



ORIGINAL ARTICLE

Exploratory Study of Epidermis, Basement Membrane Zone, Upper Dermis Alterations and Wnt Pathway Activation in Melasma Compared to Adjacent and Retroauricular Skin

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Background: Melasma is a chronic acquired focal hypermelanosis which pathogenesis has not been fully elucidated. Classical pathophysiologic studies have analysed the affected and perilesional areas, but little is known about the status of sun-protected skin, which is subjected to the same endogenous and genetic factors. **Objective:** To assess the histological characteristics of melasma compared to adjacent and retroauricular skin. **Methods:** Skin samples were collected from 10 female from: melasma, perilesional area and retroauricular. The samples were stained (haematoxylin-eosin, periodic acid-Schiff, Fontana-Masson, picosirius red, toluidine blue and Verhoeff), immunolabelled for CD34 and Wnt1. The data from the skin sites were analysed simultaneously by a multivariate model. **Results:** Melasma skin exhibited noteworthy *stratum corneum* compaction, greater collagen heterogeneity, solar elastosis, higher number of mast cells, basement membrane zone (BMZ) damage, Wnt1 expression, pendulum melanocytes, higher cellularity and vascular proliferation at the superficial dermis. *Stratum cor-*

neum compaction, collagen heterogeneity and BMZ abnormalities were variables associated to melasma that not follow a *continuum* through retroauricular to adjacent skin. Mast cell count was the variable that disclosed correlation with the most other abnormalities as well as had the greater contribution in the multivariate model. **Conclusion:** In addition to melanocyte hyperactivity, melasma skin exhibits alterations in the epidermal barrier, upper dermis and BMZ, which differ from the adjacent sun-exposed skin and retroauricular skin, indicating a distinct phenotype, rather than a mere extension of photoageing or intrinsic ageing. Mast cells appear to play a central role in the physiopathology of melasma. (Ann Dermatol 32(2) 101 ~ 108, 2020)

-Keywords-

Basement membrane, Dermis, Epidermis, Melanosis

INTRODUCTION

Melasma is a chronic acquired focal hypermelanosis that affects sun-exposed areas, particularly the face, in female. The locations of melasma in visible areas, the young age of patients and the frequent relapse (even during treatment) result in a considerable impact on the quality of life¹. Facial melasma is independently associated with miscegenation and the high occurrence within familiars (40% ~ 60%) suggests a genetic predisposition, which is best explicated by a dominant pattern of segregation^{2,3}. Although its pathogenesis has not yet been fully elucidated, it has already been established that melasma is not due only to melanocyte hypertrophy but it results from a

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global alteration of the full epidermal-melanin unit, fibroblast and endothelium, with significant abnormalities in the upper dermis and basement membrane zone (BMZ)^{4,5}. To date, no other previous research have explored all these alterations together and analysed the correlations of these variables. Moreover, the classic studies on the histology of melasma merely evaluated the affected skin and the adjacent area, and little is known about the behaviour of sun-protected skin, which is still subjected to the same endogenous and genetic factors as melasma skin.

The aims of the present study were to assess the histological characteristics of melasma skin with an integrative view and investigate the possible correlation between morphological abnormalities and epidermal expression of Wnt1 in affected skin, perilesional and retroauricular skin.

MATERIALS AND METHODS

Botucatu School of Medicine, São Paulo State University institutional committee of ethics on research approved the project (no. 1,602,185). A cross-sectional study with intra-subject control was performed, in which were included 10 adult female (> 18 years old) with facial melasma who had not been treated in the past 30 days or more, except for use of sunscreen. The participants were selected using convenience sampling from January through June-2016. Patients with systemic diseases and those who were pregnant or using anticoagulants were not included. The study design for 10 patients (30 samples) was based on the expectation of more than 50% of difference between the topographies (dependent samples), with $\alpha = 0.05$ and $\beta = 0.2^6$. Skin samples (3-mm punch) were taken from three different sites: facial melasma, adjacent healthy skin (up to 2 cm from melasma skin) and retroauricular skin (which is a relatively non sun-exposed skin and with a pattern of melanocytes distribution similarly to the face).

The samples were processed for histochemical staining techniques (haematoxylin-eosin, Fontana-Masson, periodic acid-Schiff [PAS], picosirius red, Verhoeff and toluidine blue), for immunohistochemical (goat polyclonal anti-CD 34 primary antibody, C-18, sc-7045; Santa Cruz Biotechnology, Dallas, TX, USA) and for double-labelling direct immunofluorescence (Wnt1), which objective was to analyse the anatomical differences between sites, degree of solar elastosis, amount of epidermal and dermal melanin, BMZ abnormalities, elastic and collagen fibres, pendulum melanocytes, number of mast cells and dermal cellularity, vascular components and expression of Wnt pathway. The *stratum corneum* compaction and solar elastosis were analyzed through a qualitative method (for compaction: 0, no compaction; 1, moderate, and 2, severe compaction;

for solar elastosis: 0, absent; 1, moderate, and 2, severe solar elastosis).

The BMZ damages were counted in PAS stain (mean count per high-power field: $\times 400$), totalizing approximately 13 fields in each histologic fragment. Collagen homogeneity was evaluated in picosirius red using the box-counting fractal dimension to quantify the organization of collagen fibers. It results how thick a set occupies the metric space in which it exists.

The protocol employed in the Wnt included slide deparaffinisation, antigen retrieval in citrate solution and heating cycles in a microwave oven. Non-specific protein blocking was performed with 1% milk. Histological sections were incubated with primary antibody-anti-Wnt1 antibody (ab15251; Abcam, Cambridge, MA, USA) 1:100 overnight in a wet chamber at 4°C. Following rinsing, the sections were incubated with secondary antibody conjugated to Alexa Fluor[®] 594 dye (Thermo Fisher Scientific, Waltham, MA, USA) 1:500 for 20 minutes in a wet chamber at 37°C in a dark room. Counterstaining with 4',6-diamidino-2-phenylindole was performed for nuclear labelling.

Photographs were taken in triplicate from regular interfollicular, artefact-free areas from each histological section using a Coolscope II microscope (Nikon Inc., Tokyo, Japan), resulting in tagged image file format images with 1280 \times 1024 pixels (24-bit colour). The intensity of epidermal labelling (excluding the *stratum corneum* in the instances with autofluorescence) was analysed by two different dermatologists with wide knowledge in dermatopathology, using the software ImageJ 1.51e (0-255 grey hue variation) for the various colour channels⁷. The sites of the samples were blinded during the assessment.

The data were compared among skin types using a generalised linear mixed effects model, with a robust covariance structure, a probability distribution adjusted for each sample (normal or gamma) and *post hoc* analysis using a sequential Sidak algorithm⁸.

The correlation of variables that were significant on bivariate analysis was assessed using Spearman's test and then included in an exploratory multiple correspondence analysis model^{9,10}.

The analyses were performed using IBM SPSS ver. 25.0 software (IBM Corp., Armonk, NY, USA); the significance level was set as $p \leq 0.05$.

RESULTS

The mean age (standard deviation) of the participants was 42.9 (8.9) years, the length of melasma was 16.7 (7.9) years, and all cases exhibited phototypes III or IV.

The main results of immunologic and histomorphometric

Table 1. Immunological and histomorphometric measurements related to patients with melasma per investigated skin site

Histomorphometric and immunological measurement	Melasma skin	Perilesional skin	Retroauricular skin	p-value
Haematoxylin-eosin				
<i>Stratum corneum</i> thickness (micra)	13.4 (4.2)	13.3 (3.7)	18.1 (5.3) ^{§,}	<0.01 [†]
Viable epithelium thickness (micra)	58.8 (9.7)	55.2 (10.9)	53.1 (9.6)	0.59
Total epidermis thickness (micra)	72.1 (11.0)	69.6 (15.1)	71.2 (11.9)	0.91
<i>Stratum corneum</i> compaction (qualitative) ^{*,†}	1 (1~2)	1 (0~1) [§]	0 (0) ^{§,}	<0.01 [†]
Solar elastosis (qualitative) ^{*,†}	1 (1~2)	0 (0~1) [§]	0 (0) ^{§,}	<0.01 [†]
Cellularity of the upper dermis (per ×40 field)	53.6 (7.5)	45.4 (6.2) [§]	37.7 (6.6) ^{§,}	<0.01 [†]
Fontana-Masson				
Melanin density in epidermis	0.35 (0.06)	0.32 (0.08) [§]	0.25 (0.08) ^{§,}	<0.01 [†]
Melanin density in dermis (×100)*	0.19 (0.06~0.75)	0.25 (0.20~0.35)	0.25 (0.16~0.45)	0.82
Periodic acid-Schiff				
Basement membrane lesions (per ×40 field)	2.1 (0.6)	1.7 (0.8) [§]	1.0 (0.5) ^{§,}	<0.01 [†]
<i>Pendulum</i> melanocytes (per ×40 field)*	2.7 (2.7~3.3)	1.3 (1.3~3.0) [§]	1.3 (1.0~1.7) [§]	<0.01 [†]
Verhoeff				
Elastotic material density in upper dermis	0.29 (0.07)	0.25 (0.04) [§]	0.19 (0.04) ^{§,}	<0.01 [†]
Picrosirius red				
Collagen density in upper dermis	0.63 (0.09)	0.68 (0.07)	0.66 (0.06)	0.15
Collagen homogeneity (fractal dimension)	1.53 (0.02)	1.56 (0.04) [§]	1.57 (0.03) [§]	<0.01 [†]
Toluidine blue				
Mast cell (per ×40 field)	6.4 (1.5)	3.6 (0.7) [§]	2.5 (0.8) ^{§,}	<0.01 [†]
CD34				
Blood vessels density in the upper dermis	6.8 (1.4)	4.3 (0.8) [§]	4.5 (1.5) [§]	<0.01 [†]
Wnt1 (epidermal pixel intensity)	67 (29)	54 (30) [§]	57 (32)	0.02

Values are presented as mean±standard deviation.

*Median (p25-p75). †0: no corneal compaction; 1: mild to moderate corneal compaction, 2: significant corneal compaction. ‡0: absence of solar elastosis; 1: mild to moderate solar elastosis, 2: intense solar elastosis. § $p < 0.05$ in relation to melasma, || $p < 0.05$ in relation to adjacent skin. ¶ $p < 0.05$.

analysis are disclosed in Table 1. Thinning of the *stratum corneum* in the most sun-exposed fragments (Fig. 1) and its greater compaction in the melasma lesions were remarkable. Progressive melanin pigmentation of the epidermis was detected across the skin topographies (Fig. 1). Solar elastosis was more prominent in the most sun-exposed skin, particularly the melasma lesions (Fig. 1, 2). Similarly, the dermal collagen was more heterogeneous in the papillary dermis of melasma skin than in the other sites (Fig. 2).

Pendulum melanocytes (Fig. 3) were more frequent in melasma and sun-exposed skin, as the dermal cellularity was higher, and there was a larger number (and diameter) of vascular structures (Fig. 3), mast cells (Fig. 4), and epidermal Wnt1 (Fig. 5) in melasma.

The correlations among histological variables are described in Table 2. The compaction of the *stratum corneum*, cellularity of the superficial dermis, mast cell density and solar elastosis were the variables that exhibited the strongest patterns of correlation. Mast cell count was the variable that disclosed correlation with the most other abnormalities.

The perceptual map for the multiple correspondence analysis (Fig. 6) included 13 variables and accounted for 65% of the variance of the two-component model. The topographies were well characterized by the variables. Solar elastosis, collagen heterogeneity, dermal cellularity, mast cell number, presence of pendulum melanocytes, vascular structures, abnormalities of the elastic fibres and Wnt1 expression shown the strongest correlation with the facial skin topographies. Melasma exhibited preferential association with mast cell number, superficial dermal cellularity and abnormalities of the elastic fibres. Mast cell count was the variable that disclosed greater inertia from the origin, in the three quadrants, providing the greater contribution to the model. *Stratum corneum* compaction, density of epidermal melanin, collagen heterogeneity and BMZ abnormalities were variables associated to melasma that not follow a *continuum* through retroauricular to adjacent skin.

DISCUSSION

Histological differences and Wnt/ β -catenin activation were explored among facial melasma, perilesional and retro-

auricular skin. The comparison of these three areas provides a model that allows inferences about the role that environmental stimuli (e.g., ultraviolet radiation [UVR]) and intrinsic factors, such as genetic and hormonal influences, play in the pathogenesis of melasma¹¹.

Stratum corneum compaction together with functional lipid metabolism disorder are associated to the functional integrity of the skin barrier. The difference in its compaction between melasma and perilesional skin (both sun-exposed areas), favours the hypothesis that melasma is associated

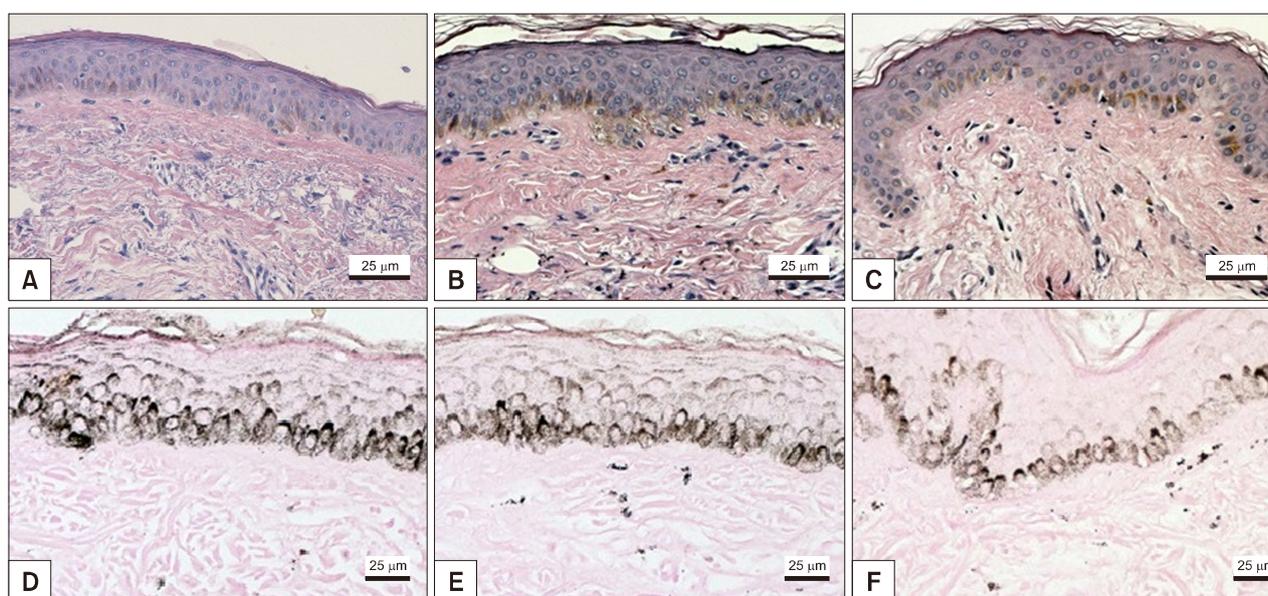


Fig. 1. Haematoxylin-eosin staining shows thinning of the *stratum corneum* in melasma (A) and adjacent (B) skin and greater compaction in the melasma sample. Solar elastosis exhibited increased progressively expression across the investigated skin types and it was greater in melasma (A) than in the adjacent skin (B) and retroauricular (C) skin. Fontana-Masson staining shows the progressive increasing in the epidermal melanin pigmentation across the skin types, which was greater in melasma (D) than in the adjacent skin (E) and retroauricular (F) skin. The melanin density in the dermis did not differ between samples. (A ~ C) $\times 100$, (D ~ E) $\times 200$.

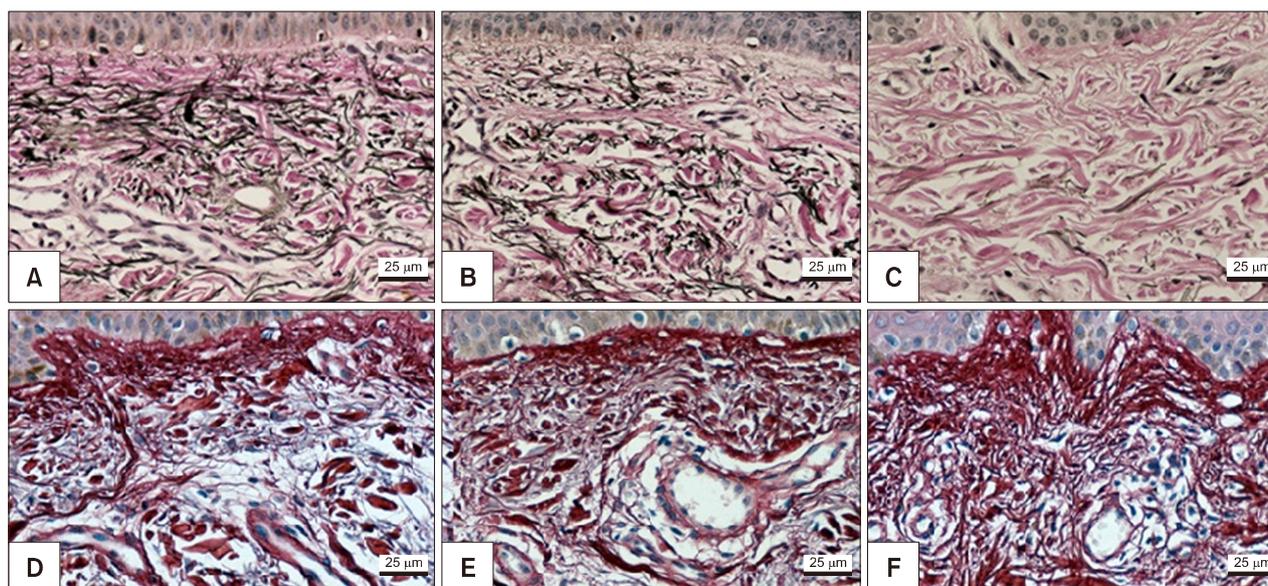


Fig. 2. Melasma (A), adjacent (B) and retroauricular (C) skin subjected to Verhoeff staining: the elastotic material is more prominent in the most sun-exposed skin, specifically melasma (A). Picrosirius red staining showed greater collagen heterogeneity in the papillary dermis of melasma (D) than in the adjacent (E) and retroauricular (F) skin. (A ~ E) $\times 200$.

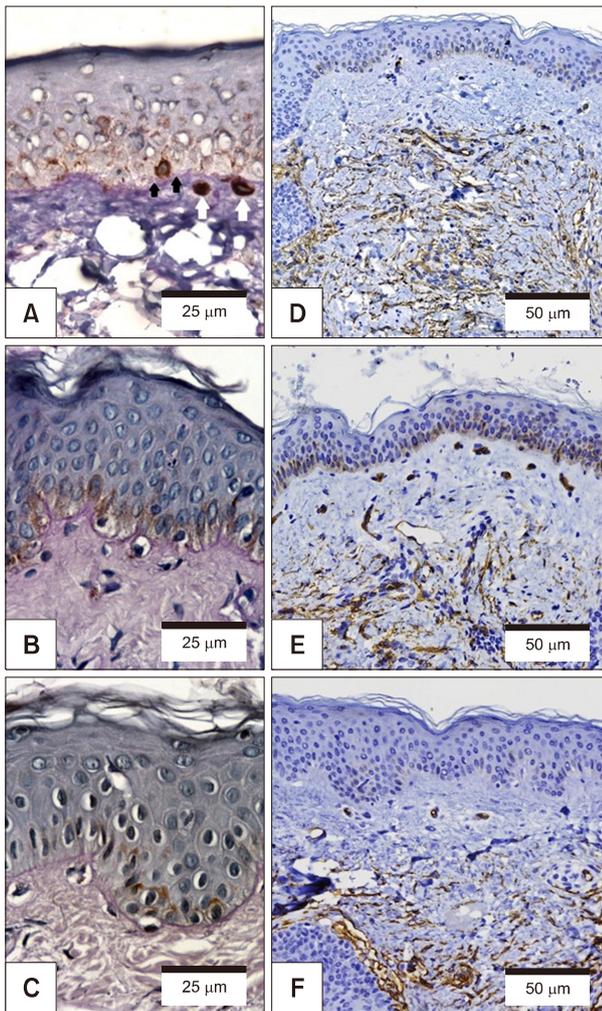


Fig. 3. Melasma (A), adjacent (B) and retroauricular (C) skin subjected to periodic acid-Schiff staining: basement membrane changes was more prominent in melasma skin (A). CD34 immunohistochemical showed larger number of blood vessels more evident in melasma (D) than in the adjacent (E) and retroauricular (F) skin. The white arrows are pointing to the *pendulum* melanocytes while the black arrows are pointing the basement membrane disruptions. (A~C) $\times 100$, (D~E) $\times 200$.

with greater damage to the skin barrier^{12,13}.

Melanogenesis in melasma involves epithelial secretion of α -melanocyte stimulating hormone (α bMSH) and activation of the Wnt pathway; nonetheless, it seems to be independent of the stimulation by ultraviolet radiation/p53, interleukin (IL)-1 α , cyclooxygenase-2/prostaglandin E2, WIF-1 and ASIP¹⁴. The melanin synthesis in melasma suggests an microenvironment that favours focal melanogenesis in an area exposed to similar UVR to the adjacent skin. In this study, the melanin density in the epidermis exhibited a correlation with the number of cells in the upper dermis, the amount of vascular structures and mast

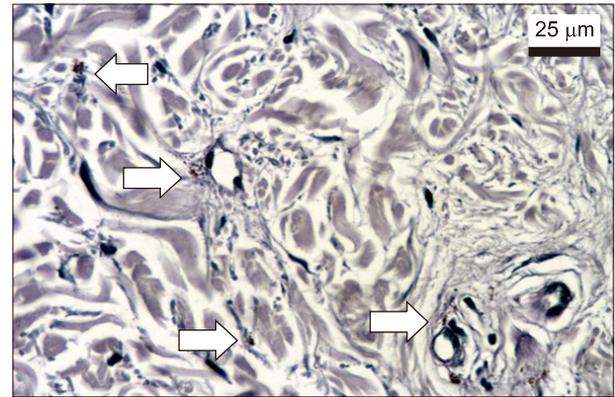


Fig. 4. Mast cells in the superficial dermis (arrows) of melasma skin, with preferential perivascular topography and metachromatic granules (purple), as assessed using toluidine blue staining ($\times 100$).

cells, which suggests that dermal stimuli play a major role in the melanogenesis of melasma.

The number of *pendulum* melanocytes exhibited significant progression as a function of the assessed skin area. The density of *pendulum* melanocytes was correlated with *stratum corneum* compaction, solar elastosis, counting of mast cells and abnormalities of the elastotic material but was not correlated with the number of BMZ failures. Experimental exposure of photoprotected skin to UVA triggers abnormalities similar to those found in melasma¹⁵. Beyond the evident epidermal abnormalities, there was a significant difference in the degree of elastosis between melasma and the adjacent skin, one might hypothesise that this change is not just a secondary epiphenomenon of photoageing but a condition relevant to the development of melasma. Solar elastosis was correlated with collagen heterogeneity, superficial dermis cellularity, counting of mast cells and dermal blood vessels, *pendulum* melanocytes, *stratum corneum* compaction and elastic fibre damage, which suggests an integrative role of the various components in the physiopathology of melasma.

Collagen heterogeneity, which was assessed through fractal dimension, reflects greater fragmentation of collagen fibers. Its correlation with solar elastosis and mast cell count in upper dermis suggests the role of ultraviolet radiation and inflammatory mediators in collagen alterations, as well as it occurs in photoageing, where the dermis disruption is clinically manifested by increased sagging and wrinkled skin.

The increased dermal cellularity in melasma lesions might be due to inflammation and oxidative damage¹¹. UVB radiation is able to induce the secretion of cytokines, such as endothelin-1, IL-1, nitric oxide, stem cell factor and α MSH,

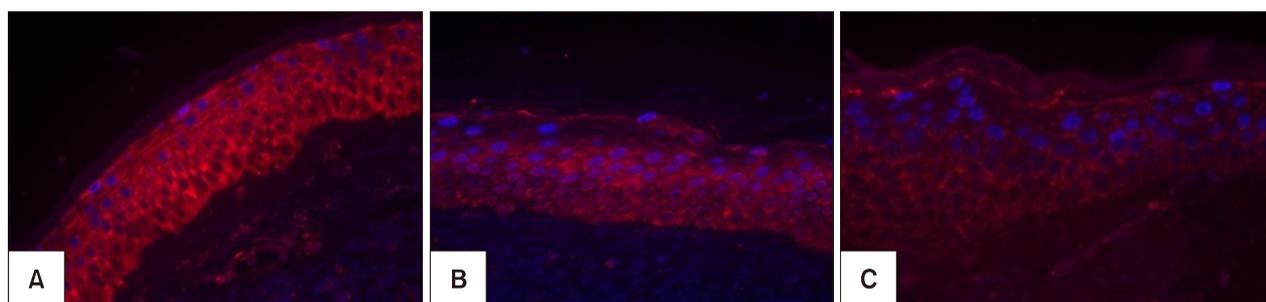


Fig. 5. Melasma (A), adjacent healthy skin (B) and retroauricular skin (C) with double-labelling double immunofluorescence for Wnt1 (red/cytoplasmic). The blue colour is related to the nucleus marker (4',6-diamidino-2-phenylindole).

Table 2. Correlation (Spearman's rho) of histomorphometric measurements regarding the patients with melasma

Histomorphometric measurement	Corn	Compact-Corn	Verh	Elastos	CollHomog	DermCell	BasMemb	Masto	VasCD34	Pendul	Wnt1
MelEpid	-0.36*	0.19	-0.05	0.23	-0.16	0.41*	0.25	0.37*	0.45*	0.04	-0.23
Corn		-0.31	-0.11	-0.23	0.02	-0.38*	-0.36*	-0.15	-0.04	-0.24	-0.07
CompactCorn			0.60*	0.57*	-0.16	0.58*	0.13	0.51*	0.15	0.60*	0.18
Verh				0.54*	-0.27	0.27	-0.15	0.46*	0.11	0.53*	-0.06
Elastos					-0.39*	0.43*	0.17	0.66*	0.41*	0.37*	0.30
CollHomog						-0.30	-0.10	-0.47*	-0.20	-0.18	0.00
DermCell							0.37*	0.70*	0.27	0.30	0.32
BasMemb								0.29	0.27	0.11	0.24
Masto									0.45*	0.49*	0.40*
VasCD34										0.26	-0.07
Pendul											0.09

Corn: *stratum corneum* thickness, CompactCorn: *stratum corneum* compaction, Verh: assessment of elastic fibres by means of Verhoeff staining, Elastos: solar elastosis, DermCell: superficial dermis cellularity, VasCD34: density of blood vessels in the superficial dermis, Pendul: pendulum melanocytes, Wnt: density of anti-Wnt antibody labelling in the epidermis, MelEpid: melanin in the epidermis, BasMemb: density of basement membrane lesions, Masto: mast cell density in the superficial dermis, CollHomog: collagen heterogeneity in the upper dermis. * $p \leq 0.05$.

which stimulate melanocyte mitosis or melanogenesis^{11,16}. The density of mast cells increases according to the degree of photodamage in sun-exposed areas¹¹. Histamine stimulates proliferation and migration of melanocytes through activation of protein kinase-A via the H2 receptor, thus behaving as a direct stimulator of melanogenesis^{16,17}. Mast cells also secrete paracrine mediators factors that induce elastin production by fibroblasts¹⁸. In addition, mast cells produce tryptase, which participate in the damage to the extracellular matrix and basement membrane, (through matrix metalloproteinases 9-collagen IV damage), what can influence the occurrence of collagen heterogeneity and pendulum melanocytes, which were correlated with the presence of mast cells in the present study^{19,20}. Our results suggest mast cells as playing a central role in the pathophysiology of melasma, and therapeutic trials with mastocyte stabilizers drugs are warranted.

The melasma samples exhibited a larger number of vascular structures, which was correlated with solar elastosis,

the presence of mast cells and epidermal pigmentation. Angiogenesis might be stimulated by UVR, fibroblast growth factor secretion, IL-8 and vascular endothelial growth factor (VEGF)^{21,22}. Studies are needed to demonstrate the direct action of VEGF in the melanogenesis in melasma^{4,23}. Endothelin-1, which is produced by endothelial and inflammatory cells, promotes the upregulation of key genes for melanogenesis, such as the genes encoding tyrosinase and the microphthalmia-associated transcription factor. The endothelium-induced melanogenic stimulus is even stronger after exposure to UVR, which reinforces its role in the pathophysiology of melasma²⁴.

The Wnt/ β -catenin pathway includes a large family of proteins with different cell functions, such as the melanoblasts migration, proliferation and induction of pigmentation²⁵. Wnt1 is a ligand of transmembrane frizzled receptors, which promote de accumulation and stabilization of β -catenin²⁶. Wnt1 was select to be analysed because it is the mainly activator of β -catenin pathway. The dynamic

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