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Article type : Original Article

TITLE:

The evolution of healthy skin to acne lesions: a longitudinal, in vivo evaluation with reflectance confocal microscopy and optical coherence tomography.

RUNNING HEAD:

The evolution of healthy skin to acne lesions

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This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/jdv.15641

Conflict of interest

The Authors have no conflict of interest to declare.

Funding sources

None

Abstract

Background: Comedogenesis is defined as the process of the development of a new comedo, which is of great importance for the understanding of acne.

Objective: To evaluate the formation and evolution of acne lesions from clinically unaffected skin of patients with mild-moderate acne to characterize the morphological changes and natural resolution by means of in vivo reflectance confocal microscopy (RCM) and dynamic otpical coherence tomography (D-OCT).

Methods: ten patients with mild-moderate acne, not assuming any topical or systemic therapy, comprised between 12 and 30 years of age, were recruited. A target area of 4x4mm of the face, without acne lesions at baseline, was selected. A set of standardized clinical pictures, RCM and D-OCT images were acquired weekly for six weeks and evaluated.

Results: Seventy full sets of clinical, RCM and D-OCT images were analyzed. The appearance of acne lesion is preceded by an increase of large bright follicles in the area corresponding to infundibular keratinization, followed by increment of inflammation parameter, such as increased of small bright cells upon RCM and vascular network upon D-OCT, which return to normal after the resolution of acute inflammation.

Conclusion: Acne skin dynamics is complex and seems characterized by the early increase in the number of dysmorphic pilosebaceous units and the hyperkeratinization of the acroinfundibulum of the pilosebaceous duct prior to the occurrence of inflammatory events

around the follicle. The processes of hyperkeratinisation and inflammatory phenomena may generate a pathologic vicious cycle, which characterizes acne through progressive worsening and a self-sustainment mechanism.

KEYWORDS:

Acne, Reflectance confocal microscopy, Optical coherence tomography, Vascularization, Therapy, Comedogenesis, Microcomedo

Introduction

Current theories on the pathogenesis of acne mainly focus on its multifactorial nature, which involves several processes: hormonal and sebum anomalies, skin bacterial activity, gut microbiome alterations, skin immunology and keratinocytes proliferation.[1–5]

Comedogenesis is defined as the process of the development of a new comedo. The understanding of this process and of the pathogenesis of the disease is of great importance for the follow-up of patients with acne.[6–8]

Several pathogenetic process have been demonstrated to play a central role in the development of new acne lesions: inflammatory molecules released into the skin[8,9]; alteration of the keratinisation process leading to comedones[10]; altered hormonal mechanisms leading to altered sebum production[3]; bacterial colonisation of the hair follicle[5]; and gut[11] or skin microbiome alterations[4]. Infundibular hyperkeratinisation have been demonstrated to cause formation of keratin plugs, which are the starting events of comedo formation.[7,10,12] They close the infundibular opening, lead to sebum accumulation in the pilsebaceous duct and to sebaceous gland alterations. Comedones are characterized by hyperproliferating ductal keratinocytes, which were demonstrated through Ki-67, keratins 6 and 16 and 3H-thymidine

labelling studies.[10,12] At present, the exact sequence of events leading to acne and how they and other factors interact remains unclear.

Altered pilosebaceous units, which can not be identified through clinical examination alone, have been described as the earliest pathologic substrate of acne.[1,10] Although histopathology is considered the optimal method to study the morphological changes associated with acne, it is not feasible for an in vivo follow-up of the same lesion during time. Recently, Reflectance Confocal Microscopy (RCM) and Dynamic Optical Confocal Microscopy (D-OCT) have been applied to the non-invasive exploration of acne at a microscopic resolution.[13–16] Previous studies demonstrated that, compared to healthy individuals, the pilosebaceous units of the clinically unaffected skin of acne patients showed larger infundibular diameter, with bright border and amorphous material inside, whilst the pilosebaceous units of healthy patients showed well-defined, roundish/elliptic infundibula, that did not contain amorphous or bright material.[13,17] Furthermore, D-OCT imaging enables to identify the alterations of the microcirculation providing information on the dermal blood flow, which is of great value in the characterization of the inflammatory process and in the determination of the functional and metabolic features of skin diseases.[14] The importance of structural and microvascular changes was demonstrated by previous studies that have shown how local changes in the peripheral blood vessels at the dermal papilla or in the interfollicular region may be associated to scarring phenomena and may be crucial for the supplying of proinflammatory cytokines controlling the inflammatory and proliferative processes.[14,18,19]

The aim of the current preliminary longitudinal study is to evaluate clinically unaffected skin of patients with mild moderate acne to characterize the in vivo morphological changes through the appearance of inflammatory lesions and its natural resolution.

Methods

Patients

The study was conducted in accordance with the ethical principles from the Declaration of Helsinki and Good Clinical Practices and, in compliance with local regulatory requirements; it was reviewed and approved by an institutional review board (N° 621015 EC 69/14).

Candidates were enrolled in the study if they were affected by mild-moderate acne (revised Leeds' score $\leq$ 2) and comprised between 12 and 30 years of age.[20] During the enrollment visit, patients were offered to receive a topical or systemic prescription for acne and those who refused any treatment were considered eligible and approached for enrollment. Study exclusion criteria included the assumption of any topical or oral medication, including oral contraceptives, in the previous month, previous diagnosis of an endocrinology disorder, or any previous facial procedures, including aesthetical or medical interventions, UV beds or sun-exposure.

The protocol represents an observational pilot study for the in vivo evaluation of microscopic changes of the skin of patients affected by acne over a six-week period, with programmed visits every week. During the enrollment visit, the investigator reported for each patient: demographic data, revised Leeds' score, previous acne therapies, clinical signs of virilization, such as hirsutism and acanthosis nigricans and reported menstrual irregularities in females.

Clinical imaging (Canon G16 and Canfield Close-up Scale®; Canfield Imaging Systems, Fairfield, NJ, USA), in vivo reflectance confocal microscopy (RCM) imaging (Vivascope 1500®, MAVIG GmbH, Munich, Germany) and dynamic optical coherence tomography (D-OCT) acquisition (VivoSight®, Michelson Diagnostics Ltd, Maidstone, Kent, UK) were performed at baseline and every week ($\pm$2 days) for the study period.

On the cheek or forehead skin, a target area of 4x4mm, without presentation at baseline of inflammatory or non inflammatory acne lesions, was selected. The target area of 4x4 mm was reported on a transparent plastic film that was modeled onto the patient's face. The plastic film was employed during the subsequent visits for the precise identification of the target area.[17]

RCM mosaics were evaluated according to a selection of features already described in previous RCM acne studies, focusing in particular on the number of normal infundibula, infundibula presenting a thickened bright border, dilated infundibula (>200 μm), and inflammatory lesions (papules and pustules), corresponding to large roundish areas with ill-defined borders, containing bright particles, inflammatory small bright cells, bright compact organized amorphous material, and inflammatory infiltrate at the periphery of the lesion.[13]

D-OCT images were processed through 3-D image reconstruction software and evaluated in accordance to the findings of the previous D-OCT study on acne, focusing in particular on the presence of increased vascular network.[14,21,22] The definitions of the RCM and the D-OCT parameters that were considered are described in Table 1.

Acquired RCM and D-OCT images were classified according to phase of development of an eventual inflammatory lesion, as assessed through clinical examination. Clinical grouping included: 1) absence of inflammatory lesions (AIL), 2) images prior to the appearance of an inflammatory lesion (PIL), 3) the presence of an inflammatory lesion (ILS) and 4) resolution of an inflammatory lesion (FRI). The process of categorization of images is represented in Figure 1.

Statistical evaluation

Mean (M) with standard deviation (SD) were calculated for continuous variable and relative frequencies were calculated for binary data. Student's t-test and Fisher exact X^2 test were calculated to determine if changes among the four groups were significant, using a p-value less than 0.05.

Results

Ten young adults, 7 females and 3 male, were enrolled. Average age was 18.6, ranging from 13 to 29 years old. All patients were affected by mild-moderate acne characterized by an average revised Leeds' score of 1.3 $\pm$ 0.5. Average VAS scores of discomfort were relatively low 2.6 $\pm$ 1.3. The median of previous acne therapies was 1 per patient (range 0-3). All females reported regular menstrual cycles, without any ovarian cyst diagnosed within the last year by any eventual gynaecological ultrasound examination. None of the female patients had signs of virilization like hirsutism, acanthosis nigricans and androgenic alopecia and previous laboratory findings of hormonal, hepatic, hematologic or renal diseases were negative.

Seventy full sets of clinical, RCM and D-OCT images were acquired and evaluated with no drop-outs or missing data in our collection. Upon clinical examination, 7 inflammatory lesions, comprising 3 pustules and 4 papules, appeared in the area of interest during the follow-up period. The average duration of the inflammatory lesions were 12 days (7-21 days). Thirty-six image sets were classified in the AIL group, 14 image sets in the PIL group, 12 image sets in the ILS group, 8 image sets in the FRI.

Mean (M), standard deviations (SD) and frequencies of the measured RCM and D-OCT parameters according to clinical groups are reported in Table 2. Student t test demonstrated a significant increase in the number of large bright follicles and a significant decrease in the number of normal follicles from AIL to PIL. The number of large bright follicles and normal

follicles then remained stable during time, throughout the ILS and FRI phases. Small bright follicles increased significantly from PIL to ILS and remained stable from ILS to FRI.

The D-OCT features of ILS phase are characterized by the presence of an increased vascular network, which return to normal after the resolution of acute inflammation (FRI) and by an increase of enlarged sebaceous glands. Representative RCM, D-OCT and the clinical images of AIL, PIL, ILS and FRI, respectively, are represented in Figure 2.

Our data show that the appearance of an inflammatory lesion (ILS phase) in acne skin is a process preceded by a statistically significant increase in large bright follicles (AIL - PIL phases) and a decrease in normal follicles (AIL-PIL and PIL-ILS). The number of small bright follicles increases from PIL to ILS and then remains stable through to the FRI phase.

Discussion

To the best of our knowledge, this is the first study that describes the in vivo evolutionary stages of acne lesions from clinically unaffected skin to lesion appearance and resolution, with RCM and D-OCT in a cohort of young patients affected by mild-moderate acne.

RCM allows a fast and reliable characterization of clinically evident acne lesions, skin conditions before their appearance, and the study of the infundibular alterations of the pilosebaceous units at a nearly histologic resolution. It permits the morphologic analysis of the pathogenic phenomena occurring in acne and the evaluation of compartment-specific changes, without requiring skin biopsy or other interventions, which may alter the morphology of the lesion. Furthermore, sequential D-OCT imaging enables to identify the alterations of the microcirculation during comedogenesis and the phases of resolution. It provides information on the dermal blood flow, which is of great value in the characterization of the inflammatory process and in the determination of the functional and metabolic features of the skin diseases.

Our data demonstrate that the appearance of an inflammatory acne lesion is preceded by an increase of large bright follicles. At this prodromal phase (PIL), the RCM and D-OCT inflammatory descriptors, such as exocytosis/dermal inflammation and increased vascular network were observed infrequently (21.4% and 35.7%, respectively). The appearance of an inflammatory lesion (ILS phase) is matched to the presence of an inflammatory infiltrate that appears into the epidermis and organizes into the dermis.[1,13,16] Of note, in the area where exocytosis is mostly represented it is possible to observe an increased presence of small bright follicles and large bright follicles. This observation may reflect a complex interplay between the hyperkeratinization process and the inflammatory phenomena. From the PIL to ILS phase, inflammation seems to additionally enhance hyperkeratinisation with the subsequent increase of small and large bright. The D-OCT features of ILS phase are characterized by the presence of an increased vascular network, which return to normal after the resolution of acute inflammation (FRI) and by an increase of enlarged sebaceous glands, which remain stable during FRI.

Similarly to the "cancerization field" that has been described for squamous cell carcinoma and actinic keratosis, acne is characterized by an "acneization field", which is constituted by an increased number of dysmorphous hair follicles also in clinically unaffected acne skin.[13,23,24] These small and large altered pilosebaceous units lead over time to important inflammatory reactions and microvascular changes. Previously reported data suggests that clinically unaffected skin of acne patients show an increased number of small bright follicles compared to patients without acne.[13,17] The current study therefore supports the following progression model: 1) an increased number of small bright follicles are present in the AIL phase, 2) several small bright follicles evolve into large bright follicles, with hyperkeratinization, that could lead to follicular occlusion phenomena, during the PIL phase, 3) an inflammatory infiltrate is recruited and gives rise to a papule, a pustule or a nodule

around which several bright follicles appear together with an enhancement of the vascular network and the enlargement of the adjacent sebaceous glands during the ILS phase, and 4) the inflammatory infiltrate reduction, due to the resolution of the inflammatory lesion, with the persistence of many small and large bright follicles and of enlarged sebaceous glands remain through the FRI phase (Figure 3).

The study limitations include the small number of enrolled volunteers and the inability to detect the eventual presence of inflammatory cells in the deep dermis with RCM and OCT, must be taken into consideration when evaluating the current study outcomes. Our data are based on dynamic observations of the skin as observed by high-resolution non invasive technology. However an in depth knowledge on the micro anatomical structure of the skin in different ages and areas of the face, differentiation in male and female, and influence of menstrual period or other environmental features is still lacking, limiting a full robustness of our observations. The possible effects of several factors, such as gender, age, diet, body-mass index, hormonal alterations and menstrual period, to the development of specific pilosebaceous morphologic alterations will be a matter of future studies in larger cohorts of patients.

Acne skin dynamics are complex and seem to constitute an organized process, characterized by the early increase in the number of dysmorphic pilosebaceous units and the hyperkeratinization of the acroinfundibulum of the pilosebaceous duct prior to the occurrence of inflammatory events around the follicle. The processes of hyperkeratinisation and inflammatory phenomena may generate a pathologic vicious cycle, which characterizes acne through progressive worsening and a self-sustainment mechanism. The microscopic changes and the characterization of the sequence of events over time in acne affected skin outlined by the current study will be of crucial importance for acne patients' management and eventual assessment of treatment efficacy.

Acknowledgments: The authors would like to thank Johanna Chester for her critical revision.

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Figure 1

Schematic representation of the process of categorization of clinical RCM and D-OCT images.

Figure 2

Clinical images (a-d), RCM (e-h) and D-OCT images (i-t) of the different stages of evolution of an inflammatory lesion, from AIL to FRI. During AIL the skin of acne affected patients shows an increased number of small bright follicles respect to patients without acne (e). During PIL several small bright follicles evolve into large bright follicles because of hyperkeratinization process or follicular occlusion phenomena (f). During ILS an inflammatory infiltrate is recruited and give rise to a papule, a pustule or a nodule. Next to the inflammatory lesion several bright follicles appear (g). During FRI the inflammatory

infiltrate disappears due to the resolution of the inflammatory lesion, but many small and large bright follicles remain (h).

D-OCT images at 150 µm depth (i-l), at 300 µm depth (m-p) and 3-D vascular reconstruction image (q-t). AIL (i,m), PIL (j,n), ILS (k,o) and FRI (l,p) at 150 µm and 300 µm depth respectively. Enlarged sebaceous glands are increased in ILS (k) and FRI (l). Vascular network is normal in AIL (q), PIL (r) and FRI (t), but is increased in ILS (s).

Figure 3

Graphical representation of comedogenesis based on RCM and D-OCT images. The three vertical diagrams represent: the development and resolution of an acne inflammatory lesion, the comedogenesis model based on RCM and D-OCT images and how RCM mosaics change from AIL to FRI.

Tables

Table 1

The RCM and the D-OCT parameters and their definitions.

Table 2

Mean (M), standard deviations (SD) and frequencies of the RCM and D-OCT parameters.

RCM features	
Normal follicle	Well-defined infundibulum with roundish/elliptic morphology, that didn't contain any amorphous or bright material. The infundibular diameter is small (less than 90 μm)
Small bright dilated follicle	The average infundibular diameter is larger than a normal infundibulum but less than 200 μm, with a bright border and usually containing a small amount of amorphous material
Large bright dilated follicle	The average infundibular diameter is larger than a small bright dilated follicle (>200 μm), with a bright border and usually containing a small amount of amorphous material
Organized inflammatory infiltrate	Large roundish areas with ill-defined borders, containing bright particles, inflammatory cells and/or bright compact organized amorphous material, and surrounded by abundant inflammatory infiltrate at the periphery of the lesion
Exocytosis and dermal inflammation	Small bright dots within the meshes of the epidermal honeycombed pattern or in the upper dermis, corresponding to inflammatory infiltrate .
OCT features	
Increased vascular network	Narrowing of the vascular network with a subsequent increase of the vascularity in the dermis
Vascular dots	Small red points corresponding to vessels oriented vertical to the surface
Vascular blobs	Large round to oval red globules corresponding to vessels oriented vertical to the surface
Branching vessels	Vessels that progressively subdivide along their course. They can be defined as 'regular' if the thickness in subsequent branches is similar for the whole length, 'arborizing' if a progressive decrease in their diameter is observed in subsequent branches, or 'bulging' if enlarged buds originate from the main structure
Enlarged sebaceous glands	Hypo-echogenic area with half-moon shape around the hair follicle, observed on the horizontal OCT images obtained at 150 μm depth.

RCM and D-OCT Parameters	*AIL*	*PIL*	*ILS*	*FRI*
Normal follicles	28.8 (SD: 5.1)	22.2 (SD: 6.2)*	12.0 (SD: 4.6)*°	16.5 (SD: 2.9)*°§
Small dilated bright follicles	15.7 (SD: 4.4)	14.8 (SD: 2.8)	20.5 (SD: 4.3)*°	19.7 (SD: 1.6)*°
Large dilated bright follicles	2.7 (SD: 1.2)	6.9 (SD: 1.7)*	8.1 (SD: 2.1) *	8.5 (SD: 2.4)*
Inflammatory papules or pustules	0 (SD: 0)	0 (SD: 0)	1 (SD: 0)	0 (SD: 0)
Dermal inflammation/Exocytosis	8.3	21.4	83.3*°	37.5
Increased vascular network	11.1	35.7	83.3*	37.5
Vascular dots (150 µm)	86.1	85.7	75.0	87.5
Vascular dots (300 µm)	100.0	100.0	100.0	100.0
Regular branching vessels (150 µm)	5.5	7.1	8.3	0.0
Regular branching vessels (300 µm)	86.1	85.7	83.3	87.5
Vascular blobs (150 µm)	5.5	0.0	8.3	0.0
Vascular blobs (300 µm)	52.7	50.0	75.0	62.5
Enlarged sebaceous glands	1.5 (0.6)	1.4 (0.5)	2.75 (1.0)*°	3.1 (0.8)*°

* p-value <0,05 compared to AIL
° p-value <0,05 compared to PIL
§ p-value <0,05 compared to ILS