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Hassan Ait El Madani
Emmanuelle Tancrède-Bohin
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Hassan Ait El Madani,[a,b] Emmanuelle Tancrède-Bohin,[c] Armand Bensussan,[b] Anne Colonna,[a] Alain Dupuy,[d] Martine Bagot,[b] and Ana-Maria Pena[a]
[a]L'Oréal Research and Innovation, Aulnay sous Bois, France
[b]Université Paris 7, Denis Diderot, Centre de Recherche sur la Peau, INSERM UMR-S-976, Paris, France
[c]L'Oréal Research and Innovation, Centre de Recherche Bioclinique, Hôpital St. Louis, Paris, France
[d]Université Rennes 1, CHU de Rennes, Service de Dermatologie, Rennes, France

Abstract. Multiphoton microscopy has emerged in the past decade as a promising tool for noninvasive skin imaging. Our aim was to evaluate the potential of multiphoton microscopy to detect topical corticosteroids side effects within the epidermis and to provide new insights into their dynamics. Healthy volunteers were topically treated with clobetasol propionate on a small region of their forearms under overnight occlusion for three weeks. The treated region of each patient was investigated at D0, D7, D15, D22 (end of the treatment), and D60. Our study shows that multiphoton microscopy allows for the detection of corticoid-induced epidermis modifications: thinning of stratum corneum compactum and epidermis, decrease of keratinocytes size, and changes in their morphology from D7 to D22. We also show that multiphoton microscopy enables *in vivo* three-dimensional (3-D) quantitative assessment of melanin content. We observe that melanin density decreases during treatment and almost completely disappears at D22. Moreover, these alterations are reversible as they are no longer present at D60. Our study demonstrates that multiphoton microscopy is a convenient and powerful tool for noninvasive 3-D dynamical studies of skin integrity and pigmentation. [DOI: 10.1117/1.JBO.17.2.026009]

Keywords: three-dimensional imaging; second harmonic generation; two-photon excited fluorescence; *in vivo* multiphoton microscopy; corticosteroid; skin; melanin.

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1 Introduction

Skin atrophy is one of the main side effects of topical corticosteroid therapy, strongly modulated by factors such as potency of the corticosteroid, frequency of application, duration of therapy, and occlusion. The mechanisms underlying corticosteroid-induced skin atrophy, as well as the detailed changes appearing in the skin following this type of anti-inflammatory local treatment, have been amply described.[1,2] Topical corticosteroids have profound effects on both the epidermis and dermis, resulting in the appearance of prematurely aged skin. They decrease the size and the proliferation ratio of keratinocytes, which results in epidermis thinning, and they induce dermis atrophy as a consequence of reduced synthesis and induced degradation of extracellular matrix components. Epidermal effects also include melanocyte inhibition, a vitiligo-like condition more likely to occur with corticosteroids under occlusion or with intracutaneous corticosteroid injection[3] and also in black populations in which topical corticosteroids are used as bleaching products.[4]

The *in vivo* characterization of corticosteroid-induced skin modifications at both cellular and extracellular levels and the dynamical study of these effects (e.g., their appearance and disappearance) require the use of 3-D noninvasive imaging methods. Optical coherence tomography[5] and confocal microscopy[6] can reveal, for example, epidermis thinning or the reduced size of the keratinocytes. However, since they lack specificity, they cannot provide access to other modifications induced in the cells or the elastic and collagen fibers networks. In that context, multimodal multiphoton microscopy seems to be an appropriate tool as it allows simultaneous visualization of different skin components with sub-μm resolution.

Multiphoton microscopy advantageously provides complementary modalities, such as two-photon excited fluorescence (2PEF) and second harmonic generation (SHG). In combination with endogenous sources of contrast, these modalities allow noninvasive imaging of tissues under natural physiological conditions, which is particularly important in the field of cosmetic and pharmaceutical research as it enables dynamical studies in the same tissue areas.

In the skin, 2PEF signals are emitted by endogenous chromophores such as NAD(P)H, flavins, keratin, melanin, or elastin whereas SHG signals, obtained from dense, noncentrosymmetrical, and ordered macromolecular structures, reveal the fibrillar collagen.[7–11] These signals can be simultaneously excited by the same infrared femtosecond (fs) laser source and spectrally separated as the fluorescence signals are red-shifted compared to the SHG signals that are emitted at exactly half the excitation wavelength.

In vivo multiphoton microscopy has already been applied to the study of skin aging,[12–14] dermatological disorders and melanoma,[15,16] as well as in cosmetic and pharmaceutical

Address all correspondence to: Ana-Maria Pena, L'Oréal Research and Innovation, Aulnay sous Bois, France. Tel: +33 1 48 68 98 27; E-mail: ampena@rd.loreal.com

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research.[17] In this paper, we evaluate the potential of multiphoton microscopy to evidence topical corticosteroids side effects within the epidermis and to provide new insights into their dynamics.

2 Materials and Methods

2.1 *Patients and Treatment*

The study involved four healthy Caucasian volunteers (two women and two men, age range 23 to 43) who had not used topical or systemic corticosteroids for at least two months before the study. Before enrolment, each volunteer received information about the investigation procedure with the DermaInspect® medical device, was fully informed about the nature of the test product, and signed an informed consent. The experimental protocol was approved by the Saint Louis Hospital ethics committee, and we followed the Declaration of Helsinki protocols.

Clobetasol propionate (0.05% cream, Dermoval®, GlaxoSmithKline, Marly-le-Roi, France) was applied under overnight occlusion for three weeks on the dorsal side of the forearm. The application zone was limited to an area of approximately 0.8 cm^2, and Tegaderm sheets (3 M Health Care, St. Paul, Minnesota) were used for the occlusion. The treated regions were identified by tracing natural anatomic marks, such as skin folds, beauty spots, and neavus on transparent plastic sheets (Monaderm, Monaco), which allowed us to investigate the same region of the skin at D0 (before the treatment), D7, D15, D22 (end of the treatment), and D60 (38 days after the end of the treatment).

2.2 *Multiphoton Imaging*

Multiphoton imaging was performed with DermaInspect® (JenLab GmbH, Jena, Germany), a CE-marked medical system integrating two time-correlated single photon-counting detectors for fluorescence lifetime imaging (FLIM) (SPC-830, Becker & Hickl, Berlin, Germany).[14,15] SHG and 2PEF were simultaneously excited by a femtosecond titanium-sapphire laser adjusted to 760 nm (MaiTai Spectra-Physics, Mountain View, California).

The treated regions were identified using marked transparent plastic sheets, and the human skin was imaged by use of a 40×, 1.3-NA oil immersion objective lens (Zeiss, Jena, Germany). A cover glass was placed at the surface of the skin, and a small drop of water was used for the optical contact between the skin and the cover glass. The excitation power was exponentially increased from 12 mW at the surface of the skin up to 47 mW at a depth of 75 μm and kept constant for imaging depths greater than 75 μm. 2PEF and SHG signals were dispatched to two time-correlated single photon-counting detectors using a dichroic mirror and appropriate spectral filters.

For each volunteer, we recorded two 2PEF/SHG z-stacks of FLIM data (see Fig. 1). Each $130 \times 130 \times 162$ μm^3 z-stack was acquired with 2.346 μm z-step and 0.255 μm pixel size, and for each pixel the multiphoton signals were integrated using only four temporal channels with an integration time of 2 ns/channel. The total acquisition time was 7.4 s per image (511×511 pixel) and 9.4 min /z-stack.

The two z-stacks of images were acquired within a small region of the skin (less than 1 mm distance between the z-stacks) situated in the central part of the 0.8 cm^2 area, identified using the marked transparent plastic sheets. We did not exactly follow the same region of $130 \times 130 \times 162$ μm^3 over time, but we approximately investigated the same central part of the 0.8 cm^2 treated region.

2.3 *Image Processing*

The 2PEF/SHG images of a z-stack of FLIM data are individually saved in an sdt file format. In order to have fast access to the data, the sdt image files were opened under ImageJ (Rasband, National Institute of Health) using an appropriate plugin (LOCI) and further processed using a macro developed with ImageJ. This macro converts the sdt image files to a more accessible file format—bmp, for example—that provides direct access to the photon numbers per pixels and allows for a rapid quantitative analysis of the whole stack of images. Two-dimensional (2-D) images were combined using ImageJ, and 3-D reconstructions were performed with Imaris (Bitplane, Zurich, Switzerland).

2.4 *Analysis of Epidermis Morphology and Qualitative Evaluation of Melanin Density*

2PEF/SHG images were combined using ImageJ to obtain montages of multimodal z-stacks and to qualitatively and quantitatively analyze the different constituents of the epidermis (see Fig. 1). We estimated the thickness of stratum corneum compactum by the number of images in which at least 50% of the image pixels are occupied by this layer. The thickness of the epidermis was estimated by the number of images between the skin surface and the top of the dermal papillae. In Fig. 1 the stratum corneum and the compactum layer correspond to images 6 to 14 and 9 to 14, respectively.

In stratum granulosum, we counted the keratinocytes in the region of interest defined by the first three images below the compactum layer, which allows for an estimation of the keratinocytes size, as there is an inverse correlation between their number and their sizes.

We also characterized the global aspect and the morphology of the keratinocytes in stratum granulosum and the melanin density. The global aspect and the morphology of the cells were qualified as normal before the corticosteroid therapy and as modified if any modifications were visible during or following the corticosteroid therapy. A qualitative evaluation of melanin density was performed using a visual assessment of the extent of white pixels that correspond to melanin in the basal layers of the epidermis. We arbitrarily defined three score levels: (0) for no or very low melanin density, (+) for moderate melanin density, and (++) for high melanin density (see Fig. 2).

2.5 *Quantitative Analysis of Melanin Density in the Basal Layers of the Epidermis*

2PEF images were processed to assess the melanin density in the basal layers of the epidermis using a macro developed under ImageJ. For each z-stack, 3-D regions of interest (ROI) of $130 \times 130 \times 21$ μm^3 were defined inside the epidermis and just above the epidermal–dermal junction (in Fig. 1 the 3-D ROI corresponds to images 18 to 27). These ROIs were further processed to obtain the volume of the pixels occupied by the melanin 2PEF signal. For that purpose, we used a similar procedure to the ones developed for fibrosis scoring and for the assessment of 3-D

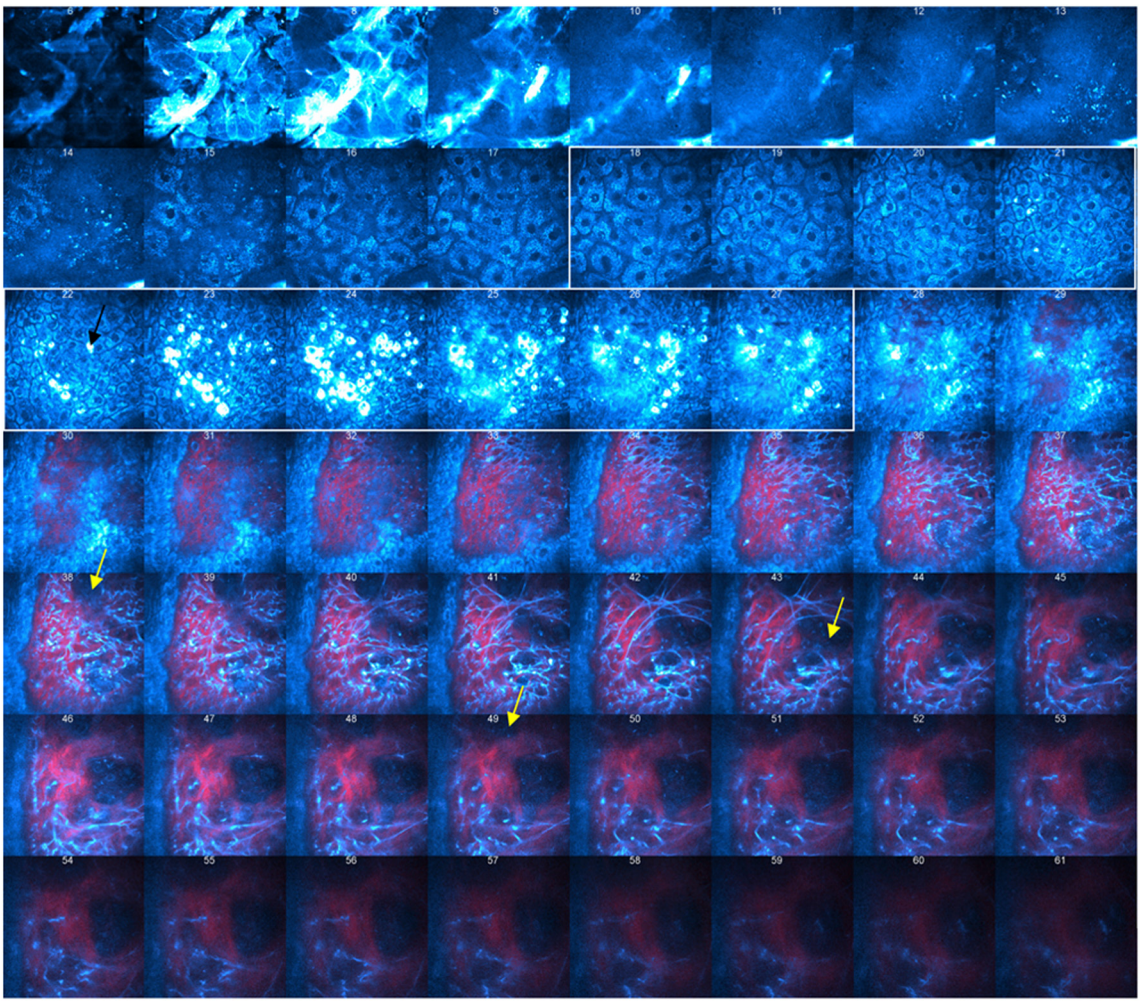

Fig. 1 Montage of a *z*-substack of combined 2PEF/SHG *in vivo* images of normal human skin.2PEF signal (cyan hot color) reveals the endogenous fluorophores distribution inside the epidermis and dermis (elastic fibers, fibroblasts) whereas the SHG signal (red color) reveals the collagen fibers distribution in the dermis. This figure shows images 6 to 61 extracted from a stack of 70 images; the image number is indicated on the top of each image. Images 6 to 9 correspond to stratum corneum disjunctum, and images 9 to 14 to stratum corneum compactum (the numbers are indicated on top of each image). The keratinocytes in the stratum granulosum appear in image 14, and melanin distribution in the basal layers of the epidermis can be seen in images 22 to 32. The black arrow in image 22 indicates a melanized keratinocyte with high melanin 2PEF intensity signal highlighted in white. Image 28 was acquired at the epidermis–dermis junction level as indicated by the onset of elastic and collagen fibers of the dermis. A blood capillary can be followed from images 35 to 61 (see yellow arrows). A typical 3-D region of interest (ROI, $130 \times 130 \times 21$ μm^3) used for the quantification of melanin volume density in the basal layers of the epidermis is depicted as a white rectangle. Acquisition time: 7.4 s/image of 511×511 pixel and 8.5 min /stack of 130×130 $\mu m^2 \times 70$ images acquired every 2.346 μm.

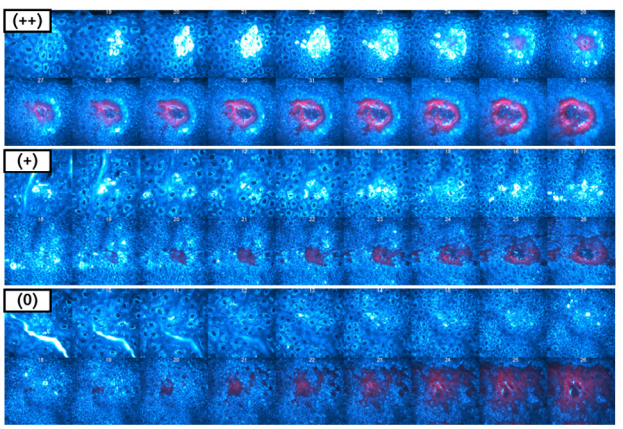

Fig. 2 Qualitative scoring of melanin density in the basal layers of the epidermis. We arbitrarily defined three score levels: (0) corresponds to no or very low melanin density images, (+) to moderate, and (++) to high melanin density images. The same imaging conditions applied as in Fig. 1.

collagen matrix remodeling.[18–20] We performed a Gaussian blur with 1 pixel sigma radius and applied a threshold equal to the maximum 2PEF signal intensity of nonmelanized keratinocytes to obtain a mask corresponding to melanin distribution [see Fig. 3(d)]. In order to choose the threshold value, we performed histograms of our images and found that in our experimental conditions the maximum 2PEF signal intensity in nonmelanized keratinocytes corresponds to 40 photons whereas in melanized keratinocytes it reaches 120 photons. The threshold value was fixed to 45 photons. We then expressed the melanin density as the ratio of the number of voxels occupied by melanin and the total number of voxels in the 3-D ROI.

2.6 *Statistical Analysis*

We performed a descriptive statistic followed by a normality test and a two-sample *t*-test. The analyses were performed using OriginPro software (OriginLab Corporation, Northampton,

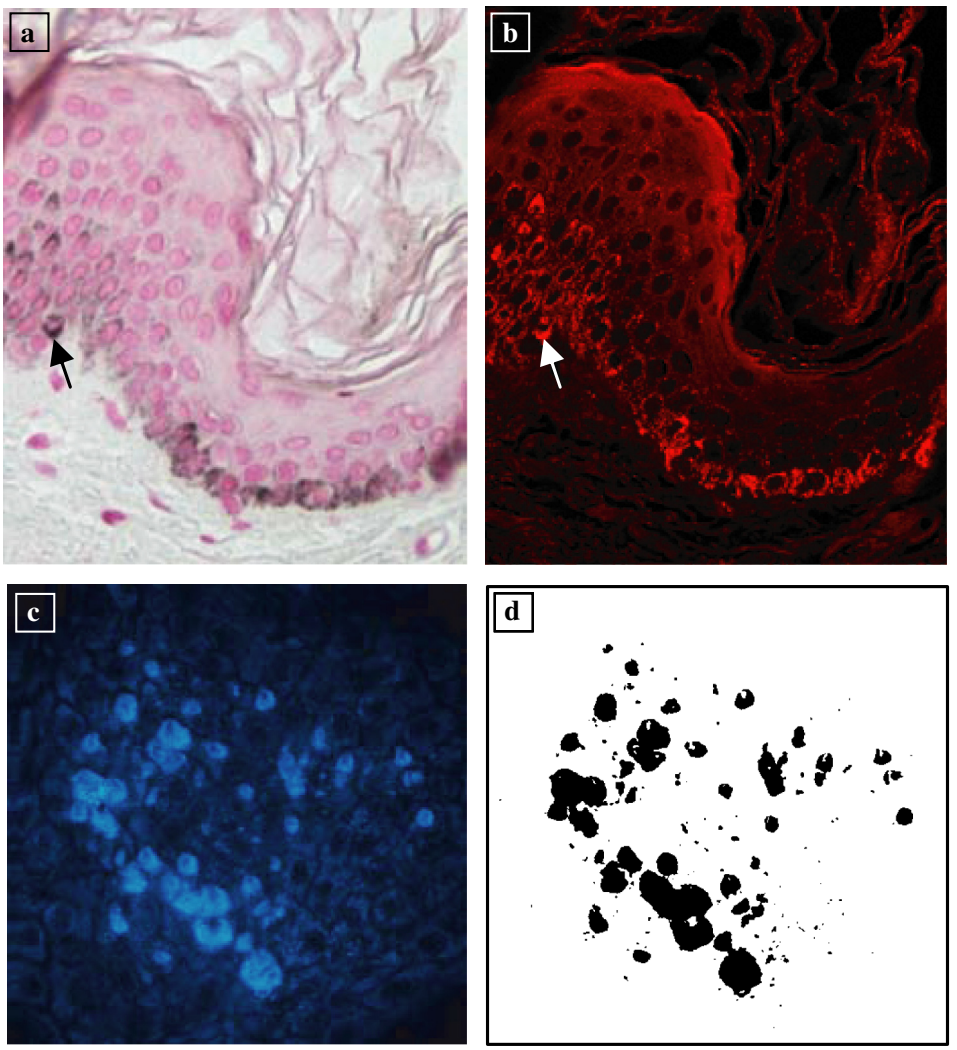

Fig. 3 Assignment of highly intense 2PEF endogenous signals in the living epidermis to melanin and image processing for quantitative analysis of melanin density. (a) Transmitted light image and (b) corresponding 2PEF image of a fixed histological section of normal human skin stained with Fontana-Masson, showing the epidermis and the upper part of the dermis (image size is 210 μm). In the living epidermis the melanin regions colored in black in the conventional histological image (black arrow) clearly colocalize with the highly intense 2PEF signal in the multiphoton image (white arrow). The biopsy was taken from a 25-year-old female volunteer included in another clinical study. (c) *In vivo* 2PEF raw image with melanized keratinocytes in the basal layers of the epidermis; (d) 2PEF mask obtained after application of a threshold. The volume of the pixels occupied by the melanin 2PEF signal was obtained from the 2PEF mask and is expressed as the ratio of the number of voxels occupied by melanin and the total number of voxels in the 3-D ROI.

Massachusetts). The quantitative parameters extracted from the multiphoton images are expressed as mean ±95% confidence intervals of the mean.

3 Results

We performed a kinetic study on four healthy Caucasian volunteers topically treated with clobetasol propionate under overnight occlusion for three weeks in a limited area of the dorsal side of the forearm. The treated region was investigated at D0 (before the treatment), D7 and D15 (during the treatment), D22 (end of the treatment), and D60 (approximately six weeks after the end of the treatment). At each time point, two z-stacks of combined 2PEF/SHG images were acquired parallel to the surface of the skin upon 760 nm excitation. The images were qualitatively and quantitatively analyzed.

3.1 *Normal Human Skin*

Typical *in vivo* multiphoton images of normal human skin are presented in Fig. 1. The images acquired from the surface of the skin up to a depth of 10 μm reveal the organization of the stratum corneum disjunctum. This superficial layer contains polygonal-shaped keratinized cells, the corneocytes, which create a high-intensity fluorescence signal that mainly arises from keratin.[11] The next 10 μm below the stratum corneum disjuctum show a decreased intensity signal and no distinguishable cell structure. This region corresponds to the stratum corneum compactum (see Fig. 1).

About 20 μm below the surface of the skin, the transition to the stratum granulosum is detected by the occurrence of living cells that present large homogenous fluorescent cytoplasms and nonfluorescent membranes and nuclei. Deeper inside, the keratinocytes of the spinous layer show decreasing cell diameter

and a decreasing ratio between the cytoplasmic and nuclear volumes. In this layer the cytoplasm fluorescence is mainly due to mitochondrial NAD(P)H.[9]

At depths between 50 and 80 μm, basal keratinocytes appear as small and polygonal cells. Some of them contain melanin, which is found in different concentrations within the cells (see Fig. 1). Under fs excitation, the highly concentrated melanin regions create a high-intensity fluorescence signal stronger than the one created by the other cellular fluorophores.[10,12,15,21–23]

The epidermis–dermis junction is revealed in our images by the appearance of the dermal papillae. These structures, as well as the deeper parts of the dermis, are mainly characterized by a 2PEF signal created by the elastic fibers and an SHG signal created by the collagen fibers. Blood capillaries and blood cells are often seen in the papillary dermis (see yellow arrows in Fig. 1).

3.2 *Skin Modifications Following Topical Corticosteroids Occlusive Treatment*

Several modifications were evidenced by *in vivo* multiphoton microscopy in the treated areas.

We regrouped the results into:

1. Qualitative observations of known corticosteroid-induced skin modifications that confirm the capability of *in vivo* multiphoton microscopy to evidence morphological features similar to the ones observed with histology or other imaging techniques.
2. New qualitative observations related to the metabolic state of the cells and to the extent of melanin in the living epidermis that are possible to evidence thanks to the specificity of multiphoton signals.
3. New quantitative observations of melanin volume density in the living epidermis.

Qualitative observations of known corticosteroid-induced skin modifications: The stratum corneum and, more precisely, the compactum layer showed a decreased thickness for all the volunteers from D7 until the end of the treatment. Most of the time, very strong modifications could be observed, as evidenced in Fig. 4(b), in which the compactum layer seems to have disappeared completely.

A decrease in the global epidermis thickness could also be observed during the treatment at D15 [see Fig. 5(b)]. The thickness of the epidermis, measured from the skin surface to the top of the dermal papillae, varied from 48 ± 4 μm at D0 to 46 ± 6 μm at D7, 37 ± 2 μm at D15, 55 ± 10 μm at D22, and 56 ± 6 μm at D60. To check whether the decrease in the epidermis thickness observed at D15 is significant, we performed a descriptive statistic followed by a normality test and a two-sample t-test. This statistical analysis confirmed that there is a significant difference between D15 and D0, D7, D22, or D60 ($p \leq 0.008$). Moreover, we observed a significant increase in the epidermis thickness at D60 as compared to D0 or D7 ($p \leq 0.023$). This increase, appearing at 38 days after the end of the treatment, could be due to an increase in the proliferation rate of the epidermal cells.

The keratinocytes in the granulosum layer were also dramatically modified from D7 until D22 for all the volunteers. The number of the granular keratinocytes in the chosen region of interest varied from 20 ± 2 at D0, 38 ± 7 at D7, 37 ± 3 at D15, 35 ± 3 at D22, and 22 ± 3 at D60, indicating a decreased cell size during the treatment [see Fig. 5(a)]. This modification is significantly different as compared to D0 and D60 ($p < 0.002$).

New qualitative observations: the keratinocytes in the granulosum layer were also characterized by irregular shape and an inhomogeneity in the fluorescence signal intensity of their cytoplasm at D22. Highly intense 2PEF signals were observed close to the nucleus. These signals were also irregularly distributed all over the cytoplasm [see Fig. 4(b)] whereas in normal skin [see Fig. 4(a)] the intensity of the cytoplasm fluorescence was rather homogenous. The increase of the 2PEF signal intensity at D22 indicates an increased concentration of the intracellular fluorophores. Furthermore, as the intracellular fluorescence is dominated by mitochondrial NAD(P)H emission upon excitation at 760 nm,[24] the increased 2PEF signal around nuclei is probably due to increased NAD(P)H concentration and thus reflects a change of the metabolic state of the cells.

Epidermal modifications also include whitening. In order to characterize this effect, we first performed a qualitative analysis of the melanin content based on a visual examination of multiphoton images (see Fig. 2). Our results show that the high-intensity fluorescence signals observed in normal skin that

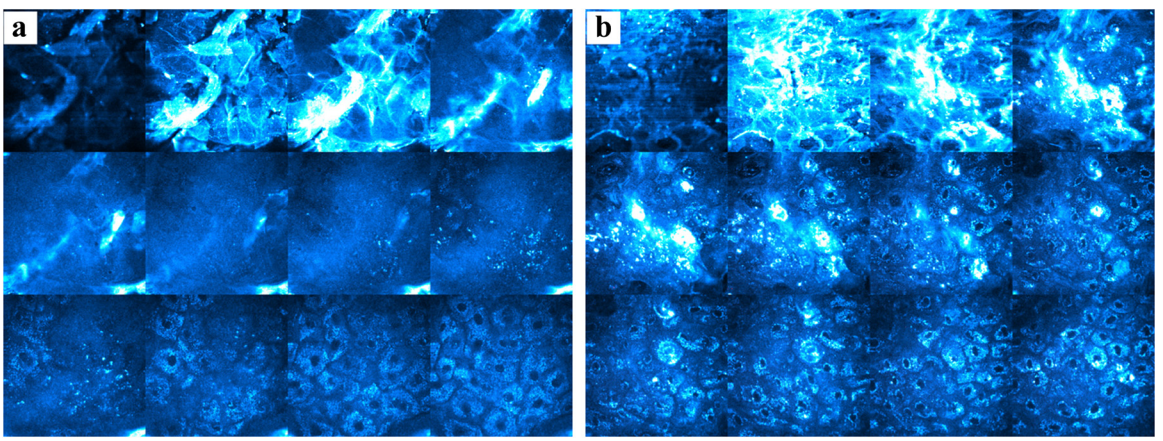

Fig. 4 Multiphoton images of (a) normal human skin and (b) skin after three weeks of topical overnight occlusive treatment with clobetasol propionate in the treated regions, of the stratum corneum compactum seem to have completely disappeared, and the morphology of the cells in the granulosum layer has changed. Substack of 130×130 $\mu m^2 \times 12$ images acquired every 2.346 μm; the same imaging conditions applied as in Fig. 1.

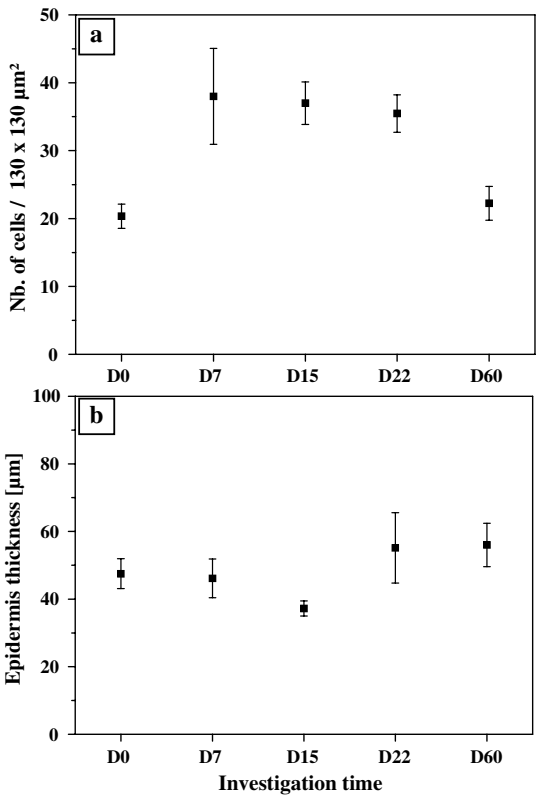

Fig. 5 Impact of clobetasol propionate treatment on the size of granular keratinocytes and epidermis thickness. (a) The number of granular keratinocytes in the chosen region of interest was dramatically modified from D7 until D22 for all the volunteers. The results show an increased cell number/image, indicating a decreased cell size during the treatment. (b) The thickness of the epidermis measured from the skin surface to the top of the dermal papillae shows a significant decrease at D15 (p-value < 0.008). Error bars correspond to 95% confidence intervals of the mean.

correspond to melanin almost completely disappeared after three weeks of corticosteroids treatment, which induced a bleaching of the treated region that was visually observed at D22 (see the multiphoton images at D22 in Fig. 6).

New quantitative observations: in the living epidermis the highly concentrated melanin regions create a high-intensity fluorescence signal stronger than the one created by the other cellular fluorophores.[10,12,15,21,23] We verified this point in a previous unpublished clinical study using conventional histological techniques, as displayed in Fig. 3. We imaged a fixed histological section of normal human skin stained with Fontana-Masson, using transmitted-light microscopy [see Fig. 3(a)] and 2PEF microscopy [see Fig. 3(b)]. Both images reveal the same structures, namely the melanin regions colored in black in the conventional histological image (black arrow) clearly colocalize with the highly intense 2PEF signal in the multiphoton image (white arrow). It validates the attribution of the highly intense 2PEF signal in the living epidermis to melanin. Based on this criterion, a more precise evaluation of the melanin changes upon corticosteroids treatment can be assessed. For that, we developed a melanin quantification procedure. We selected 3-D regions of interest (ROI) inside the basal layers of the epidermis (see Fig. 1), extracted melanin masks after image processing (see Fig. 3), then calculated a melanin volume density as the ratio of the number of voxels occupied by melanin to the total number of voxels in the 3-D ROI (see Fig. 6).

The 2PEF images and the 3-D reconstructions in Fig. 6 show an example of time-dependent evolution of melanin distribution with corticosteroids treatment for one volunteer. The melanin volume density decreased at D22 as shown in the 3-D reconstruction in Fig. 6. This result was also observed for all the volunteers. The graph in Fig. 6 shows a decrease in the mean melanin volume density after three weeks of topical occlusive corticosteroid treatment. We confirmed this result by statistical analysis of the data ($p \leq 0.03$ between D22 and D0, D7, D15, or D60).

Two months after the end of the treatment, stratum compactum and epidermis thickness, cell morphology, and pigmentation had all almost reached their baseline values (see the results obtained at D60 in Figs. 5 and 6).

4 Discussion

Skin atrophy is a well-known side effect of topical corticosteroids, and possible factors in its pathogenesis have been reviewed.[25] Both the epidermis and the dermis are affected by topical applications of corticosteroids, and several noninvasive methods, such as high-frequency ultrasound, optical coherence tomography, and confocal microscopy, are available for assessing the degree of skin atrophy *in vivo*. Nonetheless, except for the confocal microscopy that was used in one study,[6] these methods provide limited information as to the changes occurring within the epidermis.

Even if the outer layer of the skin contributes quite little to the total skin thickness, it is affected earlier than the dermis because of the high proliferative rate of the keratinocytes compared to the weak production rate of collagen and other extracellular matrix components affected by a changed m-RNA synthesis.[26] Changes in the epidermal structure may thus allow an early detection of corticosteroids side effects and yield insight into the nature of the atrophic effects in general.

Our *in vivo* multiphoton microscopy study was focused on the dynamical assessment of the corticosteroids effects appearing within the epidermis. We demonstrated the capability of this imaging technique to offer qualitative information of known corticosteroid-induced epidermis modifications and new qualitative and quantitative data that can only be provided *in vivo* with multiphoton microscopy.

We first confirmed *in vivo* the well-documented epidermal corticosteroids effects evidenced by the use of invasive techniques,[27] namely thinning of the stratum corneum compactum and epidermis with decreased keratinocytes size. The main advantage of multiphoton microscopy over histology or other invasive techniques that need biopsies to be taken is its noninvasiveness, which allows one to continuously monitor and evaluate the reversibility of the corticosteroids effects within the skin and more generally to study the dynamic effects of any other active compound that can modulate the skin components.

Additionally, multiphoton microscopy allowed new insights into the granular keratinocytes modifications upon corticosteroids therapy, showing an increased peri-nuclear fluorescence and a heterogenic intensity distribution of this signal within the cytoplasm. These type of modifications, probably due to increased NAD(P)H concentration, could reflect a change of the metabolic state of the cells, as has been shown in actinic keratosis.[28]

Moreover, we demonstrated the capability of this technique to provide new and unique insights into *in vivo* quantitative assessment of melanin content in the basal layers of the epidermis.

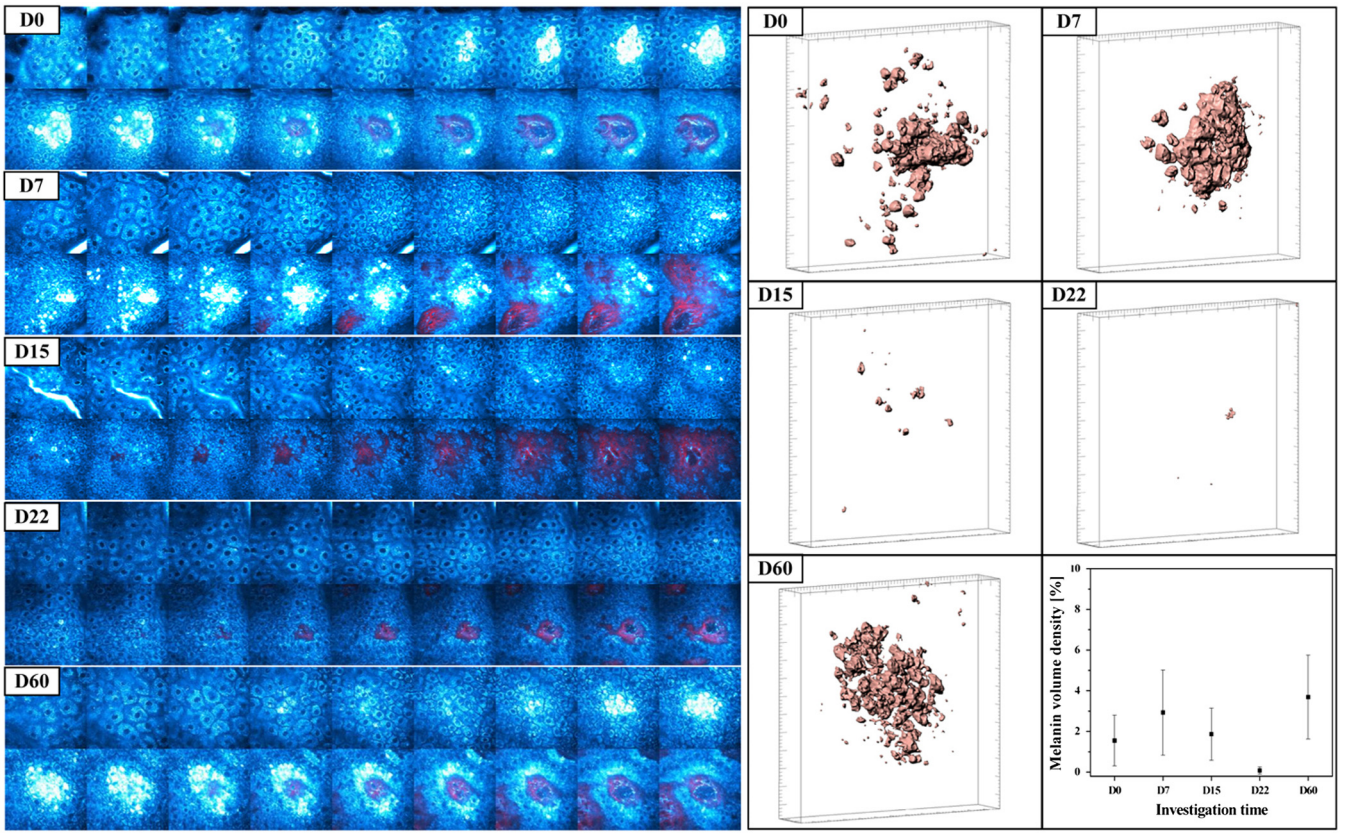

Fig. 6 Example of time-dependent evolution of melanin density with clobetasol propionate treatment in the basal layers of the epidermis for one volunteer. (left) 2PEF images used for qualitative scoring of melanin density; (right) 3-D melanin distribution before (D0), during (D7, D15, D22), and after (D60) clobetasol propionate treatment; (bottom right) mean values of melanin volume density calculated in 3-D ROIs. All the volunteers show a significant decrease in the melanin volume density at D22. Error bars correspond to 95% confidence intervals of the mean. The same imaging conditions applied as in Fig. 1.

The melanin quantification procedure implemented here allowed us to detect the decrease in the mean melanin volume density after three weeks of topical occlusive corticosteroid treatment, which was accompanied by a visual bleaching of the treated region observed at D22. Skin bleaching with cutaneous topical corticosteroids is a widespread practice in black populations and is associated with numerous cutaneous complications, sometimes leading to adrenal insufficiency.[4,29] In this case, as in other pigmentation diseases, multiphoton microscopy is actually the unique method allowing *in vivo* 3-D quantitative and dynamical assessment of melanin content.

These results demonstrate the advantage of *in vivo* multiphoton microscopy over other noninvasive imaging methods, such as OCT or confocal microscopy, that lack specificity, as their contrast mechanism relies on spatial variations of refractive indices. Stratum corneum compactum and intracellular granular keratinocytes modifications cannot be studied with these imaging methods. Melanin visualization in confocal microscopy is based on its high reflectivity. However, melanin 3-D quantification from confocal images (which has not been performed to our knowledge) is not trivial since the cellular membranes also exhibit a reflection signal with intensity similar to the one created by melanin. Moreover, multiphoton microscopy offers additional modes of contrast, as discussed in the introduction section, and provides 3-D images with better resolution than *in vivo* confocal images acquired with commercial systems such as VivaScope® (Mavig, Munich, Germany): 400 nm experimental lateral resolution instead of 1.25 μm and 2 μm axial resolution instead of 5 μm. One disadvantage of multiphoton microscopy over confocal microscopy is the field of view, which is indeed quite small. However, a large area scanning (tiles of images) is only compatible with 2-D imaging of living samples like humans or 3-D imaging of fixed samples. As 3-D image acquisition with DermaInspect® takes about 9 min, a large area scanning (tile of 4×4 z-stacks of images) will require 36 min, and the effects of volunteer movements will degrade the quality of the images. Moreover, most of the *in vivo* studies performed with the DermaInspect® system deal with the same problem. The authors of these studies have been able, however, to demonstrate that such a reduced image field is enough to evidence modifications appearing during skin aging, etc.

In our study, clobetasol propionate was topically applied under overnight occlusion for three weeks. Occlusion for this period of time with a very potent corticosteroid is a severe type of exposure that does not correspond to normal use. However, in combination with multiphoton microscopy, this type of occlusive local treatment could provide a model for the assessment of the atrophogenic potential of corticosteroids, thereby allowing their *in vivo* evaluation at the same time.

Multiphoton microscopy should also be powerful enough to monitor the evolving pattern of atrophogenicity even after open classic applications of corticosteroids, given its ability to assess the elastic and collagen fibers network changes during skin ageing.[13] Since only the papillary dermis can be imaged with multiphoton microscopy, corticosteroid-induced dermis thinning cannot be measured by this imaging technique. Several

studies performed either on histological sections of human skin[30] or directly *in vivo*[12–14] found that the SAAID (second harmonic generation-to-autofluorescence aging index of dermis) could be used to quantify skin aging. This parameter could be applied to the evaluation of topical corticosteroids effects within the dermis, but such measurement has only been performed in 2-D at a fixed imaging depth below the epidermal dermal junction. As corticosteroids modify the thickness of the epidermis, it will be challenging to estimate the SAAID in 3-D in the whole imaged dermis. For that, one could perform an epidermis dermis segmentation to extract the dermis and further process the images in order to estimate other parameters related, for example, to the shape of the epidermal dermal junction that could be modified during the treatment. Such an approach, which is beyond the aim of this paper, would moreover enable new insights into the 3-D organization and quantity changes of the collagen and elastic fibers network within the papillary dermis. For this purpose, it is necessary to implement specific 3-D image analysis tools, as well as long term clinical study models, because of the relatively later onset of the atrophy in the dermis compared to the epidermis.

5 Conclusion

In summary, we evaluated the potential of multiphoton microscopy to evidence topical corticosteroids side effects within the epidermis and to provide new insights into their dynamics.

Despite the small number of volunteers in this pilot study, our results confirm that multiphoton microscopy allows early detection of epidermis modifications following corticosteroid therapy. In order to asses the changes appearing within the dermis, we are now performing a dynamical study with a larger number of young and old healthy volunteers and working on the development of specific 3-D image processing tools. The ability of multiphoton microscopy to continuously monitor and evaluate the reversibility of the above-described corticosteroid-induced epidermis modifications demonstrates that this type of imaging technique is a convenient and powerful tool for noninvasive *in vivo* dynamical studies of skin integrity and pigmentation. The new 3-D melanin quantification procedure could easily be applied to the evaluation of active compounds that can modulate the melanin content on either human skin or engineered skin.

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