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REGULAR ARTICLE

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The influence of MC1R on dermal morphological features of photo-exposed skin in women revealed by reflectance confocal microscopy and optical coherence tomography

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Abstract

Background: The melanocortin 1 receptor (MC1R) gene is one of the major determinants of skin pigmentation. It is a highly polymorphic gene and some of its polymorphisms have been related to specific skin phenotypes, increased risk of skin cancers and skin photoageing. Currently, its contribution to changes in dermal features in photo-exposed skin is unknown.

Objective: The main objective of this study is to evaluate the potential correlation between MC1R status and specific healthy photo-exposed skin characteristics.

Materials and methods: Skin facial features were estimated by evaluation with standard digital photography with automated features count, reflectance confocal microscopy (RCM) and optical coherence tomography (OCT) in 100 healthy women. Skin of the forearms was used as a control.

Results: The study found an association between RHC MC1R polymorphisms and dermal features in photo-exposed areas being represented by increased vessel density and pixel density in OCT (P = .025 and P = .001, respectively) and increased coarse collagen in RCM (P = .034), as compared to non-RHC subjects. To our knowledge this is previously unreported. Additionally, previously reported correlations between light hair colour and pigmented spots with MC1R RHC polymorphisms have been confirmed.

Conclusions: Our results suggest the role of RHC MC1R variants in dermal variations of facial skin, as compared to non-RHC variants. To our knowledge this is previously unreported.

KEYWORDS

dynamic optical coherence tomography, melanocortin 1 receptor, reflectance confocal microscopy, skin photoageing

1 | INTRODUCTION

The MC1R gene is highly polymorphic in Caucasian populations, and as many as 200 polymorphisms have been described.[1] The MC1R gene codifies for a transmembrane G protein-coupled receptor. The main ligand of this receptor is the α-melanocyte-stimulating hormone (MSH), a preopiomelanocortin (POMC) derivative. The α-MSH activates an adenylate cyclase, leading to an increase in intracellular cyclic adenosine monophosphate (cAMP) and a transcriptional activation of the tyrosinase family of genes, thereby regulating melanin biosynthesis, leading from pro-oxidant pheomelanin to the production of photoprotective eumelanin.[2]

Several studies have demonstrated an association between MC1R polymorphisms and red hair colour (RHC) phenotype characterized by red hair, fair skin and freckles.[3–6] MC1R variants have been classified, according to their penetrance for the RHC phenotype, into strong "R" or RHC alleles, or weak-r forms.[7,8] Extensive evidence shows that all the RHC alleles yield hypomorphic proteins, impairing the activation of the cAMP pathway.[9,10]

Some MC1R polymorphisms have also been associated with pigmented spots,[11] a subjective "inferior youthful appearance"[12] and an increased risk of photoageing,[13] as compared with wild-type (wt) subjects. Furthermore, melanoma[14,15] and non-melanoma skin cancer risk[16] have been associated to specific MC1R polymorphisms. Interestingly, MC1R polymorphisms have been related to both sun sensitivity/poor tanning, after ultraviolet (UV) radiation,[17] and the decreased ability to repair UV-damaged DNA.[1]

Studying histopathologic facial skin variations related to the ageing process has previously been limited by the requirements for facial skin biopsies. Recently, novel non-invasive skin imaging tools, such as reflectance confocal microscopy (RCM) and optical coherence tomography (OCT), have made in vivo skin morphological features assessment possible, widening the scope of application to skin features analyses in other than disease-specific scenarios. RCM enables the dynamic detection of morphologic and functional skin aspects at *quasi*-histologic resolution[18] and has been recently successfully applied to epidermal and dermal changes during skin ageing.[19] OCT and dynamic OCT (D-OCT) enable the visualization of vertical and horizontal sections of morphological and microvascular skin features.[20–22] However, specific skin features of a photo-exposed area such as the face, as revealed by RCM and OCT/D-OCT, and the correlation with MC1R status have not yet been investigated.

The primary aim of the study was to correlate strong RHC MC1R (RHC group) and non-strong RHC MC1R (non-RHC, including both MC1R weak-r and wt carriers) carriers on patient clinical, comorbidities and skin morphological changes revealed through in vivo skin imaging analyses. Secondary aims included (a) a comparison with non-photo-exposed skin and (b) a subgroup morphological analysis of RHC versus (vs) weak-r carriers vs wt subjects.

2 | MATERIALS AND METHODS

2.1 | Study design

This prospective, single centre study enrolled women at the Dermatology Unit, University of Modena and Reggio Emilia between June and August 2016. A total of 100 women known to the investigators, between the ages of 30 and 60 years, were invited to participate in the current study. Inclusion criteria specified Caucasian origin, no known dermatological disorders (including personal or family skin cancer history), no history of any other previous cancers or any facial interventions, such as injection of fillers or laser procedures within the previous 6 months, or any type of facial plastic surgery.

The study protocol was approved by the local Ethics Committee (nr 33/16 prot nr 2560) and all participants provided written, informed consent. The study was conducted according to the Declaration of Helsinki Principles. Participants gave their written informed consent.

2.2 | Subject data

Subjects were instructed not to apply any facial detergents or cosmetic agents for at least 12 hours prior to the arranged dermatological examination. A standardized questionnaire was delivered to all subjects, including questions referring to personal and clinical information, such as age, sun exposure, smoking and alcohol assumption habits. Clinical examination determined the individual subjects' hair, eye and skin colours.

2.3 | Standard digital photography with automated feature evaluation

All subjects were photographed using a digital photography analysis system with automated features evaluation of the face (Canfield Scientific, Inc, New York, NY). This tool enables the estimation of characteristics of the skin that are not visible to the naked eye. The subjects' grading of facial skin texture, erythema and hyperpigmentation were recorded. Standard photography was used to assess rhytides and texture, ultraviolet (UV) light was employed for UV spots and porphyrin examination and cross-polarization light was used for brown spots and red area analyses. For each photograph, areas of skin meeting a threshold level of colour contrast to adjacent skin were defined as spots.[23] The software provides an automated evaluation quantifying the per cent area of the face comprising the spots in each of the photographs.

Results were retrieved in terms of absolute (ABS) scores, referred to the intensity of detected instances of the feature being analysed in the total size and area.[24]

2.4 | Reflectance confocal microscopy

Reflectance confocal microscopy imaging was performed with the confocal Vivascope 1500® (MAVIG GmbH, Munich, Germany),

reaching a depth of up to 250 μm, on the right cheek (5 mm below the zygomatic arch), as previously described.[19] The inner forearm of each subject was used as control. Four 3 x 3-mm mosaics with a 25-μm step were acquired for each subject, starting from 5 to 10 μm below the stratum corneum, to the first appearance of the honeycomb pattern.

Reflectance confocal microscopy features evaluated in this study included the irregular honeycombed pattern and mottled pigmentation of the epidermis, polycyclic papillary contours and sebaceous glands at the dermo-epidermal junction (DEJ) and thin, coarse or huddle collagen and elastosis at the dermis. These features were described in Supplementary Table S1. RCM images were evaluated by two expert readers, and a third one was involved in case of discordance.

2.5 | Optical coherence tomography criteria

Optical coherence tomography images were acquired through the VivoSight OCT® (Michelson Diagnostics Ltd., Orpington, UK) at the right cheek (5 mm below the zygomatic arch). The inner forearm of each subject was used as control. OCT employs a swept source tunable diode laser with a peak power of 15 mW at k = 1310 nm for non-invasive real-time skin imaging, up to a 2-mm depth. Vessel measurements were performed on horizontal (parallel to the skin surface) D-OCT images, according to "vessel density," with a 4-point scale (Figure 1) and amount of red pixels, "pixel density," with the ImageJ software[25] (Image Processing and Analysis in Java, freeware 2014 version USA) analysis applied to images, all of the same size, at 300 μm depth.[26] From the many vertical sections acquired, sections analysed were selected according to the absence of interrupting features (hair shadows, adnexal structures, etc). Features assessed at OCT and D-OCT were outlined in Table S2. OCT and D-OCT images were evaluated by two expert readers, and a third one was involved in case of discordance.

2.6 | Melanocortin 1 Receptor genotype and sequence analysis

Melanocortin 1 receptor sequencing was performed from standard blood samples, collected during the dermatological visit. Genomic DNA was extracted from whole frozen blood samples according to the manufacturer's protocol (QIAamp DNA blood kit; QIAGEN). The MC1R gene sequence (FW: 50 CCTAAGCTTACTCCTTCCTGCTTCCTGGACA 30; Rv: 50 CTGGAATTCACACTTAAAGCGCGTGCACCGC 30) was amplified by polymerase chain reaction (PCR). PCR products were analysed by direct sequencing on both strands using the Eurofins Genomic service (Eurofins Genomics). MC1R variant allele distribution was evaluated and subjects were classified as strong RHC MC1R polymorphisms (RHC group) [R151C, R160W, R142H, D294H, I155H] or non-strong RHC MC1R polymorphisms (non-RHC group); the latter subdivided according to weak-r variants and wt subjects.

2.7 | Statistical analysis

Mean and percentage frequency were used to express population characteristics. To test the effect of MC1R status (RHC vs non-RHC) on clinical comorbid and morphological features, univariate analyses were carried out. For quantitative variables, Student's *t* test was performed and for qualitative variables the Pearson chi-square test or Fisher's test (in case of values <5 in the contingency Table) were performed. Statistically significant variables ($P \leq .1$) in univariate analysis or clinically relevant variables were included in a multivariate logistic model (a stepwise method of variable selection was used to estimate a final model). The relationship between specific skin features and MC1R status was expressed as "Odds Ratio" (OR) with a confidence interval of 95%. Secondary outcomes included the comparison of statistically significant morphological features with non-photo-exposed skin morphological features according to the Pearson chi-square test. The subgroup analysis (weak-r vs wt vs RHC) included statistically significant variables (clinical and morphological features) identified in primary analysis and were assessed according to an ordered logistic regression. All statistical analysis was performed using Stata (version 11.2). Reported significance levels were two-sided, and a threshold of $P < .05$ was considered statistically significant.

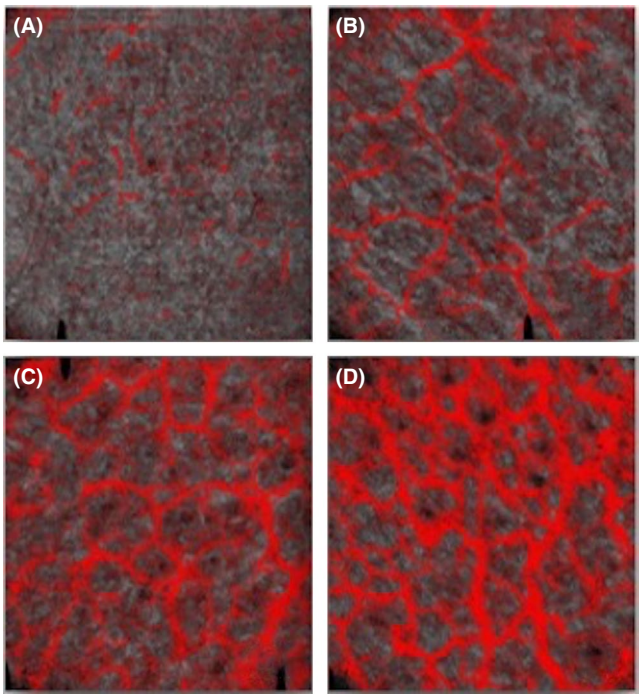

FIGURE 1 Vessel density scale. Dynamic optical coherence tomography (D-OCT) images in horizontal (parallel to skin surface) section (size 6 × 6 mm; skin depth 300 μm) showing the 4-point scale used for vessels density assessment. Increasing density is shown: absent (A), low (B), medium (C), high (D)

3 | RESULTS

3.1 | Characteristics of the study population

This study enrolled 100 women invited to participate in the study, of which three subjects were excluded following DNA analysis due to potential bias from two rare MC1R variants (D294Q and D294N).

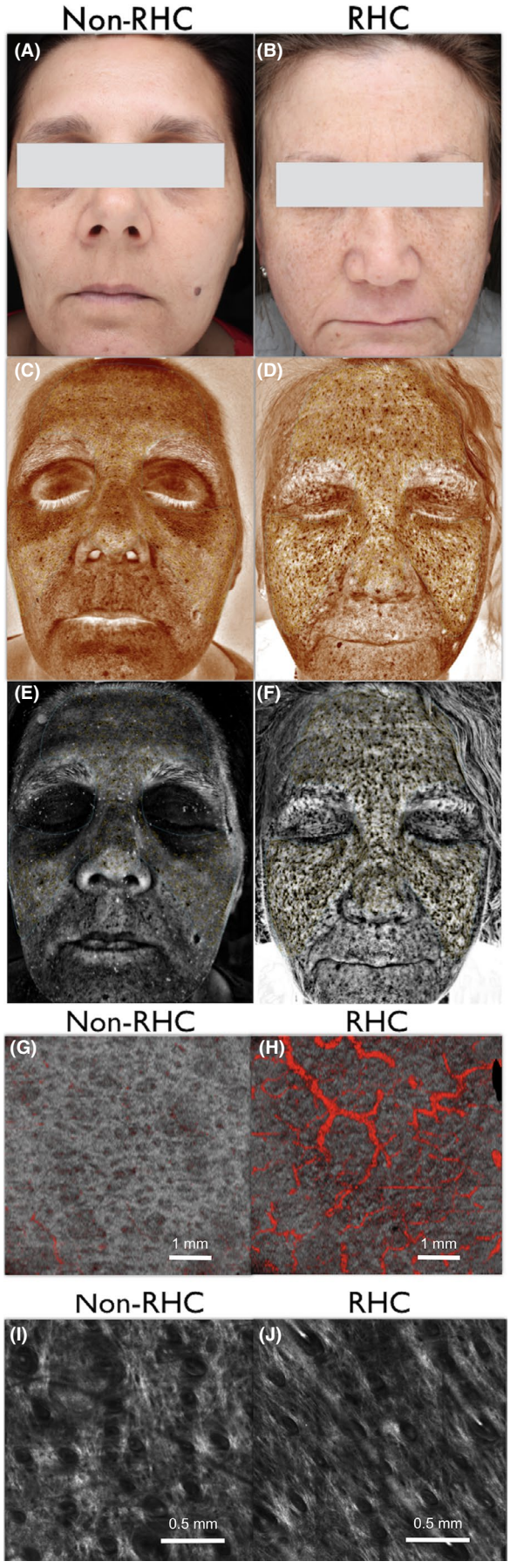

FIGURE 2 Clinical and non-invasive skin imaging features according to non-RHC vs RHC MC1R status. Pictures of 52-year-old women (A, B) clinical standard photography (C, D) UV light revealing the amount of UV spots (E, F) cross-polarized light revealing brown spots, in a MC1R non-RHC (wt) (A, C, E) and RHC carrier (R160W/V60L) (B, D, F). Dynamic optical coherence tomography (D-OCT) images (size 6 × 6 mm; scale bar 1 mm; skin depth 300 μm) showing different density of blood vessels in (G) a MC1R non-RHC and (H) an RHC carrier. Reflectance confocal microscopy images (scale bar 500 μm) showing (I) more than 50% of thin reticulated collagen in a MC1R non-RHC carrier and (J) more than 50% of coarse collagen in a MC1R RHC carrier

Therefore, a total of 97 women were included into the dataset for analysis. Subject mean age was 44.6 ± 6.7 (31-58).

Overall, seven non-synonymous MC1R gene polymorphisms were detected in 33 women (34%): all represented by amino-acid substitutions, with no deletions or insertions. A total of 32 subjects were heterozygous MC1R polymorphism carriers (wt/Polymorphism), therefore bearing one non-synonymous MC1R, while one patient carried two MC1R variants: R160W and V60L (red hair subject).

A total of 84 women were non-RHC carriers: 21 carriers of weak-r variants and 63 wt. The remaining 13 women were found to carry at least one RHC polymorphism. R151C was the most represented RHC variant, present in five women. (Supplementary Table S3).

3.2 | MC1R status and clinical/non-invasive imaging features

3.2.1 | Primary outcome

Participants' clinical features according to MC1R status were presented in Supplementary Table S4. RHC and non-RHC groups were not significantly different in terms of comorbidities (including sun exposure) and a significant association between MC1R status and hair colour was confirmed (P = .02). Automated digital facial photo features, according to the ABS scores, were presented in Supplementary Table S5. ABS scores were significantly elevated for UV and brown spots (P < .01) in the RHC group. Figure 1 (A-F) illustrated UV and brown spots of non-RHC and RHC participants with automated digital facial photographs.

At RCM analysis, morphological features according to non-RHC and RHC were similar. Although not significant, the presence of ≤50% of coarse collagen in RHC participants was over 90% compared to only 69% in non-RHC participants, Supplementary Table S6. Whilst no significant differences were found in dermal and epidermal features in OCT, D-OCT highlighted an increased vessel density (P = .038) and pixel density (P < .01) in RHC participants, Supplementary Table S7 and Figure 2 (G, H).

Significant and clinically relevant variables were included in a multivariate analysis, highlighting the correlation of UV spots (P = .012) and coarse collagen (P = .043) according to MC1R status (Figure 2 A-F,I,J, respectively). In detail, there was an 18 times increased risk

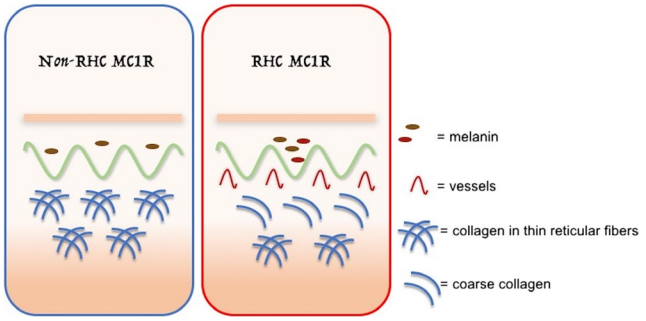

FIGURE 3 Model of photoageing according to non-RHC vs RHC MC1R status in our population. This model shows the presence of pigmented spots, increased vessel density and coarse collagen in RHC MC1R carriers, as compared to non-RHC

for >50% coarse collagen and increased presence of UV spots associated with RHC compared to non-RHC (Supplementary Table S8). Based on these results, a model of photo-exposed skin compartment characteristics, according to MC1R status, has been proposed (Figure 3).

3.2.2 | Secondary outcomes

No statistical differences were observed for the analysis in non-photo-exposed skin in RHC vs non-RHC, even for the variables identified as significantly different at primary analysis: the amount of coarse collagen (P = .761) and vessels (P = .254), data not shown. Further, no differences were found in the subgroup (weak-r vs wt vs RHC) analysis of morphological features (data not shown).

4 | DISCUSSION

Previously, the influence of MC1R polymorphisms on dermal morphological changes in photo-exposed skin was undefined.[13,27] The current study highlights that photo-exposed skin in RHC polymorphisms carriers, as compared to non-RHC, has more coarse "photoaged" collagen (evidenced in RCM) and more dense vascularity (evidenced in D-OCT).

Cutaneous morphological studies have mostly been applied to malignant lesions, in order to improve diagnostic accuracy and avoid unnecessary excisions.[28,29] However, skin changes in other cutaneous processes have been difficult to assess as biopsy, especially for the face, is often difficult to justify due to scarring risk. With the introduction of in vivo assessment tools, the deepest skin layer (dermis) can now be investigated without scarring also in healthy skin.

In the current study, a higher prevalence of coarse collagen in photo-exposed areas associated with RHC vs non-RHC subjects was underlined with RCM analysis. Coarse collagen, which has been previously associated with the skin photoageing process,[19] is visualized at RCM as coarse filamentous thick structures with a tendency to be packed. UV irradiation triggers the overproduction of matrix metalloproteinases, such as MMP1, and consequent collagen degradation. Interestingly, UV exposure is known to increase α-MSH release, and a role for α-MSH/MC1R as a regulator pathway of the MMP1 and collagen metabolism has been demonstrated,[30,31] thus supporting the potential influence of MC1R status on collagen morphology.

Univariate analysis of D-OCT assessments shows a significant association between vessel density and pixel density in photo-exposed areas for RHC carriers vs non-RHC. It has been proven that the αMSH/MC1R pathway inhibits angiogenesis through attenuation of the vascular endothelial growth factor (VEGF)/VEGF receptor 2 (R2) signalling pathway.[32] Therefore, RHC MC1R polymorphisms may lead to a dysregulation of the anti-angiogenic activity, thus contributing to an increase in vessel density and pixel density.

Clinical assessments in this study confirmed the well-known association of RHC MC1R variants with light hair and pigmented spots. Pigmented spots have been previously associated at semi-automated facial image analysis with multiple MC1R polymorphisms,[11] whilst only a non-significant trend has previously been reported for the association between a single MC1R polymorphism and clinical signs of skin photoageing.[13] However, there is a significant RHC allele heterozygote carrier effect on skin features, suggesting that variant alleles do not behave in a strictly recessive manner.[33] In the current study, the automated facial image analysis revealed a correlation with RHC and an increased prevalence of pigmented spots, with both UV light (UV spot) and cross-polarized light (brown spot). Bustamante et al[11] suggested that the increased levels of pro-oxidant pheomelanin associated with RHC may produce an increase in reactive oxygen species, leading to DNA damage. In addition, MC1R is now considered a master regulator, not only for processes involved in pigment production but also for pigment distribution throughout the skin, by means of downstream signalling.[34–36] Therefore, the authors suggest that the MC1R status may contribute to the heterogeneous melanin distribution and the appearance of pigmented spots.

Further, in the current study, the correlation between MC1R status, pigmented spots and coarse collagen seem independent of skin colour. This finding has also been reported for pigmented spots[14] and skin cancer risk,[37] therefore underlying the importance of pigmentary-independent functions of MC1R.[16] Accordingly, MC1R expression has been demonstrated in diverse cell types of both the epidermis, such as melanocytes and keratinocytes, and the dermis, such as fibroblasts and endothelium, but its function has been proven in melanocytes only.[35,38]

Melanocortin 1 receptor signalling in melanocytes has been proven to interact with many molecular pathways[35,39–41] and carriers of MC1R variants have been shown to have a de-regulated expression of a large number of genes involved in oxidative stress and DNA damage.[42] The non-invasive skin imaging in the current study suggests that MC1R status also influences dermal morphology and architectural features, such as vessel density, pixel density and the observation of coarse collagen, which may be explained by those interactions with other, as yet unstudied, pathways.

No significant differences were observed in our subgroup study (weak-r vs wt vs RHC). Some previous authors observed a minor signalling impairment associated with weak-r, or behaviour of weak-r similar to wt[43,44] leaving the precise role of the weak-r MC1R variants unknown.

However, Elfakir et al[13] previously reported the association of the weak-r V92M variant, and clinically assessed photoageing. Many differences between our study and the study of Elfakir et al[13] should be noted. Firstly, the Elfakir study included a much larger population and all subjects carrying weak-r V92M polymorphism, as a single polymorphism or in combination with other variants, were pooled together, without specifying the relative proportion of subjects carrying V92M/wt. In the current study, all patients carrying weak-r V92M polymorphism were V92M/wt only and this may explain our varying results. The included populations were also different in terms of age. Elkafir et al included an older population (range 44-70 vs 30-60 in the current study) with a clinical modality of evaluation only, compared to the addition of morphological assessment in the current study which may have identified early features of photo-exposed skin, not yet observable with clinical assessment only.

The main limitations of our study include the number of subjects enrolled and the representation of RHC compared to non-RHC carriers. However, this proportion is representative of the local population without any known dermatological disorders (especially skin cancer). Further, the population is relatively younger than other comparative studies.

In conclusion, this study confirms the influence previously observed of RHC MC1R polymorphisms on pigmented spots in healthy skin. Non-invasive in vivo skin imaging performed in this study reveals for the first time the role of RHC polymorphisms on morphological and architectural features of the dermis in healthy skin. Future studies should investigate the evolution of these observed morphological and architectural feature changes in the dermal layer in healthy skin and any potential association with skin cancer development.

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CONFLICT OF INTERESTS

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

GS, CS, GP, DCN, PF, MMa, FF, MMi have made substantial contributions to conception and design, or acquisition of data, or analysis and interpretation of data; and GS, CS, BDP, CJ, SK, GG have been involved in drafting the manuscript or revising it critically for important intellectual content. All authors gave final approval of the version to be published and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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