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Characterisation of *Cutibacterium acnes* phylotypes in acne and *in vivo* exploratory evaluation of Myrtacine®

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Abstract

Objective Our main objective was to compare *Cutibacterium acnes* (*C. acnes*) skin colonisation in patients with mild to moderate acne versus healthy controls and secondly, to evaluate a Myrtacine®-based cream on *C. acnes* total population and antibioresistant *Cutibacteria* in patients with acne.

Methods In 60 acne patients (Global Acne Severity Scale, GEA grades 2–3), of mean age 20 [15–30] years and in 24 age- and sex- matched healthy controls, forehead strips samplings were performed for microbiological analysis of comedones by colony forming unit (CFU) counts of global *C. acnes* and erythromycin (EryR) or clindamycin-resistant (ClnR) populations of *Cutibacterium* and determination of phylotypes by MALTI-TOF. Clinical evaluations of acne patients (GEA, lesion count, porphyrin fluorescence) were performed at baseline and after 56 days of twice-daily application of a Myrtacine®-based cream.

Results We first showed (i) high and similar levels of *C. acnes* colonisation in superficial pilosebaceous follicles and detection of EryR and ClnR strains in both acne and control groups; (ii) different repartition of phylotypes in acne patients versus healthy control, with a predominance of phylotype IA in acne patients and a link between phylotype IA and erythromycin resistance. Besides, after treatment with the Myrtacine®-based cream in acne patients, there was no change in *C. acnes* total load, but a significant decrease of EryR *Cutibacteria,* reduced porphyrin production by *C. acnes,* a decrease in acne severity (GEA), associated with reduced retentional and inflammatory lesions.

Conclusion *Cutibacterium acnes* colonisation was not significantly different in acne versus control groups. Phylotype IA was predominant in acne patient and in EryR *C. acnes*. A Myrtacine®-based cream significantly reduced the level of EryR *Cutibacteria in vivo* and improved acne lesions.

Conflict of interest disclosure

T. Nocera, C. Peraud and V. Mengeaud are employees from Pierre Fabre laboratories. Fonderephar and CRCINA received fees to conduct the study.

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Introduction

Very recently, there has been a paradigm shift in our understanding of the role of *Cutibacterium acnes* (*C. acnes*, formerly *Propionibacterium acnes*) in the pathophysiology of acne. Instead of resulting from *C. acnes* hyperproliferation, acne would rather result from the loss of balance between the different phylotypes of *C. acnes*, in addition to an impairment of the skin microbiome equilibrium[1,2] (B. Dréno *et al.*, this issue). Many recent studies thus focus in determining the role of *C. acnes* and its different phylotypes in inflammatory acne.

As regards acne therapy, in mild to moderate acne, topical treatments are preferred,[3–5] including antibiotics targeting

C. acnes. However, epidemiological studies have shown a marked increase in the frequency of topical antibiotic resistance in acne subjects – from 20% in 1979 to 64% in 2000 – especially resistance to erythromycin and clindamycin.[6,7] As reviewed by Dréno *et al.* (this issue), *C. acnes* resistance may be due to several contributing mechanisms, including the formation of biofilm,[8] which can act by restricting penetration of antibiotics.[7]

Myrtacine® (*Myrtus communis* extract) has been demonstrated to be efficient on *C. acnes* biofilm alone or combined with antibiotics using *in vitro* models.[9] Furthermore, by targeting the biofilm, Myrtacine® is able to potentiate the bactericidal activity of antibiotics and reduce *C. acnes* counts even with strains resistant to erythromycin, *in vitro*.[9]

With the aim to further understand the role of *C. acnes* and its different phylotypes in acne pathogenesis, as a main objective of this work, we compared total *C. acnes* load and antibiotic-resistant *Cutibacterium* populations, as well as *C. acnes* phylotype repartition in the skin of acne patients with that observed in the skin of age- and sex-matched healthy controls. In addition, as a secondary objective, we determined the effect of a Myrtacine®-based cream targeting the biofilm of *C. acnes* in an open exploratory evaluation and quantified *C. acnes* populations (total and antibiotic-resistant strains) in patients with mild to moderate acne.

Participants and methods

Study design

This study was carried out at the Dermatology Department – CHU Nantes (France) and at the Clinical Skin Research Center, Pierre Fabre, Toulouse (France), in accordance with the ethical principles of the declaration of Helsinki and the guidelines for Good Clinical Practice. The protocol was approved by the Ethics Committee (Comité de Protection des Personnes (CPP) Ouest IV) and the ANSM (French National Agency for Medicine and Health Product Safety). Each volunteer signed a written informed consent.

Participants

Acne patients were included by a dermatologist. They were aged ≥15 years with mild to moderate acne (Global Acne Severity Scale[10] (GEA) II to III), presenting at least five papules and pustules on the face (not located on the nasal pyramid), and at least six closed and open comedones on the face, including at least three on the forehead. Exclusion criteria were presence of any cutaneous lesion affecting the face apart from ongoing acne (i.e. vitiligo, psoriasis and seborrhoeic dermatitis…), or any chronic or acute progressive disease, which may interfere with the study. Patients who applied facial topical treatments during the preceding month including anti-acneic agents (retinoids, zinc, benzoyle peroxide), antimicrobial agents (antibiotics, antiseptic…), corticosteroids or nonsteroid anti-inflammatory drugs (NSAID) or patients who took systemic anti-acneic treatment during the preceding month with antibiotics (tetracycline, macrolides, macrolide derivatives) or zinc, or patients treated with NSAID, corticosteroid, or antibiotics other than anti-acneic taken during the preceding month were not included. We also excluded patients treated with oral retinoids treatment or any hormonal treatment for contraceptive or anti-acneic purpose initiated or modified during the 12 preceding weeks. During treatment, none of the aforementioned treatments was allowed.

The group of healthy controls was constituted by volunteers, free of facial or dorsal acne and of any facial dermatosis, with no history of acne nor acne treatment in the brotherhood, who did not use any local treatment nor any antibiotic, anti-inflammatory or anti-histaminic oral treatment during the four preceding weeks, were age- and sex-matched with the acne patients included in the study group.

Interventions

Three visits were performed for acne patients: at inclusion (D0), intermediate (D28 ± 3 days) and at the end of the study (D56 ± 7 days).

At baseline, microbiological sampling was performed on 24 healthy controls and 60 acne patients for microbiological analysis. Besides, in the patients only, the dermatologist performed a clinical examination of the face at D0, D28 and D56. Concomitant treatments, adverse events and treatment compliance were recorded. Finally, another microbiological sampling was realised on acne patients at D56.

Study product

The study product was a cream (Ducray Laboratory, France) containing *Myrtus communis* leaf extract. It was applied twice daily, on the whole face, after cleaning with a hygiene product (soothing foaming gel).

Outcomes

The main outcome was the comparison of the number of CFU (Colony Forming Units) of *C. acnes* and total *C. acnes* (qPCR) in healthy controls versus acne patients.

The secondary criteria were:

- The quantification of the amount of *Cutibacterium* spp. CFU resistant to Erythromycin or resistant to Clindamycin in healthy controls and acne patients;
- The determination of the *C. acnes* phylotypes among *C. acnes* and among EryR *C. acnes* in healthy controls and acne patients
- The change in total *C. acnes* load and in *Cutibacterium* spp. resistant to Erythromycin and to Clindamycin after 56 days of application of a Myrtacine®-based cream in patients with acne, by measuring the amount of CFU in noninvasive sampling of comedones at D0 and D56
- Follicular porphyrin fluorescence quantification by imaging analysis based on UV standardised photography[11] after 56 days of application of a Myrtacine®-based cream

- Determination of the evolution of retentional and superficial inflammatory lesions globally with acne severity using GEA scale[10] and through the count of lesions on the face using ECLA scale[12] at baseline, after 29 and 56 days of application of a Myrtacine®-based cream
- Safety assessment: all adverse events were documented throughout the study. Local tolerance was assessed during visits (D28, D56) using a 4-point scale from 1 = very good tolerance (no functional symptom or physical sign) to 4 = poor tolerance (functional symptom and/or physical sign leading to treatment discontinuation).

Methods of measurement

Microbiological sampling Comedone sampling of the same area of the face (located with a tracking mask) was performed with Purifying Strips (Strips Purifiants, Laboratoires Diadermine, Henkel, Boulogne Billancourt, France) applied on the forehead of subjects after humidification of the skin. After 15 min, strips were removed and transferred into 20 mL of modified reduced RTF[13] at ambient temperature and stored for less than 48 h at room temperature before microbiological analysis.

Measurement of the bacterial load of Cutibacterium *spp. (resistant or not) by culture and isolates collection* One millilitre of each sample was transferred in 9 mL of neutralising broth (10% Tween 80, Sigma; 2% lecithin, Acros; 2% saponin, Acros; 0.5% sodium thiosulfate, Sigma; in chloride-peptone buffered solution, bioMérieux) to neutralise components from the strip that could inhibit bacterial growth, and serially diluted in sterile distilled water. One hundred µL of each dilution was spread onto Columbia agar supplemented with 5% sheep blood (COS, Biomérieux, Marcy l'Etoile, France) and on COS supplemented with erythromycin (2 µg/mL; Sigma-Aldrich) and COS + clindamycin (4 µg/mL; Sigma-Aldrich). The clinical breakpoint we used for clindamycin was that defined by EUCAST[14] for the determination of bacterial resistance for anaerobic gram-positive bacilli. As the clinical breakpoint of Erythromycin has not been defined by CLSI or EUCAST, we used the clinical breakpoint proposed by Ishida *et al.* in 2008.[15] Petri dishes were incubated at 37 ± 2°C under anaerobic atmosphere (Genbox, bioMérieux) for 5 days. Morphologically different types of colonies were described and counted. *Cutibacterium* colonies identification (small, round, domed, opaque, white to off-white sometimes ß-haemolytic) was assessed by Gram staining, the incapacity to grow aerobically, and biochemical tests (API ID32A, bioMérieux).

Ten representative colonies (selection based on morphotypes and CFU counts) from COS and three colonies from COS+erythromycin and COS+clindamycin (when present) of each subject were also maintained in a cryoprotective solution at −80°C for type determination of *C. acnes*.

Measurement of the C. acnes *load by quantitative PCR (qPCR)* Bacterial DNA was extracted from 9 mL of sample with the DNA mini kit (Qiagen, Courtabœuf, France) according to the manufacturer's instruction, except for the first step where bacteria were pelleted (1452*g*, 4°C for 20 min) and suspended in 180 µL TET + lysozyme buffer (Tris 20 mmol/L, EDTA 2 mmol/L, Triton-X100, lysozyme 20 mg/mL, Sigma-Aldrich) before incubation at 30 min for 37 ± 2°C. At the end of the procedure, DNA was eluted in 200 µL of elution buffer and stored at −20°C before qPCR.

DNA samples were thawed before q-PCR and 10-times diluted to reduce the inhibitors concentration. The qPCR reaction, primers and probes were described by Miura *et al.*[16] Q-PCR was made using the Sso advanced Universal Probes Supermix 2X (Bio-Rad), on a MyiQ™ (Bio-Rad). Standard curve was made with calibrated genomic DNA (gDNA) from *C. acnes* (CIP 53.117T). Each sample was analysed in triplicates. A positive control was made for every sample (addition of 1 µL gDNA of *C. acnes* CIP 53.117T to the reaction). For each run, negative controls (without DNA) and negative extraction control (DNA extraction on a mock sample) were also made.

Determination of C. acnes *phylotypes by MALDI-TOF (Matrix Assisted Laser Desorption Ionisation-Time of Flight)* Each of the 10 representative clones of *C. acnes* (+3 resistant clones, if existing) stored at −80°C was thawed and cultured for 72 h on COS at 37°C. As a result, only phylotypes present in a proportion over 90% were detected.

The MALDI-TOF method described by Nagy *et al.*[17] was used to type *C. acnes* colonies – as well as to confirm the identification at the species level. Analyses were made with a Microflex LT MALDI-TOF (Bruker) using the MTB Compass IVD software (Bruker). Strain identification at the species level was performed with MALDI Biotyper IVD Compass software and its database (DB5989). Phylotypes were determined for each spot with a MALDI Biotyper typing software prototype for *C. acnes* developed by Bruker.

Statistical analysis

As this was a pilot exploratory study, and in the absence of data from the literature, there was no rational basis to determine the number of healthy controls. The 60 patients included allowed to use the normal distribution to describe data variability. Microbiological variables were described by quantity of *C. acnes* load, using the derived variable (log10 (CFU or GU/strip) of *C. acnes*), and per cent of subjects. The statistical comparison between healthy controls and acne patients at D0 in *C. acnes* total load and EryR *C. acnes* (log CFU) was performed by Student's *t*-test, performed at a significance level of 0.05. Comparisons of proportions of patients were tested with the chi-square test, also performed at a significance level of 0.05. Regarding the analysis of product efficacy, it was performed on the FAS

population, with quantitative variables expressed as mean and median values, standard deviation and range and qualitative variables as frequencies and percentages. Comparisons between D0 and D28 or D56 were performed using the paired Student's *t*-test or the Wilcoxon signed-rank test, at a significance level of 0.05 ($P < 0.0001$ was considered very significant).

Results

Demographic and baseline data

Twenty-four individuals with no history of acne were included as healthy controls (15 female, mean age 21.6 years). They were age- and sex-matched with sixty patients affected with acne vulgaris (85% mild acne GEA grade 2, 15% moderate acne, GEA grade 3). Patients aged 20 years (range: 15–30 years) were mostly female ($n = 37/60$). Clinical characteristics of the patients' population are described in Table 1.

Main objective: Comparison of *C. acnes* population in healthy controls and acne patients

C. acnes *load* Only two species belonging to *Cutibacterium* genus were recovered: *C. acnes* and *C. granulosum*. Mean CFU (culture) and GU (qPCR) measurements (±SD) of *C. acnes* loads both showed a high total colonisation by the species both in healthy controls (5.94 ± 1.01 log (CFU/strip) and 5.62 ± 0.76 log (GU/strip)) and in acne patients (6.33 ± 0.19 log (CFU/strip) and 5.62 ± 0.71 log (GU/strip)). No significant difference between healthy and acne groups was noted in log (CFU/strip) (*P*-value calculated by Student's *t*-test = 0.117). Concerning *C. granulosum* carriage, only three healthy controls (12.5%) versus 14 patients with acne (23.3%) were concerned.

Resistant isolates of Cutibacterium Among the 14 acne patients carrying *C. granulosum*, EryR or ClnR resistant isolates were detected in 10 patients. Furthermore, in 5 patients, *C. granulosum* was recovered only from selective agar containing antibiotics. Considering the apparent high proportion of resistant clones of *C. granulosum*, the evaluation of EryR and ClnR populations was performed on *Cutibacterium* spp.

Table 1 Demographic and clinical characteristics at inclusion

Baseline demographic characteristics	Patients FAS population *N* = 60
Sex	
Male/female	23/37
Age (years), Mean ± SD Median [Min–Max]	
Whole population, *N* = 60	20.22 [15–30]
Dermatological examination	
Presence of particular signs apart from acne	No
Evaluation of facial acne severity with GEA scale	
Grade 0–1 (no or almost no acne lesions)	0
Grade 2 (mild)	51
Grade 3 (moderate)	9
Grade 4–5 (Severe or very severe)	0

Resistance to erythromycin. Erythromycin-resistant strains (EryR) of *C. acnes* and *C. granulosum* were detected in 56.7% of acne patients at baseline ($n = 34/60$), and 54.1% of healthy controls ($n = 13/24$), without significant difference in terms of bacterial load between the two groups (5.05 ± 0.93 log CFU/strip and 4.56–0.95 log CFU/strip, respectively; $P = 0.115$).

Resistance to clindamycin. Clindamycin-resistant *C. acnes* and *C. granulosum* strains were also detected (3–6 log CFU/strip) in both acne patients (10%, $n = 6/60$) and healthy controls (12.5%, $n = 3/24$).

C. acnes *phylotype populations among healthy controls and acne patients* The analysis of *C. acnes* phylotype repartition was performed on a total of 1531 *C. acnes* isolates from healthy controls ($n = 257$) and acne patients ($n = 1274$).

Among *C. acnes* isolates collected in the patient and control groups, the repartition of *C. acnes* phylotypes showed a skewed distribution associated with acne, with a 1.54-fold increase in the proportion of phylotype IA compared with healthy samples (40% vs 26%). On the reverse, phylotypes IB-IC were 1.46-fold less frequent in acne samples (24% vs 35%). The proportion of phylotype II was similar between acne and healthy subjects (25% vs 26%). Besides, 11% (acne patients) to 13% (healthy subjects) of the *C. acnes* isolates could not be associated to any phylotype, even though the identification at the species level was assessed by biochemical tests and by mass spectrometry and typing was confirmed twice by mass spectrometry. Nevertheless, all these isolates presented similar spectral characteristics and were thus classified into the same «unknown» group. Only one isolate of phylotype III was found in each group of subjects. Concerning the diversity of phylotypes per patient, it was observed that, in the control group, 70.8% of the subjects carried less than two major phylotypes and the maximum number of phylotypes detected per subject was four. In the acne group, only 37.3% of patients carried less than two major phylotypes, and the maximum number of phylotypes was 6 ($P = 0.019$).

When considering the proportions of subjects carrying certain phylotypes, important variations were also observed between acne patients and healthy controls (Fig. 1a). In particular, it was observed that phylotype IA was carried by 73.3% of acne patients versus 50.0% of the healthy controls ($P < 0.05$). On the contrary, a higher proportion of healthy controls carried the IB_IC phylotypes (75.0%) compared with acne patients (55.0%), although the difference was not statistically significant. Proportions of patients carrying phylotypes II did not vary significantly between healthy controls and acne patients (about 45%).

Phylotypes in patients carrying an Erythromycin-resistant isolate of C. acnes To get a better idea of the phylotypes potentially involved in resistance in *C. acnes*, subpopulations of healthy controls and acne patients carrying an EryR strain were studied.

Phylotype IA was dominant among EryR strains. However, its occurrence differed from 62.5% in the healthy group to 92.6% in the acne group (Fig. 1b, $P < 0.05$). The load of phylotype IA EryR isolates was also significantly inferior in the healthy group compared with the acne group ($P < 0.05$) while the total load of phylotype IA was similar between both groups (Fig. 1c).

Secondary objective: Effects of a Myrtacine®-based cream on *C. acnes* populations in acne patients

Total C. acnes *load before and after 56 days of treatment* The change in viable and culturable *C. acnes* load in treated acne patients, assessed by culture counts, was not significantly different (−0.05 ± 0.62 log CFU/strip) between D0 (6.33 ± 0.99) and D56 (6.28 ± 1.13). Those results were confirmed by qPCR (i.e. measuring viable as well as nonviable and unculturable *C. acnes*), with a nonsignificant change in amount of *C. acnes* (0.05 ± 0.61 log GU/strip) between D0 (5.62 ± 0.71) and D56 (5.67 ± 0.77).

Antibiotic-resistant C. acnes *and* C. granulosum *load before and after 56 days of treatment* After treatment, the load in EryR *C. acnes* and *C. granulosum* (log CFU/strip) decreased from 4.95 ± 0.99 at D0 to 4.76 ± 1.05 at D56 (Wilcoxon, $P = 0.024$). In parallel, the load in *C. acnes* ClnR isolates decreased – although not significantly – from 4.62–0.85 log (CFU/strip) at D0 to 4.34 ± −0.96 log (CFU/strip) at D56.

Production of porphyrines by C. acnes *after 56 days of treatment* After treatment, reduction in porphyrines production by *C. acnes* was observed in open comedones, follicles, and inflammatory lesions, of −23% (Student's test $P < 0.0001$) in surface and −25% in the volume of fluorescence measured on the standardised pictures at D56 versus D0 (Student's test $P < 0.0001$) (Fig. 2).

Evolution of acne severity according to GEA scale after 29 and 56 days of treatment At the end of treatment, 38 of 60 subjects (63%) had experienced a highly significant ($P < 0.0001$) decrease in their severity score compared with inclusion, and they were already 18 (30%) at D28 ($P < 0.0001$) (Fig. 3a).

Evolution of retentional and inflammatory acne lesions according to ECLA grading after 29 and 56 days of treatment ECLA score significantly decreased at the intermediate

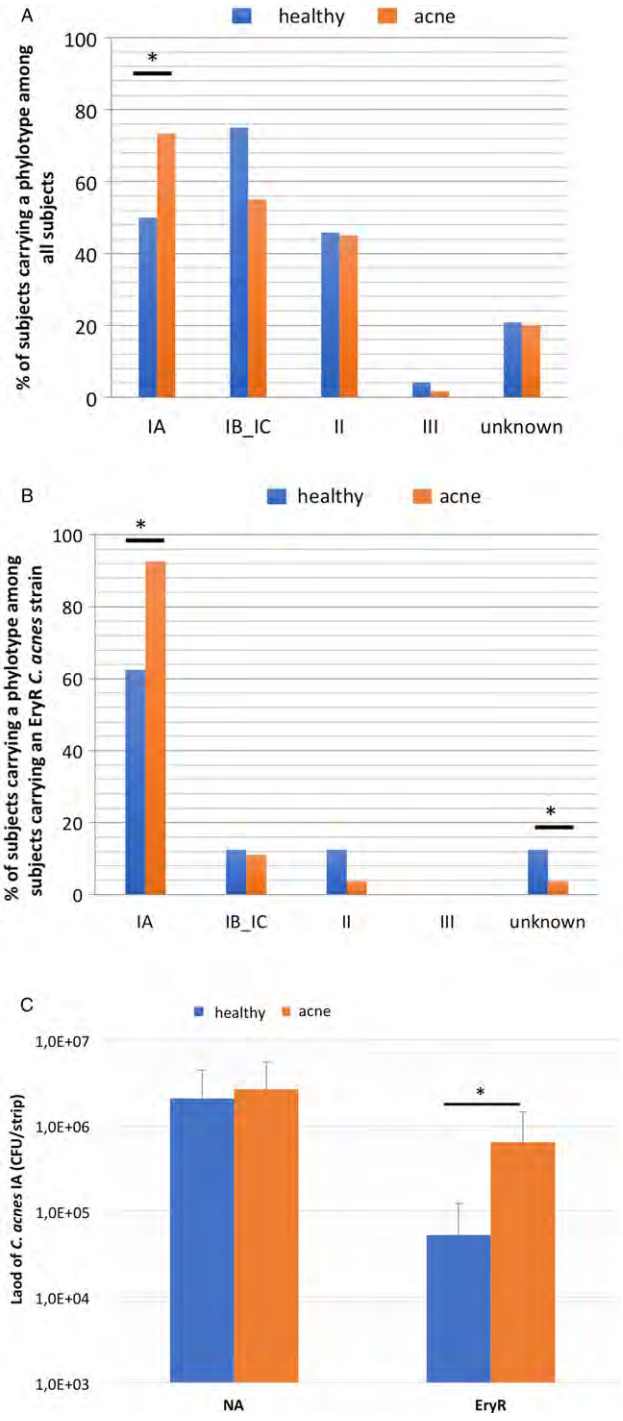

Figure 1 Comparison of *C. acnes* phylotypes in healthy and acne patients. (A) Percentage of subjects carrying different phylotypes among all subjects (*$P < 0.05$). (B) Percentage of subjects carrying different phylotypes among subjects carrying an EryR strain (*$P < 0.05$). (C) Density of phylotypes IA (NA:all, EryR: Erythromycine resistant) (*$P < 0.05$).

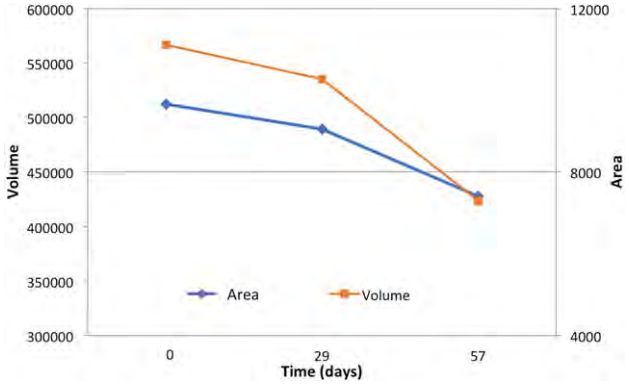

Figure 2 Reduction of porphyrines production by *C. acnes* after Myrtacine®-based cream treatment.

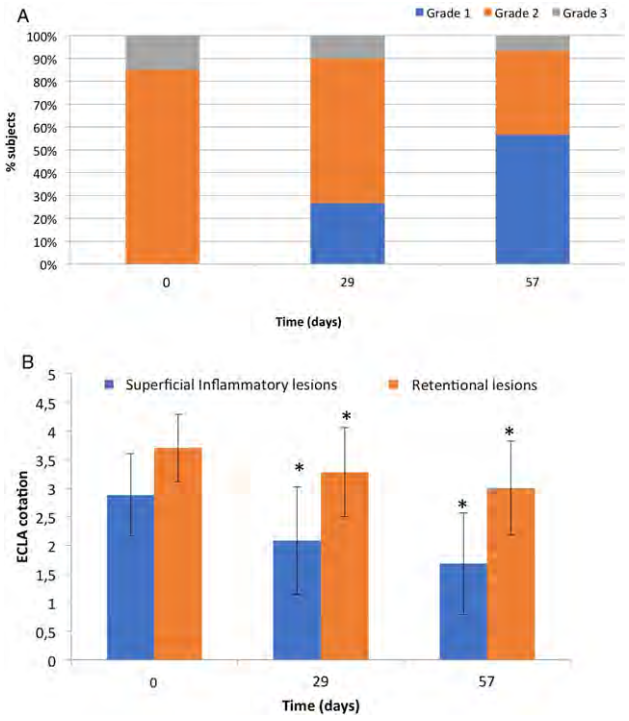

Figure 3 (A) Acne severity (GEA grades) among the 60 acne patients according to time. (B) Reduction of acne lesions (measured by ECLA) after Myrtacine®-based cream treatment *$P < 0.0001$.

visit (D28) and further at the end of treatment, for both retentional and superficial inflammatory lesions ($P < 0.0001$) (Fig. 3b). At D56 versus D0, 37 patients (62%) showed an improvement of their ECLA score for retentional lesions, including 12 with a reduction of two grades, and 52 patients (87%) had an improvement of their ECLA score for superficial inflammatory lesions, including 19 with a reduction of two grades.

Safety

There was no serious adverse event during the study and among the adverse events recorded, none was suspected to be related to the study product. Topical tolerance was rated "very good" for 100% of the subjects.

Discussion

Using new microbiological techniques, this exploratory study showed that the *C. acnes* population of forehead follicles of patients with acne and healthy controls did not differ in terms of *C. acnes* counts, confirming that acne is not associated with an over-proliferation of *C. acnes*.[7,16,18] There was also no difference between acne patients and healthy controls in terms of EryR *Cutibacterium* spp. Indeed, as recently shown in the literature, acne most likely results from a variation of *C. acnes* phylotypes in pilosebaceous follicles.[19] Likewise, in the present study, the phylotype repartition was significantly different between the two groups with the overrepresentation of type IA *C. acnes* in forehead follicles from acne patients and among EryR strains. Moreover, this study suggests that a Myrtacine®-based cream significantly reduces the level of EryR strains of *C. acnes in vivo,* in association with a decrease of acne lesions.

Our analysis of *C. acnes* phylotypes confirmed that phylotype IA is present both in healthy controls and in acne patients, although it predominates in the latter group (40% of all isolates of the acne group vs. 26% of the isolates of the healthy group). The presence of the clade IA in healthy controls and acne patients was demonstrated in several studies,[19–22] as well as its dominance in acne patients.[23–25] While analysing the dominant phylotypes of *C. acnes* in 63 patients with mild to severe acne, Paugam *et al.*[26] also observed that phylotypes IA1 and IA2 clones were carried by 58.6% of patients with mild acne, in the range of what was observed in the present study (73.3% of acne patients, Fig. 1a).

In contrast, the phylotype III, which is characterised by high pro-inflammatory potential in skin explants,[27] does not seem to be related to acne: it was found in only one acne patient and one healthy control. The phylotype III was also observed in only one individual in Paugam's study focusing on severe acne.[26]

Regarding phylotype II isolates, we observed that 45% of subjects were carriers, without any variation between healthy and acne group, a proportion similar to that observed by Fitz-Gibbon *et al.*[19] in acne or healthy patients (35–49%). These observations support the fact that phylotype II participates in the skin microbiome but is probably not relevant in the pathology of acne.[19,28]

At last, we identified a new phylotype by mass spectrometry analysis, which does not seem to be involved in acne (no variation of proportion between the two groups, Fig. 1a). However,

its proportion (more than 10%) suggests that it should be more extensively studied.

One important feature of the present study was the phylotype determination of 10 clones per subject (taking into account the apparent representativeness), which led us to study the *C. acnes* phylotype richness per patient. To our knowledge, only two papers so far have described the richness of *C. acnes* phylotypes per patient. Lomholt *et al.*[20] observed a higher number of different phylotypes per patient in healthy subjects (4) *vs.* acne patients (2). On the contrary, Barnard *et al.*[1] observed a wider "richness" in terms of *C. acnes* ribotypes in acne patients *vs.* healthy subjects. In the present study, more than 70% of healthy subjects carried two phylotypes and more than 70% of acne patients carried three phylotypes indicating a higher richness of *C. acnes* in acne patients compared to controls. Further analyses would be needed concerning the diversity of phylotypes per patient to clarify these contradictory results. Nevertheless, they are in accordance with the potential reversibility of the shift concerning phylotype IA dominance in acne patients.

Another originality of our study is the analysis of the characteristics of resistant isolates of *C. acnes.* A high percentage of individuals (from 40% to 54.1%) carried a resistant *Cutibacterium* strain, regardless of the group to which they belonged (healthy or acne), indicating the high level of resistance of that species. In UK, a steep increase in *C. acnes* resistance has been observed from 20% in 1988 to 72% of patients in 1997 and this proportion has likely increased ever since.[29] In a Colombian study in 100 patients with facial acne, *C. acnes* antimicrobial resistance was demonstrated even in isolates from patients with no previous history of antibiotic use.[30] To our knowledge, this is the first study to report the level of resistance to antibiotic of *Cutibacterium* spp. in healthy controls. Interestingly, the percentage of subjects with EryR *Cutibacterium* spp. was relatively high in both groups (more than 54%) and did not differ significantly in term of CFU load. The literature reports various levels of resistance among *C. acnes* strains: from 6.1% to 7.6% of EryR strains[31] to 98% of EryR strains,[32] with various intermediate levels.[33–38] Nevertheless, it is worth mentioning that in most of these studies, (i) sampling was performed on the skin (with swabs) and did not always take into account the pilosebaceous gland containing *C. acnes*; and (ii) one *Cutibacterium* isolate (the main) per patient was isolated by culture on nonselective agar (without antibiotics), then its sensibility to antibiotics was analysed. By contrast, in our study, *Cutibacterium* from superficial pilosebaceous follicles were directly grown on selective agar. Consequently, resistant strains, that can be present in low proportions, were recovered, while they could have been occulted by sensible strains on nonselective agar. Indeed, among the 34 acne patients carrying an EryR strain, the EryR *Cutibacterium* spp. was subdominant (≤10% of total *Cutibacterium* spp.) in 30 patients (88%; data not shown). This result suggests that resistant *Cutibacterium* spp. might exist in pilosebaceous follicles before the apparition of acne and might explain the failure of first-line antibiotic treatments. Considering the increasing levels of resistant bacteria and the effect of long-term antibiotics treatments on the microbiota,[39] this phenomenon should be investigated in more details.

Furthermore, we confirmed that the phylotype IA was clearly associated to erythromycin resistance, as a high proportion of *C. acnes* EryR isolates, especially from acne individuals, were phylotype IA strains (Fig. 1b & c), in accordance with the studies of Lomholt and Kilian[37] and of Nakase *et al.*[36]

A second part of the study demonstrated that a Myrtacine®-based cream *in vivo,* significantly reduced the level of erythromycin-resistant strains of *Cutibacterium* and this was associated with a decrease in acne lesions. These data corroborate the results obtained *in vitro* in a previous study using a dynamic model of biofilm formation, demonstrating the destructuring effect of Myrtacine® on a mature *C. acnes* biofilm formed of both sensitive and erythromycine and clindamycine-resistant strains.[9] The formation of *C. acnes* biofilm is now considered as responsible for the *in vivo* resistance of *C. acnes* to the main antimicrobials prescribed in acne vulgaris[8] (B. Dréno, this issue). We can therefore hypothesise that *in vivo,* as it was described *in vitro*, Myrtacine® may impair the formation and/or the persistence of *C. acnes* biofilm in the pilosebaceous follicle, leading to an increased sensitivity of bacterial cells to the immune system – even though the total load of *C. acnes* is not modified in the patient skin. Furthermore, as *C. acnes* biofilm formation seems associated with an increased production of virulence factors,[8] its disorganisation may also lead to a loss of virulence of bacterial cells, which may explain the decrease in erythromycin-resistant strains observed after treatment with the Myrtacine®-based product. Another hypothesis, which needs to be further explored, is that Myrtacine® might have a direct effect on the expression of virulence factors.

With the advent and progress of antibiotic resistance in all medical fields, it is of utmost interest to develop strategies, which directly overcome the mechanisms of bacterial resistance[40] and limit antibiotic use.[41] Indeed, in the last decade, bacteria resistance to antibiotics, macrolides and tetracyclines,[42,43] but also rifampicin,[44] has dramatically increased, leading to a renewed interest in nonantibiotic treatments.[45] We have also observed an improvement of acne lesions, with a 42% and 19% reduction in the score of inflammatory and retentional lesions, respectively, at D56. However, a limitation of this study lies in its open design and these results would need to be reproduced in a larger placebo-controlled study.

In conclusion, our study focused on *Cutibacterium* species only, but it will be interesting to study other species forming pilosebaceous microbiota, such as *Staphylococci.* Yeast population could also be of interest, especially *Malassezia* noticeably less present in acne patients than in healthy controls.[46]

The next step will consist in testing if a Myrtacine®-based cream is able to re-establish a balance between *C. acnes* phylotypes and potentially modify *C. acnes* expression virulence factors or factors involved in biofilm formation.

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