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ORIGINAL PAPER

In vitro modeling of unsaturated free fatty acid-mediated tissue impairments seen in acne lesions

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Abstract *Acne vulgaris* is a disease of pilosebaceous units with multifactorial pathogenesis, including hyperkeratinization, increased sebum secretion, and inflammation. Recently, it was suggested that acne subjects may have also impaired skin barrier. We hypothesized that excess unsaturated free fatty acids (UFFA) present in the sebum may cause barrier impairment associated with increased follicular stratum corneum (SC) thickening and inflammation seen in acne. Therefore, epidermal and sebaceous lipid profiles from acne and healthy subjects were analyzed and an in vitro epidermal tissue model was developed to validate this hypothesis. Significantly increased levels of free fatty acids ($p < 0.05$) were observed in skin lipids of human acne vs. healthy subjects. Exposure of human epidermal equivalents (HEEs) to the UFFA oleic acid (OA), also present in sebum, led to barrier impairment associated with increased SC lipid disorder, increased secretion of interleukin-1α (IL-1α), and excessive SC thickening. Furthermore, the expression of genes encoding for inflammatory cytokines and epidermal differentiation proteins was also increased both in acne lesions and in OA-treated HEEs. Taken together, these data are in agreement with the hypothesis that excess UFFAs in sebum of acne subjects may contribute to impaired skin barrier associated with the increased follicular SC thickness and inflammation seen in acne. Moreover, OA induces similar molecular and phenotypic changes in HEEs as those seen in acne lesions and suggests that an UFFA-treated epidermal tissue model can be used to study the UFFA-mediated pathways involved in the pathogenesis of inflammatory acne and for the development of appropriate therapies.

Keywords *Acne vulgaris* · Free fatty acids · Abnormal keratinization · Human epidermal equivalents · Oleic acid

Introduction

Acne vulgaris is a chronic inflammatory disease affecting more than 85% of adolescents and often persisting into later adulthood [22]. The pathogenesis of acne can be attributed to multiple factors, including increased sebum production, inflammation, follicular hyperkeratinization, and the proliferation of *Propionibacterium acnes* (*P. acnes*) within the follicle [25]. Among them, increased sebum production has been considered as a major etiopathogenetic factor for acne. The facial skin of patients with acne has higher sebum production and larger size sebaceous glands as compared with the facial skin of people without acne [34], higher secretion rates [13, 41], and the sebum secretion rates correlate well with the severity of acne [4]. In addition, emerging studies suggest that the alteration of the sebum composition may also be important for the development of acne, such as squalene [33], squalene peroxide [35], linoleic acid [6], essential fatty acid level in wax ester [40], and saturated fatty acid/monounsaturated fatty acid (SFA/MUFA) ratio in triglycerides [39]. Despite research showing the elevated sebum levels and its altered composition, only few studies have examined the role of sebum lipids to induce acne.

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The barrier functions of both interfollicular and intrafollicular epithelial linings are important to consider as they may be involved in the excessive keratinization associated with acne and with impaired physiological properties of the SC [44]. Yamamato et al. [51] described that male patients, age 14–26, with mild-to-moderate acne exhibited markedly higher sebum secretion, larger transepidermal water loss (TEWL), and markedly decreased SC conductance (i.e., lower SC hydration), suggesting a deficient intercellular lipid membrane which correlates with the impairment of the SC permeability barrier. A recent study on acne found that total sebum lipids were significantly higher and TEWL was higher in acne subjects than that of normal subjects [29], further suggesting that high sebum may lead to barrier impairments that contribute to higher TEWL in acne subjects.

Human sebum is composed of triglycerides, fatty acids, cholesterols, squalene, and wax esters. The free fatty acids in the sebum are mainly released from triglycerides by bacteria such as *P. acnes* in the hair follicle canal and on the skin surface [19]. Recently, Bouslimani et al. [2] further mapped the skin lipids, including the unsaturated fatty acids (i.e., OA) in larger amounts on the head, face, chest, and back of a human subject, and showed that the localization of OA mirrored the localization of the genus *Propionibacterium* in a human three-dimensional (3D) skin surface map. They also showed that significantly more OA was detected in the *P. acnes* cultures containing the triglyceride triolein. Studies on UFFAs, i.e., OA and palmitoleic acid, were shown to induce calcium influx and abnormal keratinocyte differentiation in hairless mice [17]. When OA was applied topically, it either induced ultrastructural changes on rabbit ears similar to those seen in human comedones [25] or it caused a decrease in Langerhans cells density and generation of pores on the surface of epidermal corneocytes [45]. In addition, inflammatory acne may be triggered by UFFAs to produce multiple proinflammatory cytokines such as IL-1α, tumor-necrosis factor (TNF)-α, and chemokines (e.g., IL-8) stimulating keratinocytes, sebocytes, and immune cells, which, in turn, attract neutrophils and mononuclear cells to the pilosebaceous unit [10, 21, 30, 31, 37, 38, 46–49].

The purpose of the current study was to examine the composition of sebum lipids especially pertaining to UFFAs between acne and healthy subjects and to investigate the potential role of UFFAs to induce acne. The present study showed that there were more free fatty acids in either epidermal or sebaceous lipids, including that of UFFAs in sebaceous lipids, in acne subjects as compared with the skin of healthy subjects. In epidermal equivalent cultures, topically applied UFFAs such as OA induced disordering of endogenous SC lipid acyl chains associated with a clear decrease in barrier function caused proinflammatory cytokine IL-1α release and induced SC excess keratinization mimicking symptoms present in inflammatory acne lesions. Furthermore, gene expression analysis showed increased expression of genes encoding for inflammatory cytokines and epidermal differentiation proteins both in acne lesions and in OA-treated HEEs. Taken together, these findings suggest that excess UFFAs in acne subjects may contribute to the barrier defects and inflammation associated with excessive keratinization seen in inflammatory acne.

Materials and methods

Subjects and sampling

A total of nine subjects with mild-to-moderate *Acne vulgaris* (female, age range 20–40, mean 27.8) and six healthy subjects (female, age range 18–32, mean 24.5) were included in the study. This study was to obtain human skin biopsies from the back of subjects and skin lipid samples from the face and back of acne and healthy subjects. The study protocol was approved by Allendale Investigational Review Board (Old Lyme, CT, USA) and informed consent was obtained from all individual participants included in the study. The inclusion criteria when the subjects were recruited the study included (a) subjects which have not been treated with isotretinoin for acne within the previous 6 months and (b) if individuals are taking hormone replacement therapies (HRT) or hormones for birth control, then they must have been on a stable dose for at least 3 months, and be willing to not change this medication for the duration of the study. Individuals who are not taking HRT or hormones for birth control at the start of the study must be willing to not begin use during the course of the study. The exclusion criteria included (a) individuals currently taking medications which, in the opinion of the investigator may interfere with the study (e.g., prescription steroids, prescription anti-inflammatory drugs, oral antibiotics, isotretinoin, topical drugs on the upper mid-back, etc.) or increase the risk to the subject, (b) individuals with uncontrolled metabolic diseases, such as diabetes, hypertension, hyperthyroidism, and hypothyroidism as determined by the medical history, and (c) individuals with a history or current disease or condition of the skin that the investigator deems inappropriate for participation (atopic skin disease, eczema, psoriasis, etc.). Punch biopsies were obtained under local anesthesia from the back of acne patients from lesional to nonlesional skins. The acne lesions obtained by biopsy were clinically inflammatory

acne lesions, i.e., papules. The control skin biopsies were taken from the healthy volunteers' back skin.

Sebaceous lipids

Sebaceous lipids were sampled using Sebutape® Adhesive Patches (CuDerm Corporation, Dallas, TX, USA), as described previously [33]. Briefly, the subject's forehead or back was wiped ten times with a 70% isopropyl alcohol wipe to remove any surface sebum or dirt and allowed to dry for ≥1 min. Two Sebutape® patches were applied to the forehead over the right eyebrow or to the right side of upper back. After 30 min, the tapes were removed using forceps and a second set of tapes was applied in the same position as the first tapes. After 30 min, the second set of tapes was removed and frozen (−80 °C) for lipid analysis [33]. Commercially available standards for the lipid analysis (squalene, palmitoyl palmoitate, triolein, and oleic acid) were obtained from Sigma-Aldrich (St. Louis, MO, USA), and were dissolved and diluted in chloroform:methanol (2:1) to appropriate working stock solutions before use.

Sebaceous lipids were extracted from the tapes as previously described and the individual components were separated by high-performance thin-layer chromatography (HPTLC) [33]. In brief, the extracted lipid sample was spotted on an HPTLC plate, which was developed with 80:20:2 isohexane/diethyl ether/formic acid until the solvent front was a short distance from the top. The plate was air-dried and subsequently sprayed with 0.01% primulin to visualize the free fatty acid (FFA), triglyceride (TG), cholesterol ester (CE), wax ester (WE), and squalene (SQ) bands under ultraviolet (UV) light.

Sebaceous lipids were further quantified after separation by gas chromatography with fluorescence ionization detection (GC-FID) [33]. In this analysis, each marked band was removed from the glass thin-layer chromatography (TLC) plate and was placed into a test tube with 1 mL of toluene and 2 mL of 1% sulfuric acid in methanol. The tubes were capped and incubated overnight at 50 °C. Afterwards, 5 mL of 5% sodium chloride solution was added and the samples were extracted with 2 × 2 mL isohexane. The isohexane extracts were transferred to fresh test tubes that were shaken with 3 mL of 2% potassium hydrogen carbonate, subsequently passed through an isohexane (3 mL) pre-washed sodium sulfate column, and then post-washed with 2 mL isohexane. The solvent was removed by nitrogen, and isohexane + butylated hydroxytoluene (70 µL) were added before transferring the samples to a gas chromatograph vial for subsequent analysis (Agilent 6890 Gas Chromatograph; Agilent Technologies, Inc., Wilmington, DE, USA). Values were expressed as µg lipid per tape.

Epidermal lipids

Epidermal lipids were sampled using pre-cut circular shapes (~3.5 cm diameter) of Leukoflex® tapes (BSN medical GmbH, Hamburg, Germany). The tapes were applied to the right cheek or the left side of upper back for 60 s and then were removed using forceps and stored at −80 °C until analysis. The Leukoflex tapes were extracted by shaking with 8 mL methanol:ethyl acetate (80:20) at room temperature for 1 h and evaporated to dryness under vacuum. Sample residue was dissolved in 200 µL chloroform:methanol (2:1) and the tapes were reserved for protein assay using the QuantiPro™ BCA Assay system (Sigma-Aldrich, St. Louis, MO, USA).

Epidermal lipids were quantified by HPTLC using a modification of a previously described method [37]. Two aliquots of the dissolved sample (25 and 75 µL) were applied on the HPTLC plate (Whatman™ Partisil™ Silica Gel; GE Healthcare UK Limited, Buckinghamshire, UK) using an Automatic TLC Sampler 4 (CAMAG, Muttenz, Switzerland) at a distance of 1 cm. The plate was developed using the following sequential development system: (1) dichloromethane:ethyl acetate:acetone (80:16:4) to 85 mm above application point; (2) chloroform:methanol:acetone (76:16:8) to 80 mm; and (3) hexane:chloroform:acetic acid:acetone:methanol (6:80:0.1:10:4) to 85 mm above the application point. The plates were stained with 3% copper acetate in 8% phosphoric acid and charred at 80 °C for the quantitation of cholesterol and subsequently at 160 °C for the quantitation of ceramides and FFAs. Quantification was performed against known quantities of Ceramide III standard by laser scanning densitometry. Each HPTLC plate was scanned at 425 nm (TLC Scanner 3; CAMAG) and quantified using the winCATS software (CAMAG). The optical density of each epidermal lipid band was measured and quantified against the standard calibration curve. Where both sample levels (25 and 75 µL) were inside the linearity parameters of the calibration curve, an average of the two values was reported. All results outside the standard linearity parameters were disqualified.

Human epidermal equivalent (HEE)

Human epidermal equivalents (HEE) in 9 mm inserts were purchased from MatTek Company (Ashland, MA, USA). Upon receiving, human epidermal equivalents were incubated in hydrocortisone-free, pheno-red-free MatTek assay medium overnight. The surface of each HEE tissue is ~0.64 cm^2. Ten microlitre of 5, 10% oleic acid, or 10% palmitic acid in propylene glycol/ethanol (3/7, Sigma-Aldrich, St. Louis, MO, USA) was applied on top of HEEs once per day for 2 days or 2.5 ng/mL of IL-1α was put in

the medium of HEEs for 2 days. The tissue culture media were collected after 48 h treatment for IL-1α release measured by Milliplex MAP kit (EDM Millipore, Billerica, MA, USA) following manufacturer's instruction. The HEEs were cut into two halves and one halves were harvested for histological examination, and the other halves were quick frozen in liquid nitrogen and stored in −80 °C for quantitative real-time polymerase chain reaction (qPCR) analysis. For qPCR analysis, each treatment was pooled from three halves of HEES and at least two experiments were analyzed.

Epidermal barrier assessment by trans-epithelial electric resistance (TEER) measurement of HEE

Electric resistance of HEEs was measured in a temperature-controlled laboratory room kept at 20 °C using the epithelial ohmmeter Millicell ERS (EDM Millipore, Billerica, MA, USA) at day 0 and day 2. HEEs were overlaid with 400 μL of 1× phosphate buffered saline (1× PBS) for the time required to measure electrical resistance. Two electrodes were positioned with one in the outside of the well submersed in medium and the other above the equivalent submersed in 1× PBS. The electric resistance readings were expressed in kilo-ohm (kΩ). The TEER values were normalized by the readings of day 0.

Histological analysis

HEE samples were fixed at the end of treatment in 10% neutral buffered formalin solution (Thermo Fisher Scientific, Hudson, NH, USA), dehydrated and embedded in paraffin. Tissue sections (5 μm thick) were cut perpendicular to the filter and were stained with hematoxylin–eosin. Three pictures per section were taken for each skin equivalent. The thickness of the stratum cornea and epidermis was measured using ImagePro Plus (Media Cybernetics Inc., Rockville, MD, USA).

Sample preparation, spectral acquisition, and analysis for infrared (IR) imaging

HEE samples were flash frozen immediately at the end of treatment with liquid nitrogen (N_2) to preserve sample integrity by substantially limiting ice damage. Frozen HEE samples were fixed with ice on a metal chuck stage of a Bright/Hacker 5030 Microtome (Bright Instrument Company, Huntington, UK; Hacker Instruments, Fairfield, NJ, USA). After 5 min of stabilization at −30 °C, the samples were microtomed to ∼10 μm-thick sections and transferred to calcium fluoride (CaF_2) IR windows. IR images were acquired with a PerkinElmer Spotlight 300 instrument (PerkinElmer, Waltham, MA, USA) using the transmission mode and a 6.25 μm^2 pixel size. The instrument is equipped with an essentially linear array (16 × 1) of mercury–cadmium–telluride (MCT) detector elements. 32 scans were acquired with a spectral resolution of 8 cm^{-1} and one level of zero filling yielded data encoded in 4 cm^{-1} increments. Two specimens from each treatment group were microtomed and four images ($\sim 200 \times 400$ μm^2) were acquired for each specimen. Images were analyzed using the ISys software (version 5.0 from Malvern Instruments, UK). The center of mass (COM) of the symmetric methylene stretching band was obtained following the application of a linear baseline (2865–2835 cm^{-1}).

Gene expression analysis

Total RNAs were isolated from acne nonlesional and lesional biopsies using an Qiagen RNeasy Mini kit for fibrous tissues (Valencia, CA, USA) or from HEE samples using Qiagen RNeasy kit (Valencia, CA, USA) following the manufacturer's instructions. RNA concentration was assessed using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Hudson, NH, USA).

Reverse transcription was performed using a High Capacity cDNA kit (Life Technologies Corporation, Grand Island, NY, USA). TaqMan® gene expression assays for interleukin 8 (IL8), transglutaminase 1 (TGM1), involucrin (IVL), loricrin (LOR), and polymerase (RNA) II polypeptide A (POLR2A) and TaqMan® gene expression master mix were purchased from Life Technologies Corporation (Grand Island, NY, USA). qPCR was performed using an ABI 7500 Fast Real-Time PCR system (Life Technologies Corporation, Grand Island, NY, USA). Each reaction was carried out by ribonucleic acid (RNA) from individual biopsy of clinical samples or from three HEEs combined in duplicated experiments, and normalized to the POLR2A. The fold changes were calculated in comparison with the nonlesional or vehicle control.

Statistics

Statistical analyses were performed using either two-tailed two-sample unequal variance Student t test for clinical samples (Microsoft Office Excel 2007; Microsoft, Redmond, WA, USA) or an analysis of variance (ANOVA) model with Dunnett's multiple comparison test for in vitro samples (Prism 6, GraphPad Software Inc., La Jolla, CA, USA). Differences were considered statically significant if $p < 0.05$ (*), $p < 0.01$(**), $p < 0.001$(***), or $p < 0.0001$ (****).

Results

Epidermal and sebaceous lipid profile comparison of acne and healthy subjects

Lipid analyses of epidermal lipids from the faces of acne or healthy subjects showed that higher amounts of ceramides and cholesterol and more FFAs ($p < 0.1$) were detected in epidermal lipids in acne vs. healthy subjects (Fig. 1a). Lipid analyses of sebaceous lipids from the faces of acne or healthy subjects showed that higher amounts of cholesterol esters/wax esters and squalene and significantly more FFA and triglycerides ($p < 0.05$) were measured in sebaceous lipids from the faces of acne subjects than that of healthy ones (Fig. 1b). Lipid analyses of both epidermal (Fig. 1a) and sebaceous lipids (Fig. 1b) from the backs of acne or healthy subjects also showed similar results of higher amounts of lipid components in epidermal and sebaceous lipids from acne subjects than that from healthy subjects. There were significantly more epidermal FFAs from the back in acne subjects than that in healthy subjects ($p < 0.05$) (Fig. 1a).

Sebaceous free fatty acid profiles comparisons of acne and healthy subjects

Sebaceous free fatty acid profiles comparisons of acne vs. healthy subjects showed that there were higher sebaceous total FFAs, higher UFFAs, and higher saturated free fatty acids (SFFAs) from the faces (Fig. 2a), as well as the backs (Fig. 2b) of acne vs. healthy subjects. Ratios of sebaceous total FFAs, UFFAs, and SFFAs from the faces of acne to healthy subjects were 2.18 ± 1.64, 2.23 ± 1.88, and 2.16 ± 1.55, respectively (Fig. 2c). Ratios of sebaceous total FFAs, UFFAs, and SFFAs from the backs of acne to healthy subjects were 1.83 ± 1.38, 1.60 ± 1.61, and 1.96 ± 1.29, respectively (Fig. 2c). The sebaceous UFFAs and SFFAs accounted for $\sim$30 and 70% of the total FFAs, respectively. Furthermore, SFFAs/UFFAs ratio was lower (1.94 ± 0.63) in the sebum of acne subjects as compared to that of the healthy subjects (2.11 ± 0.77), suggesting that there was a relative higher abundance of UFFAs compared to SFFAs in sebum of acne vs. healthy subjects, as shown in Fig. 2a.

Oleic acid induced thickening of stratum cornea, barrier defects, and the release of IL-1α from human epidermal equivalents

Since there were higher amounts of UFFAs detected in sebum of acne subjects, the effect of UFFAs on skin barrier in vitro was investigated. Although among UFFAs, sapienic acid (C16:1, n-10) was the highest abundant UFFA measured in the sebum of acne vs. healthy subjects (Fig. 2a), oleic acid (C18:1, n-9) was used as a representative UFFA in our in vitro studies, because it was the second abundant UFFA in the sebum of acne vs. healthy subjects (Fig. 2a) and because of its easier commercial availability. Figure 3a, b shows normal epidermal equivalents without treatment and treated by vehicle, respectively. When the UFFA oleic acid, used at the doses of 5 or 10% comparable to the levels of total sebum UFFAs found in acne subjects (Fig. 1), was applied topically on human epidermal equivalents (HEEs), it induced a dose-depend thickening of SC (Fig. 3d, e). When IL-1α (2.5 ng/mL) was included in the medium of the HEEs, IL-1α induced a similar thickening of SC (Fig. 3c) in these tissues. Figure 3f shows the quantitation of the thickness of the epidermis and SC. There were significantly thicker SCs in HEEs treated with IL-1α, with 5 and 10% oleic acid. Topical treatments of HEEs with 5 and 10% oleic acid also induced a significant IL-1α protein release from epidermal equivalents ($p < 0.001$, Fig. 3g), associated with barrier defects as reflected by a decrease in TEER ($p < 0.05$, Fig. 3h). 10% oleic acid induced a significantly higher thickening of SC ($p < 0.05$) than that of 5% oleic acid. In contrast to UFFAs, e.g., oleic acid, SFFAs, e.g., the palmitic acid, used at 10% did not induce a significant thickening of stratum corneum (Fig. 3i).

IR imaging of skin equivalents with or without oleic-acid treatment

The effect of OA on epidermal lipid orders was examined by IR imaging. Typical single pixel IR spectra of the CH stretching region (3000–2828 cm^{-1}) of the SC for vehicle-treated (control, blue) and 10% deuterated oleic acid (OA-d) in vehicle-treated (red) HEE sections are overlaid in Fig. 4a. Data analysis focused on this spectral region to qualitatively evaluate SC barrier properties as indicated by the acyl chain conformational order of the SC lipids in the HEE sections. The peak positions of the methylene stretching modes (asymmetric, $\nu_a CH_2$, at $\sim$2920 cm^{-1} and symmetric, $\nu_s CH_2$, at $\sim$2850 cm^{-1}) are widely used to monitor chain conformational order with a higher frequency indicative of more disorder.

Visible micrographs of the vehicle-treated and OA-d in vehicle-treated HEE sections (four from each section) are displayed in Fig. 4b as noted. The thin, darker SC region (top of each image) is clearly delineated from the underlying viable epidermis (VE). Since the barrier function of skin resides in the SC, IR images of the $\nu_s CH_2$ COM in the SC are displayed in Fig. 4c for the same sections, as shown in Fig. 4b. The color-coded variation in $\nu_s CH_2$ COM is obvious comparing the control (blue, more ordered lipid)

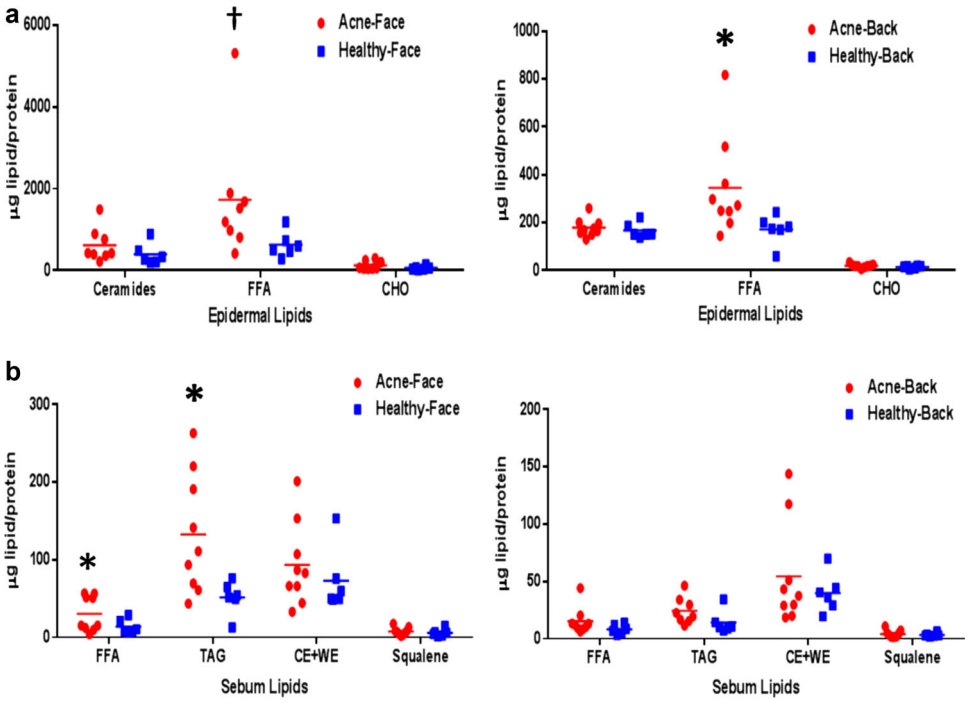

Fig. 1 Lipid profiles of acne vs. healthy subjects. **a** Epidermal lipids of acne vs. healthy subjects from face and back. Acne subjects have higher amounts of ceramides and cholesterol, and significantly more FFAs ($p < 0.05$) were detected in epidermal lipids in acne vs. healthy subjects from back. **b** Sebaceous lipids of acne vs. healthy subjects from face and back. Acne subjects have higher amounts of cholesterol esters/wax esters and squalene, and significantly more free fatty acids and triglycerides ($p < 0.05$) from the face in acne subjects than that in healthy subjects. *FFA* free fatty acids, *CHO* cholesterol, *TAG* triglycerides. *CE + WE* cholesterol esters/wax esters. † $p < 0.1$, *$p < 0.05$

and OA-d-treated (yellow–red, disordered lipid) sections, clearly indicating a decrease in barrier integrity in the latter. Similar results were obtained from the $\nu_a CH_2$ COM (not shown). Figure 4d displays the average COM in the SC (±1 SD) for each treatment. As anticipated, the average $\nu_s CH_2$ COM is $\sim$1.5 cm^{-1} greater in the OA-d-treated samples compared to the control, which indicates that the lipid acyl chains are significantly less ordered in the OA-d-treated samples.

Up-regulation of inflammatory and differentiation genes in both inflammatory acne lesions and oleic-acid-treated HEEs

Comparisons of the gene expression levels by qPCR in inflammatory acne lesions and in the oleic-acid-treated HEEs showed that there was a 17-fold increase in the mRNA expression of IL-8 in both the inflammatory acne lesions vs. the nonlesional control (Fig. 5a, $p < 0.01$), and a threefold increase in oleic-acid-treated HEEs vs. the vehicle control (Fig. 5b, $p < 0.05$). Furthermore, the expression of the genes involved in epidermal differentiation, i.e., transglutaminase 1 (TGM1), involucrin (IVL), and loricrin (LOR), was also increased sixfold ($p < 0.05$), sevenfold ($p < 0.001$), and fourfold ($p < 0.05$), respectively, in the inflammatory acne lesions vs. the nonlesional control (Fig. 5a), and threefold ($p < 0.05$), twofold ($p < 0.01$), and sixfold ($p < 0.01$), respectively, in the OA-treated HEEs vs. the vehicle control (Fig. 5b).

Discussion

Increased sebum production is one of the major factors for acne development as supported by the findings that higher sebum secretion was found in acne patients than healthy subjects [4, 13, 26, 41] and acne lesions can be improved with sebum-inhibitory agents [8, 42]. There are increasing evidences suggesting that sebum compositional changes in the context of increasing sebum production may influence acne development [6, 33, 35, 39, 40]. Therefore, examining the free fatty acids of sebum may contribute to our understanding of pathogenic factors involved in acne. Smith et al. [39] found that acne subjects on the experimental diet demonstrated increases in the ratio of saturated to monounsaturated fatty acids of skin surface triglycerides when compared to controls. The increase in the ratios of saturated to monounsaturated fatty acids correlated with the decrease of acne lesion counts [39].

The present study examined epidermal and sebaceous lipids and the composition of FFAs in sebum of acne and healthy subjects. There were significantly more epidermal FFAs detected in acne subjects either from the face ($p < 0.1$) or from the back ($p < 0.05$) through lipid analysis (Fig. 1a). Furthermore, there were significantly more FFA and triglycerides ($p < 0.05$) in sebaceous lipids from the faces of acne subjects than that of healthy ones (Fig. 1b). Although the menstrual cycle may cause variations of the amounts of surface lipids as described in the paper by MacDonald et al. [24], the findings in our studies are in agreement with, and supported by the findings

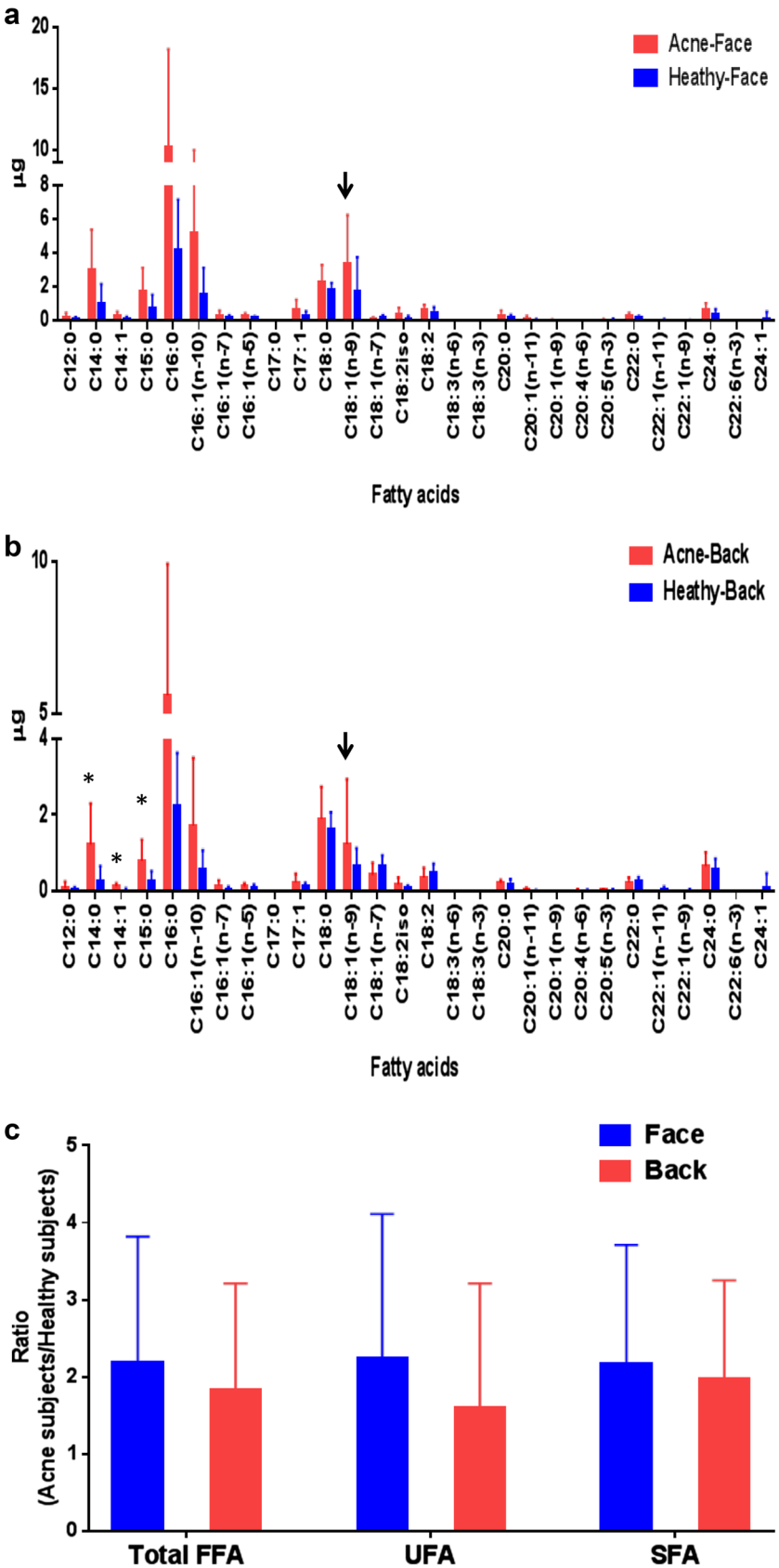

Fig. 2 Sebaceous free fatty acid (FFA) profiles of acne vs. healthy subjects from face and back. **a** Sebaceous fatty acid profiles of FFA from the faces of acne and healthy subjects. **b** Sebaceous fatty acid profiles of FFA from the backs of acne and healthy subjects. **c** Face and back ratios of sebaceous total FFA, unsaturated FFA, and saturated FFA from acne to healthy subjects. *Arrows* oleic acid (C18:1, n-9). *FFA* fatty acids, *UFA* unsaturated fatty acids, *SFA* saturated fatty acids. $^{*}p < 0.05$

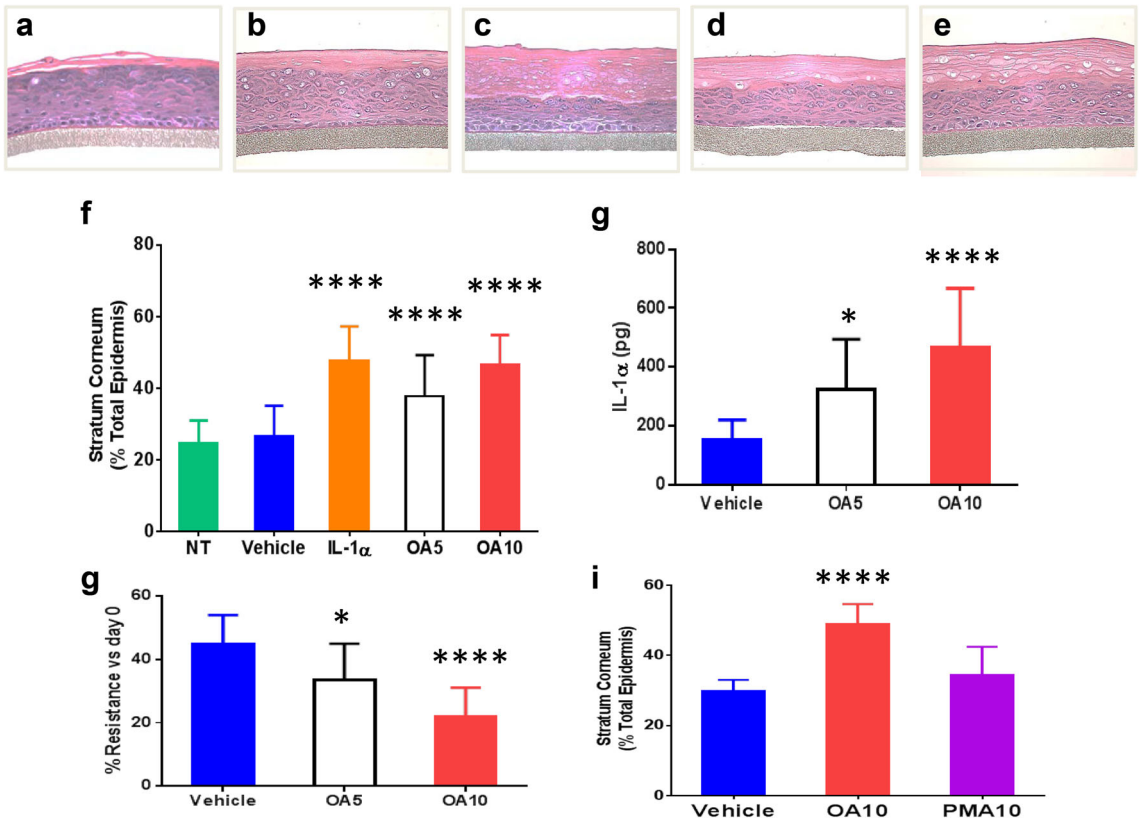

Fig. 3 Oleic acid induced stratum cornea thickening, barrier defect, and IL-1α release in epidermal skin equivalents. Histology of epidermal skin equivalent without treatment (**a**), with vehicle (**b**), with 2.5 ng/mL IL-1α in the medium (**c**), with topical oleic acid 5% (**d**), or 10% (**e**). HEE treated with IL-1α, 5 or 10% oleic acid showed an increase in thickness of stratum cornea. **f** Quantitative analysis of stratum cornea and epidermis in vehicle-, IL-1α-, or oleic-acid-treated epidermal skin equivalents. Oleic acid caused dose-dependent increase in the thickness of stratum cornea as compared to that of vehicle (****$p < 0.0001$). **g** IL-1α release increased with an increase dose of oleic-acid treatment (*$p < 0.05$, ****$p < 0.0001$). **h** Trans-epithelial electrical resistance (TEER) decreased with an increase dose of oleic-acid treatment (*$p < 0.05$; ****$p < 0.0001$). **i** Quantitative analysis of stratum cornea and epidermis in vehicle-, 10% oleic-acid-, or 10% palmitic-acid-treated epidermal skin equivalents. 10% OA caused a significant thickening of stratum cornea (****$p < 0.0001$), whereas 10% PMA did not induce the thickening of stratum corneum. *OA* oleic acid, *PMA* palmitic acid. *$p < 0.05$, ****$p < 0.0001$

published by Harris et al. [13] from an aged- and gender-matched study (that included both male and female subjects), showing that acne subjects had higher sebum secretion rates and higher amount of sebum lipids than aged- and gender-matched healthy subjects, irrespective of gender differences and their associated sex hormones levels variability. In addition, the ratios of SFFAs/UFFAs were lower in acne subjects than that of healthy subjects, which are in agreement with the finding of Smith et al. [39], and suggested that increased UFFAs might play a role in acne lesion formation. In the current study, the observed significantly more FFAs content in both epidermal and sebum lipids of acne vs. healthy subjects further supports that FFAs may contribute to acne development in the context of increasing sebum production.

The increased amounts of free fatty acids may result from *P. acne* hydrolysis of sebum triglycerides in the pilosebaceous follicle and may reflect intrafollicular *P. acnes* lipase activity [27, 32]. The skin lipids, including the UFFAs (e.g., OA), had been found to mirror the localization of the genus *Propionibacterium* in a human 3D skin surface map and there were significantly more OA detected in the *P. acnes* cultures containing the triglyceride triolein in vitro [2]. Effective doses of antibiotic therapy reduced *P. acnes* populations correlated with the amelioration of acne, in parallel to a decrease in surface FFAs [5, 9]. Conversely, agents that reduce skin surface FFAs have also shown antibacterial effect on *P. acnes* and a clinical improvement in the acne condition [36].

Studies have suggested that *Acne vulgaris* may be associated with markedly higher sebum production and larger TEWL, markedly decreased SC conductance (lower SC hydration) and epidermal barrier dysfunction. Yamamoto et al. [51] showed that acne patients had a reduced water barrier function (higher TEWL) and that the degree of SC permeability barrier impairment correlates directly

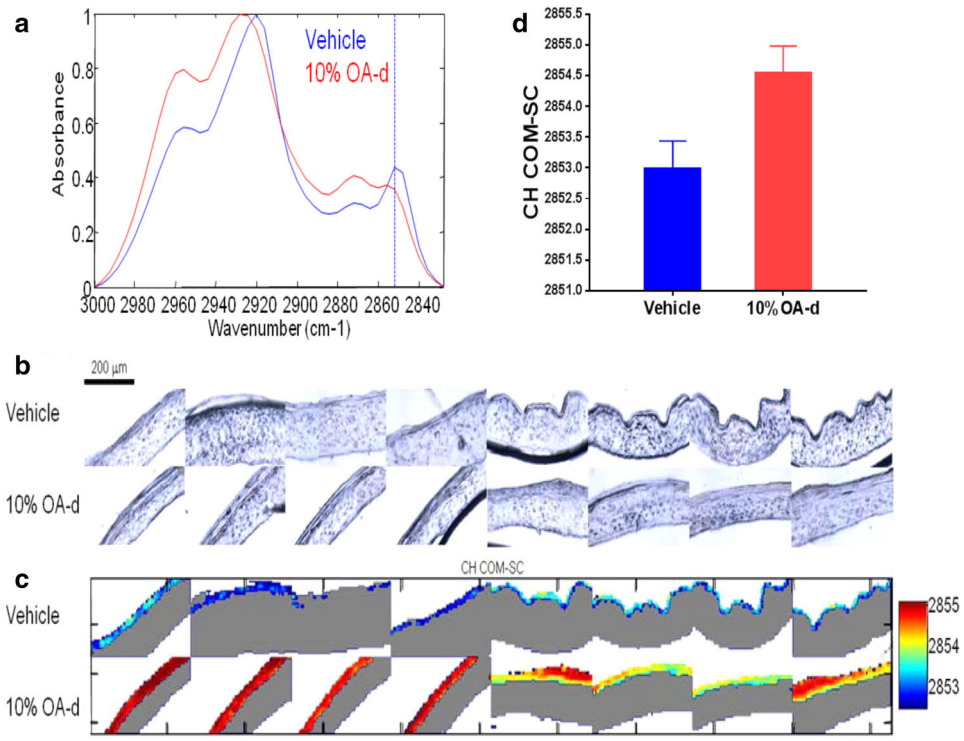

Fig. 4 IR imaging of vehicle- and oleic-acid-treated epidermal skin equivalent. **a** Overlaid, normalized single pixel spectra of the CH stretching region (3000–2828 cm^{-1}) in the stratum corneum of the HEE sections: vehicle treated in *blue* and 10% deuterated oleic acid treated in *red*. The *blue dashed line* is at 2852 cm^{-1}. **b** Visible images of HEE sections for two separate treatments (four sections each) of vehicle only (*top*) and 10% deuterated oleic acid (*bottom*). **c** IR images of ν_sCH2 COM in the SC region for the same sections shown in figure **b**. **d** Average ν_sCH2 COM values (±1 SD) of the SC region of the HEE sections for the vehicle vs. 10% deuterated oleic-acid treatment. The *scale bar* (200 µm) applies to both visible (**b**) and IR (**c**) images. *OA-d* deuterated oleic acid

with the severity of acne. Recently, Meyer et al. [29] also showed that acne subjects had higher TEWL. Furthermore, Katsuta et al. [18] showed that unsaturated fatty acids such as oleic acid greatly increased TEWL, accelerated epidermal proliferation and epidermal differentiation, and increased the level of parakeratotic corneocytes in mice, in contrast to the SFFA palmitic acid. The current study confirms these findings and provides a human in vitro model using an UFFA to cause a decrease of epidermal barrier in contrast to an SFFA, mimicking the barrier defects seen in acne lesions.

Lipid organization plays a key role in the permeability barrier function of the SC [3, 50]. The exceptional intercellular SC lipid composition yields lamellar structure with predominantly crystalline lateral phases that are more highly ordered than other biological membranes. IR imaging is well suited to evaluate lipid organization in skin and skin models providing spatially resolved characterization of lipid packing and chain conformational order [27, 52]. In the current study, the endogenous SC lipid acyl chain conformational order was mapped via the $\nu_s CH_2$ of vehicle-treated and 10% OA-d in vehicle-treated HEE sections. Deuteration of the acyl chain in OA shifts the CD_2 stretching modes to lower frequencies, so that they do not interfere with the endogenous lipid CH_2 stretching bands. A significant increase in mean frequency ($\sim$1.5 cm^{-1}) was observed for the OA-d-treated sections indicative of OA-d-induced disordering of endogenous SC lipid acyl chains associated with a clear decrease in SC barrier function.

Among human skin surface lipids, the free fatty acids may be the most irritating component of human skin surface lipid [43], and the UFFA oleic acid, found in higher amounts in the sebum of acne vs. healthy subjects in this study, has been shown to cause irritation in vitro and in vivo [1, 19]. Ingham et al. found IL-1α-like activity in open comedones from acne subjects [14]. The uninvolved skin of patients with acne exhibited increased perifollicular and follicular patterns of inflammatory cellular infiltration and inflammatory IL-1α expression similar to that of the early acne papules <6 h [16]. IL-1α has been shown to cause comedonal features, such as hypercornification, in isolated human pilosebaceous units (PSUs) in vitro [7, 11, 12]. In agreement with these data, results presented in this work showed the increase of IL-1α secretion induced by UFFAs, found in higher amounts in the sebum of acne vs. healthy subjects, and used at doses comparable to the total UFFA levels found in sebum of acne subjects [35]. Moreover, our study links the barrier damage and thickening of stratum corneum through UFFA-mediated irritation in vitro, mimicking the abnormal keratinization and the barrier defects seen in acne lesions.

In the current study, the acne lesions showed a 17-fold increase in the expression of IL-8 gene ($p < 0.01$) and the UFFA-treated HEEs also showed a threefold increase of IL-8 gene ($p < 0.05$), which is consistent with the data shown by Kelhälä et al. [20] and Trivedi et al. [46] that in acne lesions, there is an increase in expressions of inflammatory genes, such as IL-8. Furthermore, both the

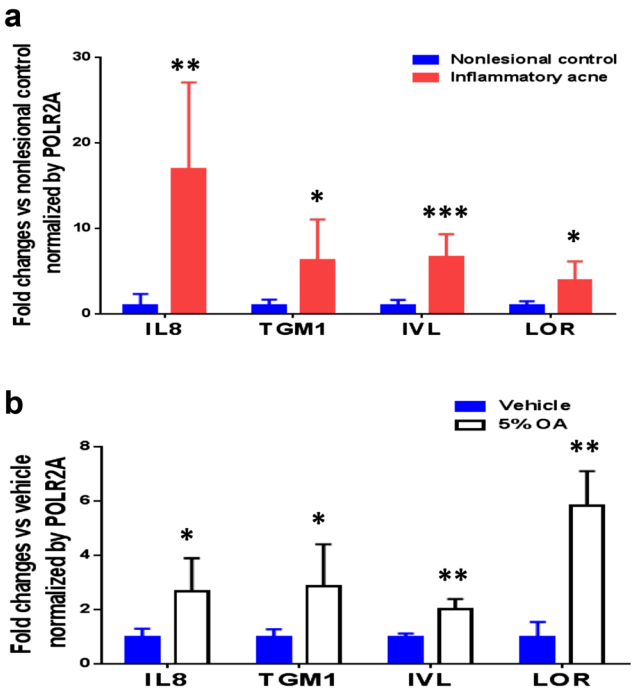

Fig. 5 Gene expressions in inflammatory acne lesions, and oleic-acid-treated human epidermal equivalents. **a** Gene expression of the inflammatory acne lesions vs. nonlesional samples. The expression of interleukin-8, transglutaminase 1, involucrin, or loricrin for inflammatory acne lesions was significantly more than that of the nonlesional control. **b** Gene expression of oleic-acid-treated human epidermal equivalents vs. vehicle control. The expression of interleukin-8, transglutaminase 1, involucrin, or loricrin for oleic-acid-treated epidermal skin equivalents was significantly more than that of the vehicle control. *IL-8* interleukin-8, *TGM1* transglutaminase 1, *IVL* involucrin, *LOR* loricrin. $*p < 0.05$; $**p < 0.01$, $***p < 0.001$

acne lesions and UFFA-treated HEEs showed an increase in the expression of genes encoding for SC proteins and cornification, i.e., transglutaminase 1, involucrin, and loricrin (Fig. 5), suggesting that the unsaturated free fatty acid, i.e., OA, may contribute to hypercornification (hyperkeratinization) in acne lesions and the UFFA-treated HEEs may mimic the acne lesions in vitro. Increasing loricrin gene expression in the UFFA-treated HEEs found in our study is consistent with the finding that topical application of oleic acid was found to increase abnormal loricrin protein expression in murine epidermis [18]. A hypothesis suggested that a reduced ability to express filaggrin due to genetic mutation correlates directly with a lesser ability to form acne lesions [44]. Previous studies have shown that there is an increase in filaggrin expression in keratinocytes lining the follicle wall within acne lesions and that *P. acnes* increased filaggrin expression in cultured keratinocytes and in explants of human skin [15, 23]. However, our gene expression analysis did not show an increase in the gene expression of filaggrin in the acne lesions vs. nonlesional skin (data not shown). Further studies are needed to provide further evidence to clearly support this hypothesis.

In conclusion, this study revealed increased fatty acids, including the UFFAs in skin lipids of acne vs. healthy subjects, and that UFFAs can induce in vitro in a human epidermal equivalent model, the barrier defects, molecular changes, and abnormal keratinization seen in acne. Taken together, these data suggest that an UFFA-treated human epidermal tissue model can be used to study the UFFA-mediated pathways involved in the pathogenesis of inflammatory acne and for the development of appropriate therapies.

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Compliance with ethical standards

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Conflict of interest WHL, MDS, and RP were employees of Johnson & Johnson Consumer Inc. when these experiments were conducted.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 2013 Helsinki declaration and its later amendments or comparable ethical standards.

References

1. Boelsma E, Tanojo H, Bodde HE, Ponec M (1996) Assessment of the potential irritancy of oleic acid on human skin: evaluation in vitro and in vivo. Toxicol In Vitro 10:729–742
2. Bouslimani A, Portoa C, Ratha CM et al (2015) Molecular cartography of the human skin surface in 3D. PNAS 112:E2120–E2129
3. Bouwstra JA, Ponec M (2006) The skin barrier in healthy and diseased state. Biochim Biophys Acta 1758:2080–2095
4. Cunliffe WJ (1989) Acne. Martin Dunitz, London
5. Cunliffe WJ, CotteriU JA, Williamson B (1972) The effect of clindamycin in acne—a clinical and laboratory investigation. Br J Dermatol 87:37–41
6. Downing DT, Stewart ME, Wertz PW et al (1986) Essential fatty acids and acne. J Am Acad Dermatol 14:221–225
7. Downie MM, Sanders DA, Kealey T (2002) Modelling the remission of individual acne lesions in vitro. Br J Dermatol 147:869–878
8. Farrell L, Strauss J, Stranieri A (1980) The treatment of severe cystic acne with 13-cis-retinoic acid. Evaluation of sebum production and the clinical response in a multiple-dose trial. J Am Acad Dermatol 3:602–611
9. Freinkel RK, Strauss JS, Yip SY, Pochi PE (1965) Effect of tetracycline on the composition of sebum in acne vulgaris. N Engl J Med 273:850–854

10. Graham GM, Farrar MD, Cruse-Sawyer JE et al (2004) Proinflammatory cytokine production by human keratinocytes stimulated with *Propionibacterium acnes* and *P. acnes* GroEL. Br J Dermatol 150:421–428
11. Guy R, Green MR, Kealey T (1996) Modeling acne in vitro. J Investig Dermatol 106:176–182
12. Guy R, Kealey T (1998) The effects of inflammatory cytokines on the isolated human sebaceous infundibulum. J Investig Dermatol 110:410–415
13. Harris H, Downing D, Stewart M, Strauss J (1983) Sustainable rates of sebum secretion in acne patients and matched normal control subjects. J Am Acad Dermatol 8:200–203
14. Ingham E, Eady EA, Goodwin CE, Cove JH, Cunliffe WJ (1992) Pro-inflammatory levels of interleukin-1 alpha-like bioactivity are present in the majority of open comedones in acne vulgaris. J Investig Dermatol 98:895–901
15. Jarrousse V, Castex-Rizzi N, Khammari A, Charveron M, Dréno B (2007) Modulation of integrins and filaggrin expression by *Propionibacterium acnes* extracts on keratinocytes. Arch Dermatol Res 299:441–447
16. Jeremy AHT, Holland DB, Roberts SG et al (2003) Inflammatory events are involved in acne lesion initiation. J Investig Dermatol 121:20–27
17. Jiang SJ, Hwang SM, Choi EH, Elias PM, Ahn SK, Lee SH (2000) Structural and functional effects of oleic acid and iontophoresis on hairless mouse stratum corneum. J Investig Dermatol 114:64–70
18. Katsuta Y, Iida T, Inomata S, Denda M (2005) Unsaturated fatty acids induce calcium influx into keratinocytes and cause abnormal differentiation of epidermis. J Investig Dermatol 124:1008–1013
19. Katsuta Y, Iida T, Hasegawa K, Inomata S, Denda M (2009) Function of oleic acid on epidermal barrier and calcium influx into keratinocytes is associated with *N*-methyl D-aspartate-type glutamate receptors. Br J Dermatol 160:69–74
20. Kelhälä HL, Palatsi R, Fyhrquist N et al (2014) IL-17/Th17 pathway is activated in acne lesions. PLoS One 9:e105238
21. Kim J, Ochoa MT, Krutzik SR et al (2002) Activation of toll-like receptor 2 in acne triggers inflammatory cytokine responses. J Immunol 169:1535–1541
22. Kraning KK, Odland GF (1979) Prevalence, morbidity and cost of dermatologic diseases. J Investig Dermatol 73:395–401
23. Kurokawa I, Mayer-da-Silva A, Gollnick H, Orfanos CE (1988) Monoclonal antibody labeling for cytokeratins and filaggrin in the human pilosebaceous unit of normal, seborrhoeic and acne skin. J Investig Dermatol 91:566–571
24. MacDonald I, Clarke G (1970) Variations in the levels of cholesterol and triglyceride in the skin surface fat during the menstrual cycle. Br J Dermatol 83:473–476
25. Maeda T (1991) An electron microscopic study of experimentally-induced comedo and effects of vitamin A acid on comedo formation. J Dermatol 18:397–407
26. Makrantonaki E, Ganceviciene R, Zouboulis C (2011) An update on the role of the sebaceous gland in the pathogenesis of acne. Dermato-Endocrinology 3:41–49
27. Marples RR, Downing DR, Kligman AM (1971) Control of free fatty acids in human surface lipids by *Corynebacterium acnes*. J Investig Dermatol 56:127–131
28. Mendelsohn R, Flach CR, Moore DJ (2006) Determination of molecular conformation and permeation in skin via IR spectroscopy, microscopy, and imaging. Biochem Biophys Acta 1758:923–933
29. Meyer K, Pappas A, Dunn K, Cula G, Seo I (2015) Evaluation of seasonal changes in facial skin with and without acne. J Drugs Dermatol 14:593–601
30. Nagy I, Pivarcsi A, Koreck A et al (2005) Distinct strains of Propionibacterium acnes induce selective human beta-defensin-2 and interleukin-8 expression in human keratinocytes through toll-like receptors. J Investig Dermatol 124:931–938
31. Nagy I, Pivarcsi A, Kis K et al (2006) *Propionibacterium acnes* and lipopolysaccharide induce the expression of antimicrobial peptides and proinflammatory cytokines/chemokines in human sebocytes. Microbes Infect 8:2195–2205
32. Nicolaides N, Wells GC (1957) On the biogenesis of the free fatty acids in human skin surface fat. J Investig Dermatol 29:423–433
33. Pappas A, Johnsen S, Liu JC et al (2009) Sebum analysis of individuals with and without acne. Dermatoendocrinology 1:157–161
34. Plewig G, Kligman AM (2000) Sebaceous glands. In: Plewig G, Kligman AM (eds) Acne and rosacea, 3rd edn. Springer, Berlin, p 70
35. Picardo M, Ottaviani M, Camera E, Mastrofrancesco A (2009) Sebaceous gland lipids. Dermatoendocrinology 1:68–71
36. Puhvel SM, Sakamoto M (1977) A reevaluation of fatty acids as inflammatory agents in acne. J Investig Dermatol 68(2):93–97
37. Puhvel SM, Sakamoto M (1980) Cytotaxin production by comedonal bacteria (*Propionibacterium acnes*, *Propionibacterium granulosum* and *Staphylococcus epidermidis*). J Investig Dermatol 74:36–39
38. Schaller M, Loewenstein M, Borelli C et al (2005) Induction of a chemoattractive proinflammatory cytokine response after stimulation of keratinocytes with *Propionibacterium acnes* and coproporphyrin III. Br J Dermatol 153:66–71
39. Smith RN, Braue A, Varigos GA, Mann NJ (2008) The effect of a low glycemic load diet on acne vulgaris and the fatty acid composition of skin surface triglycerides. J Dermatol Sci 50:41–52
40. Stewart ME (1992) Sebaceous gland lipids. Semin Dermatol 11:100–105
41. Strauss JS, Pochi PE (1961) The quantitative gravimetric determination of sebum production. J Investig Dermatol 36:293–298
42. Strauss JS, Kligman A, Pochi PE (1962) The effect of androgens and estrogens on human sebaceous glands. J Investig Dermatol 39:139–155
43. Strauss JS, Pochi PE (1965) Intracutaneous injection of sebum and comedones. Histological observations. Arch Dermatol 92:443–456
44. Thiboutot D, Del Rosso JQ (2013) Acne vulgaris and the epidermal barrier: is acne vulgaris associated with inherent epidermal abnormalities that cause impairment of barrier functions? Do any topical acne therapies alter the structural and/or functional integrity of the epidermal barrier? J Clin Aesthet Dermatol 6:18–24
45. Touitoua E, Godina B, Karla Y, Bujanoverb S, Beckerb Y (2002) Oleic acid, a skin penetration enhancer, affects Langerhans cells and corneocytes. J Control Release 80:1–7
46. Trivedi NR, Gilliland KL, Zhao W, Liu W, Thiboutot DM (2006) Gene array expression profiling in acne lesions reveals marked upregulation of genes involved in inflammation and matrix remodeling. J Investig Dermatol 126:1–9
47. Vowels BR, Yang S, Leyden JJ (1995) Induction of proinflammatory cytokines by a soluble factor of *Propionibacterium acnes*: implications for chronic inflammatory acne. Infect Immun 63:3158–3165
48. Webster GF, Leyden JJ (1980) Characterization of serum-independent polymorphonuclear leukocyte chemotactic factors produced by *Propionibacterium acnes*. Inflammation 4:261–269
49. Weerheim A, Ponec M (2001) Determination of stratum corneum lipid profile by tape stripping in combination with high-performance thin-layer chromatography. Arch Dermatol Res 293:191–199

50. Wertz PW (2013) Current understanding of skin biology pertinent to skin penetration: skin biochemistry. Skin Pharacol Physiol 26:217–226
51. Yamamoto A, Takenouchi K, Ito M (1995) Impaired water barrier function in acne vulgaris. Arch Dermatol Res 287:214–218
52. Yu G, Zhang G, Flach CR, Mendelsohn R (2013) Vibrational spectroscopy and microscopic imaging: novel approaches for comparing barrier physical properties in native and human skin equivalents. J Biomed Opt 18:061207. doi:10.1117/1.JBO.18.6.061207