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Holistic approach targeting Primary Hallmarks of Ageing to reverse it: toward a new role for Hyaluronic Acid?

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1. Introduction

Hyaluronic acid (HA) is a crucial molecule in the cosmetics industry, known for its varying effects based on molecular weight (MW). High molecular weight (HMW) HA primarily hydrates the skin surface, while medium molecular weight (MMW) HA enhances skin defense and stimulates collagen I production [1].

The understanding of biological ageing has advanced significantly, with over ten methods available to quantify it, including telomere length measurement, epigenetic clocks, and biomarker studies. An international scientific consortium has classified the Hallmarks of Ageing into three categories: Primary hallmarks, which reflect accumulated damage; Antagonistic hallmarks, which denote regulatory failures due to damage; and Integrative hallmarks, which emerge when both primary and antagonistic hallmarks cannot be compensated [2-3].

The Primary Hallmarks of Ageing are recognized as the fundamental causes of skin damage, making them ideal targets for anti-ageing strategies. Key components include genomic instability, telomere attrition [4], epigenetic alterations [5], loss of proteostasis [6], and impaired macro-autophagy. Notably, DNMT3B, a DNA methyltransferase, plays a significant role in maintaining genomic methylation patterns [7,8] avoiding cryptic transcription [9], while SIRT1, a transcriptional regulator [10,11], helps modulate telomerase activity to preserve telomere length. With ageing, the activity of the proteasome, essential for cellular protein turnover and homeostasis, declines, further complicating the ageing process [12].

An extra low molecular weight (ELMW) HA has been developed to explore additional cosmetic benefits, with preliminary studies suggesting its potential for cell penetration and new intracellular functions that may help reverse ageing.

2. Materials and Methods

2.1. DNMT3B expression in keratinocytes

Normal Human Epidermal Keratinocytes (NHEKs) were cultivated in monolayer at passage 2 for 72 hours in absence of product. These cells will be used as non senescent control.

NHEKs from this same donor were then sub-cultivated until passage 4 and then incubated for 72 hours in absence of in presence of positive reference (IL-6 at 50ng/mL) or ELMW HA at 10µg/mL diluted in culture medium.
At the end of the incubation period, nuclear proteins were extracted from cell monolayer. DNMT-3B was then quantified using a sensitive and specific ELISA kit. Proteins contained in the cell lysates (nuclear fraction) were quantified using a spectrocolorimetric method (Bradford method).

2.2. ISO-SEQ analysis to measure level of aberrant transcription of isomers

NHEKs keratinocytes have been cultivated in monolayer until reaching confluency. Cells at passage 2 were incubated for 72 hours and kept untreated as young skin cells reference.
Cells prematurely aged by replicative senescence (passage 4) were incubated for 72 hours in absence (control at passage 4) or in presence of ELMW HA at 10µg/mL.
At the end of incubation, mRNA were extracted using RNA extraction kit. Then, ISO-Seq sequencing was performed. To identify aberrant transcript isoforms, a specific data processing was used.

2.3. Sirtuin 1 expression in keratinocytes

NHEKs were cultivated in monolayer until reaching confluency. Then, NHEKs were incubated for 24 hours in absence of in presence of positive reference (rosiglitazone at 10µM) or ELMW HA at 5 and 10µg/mL diluted in culture medium. At the end of the incubation period, nuclear proteins were extracted from cell monolayer. SIRT-1 expression was then quantified using a sensitive and specific ELISA kit. Proteins contained in the cell lysates (nuclear fraction) were quantified using a spectrocolorimetric method (Bradford method).

2.4. Sirtuins activity in keratinocytes

NHEKs were cultivated in monolayer until reaching confluency. Then, NHEKs were incubated for 24 hours in absence of in presence of positive reference (rosiglitazone at 10µM) or ELMW HA at 5 and 10µg/mL diluted in culture medium. At the end of the incubation period, nuclear proteins were extracted from cell monolayer. Total sirtuins activity was then quantified using a sensitive and specific enzymatic activity measurement kit. Proteins contained in the cell lysates (nuclear fraction) were quantified using a spectrocolorimetric method (Bradford method).

2.5. PARP-1 expression and activity in keratinocytes

NHEKs were cultivated in monolayer until reaching confluency. Then, NHEKs were incubated for 24 hours in absence of in presence of positive reference (rosiglitazone at 10µM) or ELMW HA at 5 and 10µg/mL diluted in culture medium. At the end of the incubation period, nuclear proteins were extracted from cell monolayer. PARP activity was then quantified using a sensitive and specific enzymatic activity measurement kit . PARP-1 expression was then quantified using a sensitive and specific ELISA kit. Proteins contained in the cell lysates (nuclear fraction) were quantified using a spectrocolorimetric method (Bradford method).

2.6. Telomerase expression

NHEKs were cultivated in monolayer until reaching confluency. Then, NHEKs were incubated for 24 hours in absence of in presence of positive reference FK228 at 100ng/mL or ELMW HA at 10µg/mL diluted in culture medium. At the end of the incubation period, nuclear proteins were extracted from cell monolayer. Telomerase expression was then quantified using a sensitive and specific ELISA kit.

2.7. Telomerase activity

NHEKs were cultivated in monolayer until reaching confluency over 2 passages between P2 and P4 (prematurely aged). Then, NHEKs were incubated for 5 days in absence of in presence of ELMW HA at 10µg/mL diluted in culture medium. At the end of the incubation period, cells were trypsinized and rinsed with PBS. Cells pellets were collected and stored at -80°C until the DNA extraction. Telomerase activity was then quantified indirectly using qPCR analysis (QuantStudio3 Real-Time PCR System, Applied Biosystem) and targeting a specific sequence of telomere gene using a kit.

2.8. Proteasome activity and lysosome amount

NHEKs were cultivated in monolayer until reaching confluency (24 hours). Then, ELMW HA at 10µg/mL diluted in culture medium was applied for 6 hours of incubation.
After 6 hours, treatments were removed, and fresh media was added. The substrate LLVY was added to live cells and incubated at 37°C and 5% CO_2, for 1 hour. The cleavage of by the proteasome generates a fluorescent compound which becomes detectable and quantifiable using a microplate reader.
After reading the fluorescence, the medium was removed, the cells were fixed and DAPI labelling was carried out to allow the fluorescence to be normalized to the same number of cells.
For lysosome amount, after 6 hours, treatments were removed, and cells were incubated with lysosome detection probe for 1 hour and lysosomes were observed and quantified by automated fluorescence microscopy.

2.9. Clinical study

The aim of this study was to conduct a clinical evaluation of ELMW HA at 0.5% in a double-blind, randomized design. A total of 40 Caucasian women, aged between 51 and 74 years (mean age: 61.4 ± 6.9 years), with visible wrinkles and fine lines on their faces were recruited for the study. The active ingredient was tested in a cream formula at 0.5%, with a placebo formula used as a control.

All participants provided their consent for participation, and the clinical procedures complied with the Declaration of Helsinki. The study was previously ethically approved by the Internal Review Board (IRB) under the reference number 2024-001.

INCI formula

AQUA/WATER, CETYL ALCOHOL, GLYCERYL STEARATE, PEG-75 STEARATE, CETETH-20, STEARETH-20, ISODECYL NEOPENTANOATE, PENTYLENE GLYCOL, CAPRYLIC / CAPRIC TRIGLYCERIDE **± ELMW HA** ,CITRIC ACID, DIMETHICONE, SODIUM BENZOATE, AMMONIUM, FRAGRANCE ACRYLOYDIMETHYLTAURATE/BEHENETH-25 METHACRYLATE CROSSPOLYMER

Wrinkles analysis measured by VISIA CR2.3®

Using the Visia CR 2.3® from Canfield Imaging Systems, digital photography of the face was conducted at different time points, with repositioning at D0. The repositioning was controlled directly on the data-processing screen using an overlay visualization of the images at each acquisition time. The Visia CR 2.3® allows for capturing images under different types of illumination and enables rapid image capture. A series of photos taken under multi-spectral imaging and analysis captures visual information affecting the appearance of the skin. With the obtained images, wrinkles were also analyzed. A first analysis focused on expression lines, followed by a second analysis on sleep wrinkles.

The photos were taken from the front and from the left and right sides of the face at D0, D28, and D56.

Illustrative pictures
Photos were taken of each panelist from the front and the preferred sleeping side (left or right) in a normal position, that is to say sitting without head support. The photos were taken with the camera: Canon EOS - 5D Mark IV at D0, D28 and D56.

Scooring of age using Artificial intelligent (AI)
A state-of-the-art artificial intelligence algorithm was employed to assess the apparent age of volunteers through advanced image analysis techniques. This innovative approach aimed to track the evolution of their facial features over time, allowing researchers to gain insights into how these changes affect the volunteers' perceived age.

Long-term benefit using AI prediction
This study employed a predictive modeling approach to visualize the effects of an anti-aging active ingredient compared to a placebo on a virtual human face. A 3D avatar of a 60-year-old woman was created to represent the morphological characteristics typical of this age group, including skin folds, volume loss, and texture variations.
Visual and media outputs were created to facilitate synchronous comparisons between the placebo and active ingredient conditions. This included split-screen and crossfade techniques. Informative elements were integrated during post-production, including hotspots to indicate impact areas, overlay curves to display raw data, and morphological zooms on targeted regions.

3. Results

3.1. Effect on DNM3TB expression in prematurely aged keratinocytes

The following experiment has been designed to observe the expression of DNMT3B by comparing a “young” culture and prematurely aged (by replicative senescence) keratinocytes.

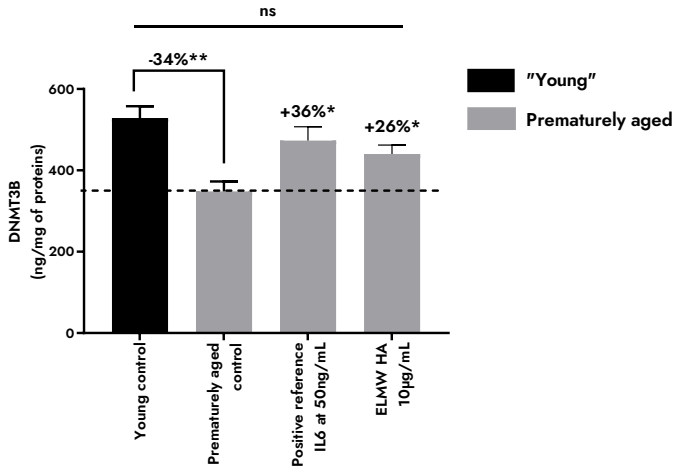


Figure 1. Quantification of DNMT3B expression in keratinocytes (young passage and prematurely aged). Student *t* test with * $p<0.05$.

In this experiment, we can osbserved that the expression of DNMT3B is significantly reduced by -34%** after replicative senescence in the prematurely aged keratinocytes, evidencing the loss of this crucial DNA methyl transferase.

In this same prematurely aged keratinocytes, the positive control has significantly succeeded in restoring its expression by +36%*. In a similar manner, ELMW HA significantly boosted the expression by +26%*.

3.2. Effect of ELMW HA on reducing aberrant transcription

We designed a unique method to assess the quantification of this cryptic transcription using ISO sequencing. The number of different unique isomers was counted and no significant difference was found between the young and prematurely-aged condition. The treatment of prematurely-aged keratinocytes induced a slight decrease of isomers numbers, suggesting a shift toward the “young” condition (*data not shown*). Then, we assessed the number of incomplete splice match (ISM) isoforms that specifically characterize the generation of aberrant transcription.

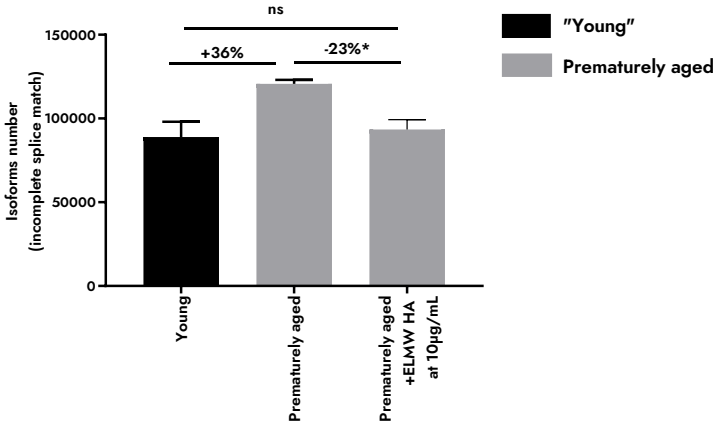
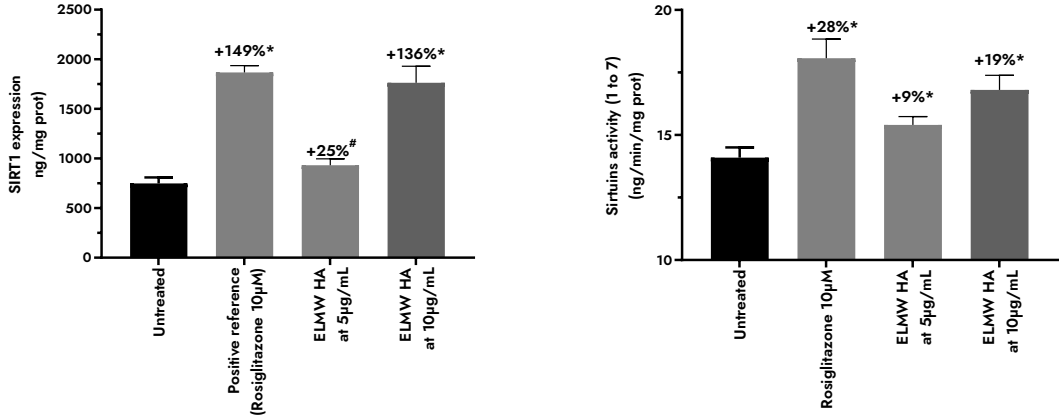


Figure 2. Quantification of isoforms number by ISO-SEQ representing aberrant transcription keratinocytes (young passage and prematurely aged). Isoforms (incomplete splice match) are given in number counted. Pairwise Student *t* test with * $p<0.05$.

3.3. Effect of ELMW HA on sirtuin 1 expression and sirtuins activities in keratinocytes

In Figure 3.a. the expression of sirtuin 1 was assed in NHEKs. ELMW HA significantly increased the SIRT1 expression up to +136%* at 10µg/mL, in a dose-dependent manner.

Secondly, to go further, we assess the enzymal activity of sirtuins in the keratinocytes (Figure 3.b). In the keratinocytes culture, the positive reference significantly activated the sirtuins (1 to 7) activities by +28%*. ELMW HA significanyly increased their activity up to +19%* at 10µg/mL in a dose-dependent manner.



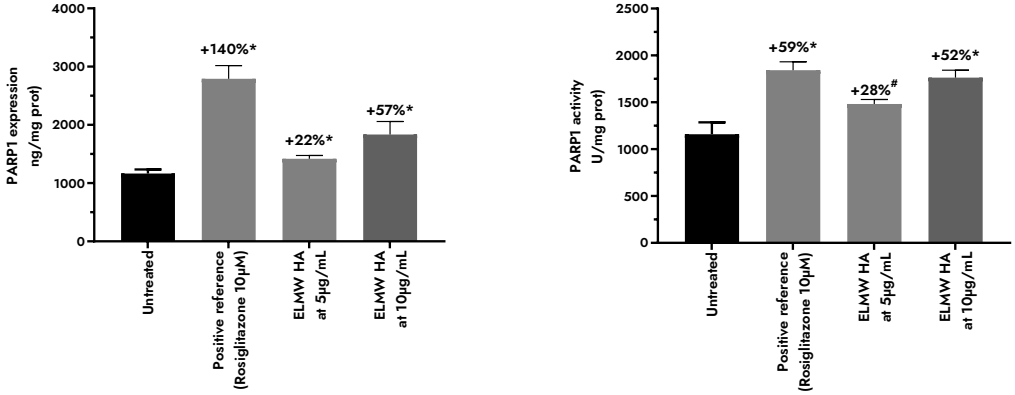
(**a**) (**b**)

Figure 3. (a) Quantification of SIRT1 expression in keratinocytes. (b) Quantification of sirtuins (1 to 7) activity in keratinocytes. Mann-Whitney test with # p<0.1 and with * p<0.05.

3.4. Effect of ELMW HA on PARP1 expression and activity

ELMW HA significantly boosted expression and activity of PARP1, in a dose-dependent manner, up to +57%* and +52%* respectively.

This effect allows us to prevent from a second Primary Hallmark of ageing, the genomic instability.

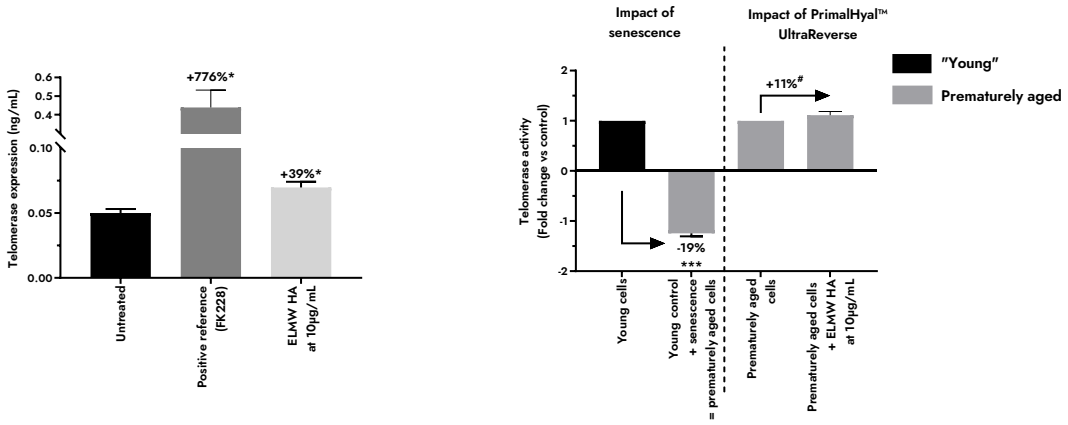


(**a**) (**b**)

Figure 4. Quantification of PARP1 expression (a) and activity (n) in keratinocytes (in absence or presence of positive reference rosiglitazone at 10µM or ELMW HA at 5 and 10µg/mL. PARP1 expression in ng/mg of proteins and activity is expressed in ng/min/mg of proteins. Statistical analysis with Mann-Whitney test with * p<0.05.

3.5. Effect of ELMW HA on telomerase expression and activity

In the study presented in Figure 5.a., we evaluated the ability to stimulate the expression of the telomerase, enzyme in charge of telomere elongation. In the second study (Figure 5.b), we evaluated the ability to stimulate the telomerase activity.



(**a**) (**b**)

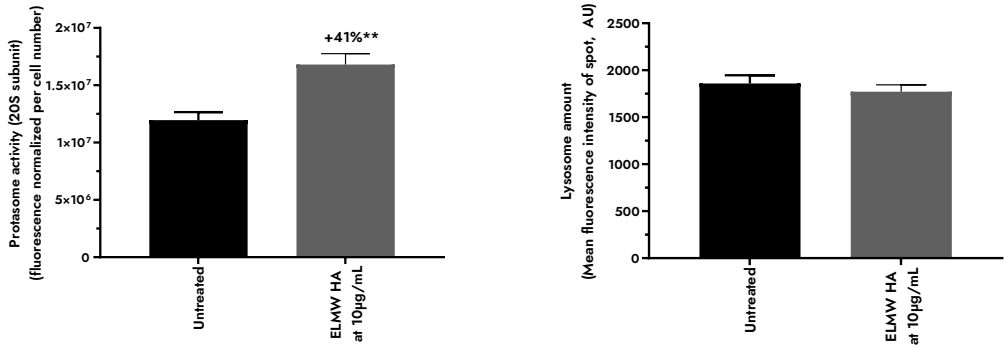
Figure 5. (a) Quantification of telomerase expression in keratinocytes. Whitney test (b) Sirtuins activity in keratinocytes. Student *t* test with $^{\#}$ p<0.1, * p<0.05 and *** p<0.001.

In Figure 5.a, we showed that ELMW HA at 10µg/mL significantly boosted by +39%* the expression of the telomerase.

In Figure 5.b., the telomerase activity has been assessed by analysing the expression of a specific gene sequence. First, impact of senescence was assessed by comparing “young” culture and prematurely aged keratinocytes (replicative senescence). The results showed that ageing has an impact since the fold change was significantly reduced by -19%***. ELMW HA was applied to prematurely aged keratinocytes and fold change was significantly increased by +11%$^{\#}$. This result evidenced a positive increase in telomerase activity.

3.6. Effect of ELMW HA on proteasome activity and lysosome amount

In this study, we assessed the proteasome activity in keratinocytes. ELMW HA significantly increased the proteasome activity by +41%**, suggesting a beneficial effect against proteostatis loss.



(**a**) (**b**)

Figure 6. (a) Quantification of proteasome activity in keratinocytes (in absence or presence of positive reference rosiglitazone at 10µM or ELMW HA at 5 and 10µg/mL. (b) Lysosome amount expressed in Mean fluorescence intensity (AU). Statistical analysis with Mann Whitney test with ** p<0.01.

ELMW HA had no effect on lysosome amount.

3.7. Effect of ELMW HA on global wrinkles

Our results demonstrated that ELMW HA significantly and progressively reduced the number of wrinkles after 28 and 56 days, with reductions of -7.6% and -14.8%, respectively. In contrast, the placebo showed a reduction in wrinkle count over time, but with lesser performance, exhibiting only -2.9% and -9% reductions after 28 and 56 days, respectively.

Interestingly, we found that ELMW HA performed significantly better than the placebo from 28 to 56 days of twice-daily application, showing an effect up to 2.6 times greater. Based on AI analysis and prediction, the ELMW HA significantly improved the ageing score.

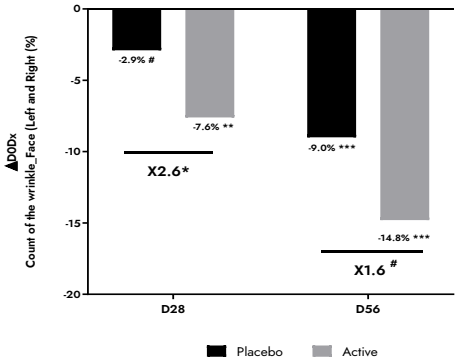


Figure 7. Evaluation of count wrinkles on all face (left and right). Wilcoxon and Mann Whitney tests with # p<0.1, * p<0.05, ** p<0.01 and *** p<0.001.

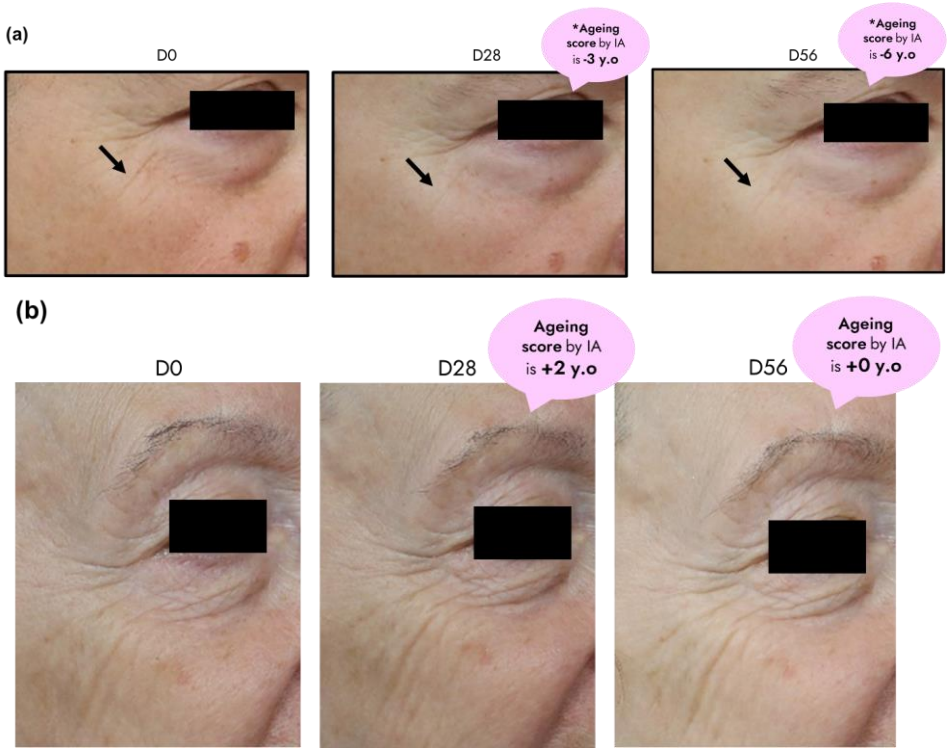


Figure 8. (a) Illustrative pictures of a volunteer 15 (56 y.o) applying cream containing 0.5% ELMW HA on face for 28 and 56 days. (b) Illustrative picture from volunteer 18 (70 y.o) applying placebo cream on face for 28 and 56 days. Ageing score was determined by IA.

Figure 9. 3D avatar generated by AI based on the clinical results obtained. Prediction of skin evolution over 5 years.

4. Discussion

ELMW HA evidenced pro-longevity benetifs by playing on 4 of the 5 primary hallmarks of ageing. It significantly enhances DNMT3B expression by 26% in prematurely aged keratinocytes, while also reducing aberrant transcription by 18.1%. It boosts SIRT1 expression and activity by 136% and 19%, respectively, and elevates PARP1 expression and activity by 57% and 52%. These results demontstrates the reverse ageing effect by protecting DNA instability and controlling a good epigenetic mechanism. Additionally, it increases telomerase expression by 39% and proteasome activity by 41%, contributing to improved cellular repair and maintenance. Both contributing to protection against telomere attrition and boosting proteostatsis which are both primary hallmarks for better longevity.

In clinical evaluation, ELMW GA led to a notable 14.8% reduction in wrinkle count among Caucasian women after 56 days. These findings collectively underscore the product's potential as a powerful anti-aging solution, effectively addressing multiple hallmarks of skin aging and promoting overall skin vitality and longevity.

5. Conclusion

A new mode of action for this extra low MW HA has been discovered, targeting 4 to 5 PHoAs, contributing to a better understanding of the anti-ageing mechanisms behind the iconic HA.

6. References

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