

Porphyr'aging: new insights into phenomena related to skin aging

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Abstract:

Introduction: The skin microbiota produces metabolites that positively or negatively affect our skin. Among these, porphyrins are secreted at high levels by *Cutibacterium acnes* and other skin bacteria. Levels of these molecules do not decrease with age, raising the question of their impact on skin aging. Protoporphyrin IX is reported to promote melanogenesis, whereas coproporphyrin III is more closely linked to skin inflamm'aging. Moreover, some of our studies revealed correlations between porphyrins, brown spots and wrinkles, that perfectly support the data from literature. Based on these observations, we hypothesized that reducing porphyrin levels on the skin could delay signs of aging.

Methods: The depth of penetration of porphyrins into the skin was measured by spectrofluorometry. A co-culture model with basal layer melanocytes and keratinocytes was developed to study the formation of dark spots and the impact of porphyrins on inflammation and melanogenesis. A transcriptomic study on fibroblasts explored how porphyrins affect the structure of the extracellular matrix (ECM) and inflamm'aging. Finally, a clinical assessment was conducted on 38 women presenting wrinkles and dark spots to evaluate the effects of an ingredient reducing porphyrin production by *C. acnes* on the appearance of wrinkles and dark spots after 2 months of twice-daily application.

Results: Porphyrins can penetrate to the epidermis's basal layer. Transcriptomic data revealed that porphyrins down-regulated genes involved in ECM structure, pluripotency, proliferation, and stem cell maintenance, while up-regulating genes involved in fibrosis and inflammation. In co-cultures, porphyrins significantly increased melanin production (+35%) and inflammation (+83%), confirming that they have deleterious effects on skin and are involved in dark spot formation. Reducing porphyrin levels 10-fold resulted in a 41% decrease in melanogenesis in co-cultures. We confirmed these results through clinical evaluation of an active ingredient that reduces *C. acnes*-mediated porphyrin production. After 2 months of application, volunteers' skin produced -13% less porphyrin, leading to a 1.7-fold decrease in invisible spots and a 2-fold decrease in visible spots along with diminished areas of fine and coarse wrinkles

(2.7-fold and 1.9-fold, respectively) relative to a placebo. Overall wrinkle count was reduced -6% by the active compared to the placebo.

Discussion and conclusion: Our results highlight for the first time the involvement of porphyrins in the emergence of visible signs of aging, which we refer to as skin aging phenomena. Indeed, previous studies involving 100 volunteers revealed porphyrin levels on skin to be stable throughout life. In vitro evaluation of porphyrins in a co-culture model and transcriptomic analysis of fibroblasts revealed how this metabolite influences skin aging, through degradation of ECM structure and formation of hyperpigmented spots in the basal layer. An extended study shed light on the biogenesis of dark spots, from the precursor in the basal layer of the epidermis, which is initially invisible, until the appearance of a visible dark spot on the skin's surface. Thus, targeting porphyrins on the surface of the skin is as an innovative strategy to support consumers by delaying the emergence of signs in the early stages of aging and throughout life.

Keywords: porphyrins ; dark spots genesis ; wrinkles

Introduction

If last decades have been rich in studies to try understanding the microbiota composition and its impact on skin, recent years' research mainly focused on the understanding of the nature of metabolites produced by the microbiota and their impact on skin. Indeed, many studies evidenced that the skin microbiota produces various metabolites that can positively or negatively affect our skin (Jiminez and Yusuf, 2023; Roux et al., 2022). A very well characterized microbiota metabolite is porphyrin. Porphyrins are small molecules (<700Da) that are able to penetrate skin cells by causing permeability changes and the cells membrane and that could also penetrate by passive diffusion due to their small size (Ayano et al., 2023; Fyrestam et al., 2015; Spittaels et al., 2021). Among porphyrins, coproporphyrin III (CPIII) is described to be mostly secreted by Gram positive bacteria, among them *Cutibacterium acnes* mostly known for its involvement in acne but that has been also evidenced to be highly present on healthy skin, depending of the phylotype (Cros et al., 2023; Surdel et al., 2017). On the contrary, protoporphyrin IX (PPIX) form is more secreted by Gram negative bacteria (Hobbs et al., 2016). Some evidences in the literature describe the protoporphyrin IX as promoting melanogenesis, melanocytes dendrity and melanosome transport through the upregulation of targets such as MITF, TRP-1, Rab27a or even Cdc42

(Lv et al., 2020), whereas coproporphyrin III is more closely linked to skin inflammation since it boosts the mRNA expression of IL-8 (Schaller et al., 2005). A previous internal clinical study on 100 volunteers highlighted the fact that porphyrins do not decrease with age contrary to what one might think, raising the question of their potential implication in skin aging.

All these data taken together suggest that porphyrins could have an impact on the apparition of some aging signs on the skin. We propose to define this newly identified aging phenomenon as "Porphy'aging".

Based on these observations, we hypothesized that reducing porphyrin levels on the skin could delay signs of aging. In order to answer this hypothesis, an active ingredient was specifically designed to reduce porphyrins production by the skin microbiota and was clinically tested on volunteers who presented wrinkles and dark spots.

Materials and Methods

Porphyrins penetration into the skin: skin explants from a 57 years old donor having undergone a breast reduction surgery were topically treated with Coproporphyrin III. After 8 hours treatment, skin explants were frozen at -80°C and longitudinally cut. 6 Raman images were recorded with a size of Y:10µm / X:120µm with a step of 5µm in both directions. Each Raman image had 3Y spectra and 23X spectra. The images pre-processing consists of spikes correction, elimination of aberrant spectra (Bad S/N ratio, saturation...) and smoothing. After data pre-processing, the parameter of skin porphyrin makers was calculated on the overall spectral image to determine the penetration and distribution of porphyrin in the skin section. The processing of corrected data maps was performed by using homemade software operates in the Matlab environment.

For the assessment of the porphyrin skin penetration, we calculated the integrated intensity of the spectral range 715 nm - 745 nm. This spectral region reflects the porphyrin fluorescence emission with 660 nm Laser excitation and is associated with the degree of the porphyrin penetration in skin section. The reconstructed spectral images based on integrated intensity of porphyrin fluorescence permit to see the level of permeation and spatial distribution of the porphyrin in the skin section until 120µm. The results were averaged over 3 measurements (n=3) performed on different but adjacent skin sections.

Transcriptomic analysis on fibroblasts: normal human fibroblasts were treated with a mix of Coproporphyrin III / Protoporphyrin IX (ratio 100:1) for 6 hours compared to the untreated control. RNA was extracted using Trizol (Thermo Fisher Scientific, Massachusetts, USA) and the RNA quality was evaluated. Reverse transcription to obtain cDNA was performed using the Verso cDNA kit (Thermo Fisher Scientific). RT-qPCR was performed on specific pre-coated plates (Bio-Rad, California, USA) designed to study the transcriptomic expression of various genes involved in dermal function. 10 ng of cDNA per well was used with CFX96 Touch (Bio-Rad) and an iTaq Universal Sybr Green supermix (Bio-Rad).

Pro-collagen I synthesis by fibroblasts: normal human fibroblasts were culture in 6-wells plates for 48 hours before being treated for 48 hours with a mix of Coproporphyrin III / Protoporphyrin IX (ratio 100:1) compared to the untreated control. Cell culture supernatant were harvested and pro-collagen I release in medium was quantified by an ELISA assay (Abcam, ab210966). Quantified amount of pro-collagen I was normalized with the number of cells that was estimated by a Crystal Violet staining.

Co-culture model of dark spots genesis: normal human keratinocytes from the basal layer were co-cultivated with melanocytes and treated with a mix of Coproporphyrin III / Protoporphyrin IX (ratio 100:1) compared to the untreated control. After 24 hours of stress with the mix of porphyrins, cell culture supernatant were harvested and IL-8 release in the medium was quantified by ELISA assay (Biotechne, D8000C). Cell medium was renewed with or without the mix of porphyrins, and after 5 days, melanin was extracted to evaluate the impact of porphyrins on melanogenesis.

C.acnes culture and coproporphyrin III dosage:

- Bacterial culture: *C. acnes* strains were grown anaerobically to stationary phase at 37 °C: *C. acnes* was seeded from a working cell bank (50 µL) into 5 mL of Reinforced Clostridium Medium (RCM) and cultured for 3 days with stirring (130 rpm) at 30 °C in a single sealed bag containing Anaerocult P and an Anaerotest strip. This pre-culture was then transferred to a 50-mL Erlenmeyer flask containing 20 mL of RCM to obtain a starting OD_{600 nm} of 0.015. Culture was pursued in an anaerobic jar with GasPak EZ and an Anaerotest strip. The culture medium was supplemented with the molecules and

extracts to be tested, dissolved in water or DMSO. Carrier alone was added to controls. Bacterial growth was assessed by measuring the optical density of culture samples at 600 nm.

- Coproporphyrin III extraction: Bacterial culture (1.4 mL) was collected and centrifuged at 18000 g, 10°C for 15 minutes. Coproporphyrin III was extracted by liquid/liquid extraction from supernatant (adapted from Hamblin 2005). The supernatant (1 mL) was extracted with 2.5 mL ethyl acetate/acetic acid (4:1, v/v). The upper phase (2.4 mL) was collected and transferred in a new tube to be evaporated to dryness in Genevac at 30°C under vacuum. Extract was suspended in 70 µL HCl 1.5 M and analyzed by LC-MS.

- Coproporphyrin III quantification by LC-MS: Quantification of porphyrins was adapted from Mancini 2015. Analysis was performed using an Ultimate 3000 system (Thermo Scientific) composed of a quaternary pump (LPG 3400 SD), an autosampler (WPS3000) and UV and MS detectors (DAD 3000 and ISQ EC, respectively). Software was Chromeleon 7.210ES. Separation was conducted on a Luna PFP column 5 µm, 100 Å, 4.6 x 150 mm (Phenomenex). Solvent A was ultrapure water at 0.1% formic acid and solvent B was acetonitrile at 0.1% formic acid. Gradient was as follows: 0 min, 25% B; 2 min, 25% B; 15 min 66% B; 15.5 min 95% B; 19.5 min, 95% B; 20, 25% B and 24 min, 25% B. Flow was 1 mL/min, column oven was 25°C and sampler at 10°C. UV detection was performed at 405 nm. ISQ EC was in full-scan negative mode at 100-1000 Da, CID was 20 V, dwell scan time was 2 s, SIM widths were set at 0.1 amu, sheet gas pressure was 80 psi, auxiliary gas pressure was 9.7 psi, sweep gas pressure was 0.5 psi, and vaporizer temperature was 550°C. Coproporphyrin III content was calculated from calibration curve of coproporphyrin III (MedChem Express) from 0.061 µM to 61.1 µM in HCl 1.5M.

Clinical evaluation: Clinical studies were carried out in compliance with the most recent recommendations of the World Medical Association ethical principles for medical research involving human subjects (Helsinki Declaration, 64th WMA General Assembly, Fortaleza, Brazil, October 2013). Participation in the study was only permitted after providing informed consent.

- INCI formula: AQUA/WATER, CETYL ALCOHOL, GLYCERYL STEARATE, PEG-75 STEARATE, CETETH-20, STEA-RETH-20, ISODECYL NEOPENTANOATE, PHENOXYETHANOL, DIMETHICONE, FRAGRANCE ± ACTIVE

- Panel description: A placebo-controlled, double blind clinical study was carried out on 38 volunteers aged between 45 and 73 years old and presenting invisible spots, pigmented spots and wrinkles on the

face and the upper chest. The subjects were randomly divided in two equal groups: one applied a placebo cream, while the other applied a cream containing the active ingredient. Each group of volunteers applied the cream every day in the morning and evening for 56 days.

- Skin analysis: VISIA CR2.3® was used to measure the evolution of porphyrin count, invisible spots count and wrinkles area at the beginning of the study and after 56 days of application of a cream containing or not the active ingredient. On the same kinetic, Siascope® was used to measure melanin content in spotted area on the face.

Results

First, we explored the capacity of porphyrins to penetrate into the skin to understand where they can have an effect into the skin. By following porphyrins signal by Raman spectroscopy, we evidenced that porphyrins can penetrate into the skin until 65µm depth. This depth corresponds to the epidermis' basal layer, and even to the upper dermis, depending of the age of volunteers and of the skin area (Lintzeri et al., 2022) (Figure 1).

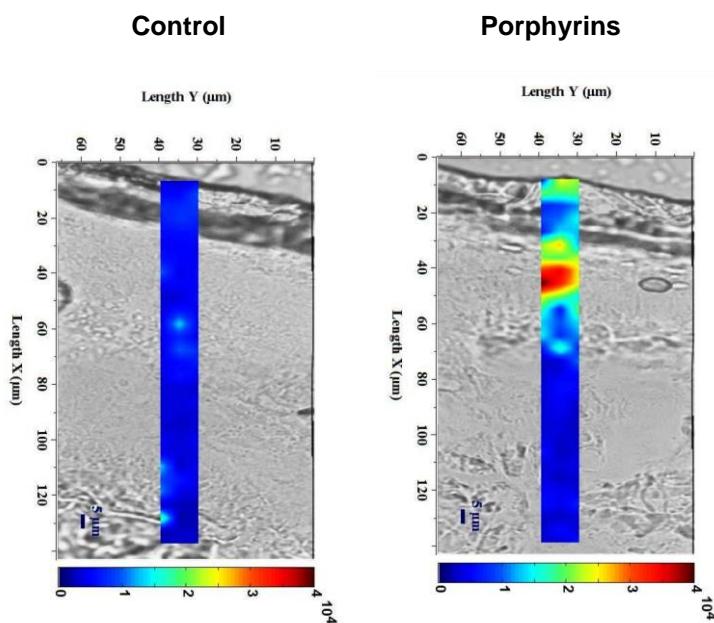


Figure 1: Tracking of exogenous topically applied porphyrins penetration into the skin after 8 hours of application, by Raman spectroscopy.

After evidencing the capacity of porphyrins to penetrate until the upper dermis, we were interested by understanding the potential impact of porphyrins on the dermis. For that, fibroblasts from a 46 years old donor were stressed for 6 hours with a mix of coproporphyrin III and protoporphyrin IX. Transcriptomic

data revealed that porphyrins down-regulated many genes involved in cell proliferation, dermoepidermal junction, extracellular matrix structure, DNA repair, enzymatic antioxidant defences, pluripotency and telomere maintenance as represented in the Table I. These results highlighted the deleterious effect that porphyrins can have on the dermis when penetrating into the skin.

Pathway	Genes	Average modulation	p value
Angiogenesis	VEGFA	-74%	0.0055
Cell proliferation	FGF2, IGFBP3	-67%	<0.05
Dermo-epidermal junction	COL7A1, COL4A1	-49%	<0.05
Extracellular matrix	CYR61, CTGF, FBN2, FBN1, HPSE, HSPG2, TIMP2, MMP3, FBLN5, ELN, SPARC, FMOD, COL1A1, SDC1, LOXL2, VCAN	-48%	<0.05
DNA repair	DDIT3, GADD45A, XPC	-76%	<0.05
Enzymatic antioxidant defenses	SOD2, GPX1	-34%	<0.05
Fibrosis	TGFB1	-54%	0.0002
Gene silencing	SIRT2	-47%	0.000004
Melatonin receptor	RORA, NQO2	-52%	<0.05
p53 regulator	SIRT1	-39%	0.0025
Pluripotency transcription factor	NANOG, POU5F1	-59%	<0.05
Neurotrophin	NTF3, NGF, BDNF	-114%	<0.05
Telomere maintenance	TERT	-450%	0.0112
Transcription factor	MYC	-50%	0.0019

Table I: Significant genes modulation in fibroblasts following 6 hours of porphyrins stress relative to the untreated condition.

To complete these observations, the type I pro-collagen synthesis by fibroblasts was evaluated following exposure to increasing doses of porphyrins. The results presented in the Figure 2 confirmed the transcriptomic results as an increasing exposure to porphyrins significantly reduced the type I pro-collagen production by 20% compared to the untreated condition. Taken altogether, these results highlight the potential deleterious effect of porphyrins on skin aging, more precisely on wrinkles formation.

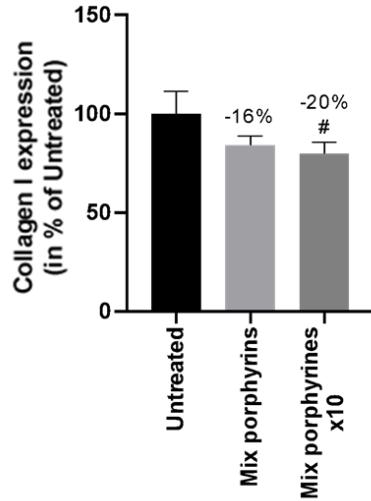


Figure 2: Evaluation of the impact of porphyrins on fibroblasts' capacity to synthetize type I pro-collagen. Quantification was obtained by an ELISA dosage in cell culture supernatant.

After showing the consequences of porphyrins penetration into the skin until the upper dermis, we were also interested by studying the potential impact on the basal epidermis. For that, basal keratinocytes were co-cultured with melanocytes in order to mimic the basal layer of the epidermis. This co-culture was treated for 5 days with a mix of porphyrins, interleukin-8 (IL-8) was quantified in the cell culture supernatant and melanin extracted from cells. Through this experiment, we evidenced that the porphyrins application on co-culture significantly increased the melanin production by 35% and increased the inflammation by 83% through the IL-8 release (Figure 3). These results are consistent with the literature that specifically describe the protoporphyrin IX as increasing the melanogenesis through the activation of the cGMP/Pkg pathway that induces the increase of tyrosinase activity and boosts the expression of proteins involved in melanogenesis, melanocyte dendricity and melanosome transport, such as MITF, TRP-1, TRP-2, Rab27a and CDC42 (Lv et al., 2020). On the top of that, these results also confirm Givaudan's previous clinical results that evidenced a significant correlation between the porphyrins count and the brown spots count on skin.

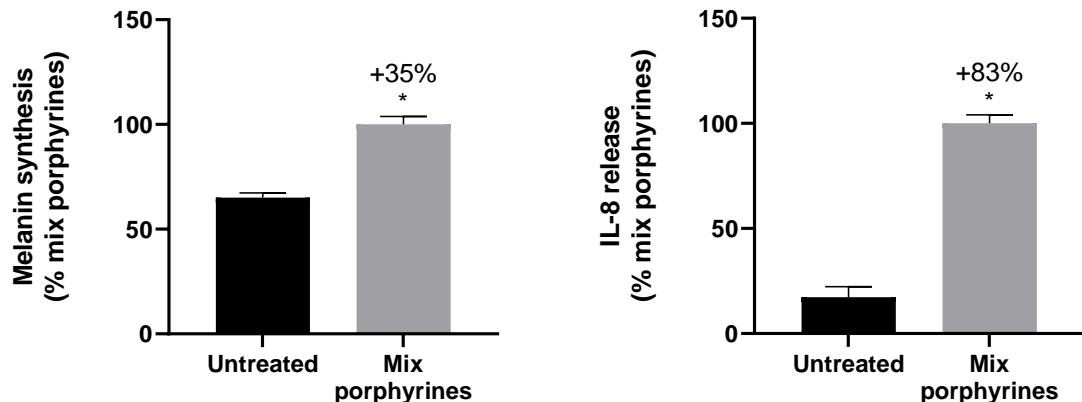


Figure 3: Evaluation of the impact of porphyrins on melanin synthesis (left figure) and IL-8 release (right figure) in a keratinocytes - melanocytes coculture. Melanin synthesis was quantified in cell lysate and IL-8 quantification was obtained by an ELISA dosage in cell culture supernatant.

Some data in the literature also describe that protoporphyrin IX is able to interact with the mitochondrial 18kDa peripheral-type benzodiazepine receptor (PBR) (Wendler et al., 2003). Interestingly, when a molecule like diazepam activates the PBR, this results in the upregulation of MITF, Rab27a, Myosin Va, Rab17 and CDC42, leading to the activation of melanogenesis and melanosome transport (Lv et al., 2019). By discovering the similarities of targets described to be activated by protoporphyrin IX and by the interaction of diazepam with PBR, we wondered if protoporphyrin IX could activate melanogenesis through interaction with this receptor. To answer this question, a new co-culture model of keratinocytes and melanocytes was treated with porphyrins alone or in combination with the molecule PK11195 that is a PBR antagonist. In this model, the application of the porphyrins mix stimulated the melanin synthesis by 35% (Figure 4), confirming the previous results (Figure 3) and the robustness of the model. Contrariwise, the combination of porphyrins mix with PK11195 PBR antagonist totally inhibited melanin synthesis by 57% compared to the porphyrins mix alone. These data confirm our hypothesis that porphyrins could stimulate melanin synthesis through an interaction with PBR that is located at the external membrane of mitochondria. The results are also consistent with the fact that porphyrins, due to their low molecular weight (<700Da, (Fyrestam et al., 2015)) and their ability to permeabilise cell membrane from keratinocytes (Ayano et al., 2023; Spittaels et al., 2021), are able to penetrate inside the cells to interact with PBR at the mitochondrial external membrane.

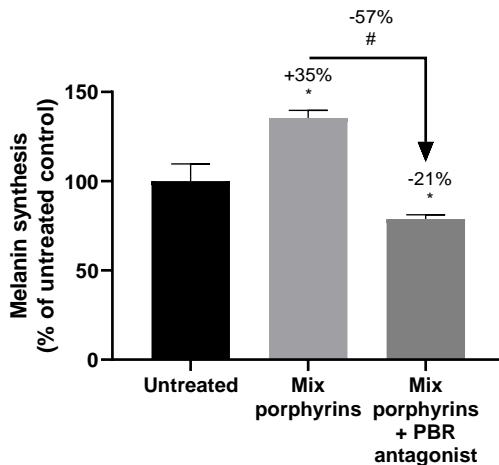


Figure 4: Evaluation of the impact of porphyrins on melanin synthesis in presence of PBR antagonist to elucidate the mode of action of porphyrins.

By showing that porphyrins effectively stimulate melanogenesis in co-culture of keratinocytes and melanocytes, we suggested that reducing porphyrins could be an efficient strategy to decrease melanogenesis. For that, we treated a co-culture with our well-established mix of porphyrins, and reduced the dosage by a 2- and a 10-fold. Reducing porphyrin levels 10-fold resulted in a significant 41% decrease of melanogenesis in co-cultures, reinforcing our hypothesis (Figure 5).

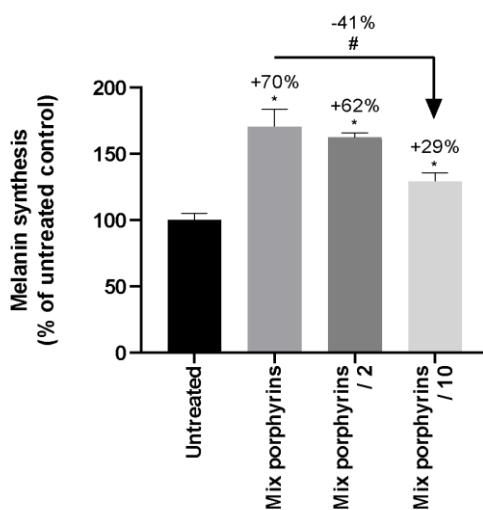


Figure 5: Evaluation of the impact of porphyrins reduction on melanin synthesis.

A specific screening on *C.acnes* strains that are associated with healthy and non-acneic skin allowed identifying a specific active ingredient able to significantly reduce coproporphyrin III production by 53% compared to the control (Figure 6). This active ingredient was selected for its capacity to reduce

porphyrins production by *C.acnes* and was then clinically tested to evaluate the benefits of this porphyrins reductions on aging signs.

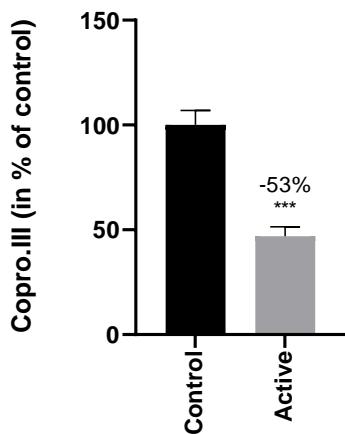


Figure 6: Evaluation of the impact of an active ingredient on porphyrins productions by healthy and non-acneic strains of *C.acnes*.

After 2 months of application on face from volunteers who presented wrinkles and hyperpigmented spots, we evidenced that the active ingredient produced 13% less porphyrins compared to placebo application (Figure 7). Thus, the *in vitro* capacity of the active ingredient to inhibit porphyrins production was confirmed until clinical level and encouraged us to purchase the investigations with this ingredient.

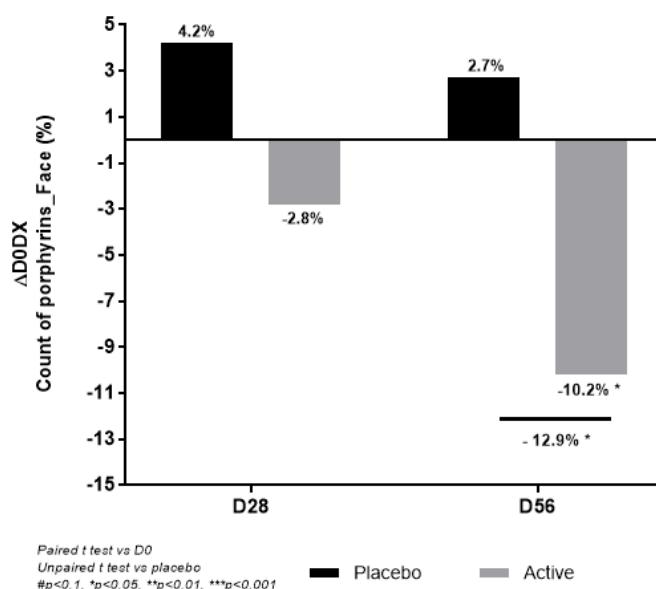


Figure 7: Evaluation of the impact of an active ingredient on porphyrins count on the skin after 28 and 56 days of application versus placebo.

We evidenced *in vitro* that a reduction of porphyrins could lead to a reduction of melanogenesis (Figure 5). We were interested by verifying it through a clinical study. As our active ingredient was able to reduce the porphyrins count on the skin from volunteers, we went further by evaluating its impact on invisible and visible spots. Indeed, we showed a 1.7-fold decrease in invisible spots count, and a 2-fold decrease of melanin content in visible spots after 56 days versus placebo (Figure 8). These results confirmed that reducing porphyrins amount on the skin can lead to a reduction of invisible spots until a reduction of visible spots for a corrective effect. As invisible spots constitute a kind of precursor for future visible spots, we can conclude that reducing porphyrins and invisible spots represent a preventive action to reduce the apparition of future visible spots.

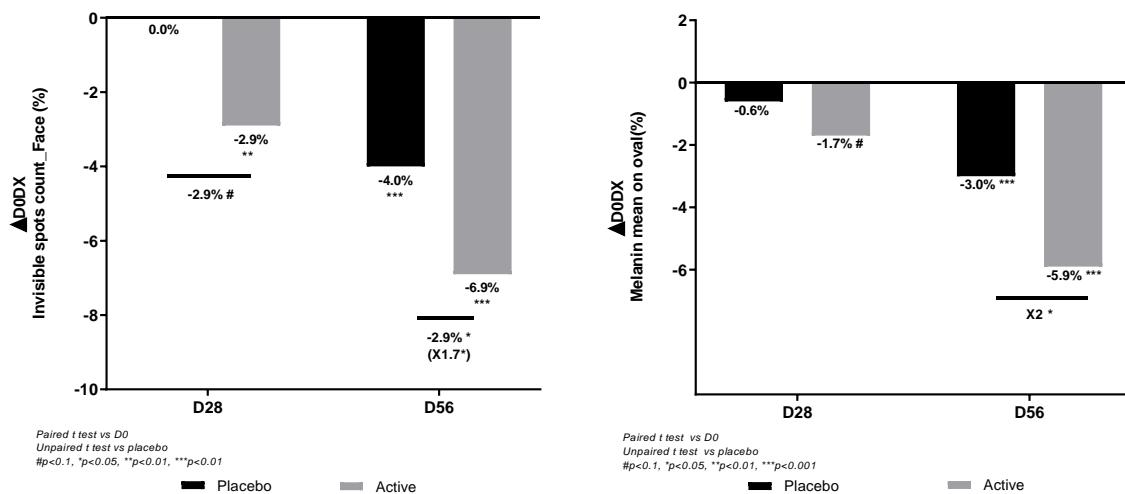


Figure 8: Evaluation of the impact of an active ingredient on invisible spots count (left) and melanin mean in the visible spots (right) after 28 and 56 days of application versus placebo.

Finally, as our *in vitro* data showed the deleterious effect of porphyrins on collagen synthesis by fibroblasts, we wanted to evaluate if the clinical reduction of porphyrins would lead to an improvement of wrinkles appearance. To answer this question, we clinically evaluated the fine and coarse wrinkles on the same clinical study that previously evidenced a reduction of porphyrins. The VISIA analysis highlighted diminished areas of fine and coarse wrinkles (2.7-fold and 1.9-fold, respectively) relative to placebo after 56 days of application (Figure 9). These data confirmed that reducing porphyrins amount on the skin can be helpful to the skin finding back its capacities to synthetize type I collagen, and thus reducing wrinkles appearance.

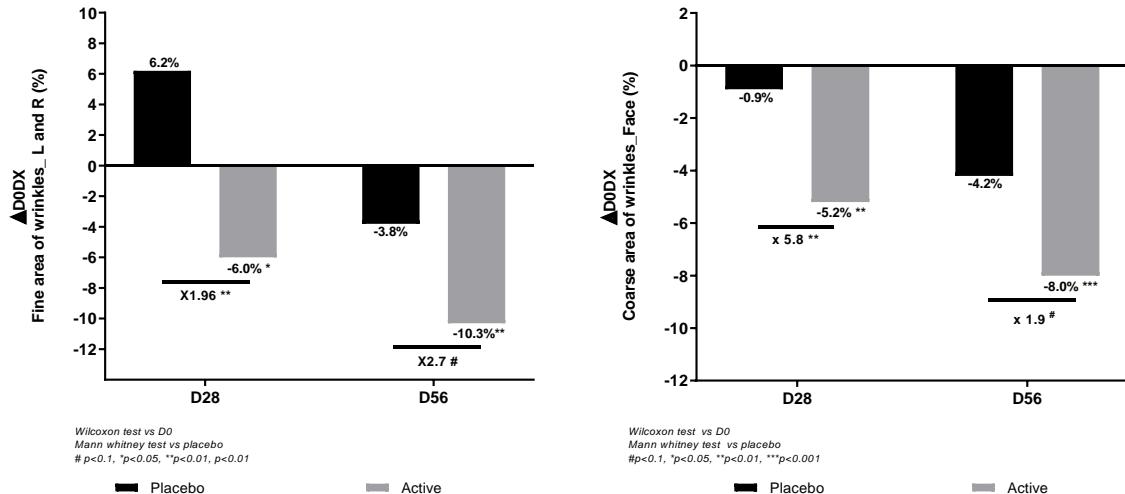


Figure 9: Evaluation of the impact of an active ingredient on fine (left) and coarse (right) wrinkles after 28 and 56 days of application versus placebo.

Discussion

Our results highlight for the first time the involvement of porphyrins in the emergence of visible signs of aging, among them wrinkles and dark spots. Indeed, previous studies involving 100 volunteers revealed a significant and positive correlation between porphyrins quantity on skin and the prevalence of wrinkles, invisible spots and dark spots (unpublished data). The amount of porphyrins on skin doesn't decrease with age, supporting a potential involvement in the apparition of aging signs. To confirm this hypothesis, a first *ex vivo* evaluation was performed and evidenced that porphyrins are able to penetrate in the skin until the upper dermis, confirming a colocalisation between porphyrins and melanocytes. Moreover, it is also described that *C.acnes* is also present in skin appendix (hair follicles, sebaceous glands...), reinforcing this observation that porphyrins can be in contact with fibroblasts from the upper epidermis. *In vitro* evaluation of porphyrins impact on fibroblasts was performed through a transcriptomic analysis and revealed how this metabolite influences skin aging, through degradation of ECM structure. A co-culture model of keratinocytes and melanocytes also evidenced the involvement of porphyrins in the formation of hyperpigmented spots in the basal layer through an interaction with PBR. The reduction of porphyrins concentration in this co-culture model evidenced a reduction of porphyrins-induced melanogenesis, and reducing porphyrins on the skin appeared as an efficient and innovative strategy to reduce the melanogenesis inside the skin, and on a longer term to reduce the formation of dark spots. The development of an active ingredient capable of inhibiting porphyrins production by the skin

microbiota confirmed this hypothesis: the clinical evaluation of this ingredient showed a significant reduction of porphyrins on the skin, that led to a significant reduction of invisible spots, dark spots and wrinkles relative to placebo. These results not only demonstrate that targeting porphyrins is an effective approach to reducing the appearance of aging signs but also emphasize the potential of counteracting the Porphyr'aging phenomenon as an innovative strategy for preventive aging.

Conclusion

To conclude of this study, we confirmed that targeting Porphyr'aging is as an innovative strategy to support consumers by delaying the emergence of signs in the early stages of aging and throughout life.

Acknowledgments

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Conflict of Interest Statement

The authors declare no conflicts of interest

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