Skin penetration of high molecular weight HA thanks to a breakthrough vectorization system

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Abstract

Background: Hyaluronic acid (HA) is an iconic cosmetic active ingredient, owing multiple skin care benefits according its molecular weight (MW). Here, we developed an innovative vectorization technology in order to drive HMW HA into skin through better skin penetration to get optimized HA benefits for cosmetic application.

Methods: Skin penetration analysis was done on e-vector-HA versus HA alone by Raman spectroscopy. We conducted several experiments in order to elucidate the mode of action of e-vector-HA. First, HA's MW was determined by Size Exclusion Chromatography. Then, we studied the sensitivity to hyaluronidases *in tubo*. Finally, electrical potential was studied by Zeta potential analysis. We explored the benefits of e-vector-HA by evaluating the smoothing effect on skin explants. Finally, a clinical study was conducted to measure the benefit of e-vector-HA on crow's feet surface and skin mattifying by ColorFace® analysis.

Results: Skin penetration study evidenced an accumulation of HA alone at the skin surface while e-vector-HA penetrated. The MW analysis revealed that the HA conserved the same MW after the process. The resistance test to hyaluronidases evidenced that HA alone and e-vector-HA had the same sensitivity. Finally, the evaluation of zeta potential evidenced that e-vector-HA has a more negative electrical potential than HA alone. The study on skin explants evidenced a clear smoothing effect with e-vector-HA and the clinical study evidenced a significant reduction of fine lines and a mattifying effect.

Conclusion: By using an innovative vectorization technology, we showed for the first time that HMW HA are able to penetrate into the skin, enlarging the clinical benefits of this iconic cosmetic ingredient.

Keywords: Hyaluronic acid; High molecular weight; Skin vectorization; Skin attraction; Smoothing

Introduction.

Skin benefits of Hyaluronic Acid (HA) is well-known today and considerated as an iconic cosmetic active ingredient [1][2]. According to its molecular weight, skin benefits are various and are dependent to their penetration depth [3]. Indeed, High Molecular Weight of Hyaluronic Acid (HMW HA) is known to remained at the surface and provide film-forming property, bringing protection and hydration to the skin [3].

Aiming to bring the properties of HMW HA deeper into the skin, we developed an innovative active ingredient based on vectorization process: e-vector-HA. We worked on the electrostatic properties of the HA complexed in clay in order to drive HA deeper into the skin [4][5].

On this study, we first evaluated the skin penetration enhancement of e-vector-HA in comparison with HMW HA then we confirmed our hypothesis by eliminating each question that could be asked regarding the skin penetration enhancement. Finally, we evaluated the skin benefits brought by the vectorization process on skin smoothing properties and skin brightness reduction at *ex vivo* level and clinical level.

Materials and Methods.

Skin penetration analysis by Raman spectroscopy

Human skin explants from a 47 years old donor were prepared and kept in survival medium (MIL215001, Biopredic) for 24 hours at 37°C and 5% CO_2. The next day, e-vector-HA at 10% (corresponding to 1% of HMW HA) and HMW HA at 1% in distilled water were topically applied and incubated for 8 hours at 37°C and 5% CO_2 before skin penetration analysis. The untreated condition did not receive any treatment. After the end of incubation, the skin surface was cleaned in order to eliminate any excess of the product. The skin explants

were then frozen at -80°C and cut longitudinally using a cryotome with a thickness of 20µm. For each explant, 3 tissue sections were selected and deposited on a CaF2 support for Raman imaging analysis for a total of 9 Raman images per condition. 3 other adjacent sections of 7µm thickness were prepared for an Hematoxylin & Eosin staining.

Raman images have a size of Y: 10µm / X: 100µm with a step of 5µm in X and 5µm in Y. Each Raman image has 3Y spectra and 21X spectra (63 spectra per image). Acquisitions were obtained with a laser wavelength at 600nm, an objective at 100X and a spectral range from 400 to 4000 cm^{-1}.

In order to ensure reproducibility of the measurements; before each use, the Raman spectrometer is calibrated with silicon which gives a Raman peak at 520.7 cm^{-1}.

A pre-processing of Raman images was made by eliminating aberrant spectra (fluorescence, burning, saturation), correcting the baseline, applying a spectral smoothing and despike and a spectral normalization.

The processing of corrected data maps was performed by using homemade software based on least squares fitting method that operates with Matlab software. This method involves mathematical modeling of reference spectra in the overall spectral image to determine the contribution and distribution of these spectra within the image. In this study, we used as reference spectra, the average spectra of hyaluronic acid and clay.

Impact of used process on the molecular weight

Size Exclusion Chromatography (SEC), is used for determination of the molecular weight. The molecule, perfectly soluble, is injected into a Gel Permeation column containing a distribution of pore sizes which separate the polysaccharides by size. The size-separated chains are measured with different detectors regarding their retention time in the column. After calculation, the molecular weight distribution, along with molecular weight moments (number-, weight-, viscosity-, averaged molecular weights) could be done. The detectors measure the Refractive Index, Intrinsic Viscometry and Low and Right Angle Laser Light Scattering.

Sensitivity to hyaluronidases

The e-vector-HA, HMW HA and clay alone were prepared in distilled water at similar concentrations in presence or absence of hyaluronidase solution (Sigma-Aldrich) at 8 UI/ml. After 16 hours of incubation in an oven at 55°C, the solutions were collected and analysed in HPLC/UV system after LC/SEC.

Zeta potential measurement

E-vector-HA and HMW HA were diluted in distilled water and added into DTS 1070 cell (Malvern Panalytical). After complete immersion of the electrodes, the cell is inserted in the Zetameter (zetasizer Nano Z, Malvern Panalytical). An average of 10 measurements is then calculated by the software Zetasizer), giving the zeta potential of the product.

Evaluation of smoothing effect of the skin surface

Human skin explants from a 55 years old donor were prepared and kept in survival medium for 24 hours at 37°C and 5% CO_2. The next day, e-vector-HA at 1% (corresponding to 0.1% of HMW HA) and HMW HA at 0.1% in distilled water were topically applied and incubated for 72 hours at 37°C, 5% CO_2 and under reduced hygrometry (20% of relative humidity). The untreated condition did not receive any treatment.

At the end of incubation, the smoothing effect was then analysed with a Scanning Electron Microscopy (SEM).

Clinical evaluation

We carried out a clinical study in double blind and placebo controlled condition on one group of 39 volunteers aged from 35 to 55 years old divided in two groups (mean age 49±5 years old). Volunteers has been recruited according to inclusion criteria which are having dry skin on cheek and fine line on crow's feet area. All the subjects participating in the study gave their informed consent signed at the beginning of the study. The study followed and was in compliance with the tenets of the Declaration of Helsinki.

Volunteers twice daily applied a cream containing 1% of e-vector-HA or placebo in full face for 28 days.

The smoothing property was analysed by ColorFace® based on crow's feet wrinkles surface analysis and the mattifying effect was measured by ColorFace® using skin brightness parameter, at D0, T1h, T6h, and D28.

INCI FORMULA

AQUA/WATER, CETYL ALCOHOL, GLYCERYL STEARATE, PEG-75 STEARATE, CETETH-20, STEARETH-20, ISODECYL NEOPENTANOATE, ±BENTONITE, SODIUM HYALURONATE, PHENOXYETHANOL, GLYCERIN, DIMETHICONE, FRAGRANCE

Statistical analysis

For in vitro and ex vivo studies, all results are presented as mean ± standard error of mean (SEM) of three independent triplicates. In vivo data are more expressed in percent of variation according to D0.

A Shapiro Wilk test was used to verify whether the raw data followed the Gaussian Law. In case of Normally-distributed data, the mean values were compared using either an unpaired t test (≤ 2 groups) or One-way ANOVA followed by post-hoc test (≥ 2 groups). In case of non-Normally-distributed data, a Kruskal-Wallis test followed by a Mann-Whitney U test was used for unpaired values and a Wilcoxon test for paired values.

In all cases, results were considered as significant with $p<0.1$ with #, $p<0.05$ with *, $p<0.01$ with ** and $p<0.001$ with ***.

Results

Enhanced skin penetration of high molecular weight hyaluronic acid thanks to an innovative vectorization technology

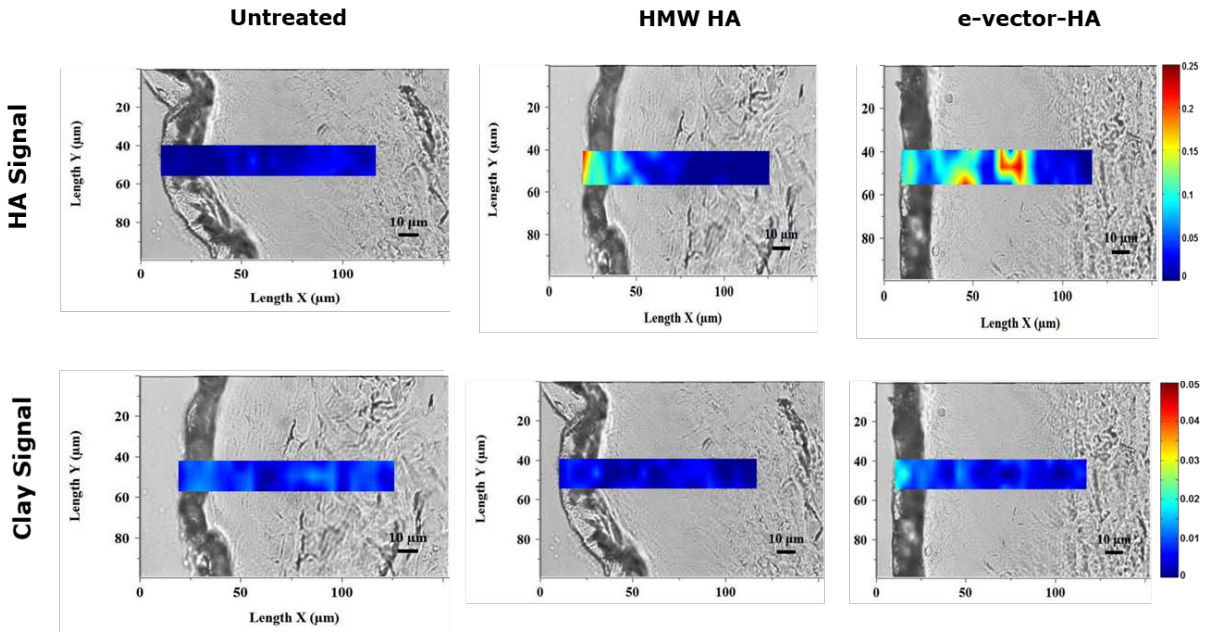

Figure 1: Skin penetration of HMW HA and e-vector-HA after 8 hours of incubation on living skin explants. Top panel: Raman spectroscopy of HA signal. Below panel: Raman spectroscopy of clay signal.

The signal of hyaluronic acid was followed in the conditions treated with the two types of HA and in the untreated condition. The results of skin penetration analysis by Raman micro-imaging demonstrated clearly that the vectorization of HMW HA improved the penetration of this latest (e-vector-HA condition) (Figure 1). The e-vector-HA reached 70µm depth in contrary of HMW HA which stayed at the surface.

<u>Elucidation of mode of action of e-vector-HA</u>

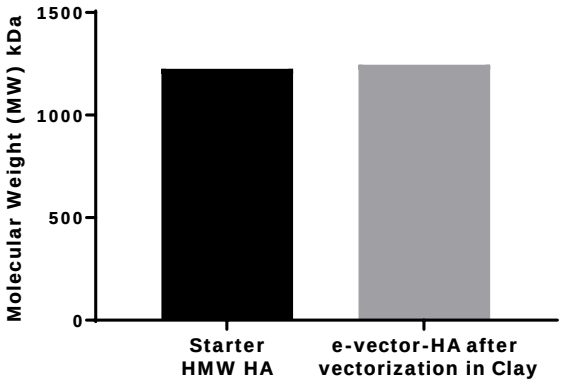

Figure 2: Measurement of Molecular weight of HMW HA before and after the vectorization process.

Molecular weight of HA was measured before and after the vectorization process in order to verify if it was impacted during it. Thanks to the results, we can confirm that this process did

not impact the molecular weight of HA (Figure 2). We can say that e-vector-HA is a vectorised HMW HA.

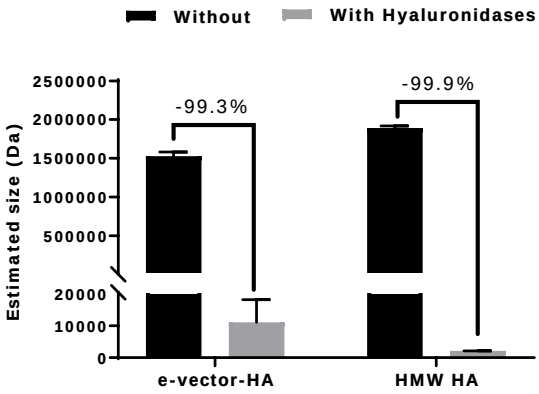

Figure 3: Measurement of molecular weight of HMW HA and e-vector HA before and after incubation in presence of hyaluronidases

Sensitivity to hyaluronidases was a hypothesis to verify consequently in order to confirm that the enhancement of skin penetration was not due to degradation by hyaluronidases. The products were exposed in tubo to hyaluronidases and then the molecular weight was estimated thanks to size exclusion chromatography (LC/SEC).

The results demonstrated that both products reacted in the same manner and were sensitive toward hyaluronidases (Figure 3).

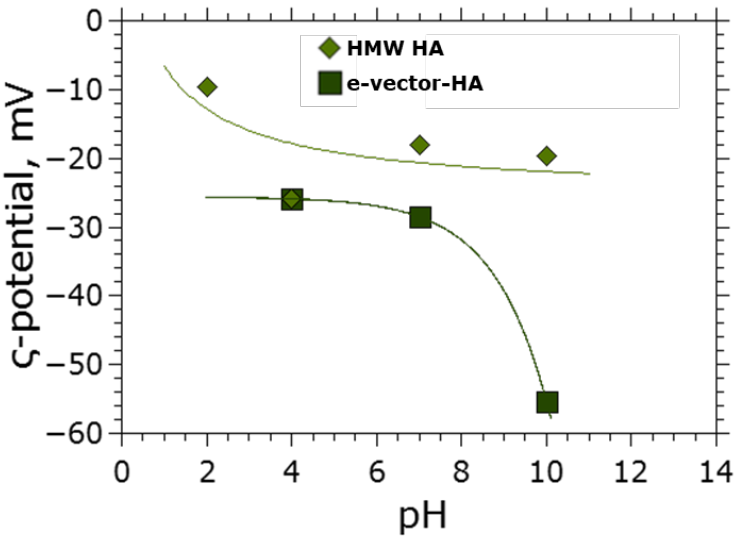

Figure 4: Zeta potential measurement of HMW HA and e-vector-HA

Measurement of Zeta potential demonstrated that HMW HA and e-vector-HA did not have the same electrostatic potential and that e-vector-HA was more negative and sensitive to a variation of pH.

Skin benefits of e-vector-HA

Enhancement of deeper penetration of HMW HA let us assumed that higher skin benefits were expected with e-vector-HA.

E-vector-HA was topically applied on skin explants for 3 days incubated in low hygrometry in order to mimic a dry atmosphere. Skin smoothing effect was evaluated with Scanning Electron Microscopy (SEM) and analysis of images revealed that in presence of e-vector-HA completely "smooth out" the *stratum corneum* (Figure 5). Indeed, we can observe that the corneocytes were no longer distinguishable in comparison to the untreated, HMW HA and clay conditions.

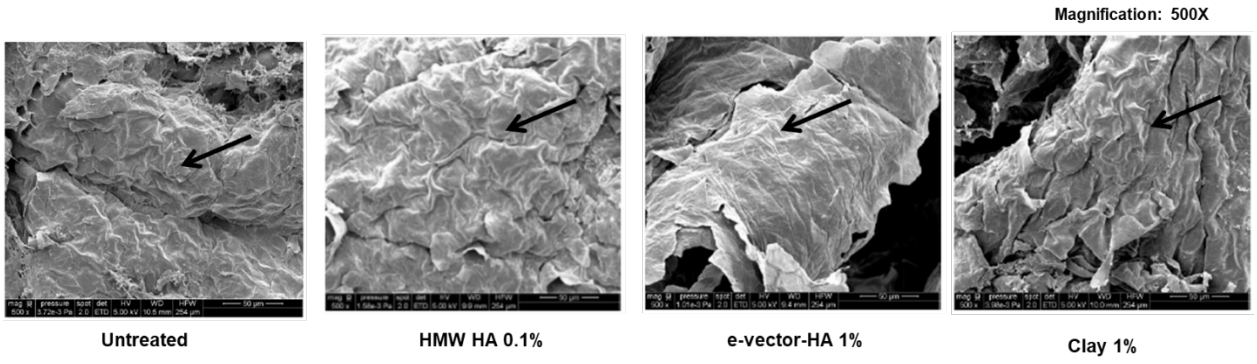

Figure 5: Representative images of the skin surface after topical treatment with the active ingredients.

The smoothing effect was then evaluated at clinical level by analysing the crow's feet surface after 1 and 6 hours and 28 days of application. The active effect was evaluated in comparison with placebo cream.

Table 1: Impact of product with or without e-vector-HA at 1% on reduction of crow's feet surface measurement by ColorFace® after 1 and 6 hours of one application and after 28 days of application. Statistical analyses were performed using Wilcoxon test and Mann Whitney test.

	Active			**Placebo**			**Mann Whitney *versus* placebo**
	Mean +/- SD (arbitrary unit)	**Average variation (%) vs D0**	**Wilcoxon test versus D0**	**Mean +/- SD (arbitrary unit)**	**Average variation (%) vs D0**	**Wilcoxon test versus D0**	
D0	2016 ± 1428			1778 ± 1516			
T1h	1651 ± 1141	-18.5%	$p<0.01$ **	1758 ± 1602	-1.1%	ns	$p<0.05$ *
T6h	1582 ± 1069	-21.5%	$p<0.05$ *	1689 ± 1633	-5%	ns	$p<0.1$ #
D28	1466 ± 1054	-27.3%	$p<0.001$ ***	1696 ± 1658	-4.6%	ns	$p<0.05$ *

The results demonstrated an immediate and significant effect after 1 hour and 6 hours of one application (Table 1). The surface of crow's feet was reduced down to 21.5% in comparison to T0 and the effect was significant in comparison with placebo.

After 28 days of application, the crow's feet surface was significantly reduced down to 27.3% and the result was significant in comparison with placebo. Illustrative pictures are presented below in Figure 6.

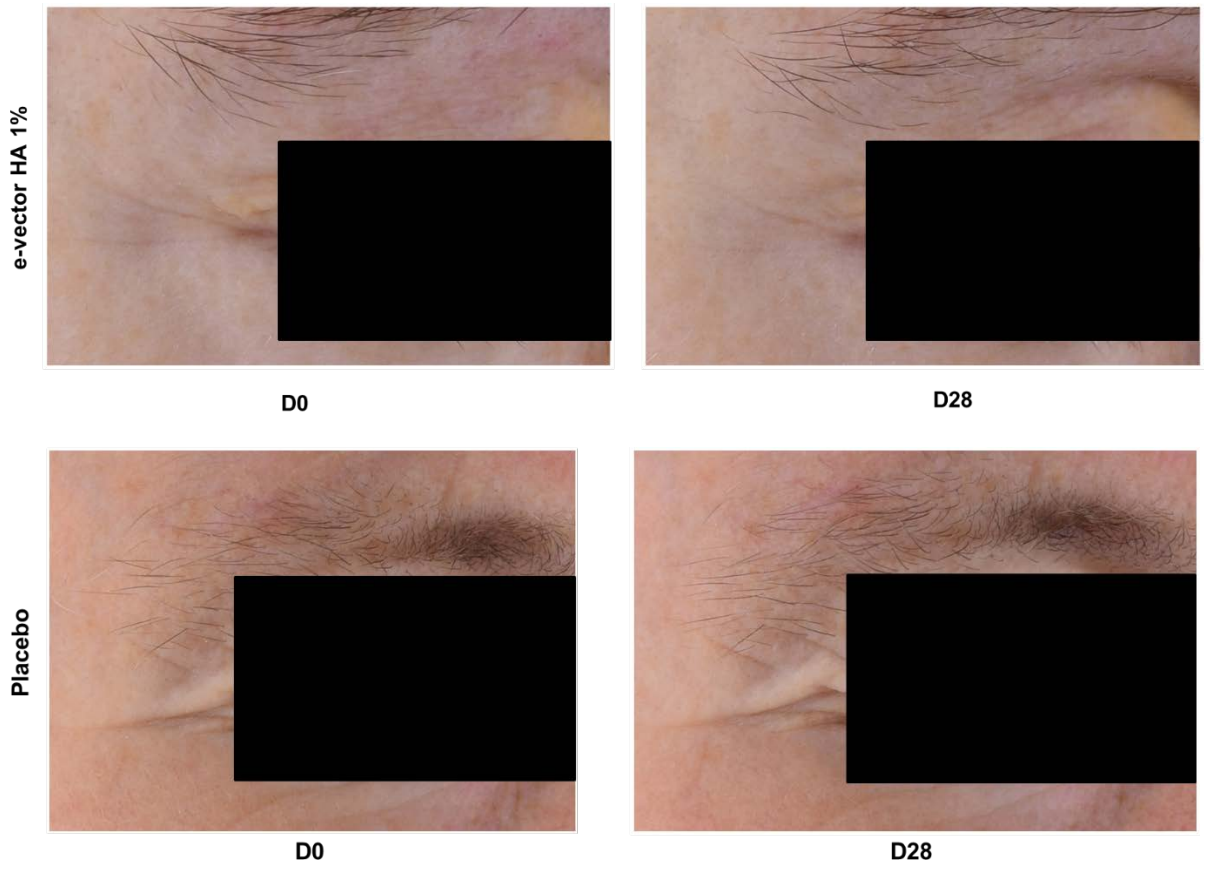

Figure 6: Illustrative pictures of Volunteer n°16 (up) and Volunteer n°1 (down) taken at D0 and D28.

The benefits brought by the presence of clay inside e-vector-HA was evaluated by analysing the skin brightness on the same panel of volunteers.

Table 2: Impact of product with or without e-vector-HA at 1% on reduction of skin brightness measurement by ColorFace® after 28 days of application. Statistical analyses were performed using Wilcoxon test and Mann Whitney test.

	Active			Placebo			Mann Whitney versus placebo
	Mean +/- SD (arbitrary unit)	Average variation (%) vs D0	Wilcoxon test versus D0	Mean +/- SD (arbitrary unit)	Average variation (%) vs D0	Wilcoxon test versus D0	
D0	19.79 ± 7.47			20.43 ± 8.09			
T1h	14.49 ± 5.48	-27.4%	$p<0.001$ ***	17.08 ± 8.27	-16.4%	$p<0.01$ **	ns
T6h	15.76 ± 7.87	-21.1%	$p<0.001$ ***	17.87 ± 8.49	-12.5%	ns	$p<0.05$ *
D28	17.84 ± 7.83	-9.9%	$p<0.1$ #	20.48 ± 7.1	0.3%	ns	$p<0.05$ *

The results demonstrated that e-vector-HA showed significant mattyfing effect after 1 hour and 6 hours of one application (-27.4% and -21.1%) (Table 2). The effect was significant in comparison to placebo after 6 hours. Then, the effect was prolonged and maintained after 28 days of application. Illustrative pictures are presented in Figure 7 below.

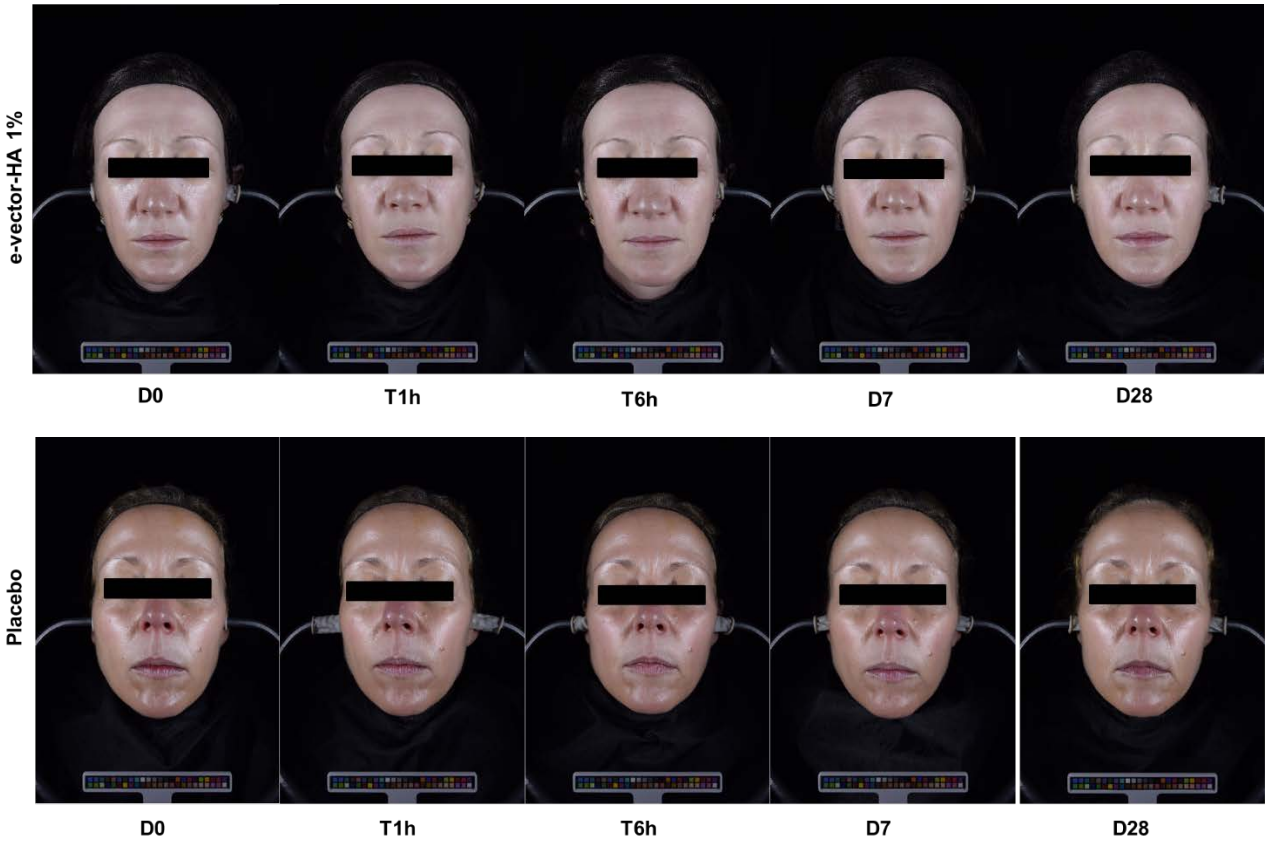

Figure 7: Illustrative pictures of Volunteer n°22 (up) and Volunteer n°5 (down) taken after 1 and 6 hours of one application and after 28 days of twice-daily application of the cream containing or e-vector-HA at 1%.

Discussion.

E-vector-HA is a vectorized HMW HA into clay which provides higher skin penetration and optimized cosmetic benefits to the skin.

Through this study, we wanted to demonstrate that our innovative process brought higher benefits to the skin.

The analysis of skin penetration using micro-imaging Raman spectroscopy evidenced that e-vector-HA penetrated deeper into the skin and reached 70µm of depth (Figure 1). The elucidation of mode of action bring us to conduct several studies to finally confirm that the electrostatic potential of e-vector-HA was involved in the enhancement of skin penetration

(Figure 4). Indeed, we demonstrated that the size of HA was not impacted after the vectorization process (Figure 2) and that natural inner hyaluronidases were not the cause either since both products reacted in the same manner toward their enzymatic activity (Figure 3).

After elucidation of mode of action of our innovative process, we verified the skin benefits brought by a deeper penetration of HMW HA. We demonstrated on skin explants incubated in low hygrometry that the active was able to protect and smooth the skin in a visual manner thanks to SEM. The smoothing effect was then evidenced in a clinical study in which we demonstrated a significant reduction of crow's feet surface after application of the active. The results were immediate (after 1 hour and 6 hours) and prolonged after 28 days of application. The effect was significant in comparison to the placebo.

In the same study, we wanted to evaluate the benefits brought by the clay [6][7]. We evidenced a significant reduction of skin brightness after one application and after 28 days of application. Indeed, the presence of clay in e-vector-HA add a mattifying property to the others related to hyaluronic acid.

Conclusion.

Our innovative process of vectorization led us to create a HMW HA able to penetrate deeper into the skin and bring optimized benefits for cosmetic use. Indeed, -vector-HA smooths the skin by reducing fine lines of the crow's feet to finally bring anti-ageing benefits.

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Conflict of Interest Statement. NONE

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