J. Cosmet. Sci., **68,** 183–194 (March/April 2017)

Evaluation of molecules or extracts modulating seborrhea and its consequences, using normal human culture of sebocytes and keratinocytes, skin explants models and *in vivo* methods: a case study

PHILIPPE MONDON, ROBERTO DAL TOSO,
CAROLINE RINGENBACH, LAURENT LAVAISSIÈRE,
EMMANUEL DORIDOT, ÉMILIE OUVRAT, SANDRA BRAHIMI,
SEDERMA, 78612 Le Perray-en-Yvelines, France (M.P., R.C., L.L., D.E., 0.E., B.S.), IRB, 36077 Altavilla Vicentina, Italy (D.T.R.)

Accepted for publication August 31, 2016.

Synopsis

Skin produces sebum through sebocytes. Hyper-seborrhea creates conditions for the development of inflamed cutaneous alterations through bacteria colonization triggering dead cell accumulation and pro-inflammatory mediator release. Study of sebum production, its modulation, and its consequences requires complementary *in vitro* models in order to evaluate the effect of molecules on cell metabolisms. Clinical studies need to be performed to confirm *in vitro* results. Effects of phenylpropanoids, obtained by elicitation and purification from plant cell culture of *Syringa vulgaris* (CCSV), were studied on sebocytes, keratinocytes, and explants, all derived from normal human skins. Normal human sebocytes (NHSs) expressed markers such as cytokeratin-7, cytokeratin-4, and perilipin-2 (PLIN-2) (1); the latter being colocalized with lipid droplets. Lipid droplets clearly appeared and their size increased rapidly when lipogenic agents were used. NHS, normal human keratinocytes (NHK), and explants reacted to presence of bacterial fragments which trigger pre-inflammatory mediator release. CCSV reduced lipid storage and release of pre-inflammatory mediators in NHS, NHK and explants. CCSV also reduced *P. acnes* growth and triggered beta-defensin-2 and cathelicidin synthesis by NHS, two natural antimicrobial peptides. On volunteers, sebum production, inflamed blemishes, and retentional lesions were significantly reduced after 1 month treatment with CCSV.

INTRODUCTION

Sebaceous glands are found in the skin of almost all mammals and are present all over the human body except palms of the hands and soles of the feet. The glands are numerous on the face, where 400–900 glands/cm^2 can be found; ears, scalp, and upper part of the trunk are also rich in glands. Their main function is to produce sebum, a mixture of relatively

Address all correspondence to Pascaline Criton at pascaline.criton@sederma.fr.
Presented at the 28th Congress of IFSCC, October 27–30, 2014, Paris, France.

neutral lipids which are synthesized *de novo* by sebocytes. Immature sebocytes are located at the periphery of sebaceous glands and are flattened cells without lipids. When sebocytes progress to the center of the gland, they mature and increase in size due to lipid synthesis. Ultimately, as the cells differentiate, they disintegrate and release their lipid content into the infundibulum. Human sebum contains cholesterol (1.5–2.5%), cholesterol esters (3–6%), squalene (12–20%), free fatty acids (15–30%), triglycerides (30–50%), and wax esters (26–30%) (2).

Overproduction of sebum is a common scheme for teenagers all around the world; however, it is also widely distributed in the general population whatever the age, the gender, and the geographical zone. It makes the skin look oily and shiny and renders people less attractive; in addition, it creates conditions for the development of inflamed cutaneous alterations (3). Balance of lipids, such as linoleic and arachidonic acids, is usually modified; these acids act directly or indirectly on lipid storage increase through Peroxisome Proliferator Activated Receptors (PPARs) and perilipin expression. They also trigger production of inflammatory mediators such as interleukins (IL-6 and IL-8) and prostaglandin E2 (PGE-2) (4, 5). These mediators, among others, are key players in the increase of lipid synthesis by sebocytes, in the proliferation of microorganism such as *Propionibacterium acnes,* in keratinocyte hyper-proliferation, and in macrophage invasion of nearby tissues.

The lack of an ideal animal model comparable to human sebaceous glands was surmounted by the development of experimental models such as SEB-1, SEB-E6E7, and SZ95 cell lines all immortalized by SV40 (6,7) or using extracted human sebaceous glands. Growth of primary normal human sebocytes (NHSs) in monolayer was achieved by various teams; however, these models could not be multiplied because cells loose quickly their features. So, this model remains a challenge in order to evaluate molecules with a nontransformed cell.

This paper relates use of NHS to study modulators of lipid storage and variation of pro-inflammatory mediator synthesis within these cells. Among products that were evaluated, a plant cell culture extract of *Syringa vulgaris* (CCSV) showed significant effects on both lipid storage and on bacterial-induced pro-inflammatory mediator release. Complementary tests performed on NHK, macrophages, and skin explants confirmed the interest of CCSV for reduction of PGE-2, IL-6, and IL-8 induced by lipopolysaccharide (LPS) or *P. acnes*. Moreover, CCSV was evaluated on volunteers with greasy skin, where it reduced sebum synthesis and consequences of over lipid production (skin blemishes, hyperkeratinization).

MATERIALS AND METHODS

CELL CULTURE, SKIN EXPLANTS, AND REAGENTS

NHS. Mycoplasmas-free NHS cells, derived from facial sebaceous gland (woman, 26 years) and obtained in compliance with the procedures and international standards for donation and collection of human tissue used for cell isolation were used. They were amplified and subcultured in appropriate and defined cell culture medium containing 2–10% of fetal calf serum (FCS, Gibco®, Thermo Fisher Scientific Inc., Waltham, MA). Various cell medium were tested for NHS growth and lipogenesis assays, such as Dulbecco's Modified Essential Medium (DMEM), Dulbecco's Modified Essential Medium and Ham's F-12 Medium (DMEM-F12), Keratinocyte Serum-Free Medium (KSFM), Medium 154 (M154) completed with antibiotics

(100 U/ml penicillin, and 100 mg/ml streptomycin), 50 µg/ml EGF, and FCS. Mycoplasma removal treatment was achieved using Plasmocure® kit (Invivogen®, San Diego, CA) and followed regularly (MycoAlert® mycoplasma detection kit, Lonza®, Basel, Switzerland). For lipid detection, proliferative NHSs were seeded in 96-well flat and black bottom plate (Becton®, Rungis, France). When the cells reached confluence, contact between cells and compounds was performed for 24–72 h. Cell number and lipid synthesis were estimated, respectively, using fluorescein diacetate (FDA) and Red Nile fluorescent probes (both from Sigma®-Aldrich®, St. Louis, MO) (8). Results were obtained using a fluorescent reader (FLUOstar®, BMGLabteck®, Ortenberg, Germany) equipped with appropriate filters. For lipid *in situ* labelling, cells were fixed with Histochoice® (Clinisciences®, Nanterre, France) then incubated with Oil Red O® (Sigma®) solution and observed under microscope. For protein *in situ* labelling, cells were fixed with Histochoice® then permeabilized with 0.1% Triton® X-100 (Sigma-Aldrich®) in phosphate-buffered saline and blocked in 1% bovine serum albumin/0.05% Tween® 20 (Sigma-Aldrich®). Primary antibodies were as follows: anti-K7, anti-K4, anti-filaggrine, anti-involucrine, and anti-PLIN-2 (Santa Cruz Biotechnology®, Eurogentec®, Dallas, TX) (1). Species-specific secondary antibodies were conjugated to Alexa-Fluor® 488 (Life Technologies®, Liège, Belgium). Nuclei were counterstained with propidium iodide or Hoechst dye (Sigma-Aldrich®, Carlsbad, CA). For pro-inflammatory studies, cells received for 24 h both *Escherichia coli* LPS (Aldrich®) and CCSV (IRB®, Altavilla Vicentina, Italy) for 3 days. Release of PGE-2 was evaluated using EIA assay (Cayman®). AntiMicrobial Peptide (AMP) cathelicidin and beta-defensin-2 (hBD2) were measured after 3 days from cell lysates using ELISA assays (Hycult Biotech®, Peprotech®, Peprotech, Rocky Hill, NJ). Results were normalized with cells quantification through Hoescht 33258 (Uden, The Netherlands) method. Collagen lattices were prepared according to the method of Bell *et al.* (9). The collagen solution and normal human dermal fibroblast (NHDF), or NHS suspensions, were blended simultaneously for lattice formation. NHDF medium was DMEM with penicillin, streptomycin, and glutamine (DMEMc), whereas NHSs were in their medium, both containing FCS (10%). The lattice diameter was measured every day. Equivalent skins were prepared according to the method of Carlson *et al.* (10). NHS or NHK were seeded onto NHDF containing lattice in order to evaluate their respective capacities of formation of an epidermis with its stratum corneum. All studies were performed at 37°C in 5% CO_2.

Normal human keratinocytes (NHKs, CELLnTEC®, Bern, Switzerland) in KSFM with Bovine Pituitary Extract and 2.5 µg/ml Epidermal Growth Factor (EGF) (Gibco®) were seeded in plastic vessel until confluence was reached. Pro-inflammatory studies were performed as mentioned earlier. Release of PGE-2, IL-6, and IL-8 was measured in cell culture supernatants using Enzyme ImmunoAssay (EIA)/ELISA assays (Cayman® Cayman Chemical, Ann Arbor, MI) and Pelikine®, Sanquin, Amsterdam, The Netherlands); results were normalized as mentioned earlier. All studies were performed at 37°C in 5% CO_2.

For macrophages studies, RAW264.7 murine macrophages were cultivated up to confluence in DMEMc containing 10% FCS. They were treated according to Kim *et al.* (11) with LPS then with CCSV. The amount of nitrite was measured in cell culture supernatant using the Griess reagent (Cayman®). All studies were performed at 37°C in 5% CO_2.

Skin explants (0.5 cm^2, Biopredic®, Biopredic® International, Saint Grégoire, France) were obtained from abdominal region of a Caucasian woman (52 years). Upon receipt, they were transferred into culture media (MIL305, Biopredic®) and cultivated at 37°C in 5% CO_2. For studies, explants were in contact with culture media containing *P. acnes*

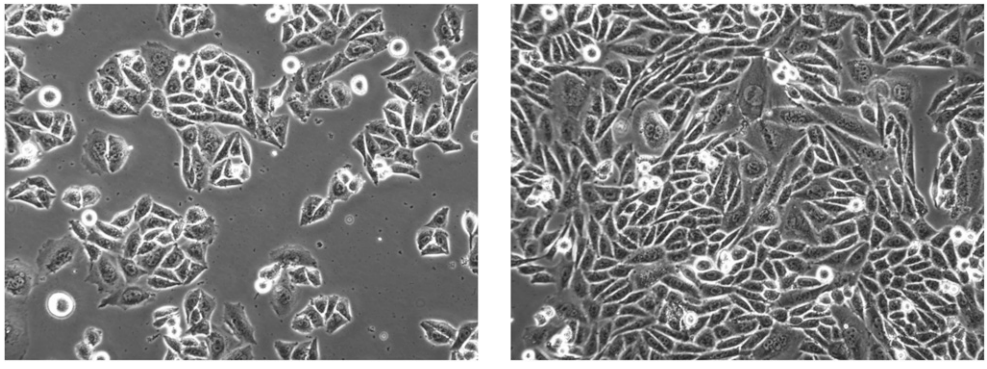

Figure 1. NHS at passage 7 in NHS-7 medium. Left: 2 days after seeding; right: 5 days. ×250.

inactivated cell extract (Sederma®, Sederma, Le Perray en Yvelines, France), prepared by freezing/thawing cycles. Cream containing 0.8 mM of CCSV (*i.e.,* 0.8 mM phenylpropanoids) was applied on skin surface; a placebo cream was applied as control. After 5 days of a daily topical application, release of PGE-2, IL-6, and IL-8 was measured as mentioned earlier, or explants were prepared for immunohistochemistry, being frozen and vertically sectioned (7–10 µm) using a cryostat (Leica® CM15105, Leica Biosystems Inc. Buffalo Grove, IL). Presence of Kallikrein-related peptidase 5 (KLK5), formerly known as stratum corneum tryptic *enzyme* (SCTE), a desquamation enzyme, which enables corneodesmosome division and corneocyte release to take place, was evaluated through immunolabelling performed using anti-SCTE antibody (Abcam®, Cambridge, MA). All studies were performed at 37°C in 5% CO_2. Statistical analysis values were expressed as means ±SDM of the results for at least three experiments. Anova and Student *t* test were used for comparisons; *p* values <0.05 were considered statistically significant.

IN-VIVO STUDIES

Several studies were performed between March 2013 and December 2013 to establish the *in vivo* efficacy of CCSV. Each individual study lasted 1 month. For each study and according to the protocol, volunteers applied by themselves a cream containing 0.8 mM CCSV and/or its vehicle. All the applications were performed on a daily basis in normal conditions of use. Each volunteer acted as his own control. Written informed consent was obtained from all participants. Medical control and noninvasive methods were used. Clinical studies comply with the latest recommendations of the World Medical Association (Declaration of Helsinki, 1964, and its successive updates) and with the French law 2004-806

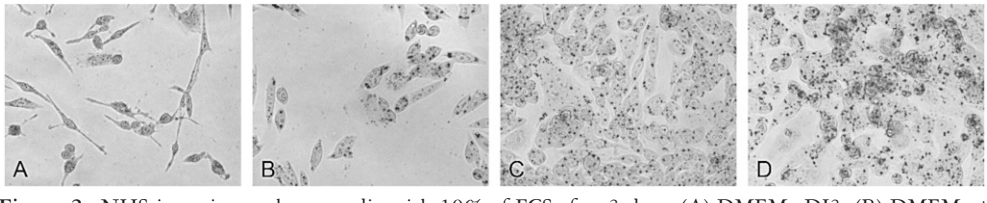

Figure 2. NHS in various culture media with 10% of FCS after 3 days: (A) DMEMc DI3, (B) DMEMc + EGF, (C) KSFM/BPE + EGF, (D) NHS7; Red Oil staining, ×500.

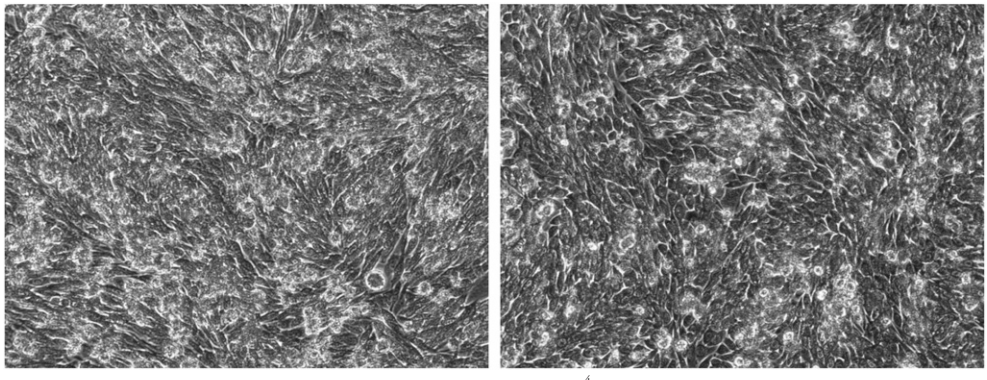

Figure 3. NHS in NHS-7 culture medium + linoleic acid 10^{-4} M at passage 8 (left picture) and 20 (right picture). Lipids are labeled using Red Oil; ×250.

dated August 9, 2004, concerning public health. Moreover, our studies follow the spirit of good clinical practices (ICH E6 GCP, 1996). As a noninterventional study, neither advice of an ethic committee nor submission to competent authorities are required. However, an internal ethic committee was asked to allow these studies.

For sebum production study, 23 volunteers (women, [19–32 years] mean: 24.5 years) were recruited, with oily skin and acne prone skin, some regularly had acne-type blemishes on the face. Cream containing 0.8 mM CCSV was applied daily onto hemiface, whereas the other hemiface received the corresponding placebo cream. Modulation of sebum production and number of active glands were evaluated using Sebufix® (Courage & Khazaka®) and image analysis through Mountains Map® software (Digital Surf®). Measurements were done one night after the last application.

Skin blemishes were evaluated on several panels (Sederma®, Cosmetest®) due to the difficulty in recruiting volunteers with blemishes in a sufficient number and intensity. Cream applications were the same as the precedent test. Blemishes were evaluated on standardized pictures made under cross polarized lighting with a photographic bench (Orion Concept®). The skin specific software Framescan® (Orion Concept®) was used to quantify blemishes based on redness threshold.

Hyperkeratosis was performed at BioEC® using cyanoacrylate biopsies on volunteers with oily skin and comedones on face (women and men, [18–45 years] mean: 32 years) after a daily application of a cream containing CCSV. Assessment was achieved after

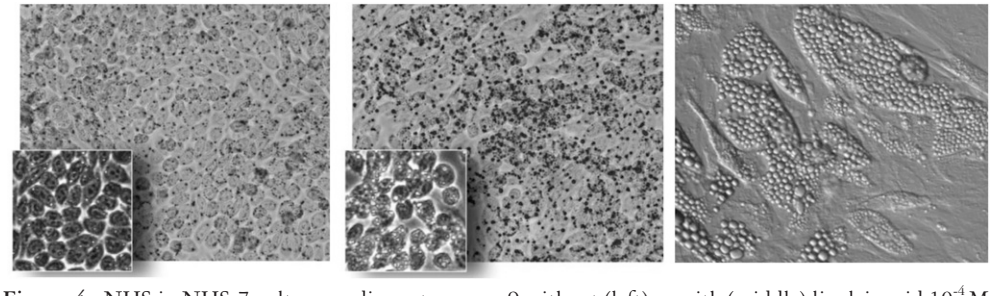

Figure 4. NHS in NHS-7 culture medium at passage 9 without (left) or with (middle) linoleic acid 10^{-4} M. Lipids are labeled using red oil; ×250. Right picture: human adipocytes.

Table I
Lipid Modulation in NHS Under Various Concentrations of CCSV. Lipids are Labeled Using Red Nile

	AFU[a]—Red Nile/10^6 cell	Variation (%)
Control solvent (mM)	49 727 ± 8202	Reference
CCSV 0.16	46 945 ± 2389	-5.6; nsd
CCSV 0.48	31 551 ± 1038	-36.6; $p < 0.05$
CCSV 0.8	20 936 ± 2213	-57.9; $p < 0.01$

[a]AFU: arbitrary fluorescent units; no cell toxicity was observed as compared to control.

Hemalun/Congo red staining and observations with microscope of orange masses at the pore opening. Perceived effect study was done on 100 volunteers (women and men, [18–30 years] mean: 24 years) with oily skin and acne-prone skin and regularly occurring pimples. Self-evaluations were performed by volunteers on cosmetic qualities and activities of the CCSV cream after 1 month of daily application on face. Statistical studies were performed using the Student's *t* test or if necessary, a Wilcoxon nonparametric test. In both case, bilateral tests were performed on paired series. χ^2 test was used for self-evaluations.

RESULTS

CELL CULTURE AND SKIN EXPLANTS

NHSs have various features and size in culture. They present mainly an epithelial morphology, but have a specific polygonal shape while some cells are rounded; once anchored, they grow as islets, spread on plastic within few days and increase their lipid storage (Figure 1). Cells did not grow without FCS, 5% FCS being the lowest concentration for NHS growth whatever the medium used. Cells grew and stored lipids in DMEM-F12 and KSFM + BPE, both completed with 25–50 µg/ml EGF, whereas proliferation was weaker than latter with DMEM or M154 (±EGF). DMEMc with dexamethasone, insulin, indomethacin, and IBMX (DMEMcDI3), used for pre-adipocyte differentiation studies, was toxic for NHS (Figure 2). A specific blend of medium and supplements, referred as NHS-7 medium, led to a maximum population doubling time and preserved lipid storage capacities up to 20 passages (Figure 3).

Moreover, NHS multiplied less in KSFM + BPE media containing high calcium content (0.8–1.8 mM) than in same media with low calcium concentration; high calcium concentrations triggered in NHS very low differentiation features as obtained with NHK. Weak involucrin and filaggrin labelling were observed, in agreement with Lee *et al.* (12).

Table II
PGE-2 Release by NHS in Contact with LPS and Under Treatment with CCSV. No Cell Toxicity was Observed as Compared to Control

PGE-2 (pg/10^6 cell)	Control (solvent)	CCSV 0.48 mM
No LPS	75.5 ± 6.1	38.6 ± 6.4
LPS 1.2 µg/ml	124.0 ± 6.3	88.5 ± 9.2
LPS 2. 5 µg/ml	156.2 ± 14.4	82.4 ± 11.4

Table III
PGE-2, IL-6, and IL-8 Release by NHK in Contact for 24 h with LPS. No Cell Toxicity Was Observed

	PGE-2 (pg/10^6 cell)	IL-6 (pg/10^6 cell)	IL-8 (pg/10^6 cell)
No LPS	31.8 ± 4.3	2.6 ± 0.8	278 ± 53
LPS 1.2 µg/ml	111.6 ± 13.3	4.3 ± 0.2	368 ± 38
LPS 2.5 µg/ml	275.5 ± 88.4	3.9 ± 0.4	476 ± 52
LPS 5 µg/ml	402.5 ± 73.3	7.0 ± 0.5	861 ± 113
LPS 10 µg/ml	810.9 ± 112.6	33.2 ± 3.2	2483 ± 253

Collagen-I coating, which helps NHK adhesion on plastic, did not modify significantly neither anchorage of NHS, *versus* noncoated plates, nor proliferation.

NHSs or NHDFs were embedded in collagen gels, respectively, in NHS-7 or in DMEMc both with 10% FCS, gel contraction was followed for 2 days. No contraction was observed with NHS (size reduction at day 2 vs. T0: 3%, nsd), whereas a quick and expected contraction was observed with NHDF after 1 day (size reduction vs. T0: 52%, $p < 0.01$).

NHSs or NHKs were seeded on NHDF containing collagen lattice in KSFM + BPE and EGF media. Epidermis formation and differentiation were followed for 14 days using histology. No cell spreading was observed with NHS, whereas NHK produced as expected an epidermis with its stratum corneum. When NHSs seeded on collagen lattice were cultivated in NHS-7 medium, cells spread horizontally first, then a multilayered "epidermis" was observed; however, it was composed of small, rounded cells. No specific differentiation features were observed (*i.e.,* neither spinous nor granular layers and no stratum corneum).

Increase in size of NHS lipid droplets was observed using Red Oil staining depending on incubation time, inducers, serum concentration. NHS droplets are as numerous as in human adipocytes but, conversely to adipocytes, they still remain small whatever the media used; moreover, NHSs are smaller cells than adipocytes (Figure 4).

As expected, lipid droplets are labelled with an anti-PLIN-2 antibody. Cells are positive for cytokeratin-7, and to a lesser extent for cytokeratin-4, which are, respectively, early and late differentiation markers of NHS (13,14). Rosiglitazone (0.1–10 µM), arachidonic acid (1–100 µM), linoleic acid (0.1–50 µM) all increased lipid storage into NHS from +44% to +665% (all $p < 0.01$, vs. solvent). Conversely, CCSV (0.16–0.80 mM) reduced lipid storage in a dose-dependent manner ($p < 0.01$, vs. solvent; Table I).

NHS produces PGE-2, which can be enhanced in a dose-dependent manner with LPS (1.2–2.5 µg/ml; Table II). CCSV (0.48 mM) was evaluated in parallel, showing an inhibition of PGE-2 release without or with LPS. These inhibitions reached, respectively, 49%

Table IV
PGE-2, IL-6, and IL-8 Release by NHK in Contact with LPS and CCSV. No Cell Toxicity Was Observed

		PGE-2 (pg/10^6 cell)	IL-6 (pg/10^6 cell)	IL-8 (pg/10^6 cell)
No LPS	No CCSV	31.8 ± 4.3	2.6 ± 0.8	278 ± 53
	CCSV 0.48 mM	23.1 ± 4.3	0,9 ± 0,13	121 ± 16
LPS 1.2 µg/ml	No CCSV	111.6 ± 13.3	4.3 ± 0.2	368 ± 38
	CCSV 0.48 mM	41.5 ± 5.5	1.68 ± 0.15	203 ± 15

Table V
PGE-2, IL-6, and IL-8 Release by Skin Explants in Contact for 24 h with LPS or *P. acnes*

	PGE-2 (pg/ml)	IL-6 (pg/ml)	IL-8 (pg/ml)
Control	220 ± 40	442 ± 30	278 ± 69
LPS 5 µg/ml	1635 ± 280	3038 ± 872	6650 ± 1019
P. acnes fragments	1569 ± 523	2419 ± 752	2079 ± 820

and 47% (no LPS and 2.5 µg/ml LPS, both $p < 0.01$). Similar results were observed with 0.16 and 0.8 mM CCSV.

Results showed that proinflammatory mediators are produced by NHK in response to stress with a dose-dependent manner. NHK, in contact with LPS (1.2–10 µg/ml), released PGE-2, IL-6, and IL-8 (Table III). PGE-2 release enhanced significantly by 3.5× and 25.5×, whereas IL-6 release was triggered by 1.65× and 12.7×, and IL-8 release was significantly enhanced by 1.3× and 8.9×, respectively, for 1.2 and 10 µg/ml. CCSV (0.48 mM) was evaluated in parallel without and with LPS; it significantly decreased the release of PGE-2, respectively, by -27% ($p < 0.05$) and 63% ($p < 0.01$), IL-6 -65% and -61%, (both $p < 0.01$) and IL-8 by -57% and -45%, (both $p < 0.01$); Table IV).

Pro-inflammatory mediators release was also studied on skin explants. Induction of PGE-2, IL-6, and IL-8 by skin explants was observed in contact with bacterial fragments. LPS and *P. acnes* fragments enhanced significantly PGE-2 release by x7.4 and x7.1, IL-6 by x6.7 and x5.5, and IL-8 by x23.9 and x7.5 (Table V). CCSV (0.8 mM) was evaluated in parallel; it inhibited the strong enhancement of PGE2 due to LPS or *P. acnes* fragments and maintained these release to their control level, same observation was made for IL-6, CCSV stabilizing the cytokine production to a basal level. For IL-8 release, CCSV was prone to regulate more efficiently its production: by -90% under LPS stimulus and by -34% with *P. acnes* (both $p < 0.01$; Table VI).

These results, obtained with NHS, NHK, and explants confirmed that bacterial fragments both from *E. coli* and *P. acnes* triggered proinflammatory mediators and indicated that CCSV controls their stress increase production.

Since Al Shobaili *et al.* (15) did show a correlation with acne vulgaris and oxidative and nitrosative stress, a study was performed on RAW 264.8 activated with LPS, in order to evaluate the control of NO release owing to CCSV. Results (Table VII) showed a dose-dependent decrease of NO release by -41% and -56% (both $p < 0.01$), respectively, with 0.16 and 0.48 mM CCSV.

It was shown that sebaceous glands and sebocytes participate to innate immunity by producing AMP such as β-defensin-2 and cathelicidin (16,17). AMPs were studied on NHS

Table VI
PGE-2, IL-6, and IL-8 Release by Skin Explants in Contact with LPS and CCSV

		PGE-2 (pg/ml)	IL-6 (pg/ml)	IL-8 (pg/ml)
LPS 5 µg/ml	Placebo cream	1635 ± 280	3038 ± 872	6650 ± 1019
	Cream with CCSV 0.8 mM	117 ± 33	390 ± 231	659 ± 259
P. acnes	Placebo cream	1569 ± 523	2419 ± 752	2079 ± 820
	Cream with CCSV 0.8 mM	171 ± 48	427 ± 216	1392 ± 518

Table VII
NO Release by RAW 264.8 in Contact with LPS and CCSV

	NO ($\mu M/10^6$ cell)
Control	49 ± 2.0
CCSV 0.16 mM	29 ± 2.0
CCSV 0.48 mM	22 ± 1.0

as they constitute a natural front line of defense against bacteria, virus, and fungi proliferation. We showed that NHS produced more cathelicidin and hBD2 in presence of CCSV than in the negative control. These two natural small proteins, with broad spectrum antimicrobial activity, were increased in a dose response manner (Table VIII) by 0.16 and 0.48 mM CCSV, respectively, by +17% and +39% (both $p < 0.01$) and by +25% and +47% (nsd; $p < 0.01$). These increases were in line with pro-inflammatory modulations (Tables II–VI).

IN-VIVO STUDIES

Sebum production and number of active glands were evaluated after 1 month of application of placebo cream or cream containing 0.8 mM CCSV. The analysis of the results demonstrated that placebo did not induce a variation whatever the studied parameter. In contrast, use of 0.8 mM CCSV for 1 month led to a significant decrease both in the quantity of sebum produced and in the number of active glands whether compared with T0 (-15% for both) or placebo: -18% ($p < 0.05$) and -15.8% ($p < 0.05$), respectively (Table IX).

Hyperkeratinization was evaluated through two complementary methods: SCTE labeling into skin explants and cyanoacrylate biopsies of stratum corneum. Results showed an increase (+40%) in SCTE labeling on skin sections after a daily application of cream containing 0.8 mM CCSV versus placebo cream (19.75 ± 1.75 vs. 14.1 ± 1.6; $p < 0.01$ vs. placebo). Moreover, microscopic assessment of the hyperkeratosis performed after staining (see materials) indicated that 83% of responders (10/12) experienced a decrease in their keratosis, whereas two of them experienced an increase.

Skin blemishes were evaluated on several panels due to the difficulty in recruiting volunteers with blemishes (see Materials). In the first study, there was an observed 38% decrease in the surface area of red blemishes (46.0 ± 28.2 → 28.6 ± 22.1; $p < 0.01$ vs. placebo, n = 12; Figure 5) and a high response level of 83%. In contrast, applying a placebo for 1 month led to no improvement of skin appearance. First results were confirmed in a second test (Cosmetest®). Dermatologist counting of blemishes indicated a 48%

Table VIII
Cathelicidin and hBD2 Variation by NHS in Contact with CCSV. No Cell Toxicity Was Observed as Compared to Control

	Cathelicidin (pg/ml/10^6 cell)	hBD2 (pg/ ml/10^6 cell)
Negative control	342 ± 8	42.0 ± 11.1
CCSV 0.16 mM	401 ± 23	52.5 ± 8.1
CCSV 0.48 mM	474 ± 29	61.6 ± 4.1

Table IX
Sebum Production and Number of Active Glands after 1 Month, n = 23 vol.
Placebo Cream or Cream with 0.8 mM of CCSV

	Sebum production (spot area in mm²)		Variation 1 m vs. T0	Number of active glands		Variation 1 m vs. T0
	T0	T1 m		T0	T1 m	
Placebo	1.45 ± 0.85	1.49 ± 0.98	+2.8%; nsd	341 ± 155	345 ± 158	+1.2%; nsd
CCSV	1.51 ± 0.92	1.28 ± 0.86	-15,2%; $p < 0.05$	384 ± 157	328 ± 154	-14.6%; $p < 0.09$
Significance CCSV vs. placebo		$p < 0.05$		–	$p < 0.05$	

decrease in the number of inflamed skin blemishes (7.08 ± 3.50 → 3.67 ± 2.64; $p < 0.01$ vs. placebo, n = 12). In parallel, placebo cream applied for 1 month did not significantly improve skin appearance, confirming first results. Third evaluation (Cosmetest®) showed a 21% decrease in the number of retentional lesions (13.65 ± 11.96 → 10.78 ± 9.57; $p <$ 0.01 vs. T0, n = 23, dermatologist). Conversely, there was no significant improvement on the placebo side.

A self-evaluation questionnaire was filled by a panel of 100 women and men (mean: 24 years, [18–30 years]) claiming to have acne-prone oily skin and regularly occurring pimples. They analyzed the cosmetic qualities and activities of a cream containing 0.8 mM CCSV applied once a day for 1 month. Figure 6 shows results demonstrating the positive perception of the product and highlighting the multiple effects perceived by the volunteers (*e.g.*, less redness, less shininess, less severe pimples).

DISCUSSION/CONCLUSION

Complementary biological models and methods are of interest for the study of highly complex skin phenomena such as seborrhea and its consequences. *In vivo* studies are essential to validate the effect of active molecules but clinical evaluations are not appropriate for the selection of candidates. However, only a few sebocyte models now exist to screen and to study the effect of molecules aside from cell lines. The development of a NHS primary culture allows focusing more physiologically on lipid metabolism under the contact of both positive and negative modulators of lipid synthesis such as linoleic

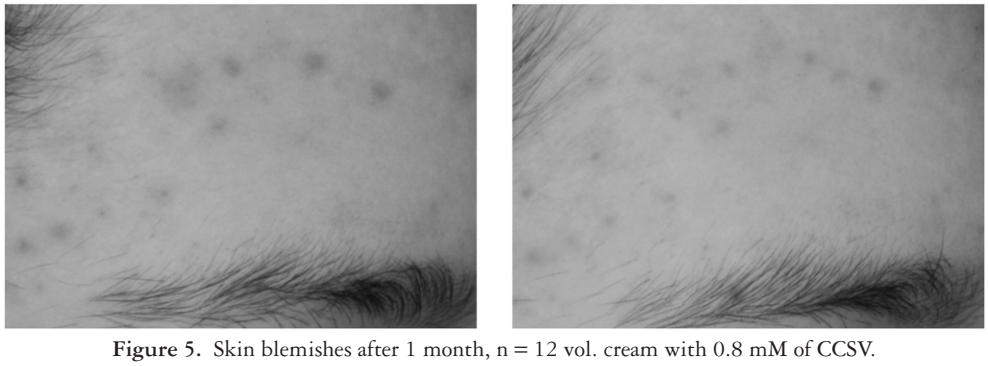

Figure 5. Skin blemishes after 1 month, n = 12 vol. cream with 0.8 mM of CCSV.

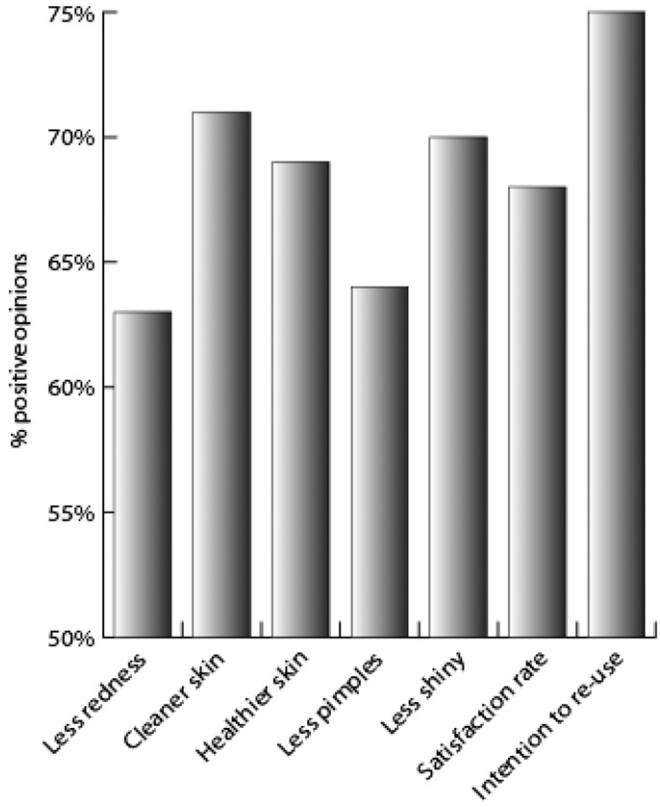

Figure 6. Percent of satisfied opinions after 1 month, n = 100 vol. cream with CCSV 0.8 mM. All bars indicate a significant variation versus negative opinions with $p < 0.01$ (χ^2 test).

acid, arachidonic acid, rosiglitazone, and CCSV. Moreover, the release of proinflammatory mediators under various external stresses (lipids and bacterial fragments) could be easily followed and results were compared to data obtained with keratinocytes, macrophages, both neighboring cells of sebocytes, or explants models. CCSV was studied here with various skin-derived cell models and *in vivo* results indicated its interest as a bioactive ingredient for the control of hyperseborrhea and its negative consequences.

REFERENCES

(1) M. Dalhoff, E. Camera, M. Picardo, C. C. Zouboulis, L. Chan, B. H. Chang, and M. R. Schneider, PLIN2, the major perilipin regulated during sebocyte differentiation, controls sebaceous lipid accumulation in vitro and sebaceous gland size in vivo., *Biochem. Biophys. Acta.*, **1830**, 4642–4649 (2013).
(2) Ottaviani M., Camera E., and Picardo M., Lipid Mediators in Acne, *Mediat. Inflamm.,* **2010**, 1–6 (2010).
(3) M. Picardo, M. Ottaviani, E. Camera, and A. Mastrofrancesco, Sebaceous gland lipids, *Dermato-Endroc.,* **1**, 68–71 (2009).
(4) N. Akimoto, T. Sato, C. Iwata, M. Koshizuka, F. Shibata, A. Nagai, M. Sumida, and A. Ito, Expression of perilipin A on the surface of lipid droplets increases along with the differentiation of hamster sebocytes *in vivo* and *in vitro, J. Invest. Dermatol.,* **124**, 1127–1133 (2005).
(5) B. T. Toth, A. Olah, A. G. Szollosi, G. Czifra, and T. Biro, Sebocyte makeup: Novel mechanism and concepts in the physiology of the human sebaceous glands, *Pflugers Arch-Eur J. Physiol.,* **461**, 593–606 (2011).

(6) D. Thiboutot, S. Jabara, J. McAllister, A. Sivarajah, K. Gilliland, Z. Cong, and G. Clawson, Human skin is a steroidogenic tissue: steroidogenic enzymes and cofactors are expressed in epidermis, normal sebocytes, and an immortalized sebocyte cell line (SEB-1), *J. Invest. Dermatol.,* **120,** 905–914 (2003).
(7) C. Zouboulis, C. H. Seltmann, H. Neitzel, and C. E. Orfanos, Establishment and characterization of an immortalized human sebaceous gland cell line (SZ95), *J. Invest. Dermatol.,* **113,** 1011–1020 (1999).
(8) P. Greenspan, E. Mayer, and S. D. Fowler, Nile red: A selective fluorescent stain for intracellular lipid droplets, *J. Cell Biol.,* **100,** 965–973 (1985).
(9) E. Bell, B. Ivarsson, and C. Merrill, Production of a tissue-like structure by contraction of collagen lattices by human fibroblasts of different proliferative potential in vitro, *Proc. Natl. Acad. Sci. U.S.A.*, **76,** 1274–1278 (1979).
(10) M. W. Carlson, A. Alt-Holland, G. Egles, and J. A. Garlick, Three dimensional tissue models of normal and diseased skin, *Curr. Protoc. in Cell Biol.,* Chapter Unit:19.9, Vol. 41,19.9 .1-19.9.17. (2008)
(11) J. Kim, M. T. Ochoa, S. R. Krutznik, O. Takeuchi, S. Uematsu, A. J. Legaspi, H. D. Brightbill, D. Holland, W. J. Cunliffe, S. Akira, P. A. Sieling, P. J. Godowski, R. L. Modlin, Activation of toll-like receptor 2 in acne triggers inflammatory cytokine responses, *J. Immunol.,* **169,** 1535–1541 (2002).
(12) W. J. Lee, K. H. Park, H. W. Cha, M. Y. Sohn, K. D. Park, S. J. Lee, D. W. Kim, The expression of involucrin, loricrin and filaggrin in cultured sebocytes., *Ann. Dermatol.,* **26,** 134–137 (2014).
(13) E. Hinde, I. S. Haslam, M. R. Schneider, E. A. Langan, J. E. Kloepper, C. Schramm, C. C. Zouboulis, and R. Paus, A practical guide for the study of human and murine sebaceous glands *in situ, Exp. Dermat.,* **22,** 631–637 (2013).
(14) C. C Zouboulis, L. Xia, B. Korge, H. Gollnick, and C. E. Orfanos, "Cultivation of human sebocytes *in vitro*: Cell characterization and influence of synthetic retinoids," in *Retinoids: 10 Years On*, J. H. Saurat. Ed. (Karger, Basel, 1991), pp. 254–273.
(15) H. A. Al-Shobaili, A. A. Alzolibani, A. A. Robaee, A. R. Meki, and Z. Rasheed, Biochemical markers of oxidative and nitrosative stress in acne vulgaris: Correlation with disease activity, *J. Clin. Lab. Anal.*, **27,** 45–52 (2013).
(16) D. Y. Lee, K. Yamasaki, J. Rudsil, C. C. Zouboulis, G. T. Park, J. M. Yang, and R. L. Gallo, Sebocytes express functional cathelicidin antimicrobial peptides and can act to kill propionibacterium acnes, *J. Invest. Dermatol.*, **128,** 1863–1866 (2008).
(17) M. R. Benakanakere, Q. Li, A. V. Singh, J. Zhao, and D. F. Kinane, Modulation of TLR2 protein expression by miR-105 in human oral keratinocytes, *J. Invest. Dermatol.,* **284,** 23107–23115 (2009).