Inicialmente, procedeu-se a desparafinização das lâminas em banhos de xilol, realizados três vezes com duração de 5 minutos cada. Em seguida, as lâminas foram hidratadas por meio de banhos sucessivos em álcool absoluto (três vezes), álcool 100%, álcool 95%, álcool 70% e, finalmente, água destilada.

Para a recuperação antigênica, as lâminas foram mergulhadas em solução de tampão citrato 10 mM (pH 6,0) e submetidas à Câmara de Pressão. Após esse procedimento, as lâminas foram resfriadas em temperatura ambiente por 20 minutos e lavadas três vezes com solução PBS (5 minutos cada).

O bloqueio da peroxidase endógena foi realizado com incubação das lâminas em solução de H_2O_2 a 3% em PBS, durante 5 minutos, mantidas em ambiente escuro. Para bloquear ligações inespecíficas, as lâminas foram submersas em solução de leite desnatado a 1% em PBS por no mínimo 1 hora.

Posteriormente, realizou-se a incubação com o anticorpo primário Anti-Aryl hydrocarbon receptor antibody [EPR7119(N)(2)], abcam ab190797, diluído na proporção 1:100 em solução de albumina bovina sérica (BSA) a 1%. Foram aplicados 70 μ L do anticorpo diluído sobre cada lâmina, as quais foram acondicionadas em uma bandeja, incubadas em câmara úmida e mantidas em geladeira a 4°C “overnight”.

Após a incubação com o anticorpo primário, as lâminas foram lavadas três vezes em solução PBS (5 minutos cada) e incubadas com o kit de polímero Nichirei, conforme as orientações do fabricante. Na sequência, realizou-se a incubação com a solução de 3’3’-diaminobenzidina tetrahidrocloreto (DAB) (Sigma Chemical CO, MO, EUA), aplicando-se 70 μ L por lâmina, com duração de 5 minutos. A contracoloração foi

realizada com hematoxilina de Harris a 50%. Em seguida, as lâminas foram desidratadas em álcool 70%, 95% e 100% (três vezes cada), finalizando com banhos em xilol (três vezes). Por fim, as lâminas foram montadas com lamínulas (24x32 mm), utilizando resina sintética Permount (FISHER, EUA).

Após cada etapa do protocolo, com exceção do bloqueio de ligações inespecíficas, as lâminas foram lavadas três vezes em solução de PBS 0,1 M (pH 7,2), durante 5 minutos cada.

Após subseqüentes testes com as concentrações dos anticorpos em pele sã (teste), o anticorpo foi validado em seu controle positivo e negativo, células HeLa, conforme orientação do fabricante do anticorpo, a exemplo das figuras 1 e 2 .

O mesmo foi realizado na imuno-histoquímica para Ki67 (anticorpo monoclonal clone MIB1, Dako, M7240 , Glostrup, Denmark – 1/50).

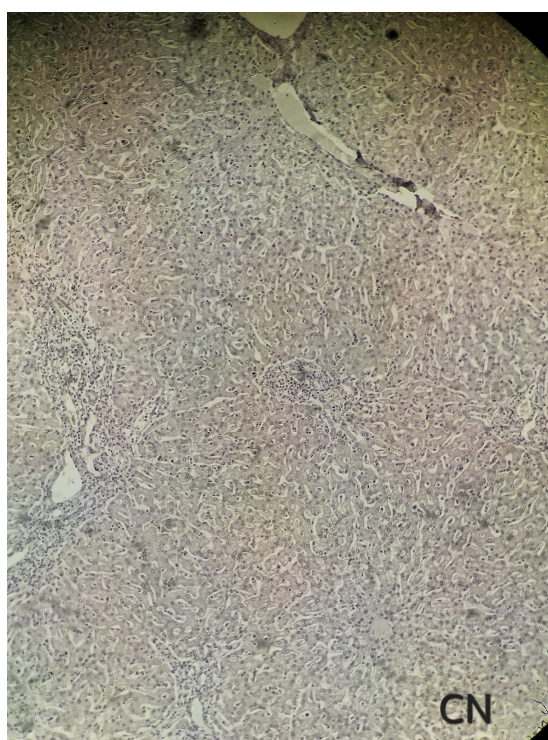


Figura 1: Marcação negativa do anticorpo anti-receptor de hidrocarboneto arílico em tecido hepático.

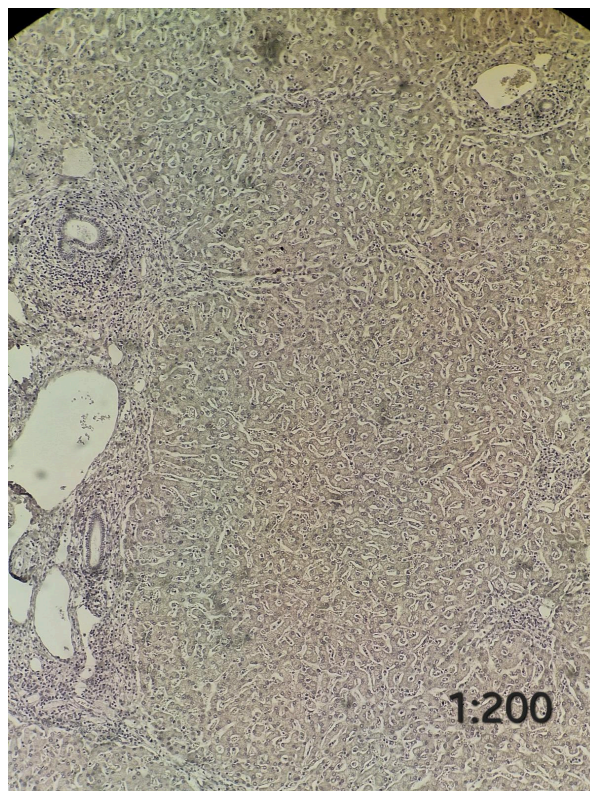


Figura 2: Marcação positiva do anticorpo anti-receptor de hidrocarboneto arílico 1:200, em tecido hepático.

APÊNDICE 3: Artigo “Update on Melasma - Part I: Pathogenesis”

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REVIEW

Update on Melasma—Part I: Pathogenesis

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ABSTRACT

Melasma is a multifactorial dyschromia that results from exposure to external factors (such as solar radiation) and hormonal factors (such as sex hormones and pregnancy), as well as skin inflammation (such as contact dermatitis and esthetic procedures), in genetically predisposed individuals. Beyond hyperfunctional melanocytes, skin with melasma exhibits a series of structural and functional alterations in the epidermis, basement membrane, and upper dermis that interact to elicit and sustain a focal hypermelanogenic phenotype. Evolution in the

knowledge of the genetic basis of melasma and the cutaneous response to solar radiation, as well as the roles of endocrine factors, antioxidant system, endothelium proliferation, fibroblast senescence, mast cell degranulation, autophagy deficits of the melanocyte, and the paracrine regulation of melanogenesis, will lead to the development of new treatments and preventive strategies. This review presents current knowledge on these aspects of the pathogenesis of melasma and discusses the effects of specific treatments and future research on these issues.

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Key Summary Points

The incidence of melasma is increasing around the world, while curative treatments are not available.

The reasons for the persistence of focal hypermelanogenesis in skin with melasma are not fully understood.

Melasma results from the interaction of sun exposure, hormonal stimuli, altered oxidative status, and upper dermal abnormalities in genetically predisposed individuals.

The role of senescent fibroblast, melanocytes with impaired autophagy, mast cells, endothelium, keratinocytes, and upper dermal and basement membrane collagen degradation as a model for melasma pathogenesis is discussed.

INTRODUCTION

Melasma is a frequent chronic acquired focal hypermelanosis that affects photoexposed areas in adults, especially women of reproductive age. While skin pigmentation from sun tanning and postinflammatory hyperpigmentation fades spontaneously after the stimulus cessation; in melasma, this reduction usually does not occur. Melasma evolves from alterations in several skin layers and cell types to hyperfunctional melanocytes, which produce and transfer mature melanosomes to the whole epidermis [1].

This review aims to collate current findings on the pathogenesis of melasma. In the following sections, we reinforce the concept of melasma as a multifactorial disorder, and discuss multiple aspects that interact in its pathogenesis, regarding genetics, solar radiation,

endocrine stimuli, oxidative status, as well as morphofunctional alterations. Finally, despite its incomplete understanding, we present a pathogenic model leading to the incorporation of all these elements. This article is based on previously conducted studies and does not contain any new studies with human participants or animals performed by any of the authors.

GENETICS

Melasma is a multifactorial disorder characterized by a cutaneous phenotype that results from exposure to external factors (such as solar exposure) and hormonal factors (such as sex hormones and pregnancy), as well as skin inflammation, in genetically predisposed individuals. The genetic basis of melasma is supported by its occurrence in first-degree relatives, as reported by 41–61% of patients in Brazil, including identical twin sisters, while the general expected prevalence in adults in the same region is 16–28%. Familial melasma is associated with prolonged duration and a reduced probability of induction by hormonal contraceptives [2–5].

Skin pigmentation follows a polygenic pattern of inheritance, which explains the phenotypic differences in mammalian species and human population groups. However, there are no animal models for melasma, although postinflammatory pigmentation can be induced in other species [6].

The evaluation of 67 Brazilian families with melasma, using a complex segregation model, demonstrated a genetic component (major gene) that follows autosomal dominant inheritance [7]. Some populations, including Middle Easterners, East Asians, Indians, African Americans, and Latin Americans, are more affected by melasma than others, such as North Europeans, Aboriginal Australians, Amerindians, and populations from Sub-Saharan Africa. A genomic ancestry study suggested that Brazilian women of mixed ancestry or with genes related to African ancestry are more affected by facial melasma [8].

A transcriptomic analysis comparing facial melasma and adjacent skin identified 279 differentially expressed genes. Genes related to melanogenesis (*TYR*, *TYRP1*, and *MLANA*) and the transfer of melanosomes (*Myosin5a* and *GDA*) are upregulated in melasma, as are those related to a subset of Wnt/ β -catenin pathway modulators (*Wnt5a*, *sFRP2*, *LGR5*, and *WIF1*), prostaglandins (*PTGIS* and *PTGS1*), repair (*SERPINE3*), and angiogenesis (angiopoietin-like 1 and 2, heparanase, and *MMP2*). In contrast, genes related to lipid metabolism (*PPAR α* , *ALOX15B*, *DGAT2L3*, and *PPARGC1A*) and *VEGFA* are downregulated [9].

Another transcriptomic analysis identified 334 differentially expressed genes. Genes related to the peroxisome proliferator-activated receptor (PPAR) pathway (*ADIPOQ*, *FABP4*, *PLIN1*, and *LPL*) are downregulated, but *GDA* and *INA*, associated with melanocyte dendricity, are upregulated, as are genes involved in the functions of the stratum corneum (*S100A8*, *SPRR2A*, *SPRR2B*, and *KLK6*) and those related to melanogenesis (*TYR*, *TYRP1*, and *sFRP2*), the suppression of tyrosine degradation (*NQO1*), and *PDZK1*, which can mediate estrogen-induced melanogenesis [10].

A comparison of facial melasma in retroauricular skin revealed downregulation of *H19*. Despite transcribing a noncoding RNA, mixed (melanocyte-keratinocyte) *H19*-gene-depleted cultures demonstrated increased melanogenesis and melanin transfer to keratinocytes, whereas melanocytes in monoculture did not, and estrogen-treated *H19*-depleted mixed cultures exhibited increased tyrosinase expression [11]. Moreover, lower expression of miR-675, an *H19* micro-RNA (miRNA) that targets *MITF*, is found in skin with melasma, and miR675 has been proven to target cadherin-11 (*CDH11*), whose expression in fibroblasts and keratinocytes induces damage in the basement membrane (BM) and melanogenesis [12]. There is also upregulation of the *PDZK1* gene in melasma. *PDZK1* is a member of the Na⁺/H⁺ exchanger regulatory factor and leads to increased melanogenesis and melanosome transfer to keratinocytes [13].

The most potent regulator of eumelanogenesis is the melanocortin type 1 receptor (MC1R),

which is transcribed from a highly polymorphic gene responsible for multiple phenotypes of skin and hair color, as well as skin sensitivity to ultraviolet radiation [14]. In melasma, *MC1R* polymorphism, as characterized by substitution of guanosine for adenosine at codon 92 (Val92Met), is prevalent in Javanese women when compared with controls [15].

Primary cultures of fibroblasts from facial melasma and adjacent skin have revealed upregulation of the *WNT3A*, *EDN3*, *ESR2*, *PTG2*, *MMP1*, and *SOD2* genes and downregulation of *COL4A1*, *CSF2*, *DKK3*, *COL7A1*, *TIMP4*, *CCL2*, and *CDH11* in melasma. These genes are related to proinflammatory melanogenic factors and repair deficits, corresponding to a phenotype that can contribute to upper dermal damage and sustained melanogenesis [16].

All these findings support melasma as a multifactorial disorder that emerges in genetically susceptible individuals. Further studies should demonstrate the possibility of precise screening for such individuals to enable early detection and the implementation of rigorous preventive measures.

SOLAR RADIATION

As melasma only occurs in photoexposed skin, has late onset in darker phototypes, worsens following sun exposure, and is more prevalent in intertropical countries, sun exposure is considered to be the most significant environmental factor in its pathogenesis [4].

Any radiation can interact with biological tissue, having effects that vary with the wavelength, intensity, skin penetration, exposure regimen, and individual susceptibility (such as skin phototype and body site). However, in melasma, the exact role of different wavelengths and the effect of their combination are not completely understood. Ultraviolet radiation (UVR) directly stimulates melanogenesis in melanocytes and affects keratinocytes, mast cells (MC), and fibroblasts, which paracrinally regulate melanogenesis. However, different effects are elicited in skin by radiation of various wavelengths [17].

UVB has fundamental effects on the epidermis and BM, while UVA extends to the upper dermis. Chronic UVR exposure leads to photoaging, oxidative stress, and inflammation that contribute to the sustained melanogenesis observed in melasma [18]. It also induces expression of p53 in keratinocytes, prompting synthesis of proopiomelanocortins [such as adrenocorticotrophic hormone (ACTH), melanocyte stimulating factor (MSH), and β -endorphin] and laminin-332 that paracrinally stimulate melanogenesis [19]. UVB increases the cytocrine activity of melanocytes, leading to more effective melanosome transfer to keratinocytes [20]. It also contributes to the degradation of heparan sulfate chains in the BM, enhancing the transfer of melanogenic stimuli from the dermis to the epidermis [21]. Finally, UVB induces the release of inflammatory mediators, such as prostaglandins and vascular endothelial growth factor (VEGF), which stimulate endothelial proliferation. In addition, neuropeptides such as calcitonin gene-related peptide (CGRP), which also trigger melanogenesis and melanocyte dendricity, are induced by UVR [22].

Compared with UVB, UVA is far less erythemogenic, but it is more effective in inducing pigment darkening (immediate and persistent) and delayed tanning, especially in people with dark skin [17]. Unlike UVB, the effect of UVA on key skin biomolecules is not direct. The energy absorbed by chromophores is transformed to generate reactive species that lead to oxidative stress [23]. Beyond the skin, UVA exposure can also induce systemic oxidative stress [24].

UVR causes epidermal release of endothelin, nitric oxide, leukotrienes, and prostaglandins, which promote the increase of melanocyte dendrites and upregulation of the *TYR* gene [25, 26]. In the upper dermis, fibroblasts release several melanogenic soluble factors such as keratinocyte growth factor (KGF), interleukin (IL)-6, tumor necrosis factor (TNF)- α , stem cell factor (SCF), basic fibroblast growth factor (bFGF), hepatocyte growth factor (HGF), and granulocyte macrophage colony-stimulating factor (GM-CSF) and produce secreted frizzled-related protein 2 (sFRP2), regulating the Wnt/ β catenin pathway, which is involved in

melanogenesis [27]. Chronic sun exposure also induces a senescent phenotype in fibroblasts, with active secretion of melanogenic and proinflammatory factors [28]. MCs degranulate under thermal, physical, and UVR stimuli, releasing bioactive mediators that induce melanogenesis and contribute to damage to the upper dermis and BM [29]. Compared with age- and phototype-matched healthy controls, women with melasma have lower erythematous dosage induced by UVB and UVA, indicating a UV-sensitive phenotype [30].

Visible light (VL) is nonionizing radiation that penetrates the deep dermis and subcutis. Pigmentation is found only in darker phototypes (III–VI) after high doses of VL exposure, and only shorter wavelengths (420–470 nm, blue and violet) can induce pigmentation through the activation of opsin 3 (OPN3) receptors in melanocytes [31]. While OPN3 is not overexpressed in skin with facial melasma compared with adjacent skin, use of tinted sunscreens (with iron oxides), which block short VL wavelengths, enhances the depigmenting effect of hydroquinone and hinders melasma pigmentation in summer [32, 33].

There are synergistic effects of long-wavelength UVA and VL on skin pigmentation and erythema [34]. In ordinary daily activities, UVA and VL are the types of solar radiation to which individuals are most exposed. Nonetheless, no available commercial sunscreen provides full protection within this range. Even sunscreens containing iron oxides demonstrate a drop in radiation absorption from 400 nm [33].

In facial skin explants subjected to minimal melanogenic doses of UVB, UVA, and VL, there is no difference in the increase in epidermal melanin density for each wavelength between melasma and adjacent skin. Nevertheless, coarser granulation of the epidermal melanin and greater density of the upper dermal melanin are evidenced in melasma skin after UVA irradiation [35].

The low-dose UVA, UVB, and blue-violet radiation experienced during ordinary indoor activities far from a window and illumination from interior lamps and electronic devices are irrelevant for skin pigmentation [35–37].

Infrared (IR) is nonionizing radiation that accounts for half of the solar spectrum and is perceived as heat [38]. Its ability to induce erythema and skin pigmentation is evidenced in erythema ab igne [39]. IR exposure has been indicated as a cause of matrix metalloproteinase 1 (MMP1) activation, vasodilatation, proinflammatory cytokine release, direct cytotoxicity, and increased oxidative stress and DNA damage, leading to photoaging. Notably, most studies on the effects of IR radiation in skin have been performed in vitro, using single exposures to artificial sources, dissimilar to the pattern of daily sun exposure in real life [17]. To date, no systematic investigation has examined the role of solar IR radiation in melasma. However, a recent survey revealed the association between disease severity and exposure to occupational heat [40].

The most important factor attributed to melasma development and aggravation is daily sun exposure, especially due to the failure to completely block all solar radiation involved in melanogenesis (UVB, UVA, and VL) owing to unsatisfactory use of sunscreens in real-life situations [41].

ENDOCRINE STIMULI

Female sex hormones are well-known risk factors for the development of melasma, and its preponderance in fertile women reinforces this hypothesis. Hormonal imbalances due to pregnancy, ovarian tumors, hormonal replacement therapy, and hormonal contraceptives stimulate melanogenesis [42]. However, the prevalence of melasma following hormonal stimuli varies: 14.5–56% of melasma cases occur in pregnant women, while 11–46% are associated with hormonal contraceptive use [2, 4, 5, 43, 44].

There is insufficient knowledge regarding the role of serum hormone levels in patients with melasma, and different studies have provided contradictory results. The serum levels of estradiol, luteinizing hormone (LH), follicle-stimulating hormone (FSH), and prolactin in the early menstrual cycle are increased in Indian women with melasma compared with controls [45].

Similarly, estradiol levels in Pakistani women with melasma are higher than in controls [46]. However, lower serum levels of testosterone, LH, free triiodothyronine (FT4), thyroid stimulating hormone (TSH), estradiol, and progesterone have been found in Indian women with melasma than in controls, although no hormone levels have been correlated with melasma severity [47]. In Puerto Rico, a comparison of women with melasma versus controls evidenced lower estradiol but higher LH levels in women with melasma [48].

Beyond ovarian and placental hormones, pregnancy promotes the production of pituitary hormones, including LH, FSH, and MSH, which lead to transcription of tyrosinase and dopachrome tautomerase, increasing physiologic pigmentation and melasma, mainly during the third trimester [49].

Administration of hormones, including topical estrogens, has been reported to trigger melasma [50]. Hormone replacement therapy during the menopause has been associated with extrafacial melasma [51]. Nevertheless, a Brazilian study enrolling women with extrafacial melasma and controls revealed no association with menopause, oral contraceptive use, pregnancy, or hormone replacement therapy [52].

Melasma in men was first reported following hypogonadism, with high LH and FSH and low testosterone levels [53]. A young man developed facial melasma after ingestion of a gonadotropic stimulant, which increased LH levels [54]. Another young man presented melasma associated with the use of finasteride for androgenetic alopecia, which decreased dihydrotestosterone (DHT) levels but increased the testosterone available for peripheral transformation in estrogen [55].

Higher LH and lower testosterone levels have been evidenced in Indian men with melasma in comparison with controls [56]. However, another comparative study found no differences in LH, FSH, TSH, testosterone, progesterone, estradiol, MSH, or dehydroepiandrosterone sulfate (DHEAS) levels [57].

Estrogen and progesterone have been associated with melasma because their effects on the skin are mediated by nuclear receptors, such as

estrogen receptors- α (ER1s) and β (ER2s) and progesterone receptors (PRs) [58]. In facial skin, ER2s are more widely distributed than ER1s, in contrast to the breast and abdomen [59]. Although both receptors have affinity for estradiol, they elicit different cellular responses regarding the epithelial-to-mesenchymal transition by genomic and nongenomic pathways [60].

Estradiol promotes epithelial proliferation through phosphorylation of Extracellular signal-regulated kinase (ERK)1-2/mitogen-activated protein (MAP) kinases and activation of the Wnt/ β -catenin pathway in keratinocytes [61]. Furthermore, estrogens lead to increased epithelial production of KGF, which also stimulates melanogenesis [62].

Estradiol influences skin thickness by stimulating fibroblasts and collagen synthesis [63]. Estrogens directly mediate melanogenesis through ER2 activation in melanocytes. Human melanocytes cultured with estrogens show increased MC1R expression, promoting upregulation of *MITE*, *TRP1*, and *TRP2* through the blockade of protein kinase A (PKA). However, addition of ER2 antagonists inhibits melanogenesis [11].

In comparison with adjacent skin, melasma-affected skin presents increased expression of ER2 in the epidermis and fibroblasts in the upper dermis [16, 64].

The role of progesterone and PRs in melanogenesis and melanocyte proliferation is contradictory [65, 66]. In melasma skin, the epidermal PR expression is increased in comparison with adjacent skin [64]. However, progesterone exhibited no effect on tyrosinase activity and demonstrated, after UVR exposure, inhibitory effects of melanocyte proliferation and estrogen-mediated melanogenesis [66].

Regarding other endocrine alterations, thyroid abnormalities have been linked with melasma. However, the results of such studies are controversial, and none has been performed with an adequate methodology to support a reasonable hypothesis on these alterations in the pathogenesis of melasma.

The prevalence of thyroid disorders in Brazilian women with melasma is similar to that expected by age [2]. Among Indian patients

with facial melasma, only 7.5% reported hypothyroidism while 0.9% reported polycystic ovary syndrome [40].

A comparison of Iranian women with melasma versus controls revealed no difference in TSH and thyroxine (T4) levels but a greater proportion of abnormalities in triiodothyronine (T3) levels in the melasma group [67]. Another investigation in Iran, comparing women with melasma and controls, found no difference in TSH or T4 levels, but abnormal levels of T3 and anti-thyroid peroxidase antibodies (TPO) were more frequently identified in the melasma group [68].

An assessment of Brazilian women with melasma demonstrated no abnormalities in thyroid hormones (TSH and fT4), prolactin, estradiol, FSH, or LH [69]. Nevertheless, a cross-sectional study in Turkey evaluating women with melasma and controls revealed higher levels of TSH, fT4, and anti-thyroglobulin antibodies, in melasma [70].

An Iranian study comparing women with melasma and controls found no differences in LH, FSH, DHEAS, prolactin, testosterone, or 17-hydroxyprogesterone, but a higher prevalence of ovarian cysts was observed in the melasma group [71].

These controversial results concerning hormonal stimuli may be attributable to the fact that most case series and cross-sectional studies have been conducted after the onset of melasma, when the endocrine imbalance that triggered the disease cannot be synchronously represented. Moreover, melasma can evolve from a focal hypersensitivity to induce hormonal effects, which are not the result of an endocrine disorder.

Endocrine stimuli, especially estrogens, are involved in the pathogenesis of melasma, and the pigmentary system is sensitive to several hormones. However, the role of local sensitivity versus endocrine alterations in melasma are not well established, although it is fundamental to the development of hormonal-based interventions in these patients.

OXIDATIVE STATUS

Exposure to oxidative stressors (such as UVR, air pollution, physical exercise, and sleep deprivation), even under normal conditions, can cause reactive oxygen species (ROS) to be produced in the skin. However, several physiological antioxidant mechanisms can neutralize their effects [72].

The lipid peroxidation of cell membranes is one of the main pathways of tissue damage caused by oxidative stress, and malondialdehyde (MDA) is the final product of this mechanism. However, some antioxidant substances can inhibit the oxidative damage caused by free radicals [73]. A strong negative correlation has been found between plasma glutathione (GSH) and the severity of melasma, suggesting that it represents a high-oxidative-stress condition, which leads to GSH depletion [74]. Plasma levels of superoxide dismutase (SOD) and GSH peroxidase activity are higher in patients with melasma than controls, but carbonyl levels are low [75]. Serum levels of MDA are also high among patients with melasma. In addition, there is a correlation between serum MDA levels and the clinical severity of melasma [76].

Inducible nitric oxide synthase (iNOS) is the primary source of nitric oxide (NO) in melasma and other inflammatory disorders. UVB stimulates the phosphatidylinositol 3-kinase/Akt pathway and nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B), which induces iNOS expression in keratinocytes, leading to paracrine NO activation of tyrosinase in melanocytes. In melasma skin, iNOS is overexpressed in keratinocytes in the basal layer, in comparison with adjacent skin [77, 78].

Besides being a key regulator of the circadian rhythm and a potent scavenger of free radicals, melatonin is an indirect antioxidant. It stabilizes cell membranes, making them more resistant to oxidative damage by stimulating other antioxidant enzymes, such as SOD, glutathione peroxidase, and GSH reductase. Melatonin also inhibits UV-light-mediated synthesis of iNOS, and it can influence the metabolism of MSH, estrogen, and progesterone [79]. Serum levels of melatonin and catalase are lower among

patients with melasma compared with controls, while serum levels of protein carbonyl and NO are higher [80]. Notably, a preliminary report has indicated promising results after topical and oral use of melatonin in melasma [81]. Finally, as sleep disturbances contribute to oxidative stress and melatonin dysregulation, the investigation of sleep disorders is warranted in melasma, and melatonin should be investigated as a potential adjuvant in the treatment of melasma [82].

The upper dermis of melasma-affected skin presents signs of oxidative stress, such as overexpression of p38, in comparison with adjacent skin [83]. Furthermore, melanin releases ROS after sun exposure, and melanogenesis is an intracellular oxidative process [84]. Several effective treatments for melasma are antioxidants, such as topical vitamin C, niacinamide, cysteamine, kojic acid, phytic acid, and oral pycnogenol [85]. These findings motivate the investigation of the mechanisms that drive the oxidative imbalance in melasma to explore therapeutic and preventive strategies.

Oxidative stress is the final consequence of several forms of damage and can also result from failure of the antioxidant system. In melasma, either local or systemic oxidative stress can be recognized and is associated with disease severity, but precise knowledge on the factors leading to (local and systemic) oxidative stress could lead to the development of effective interventions targeting the pathogenesis of this disease.

FUNCTIONAL ALTERATIONS

Skin with melasma exhibits several functional alterations that exceed those of photoaged skin and interfere with skin homeostasis. The melanin index, erythema index, and pH are higher in skin with melasma compared with adjacent photoexposed skin. However, no biophysical skin properties differ among so-called epidermal, dermal, and mixed melasma.

The skin barrier is compromised in melasma, as the stratum corneum (SC), although highly hydrated, is thinner than perilesional skin. The transepidermal water loss (TEWL) and the

amount of sebum do not differ between skin with melasma and adjacent skin. However, after skin injury caused by tape stripping, the TEWL was found to be increased in skin with melasma while the barrier recovery was delayed [86].

Thinning of the SC is a common finding in photoaged skin, and it correlates with delayed skin barrier recovery, as seen in melasma [87]. This finding is contrary to expectations in darker skin phototypes, which exhibit better barrier recovery, thicker SC, and lower pH than fair skin [88].

In general, total lipids, phosphatidic acid, phosphatidylserine, and ceramides are increased in melasma, possibly as a compensatory mechanism to preserve skin barrier function. Moreover, some key lipids have low expression with high melanocyte activation, suggesting that the repair of the damaged skin barrier may represent an effective additional treatment for melasma [89].

Human melanogenesis is a complex process mediated by paracrine, autocrine, and environmental stimuli, involving hundreds of genes and several signaling pathways that operate at transcriptional and posttranscriptional levels. However, these intricacies lie beyond the scope of this manuscript [90]. Regarding melasma, several melanogenic pathways sustaining skin pigmentation have been suggested (Fig. 1a).

MC1R, which is increased in melanocytes and keratinocytes in melasma, and its agonist α -melanocyte stimulating hormone (α -MSH) but not its antagonist agouti-signaling protein (ASIP), are secreted by the epidermis [83]. MC1R activation leads to transcription of several genes, including *MITF*, a major regulator of melanogenesis, which controls the expression of enzymes, such as tyrosinase, tyrosinase-related protein 1 (TYRP1), and TYRP2 [90]. Thus, classic treatments of melasma (such as hydroquinone and thiamidol) target tyrosinase inhibition, but melanogenesis represents the end of a complex underlying process, which can explain the frequent disease relapse [91].

Several growth factors with melanogenic activity are secreted by keratinocytes and fibroblasts in melasma. UVR stimulates fibroblasts to release HGF, NGF β , SCF, and bFGF [92]. In vitro studies have found an accumulation of

melanin in basal keratinocytes when epidermal tissue is incubated with UV-treated fibroblasts, suggesting a pigmentary role of fibroblasts in melasma [92]. SCF expression is increased in melasma dermis compared with nonlesional dermis; despite no difference in these epidermises, c-KIT is increased in the epidermis of melasma [26]. Moreover, KGF secreted by fibroblasts accumulates in the epidermis of melasma skin [93].

The Wnt/ β -catenin pathway participates in melanoblast migration and proliferation, and the induction of pigmentation [94]. Wnt1 is its main activator through the frizzled receptor and promotes β -catenin accumulation and stabilization [95]. There is greater epidermal expression of Wnt1 in melasma skin than in adjacent healthy skin or photoprotected areas; additionally, Wnt1 correlates with the MC density in the upper dermis [1]. In addition, sFRP2 is overexpressed in the epidermis and around fibroblasts in melasma [27].

PAR2 is a member of the G protein-coupled receptor family and is activated by different stimuli, such as MC tryptase, KGF, α -MSH, factor VIIa, and factor Xa. The activation of a PAR2 receptor in keratinocytes mediates melanosome transfer and increases the production of SCF, which culminates in melanin synthesis [90].

Endothelins are potent vasoconstrictors produced by endothelial cells and keratinocytes after minimal UVB exposure [96]. They induce melanogenesis directly by binding to the endothelin B receptor (EDNRB), a specific receptor on melanocytes [97]. The expression of EDNRB and c-KIT in melanocytes is also induced by UVB [96]. Endothelin-1 (ET1), rather than other factors secreted by dermal endothelial cells, such as NO, leukotrienes, and VEGF, has been indicated as the mediator responsible for the activation of signaling pathways in melanogenesis. EDNRB triggers an enzymatic phosphorylation cascade of microphthalmia-associated transcription factor (MITF), causing upregulation of tyrosinase and dopachrome tautomerase via mitogen-activated protein kinases (MAPKs), ERK1/2, and p38 [97]. The role of endothelins and PAR2 activation in melasma has yet to be clarified, although tranexamic acid reduces ET1 in melasma.

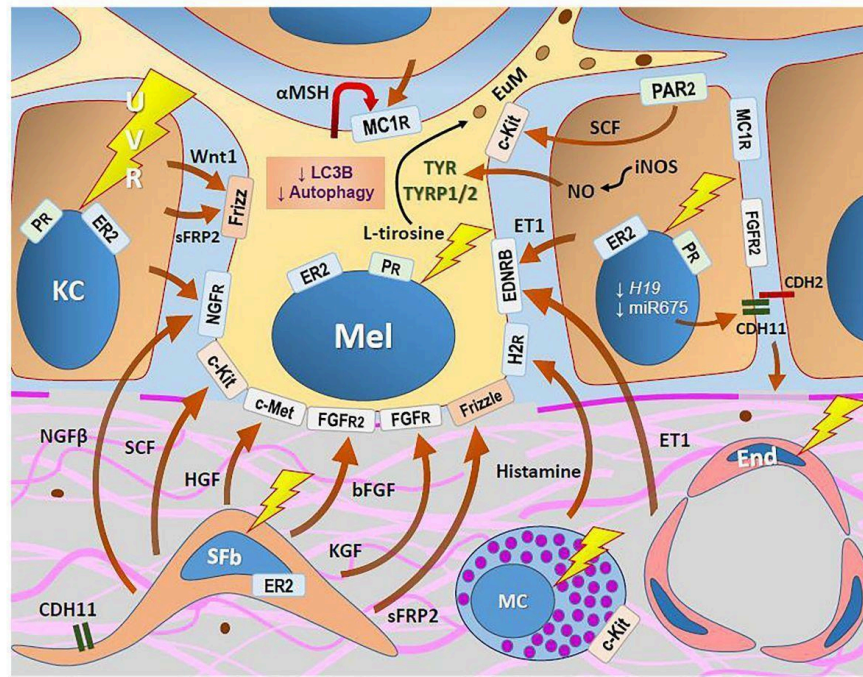


Fig. 1 Theoretic model of melanogenic pathways involved in melasma. Melanocytes (Mels) are hyperfunctional, promoting eumelanogenesis (Eum) due to paracrine and autocrine stimuli. UVR elicits melanogenic, oxidative, and inflammatory responses in the epidermis and upper dermis. Melanocortin (α MSH) and its receptor (MC1R) are increased in keratinocytes (KCs) and Mels. Hormonal stimuli mediate melanogenesis through the nuclear receptors of estrogen- β (ER2) and progesterone (PR). Several growth factors, which are also melanogenic, are actively released by senescent fibroblasts (SFbs), including nerve growth factor type β (NGF β), SCF, HGF, bFGF, KGF, and sFRP2. Endothelin-1 (ET1) is secreted by the endothelium (End) and KCs after UVR exposure. Mast

cells (MCs) release histamine under paracrine stimulation and UVR. Protease-activated receptor-2 (PAR2) stimulates melanocyte dendricity and melanosome phagocytosis by KCs and induces the release of SCF. In melasma, Mels present diminished autophagy ($\downarrow$ LC3B-microtubule-associated proteins 1A/1B light chain 3B), which stimulates melanogenesis. In addition, the lower expression of miR-675, a MITF-targeted micro-RNA, is associated with greater expression of cadherin-11 (CDH11) in KCs and fibroblasts, which contributes to basement membrane and upper dermal damage. Nitric oxide (NO), produced by inducible nitrogen oxide synthase (iNOS) and Wnt1, is increased in the epidermis in melasma

UV-induced cyclooxygenase-2 (COX-2) expression prompts synthesis of PGE2 by keratinocytes, which mediates skin inflammation and cell proliferation. In vitro studies have indicated that COX-2 knockdown in melanocytes decreases the expression of tyrosinase,

TRP-1, TRP-2, gp100, and MITF and reduces tyrosinase enzyme activity [98]. However, there is still no evidence to support the differential expression of PGE2 as a major pathogenic factor in melasma [83].

Autophagy is a catabolic cytoplasmic process that degrades abnormal proteins and damaged macromolecules [99]. It can be induced by starvation, hypoxia, oxidative stress, and UVR exposure [99]. The participation of autophagy in skin pigmentation is suggested by the evidence of impaired autophagy in premature skin aging [99]. Furthermore, there are differences in autophagic activity regarding ancestry, and keratinocytes from Caucasian individuals exhibit higher autophagic activity than those from African American individuals [100]. In cell cultures, autophagy-deficient melanocytes retain mature melanosomes and release chemokine ligands (CXCL1/2/10/12), which are associated with the induction of pigmentation and expression of MMP3 and 13 [101]. LC3 is a protein that participates in all phases of autophagy, and melanocytes from the basal layer in melasma have been found to present low expression of microtubule-associated proteins 1A/1B light chain 3B (LC3B), suggesting deficits in the autophagy process [102].

Fibroblast senescence is well documented in aged skin, especially following UVR exposure [103]. The dermis of melasma is prominent in senescent fibroblasts, which are less fusiform and have a lower mitotic rate; moreover, these cells present a proinflammatory and melanogenic secretory profile (e.g., SCF, HGF, and NGFβ) [16, 28]. Therefore, strategies that interfere with their secretory phenotype and eliminate senescent cells can reduce melasma recurrence and improve pigmentation [104].

Finally, a proteomic study of skin with melasma identified 29 differentially regulated proteins involved in energy metabolism, cell transport phenomena, control of melanogenesis, hemostasis, repair, and responses to oxidative stress [105].

All these functional alterations of the skin of melasma reinforce a localized phenotype that involves not only hypermelanogenesis but also changes in the whole epidermis and upper dermis, as discussed below.

MORPHOLOGIC ALTERATIONS

The epidermis in melasma presents morphologic alterations beyond hypermelanosis. The SC is more compact than in adjacent skin, and the granular layer is atrophic, with ridge flattening and epidermal thinning. Basal keratinocyte nuclei display larger sizes, polarization loss, irregular shapes, hyperpigmentation, and chromatin heterogeneity (Fig. 2a) compared with neighboring skin [1, 106]. Microneedling promotes early restoration of the epidermal thickness and increases Ki67 in patients with facial melasma [107].

The most important morphologic element in melasma is the increased epidermal density of eumelanin in all layers, including the SC (Fig. 2b), in which there is greater melanin degradation compared with adjacent skin [108].

Melanosomes transferred across the epidermis of melasma are larger, more mature, and more numerous than those in adjacent photoexposed skin. These alterations are independent of the skin phototype (Fig. 3) [109]. Low-fluence (laser toning) technologies target intraepidermal melanosome destruction with minimal thermal damage and accelerate the clinical results of conventional melasma treatments; however, they do not target the underlying alterations that maintain the stimuli-induced pigmentation [110].

The melanin density in the upper dermis is approximately 50–100 times lower than in the epidermis (Fig. 2b). Moreover, there is no difference in the amount of dermal melanin between melasma skin and adjacent photoexposed skin, but it is more intense than in photoprotected skin [1]. Upper dermal melanin increases with photodamage, and it is constitutively more prominent in darker phototypes.

The role of dermal melanin in melasma regarding clinical pigmentation and its role in treatment resistance is still a matter of discussion. Dermal melanin does not differ between epidermal and dermal melasma, as assessed by a Wood's lamp, highlighting that the differences observed in this clinical classification are not supported by histologic findings [111, 112]. In a study of 56 patients with melasma, only 7 (12%)

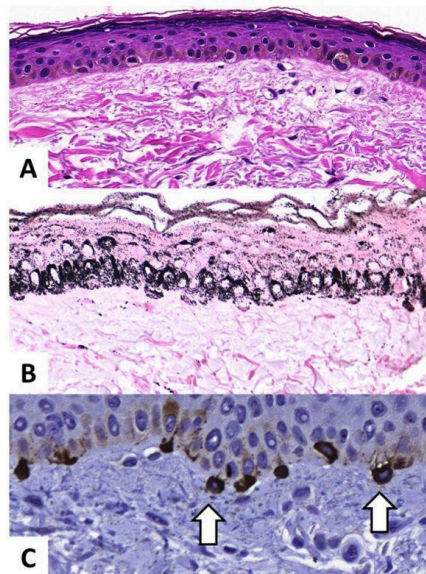


Fig. 2 Histologic images of facial melasma. **A** Atrophic epidermis with a thin stratum corneum, hypogranulosis, and polarization loss of the nuclei in the basal layer. Upper dermis revealing solar elastosis and overall unstructured collagen fibers (hematoxylin and eosin, 100 $\times$). **B** Dense and homogeneous melanin pigmentation with coarse melanosomes in all epidermal layers, including the stratum corneum, and extracellular melanin granules in the upper dermis (Fontana–Masson, 400 $\times$). **C** Atrophic epidermis with hypertrophic melanocytes (in brown) with prominent dendrites and melanocytes protruding into the dermis (pendulum melanocytes, arrows) and losing contact with the basal layer (Melan-A, 400 $\times$)

presented increased dermal melanin and melanophages compared with patients with perilesional skin [113]. Other controlled series have found no difference in dermal melanin between melasma skin and perilesional skin [109, 114]. However, an Indian series found that pigmentary incontinence and dermal melanophages

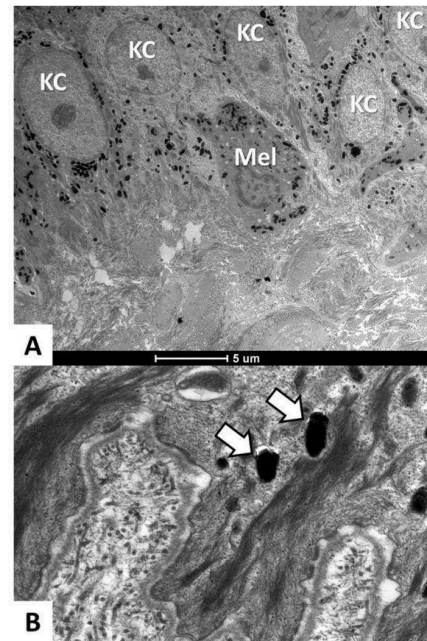


Fig. 3 Transmission electronic microscopy of facial melasma. **A** Intense distribution of mature melanosomes in the epidermis (KC, keratinocyte; Mel, Melanocyte). Sparse extracellular melanosomes in the upper dermis. **B** Mature and large melanosomes (type IV) in the cytoplasm of a keratinocyte from the basal layer (white arrows)

are more frequent in melasma skin than adjacent skin. Notably, in that study, dermal melanin was evidenced in 77% of participants and classified as epidermal melasma, without differentiation between dermal and mixed [115]. Furthermore, despite the effective bleaching of melasma lesions after treatment with a triple combination, microneedling, or tranexamic acid, there is no difference in dermal melanin compared with pretreatment [116].

Melanocytes increase in volume and their dendrites are more prominent in the basal layer

of melasma skin (Fig. 2c) than in adjacent photoexposed skin. However, there is no evidence of a substantial increase in melanocyte density, as seen in solar lentigines [109, 113, 117].

Pendulum melanocytes protrude from the basal layer into the dermis (Figs. 2c, 3a) [118]. They are more frequent in melasma skin than in adjacent skin, and there is a correlation with the compaction of the SC, number of MCs, and solar elastosis [1]. However, they are not correlated with BM zone failures, suggesting that the presence of these cells is a result of chronic exposure to UVR (especially UVA1). However, as normal melanocytes do not survive in the dermis and pendulum melanocytes lose the connection to the epidermis, they are not involved in epidermal pigmentation or active dermal melanogenesis [119]. As some effective treatments for melasma (such as oral tranexamic acid) reduce the number of pendulum melanocytes, clarification of the role of these cells in the pathogenesis of melasma may be key to developing new therapeutic strategies [116].

Disruptions and gaps are more frequent in the BM in melasma skin than in adjacent healthy skin (Fig. 4). Moreover, the lamina densa is thinner, and there is a loss of anchoring fibrils from lamina lucida [120]. Structural damage to the BM facilitates the traffic of dermal cytokines to the epidermis. This may be promoted by increased activity of MMP2 and MMP9, causing degradation of collagen types IV and VII [120]. Keratinocyte expression of CDH11 has been associated with BM damage in melasma [12]. Microneedling promotes early restoration of BM damage in patients with facial melasma [107].

It has been suggested that the alterations in the upper dermis are responsible for the maintenance and recurrence of melasma. Dermal paracrine regulation of melanogenesis by mechanisms that are not completely known causes the permanent stimulation of epidermal pigmentation, which is a UVR-induced process related to the oxidative cell aging cycle (as senescent fibroblast) involving feedback by cytokines [96, 121].

Solar elastosis (SE) is more prominent in melasma skin (Fig. 5a) than in adjacent photoexposed skin. It largely results from the

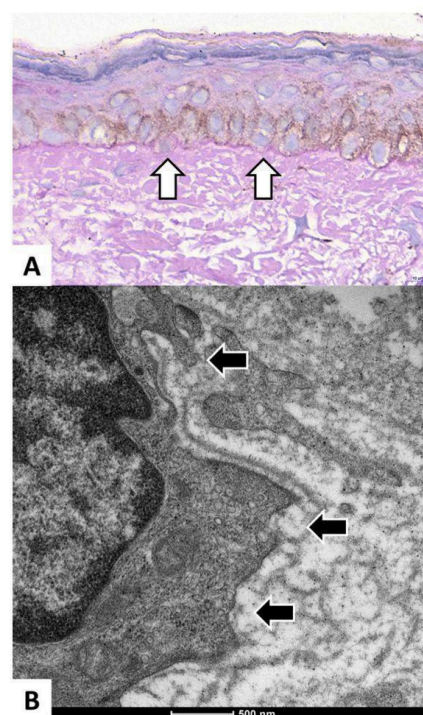


Fig. 4 Facial melasma. **A** Periodic acid-Schiff staining (400 $\times$) evidencing thinning and several discontinuities in the basement membrane (white arrows). **B** Transmission electronic microscopy of the dermoepidermal junction under a melanocyte revealing complete interruption of the lamina densa, structural damage, and loss of anchoring fibrils in the lamina lucida (black arrows)

activation of metalloproteinases in the upper dermis due to UVR, MC activation, and the senescent fibroblast secretory phenotype [1, 113, 118]. SE is not considered a secondary epiphenomenon of photoaging but a condition characteristic of melasma development. The severity of SE is correlated with collagen heterogeneity after its fragmentation, superficial dermis cellularity, increase of MCs and dermal blood vessels, pendulum melanocytes,

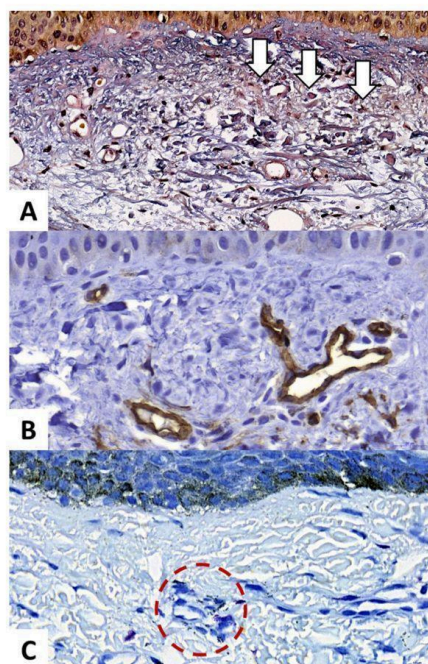


Fig. 5 Facial melasma. **A** Histologic image (Herovici, 200 $\times$) revealing upper dermis collagen fiber fragmentation with loss of structure and elastinization (solar elastosis, arrows). **B** Immunohistochemistry image of facial melasma (CD34, 400 $\times$) evidencing upper dermis endothelial proliferation (brown structures). **C** Histologic image (Toluidine Blue, 400 $\times$) evidencing mast cells in the upper dermis, especially in the perivascular areas (dashed circle)

SC compaction, and elastic fiber damage, suggesting the integration of various components in the pathogenesis of melasma [1, 122].

Skin with melasma presents an increased density of blood vessels (Fig. 5b) with an increased expression of VEGF, which is the likely angiogenic factor involved. The endothelial proliferation is also correlated with the intensity of melasma pigmentation [22, 97]. Pycnogenol, tranexamic acid, and vascular

lasers improve melasma by reducing vascular proliferation [22, 123].

Perivascular MCs are increased in the upper dermis of melasma skin (Fig. 5c) in comparison with adjacent photoexposed skin, in which MCs are increased in contrast to retroauricular skin [1]. Patients with melasma treated with oral tranexamic acid have presented decreased MC counts in the upper dermis [124]. Moreover, women with melasma treated with oral keto-tifen and famotidine have presented clinical and colorimetric improvement, superior to placebo, supporting the role of these cells in the pathogenesis of melasma [125].

MCs are not usual in normal photoprotected skin, as they are effector cells in allergic reactions and participate in tissue remodeling and repair. Their migration to the melasma dermis is mediated by SCF, secreted by senescent fibroblasts (Fig. 6) [26]. MC activity is influenced by environmental effects, and MCs degranulate with stimuli, such as heat, pressure, and UVR exposure. Inflammation and neurokinins also induce the release of a variety of biologically active mediators (such as heparin, bradykinin, serotonin, thromboxane, prostaglandins, and leukotrienes). The most relevant is histamine, which is a predominant component in cytosolic granules [29]. Histamine appears to play a central role in melanogenesis; human melanocytes treated with histamine undergo morphological changes and increased tyrosinase activity. These effects are completely inhibited by an H2 antagonist but not by an H1 antagonist [126].

In addition, MCs release enzymes that have a local tissue effect. The most important is tryp-tase, which contributes to the activation of MMPs and collagen degradation in the upper dermis. These active enzymes degrade type IV collagen and damage the BM [106].

MCs can induce vascular proliferation by secreting angiogenic factors, including VEGF, fibroblast growth factor-2, and transforming growth factor- β (TGF- β). These findings suggest that MCs are associated with UVR-induced chronic photoaging and promote SE, BM damage, and vascular proliferation, all of which are predominant features of melasma skin [106].

The role of sebocytes in melasma is still not established. Sebaceous glands are a well-known

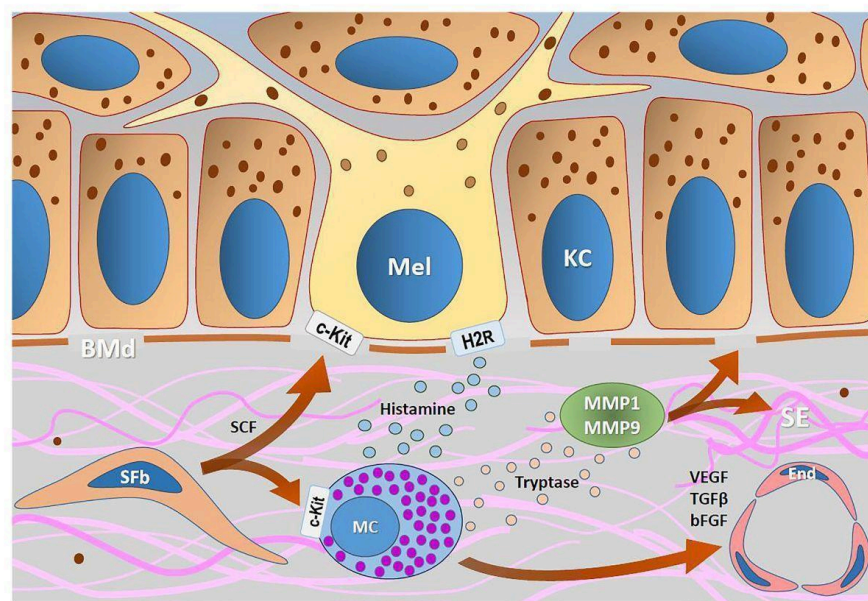


Fig. 6 Schematic representation of the interaction between senescent fibroblasts (SFbs) and mast cells (MCs) in melasma. Histamine stimulates melanogenesis directly through H2-receptors (H2Rs) in melanocytes (Mels). SCF is overexpressed in melasma, which influences MC survival, migration, and activation; it binds to the c-KIT receptor, inducing melanogenesis and the Mel cell

cycle. Tryptase activates metalloproteinases (MMP1 and MMP9), which degrade type I and IV collagens, leading to extracellular matrix degradation (solar elastosis; SE) and basement membrane damage (BMD). MCs also induce endothelial (End) proliferation by secreting VEGF, bFGF, and TGF- β

source of VEGF, IL-1, and IL-6. Sebaceous gland cells exposed in vitro to UVA induced production of α -MSH, endothelin 1 (EDN1), b-FGF, SCF, and inflammatory cytokines and mediators. Furthermore, sebocyte-conditioned media increased pigmentation in melanocytes. In vitro, melanocytes cocultivated with sebocytes present greater dendricity and proliferation rates, suggesting that sebaceous glands may regulate melanin pigmentation [127, 128]. Although centrofacial melasma is more frequent, high-density sebaceous areas, such as the nose tip, glabella, and chin, are not involved in most cases.

Actually, damage in the basement membrane zone (BMZ), solar elastosis, and increased MC are also found in photoaged skin, and as melasma affects photoaged skin, it was suggested that melasma is a photoaging disorder [18, 129]. Nevertheless, in a comparison among melasma, adjacent photoexposed and retroauricular skin, despite the continuum between these findings at these sites, alterations in skin barrier, pendulum melanocytes, upper dermal collagen fragmentation, and the amount of CD34 cells were greater in melasma but did not differ between photoexposed and retroauricular skin, leading to the hypothesis of melasma as an

individualized phenotype that emerges from photoaged skin [1].

In addition, further studies on melasma pathogenesis should use adjacent photoaged skin, since the histological architecture, melanocyte density, melanophages, MC, hair follicles, and functional measures show major differences when comparing facial and extrafacial skin [130].

MISCELLANEOUS

Melasma has been associated with other triggers, such as drugs, esthetic treatments, contact dermatitis, and stressful events.

The role of topical drugs and cosmetics in melasma has been reported, but this hypothesis remains unconfirmed. In a study including patients with melasma who were patch tested, a cosmetic and fragrance series elicited positive reactions in 43%, but sunscreen series did not elicit any positive reaction. The high frequency of positivity suggests cosmetic contact sensitivity as a possible trigger for melasma [131]. In Northern India, mustard oil is commonly used on the face as an emollient and on the scalp for hair growth. It is a known photosensitizer that can act as a melasma trigger in predisposed individuals [57].

Acute stressful events have been associated with the development or worsening of melasma [132]. Notably, stress-related hormones, such as α -melanocyte-stimulating hormone, are melanogenic through MC1R activation, as occurs in Addison's disease, although physiologic pituitary production has no effect on skin pigmentation. When compared with matched controls, a greater proportion of women with melasma uses antidepressant and anxiolytic drugs, and their scores of anxiety and depression are higher [5]. Other neuropsychological alterations in patients with melasma are low self-esteem, poor sleep quality, and higher accuracy in recognizing facial expressions of fear, suggesting differentiated brain processing of emotions [132, 133].

Melasma has been reported after peelings, intense pulsed light, and ablative treatments, which are all known to be triggers of

postinflammatory hyperpigmentation. The association of these two conditions is well documented, and postinflammatory hyperpigmentation is 2.8 times more frequent in patients with melasma [134]. Melasma is also associated with lentigines and nevi, suggesting a predisposition to melanocytic proliferation in these patients [135].

Urban and industrialized societies are associated with greater exposure to air pollutants, leading to a significant health burden that includes dermatologic disorders [136]. The primary mediator of air pollution effects in the skin is the aryl hydrocarbon receptor (AhR), which is activated by aromatic hydrocarbons that are widely present in vehicular smoke. The AhR participates in the cell cycle and melanogenesis through upregulation of tyrosinase in the melanocytes. UVR exposure generates 6-formylindolo[3,2-*b*]carbazole, which is a high-affinity ligand and endogenous activator of the AhR transcription factor [137, 138]. Actually, the incidence of melasma seems to be increasing worldwide, especially in countries with greater indexes of air pollution [139].

Melanogenesis can be regulated by epigenetic factors, such as miRNAs, DNA methylation, and posttranscriptional controllers [140, 141]. Lower expression of miR-675 and miR-1299 has been demonstrated in skin with melasma compared with the adjacent area [142, 143].

Finally, ocular irradiation with UVR induces the central release of α -melanocyte-stimulating hormone from the hypothalamus [144]. Though the pigmentary potential of this extracutaneous pathway has not been evidenced in humans, it is well defined in mice, in which UVB irradiation of the eye stimulates epidermal melanocytes and melanogenesis [145, 146].

All these factors that play a role in skin pigmentation need to be studied systematically in melasma, since the relapse of the disease after treatment and adequate sun protection can be promoted by several other factors.

CONCLUSIONS

Despite its high prevalence and the demand for dermatologic care, the mechanisms that lead to the sustained pigmentation in melasma are not completely understood. Beyond individual genetic susceptibility, several phenotypic alterations have been identified in the epidermis and upper dermis in melasma, especially related to the deficit of autophagy in melanocytes, and the senescence of fibroblasts. Moreover, the role of endocrine factors and oxidative stress are matters for future investigation regarding their systemic and local (in the skin microenvironment) actions, as well as how radiation of different wavelengths interferes with melanogenesis in melasma.

The pigmentary response in melasma is related to the interaction of multiple factors. As dermatologic science better understands the regulation of normal melanogenesis, photoaging, and the pathogenesis of melasma, new pathways will emerge as possible targets for effective treatment and preventive strategies.

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APÊNDICE 4: Artigo “Update on Melasma - Part II: Treatment”

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REVIEW

Update on Melasma—Part II: Treatment

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ABSTRACT

Melasma is a prevalent chronic relapsing pig-
mentary disorder that affects photoexposed
areas, especially in women of childbearing age.
Although there is currently no curative

treatment available for melasma, this manu-
script critically reviews the knowledge regarding
photoprotection, topical and oral therapies, and
procedures such as peeling, laser, and micro-
needling that represent the main strategies for
control and prevention of this disease. As the
pathogenesis of melasma is not entirely under-
stood, there are prospects for the development
of new therapeutic strategies that might act on
the pathways that promote sustained pigmen-
tation rather than merely decreasing melanin
synthesis and removing melanin from the
epidermis.

Keywords: Melasma; Peeling; Sunscreen; UV
radiation; Laser

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Key Summary Points

Melasma is a frequent relapsing pigmentary disorder that significantly impacts quality of life, and no curative treatment is yet available.

Photoprotection against UVB, UVA, and blue-violet visible light (e.g., broad-spectrum tinted sunscreen) is essential for successful treatment, improving pigmentation, preventing relapse, and preventing disease in high-risk individuals.

Topical therapies leading to tyrosinase inhibition are the mainstay of treatment; hydroquinone and triple combination cream are the reference drugs.

Oral therapies (e.g., tranexamic acid) and microneedling can be associated with topical therapies to synergistically increase their efficacy.

Superficial peelings and low-fluence (e.g., Q-switched) laser therapy increase melanin removal from the epidermis, accelerating clinical results.

INTRODUCTION

Melasma affects photoexposed areas, especially in women of reproductive age, but no curative treatment is available, significantly impacting the quality of life, including low self-esteem that leads to social contingencies, anxiety, and depressive symptoms [1–3]. While melasma induced by pregnancy or photosensitizing drugs has a better outcome after the causative factor ceases, most patients experience a chronic disorder, with seasonal variation and relapses after successful treatment, requiring post-treatment maintenance. In general, melasma fades with aging, and it disappears in most women after menopause, although extrafacial melasma can persist longer [4, 5]. Despite the high prevalence

of melasma and the variety of treatments available, there are few large-sample randomized controlled trials to support a robust hierarchy of treatments. This review aims to present and critically discuss the main treatment strategies for melasma.

Sun Protection

Along with pregnancy, direct sun exposure is the leading risk factor for melasma, reported by 27–51% of patients as a trigger and 84% as a factor of clinical impairment [6, 7]. Patients with melasma usually report occupational or intentional sun exposure. Thus, rigorous sun protection measures should be conditionally recommended for any patient submitted to treatment and post-treatment maintenance.

As melanogenesis in melasma can be induced by UVB, UVA, or short-wavelength visible light (VL), patients with melasma must be aware of the type of sun exposure in ordinary, daily situations. Large amounts of radiation reach the skin even in the shade or near windows, while UVA and VL pass through windshields and window glass. Moreover, these wavelengths occur at melanogenic levels from 08:30 until 17:30 in intertropical areas [8, 9].

As most sunscreens underperform in real life due to the low amount of product used or insufficient reapplications, especially for UVA and VL protection, behavioral measures and self-awareness regarding sun exposure should be reinforced for patients with melasma [10–12]. Additionally, dark-skinned people are less adherent to routine sun protection, sunscreen use, and reapplication, although they are more affected by melasma than fair-skinned individuals, thereby hindering the effectiveness of treatment [13].

Among behavioral measures for sun avoidance, topical sunscreens play a significant role in melasma treatment and should be tailored for the best performance in pigmentary disorders [14]. Nevertheless, the photoprotectors used in commercial topical sunscreens differ broadly among countries due to different regulatory recommendations from health agencies.

The UVB protection offered by sunscreens is determined worldwide by the *in vivo* sun protection factor (SPF). For routine daily activities, SPF-30 sunscreen can prevent skin redness and photoaging [15, 16]. Nonetheless, most patients usually apply up to 50% of the recommended layer of topical sunscreens on their skin, so products with higher SPF values (e.g., ≥ 50) are recommended for patients with pigmentary disorders [17].

UVA radiation is highly melanogenic and reaches high levels early in the morning (even on cloudy days), passing through ordinary glass, but does not induce erythema. UVA is the main waveband that demands attention in melasma treatment and post-treatment maintenance. Protection against UVA is usually determined by the *in vivo* persistent pigment darkening (PPD) factor. However, different regulatory agencies use various nomenclatures for UVA protection [15]. As the accomplishment of high PPD values compromises the cosmetic nature of topical sunscreens, the PPD range of 20–30 is indicated in pigmentary disorders to guarantee patient adherence.

Although VL radiation (especially in the high-energy blue-violet range) can induce melanogenesis, there is still no validated method for assessing the efficacy of sunscreens to protect against this waveband [15, 18]. Tinted sunscreens are indicated to protect against VL, while iron oxide salts seem to provide better performance in melasma. Moreover, although intense heat leads to melanogenesis, there is no systematic study on solar heat (infrared radiation [IR]) in melasma, nor are validated methods to assess IR protection available. Thus, workers submitted to intense heat as cooks, bakers, metallurgists, glassmakers, and drivers should be advised of the role of intense heat in the chronicity of melasma.

Despite the importance of sun protection in the treatment of melasma, few systematic studies have explored sunscreens in treating and preventing melasma relapse. The assessment of placebo groups in randomized controlled trials for melasma allows for measuring the efficacy of broad-spectrum tinted sunscreens, accounting for a 10–20% melasma area and severity index (MASI) reduction in 8 weeks

[19]. After 12 weeks of using a broad-spectrum sunscreen (SPF19 PA+++), there was a 26.9% reduction in the MASI in India [20]. Notably, the use of tinted (with iron oxide) broad-spectrum sunscreen reduced the post-treatment relapse of melasma in 39 French women [21].

Regarding high-risk populations, 200 women from Morocco during early pregnancy were solicited to use a broad-spectrum sunscreen (SPF 69, PPD 28). The incidence of melasma was 2.7% after 12 months of follow-up, lower than expected (53%) in this high-risk population [22]. Furthermore, 68 Mexican patients with melasma applied topical 4% hydroquinone (HQ) for 8 weeks; however, they were randomized to use an ordinary SPF-50 sunscreen or a broad-spectrum tinted sunscreen with iron oxide, which promoted greater UVA and VL protection. The mean reduction of MASI was 62% for the ordinary sunscreen group and 78% for the broad-spectrum and near-VL sunscreens [23].

Cosmetic makeup can be recommended to camouflage melasma. However, the photoprotection provided by multifunctional products (e.g., moisturizers, powders) is not as good as that offered by standard topical sunscreens, which should be applied under the makeup. Indeed, there is still no robust evidence that adding topical antioxidants (e.g., vitamin C, vitamin E, *Polypodium leucAtomos*) or depigmenting agents (e.g., butylresorcinol, glabridin) to the composition of topical sunscreens increases the efficacy of treatments for melasma [17]. Thus, the role of oral antioxidants (misnamed “oral photoprotectors”) is also discussed below.

Finally, some cosmetics, photosensitizing drugs (e.g., phenothiazines, sulfonamides, sulfonyleureas, thiazide diuretics, tetracyclines, tricyclic antidepressants, promethazine, piroxicam), and steroid hormones (e.g., contraceptive pills, estrogen replacement therapies) increase the melanogenic effect of sun exposure and should be substituted to enable successful melasma treatment [24–27]. Since melasma has a definitive genetic background, adoption of preventive measures such as sun protection and avoidance of contraceptive pills should be

recommended in adolescent girls from families with melasma [28].

Topical (Self-Applied) Treatments

Hydroquinone and Triple Combination (TC)

HQ is a phenolic compound with antioxidant properties that interferes with melanogenesis by inhibiting tyrosinase. It also inhibits DNA and RNA synthesis and can alter the formation of melanosomes, causing selective damage to melanocytes. These properties suppress the melanocyte metabolic process, inducing a gradual decrease in melanin production. The effectiveness and toxicity of topical HQ depend on the final product's concentration, vehicle, and chemical stability.

Compounding pharmacies in USA can make up to 12% HQ; over-the-counter HQ (3% and 2%) products are no longer available. Generally, HQ is used as 2–5% cream and can be applied twice a day when starting therapy, but is usually prescribed for use at bedtime. The depigmenting effect can be seen after 5–8 weeks, and the treatment can be continued for up to 1 year [29]. In melasma, the mean reduction of the modified MASI (mMASI) by treatment with a broad-spectrum sunscreen and topical 4% HQ is expected to be 30–40% after 8 weeks and 45–55% after 16 weeks [30, 31].

The efficacy of HQ is optimized when associated with retinoids and topical corticosteroids. In 1975, Kligman and Willis described the use of the association of 0.1% tretinoin, 5% HQ, and 0.1% dexamethasone in skin lightening [32]. Currently, the association of 0.05% tretinoin with 4% HQ and 0.01% fluocinolone acetonide (triple combination [TC]) is considered the gold-standard topical treatment for melasma owing to its potent and rapid whitening effect [33, 34]. All measures of effectiveness and safety indicate that intermittent use of TC for up to 6 months is recommended. In melasma, the reduction of mMASI by treatment with a broad-spectrum sunscreen and topical TC is expected to be 30–40% after 8 weeks and 50–60% for 16 weeks [35–37].

The use of HQ and TC can promote adverse effects, such as allergic and irritative contact

dermatitis, confetti-like leukoderma, redness, and telangiectasias. Exogenous ochronosis was reported due to prolonged use of high concentrations of HQ. Although topical HQ has been prohibited in some countries, there is no substantial evidence of risk from topical use for treatment of melasma. However, neither HQ nor tretinoin should be used during pregnancy [38–40].

Azelaic Acid

Azelaic acid, or 9-carbonodicarboxylic acid, is a nonphenolic compound derived from the yeast *Pityrosporum ovale*. It is a weak tyrosinase inhibitor of melanocytes and mitochondrial oxidoreductases, reducing the DNA synthesis of normal and hyperfunctioning melanocytes. Azelaic acid is safe for use in pregnant and breastfeeding patients and is generally well tolerated since the stinging and itching that follows its application fade in a few minutes. Azelaic acid is generally used as 20% cream twice daily for melasma, reducing the MASI by 50% after 8 weeks [41].

Niacinamide

Niacinamide, or nicotinamide, is a biologically active form of niacin (vitamin B3) found in the root of many vegetables and yeast. It is an essential precursor of coenzymes such as nicotinamide adenine dinucleotide (NADH) and nicotinamide adenine dinucleophosphate (NADPH) molecules with a potent antioxidant action [42]. In vitro studies have shown that niacinamide significantly decreases the transfer of melanosomes to keratinocytes, interfering in the cell signaling pathway between keratinocytes and melanocytes, decreasing melanogenesis [43]. Topical niacinamide stabilizes the epidermal barrier function with increased protein and ceramide synthesis and keratinocyte differentiation, improving skin microrelief and attenuating fine wrinkles. Moreover, it promotes an antiinflammatory effect on the skin [44]. Niacinamide is safe for use in pregnant and breastfeeding patients and is generally applied alone as 4–5% cream twice daily or in association with other lighteners, such as kojic acid and arbutin. In melasma, 4%

niacinamide has led to a 62% reduction of hemi-MASI (hMASI) after 8 weeks [45].

Cysteamine

L-Cysteamine is an aminothioli compound with antioxidant and depigmenting properties due to the inhibition of peroxidase and tyrosinase enzymes. It also acts as a chelator of iron and copper and increases intracellular glutathione levels. L-Cysteamine can be found naturally as an intracellular degradation product of L-cysteine.

Two randomized, double-blind, placebo-controlled studies with 50 and 53 participants tested a fine layer of 5% cysteamine cream on the face at night, leaving it up to 3 hours for 16 weeks, achieving reductions of the MASI by 50% and 59%, respectively [46, 47]. Moreover, a randomized, evaluator-blinded clinical trial including 40 Brazilian women with facial melasma who underwent broad-spectrum tinted sunscreen and nightly application of 5% cysteamine or 4% HQ for 16 weeks resulted in an MASI reduction of 32% and 52%, respectively [31]. Another comparison of cysteamine and 4% HQ for 16 weeks in Australia resulted in 21% and 32% mMASI reduction, respectively [48]. Furthermore, in a randomized trial of 50 women with melasma, 5% cysteamine was compared with a modified TC (4% HQ, 0.05% retinoic acid, and 0.1% betamethasone) for 16 weeks, achieving a 51% and 42% reduction of MASI, respectively.

Thiamidol

Thiamidol (isobutylamido thiazolyl resorcinol) is a new, potent tyrosinase inhibitor proven to prevent UVB-induced hyperpigmentation in human skin [49]. After 24 weeks of thiamidol 0.2% applied twice daily, 23 women with facial melasma experienced a mean MASI reduction of 26%. A randomized controlled trial including 50 Brazilian women with melasma compared a double layer of thiamidol 0.2% twice daily versus nightly 4% HQ for 12 weeks with a broad-spectrum tinted sunscreen. The reduction of mMASI was 43% and 33%, respectively.

Other Topical Agents

Several promising topical products are under consideration for melasma treatment [50]. However, few have been tested in randomized controlled trials using MASI scores as the primary outcome, limiting the evaluation of their efficacy and the definition of their role among available treatments.

The most active presentation of vitamin C is L-ascorbic acid, usually prescribed for melasma at concentrations of 5–20%. As it is highly unstable, several compounds have been developed for skin bleaching and melasma [51]. Vitamin C inhibits melanogenesis by interacting with the copper in tyrosinase, depleting dopaquinone. It is commonly associated with other lighteners, such as kojic acid and arbutin.

Kojic acid is obtained from various fungi such as *Aspergillus oryzae*, *Penicillium* spp., and *Acetobacter* spp. It also binds to the copper in tyrosinase, blocking its activity and eumelanogenesis. In the treatment of melasma, kojic acid is used in a concentration ranging from 1% to 4% in combination with other lighteners. Its effect begins after 2–4 weeks of continuous use, with progressive improvement up to 6 months [52].

Arbutin is an HQ derivative extracted from the grape *Uva ursi folium*, also found in the leaves of blueberries and cranberries. It inhibits tyrosinase and prevents the maturation of melanosomes. In melasma, it is usually used at concentrations ranging from 1% to 6% in combination with other lighteners and procedures [53].

Glycolic acid is an alpha hydroxy acid whose effect is dependent on its pH and concentration. It reduces the cohesion of corneocytes at the junction of the stratum corneum and granulosum, which leads to thinning of the epidermis with continued use. This agent facilitates the penetration of other active principles and promotes melanin's dispersion by increasing keratinocyte cell turnover [54]. For the treatment of melasma, glycolic acid is usually associated with other lighteners, such as kojic acid and HQ, in creams at concentrations from 4% to 10% [55]. The main adverse effects arise from the primary irritation. There may also be

temporary erythema and burning shortly after application.

Clobetasol is a potent topical steroid that promotes rapid clearing of melasma pigmentation. The mechanism of depigmentation promoted by corticosteroids has not yet been elucidated, and the clearance promoted is usually of short duration. Moreover, the cream cannot be administered for extended periods due to its local side effects, although clobetasol can be used for a short cycle followed by another treatment with a better safety profile. Sequential treatment with topical clobetasol for up to 8 weeks followed for topical bleaching agents can promote a faster result when compared with the usual bleaching agent [56, 57].

Ten patients with facial melasma from India were treated with 0.05% clobetasol propionate cream topically twice daily for 4 weeks, followed by use once daily associated with sunscreen for another 4 weeks. All participants presented pigmentation fading after 2 weeks, which was more noticeable after 4–6 weeks. However, three (30%) had to stop the treatment after 4 weeks because of local atrophy and telangiectasia [57].

In a split-face trial performed in India, participants with facial melasma were prescribed twice-daily application of 20% azelaic acid cream to half the face for 24 weeks. On the other half of the face, they applied 0.05% clobetasol propionate cream for 8 weeks only, followed by 20% azelaic cream for the next 16 weeks. For the 30 patients who completed the study, the side that received the sequential therapy presented substantial lightening, with mild and transient side effects [56].

Topical tranexamic acid (TXA) performs worse than its oral and intradermal regimens. Moreover, TXA has a variety of formulations, vehicles, and regimens of treatment that increase the variability of the results and hinder a suitable evaluation of its role in melasma treatment [58]. A vehicle-controlled study using liposomal 5% TXA for 12 weeks in Thailand resulted in a 51% reduction in MASI, despite no difference between groups [58].

A randomized split-face study of 100 Egyptian women compared liposomal 5% TXA cream twice daily with nightly 4% HQ for

12 weeks, which resulted in an hMASI reduction of 46% and 45%, respectively [59]. Another randomized trial with 60 Iranian women compared 5% TXA twice daily with 3% HQ for 12 weeks, resulting in a 52% and 48% mMASI reduction, respectively [60]. Furthermore, a randomized study compared topical TXA with a modified TC for 8 weeks in India, resulting in 5% and 30% MASI reduction, respectively [61].

Metformin inhibits melanogenesis by reducing intracellular cyclic adenosine monophosphate (cAMP) accumulation, but its low lipophilicity obligates its use in high concentrations for topical efficacy [62]. Topical metformin 30% lotion twice daily was compared with TC for 8 weeks in a randomized controlled study involving 40 Egyptian patients with melasma. The decrease in MASI was 56% and 57%, respectively [63]. Meanwhile, there is no robust evidence of the association of melasma with insulin resistance [64].

Methimazole is an oral antithyroid drug that has recently attracted attention due to its depigmenting effect by inhibiting peroxidase, but it has also been shown to exert inhibitory effects on tyrosinase in experimental studies. Topically, it is noncytotoxic to melanocytes, and it does not promote changes in serum thyroid stimulating hormone (TSH) levels [65]. A double-blind study evaluated the efficacy and safety of 5% methimazole once a day versus 4% HQ for 8 weeks in 50 Iranian women with melasma. The reduction of mMASI was 25% and 76%, respectively [66].

Melatonin is a hormone synthesized and secreted by the pineal gland, with a powerful antioxidant role that regulates the circadian rhythm. It reduces UV-induced free radicals and inhibits the alpha-melanocyte-stimulating hormone [67]. Since systemic oxidative stress is increased in patients with melasma, serum levels of melatonin and catalase are significantly lower [68]. After 90 days of topical melatonin 5% twice daily combined with sunscreen for 90 days, the MASI decreased by 31% compared with HQ 4% and sunscreen, which achieved a decrease of 37% [69]. As sleep disorders are a major source of oxidative stress, oral melatonin can play a special role in the

treatment of patients who refer poor sleep quality [70].

Malassezin is a natural indole compound produced by *Malassezia furfur*, reported to induce melanocyte apoptosis, dose-dependent induction of apoptotic markers, and decreased melanin synthesis in melanocyte cultures. In vitro tests also demonstrated that malassezin is not a tyrosinase inhibitor, but a highly active agonist of the aryl hydrocarbon receptor with key homeostasis functions that may impact the mechanism of pigmentation. In a double blind, dosing range study, the group that used topic malassezin twice a day for 8 weeks had a 30.13% reduction in the severity of hyperpigmentation, compared with 16.67% in the placebo group. There were no relapses during 8 weeks of follow-up [71, 72].

In 2017, Cohen proposed a novel (topically administered) double therapy for melasma that combine an anti-estrogen and a vascular endothelial growth factor inhibitor. The anti-estrogen could be either a selective estrogen receptor modulator (such as tamoxifen or raloxifene) or an aromatase inhibitor (such as anastrozole, letrozole, or exemestane). The vascular endothelial growth factor inhibitor would be bevacizumab [73–75]. Investigation of this novel approach in clinical trials is warranted.

Systemic Treatments

Tranexamic Acid (TXA)

TXA is a synthetic lysine-analog antifibrinolytic agent that competitively inhibits the activation of plasminogen to plasmin. It promotes the inhibition of tyrosinase activity by blocking the interaction of melanocytes and keratinocytes via the inhibition of the plasminogen/plasmin system. TXA also decreases mast cell activity and inhibits fibroblast growth factors [76, 77].

In a randomized controlled trial with 44 women, oral 250 mg TXA or placebo was given twice daily for 3 months, leading to a 49% reduction of MASI, compared with 18% in those who used sunscreen alone [78]. However, there was a notable relapse of melasma in the 3 months that followed TXA interruption.

Oral TXA also improves the performance of adjuvant creams such as TC cream in melasma, with earlier clinical response [79]. In a randomized trial, the group that received oral TXA, 250 mg, twice daily for 60 days associated with a broad-spectrum sunscreen and TC cream presented a 64% reduction of mMASI, while the group that received a broad-spectrum sunscreen and TC presented only a 12% mMASI reduction [80, 81]. A relapse of melasma was observed in the months following TXA interruption, even with sunscreen and TC maintenance.

Regarding TXA dosage, patients with severe melasma were randomized to receive a daily dose of 500 mg, 750 mg, 1000 mg, or 1500 mg for 8 weeks. All four doses were effective, and there were no relevant differences in the MASI or melanin index reductions among them [82], although faster results were achieved with higher dosages [83]. As long as the prothrombotic effects are dose dependent, the lower dose offers a favorable result, as fewer patient side effects and risks are preferable. Despite its off-label indication, TXA is usually prescribed as 250 mg capsules twice daily for 2–4 months in melasma.

A retrospective analysis enrolled 561 patients with different treatment regimens. Adverse events occurred in 40 (7.1%). Most were transient, but one (0.2%) developed deep vein thrombosis 6 weeks after starting therapy. She was later diagnosed with familial protein S deficiency [84].

The primary reported side effects of TXA are back pain, musculoskeletal pain, oligomenorrhea, headache, urticarial rash, and abdominal cramps. Contraindications to TXA are renal dysfunction, malignancy, cardiovascular or respiratory disease, current anticoagulant therapy, (personal or familial) history of thromboembolic disease (including deep vein thrombosis, pulmonary embolism, arterial thrombosis, stroke, and subarachnoid hemorrhage), and pregnancy. Other factors (e.g., hormonal contraception or replacement therapy, smoking, and long-distance travel) should be considered as potential exclusion criteria. There are no biochemical markers of TXA-related thrombotic events, so careful anamnesis should be performed to identify the main risk factors [85].

Pycnogenol

Pycnogenol is a standardized extract obtained from the bark of the French maritime pine *Pinus pinaster*. It contains monomeric phenolic compounds (catechin, epicatechin, and taxifolin) and condensed flavonoids (procyanidins), with antioxidant and antiinflammatory activities capable of stimulating the synthesis of the enzyme-induced nitric oxide synthase and antityrosinase activity leading to the suppression of melanin biosynthesis. In vitro, pycnogenol has antioxidant properties that are more potent than vitamins E and C. In addition, it recycles vitamin C, regenerates vitamin E, and reduces the skin erythema induced by UVB [86]. The usual dosage prescribed is 75–150 mg daily in melasma, divided into two to three doses for 2–4 months. The side effects are negligible.

Oral pycnogenol 50 mg twice daily has been suggested to treat melasma, reducing 58% of MASI in 31 Brazilian women after 90 days [87]. Another case series enrolled 30 Chinese women with melasma, who took 25 mg pycnogenol three times daily for 30 days, leading to a 32% mean area reduction and a 22% intensity reduction of facial melasma [86]. Furthermore, a randomized controlled trial evaluated the addition of 150 mg oral pycnogenol (or placebo) to a broad-spectrum tinted sunscreen and TC in 44 women with melasma. At 60 days, the reduction of mMASI was superior for the group who took oral pycnogenol, at 49% versus 34% [37].

Other Systemic Agents

Polypodium leucatomos (PL) is an over-the-counter medication, marketed as Heliocare in USA. It is a fern of the Polypodiaceae family, native to Central and South America. It has been described as an antiinflammatory supplement for treating inflammatory dermatoses and reducing sunburn severity. Its exact mechanism of action is unknown, but it is a potent antioxidant and seems to work to maintain the extracellular matrix's structural integrity, which is usually affected by ultraviolet radiation [88, 89]. These properties have led to the hypothesis of its efficacy for the treatment of melasma. However, a trial involving 40 Hispanic women with

moderate to severe melasma using sunscreen and randomized to receive oral placebo or 720 mg PL extract daily for 12 weeks revealed no difference in MASI reduction [90].

Another study included 33 Asian women with melasma using topical 4% HQ cream and broad-spectrum sunscreen randomized to take PL 480 mg/day or placebo for 12 weeks. At 56 days, the decrease in MASI was more significant in the PL group (49.4%) than placebo (32.6%). Nevertheless, the reduction in the melanin index, erythema index, quality of life scores, and blinded global photographic improvement did not differ between the groups [91]. These contradictory results warrant new systematic studies to investigate the efficacy of PL in treating melasma.

Carotenoids are natural pigments present in several plants that act as stabilizers of unstable molecules due to their ability to reversibly chelate reactive oxygen species and other free radicals, absorbing the energy of these molecules and dissipating it in the form of heat. In the skin, photoprotection by carotenoids occurs by direct absorption of UVA, UVB, and VL by acting against the action of UVR on exposed molecules, which become unstable and potentially hazardous to the DNA molecules and cellular proteins. In addition, they can activate antioxidant and antiproliferative enzymatic pathways in response to UVR exposure [92]. Oral fucoxanthin (10 mg/kg) reduced experimentally UVB-induced skin pigmentation in guinea pigs, probably due to suppression of proinflammatory cytokine prostaglandin E₂ (PGE₂) synthesis and receptors involved in melanogenesis [85]. Despite the plausibility of its use, there have been no randomized controlled trials of oral carotenoids in melasma to date.

Glutathione has been purposed to treat melasma due to its antioxidant properties, leading to tyrosinase inhibition. Moreover, it increases intracellular cysteine levels and N-acetylcysteine, shifting melanogenesis from eumelanin to pheomelanin. It is prescribed worldwide for skin discoloration by an oral or intravenous route. A randomized placebo-controlled study with 60 young Thai medical students taking 250 mg twice daily oral

glutathione for 4 weeks provided a consistent decrease in the melanin index and a reduction of lentigines [93]. Thus, randomized controlled studies using oral antioxidants with glutathione or *N*-acetylcysteine for melasma are warranted.

Oral lycopene-rich tomato extract proved to increase the serum levels of superoxide dismutase and contribute with topical 4% hydroquinone to mMASI reduction, after 12 weeks [94].

Chemical Peelings

Chemical peelings accelerate the turnover of epidermal keratinocytes, classified as superficial, medium, and deep according to the skin layer and histological level reached. The effects can include epidermal remodeling, keratinocytes from the corneal layer, melanin elimination, and dermal reorganization due to the inflammatory process and production of cytokines, which stimulate fibroblasts and increase collagen and elastin synthesis [95]. Moreover, there are many indications for medical peelings in cosmetic dermatology. With melasma, they are usually associated with medical care, such as TC and HQ plus a broad-spectrum tinted sunscreen, and other procedures, such as micro-needling, for better results [96, 97].

Chemical peels are not the first-line therapy, but they represent an adjuvant measure. Notably, superficial peeling in serial sessions should be performed for melasma. The effects are dependent on keratinocyte turnover and melanin distribution [98, 99]. The most commonly used agents for chemical peeling are (1) 15% or 20% trichloroacetic acid (TCA) in aqueous solution, gently applied with gauze and not removed or neutralized; (2) 30%, 50%, or 70% glycolic acid (GA) in gel, applied with gloved fingers and removed (after erythema or stinging) or neutralized (if frosting) with 10% sodium bicarbonate; (3) 20% or 30% salicylic acid (SA) in ethanol, applied with gauze and not removed; and (4) Jessner's solution (JS), which is composed of 14 g resorcinol, 14 g salicylic acid, and 14 mL lactic acid (85%) in ethyl alcohol 95%, applied with gauze and not removed.

The results may be mild, moderate, or marked, but no additional reduction in skin pigmentation may be observed. GA is the agent most widely cited in literature about chemical peelings and melasma [100]. Full-strength lactic acid (LA) solution, in concentrations of 92% or 82%, was reported in two studies as an alternative to be applied with gloved fingers just over the lesional area and removed after erythema or stinging. Its efficacy seemed similar to that of JS [101, 102].

Retinoic acid (RA) or tretinoin, in concentrations of 1%, 3%, and 5% in ethanol/propylene glycol, was first reported in 2001 as an agent for serial superficial peelings and indicated for melasma and photoaging [103, 104]. It is a yellow-colored dispersion and should be maintained on the skin for up to 6 h (washed at home), and a brown dye is added for facial use with no influence on the peelings' effect. The expectation is to increase keratinocyte turnover and eliminate preformed melanin within the epidermis. However, RA is a volatile formulation, degraded when exposed to VL, UV, and O₂. Thus, a powerful antioxidant system is needed.

To date, the best vehicle and ideal concentration for RA remain unknown. Pharmacotechnical studies have shown greater retention of high-strength formulations in the corneal layer when compared with traditional 0.05% RA cream. Thus, high concentrations have no apparent advantage since they are more difficult to homogenize, increasing the risk of crystallization and reducing skin penetration [105].

An evidence-based review, including 40 studies and 2912 patients, concluded that GA peeling is not more effective than HQ 4% [106]. Furthermore, another review, including grades of recommendation, concluded that all superficial peelings might be used in melasma as an additional or maintenance therapy, with mild to moderate efficacy. The authors also demonstrated no significant advantage of one agent over another and recommended a choice based on comfort level, the experience of the dermatologist, and the safest strength [107].

In contrast, a recent network meta-analysis evaluated the efficacy of all currently available

Table 1 Randomized and controlled trials, prospective and comparative studies about chemical peelings in melasma

Author, year	Study design	Population	Interventions, efficacy parameters duration, follow-up	Results
Kalla, 2001	NR, comparative	N = 100 Indian patients M:F = 1:1.6 2 groups (A, B)	A = 6855–75% GA B = 32 10–15% TCA Parameters not specified Till significant improvement Follow-up = 3 months	TCA: faster response, but more relapse and hyperpigmentation
Hurley, 2002	R, IB, split-face	N = 21 Hispanic patients (18 completed) phototypes IV and V; 2 groups (A, B)	A: 4% HQ cream twice daily—8 weeks B: 4% HQ cream twice daily + 20%–30% GA, every 2 weeks (20% for 4 weeks, 30% for 4 weeks) MASI, Mexameter, subjective 8 weeks No follow-up	Significant reduction in both groups, with no difference
Sarkar, 2002	Open, pilot, comparative, prospective	N = 40 Indian patients Photo types III–V M:F = 18:22 2 groups (1, 2)	1 = 20: MKF daily + serial GA (30–40%), 3 weeks interval 2 = 20: MKF daily 21 weeks No follow-up	Significant improvement in both groups, more rapid and greater in group 1
Khunger, 2004	Open, pilot, split-face	N = 10 female Indian patients	1% RA daily X 70% GA, weekly, 12 weeks mMASI, photograph No follow-up	Significant decrease in both sides, with no difference
Dogra, 2006	R	N = 50 female Pakistan patients 2 groups (A, B)	A = 50% GA B = 20% TCA + 5% HQ and 0.025% RA 3 peels, every 3 weeks MASI, photographs No follow-up	Same efficacy, tolerability to GA better than to TCA
Macedo, 2006	Open, comparative, split-face prospective	N = 8 female Brazilian patients	10% GA/4% HQ cream on both sides of the face + 4 bi-weekly 70% GA to one side X peeling vehicle on the other side, Clinical pictures assessed by 3 independent dermatologists No follow-up	Similar improvement on both sides; 70% GA did not produce additional reduction in pigmentation

Table 1 continued

Author, year	Study design	Population	Interventions, efficacy parameters duration, follow-up	Results
Sharquie, 2006	NR, split-face, comparative	N = 30 Iraqi women (26) and men (4) 24 completed (20 women, 4 men) phototype IV 2 groups (1, 2)	1 = 15—92% LA 2 = 15 JS every 3 weeks Till desired response 2 to 5 sessions MASI Follow-up = 6 months	Significant improvement on both sides, with no difference; no side effects
Erbil, 2007	RCT	N = 25; 15 = cases 10 = controls	Cases: serial GA 20 → 35 → 50 → 70% every other week + daily 20% AA cream (b.i.d.) + adapalene 0.1% gel Controls: daily 20% AA cream (b.i.d.) + 0.1% adapalene gel MASI 20 weeks 20 weeks	Good response for all, significantly better for group peels, with transient erythema and PIH in 3 patients
Soliman, 2007	R, comparative	N = 30 female patients, phototypes III and IV 2 groups (A, B)	A = 15 20% TCA B = 15 20% TCA + topical 5% ascorbic acid + 0.05% RA gel and 4% HQ cream once daily MASI and patients' global response 6 weeks 12 and 16 weeks	Improvement and maintenance during follow-up A = 10 patients (67%) B = 13 patients (87%) No significant

Table 1 continued

Author, year	Study design	Population	Interventions, efficacy parameters duration, follow-up	Results
Garg, 2008	R, SB	N = 60 Indian patients phototype IV (50 completed) 3 groups (A, B, C) M:F = 1:6.5	A = 15 GA 20% → 50% B = 17 0.025% RA + GA 20% → 50% C = 18 2% HQ + GA 20% → 50%) 2-weeks interval, MASI No follow-up	All improved, most significant in C A > B > C
Safoury, 2009	NR, SB, prospective split-face	N = 20 women phototypes III and IV	15% TCA on both sides X mJS on one Side 10 days interval MASI 10 weeks 8 weeks	Significantly higher improvement with combination, but significant discomfort
Kumari, 2010	NR, comparative	N = 40 Indian women 2 groups (1, 2)	1 = 20 12% GA or 0.1% RA cream, at night, previously, for 2 weeks + 20–35% GA 2 = 20 10–20% TCA every 15 days MASI Photographs 12 weeks No follow-up	Similar efficacy MASI reduction: 79% for GA 73% for TCA No difference Fewer side effects for GA compared to TCA
Ikknur, 2010	Single-blind, randomized, right-left of the face	N = 31 Turkish patients (24 completed)	12 serial peel GA X AFA. 2 weeks interval. mMASI 6 months. No follow-up	Significant decrease of mMASI No difference AFA—less irritating

Table 1 continued

Author, year	Study design	Population	Interventions, efficacy parameters duration, follow-up	Results
Kodali, 2010	RCT, split-face, prospective	N = 20 Latin American women (18 completed)	20%—30% SA every 2 weeks 4 peels on one side of the face X 4% HQ cream to both sides, twice daily Narrowband reflectance spectrophotometry No follow-up	Significant reduction in pigment intensity, no difference between sides 20% to 30% SA peels are were not effective for melasma
Faghihi, 2011	RCT, DB, split-face	N = 63 patients (male and female)	70% GA X 1% RA peel, 4 sessions 2 weeks interval, MASI 8 weeks No follow-up	No difference RA 1% more tolerable
Puri, 2012	NR, comparative	N = 30 Indian patients M:F = 1:6.5 2 groups (A, B)	A: 15% TCA B: 35% GA peel every 3 weeks MASI 15 weeks No follow-up	Same efficacy, TCA: more side effects
Sobhi, 2012	SB, right-left comparison	N = 14 Egyptian women, phototypes IV-V	Right side: 70% GA 6 sessions X Left side: nanosome vitamin C applied by iontophoresis MASI Photographs evaluated by two single-blinded physicians No follow-up	Both sides improved Nanosome vitamin C was better Few and transient side effects

Table 1 continued

Author, year	Study design	Population	Interventions, efficacy parameters duration, follow-up	Results
Mahajan, 2015	RCT, DB	N = 40 Indian patients (38 completed) 2 groups (1, 2)	1 = 20 just TC 2 = 20 serial GA peel 2-weeks interval + 20% AA cream MASI, digital photography, VAS 12 weeks 12 weeks	Significant reduction in MASI with no difference between groups
Sarkar, 2016	R, comparative	N = 90 Indian patients 3 groups (A, B, C)	Before peels: 4% HQ + 0.05% RA cream, 4 weeks A = 35% GA B = SM 20% AS / 10% mandelic acid C = phytic acid peels. every 14 days MASI Photography 12 weeks Follow-up = 20 weeks	MASI reduction: A = 62.36% B = 60.98% C = 44.71% 35% GA and SM were equally efficacious and safe and more effective than phytic acid peels SM was better tolerated
Choudhary, 2017	R, parallel control, comparative, SB	N = 36 female patients 12, melasma 12, photomelanosis 12, post acne pigmentation, 3 groups (1, 2, 3) (4 patients each)	1. 20% SA every 2 weeks 2. 50% GA every 3 weeks 3. 15% TCA every 3 weeks 6 sessions Investigator's Global Improvement Assessment, Patient's satisfaction grading scale based in photographs No follow-up	Improvement (all together) marked = 27.77% (10/36) moderate = 36.11% (13/36) mild = 36.11% (13/36) No difference All well tolerated

Table 1 continued

Author, year	Study design	Population	Interventions, efficacy parameters duration, follow-up	Results
Abdel-Meguid, 2017	RCT, IB, split face, right-left, assessor-blinded	N = 24 female Egyptian patients, phototypes IV-V	20%-25% TC + JS X 20%-25% TCA alone 6 sessions 2 weeks interval MASI photographs No follow-up	Significant decrease in MASI for both regimens; JS + TCA: more effective

RCT randomized controlled trial, *R* randomized, *NR* non-randomized, *SB* single-blind, *DB* double-blind, *IB* investigator-blind, *TC* triple combination (2%, HQ, 0.05%, RA, 0.01%); Fluocinolone), *AA* azelaic acid, *mMASI* modified Melasma Area and Severity Index, *VAS* Visual Analog Scale, *PIH* post-inflammatory hyperpigmentation, *MKF* modified Kligman formula, *M:F* Male:Female

treatments for melasma. Chemical peels were not cited as a therapeutic option due to the paucity of high-quality randomized controlled trials [108]. Adverse effects are rare, but the primary concern is the risk of hyperpigmentation, especially in dark-skinned individuals [99].

Twenty-one randomized and comparative studies about chemical peels for melasma were identified (Table 1). In general, no relevant differences were observed in the comparisons. Nonetheless, it is important to highlight the high heterogeneity and limitations of the studies, such as (1) low to moderate methodological quality, (2) variation in a selected population, (3) a lack of inclusion and evaluation in different skin types, (4) small sample sizes, (5) variable designs, (6) types of chemical peels, (7) deficient efficacy measures, (8) short-term or no follow-up, (9) attrition rate, (10) heterogeneities in the grouped results, and (11) poor conclusions. Nevertheless, GA is considered more effective and safer than TCA peeling for melasma [99, 106], while JS and RA peels are low-cost and safe adjuvant procedures for melasma. Moreover, modified JS (17% LA, 17% SA, 8% citric acid, and ethanol) may reduce allergic reactions to resorcinol and post-inflammatory hyperpigmentation [109]. However, additional well-designed studies are needed.

Microneedling

Microneedling was first developed to treat acne scars and skin laxity. Nevertheless, an improvement in post-inflammatory hyperpigmentation and melasma was perceived in these patients [110]. When performed with a roller, providing a moderate skin injury (diffuse erythema and some bleeding spots) promoted an early improvement of melasma [111]. This technique, associated with TC and broad-spectrum sunscreen for 8 weeks (Fig. 1), better promoted a superior mMASI reduction (48%) than TC alone and proved to prevent further relapsing [81, 112].

The mechanism of microneedling action is not yet entirely known. After the procedure, an early keratinocyte proliferation occurs, increasing melanin transcutaneous elimination. In addition, microneedling improves patterns of skin photoaging, such as solar elastosis and pendulum melanocytes, while restructuring damaged basal membranes [113]. Microneedling is also a safe procedure to perform on skins of color [106].

Many studies have cited microneedling to increase topical bleaching agent penetration. However, the best endpoint for penetration is not unanimous. Furthermore, the bleaching

effect of the product depends on the skin penetration and frequency of use. Therefore, the punctual drug delivery of a bleacher on a biweekly or monthly basis of microneedling was not beneficial. On the contrary, most of these agents were not developed for intradermic use. Thus, their safety profile is unknown. In addition, the lateral and deep penetration of these substances after microneedling occurs erratically, making the adverse effects of a possible systemic absorption unpredictable [114].

Radiofrequency microneedle was reported as an effective treatment for refractory melasma, although no large randomized controlled studies have been performed [115–117].

Intradermotherapy and Platelet-Rich Plasma

A significant challenge in treating melasma is the low permeability of water-soluble substances through the hydrophobic layer of the stratum corneum. With intradermotherapy, medications are delivered directly into the dermis, resulting in a higher drug concentration in the target area with smaller volumes. Consequently, the effect is greater and longer-lasting, with fewer adverse events. For intradermal injection, substances must be solubilized in sterile water, and mixtures should be avoided,

with different substances injected via separate syringes.

TXA is the most studied substance in intradermotherapy for melasma. Injections are performed by a 28-gauge to 30-gauge syringe at 1 mm depth and 1 cm distance between each point. In clinical trials, 3–12 sessions were performed with an interval of 1–6 weeks between them. There was a reduction in the severity score, which varied between 33.3% and 81.2%. Different concentrations of TXA were used (4–100 mg/cc), but higher concentrations did not appear to improve the skin lightening. Notably, lower concentrations relapsed more, while higher concentrations were more painful. Intradermotherapy with TXA appears to lighten the skin more slowly but has similar efficacy to oral treatment, with a better safety profile [118]. Although intradermotherapy is likely to promote melasma improvement, controlled studies are scarce despite the wide use of vitamin C and glutathione products.

Platelet-rich plasma (PRP) seems to be another promising technique for treating melasma, although only a few controlled studies have compared PRP with standard therapies. No consensus exists regarding a standardized method of PRP preparation or the concentration of platelets. Platelets contain platelet-growth factor, transforming-growth factor (TGF) β 1 and β 2, and epidermal growth factor. These substances act on pigment metabolism and inflammation and can repair the altered dermal structures of melasma.

In a meta-analysis of PRP for melasma involving 395 adults in ten clinical trials, there was a significant reduction in the mMASI score after the procedure [119]. In addition to intradermal injection, PRP can be used after microneedling, with similar results between the two techniques. The number of PRP sessions ranged from three to six, held monthly or semi-monthly. Minimal side effects were seen, such as temporary erythema, edema, and pain at the injection site.



Fig. 1 Facial melasma in a 48-year-old type V Fitzpatrick woman treated with tinted sunscreen, triple combination at bedtime, oral tranexamic acid 250 mg every 12 h and two microneedling sessions with a 30-day interval between sessions. A: before and B: after 2 months of treatment

Laser and Light Technologies

Multiple clinical trials have explored the use of laser and light technologies to treat melasma. However, due to the heterogeneity of the study design (clinimetric parameters used as outcomes, the number of sessions, treatment intervals, and duration of follow-up), the diversity of device settings, and melasma characteristics, comparing these results is complicated [120]. In clinical practice, the laser that targets melanin most used for treating melasma is the Q-switched Nd-YAG in toning mode. The Q-switching technique produces pulses with an extremely high peak of energy delivered in a short time (nanoseconds). The result is destruction of melanin by a photoacoustic effect [121, 122]. The toning mode, in turn, uses low fluence (up to 3 J/cm^2) and larger spot size in sessions with short intervals (1–6 weeks). Hence, there is no destruction of melanocytes or keratinocytes in the epidermis but rather the intracellular melanosomes (subcellular selective photothermolysis), leading to less inflammation and consequently less post-inflammatory hyperchromia and hypochromia in confetti. However, even with this safer technique, there are reports of dyschromia after procedures with short intervals [123].

Recently, the industry has developed lasers whose pulse duration is picoseconds at different wavelengths: 532, 755, and 1064 nm. The energy, delivered in an even shorter time, reduces the photothermal effect and increases the photoacoustic effect in the treated tissue. However, the superiority of picosecond lasers compared with nanosecond lasers in the treatment of melasma may not be well established [124, 125].

Besides pigment, lasers that target water are also used for treating melasma. Ablative fractional lasers (CO_2 and Er:YAG) vaporize the keratinocytes with more melanin and the hyperfunctioning melanocytes from melasma. Moreover, the healing of the skin around the coagulation columns improves the photoaged environment of the upper dermis of melasma, possibly reducing the paracrine stimulus to the melanocytes.

However, ablative lasers have a high thermal effect, resulting in dyschromia. Thus, short pulses with low energy density are recommended to reduce this complication [126]. Nonablative lasers, in turn, produce columns of coagulative damage within the dermis, keeping the stratum corneum intact, which generates less inflammation. One nonablative laser, the 1927 nm thulium device, appears to be promising for treating melasma because its penetration is around the dermal–epidermal junction [127].

Hypervascularization is an epiphenomenon of the ionizing radiation damage in melasma while participating in the maintenance of hyperpigmentation. Hence, a pulsed dye laser (585 nm) can be used in melasma, especially when telangiectasias are visualized. When treating the vessels, the pulsed dye laser decreases the stimulus to melanocytes and subsequently the recurrence of the spots. Due to the risk of post-inflammatory hyperchromia, it should not be used in higher ($\geq \text{IV}$) phototypes [128].

Dermabrasion

Superficial mechanical abrasion of the skin using a micromotor and a rotating diamond probe has been proposed to cure melasma. Indeed, gentle dermabrasion restores the whole epidermis and upper dermis. Although transient post-inflammatory hyperpigmentation is expected, long-term residual hypochromia is verified after dermabrasion for acne scars. For example, a long-term follow-up of 410 Thai women with melasma treated with dermabrasion evidenced a 95% sustained effect for 5 years and a low rate of adverse effects. However, these results were never repeated through a controlled trial [129]. Nevertheless, microdermabrasion with aluminum oxide has no direct role in melasma treatment since it only affects the stratum corneum but may facilitate the permeation of products [130].

CONCLUSIONS

The treatment of melasma is challenging, as the spots are not cleared completely in most patients and recurrences are frequent. The first-line treatment is rigorous daily sun protection and the association of tinted sunscreen with topical bleaching agents, which may have a different action on melanogenesis.

In severe and/or resistant cases, oral drugs should be prescribed. Tranexamic acid has the best evidence for treating melasma, however it is a thrombophilic drug and thus should be prescribed in select patients. There is also accumulating evidence for the use of pycnogenol as a systemic adjuvant in the treatment of melasma.

Procedures also are helpful to treat melasma. Microneedling is a low-cost technique with few adverse effects that complements topical treatment. Chemical peels act mainly via epidermal renewal. Lasers and light-based technologies should be used with maximum care as heat induces post-inflammatory melanogenesis.

The combination of topical bleaching agents with oral medications and procedures can provide faster results, although strategies for the prevention of further relapses are poorly studied.

FUTURE RESEARCH

Intense research is being carried out on new and more effective melasma treatments and the combination of different mechanisms of action of the available drugs. Strategies that reduce local and systemic oxidative stress, stabilize upper dermis mast cells, decrease melanogenesis without melanocyte toxicity, remove epidermal melanin without inflammatory stimulus, revert senescence, and induce autophagy are considered promising. Thus, developing effective treatment and preventive strategies in melasma is a work in progress that depends on a better understanding of its pathogenesis. Meanwhile, sun protection associated with treatments that reduce melanogenesis, increase epithelial melanin turnover, and repair the

upper dermis is the mainstay of therapeutic strategies.

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Compliance with Ethics Guidelines. This article is based on previously conducted studies and does not contain any new studies with human participants or animals performed by any of the authors. The patient in Fig. 1 gave informed consent for the publication of the image.

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APÊNDICE 5: Ilustrações de apoio

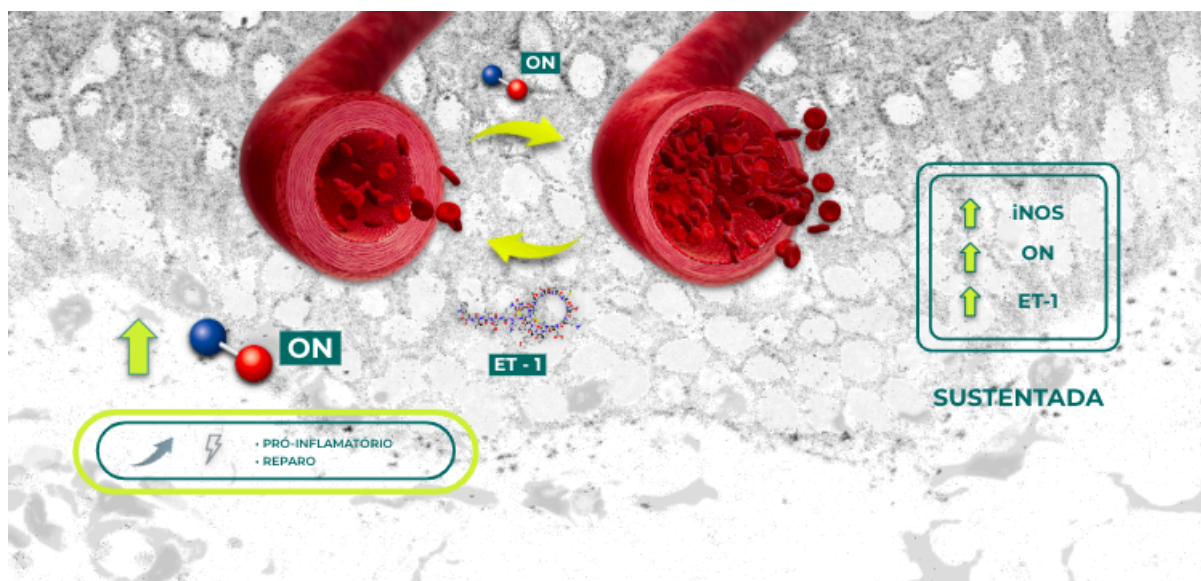


Ilustração 1- Mecanismo de aumento da produção de endotelina-1 (ET-1) na pele com melasma: em um ambiente pró-inflamatório e de reparo, como presente na pele com melasma, há liberação de óxido nítrico (ON), potente vasodilatador. A pele com melasma já mostrou aumento na concentração de óxido nítrico sintase induzível (iNOS), o que acarreta em maior produção de ON e justificaria um aumento da produção de ET-1, que é vasoconstritora, como forma de contrabalancear a vasodilatação promovida pelo ON.

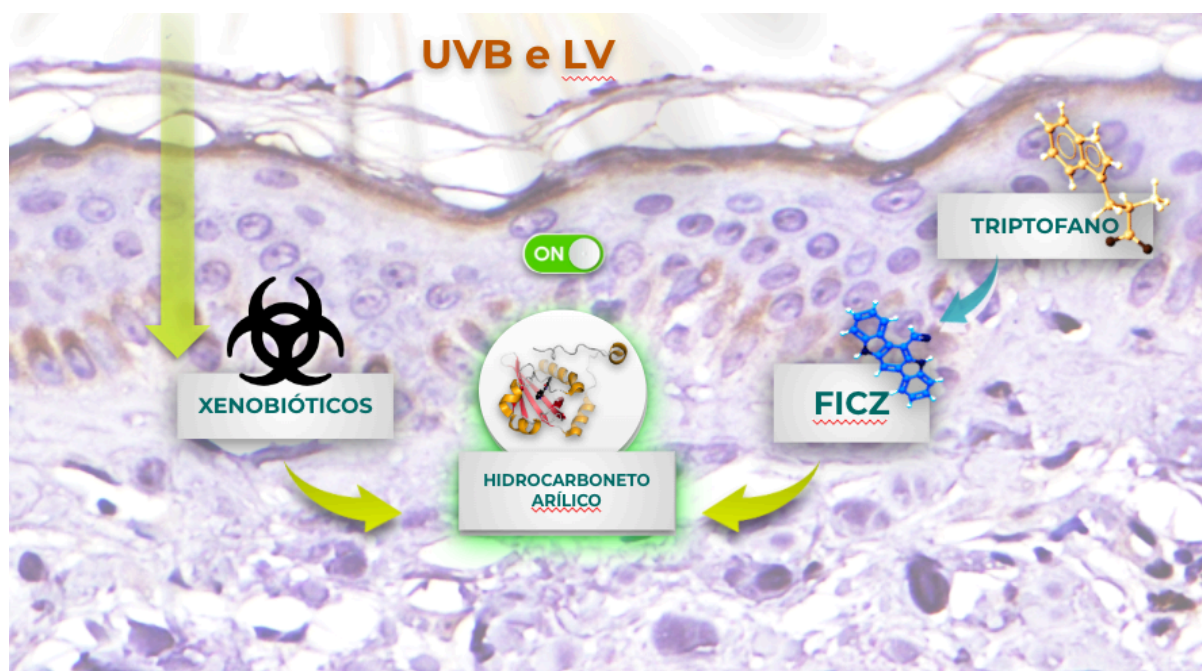


Ilustração 2- Sinergia entre a poluição atmosférica e radiação ultravioleta na ativação do receptor de hidrocarboneto arílico (AhR): o AhR pode ser ativado por elementos xenobióticos que alcançam diversas camadas da pele através de penetração por vias intercelulares, intracelulares ou pelas aberturas das unidades pilossebáceas. Elementos endógenos também são capazes de ativar o AhR, como o formindolo-(3,2-b)-carbazol (FICZ), um fotoproducto do triptofano, gerado a partir da exposição deste aminoácido à radiação ultravioleta B (UVB) e luz visível (LV) solar. O FICZ é um ligante de alta afinidade do AhR.

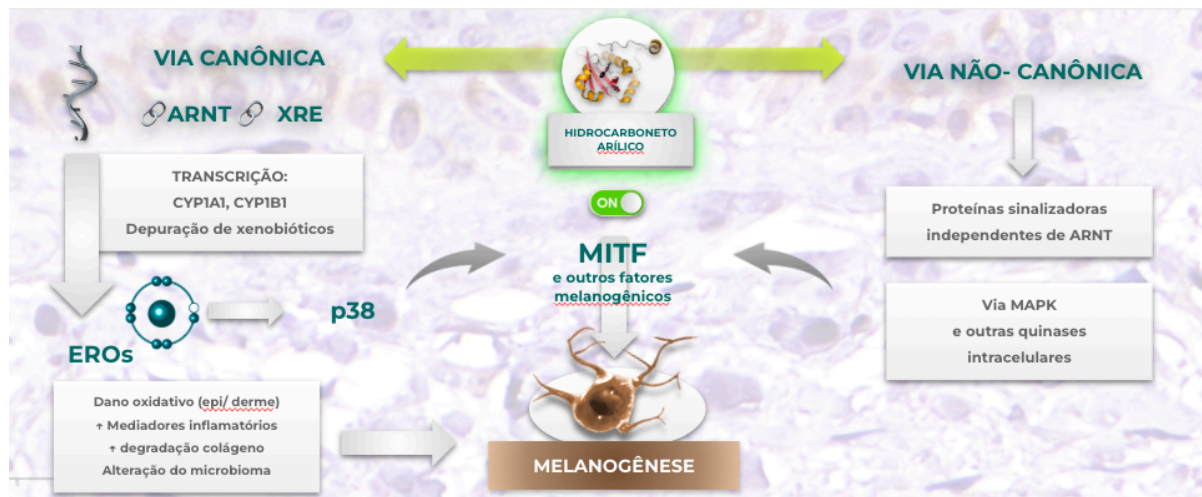


Ilustração 3- Ativação de vias melanogênicas a partir da ativação do receptor de hidrocarboneto arílico (AhR): uma vez ativado, o AhR pode seguir a via canônica ou não-canônica. Na via canônica, sofre translocação nuclear, onde interage com o parceiro de heterodimerização ARNT (*Aryl Hydrocarbon Receptor Nuclear Translocator*), formando um complexo que se liga a elementos de resposta específicos no DNA, como o elemento de resposta a xenobióticos (XRE). Isso resulta na transcrição de genes como CYP1A1 e CYP1B1, envolvidos na depuração de xenobióticos e que influenciam a síntese de melanina, podendo exacerbar hiperpigmentação do melasma. O processo de depuração de xenobióticos é gerador de espécies reativas de oxigênio (EROs), o que eleva a expressão de p38, levando a aumento da expressão de fatores pró-melanogênicos, como o MITF (*Microphthalmia-Associated Transcription Factor*), que também promovem aumento da síntese de melanina. O dano oxidativo eleva mediadores inflamatórios, à degradação de colágeno e leva também a alterações no microbioma cutâneo, elementos que favorecem a melanogênese.

Já a via não canônica envolve interações diretas ou indiretas do AhR com outras proteínas sinalizadoras, independentemente de ARNT, incluindo a ativação de quinases intracelulares e vias como a MAPK (*Mitogen-Activated Protein Kinase*). Estas vias podem promover a expressão de fatores pró-melanogênicos, como o MITF.