Cutibacterium acnes-derived extracellular vesicles promote acne-like phenotypes in human keratinocytes and sebocytes

Bize, Cécile[1*]; Vincent, Gaëlle[1]; Martin, Patricia[2]; Fontanié, Maxime[2]

[1] Seppic Research & Innovation, France
[2] Vibiosphen, France

***** Cécile Bize, 50 boulevard National, 92257 La Garenne Colombes Cedex, France, +33 (0)5 63 72 64 16, cecile.bize@airliquide.com

Abstract

Background: Among the multiple commensal microorganisms present in the healthy skin flora, *Cutibacterium acnes* (*C. acnes*) is a ubiquitous Gram-positive aerotolerant anaerobic bacterium belonging to the Actinobacteria phylum, that predominantly resides deep within the sebaceous follicle, in contact with keratinocytes. Like mammalian cells, in addition to soluble factors, most Gram-negative and -positive bacteria release extracellular vesicles (EVs) which can be involved in the intercellular communication within or between living organisms.

Methods: EVs derived from *C. acnes* (DSM1897) were isolated by ultrafiltration. A fraction of the EVs were labeled with the fluorescent dye Vybrant DiI to evaluate EVs internalization in human cells. Then, EVs were added in the culture medium of primary human keratinocytes or sebocytes derived from iPS. Production of IL-8 and TNF-α in culture supernatants, and of filaggrin, β-defensin 2 in cell lysates were analyzed. Autophagy (LC3b) and sebum production were evaluated in sebocytes. (Bodipy staining).

Results: *C. acnes*-derived EVs induced acne-like phenotypes in primary human keratinocytes, such as increased secretion of inflammatory cytokines and dysregulated epidermal differentiation. Indeed, EVs significantly induced inflammatory cytokine IL-8 production and dysregulated epidermal differentiation by increasing filaggrin protein expression. Moreover, EVs stimulated the production of antimicrobial peptides (β-defensin 2) by keratinocytes. EVs also stimulated the production of IL-8 and TNF-α on human sebocytes derived from iPS. This inflammation induced by *C. acnes*-derived EVs is a typical component of acne. Finally, EVs induced both sebum production and autophagy (LC3b) on sebocytes.

Conclusion: Our study suggests that *C. acnes*-derived EVs efficiently induce not only inflammatory responses in keratinocytes and sebocytes, but also epidermal deformation, that is, acne-like hallmarks.

Keywords: extracellular vesicles; *C. acnes*; keratinocytes; sebocytes.

Introduction.

Human skin is naturally covered with a population of microorganisms, specialized or opportunists, so called skin microbiota.

Among the multiple commensal microorganisms present in the healthy skin flora, *Cutibacterium acnes* (previously named *Propionibacterium acnes*) is a ubiquitous Gram-positive aerotolerant anaerobic bacterium belonging to the Actinobacteria phylum, that predominantly resides deep within the sebaceous follicle, in contact with keratinocytes. Specific metabolic features allow *C. acnes* to colonize the hostile lipid-rich sebaceous follicle environment. In particular, it can degrade triglycerides present in sebum to generate short-chain fatty acids that can irritate the follicular wall and induce inflammation which subsequently leads to cutaneous infections [1]. The most well-known skin ailment associated with *C. acnes* is acne vulgaris. Furthermore, the severity of acne might not only be due to a specific *C. acnes* strain but also to host and environmental factors. *C. acnes* infection induces keratinocyte activation and stimulates production of proinflammatory cytokines such as interleukins (IL-8, IL-1β and IL-12) and tumor necrosis factor-α (TNF-α). The major factors contributing to acne are the hypercornification of the outer root sheath and the pilosebaceous duct, an increased sebum production and, potentially, the overgrowth of *C. acnes* and biofilm formation [1, 2].

Like other bacterial species, *C. acnes* shows phenotypic and genotypic diversity. The species has been subdivided into three types, I–III and two subtypes IA and IB. In patients with severe acne, there is a loss of diversity of these phylotypes, with a clear predominance of the IA1 phylotype, compared to healthy individuals.

Although *C. acnes* is predicted to play roles in acne pathogenesis, the exact mechanism of action at the molecular level has not been clarified.

Bacteria secrete diverse factors to communicate with or evoke cellular responses from target cells. Like mammalian cells, in addition to soluble factors, most Gram-negative and -positive bacteria release extracellular vesicles (EVs). By harboring diverse proteins, lipids, nucleic acids, and metabolites originating from the parent cells, EVs transfer biologically active molecules to neighboring and distant cells for communication and influence [3]. In particular, bacterial EVs are known to mediate pathophysiological functions in bacteria–bacteria and bacteria–host interactions.

In this context, we examined whether *C. acnes* (phylotype IA1, DSM1897) secretes EVs and whether these EVs can be involved in the development of acne vulgaris. For this purpose, we investigated the effects of *C. acnes*-derived EVs on both human keratinocytes and sebocytes.

Materials and Methods.

EVs production

C. acnes (DSM1897) strain was grown in Brain Heart Infusion (BHI) and incubated at 37°C in anaerobic conditions. Then, EVs were isolated by ultrafiltration and stored at +4°C until use. The concentration of EVs was estimated using protein concentration determination with the BCA assay.
A fraction of the EVs was labeled with the lipophilic fluorescent dye Vybrant DiI cell-labeling solution for 30 min at 37 °C [4].

Electronic microscopy analysis

Isolated EVs from *C. acnes* were applied to 400-mesh copper grids and negatively stained with 2% uranyl acetate. Electron micrographs were recorded with a Hitachi HT7700 transmission electron microscope at an accelerating voltage of 75 kV.

Internalization of EVs

Fluorescent EVs were added in the culture medium of keratinocytes or sebocytes. EV internalization was studied by fluorescent microscopy.

Evaluation of EV effects on keratinocytes

EVs from *C. acnes* were added in the culture medium of primary human keratinocytes for 24h or 48h. Production of IL-8 and TNF-α proteins in culture supernatants, as well as filaggrin and β-defensin 2 in cell lysates, were assayed by ELISA and normalized with regard to the quantity of total proteins. Modulation of filaggrin expression was also evaluated by immunofluorescence.

Evaluation of EVs effects on sebocytes

Sebocytes (Phenocell) were treated with EVs from *C. acnes* for 24h or 48h. Production of IL-8 and TNF-α proteins in culture supernatants was assayed by ELISA and normalized with regard to the quantity of total proteins.
Expression of autophagy was also evaluated by immunofluorescence with LC3b autophagic probe.
Effect of EVs on lipid secretion was determined using bodipy staining.

Results.

C. acnes secretes EVs

Morphological assessments using transmission electron microscopy revealed spherical and closed membrane structure images (Figure 1). This indicates that C. *acnes* (phylotype IA1) spontaneously releases EVs exhibiting similar morphology and size to previously described bacteria-derived EVs in the extracellular environment [5, 6].

Based on transmission electron microscopy image analysis, the size of *C. acnes*-derived EVs was in the range of 30-100 nm, which is in agreement with literature [5, 6].

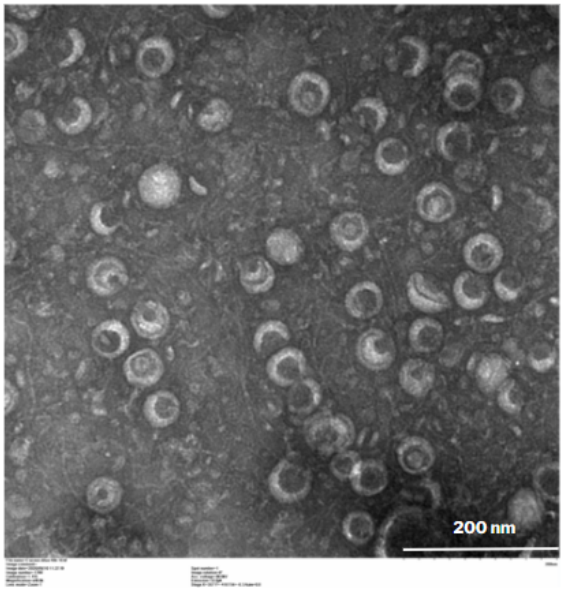


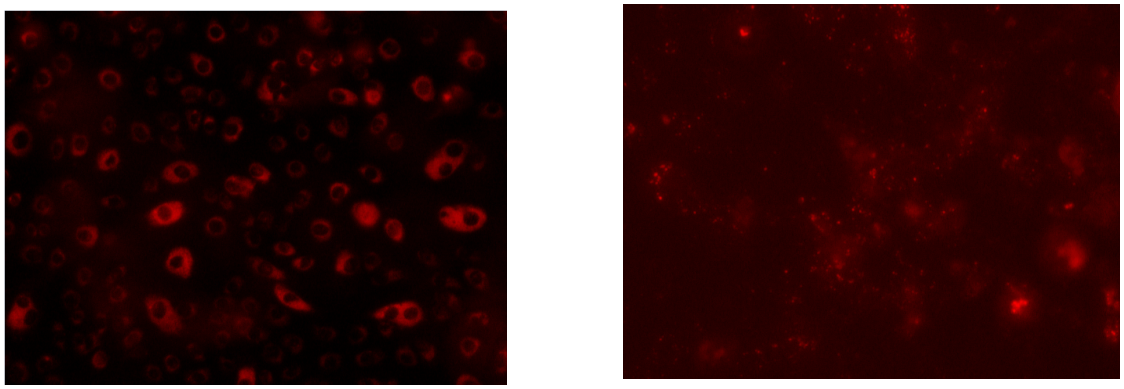
Figure 1 : Representative TEM image of EVs from *C. acnes*. Scale bar, 200 nm

C. acnes-derived EVs internalization in human epidermal keratinocytes and sebocytes

To examine whether EVs could be internalized into primary human epidermal keratinocytes and human sebocytes (derived from iPS), we labeled EVs with DiI, a fluorescent dye incorporated into membranes, and traced the intracellular localization of EVs [4].

This revealed that EVs were located in the perinuclear area (Figure 2), indicating that EVs were endocytosed into cells. Internalization seemed to be slower into sebocytes than into keratinocytes.

A



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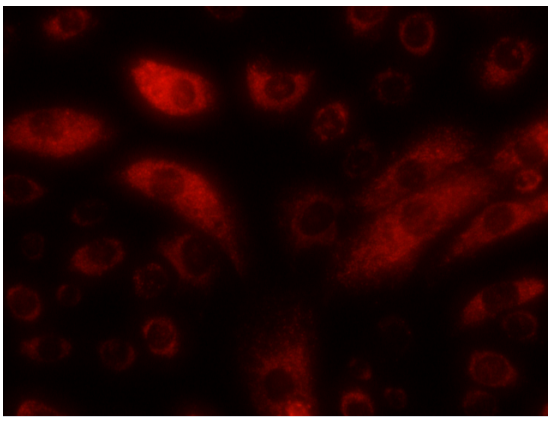

Figure 2: (A) Isolated EVs were labeled with DiI (red) and used to treat both keratinocytes (on the left) and sebocytes (on the right) for 48h (magnification x10); **(B)** Internalization of labeled-EVs into keratinocytes after 48h treatment (magnification x10)

EVs increase the expression of proinflammatory cytokines in both human keratinocytes and sebocytes

On the basis of earlier reports that inflammation is a fundamental process throughout the development of acne lesions and that *C. acnes* is considered a main causative inflammatory agent, we hypothesized that EVs may evoke inflammatory responses [7, 8]. To examine whether EVs stimulate the secretion of inflammatory cytokines, keratinocytes and sebocytes were treated with EVs, and the culture supernatants were analyzed.

IL-8 was significantly increased in the supernatant of EV-treated keratinocytes compared with supernatant from non-treated cells. However, TNF-α production was not affected by EV treatment on keratinocytes (Figure 3A).

Both IL-8 and TNF-α secretion were increased in sebocyte supernatant (Figures 3B and 3C).

These results suggest that EVs derived from *C. acnes* upregulate the expression of proinflammatory cytokines in the human epidermis, thereby participating in inflammatory responses.

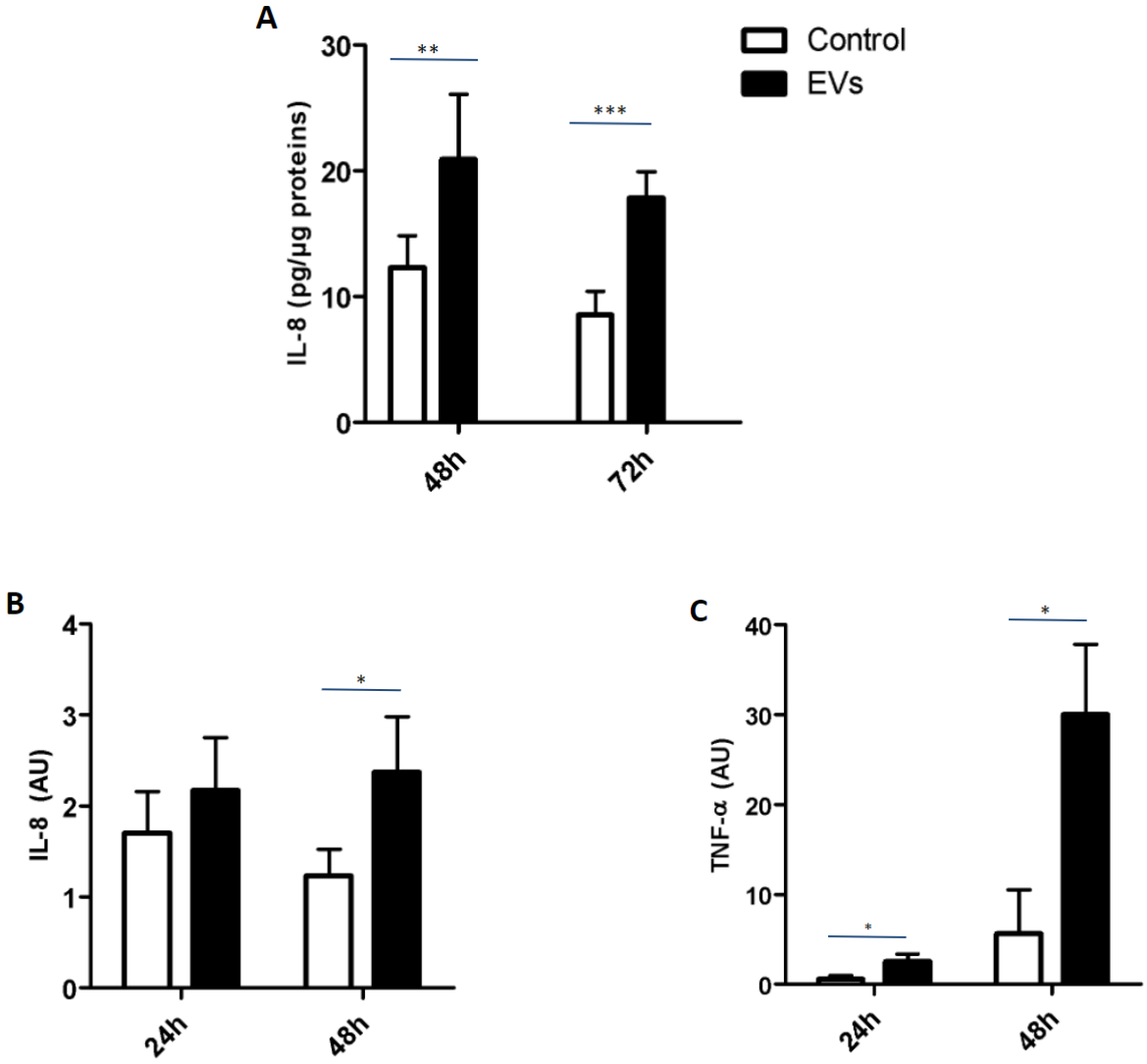

Figure 3: (A) Secreted protein levels of IL-8 in keratinocytes treated with *C. acnes*-derived EVs (protein normalization); **(B)** Secreted protein levels of IL-8 and **(C)** of TNF-α in sebocytes treated with *C. acnes*-derived EVs (Hoechst normalization) *: $p<0.05$; **$p<0.01$; ***: $p<0.001$

<u>EVs induce dysregulation of epidermal differentiation</u>

In addition to the induction of inflammatory factors, *C. acnes* influences the differentiation of keratinocytes by altering the expression of differentiation markers [9, 10].

Hyperkeratinization is a precedent step for *C. acnes* colonization in the development of acne lesions, suggesting a positive feedback loop between keratinocytes and *C. acnes* in acne pathogenesis. We therefore investigated whether EVs affected epidermal differentiation. Modulations in filaggrin expression were evaluated by ELISA (on cell lysates) and immunofluorescence (on fixed cells).

EVs significantly affected the expression of filaggrin (Figures 4 and 5), an epidermal marker related to differentiation and so could result in epidermal deformation including hyperkeratinization, similar to the phenotypes observed in acne lesions.

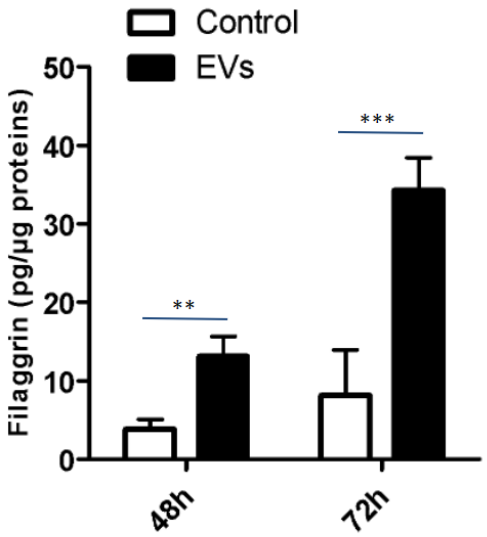

Figure 4: Filaggrin expression of keratinocytes exposed to EVs from *C. acnes* (ELISA) - **: p<0.01; ***: p<0.001

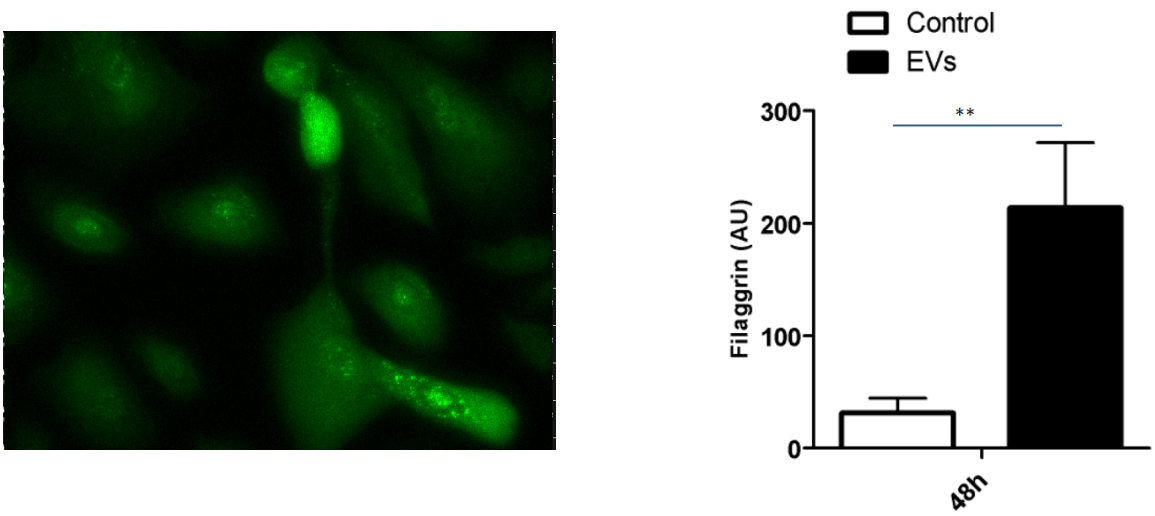

Figure 5: Filaggrin expression of keratinocytes exposed to EVs from *C. acnes* (immunofluorescence) - **: p<0.01

C. acnes-derived EVs induce AMPs production in keratinocytes

Acne, as inflammatory conditions of the skin, has shown an altered expression of AMPs (antimicrobial peptides).

In particular, human beta-defensin (hBD-2) is expressed in the pilosebaceous unit, increases in acne lesions and is upregulated by *C. acnes* [11].

In vitro observations suggest that *C. acnes* stimulates hBD-2 expression in keratinocytes and sebocytes [12-14]. Therefore, AMPs may act as a beneficial factor against *C. acnes* but the reaction may also stimulate more inflammation in acne.

To determine whether EVs stimulate the secretion of hBD-2, keratinocytes treated with EVs were lysed and analyzed.

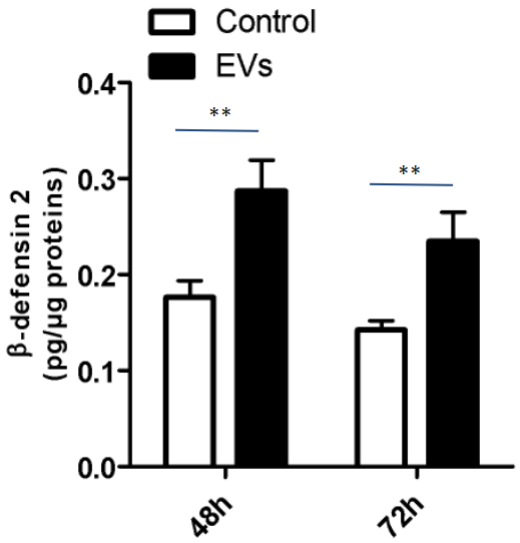

Figure 6 : β-defensin-2 expression by keratinocytes exposed to EVs from *C. acnes* (ELISA)
**: p<0.01

This showed that EVs incubation stimulated hBD-2 production by keratinocytes (Figure 6).

C. acnes-derived EVs induce lipid production in sebocytes

The increase of sebum production is one of the pillars that interplay for the development of acne.

Sebocytes were treated with *C. acnes*-derived EVs and Bodipy, a probe that specifically binds to the fat droplets, was used to evaluate sebocyte lipid production. Image analysis showed that EVs from *C. acnes* induced lipid production in sebocytes (Figure 7).

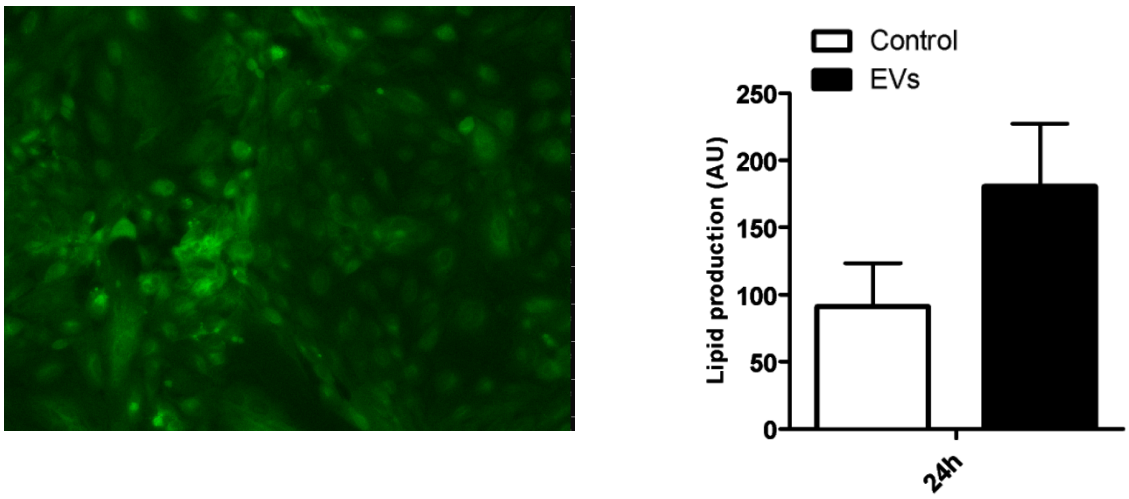

Figure 7 : Lipid production induced by *C. acnes*-derived EVs in sebocytes (Bodipy staining)

EVs induce autophagy in sebocytes

Sebum is the holocrine secretion involving an autophagic process [15]. As EVs from *C. acnes* induce lipid production in sebocytes, we aimed to determine whether these EVs play a role in the sebocyte autophagic process. The expression of LC3b, the autophagosome marker was quantified by immunofluorescence.

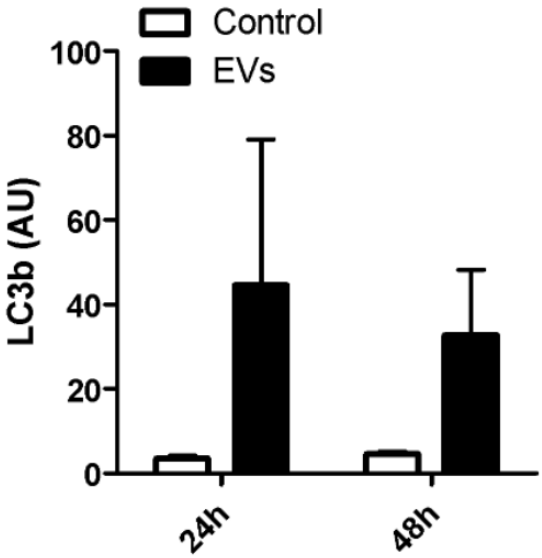

Figure 8 : Evaluation of the effect of *C. acnes*-derived EVs on sebocyte autophagy

This showed that EVs from *C. acnes* induced autophagy in sebocytes: LC3b labeling was increased (Figure 8).

Discussion.

The skin is colonized by diverse types of microorganisms that are often related to skin disorders [16]. Microorganisms secrete extracellular factors to communicate with and modulate host cells [17]. Among the secreted extracellular factors, EVs can mediate intercellular communication within or between living organisms [18, 19].

Acne is multifactorial, and interacting mechanisms involved are not fully known. What is known is that its pathogenesis focuses on the pilosebaceous unit where four pillars interplay for the development of acne: an increase of sebum generation, the duct hyperkeratinization, a rise of anaerobic bacteria, and an inflammatory response.

As *C. acnes* has been thought to be involved in the development of acne, we investigated the pathogenic roles of EVs derived from *C. acnes*.

Results obtained indicate that *C. acnes* naturally secretes EVs that can be internalized by cutaneous cells.

These EVs can influence keratinocytes and sebocytes by increasing the production of inflammatory cytokines. Here we showed that EVs significantly increase the secretion of IL-8 in both keratinocytes and sebocytes. Moreover, EVs also significantly induced an increase of TNF-α in sebocytes. This suggests that *C. acnes*-derived EVs may play pivotal roles in the early stages of the development of acne vulgaris by initiating inflammatory cytokine cascades in both keratinocytes and sebocytes.

Moreover, this study revealed that EVs induced significant dysregulation of the expression of epidermal markers such as filaggrin, resulting in epidermal hyperkeratinization similar to the phenotypes observed in acne lesions. Hyperkeratinization can create a preferable anaerobic environment for the colonization of *C. acnes*.

EV-induced inflammation can lead to the hyperproliferation of keratinocytes, and then participate in the hyperkeratinization of the pilosebaceous unit.

There is increasing evidence that host antimicrobial peptides (AMPs) may play a role in the pathogenesis of acne [20, 21]. The induction of AMPs in acne brings up the question whether increased levels of AMPs in acne is an essential and beneficial part of a concerted defense reaction against *C. acnes* or whether this defense reaction triggers inflammation in acne.

In our study, EVs incubation stimulated hBD-2 production by keratinocytes.

It is likely that the induced expression of hBD-2 in the acne lesion is first of all conceived as an antimicrobial defense response against *C. acnes*. This is strengthened by a study reporting that hBD-2 exhibits antimicrobial activity against *C. acne*s [14]. However, the question remains open whether the use of AMPs may promote inflammatory effects.

Furthermore, *C. acnes* has been implicated in sebum generation. Not only EVs significantly increase sebum production but also sebocyte autophagy which is essential for holocrine sebum secretion.

Collectively, these results suggest that EVs induce acne-like phenotypes. Therefore, inhibiting the release of EVs from *C. acnes* or targeting EV-mediated signaling pathways could represent an alternative method for alleviating acne occurrence and phenotypes.

Conclusion.
In summary, our study suggests that lipid bilayer-enclosed and nanosized *C. acnes*-derived EVs efficiently induce not only inflammatory responses but also epidermal deformation, that are, acne-like phenotypes.

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Conflict of Interest Statement. Vibiosphen has received honoraria from Seppic for *C. acnes* culture and EVs isolation.

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