IFSCC 2025 full paper (IFSCC2025-1783)

"Ubiquitination - A novel target for a universal anti-aging strategy highlighted by mass spectrometry analysis and applied to a natural ingredient"

Annabelle L'Hermitte [1,*], Lucie Cardinaud [1] and Perrine Cancellieri [1]

[1] Hallstar Beauty, Mauguio-France
* Annabelle L'Hermitte, 50 Rue du Rajol, 34130 Mauguio – France – Tel : +33 4 67 50 72 69, annabelle.lhermitte@hallstar.com

Abstract
While beauty inclusivity is widely discussed, many consumers still have difficulty finding products that suit their skin type. This study investigates the potential of targeting ubiquitin and the ubiquitination process as a novel cosmetic strategy. Ubiquitin, a small globular polypeptide present in all human eukaryotic cells, is central to ubiquitination—a key post-translational modification of proteins, that influences their fate, function, and turnover. Our research allowed us to identify betulin and betulinic acid as potent ubiquitination modulators, prompting further exploration of Birch Bark Extract (BBE) in skin care. BBE was found to significantly stimulate skin ubiquitination and consequently degradation pathways of misfolded, denatured or obsolete proteins by regulating the Ubiquitin-Proteasome System (UPS) and autophagy. These cellular clearance processes decline with age and are impacted by external stressors like UV radiation and pollution. BBE aims to preserve their function, thus preventing visible signs of skin aging. Clinical trials with a 1% BBE concentration demonstrated efficacy on a multi-ethnic panel, with significant improvements in facial wrinkles, skin radiance, complexion uniformity, and resurfacing effects. The findings suggest BBE as a promising ingredient for inclusive skin care, offering broad benefits across diverse skin types.

Keywords: ubiquitin, ubiquitomics, mass spectrometry, betulin

1. Introduction

While beauty inclusivity is widely discussed, many consumers still have difficulty finding products that suit their skin type. What if we could find an active ingredient with maximum benefits for everyone's skin? The aim of our study was to determine if acting on ubiquitin and ubiquitination process could be an effective and universal cosmetic strategy.

Ubiquitin is a small globular polypeptide present in all men and women's eukaryotic cells. It is at the heart of ubiquitination process, a key post-translational modification that determines the fate, function, and turnover of most cellular proteins. Ubiquitination is the attachment of ubiquitins to a target protein. There are several types of ubiquitination depending on the type of linkages formed by ubiquitins. Indeed, each ubiquitin possesses 8 amino acids called functional residues: 1 methionine (M1) and 7 lysines (K6, K11, K27, K29, K33, K48 and K63).

These residues enable linkages between two ubiquitins to form polyubiquitin chain, or between a ubiquitin and its target protein [1].

After having previously identified betulin and betulinic acid as potent modulators of ubiquitination, an exploratory study was used to determine the role and regulation of ubiquitination in the skin. We then showed that a natural ingredient, Birch Bark Extract (BBE), maintains cell proteostasis by targeting specific ubiquitination pathways. Indeed, it regulates the two main cellular clearance processes, Ubiquitin-Proteasome System (UPS) and autophagy [2,3].

The functionality of the UPS and autophagy is gradually impaired during the aging process [4-7]. Additionally, these cellular clearance processes can be affected by external factors, such as UV irradiation [8] and exposure to pollution and volatile organic compounds (VOC) [9]. By stimulating them, BBE aims to preserve their function, helping to prevent the appearance of skin aging signs. Consistent with these findings, clinical efficacy at a 1% dose was demonstrated on a multi-ethnic panel, with significant improvements in facial wrinkles, skin radiance, complexion uniformity, and resurfacing effects.

2. Materials and Methods

First, we conducted an enzyme activity screening test, based on Förster Resonance Energy Transfer, to identify natural compounds able to stimulate on UBE1 (Ubiquitin-activating enzyme E1), the key enzyme involved in the first stage of protein ubiquitination. We identified a plant, the birch tree bark (*Betula alba* var. *pendula* Roth.), containing these compounds and submitted the corresponding extract to an in vitro Enzyme-Linked Immunosorbent Assay (ELISA) on Normal Human Adult Primary Epidermal Keratinocytes (HEKa) to evaluate its effect at cellular level on the increase in total ubiquitinated proteins. Cells were incubated 8 hours with 5 µg/mL BBE treatment and rapeseed oil was used as a control.

Then, we designed a groundbreaking study with an expert laboratory in ubiquitomics. In this study, we utilized TUBE-Based Mass Spectrometry Ubiquitomics to elucidate changes in ubiquitination types and the targeted proteins that were ubiquitinated (TUBE: Tandem Ubiquitin Binding Entities). The assay was performed on reconstructed skin models (EPiDerm FT) which were left untreated or topically treated with 1% BBE twice a day for 5 days.

Finally, to assess its effects on skin wrinkles, roughness, tone and radiance, a randomized double-blind clinical study was conducted on a multi-ethnic panel of 33 volunteers (43% Caucasian, 36% African descent, 21% Asian, 79% females, 21% males), aged between 31 to 68 years old. 1% plant extract was applied to one side of the face and a placebo to the other side, twice daily for 28 days.

3. Results and Discussion

3.1. Selection of UBE1 natural activators

This screening assay enabled us to identify two natural activators of UBE1. Indeed, as shown in Figure 1, betulinic acid significantly enhances UBE1 activity by 317% at 1.69 µg/mL and betulin increases UBE1 activity by 27% and 50% at respectively 0.515 µg/mL and 515 µg/mL. UBE1 serves as the initial enzyme in the ubiquitination process. It activates ubiquitin by forming a covalent bond with it, effectively priming ubiquitin for subsequent transfer to other proteins involved in the ubiquitination pathway. Therefore, we chose to develop our active ingredient from the birch tree bark (*Betula alba* var. *pendula* Roth.) as it is one of the richest plants in betulin and betulinic acid, potentially able to stimulate protein ubiquitination.

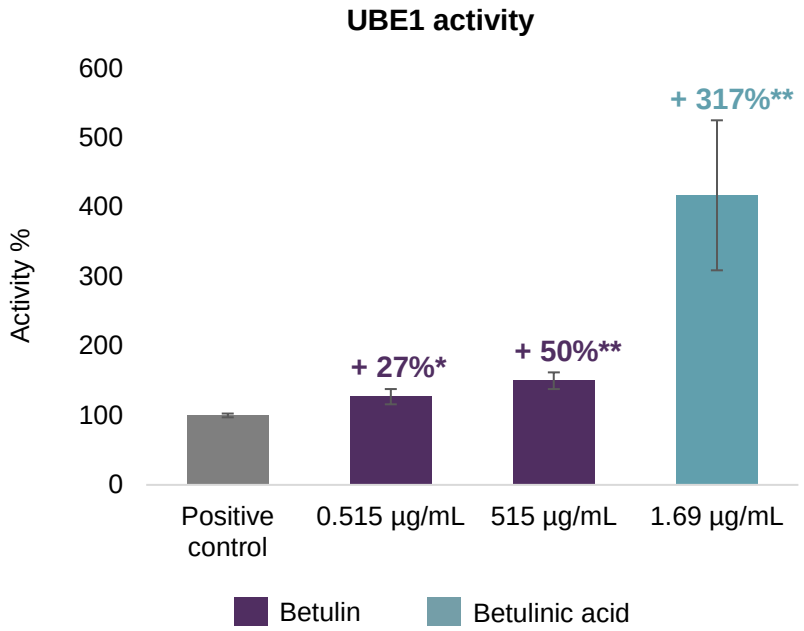

Figure 1. Betulin and betulinic acid significantly increases UBE1 activity based on fluorometric measurements. Results were obtained with the average of two independent analysis (n=2). Positive control corresponds to 100% of enzymatic activity (no sample). Student's t-test: *vs* control significant with *0.05<p<0.1,**p<0.05.

3.2. Confirmation that BBE increases ubiquitinated proteins in skin cells

The study using HEKa showed that BBE exhibits a significant 19-fold* increase in ubiquitinated proteins compared to the control condition (rapeseed oil) after a 5 µg/mL treatment. Significance of the result was measured with one-way ANOVA test: *vs* rapeseed oil, significant with *p<0.005. Results were obtained with the average of two independent analysis for BBE and three for rapeseed oil.

Ubiquitination of proteins is typically followed by UPS degradation or autophagy and the short treatment duration of 8 hours enabled us to study the increase in total ubiquitinated proteins upstream of their proteolysis. This result confirms that the molecules extracted from birch bark activate ubiquitination in skin cells.

3.3. Identification of ubiquitination types modulated in skin and regulated proteins

This study presents the first ubiquitome profiling ever conducted in human skin, unlocking the complex landscape of ubiquitination signals involved in cutaneous homeostasis. A TUBE-Based Mass Spectrometry Ubiquitomics assay was performed to assess relative proportions of ubiquitin linkage types detected in the skin. The results of the untreated skin indicated the presence of six distinct types of ubiquitin linkages, with the following three being the most predominant: K48 (91%) > K11 (5%) > K63 (4%), Figure 2.

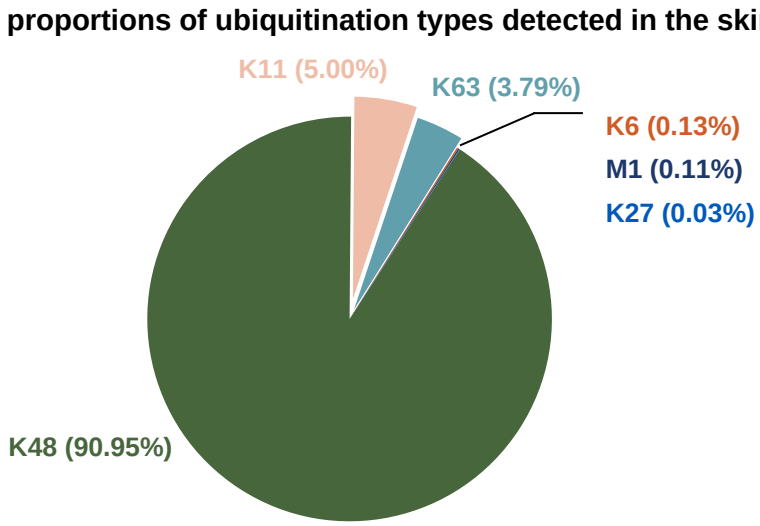

Figure 2. Untreated skin results generated after TUBE-Based Mass Spectrometry followed by bioinformatics analysis. Results were obtained with an average of two or three independent analysis (n=2 or 3).

Then, 1% BBE treatment was applied twice a day for 5 days. Results were obtained with an average of two or three independent analysis (n=2 or 3). Significance of the result was measured with one-way ANOVA test: BBE *vs* un-treated skin, significant with *p<0.05, **p<0.01. Following BBE treatment, we measured a significant decrease of 3 types of ubiquitination linkage types: K48 (-94%*), K11 (-75%**) and K6 (-74%**). It confirms that BBE activates most skin ubiquitination signals. The reduction in these 3 linkages is due to their degradation by the cellular proteolytic systems as these linkages are involved in addressing ubiquitinated proteins to the UPS and/ or stimulating autophagy [1,2]. Indeed, the longer duration of treatment compared to the previous test, Part 3.2., allowed us to obtain a snapshot of the enhanced UPS and autophagy activity.

In a second phase, a comprehensive ubiquitome characterization was performed to identify the proteins that underwent ubiquitination following BBE treatment compared to the untreated condition, Figure 3.

Ubiquitinated proteins identification, statistical analysis of BBE *vs* untreated skin

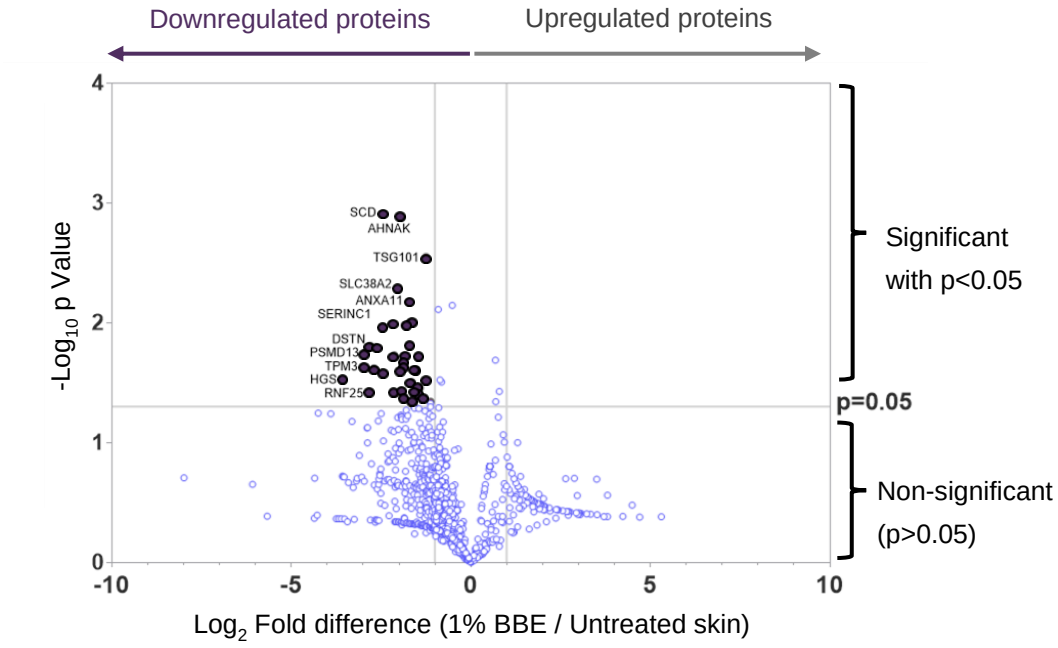

Figure 3. Volcano plot of skin ubiquitinated proteins after 1% BBE treatment twice a day for 5 days or skin left untreated. Results were generated after TUBE-Based Mass Spectrometry followed by bioinformatics analysis. 44 proteins labelled in dark purple circles were significantly decreased. Student's t-test: *vs* untreated skin.

BBE treatment resulted in the significant decrease in the level of 44 proteins compared to the non-treated skin tissue, indicating that these proteins have been ubiquitinated and addressed to clearance pathways. It demonstrates the stimulation of autophagy and UPS, leading to multiple clinical benefits.
These proteins are mainly associated with protein digestion pathways, endocytosis, lysosome as well as extracellular matrix-receptor interactions. Therefore, proteins that enable and are involved in these cellular clearance mechanisms. For instance, CLTC, HSPA2, and HGSNAT are respectively involved in autophagosome formation, the delivery of ubiquitinated proteins to UPS and phagophores, and the lysosomal degradation of specific extracellular matrix components [10-13].

3.4. Clinical efficacy on a multi-ethnic panel at 1% dose

To assess the effects of 1% BBE on skin wrinkles, roughness, tone and radiance, a randomized double-blind clinical study was conducted on a multi-ethnic panel of 33 volunteers.

First, wrinkles reduction was evaluated by volunteers scoring according to a 10 degree-scale. After 28 days of application, BBE significantly reduces skin wrinkles by -31%** (-30%$^{\#}$ *vs* placebo) for the Caucasian panelists, by -37%* (-66%$^{\#}$ *vs* placebo) for the Asian (Chinese) subjects and by -35%** (-45%$^{\#}$ *vs* placebo) for the volunteers of African descent, Figure 4.

Then, after 28 days of use and when considering:
- the gender, BBE significantly reduces wrinkles by -33%** on females (-36%$^{\#\#}$ *vs* placebo) and by -38%* on males (-76%$^{\#}$ *vs* placebo).
- the global panel, BBE significantly erases wrinkles by -34%** (-42%$^{\#\#\#}$ *vs* placebo).
Significance of the result was measured with student's t-test: *vs* D1 (baseline), significant with *p<0.05, **p<0.0001, *vs* placebo, significant with $^{\#}$ p<0.05, $^{\#\#}$ p<0.01, $^{\#\#\#}$ p<0.001.

No significant effect was noticed with the placebo regardless of ethnic group or gender.

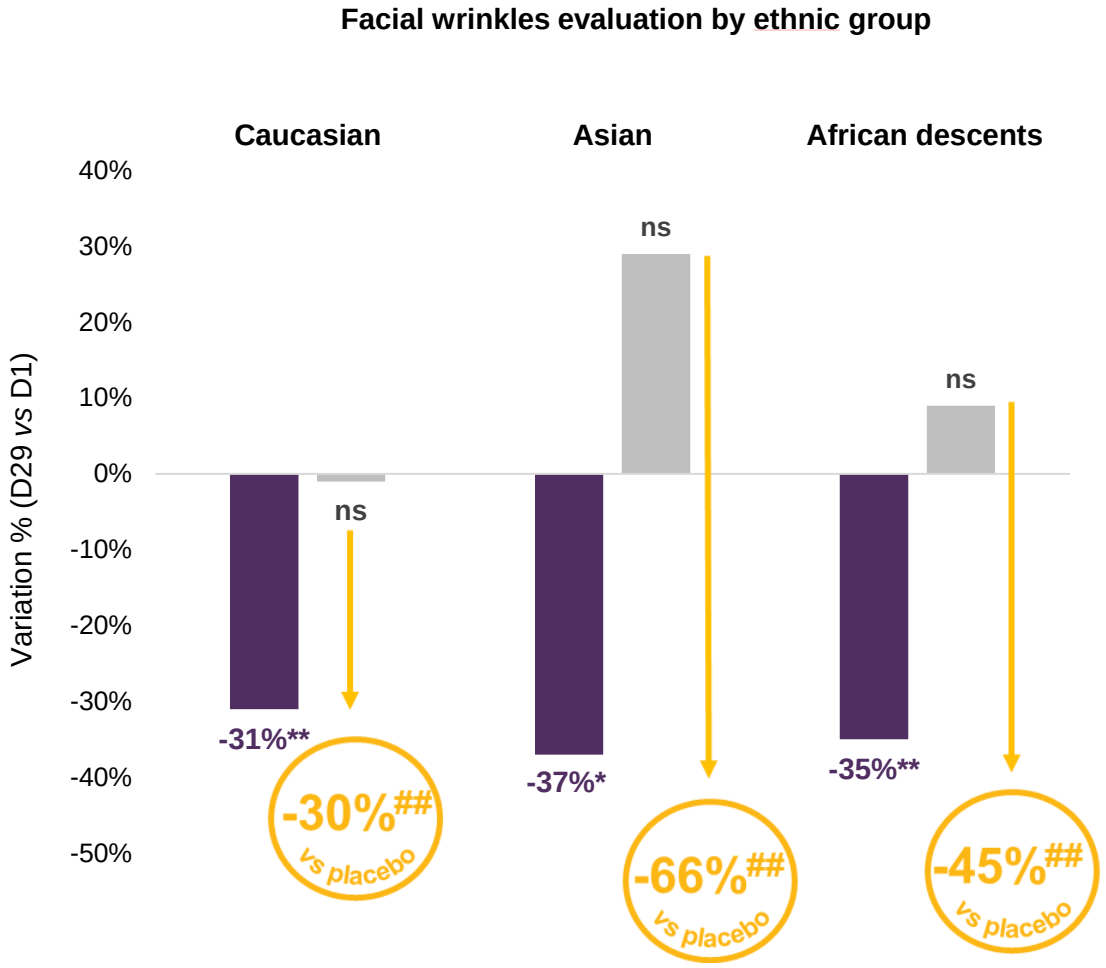

Figure 4. Variation (%) between D29 and D1 of wrinkles reduction for placebo (in grey) and 1% BBE (in purple) after volunteers scoring. Results were analyzed by ethnic groups. Student's t-test: vs placebo, significant with $^{\#}$ p<0.05, $^{\#\#}$ p<0.01, *vs* D1 (baseline), significant with *p<0.05, **p<0.01, ***p<0.0001.

The high-resolution images, Figure 5, demonstrate the anti-wrinkle effect of BBE after 14 days and 28 days of application.

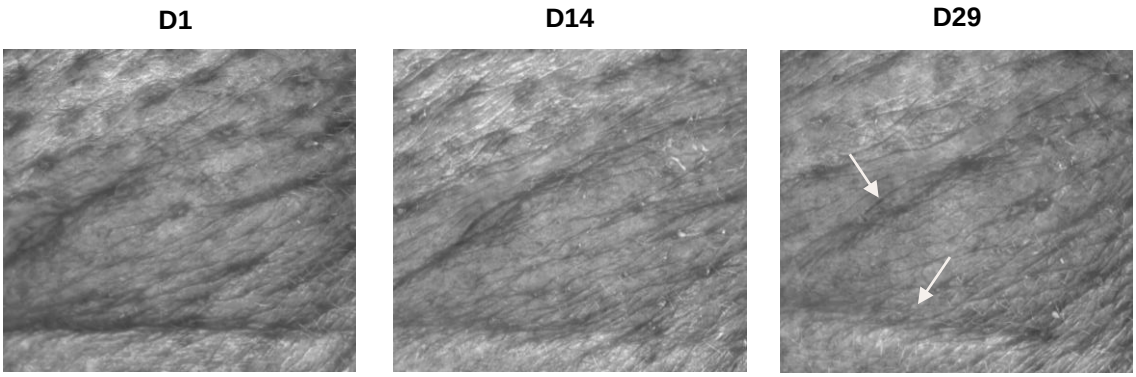

Figure 5. High-resolution images of crow's feet taken with VisioScan™. 1% BBE treatment was applied two times a day for 28 days. Measurements were performed at day 1 before treatment, 14 days and 29 days. Images obtained from a 43-year-old male of Asian (Chinese) descent, Fitzpatrick skin phototype III, presenting with normal and sensitive skin characteristics.

Then improvements in skin radiance, complexion uniformity, and resurfacing effects were also evaluated, Figure 6. Double scorings were carried out by a technical expert and by the volunteers at D29, according to 10 degree-scales. BBE significantly and respectively:

- increases skin color homogeneity by +19%*** and by +35%***
- enhances radiance by +14%* and by +28%**
- removes roughness by -16%* and by -20%**

Significance of the result was measured with student's t-test: *vs* control significant with *0.05<p<0.1, **p<0.05, ***p<0.001. No significant effect was noticed with the placebo.

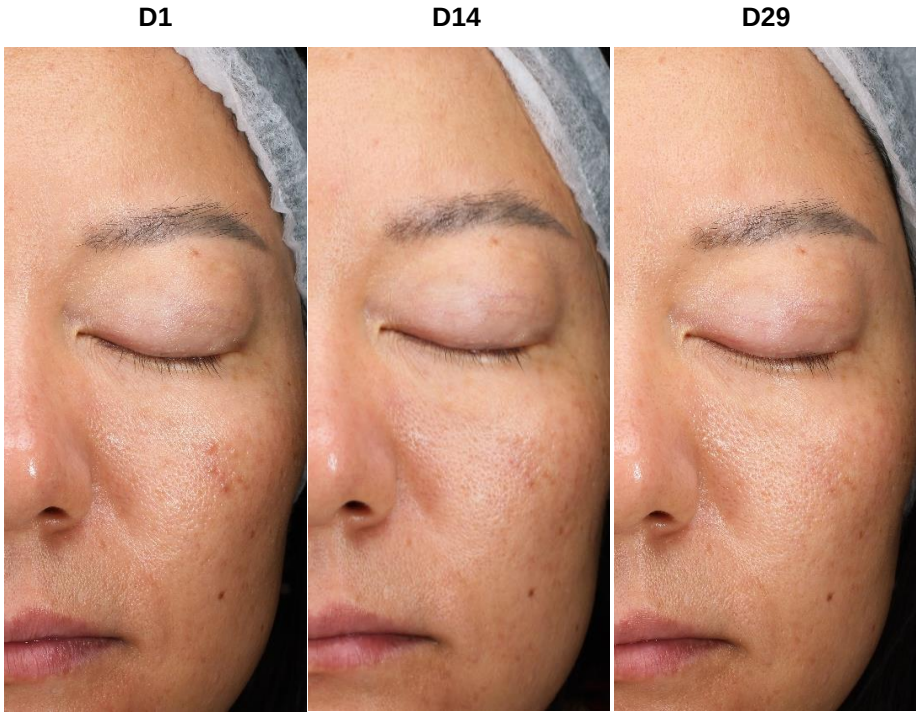

Figure 6. High-resolution digital photographs of whole face taken with VISIA®. 1% BBE treatment was applied two times a day for 28 days. Measurements were performed at day 1 before treatment, 14 days and 29 days. Images obtained from 39-year-old women of Asian (Chinese) descent, Fitzpatrick skin phototype II, presenting with mixed oily and sensitive skin characteristics.

5. Conclusion

Interestingly, several studies suggest that impaired protein clearance mechanisms contribute significantly to the aging process in human skin. Maintaining the activity of the UPS and autophagy pathways may therefore help delay skin aging. Our results show that BBE is an effective ubiquitination activator which stimulates K48, K11 and K6 ubiquitinations that is a good strategy for acting on cellular proteolytic systems and visibly improve the skin's appearance. Clinical benefits of 1% treatment BBE have been validated on a multi-ethnic panel. To further support this innovative concept, quantifying carbonylated proteins—a recognized marker of irreversible oxidative protein damage—could provide deeper insight into BBE's protective effects against oxidative stress. Since carbonylation impairs protein structure and function, its clearance via proteasome and autophagy is essential for maintaining proteostasis and skin health [6].

6. References

[1] George Vere, Rachel Kealy, Benedikt M. Kessler and Adan Pinto-Fernandez. Ubiquitomics: An Overview and Future. Biomolecules (2020), 10, 1453.
[2] Yong Tae Kwon and Aaron Ciechanover. The Ubiquitin Code in the Ubiquitin-Proteasome System and Autophagy. Trends in biochemical sciences (2017), 42(11), 873-886.
[3] Yongrong Liao, Izabela Sumara, Evanthia Pangou. Non-proteolytic ubiquitylation in cellular signaling and human disease. Communications Biology (2022), 5(1), 114.
[4] David Vilchez, Isabel Saez, Andrew Dillin. The role of protein clearance mechanisms in organismal ageing and age-related diseases. Nature Communications (2014), 5, 5659.
[5] María Carolina Barbosa, Rubén Adrián Grosso, Claudio Marcelo Fader. Hallmarks of Aging: An Autophagic Perspective. Frontiers in endocrinology (2019), 9, 790.
[6] Ioanna Korovila, Martín Hugo, José Pedro Castro, Daniela Weber, Annika Höhn, Tilman Grune, Tobias Jung. Proteostasis, oxidative stress and aging. Redox biology (2017), 13, 550-567.
[7] Ines Martic, Pidder Jansen-Dürr, Maria Cavinato. Effects of Air Pollution on Cellular Senescence and Skin Aging. Cells (2022), 11, 2220.
[8] Anne-Laure Bulteau, Marielle Moreau, Carine Nizard, Bertrand Friguet. Impairment of proteasome function upon UVA- and UVB-irradiation of human keratinocytes. Free Radical Biology and Medicine (2002), 32(11), 1157-1170.
[9] Marlène Dezest, Mickael Le Bechec, Laurent Chavatte, Valérie Desauziers, Benoît Chaput, Jean-Louis Grolleau, Pascal Descargues, Carine Nizard, Sylvianne Schnebert, Sylvie Lacombe, Anne-Laure Bulteau.Oxidative damage and impairment of protein quality control systems in keratinocytes exposed to a volatile organic compounds cocktail. Scientific reports (2017), 7(1), 10707.
[10] Anna Young. Structural insights into the clathrin coat. Seminars in cell & developmental biology (2007), 18(4), 448-58.
[11] Brinda Ravikumar, Kevin Moreau, Luca Jahreiss, Claudia Puri, David C Rubinsztein. Plasma membrane contributes to the formation of pre-autophagosomal structures. Nature cell biology (2010), 12(8), 747-57.
[12] María Rosario Fernández-Fernández, Marcos Gragera, Lissette Ochoa-Ibarrola, Lucía Quintana-Gallardo, José María Valpuesta. Hsp70 - a master regulator in protein degradation. FEBS Letters (2017), 591(17), 2648-2660.
[13] Ruisheng Xu, Yingjie Ning, Fandong Ren, Chenxia Gu, Zhengjiang Zhu, Xuefang Pan, Alexey V Pshezhetsky, Jingpeng Ge, Jie Yu. Structure and mechanism of lysosome transmembrane acetylation by HGSNAT. Nature structural & molecular biology (2024), 10, 1502-1508.