Active Beauty
Scientific File

PrimalHyal™ Ultrafiller
The dermal fillers challenger

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1. Focus on product

1.1. Hyaluronic Acid and its key role in organism

Our body naturally contains an average of 15 grams of **hyaluronic acid** (HA), found in every part of our organism: skin, joints, scalp, eyes, soft tissues, and synovial fluid (Papakonstantinou, et al., 2012). Due to its chemical nature, HA can hold up a great quantity of water, but its turnover time is very short, with approximately a third of our body HA renewed everyday (Stern, 2004). In each body location, HA serves different functions, correlated to its molecular weight (MW): high MW HA has more of a water retention function, while lower MW HA enhances biological activities, and plays a role of signal molecule. These physiological roles of HA in skin include:

- moisturising and holding water (Masson, 2010),
- maintaining skin viscoelasticity and tonicity by creating a hydrogel in the extracellular matrix (Cheolbyong, et al., 2016),
- improving cell multiplication and communication through CD44 receptors (Toole, 2004; Gomes, et al., 2004; Lingli, et al., 2006) ,
- facilitating cellular mobility and viability by transportation of nutrients (Gallorini, et al., 2017).

1.2. Ageing processes and dermal fillers

Upon **chronological ageing**, the metabolic efficiency of skin cells decreases, lowering their capability to produce major constituents of the extracellular matrix. Due to its high degradation rate, HA is among the first matrix polymer to disappear, leading to the first signs of ageing (fine lines, wrinkles). Meanwhile, antioxidant defences of ageing cells are strongly reduced, making them more sensitive to free radicals.
In parallel, **extrinsic ageing** is driven by daily expositions to a set of environmental aggressions: UV, blue light, pollution, chemicals or repeated mechanical stresses. All of these factors result in the production of Reactive Oxygen Species (ROS), damaging cells components and inducing an overexpression of matrix metalloproteinases (MMP), fostering the degradation of extracellular matrix fibres (such as collagen).

A majority of aesthetic dermal fillers is based on HA, as it is a native component of skin with high volume filling properties (water binding capacities). However the use of these dermal fillers requires local injections with possible complications, and despite their cost, they cannot restore skin natural protection.
It was therefore crucial to find an alternative to protect the skin and its constituents along our lifetime, but also to restore some of its key properties when possible, and get rid of our wrinkles.

2. Mode of action

To answer this unmet need, Active Beauty experts have designed PrimalHyal™ Ultrafiller, a new generation of topical HA, by optimizing the bioavailability of a specific MW of HA, thanks to its full acetylation.
PrimalHyal™ Ultrafiller behaves as a filler by penetrating deeply into the skin, quickly reducing wrinkles. It also stimulates skin epidermis and dermis metabolisms to:

- Protect the extracellular matrix by decreasing collagenases production and collagen degradation, even under photo-pollution,
- Restore the antioxidant defences through the sequestosome pathway (Riz, et al., 2016; Katsuragi, et al., 2015), helping skin to fight against free radicals.

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Four clinical tests have highlighted the unique consumers benefits of PrimalHyal™ Ultrafiller:

- Flash filler effect: decrease of wrinkles in only 1 to 6 hours,
- Anti-ageing effect: restore skin antioxidant potential, reduce nasogenian wrinkles and improve skin texture.

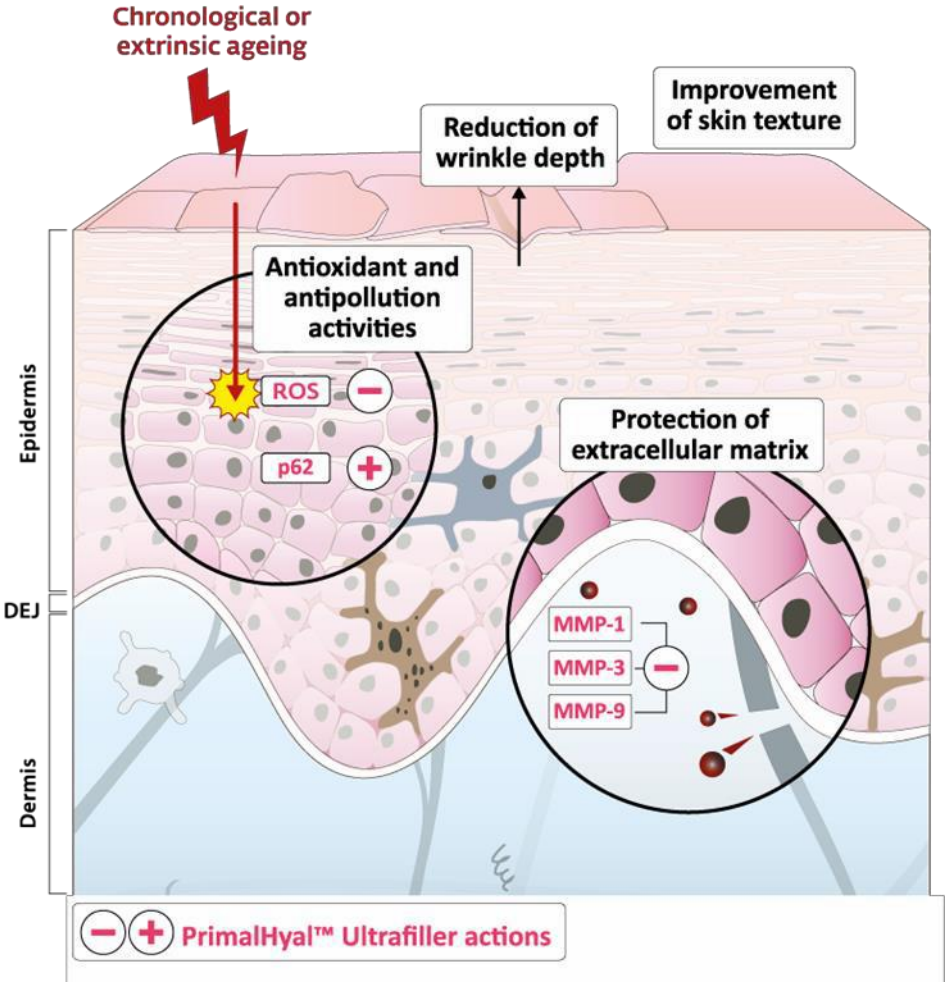

Figure 1: Mode of action PrimalHyal™ Ultrafiller

3. Material and Methods

3.1. Skin penetration

The analysis was performed on Caucasian donor aged of 37 years old. 3 explants of frozen abdominal skin (1.5 to 2 cm2) from the same donor were cut out and distributed as follows (1 explant per condition):

- 1 explant for the negative control (without treatment)
- 1 explant for PrimalHyal™ Ultrafiller 1% (T 8h)
- 1 explant for non-acetylated form of sodium hyaluronate 1% (T 8h) (PrimalHyal™ 20)

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The products were applied to the surface of the explants and then incubated at 32 °C for 8h. After the incubation phase (T8h), the surface of the explants was cleaned to eliminate any excess of the product. An explant without any treatment was used for the negative control.

The explants were frozen at -80°C and then cut longitudinally using a Cryotome with a thickness of 20 µm. For each explant, 3 tissues sections were selected and deposited on a CaF2 support for Raman imaging analysis. In total 9 images Raman (3 sections per explant (n = 3) and one image per section) have been recorded.

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 3 Y spectra and 21 X spectra (63 spectra per image).

3.2. Resistance to hyaluronidase

3.2.1. Method of molecular weight determination

In order to evaluate the effectiveness of different ways of stress on the tested products, an analytical method has to be developed.

Standard solutions, prepared in mobile phase, have been injected in the chromatographic conditions set up.

An example of profile is presented hereafter:

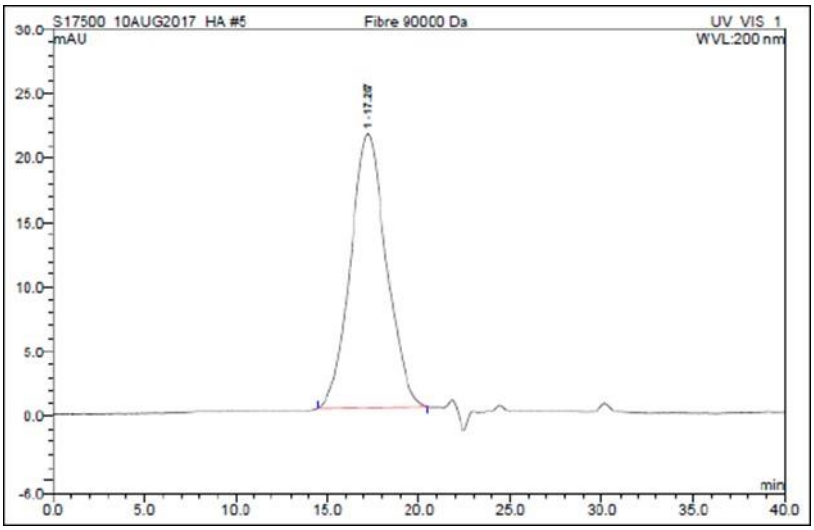

Figure 2: Chromatogram obtained with Fiber at 90 000 D

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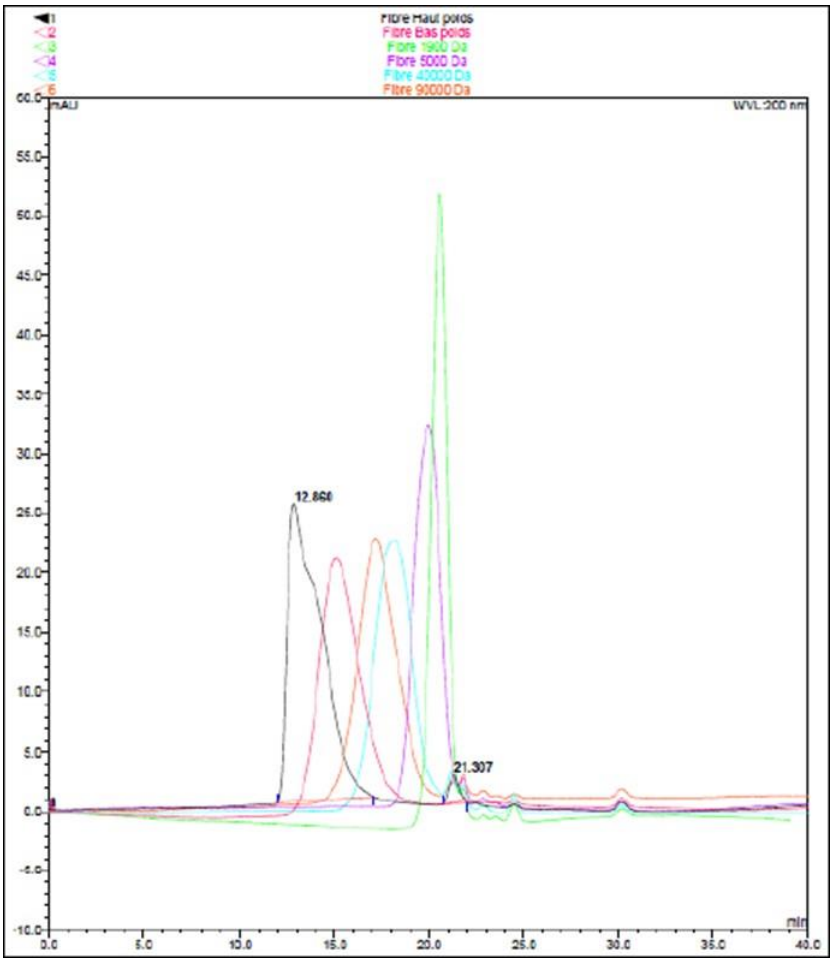
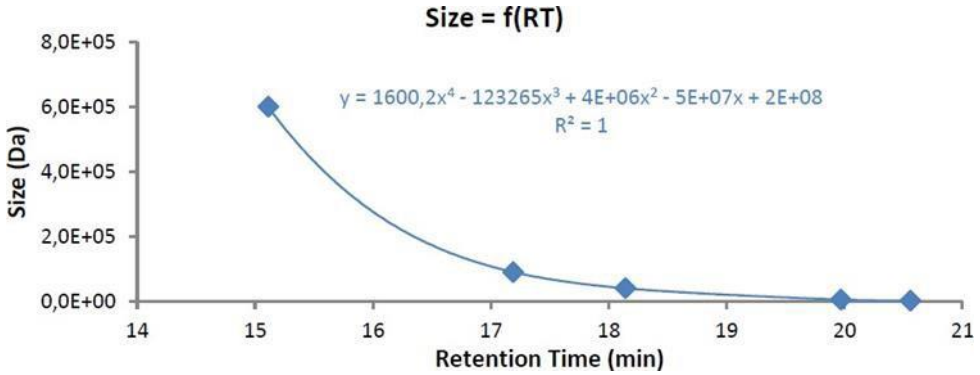


Figure 3: Overlay of all analysed fibers

The following correlation between retention time and size has been established:



A correlation between retention time and size of HA was determined. It can be used for the next step to evaluate the behaviour of HA under stress exposure.

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3.2.2. Enzymatic exposure

Samples were, then, prepared and analyzed with the chromatographic conditions previously described. Three preparations per raw material in different conditions were prepared.
Sample preparation was realized as following:

- Into a glass tube, accurately weigh about 40 mg of product (PrimalHyal™ Ultrafiller or non-acetylated form) and complete with 2 ml of water.
- Mix well for 5 minutes. This protocol was applied:
- Add 300 µl of the solution containing the tested product (PrimalHyal™ Ultrafiller or non-acetylated form) into a Head Space vial in which a hyaluronidase solution (8 UI/ml) was previously introduced. The unstressed condition was performed without hyaluronidase.
- Mix well and put the vial at 55°C in an oven for 16h and let it cool at room temperature

Extract PrimalHyal™ Ultrafiller or non-acetylated form using the mobile phase and centrifuge at 4500 rpm for 10 minutes. Collect the supernatant. A dilution was performed for the analysis.

3.3. Transcriptomic screening

Normal human dermal fibroblasts (NHDFs) and normal human epidermal keratinocytes (NHEKs) were seeded respectively at 250 000 and 300 000 cells per wells in 6-wells plates. After 72h of culture for NHDFs and 48h for NHEKs, cells were stimulated for 24h with PrimalHyal™ Ultrafiller or with HA non-acetylated as control at 0,1 mg/ml for NHDF and 1mg/ml for NHEK in free medium. After 24h of treatment, RNA were extracted according to Trizol method. RNA quality was controlled and a reverse transcription was performed to obtain cDNA. RT-qPCR was made on specific plates designed to study transcriptomic expression of different genes involved in dermis biology for NHDFs and in epidermis biology for NHEKs with 10 ng of cDNA per well. The results of gene expression obtained with fibroblasts were normalized according to POLR2A (RNA polymerase II, subunit A) housekeeping gene and those obtained with keratinocytes were normalized according to IPO8 (Importin-8). We reminded the genes presenting a significant modification according to untreated and treated with sodium hyaluronate conditions.

3.4. Proteomic investigation on skin explants

3.4.1. Skin explants culture

The product PrimalHyal™ Ultrafiller was applied at 0.1% on the skin explants surface.
We use NativeSkin® skin explant, a biopsy of human normal skin embedded in a solid and nourishing matrix. The epidermal surface is maintained in contact with air to allow topical application. The donor is a caucasian women aged of 59 years old. After 5 days of treatment, the treatment has been removed and the half of each 3 explants per condition was collected and kept on ice until the immediate delivery to proteomic analysis.

3.4.2. Proteomic analysis by LC-MS/MS

Samples were extracted and prepared for analysis using OxiProteomics' standard protein extraction method adapted for skin explants.
Mass spectrometry based proteomics analyses were performed (Label-Free LC-MS/MS; LTQ Orbitrap Velos) and label-free Mascot (Matrix Science) software package was used for analyzing large-scale data sets.
Data mining and bioinformatic analyses were performed using the Ingenuity Pathway Analysis software (version 2.0; Ingenuity Systems, Mountain View, CA, USA).

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3.5. Measurement of TRXR1 expression *in vitro*

NHEKs were seeded in a 24-well plate on glass coverslips coated with collagen at 80 000 cells per well in complete medium. After 24h of culture, NHEK were pre-stimulated with PrimalHyal™ Ultrafiller or non- acetylated form at 1mg/ml in free medium for 2h at 37°C. Then, an oxidative stress was induced with H2O2
500µM treatment for 15 minutes at 37°C and reaction was stopped by changing medium containing PrimalHyal™ Ultrafiller or non-acetylated from at 1mg/ml in free medium for 24h at 37°C.
After 24h, NHEKs were rinsed two times with PBS and fixed with paraformaldehyde (PFA) 2% for 10 minutes at room temperature. After three rinses with PBS, NHEKs were permeabilized with a solution containing PFA 2% and triton 0.5% for 5 minutes at room temperature. Three new rinses with PBS and NHEKs' aspecific sites were blocked with bovine serum albumin (BSA) 3% and then were incubated with primary antibody against TRXR1 diluted at 1/100 in BSA at 0.3% overnight in a humidified room at 4°C.
The next day, NHEKs were rinsed three times with wash solution and incubated with secondary antibody anti-rabbit AlexaFluor488 1/500 in BSA 0.3% for 2h at room temperature in humidified room in the dark. Three rinses with wash solution and three rinses with PBS and coverslips were mounted on glass blade with DAPI and conserved at 4°C until taking pictures with a fluorescent microscope.

3.6. Measurement of ROS production *in vitro*

NHEKs were seeded in a 96-well plate pre-coated with collagen at 20 000 cells per well in complete medium. After 24h of culture, cells were treated with PrimalHyal™ Ultrafiller at 1mg/ml or non-acetylated form at 1mg/ml or untreated (negative control), for 24h at 37°C. Then, cells were incubated with DCFH-DA probe at 50µM for 30 to 45 minutes at 37°C and rinsed two times with PBS. Half of the wells per condition were kept in basal condition with PBS and the others were stressed with Tert-Butyl Peroxide 5mM. Fluorescence intensity was measured for 1 hour at room temperature with excitation at 488nm and emission at 525nm.

3.7. Impact on outdoor pollution in presence of UV

3.7.1. Skin explants treatment

Human skin explants of an average diameter of 14 mm (±1mm) were prepared on an abdominoplasty coming from a 56-years old caucasian woman (reference: P1839-AB56). The explants were kept in survival in BEM culture medium (BIO-EC's Explants Medium) at 37°C in a humid, 5 %-CO2 atmosphere.
On day 0 (D0), the products containing PrimalHyal™ Ultrafiller at 0.1% or non-acetylated form were topically applied on the basis of 2 µl per cm², and spread using a small spatula.
The control explants "untreated" did not receive any treatment expect the refreshing of the culture medium. The culture medium was refreshed entirely (2 mL) on D1 after pollutants exposure.

3.7.2. Outdoor photo-pollution exposure

The outdoor pollutant is composed of heavy metals and hydrocarbure coupled to UV exposure.
On D1, 24h after treatment with the tested products, the explants were placed on the PolluBox® system with 900 µl per well of HBSS, and exposed by vaporization to a mixture of hydrocarbons + heavy metals supplemented with NaCl 0.9% (150 µl of NaCl 0.9% per ml of pollutant solution) for 1.5 hour, as shown in the figure below.

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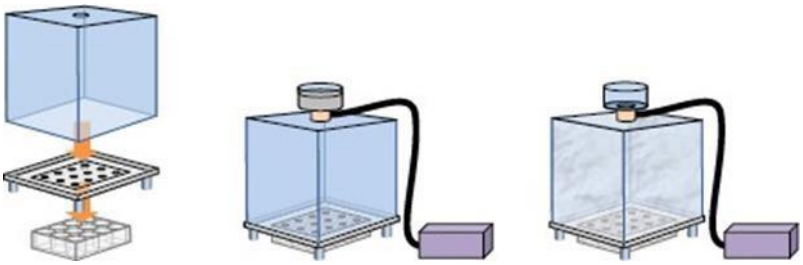

After the pollutant exposure, the explants received a UVA irradiation at the dose of 13,5 J/cm2 (3 minimal erythema dose, MED) using a Vilbert Lourmat simulator RMX 3W.
In parallel, the explants of the unexposed explants were placed outside the incubator in a 12-well plate with 1 ml of HBSS per well, in the dark and without exposure to pollution.
At the end of the exposure, all the explants were put back in the incubator under standard culture conditions in 2 ml of fresh BEMc per well.

3.7.3. Sequestosome 1 (P62) expression after outdoor photo-pollution exposure

Histological processing

5-µm-thick sections were made using a Leica RM 2125 Minot-type microtome, and the sections were mounted on Superfrost® histological glass slides.
The frozen samples were cut into 7-µm-thick sections using a Leica CM 3050 cryostat. Sections were then mounted on Superfrost® plus silanized glass slides.
The microscopical observations were realized using a Leica DMLB or Olympus BX43 microscope.
Pictures were digitized with a numeric DP72 Olympus camera with Cell^D storing software.

Sequestosome P62 immunostaining

p62 immunostaining has been realized on paraffinized sections with a polyclonal anti-p62 antibody (Novus, ref. NBP1-48320) diluted at 1:400 in PBS, BSA 0.3% and Tween 20 (0.05%) for 1h at room temperature using Vectastain Kit Vector amplifier system avidin/biotin, and revealed by VIP, a substrate of peroxidase (Vector SK-4600).
The staining was performed using an automated slide-processing system (Dako, AutostainerPlus).
The immunostaining was assessed by microscopical observation.

3.7.4. MDA production after outdoor pollution exposure

The MDA (malondialdehyde) assay was realized with an enhanced method of the TBARs assay. The MDA was assayed in HBSS medium by addition of TBARs solution (thiobarbituric acid, hydrochloric acid and tricholoroacetic acid) and placed in a water bath (80°c for 15 minutes). A lot of substances (like glucose…) which are not related with lipoperoxidation, react with thiobarbituric acid (ThioBarbituric Acid Reagents = TBARS), so to enhance the specificity of the assay, the MDA was extracted by a liquid/liquid extraction with butanol. The MDA in butanol was measured in spectrofluorimetry (excitation: 515 nm, emission: 550 nm) using a Tecan Infinite M200 Pro microplate reader.
Using this assay, MDA contained in the culture medium BEMc on day 2 (24 hours after pollution exposure) was measured for the 4 explants per batch. The MDA concentration was expressed in nmol/l.

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3.7.5. MMP-1 expression analysis by immunostaining after outdoor photo-pollution exposure

MMP1 immunostaining has been realized on paraffinized sections with a monoclonal anti-MMP1 antibody (R&D systems, ref. MAB901) diluted at 1:25 in PBS, BSA 0.3% and Tween 20 (0.05%) for 1h at room temperature using Vectastain Kit Vector amplifier system avidin/biotin, and revealed by VIP, a substrate of peroxidase (Vector SK-4600).
The staining was performed using an automated slide-processing system (Dako, AutostainerPlus).
The immunostaining was assessed by microscopical observation.
The signal was quantfied in the epidermis and the dermis.

3.7.6. MMP-9 expression analysis by immunostaining after outdoor photo-pollution exposure

MMP-9 immunostaining has been realized on frozen sections with a polyclonal anti-MMP9 antibody (Novus Biologicals, ref. NB600-1217) diluted at 1:400 in PBS, BSA 0.3% and Tween 20 (0.05%) for 1h at room temperature and revealed by AlexaFluor488 (Lifetechnologies, ref. A11008). The nuclei were counter- stained using propidium iodide.
The staining was performed using an automated slide-processing system (Dako, AutostainerPlus).
The immunostaining was assessed by microscopical observation.
The signal was quantified in the epidermis and the dermis.

3.8. Anti-oxidant activity at the clinical level

3.8.1. Description of the creams used

AQUA/WATER, SODIUM ACETYLATED HYALURONATE, CAPRIC/CAPRYLIC TRIGLYCERIDE, CETEARYL WHEAT STRAW GLYCOSIDES, CETEARYL ALCOHOL, PHENOXYETHANOL, METHYL PARABEN, PROPYL PARABEN, ETHYL PARABEN, DIMETHICONE, FRAGRANCE, HEXYL CINNAMAL, BUTYLPHENYL METHYLPROPIONAL, CITRONELLOL, ALPHA ISOMETHYL IONONE, HYDROXYISOHEXYL 3-CYCLOHEXENE CARBOXALDEHYDE SODIUM HYDROXIDE

3.8.2. Description of the panel

A double blind clinical evaluation was carried out on 20 volunteers (women aged from 18 to 65 years old with an age mean 47 years old) having caucasian phototype from II to IV according Fitzpatrick classification. All the subjects participating in the study gave their informed consent signed at the beginning of the study. The cream containing the active was compared to control area and vitamin E at 2% as positive reference. The volunteers applied the products on thighs for 28 and 56 days according to the measurement including LPO (lipiperoxiation) production after UV exposure and FRAP (Ferric Reducing Antioxidant Parameter) permitting to analyse the antioxidant property of the active ingredient.

3.8.3. Skin treatment

The skin site chosen to evaluate product efficacy are thighs. Products are applied twice a day (2 mg/cm2, morning and evening) during 56 days over the skin of the anterior surface of the thights in 3 different are- as (15x20cm = 300cm2 each one).
Sites of products application (negative/untreated area, test Active product, and positive control/2% Vitamin E cream treated areas) are randomized. Products are applied by the subject at home, on the morning and in the evening on clean (after bath/shower).

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3.8.4. Skin irradiation and stripping

UV radiation and skin stripping

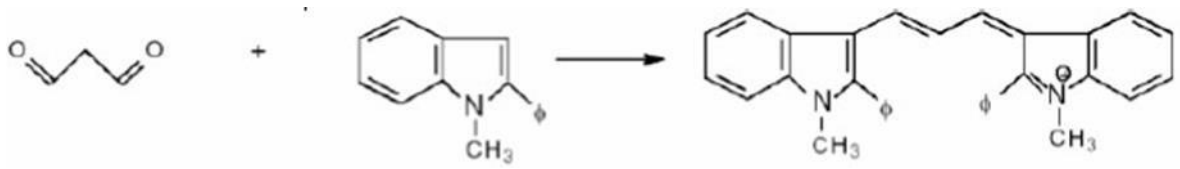
The source of UVA radiation is a Multiport 601 Solar simulator (Solar® Light Co. Inc., PA). The spectral quality complies with the acceptance limits ISO 24442:2011 and the JCIA PPD method (version dated 15.11.1995). The output from the UV solar simulator is stable, uniform across the whole output beam and suitably filtered to create a spectral quality that complies with the required acceptance limits. The dose of UVA radiation applied is adjusted with a model PMA2100 radiometer (Solar® Light Co. Inc.) equipped with a UVA DECTOR PMA 2113 LLG (Solar® Light Co. Inc.). Exposure is 5J/cm2. Wavelength is 320-390 nm
At baseline (D0) a small skin area within each treated zone (1/2/3) is subjected to skin stripping (upper skin layer removal by repetitive application and removal of Corneofix®, a special adhesive foil collecting corneocytes). Skin strips are immediately stocked at -80°C until the test end.
For the LPO assay all the stripped skin sites on the two legs are then irradiated with UVA rays (5 J/cm2). The stripping procedure on the irradiated sites are repeated after 4h (T4) and 24h (T24) from the UVA irradiation both at D0 and at D28.
Volunteers use the products until D56 for the evaluation of the skin antioxidant capability at the end of the treatment. Then, the products to be tested and instructions concerning their use are given to each volunteers. The date for the following checks are fixed after 28 (D28) and 56 (D56) days of product use.
Volunteers are asked to perform the last application of the tested product at least 8 hours prior the check.

Skin stripping is performed using Corneofix® foils (Courage+Khazaka electronic GmbH). The technique allows to collect different layers of *stratum corneum*

- Strips are collected for LPO after D0 and D28 of application;
- Strips are collected for FRAP after D0, D28 and D56 of application. Skin strips are stored at -80°C after their collection for LPO and FRAP assays.

LPO measurement

Malonyl di aldheyde (MDA) and 4-hydroxynonenal are the two main products of lipid peroxidation. Their concentration in a biological system is a good index of its lipo-peroxide damage. To determine the lipo-peroxides levels it was assayed the method tested by Erdelmeier and collaborators (1998): the assay is based on the capability of a cromogen, N methyl 2 phenylindole (NMPI), to react with MDA at 45°C and acid pH to produce a stable chromophore that has an absorption peack at 586 nm.



The lipo-peroxide levels are measured after the induction of unstable hydroperoxides decomposition, produced in the oxidative processes by means of a pro-oxidant agent (CuSO4 500 mM).

FRAP measurement

The Ferric Reducing Antioxidant Parameter (FRAP) is a direct measure of the total reductive power of a biological matrix and an indirect index of the capability of the considered system to resist to the oxidative damage. FRAP uses the antioxidants in the biological system as reductive agent in a colorimetric method based on redox reactions (Benzie & Strain, 1996).

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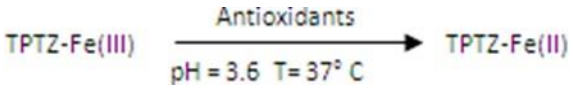

The reduction at acid pH of the complex TPTZ-Fe(III) in the ferrous form (Fe(II)) is characterised by an intense blue colour. The reaction is monitored by measuring the solution absorbance at 595 nm. The recorded absorbances are compared to a Fe(II) standard curve of known values. The result is directly proportional to the total reductive power of the antioxidant in the reaction mix.

3.9. Analysis of MMPs inhibition *in vitro*

The experiment has been done on fibroblasts from a woman of 52 years old (abdominoplasty). NHDFs were seeded in 12-wells plates at reason of 0.15 million cells per well in complete medium containing DMEM and 1 % ATB (penicillin and streptomycin). At confluence, cells were washed with PBS and starved by fetal calf serum (FCS) privation for 24h in DMEM. Cells were pre-incubated with PrimalHyal™ Ultrafiller or non acetylated form at 0.1 mg/ml for 2h at 37°C and were then exposed or not to oxidative stress (H2O2 200µM) for 2h at 37°C. The culture medium was replaced by basal medium and cells were incubated for 48h at 37°C. 48h after stress oxidative induction, conditioned media were collected and stocked at -20°C. Supernatants were then used to analyze the MMP-1 and MMP-3 release by Luminex analysis (Human MMP premixed Magnetic Luminex Performance Assay, Ref panel: 1307e319 (MMP-1, MMP-3)).

3.10. Analysis of MMPs inhibiton on skin explants

3.10.1. In presence of chronologic aging

In order to confirm the *in vitro* investigation, we performed specific immunostaining skin explant from donor aged to 59 years old treated with 0.1% PrimalHyal™ Ultrafiller for 5 days.
After the culture, skin explants were fixed in formalin and embedded in paraffin. Makers of interest were detected by immunofluorescence. The biomarkers expressions were analyzed by fluorescence microscopy (DM5000B - Leica Microsystems), 10 images were acquired by biomarker.
Height biomarkers were analyzed:

- MMP1
- MMP9

The fluorescence was quantified for each picture by software imaging (Image J) in the dermis only. The region of interest was delimited and the total fluorescence intensity was calculated then reported to the total area of interest. The specific labeling is in gray and the nuclei are in blue by a DAPI labeling (Scale bars 100µm).

3.11. Matrix degradation

3.11.1. DQ-collagen type I

The experiment has been done on fibroblasts from a woman of 52 years old (abdominoplasty). NHDFs were seeded in 12-wells plates at reason of 0.15 million cells per well in complete medium containing DMEM and 1 % ATB (penicillin and streptomycin). At confluence, cells were washed with PBS and starved by fetal calf serum (FCS) privation for 24h in DMEM. Cells were pre-incubated with PrimalHyal™ Ultrafiller or non acetylated form at 0.1 mg/ml for 2h at 37°C and were then exposed or not to oxidative stress (H2O2 200µM) for 2h at 37°C. the culture medium were replaced by basal medium and cells were incubated for 48h at 37°C. 48h after stress oxidative induction, conditioned media were collected and stocked at -20°C. Supernatants were then used to analyze the collagen degradation mediated by the active MMPs presents inside the samples using the DQ-collagen (DQ(TM) COLLAGEN, TYPE I F, 10562903, Invitrogen). Briefly, 100 µL of each sample were deposited

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on corresponding wells. A mixture of Reaction buffer containing 50mM Tris-HCL, 0.15 M NaCl, 5 mM CaCl2 and 0.2 mM Sodium Azide at pH 7.6 and the QD- collagen at 50µg/ml was prepared and added at each wells. Plate was incubated 4h at room temperature in darkness and emitted fluorescence corresponding to type I collagen degradation was measured by Micro- plate Reader (TECAN) with absorption maxima at 495nm and fluorescence emission maxima at 515 nm.

3.11.2. Zimography *in situ*

A 24-well plate was coated with 50µl per well of gelatin containing 2,5% of DQ-collagen and incubated 2h at room temperature. NHDFs were seeded at reason of 15 000 cells per well in DMEM supplemented with 1% FCS and incubated overnight at 37°C, 5% CO2. Next day, NHDFs were pre-incubated with PrimalHyal™ Ultrafiller or non-acetylated form at 0.1mg/ml for 2h at 37°C. Cells were next stimulated with PMA at 20ng/ml for 36h. Following, pictures were taken with a fluorescent microscope to detect the collagen degradation around the cells.

3.12. Clinical investigation of anti-aging efficacy of PrimalHyal™ Ultrafiller

3.12.1. Description of the creams used

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3.12.2. Flash anti-wrinkles effect on crow's feet and nasogenian area

Description of the panel

A double blind and placebo controlled clinical evaluation was carried out on 21 volunteers (women aged from 42 to 70 years old with a age mean 63 ± 2.16 years old) having wrinkles on the crow's feet and in the nosalabial area. All the subjects participating in the study gave their informed consent signed at the begining of the study. The cream containing the active was compared to placebo during this study. The volunteers applied the products on hemiface at D0 and the flash anti-aging efficacy was measured by profilometery and VISIA analysis after 1h and 6h of application in comparison with D0. The active was tested at 0.2% in this study.

Crow's feet wrinkles analysis by profilometry

The objective of the QUANTIRIDES® software is to analyze, quantify and characterize the wrinkles of a silicone resin impression (SILFLO). A negative replica of the wrinkles of the cutaneous surface is illuminated by a grazing light (35 °). This generates shadows behind each ride. The acquisition of this image and therefore of these shadows is achieved by means of an IEEE high resolution digital camera. The QUANTIRIDES® software analyzes the images obtained and determines the different characteristics of the wrinkles (surface, length, depth). An area of 1.4 cm² is studied.
The studied parameters are the total wrinkled surface, the number and average depth of the micro-relief wrinkles, middle wrinkles and deep wrinkles.
The micro-relief wrinkles have a depth of less than 55 µm. Average wrinkles have a depth between 55 and 110µm and deep wrinkles have a depth greater than 110µm (up to 800µm).
We can also study the number and the average length of the grooves for micro-relief from 0 to 0.5mm, for average wrinkles from 0.5 to 2mm and for deep wrinkles from 2 to 10mm.

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Silicone polymer replicas were taken on the crow's feet of volunteers before use of the product and at each control carried out during the study.

Nasogenian wrinkles analysis by VISIA CR2.3

With VISIA® CR2.3, digital photos of the face were performed at D0, T+1h and T+6 hours. Three types of photos are taken:

- Right profile
- Left profile
- Full face

Different parameters can be analyzed with the VISIA®: Spots, Wrinkles, Texture, Pores, UV Spots, Brown spots, Red areas and Porphyrins.
The VISIA® CR 2.3 was used in this study

In this study, will be analyzed skin texture and wrinkles data's (crow's feet and nasolabial)

Self-assessment questionnaire

Assessment of the sensation felt, efficacy and cosmetic quality of the product was performed through a self-assessment questionnaire completed by volunteers after 1 hour and also after 6 hours of product application during the study

3.12.3. Anti-wrinkles efficacy on nasaogenian area after 1 month of application

Description of the panel

A double blind and placebo controlled clinical evaluation was carried out on 20 volunteers (women aged from 35 to 70 years old with a age mean 56 ± 6.69 years old) having wrinkles on the nosalabial area. All the subjects participating in the study gave their informed consent signed at the begining of the study. The cream containing the active was compared to placebo during this study. The volunteers twice daily applied the products on hemiface during 1 month (morning and evening). The anti-aging efficacy was measured by VISIA CR analysis after D0 and 28 days (D28) of application. The active was tested at 0.1% in this study.

Nasogenian wrinkles analysis by VISIA CR

With VISIA® CR2.3, digital photos of the face were performed at D0 and D28. Three types of photos are taken:

- Right profile
- Left profile

Different parameters can be analyzed with the VISIA®: Spots, Wrinkles, Texture, Pores, UV Spots, Brown spots, Red areas and Porphyrins.
The VISIA® CR 2.3 was used in this study. In this study, will be analyzed wrinkles data's (nasolabial area)

3.12.4. Improvement of skin texture after 2 months of application

Description of the panel

A double blind and placebo controlled clinical evaluation was carried out on 30 volunteers (women aged from 50 to 70 years old with a age mean 57 ± 9 years old) having wrinkles on the face. All the subjects participating in the study gave their informed consent signed at the beginning of the study.

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The cream containing the active was compared to placebo during this study. The volunteers twice daily applied the products on hemiface during 2 months (morning and evening). The skin texture was measured by VISIA CR analysis after D0 and 56 days (D28) of application. The active was tested at 0.1% in this study.

Skin texture analysis by VISIA CR

With VISIA® 6th generation, digital photos of the face were performed at D0, and after 56 days (D56). Two types of photos are taken:

- Right profile
- Left Profile

Different parameters can be analysed with the VISIA®: Spots, Wrinkles, Texture, Pores, UV Spots, Brown spots, Red areas and Porphyrins. In this study, we focused on skin texture analysis.
The VISIA® 6th generation was used in this study. In this study, will be analysed skin texture.

Self-assessment questionnaire

Assessment of the sensation felt, efficacy and cosmetic quality of the product was performed through a self-assessment questionnaire completed on Eval & Go by volunteers after 27 days and also after 55 days of product application during the study.
Eval & GO is a SaaS application of feedback management. It permits to create On-line surveys, to publish them by link or by email, to collect the answers and analyze the results in real time. Access to the service is via an internet connection and a recent web browser (Internet Explorer 9+, Chrome, Safari, Firefox). It is on the basis of subscriptions without installation on computers. Any use of Eval & GO software or services is subject to the terms and conditions.

3.13. Statistical analysis

All the *in vitro* and *ex vivo* studies were performed at minima in triplicat. The statistical analysis was per- formed using t student test with * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.
Regarding the clinical investigation, the statistical analysis was done using t student test parametric or non parametric test (ANOVA) according the studies. Similarly we obtained significant results with *$p<0.05$, ** $p<0.01$ and ***$p<0.001$.

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4. Results

4.1. Impact of acetylation on sodium hyaluronate

In order to define the benefit of acetylation, we analyzed the benefit on skin penetration and resistance to the hyaluronidase relative to PrimalHyal™ Ultrafiller form to the non-acetylated form by Raman spectroscopy and measuring the degradation of hyaluronic acid. In second part, we performed transcriptomic analysis on the both product in order to identify a potential new biological activity bring to the acetylation.

4.1.1. Skin penetration

No Raman signal specific to the reference spectra of PrimalHyal™ Ultrafiller or not products was detected in the negative control sections at T0h and at T8h (left panel figure 4).
At T8h the non-acetylated form penetrated at the level of the skin up to 90µm of depth and its signal was important until 50 µm of depth in the epidermis beyond this depth its presence was less important.
At T8h PrimalHyal™ Ultrafiller penetrated at the level of the skin until to 100µm of depth and its signal was detected mainly between 80 and 100µm deep in the epidermis at T8h.
The two products penetrate the epidermis. On the other hand, they have a very different distribution pro- file. This can be explained by the hypothesis that PrimalHyal™ Ultrafiller could have a higher diffusion kinetics than non-acetylated form (figure 4).

The acetylation improves the skin penetration of sodium hyaluronate.

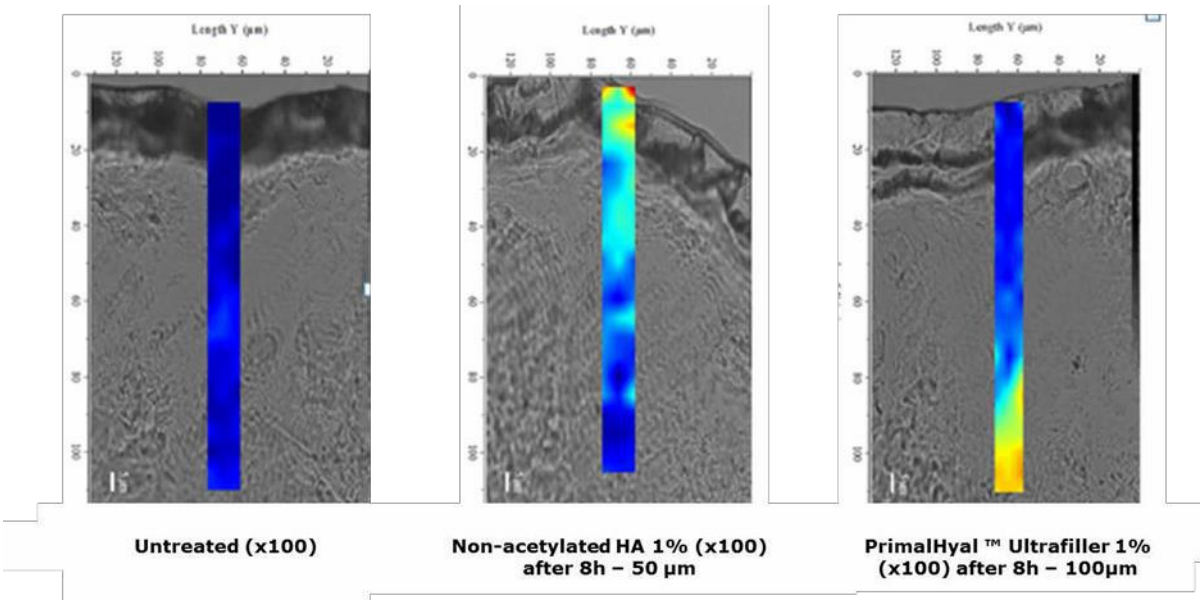

Figure 4: Skin penetration of PrimalHyal™ Ultrafiller and it non-acetylated from after 8h

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4.1.2. Resistance to hyaluronidase

The resistance to the hyaluronidase of the both products was analyzed by HPLC after hyaluronidase exposure for 16h. We used the chromatographic profile in order to estimate the size of molecule in the different experimental conditions
The chromatographic profile of PrimalHyal™ Ultrafiller (retention time) is the same under enzyme exposure condition, it means that active ingredient is not sensitive to the hyaluronidase. Consequently, we observed nearly not degradation of PrimalHyal™ Ultrafiller after hyaluronidase exposure (table 1).
The chromatographic profile of sodium hyaluronate (retention time) is not the same under enzyme expo- sure condition. It means that the non-acetylated form of sodium hyaluronate is sensitive to the enzymatic exposure. Consequently, we observed drastic and significant degradation of sodium hyaluronate after hyaluronidase exposure (table 1).

	Basal		With hyaluronidase		
	Mean	SEM	Mean	SEM	p value (versus basal)
PrimalHyal™ Ultrafiller	16920	119	15689	0	p<0.01
Non-acetylated form	21046	156	1644	2	p<0.001

Table 1: Impact of hyaluronidase on PrimalHyal™ Ultrafiller and non acetylated form

As observed on the figure 7, the acetylation of sodium hyaluronate significantly protects it against enzymatic activity of hyaluronidase while the non-acetylated form is very sensitive.
The acetylation permit to prolong the half-life of PrimalHyal™ Ultrafiller into the skin.

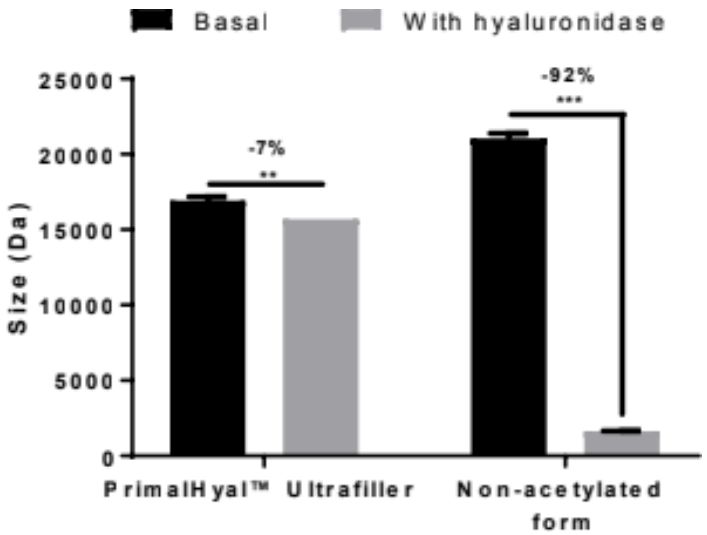

*Figure 5: Effect of hyaluronidase activity on PrimalHyal™ Ultrafiller versus non-acetylated form. T student's test ** p<0.01, *** p<0.001.*

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The acetylation permits to prolong the half-life of PrimalHyal™ Ultrafiller into the skin. Indeed, the half-life of sodium hyaluronate is to 9h while that of acetylated form is increased to 114h on the base of these results. We observed an improvement and prolongment of half-life of 13 times thank to acetylation of sodium hyaluronate.

4.1.3. Transcriptomic screening

Transcriptomic analysis was performed on fibroblasts and keratinocytes in order to identify the potential bioactivity of the product on dermis and epidermis.
We designed a plates specific to the epidermis function with 92 genes including some genes involved in epidermal differentiation process, inflammation, antioxidant defenses, cell proliferation, and others mechanism related to epidermal functions. Similarly we designed another specific plate targeting 91 genes specific of dermis function including matrix components, genes involved in matrix remodeling mechanism, in wound healing process and others permitting to cover the main biological functions in the dermis. Each plate included three housekeeping genes in order to normalize the results and permitting a multi-plates comparison.

The transcriptomic analysis was performed on NHEKs after stimulation with PrimalHyal™ Ultrafiller and the results were analyzed in comparison with untreated condition and non-acetylated hyaluronate. The modification of gene expression with PrimalHyal™ Ultrafiller showed an increase of antioxidant defenses with an significant up-regulation of TXNRD1 (Fc=+1.7) and AKR1C3 (Fc=+2.1) expression, coding respectively for Thioredoxin reductase 1 and Aldo-keto reductase family 1 member C3, two antioxidant enzymes. This confirmed that PrimalHyal™ Ultrafiller has a potent antioxidant activity. Finally, PrimalHyal™ Ultrafiller also significantly increased NQO1 and HMOX1 expression (Fc=+2.51 and Fc=+3.01 respectively) which are two strong antioxidant enzymes regulated by NRF-2 pathway, suggesting a potential active anti-pollution role (figure 6).

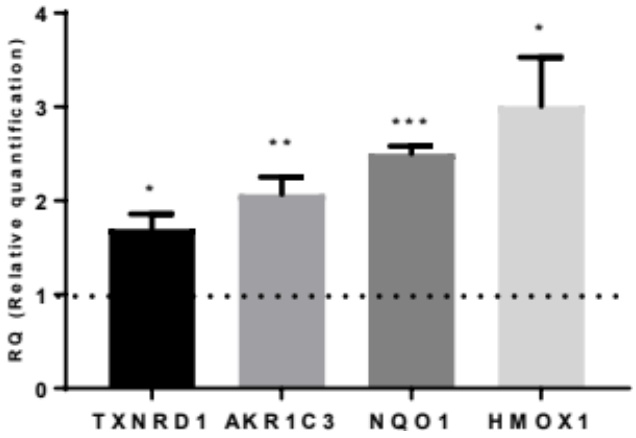

*Figure 6: Transcriptomic analysis on epidermis plate with PrimalHyal™ Ultrafiller at 1mg/ml for 24h. t student test * p<0.05, ** p<0.01, *** p<0.001.*

The transcriptomic analysis was performed on NHDFs after stimulation with PrimalHyal™ Ultrafiller and the results were analyzed in comparison with untreated condition and non-acetylated hyaluronate. We obtained interesting modifications induced by PrimalHyal™ Ultrafiller. Among them, we specifically identified some genes involved in matrix remodeling and especially matrix metalloproteinases (MMPs). Indeed, MMP- 1, MMP-3 and MMP-9 are three collagenases that are significantly decreased in their gene expression with a fold change of -2, -1.9 and -1.3 respectively (figure 7).

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These results suggested that PrimalHyal™ Ultrafiller could have an anti-aging activity through the protection of matrix from degradation that is associated to wrinkles formation with the aging.

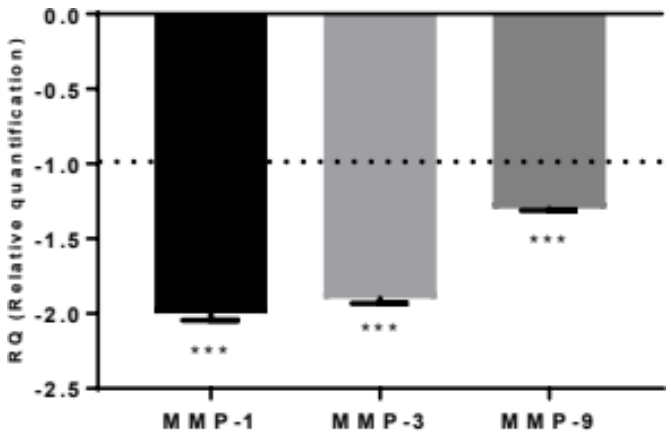

*Figure 7: Transcriptomic analysis on dermis plate with PrimalHyal™ Ultrafiller at 0.1mg/ml for 24h. T student's test *** p<0.001.*

In conclusion the transcriptomic analysis permit to identify two potential bioactivities of PrimalHyal™ Ultrafiller that are an anti-aging activity through a protection of intra dermal matrix and an antioxidant activity that can potentially be efficient against oxidative stress and pollution.

4.1.4. Proteomic investigation on skin explants

A proteomic study was performed on one donor aged of 59 years old after 5 days of application in topical. The aim of this study is to do an exploratory analysis on skin explants in order to identify the most relevant biological property of PrimalHyal™ Ultrafiller. After full proteomic analysis, we identified three main biological functions as very modulated by the product after 5 days of application including an improvement of dermo-epidermal junction attachment, matrix protection activity and an anti-oxidant activity (figure 8).

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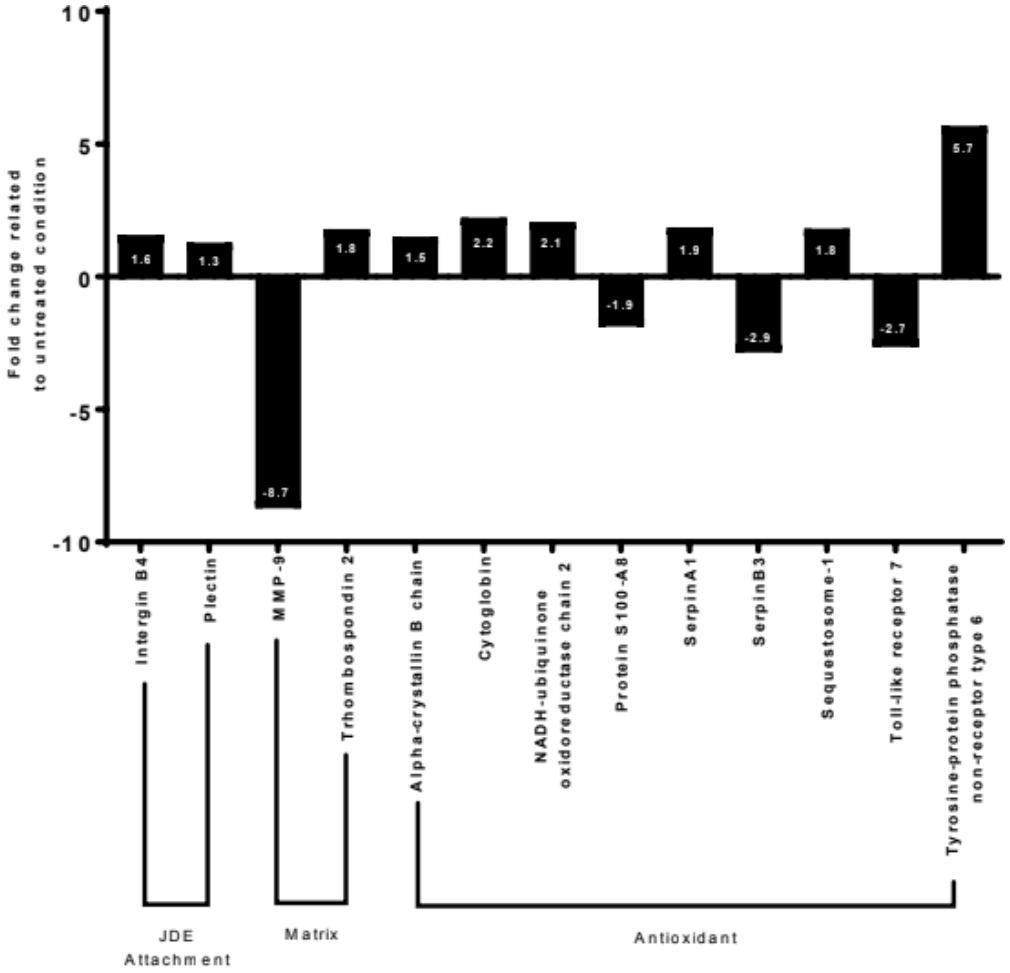

Figure 8: Proteomic analysis from skin explants treated with PrimalHyal™ Ultrafiller 0.1% for 5 days (59 years old).

The proteomic results demonstrated that PrimalHyal™ Ultrafiller could have an efficient anti-oxidant activity as observed by various proteins up or down–regulated after the application of the product for 5 days. All are and evidenced the improvement of anti-oxidant defense.
Among them, sequestosome-1 also named p62 seems to be an interesting target linked to the anti-oxidant/pollution response. During the oxidative stress, the KEAP1/Nrf-2 interaction is impaired due to a modification of KEAP1 conformation linked to the oxidation of cysteines. Free Nrf-2 is then trans-located in the nucleus where it triggers the gene expression of anti-oxidant enzymes such as HMOX-1, NQO1, TRXRD1, p62 and many others. p62 newly synthetized following the Nrf-2 activation is able to play positive retrocontrol on this system by the direct interaction with KEAP-1 therefore blocking its interaction with Nrf- 2.
Another key actor on the regulation Nrf-2 pathway is the Thioredoxin Reductase 1 (TRXR1) which is involved in Trx system (Thioredoxin system). Indeed, previous works described that TRXR1 is able to regulate the activation of Nrf-2 and lead to redox homeostasis, defense against oxidative stress, and regulation of redox signaling pathways (Cebula, et al., 2015).

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4.1.5. Conclusion

We demonstrated that acetylation promoted a relevant improvement of skin penetration and prolong the half-life of the molecule thank to an increase of hyaluronidase resistance to degradation.
The transcriptomic screening of PrimalHyal™ Ultrafiller performed on normal human keratinocytes and fibroblasts in comparison with non-acetylated form permit to identify two key biological pathways potentially modulate by the active:

- Antioxidant activity by the over-expression of antioxidant defenses.
- Anti-aging activity though the capacity to protect the skin against matrix degradation

4.2. Efficacy at the epidermis level

4.2.1. Measurement of TRXR1 expression *in vitro*

We developed a specific *in vitro* model inducing oxidative stress permitting to evaluate the antioxidant activity of the compound on keratinocytes. Our previous results suggested that TRXR1 could be positively regulated by PrimalHyal™ Ultrafiller. We analyzed its expression in basal or after oxidative stress. Our results showed that PrimalHyal™ Ultrafiller induced a significant increasing of TRXR1 expression in basal condition and after oxidative stress. In contrast, non-acetylated forme didn't increase thioredoxin reductase expression (figure 9).
Consequently, these results showed that the effect of PrimalHyal™ Ultrafiller on thioredoxin reductase expression is linked to the acetylation. This overexpression of thioredoxin reductase after PrimalHyal™ Ultrafiller treatment confirmed the antioxidant activity.

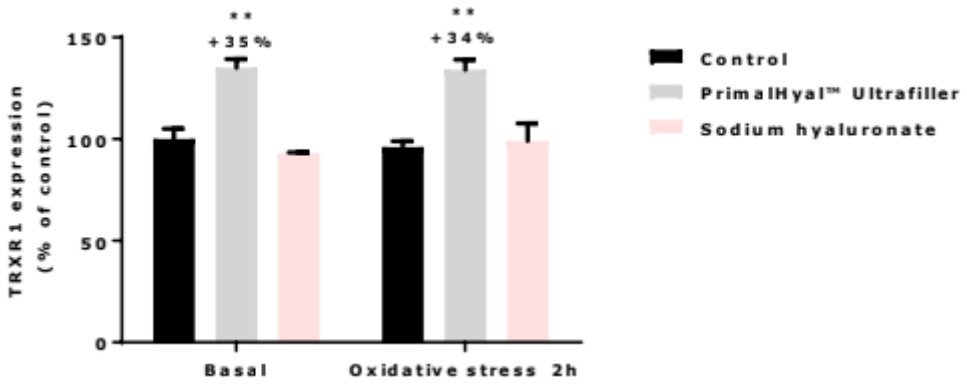

*Figure 9: TRXR1 expression in presence of PrimalHyal™ Ultrafiller or non-acetylated in basal condition or in presence of oxidative stress on NHEKs. The histogram shows the quantification of immunofluorescence. T student's test ** p<0.01.*

4.2.2. Measurement of ROS production *in vitro*

In order to confirm that this increase of antioxidant enzymes expression could be linked to efficient antioxidant activity we measure the impact of PrimalHyal™ Ultrafiller on ROS production in basal and in presence of oxidative stress.

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In basal condition PrimalHyal™ Ultrafiller and non-acetylated form didn't decrease the ROS production. In oxidative stress condition, we noticed a significant decrease of ROS production with PrimalHyal™ Ultrafiller (–30% *). This effect was not observed with the non-acetylation form proving the acetylation confers an antioxidant property to PrimalHyal™ Ultrafiller (figure 10).

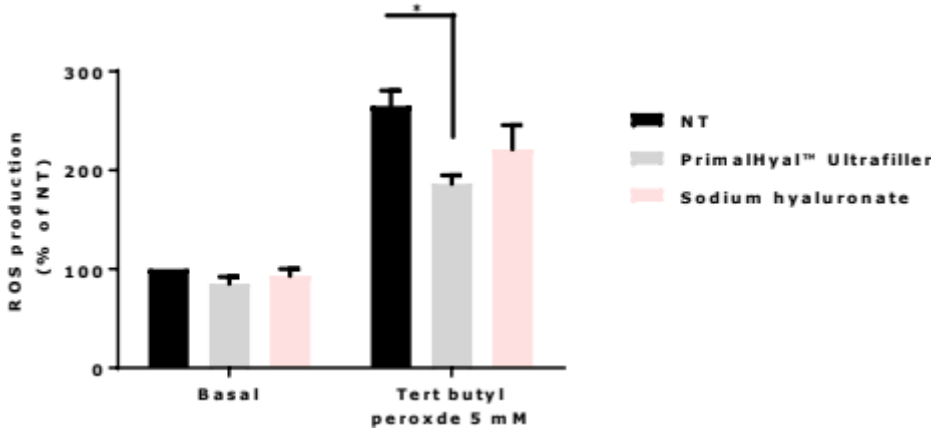

*Figure 10: ROS production assessment after incubation with PrimalHyal™ Ultrafiller or non-acetylated form in basal or in presence of oxidative stress mediated by Tert Butyl Peroxide on NHEK. T student's test * p<0.05.*

4.2.3. Impact on outdoor pollution in presence of UV

In the previous data, we demonstrated that PrimalHyal™ Ultrafiller has an antioxidant activity able to reduce the ROS production *in vitro* and the over-expression of antioxidant enzymes able to improve the skin defense against oxidative or pollutant stress. Among them, we identified the sequestosome-1/p62 as a key factor in the pollution defense which is specifically up-regulated by PrimalHyal™ Ultrafiller.

In order to demonstrate the efficacy of PrimalHyal™ Ultrafiller to control the oxidative stress or pollutant stress, we performed an *ex vivo* study in presence of outdoor pollutant exposure composed to heavy metals and hydrocarbure coupled to UVA exposure. In this study, we used the pollubox from BioEC which permit to exposure the skin explant to the pollution by a polluted cloud and to irradiate them in order to reflect the real life conditions of outdoor pollution exposure. In first, we focused on the impact of outdoor pollution on p62 (sequestosome-1) expression by immunostaining on skin explants. In second step, we quantified the MDA (Malondialdehyde) production in the supernatant in order to prove the efficient anti- oxidant activity. Indeed, MDA production is considered as oxidative stress maker from skin.

Sequestosome 1 (p62) expression after outdoor photo-pollution exposure

Using specific immunostaining, we observed a significant over-expression of sequestosome-1/p62 (+17%*) after 2 days of topical application of PrimalHyal™ Ultrafiller. This result demonstrated that PrimalHyal™ Ultrafiller boosted the production of anti-oxidant defenses on skin explants (figure 11 A).
In presence of outdoor pollution associated to UV exposure, the sequestosome-1/p62 was increased as the consequence of stress exposure. 24h of pre-treatment with PrimalHyal™ Ultrafiller before the outdoor photo-pollution exposure lead to a significant over-expression of sequestosome-1/p62 (+40% *) demonstrating an increase of anti-oxidant defences permitting to control the effect of outdoor photo-pollutant (figure 11).

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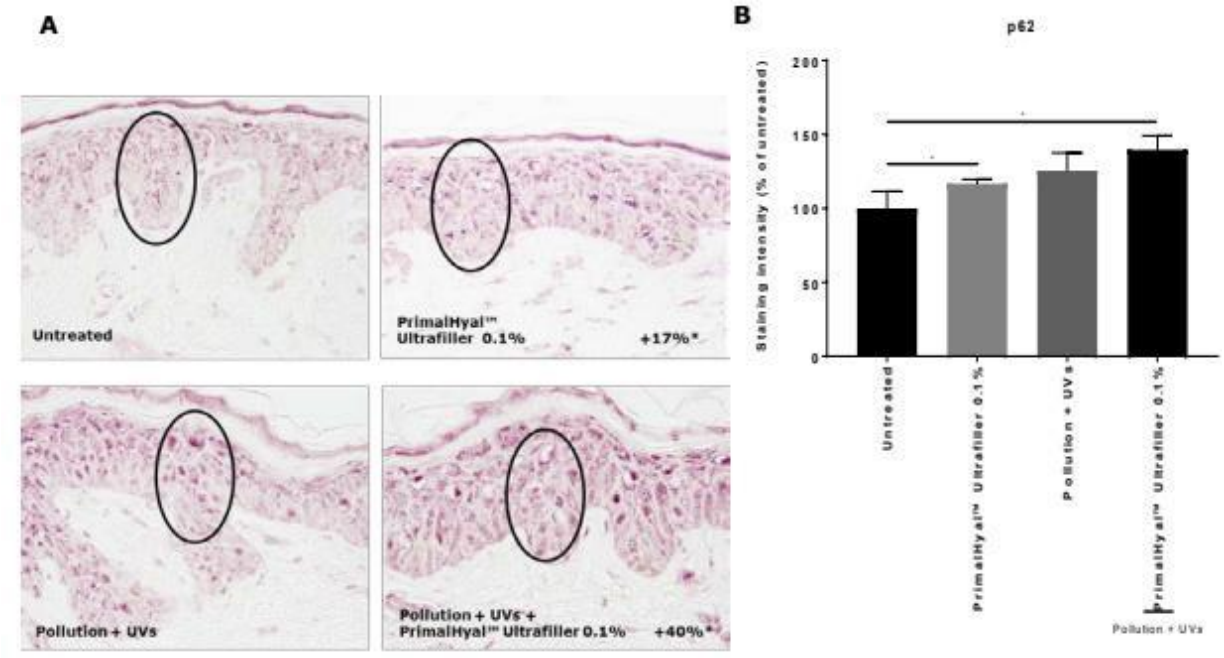

*Figure 11 : Influence of PrimalHyal™ Ultrafiller on sequestosome-1/p62 expression after outdoor pollutant exposure (pollution + UVs). A. Specific immunostaining targeting sequestosome-1/p62 (violet staining) without PrimalHyal™ Ultrafiller in presence or not of outdoor photo-pollution. B. Quantification of sequestosome-1/p62 immunostaining was represented in the histogram expressed in percent of untreated condition. T student test * p<0.05.*

MDA production after outdoor pollution exposure

In second step, we measured the MDA production in the supernatant in order to confirm that this over- expression of sequestosome-1/p62 could lead to an efficient antioxidant defences.
Photo-pollution induced a significant production of MDA as a consequence of uncontrolled oxidative stress in this condition. This results confirmed that the up-expression of sequestosome-1/p62 with photo- pollution observed in figure 12 is a consequence of stress. In contrast, a pre-treatment with PrimalHyal™ Ultrafiller induced significant reduction of MDA production (-29% *) while the non-acetylated form didn't reproduce the same efficacy. These results demonstrated that PrimalHyal™ Ultrafiller induced an efficient antioxidant protection against outdoor photo-pollution through the sequestosome-1/p62 over-expression as evidenced by the significant decreased of MDA production (figure 12).

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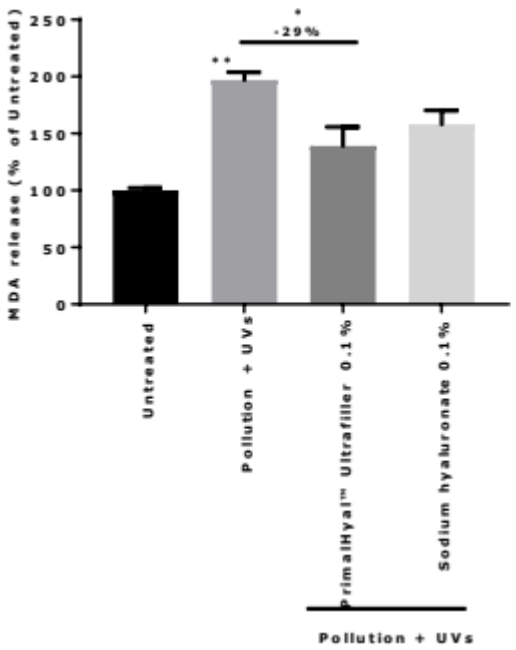

*Figure 12: Quantification of MDA production in presence of PrimalHyal™ Ultrafiller under outdoor photo-pollution exposure. T student test * p<0.05, ** p<0.01.*

4.2.4. Anti-oxidant activity at the clinical level

In order to determine the clinical antioxidant efficacy of PrimalHyal™ Ultrafiller, we evaluated its impact on Lipid peroxidation (LPO) after UV exposure after 28 days of application and the global antioxidant power of the skin through the FRAP measurement after 28 and 56 days of application.

Impact on LPO after UV exposure *in vivo*

In this study, we evaluated the basal level of LPO after UV exposure at D0 and at D28 after 1 month of application cream containg PrimalHyal™ Ultrafiller at 0.1% compared to control and vitamin E area.
Control represents the basal level of LPO found in untreated condition. The results showed a significant increase of LPO production at D28 related to D0. Vitamin E is used like an antixodiant reference in this study and showed a significant production of LPO relative to D0 but this production was significantly reduced in comparison of control demonstrating the antioxidant efficacy of the vitamin E. Similarly to Vitamin E, the area treated with a cream containing 0.1% PrimalHyal™ Ultrafiller demonstrated a significant production of LPO relative to D0 but it is significantly decrease in comparison of control value. These results evidenced that PrimalHyal™ Ultrafiller have a significant antioxidant activity controling the skin oxidation as observed by the production of LPO after UV exposure (figure 13 and table 2).

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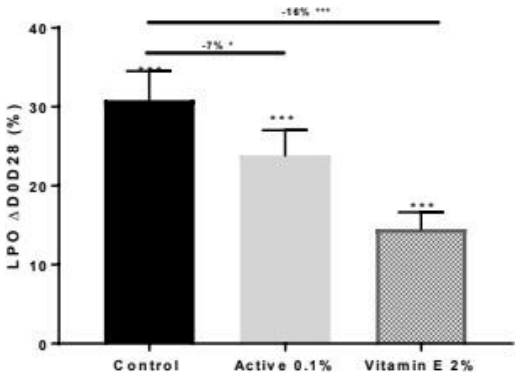

*Figure 13: Quantification of LPO after UV exposure following D28 of treatment with PrimalHyal™ Ultrafiller at 0.1% of vitamin E at 2%. Statistical analysis was performed using t student's test with * p<0.05, ** p<0.01 and *** p<0.001.*

	ΔD0D28 (%) ± SEM	p value versus D0	p value versus control
Control	31 ± 3.6	p<0.001	
PrimalHyal™ Ultrafiller 0.1%	23.9 ± 3.1	p<0.001	p <0.05
Vitamin E 2%	14.6 ± 2.1	p<0.001	p<0.001

Table 2: Quantification of LPO after UV exposure following D28 of treatment with PrimalHyal™ Ultrafiller at 0.1% of vitamin E at 2%. Statistical analysis was performed using t student's test.

Measurement of antioxidant power of the skin *in vivo* (FRAP)

In parallel to the LPO measurement, we evaluate the global antioxidant power of the skin thank to the FRAP measurement after 28 days and 56 days of application of cream containing PrimalHyal™ Ultrafiller at 0.1% or vitamin E at 2%.
Control represents the basal level of antioxidant power of the skin after D28 and D56. The results showed without products the global antioxidant power of the skin is stable until 56 days.
A treatment with Vitamin E at 2% used here as a positive antioxidant molecule is able to significantly improve the antioxidant power of the skin after D28 and D56 relative to D0 and control.
Similarly to Vitamin E, the application of cream containing PrimalHyal™ Ultrafiller at 0.1% for 28 and 56 days promoted an improvement of global antioxidant power of the skin with a significant effect after D56 relative to D0 and control (table 3 and figure 14).

	ΔD0D28			ΔD0D56		
	ΔD0D28 (%) ± SEM	p value versus D0	p value versus control	ΔD0D56 (%) ± SEM	p value versus D0	p value versus control
Control	-3.7 ± 4.8	ns		-0.6 ± 3.5	ns	
PrimalHyal™ Ultrafiller 0.1%	3.2 ± 1.9	ns	ns	19.4 ± 2.3	ns	p <0.001
Vitamin E 2%	29.8 ± 5.1	p<0.001	p<0.001	33.5 ± 4.6	p<0.001	p<0.001

Table 3: Analysis of global antioxidant power of the skin by FRAP measurement after D28 and D56 in presence of cream containing PrimalHyal™ Ultrafiller at 0.1% of vitamin E at 2%.

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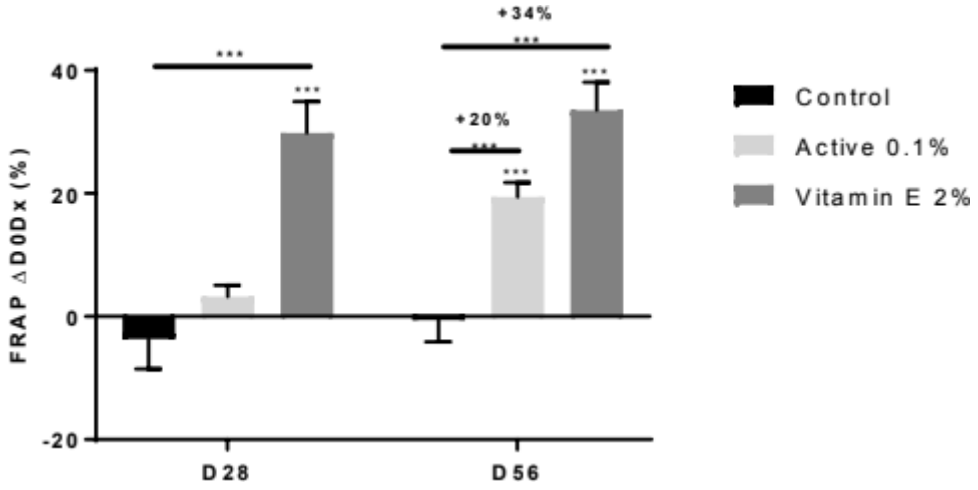

*Figure 14: Analysis of global antioxidant power of the skin by FRAP measurement after D28 and D56 in presence of cream containing PrimalHyal™ Ultrafiller at 0.1% of vitamin E at 2%. Statistical analysis was performed using t student test with *** p<0.001.*

4.2.5. Conclusion on the effect of PrimalHyal™ Ultrafiller on epidermis

In this part we demonstrated the efficacy of PrimalHyal™ Ultrafiller on antioxidant and antipollutant defense through:
At *in vitro* level:

- increase of TRXR1 expression from NHEK up to 35% in basal condition and under oxidative stress
- that is correlate to efficient decrease of ROS production with -30% under oxidative stress At *ex vivo* level:
- an increase of sequestosome p62 expression on basal condition +17% and under photo-pollution exposure with +40%
- that is correlated to a significant decrease of MDA production -29% confirming the antipho-topollution efficacy thank to the increase the antioxidant defence as a "vaccine like" effect.

At clinical level:

- significant decrease of LPO production after UV exposure with -7%
- correlated to an increase of global antioxidant power of the skin as observed by the FRAP measurement with +20%

4.3. Efficacy at the dermis level

Different studies were performed in order to confirm the previous investigations which suggest that PrimalHyal™ Ultrafiller could have an anti-aging efficacy through a matrix protection activity.

4.3.1. Matrix protection by MMPs inhibition *in vitro*

We developed an « aging » like model with over-expression of MMP-1 and MMP-3 expression reflecting the phenomenon observed with the aging and responsible for the wrinkles formation.
In the figure 15, we observed an increase of MMP-1 and MMP-3 production after oxidative stress by two fold. This effect was significantly inhibited by the treatment with PrimalHyal™ Ultrafiller at 0.1

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mg/ml. These effects were significant compared to no active (Basal) and non-acetylated hyaluronate conditions. Non-acetylated hyaluronate induced a slight but non significant decrease of MMP-1 and MMP-3 releasing.
These results confirmed that PrimalHyal™ Ultrafiller was able to inhibit the MMP-1 (-40%*) and MMP-3 (- 37% *) releasing and could protect the cell against matrix degradation mediated by oxidative stress (figure 15A and B).

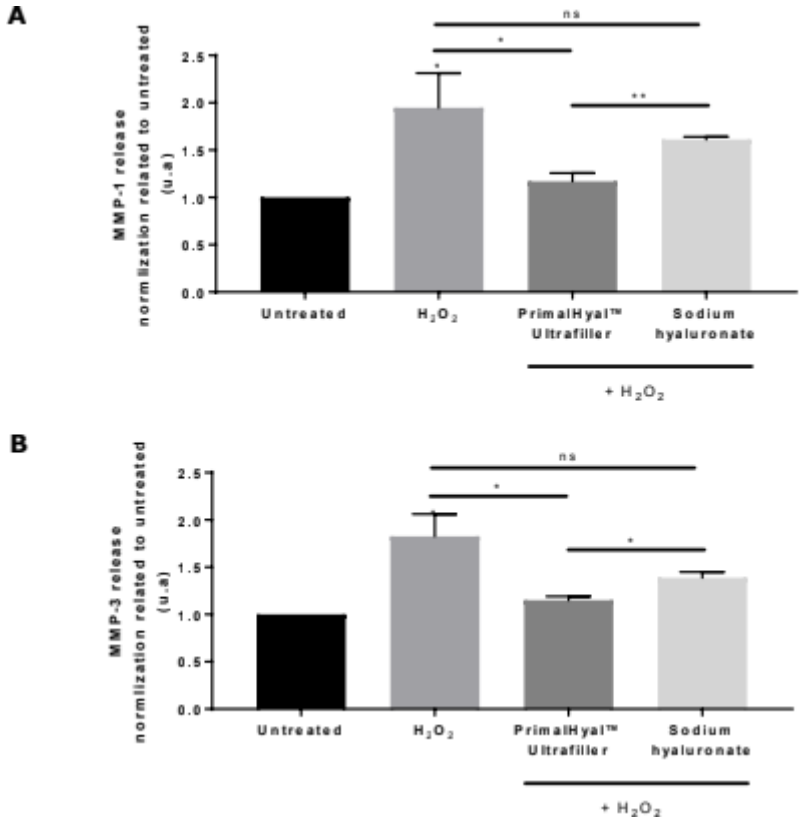

*Figure 15: Quantification of MMP-1 (A) and MMP-3 (B) after oxidative stress in presence of PrimalHyal™ Ultrafiller or non-acetylated form on FDH. Statistical analysis was performed by T student test with * p<0.05, ** p<0.01.*

4.3.2. Matrix protection by MMPs inhibition in chronobiologic aging *ex vivo*

In *ex vivo* study, we studied the anti-aging related to matrix protection activity of PrimalHyal™ Ultrafiller after 6 days of treatment topically applied on human skin explants from the donor aged to 59 years old. We observed after 6 days of culture a drastic decrease of MMP-1 and MMP-9 expression in dermis on skin explants treated with 0.1% PrimalHyal™ Ultrafiller with -18% ** and -27% ** respectively.
These two proteases are specifically involved in dermal matrix degradation and actively participated to wrinkles formation (figure 16).
These results demonstrated that PrimalHyal™ Ultrafiller actively protects the dermal matrix from potential degradation mediated by MMPs.

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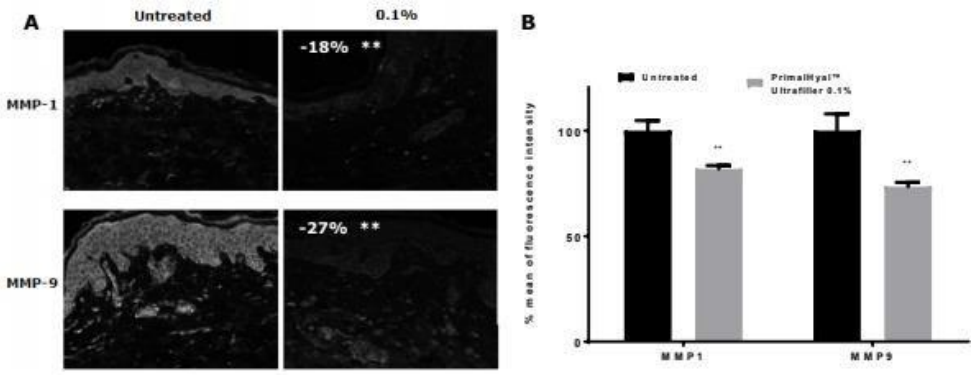
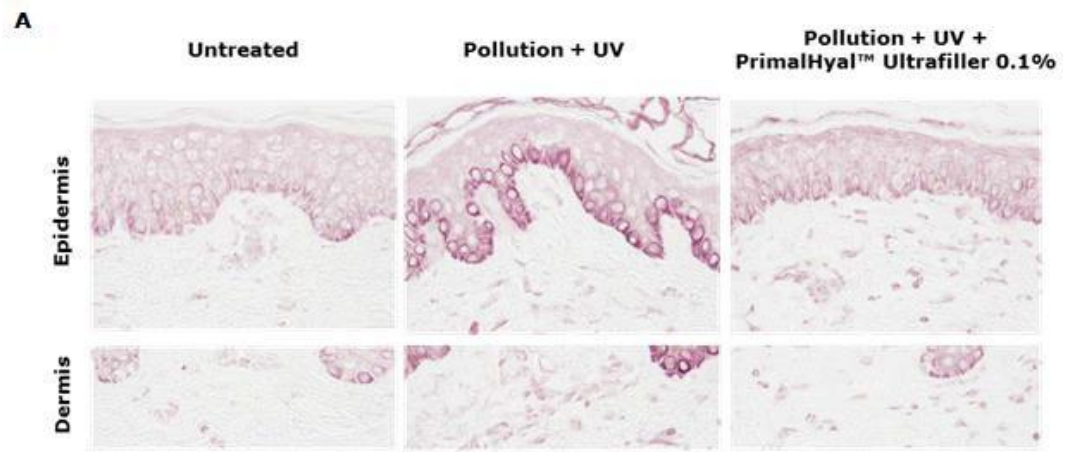


*Figure 16: Impact of PrimalHyal™ Ultrafiller on MMP-1 and MMP-9 expression ex vivo. A.specific immunostaining of MMP-1 and MMP-9 from skin explants after treatment with PrimalHyal™ Ultrafiller at 0.1% for 6 days. B. quantification of MMP-1 and MMP-9 expression in the dermis by image analyzer image J. Statistical analysis was per- formed by T student test with ** p<0.01.*

4.3.3. Matrix protection by MMPs inhibition in extrinsic aging *ex vivo*

After to have study the impact of PrimalHyal™ Ultrafiller on chronologic aging, we are interested to observe its impact on the effect of the premature aging induced by an external environmental stress as outdoor photopollution. MMP-1 and MMP-9 are two key proteases involved in the collagen degradation that is the most important extracellular matrix component in the dermis.
Regarding the effect on MMP-1, it presents a low expression at the basal level. The outdoor photo-pollution induced an increase of its expression in epidermis and dermis. A pre-incubation with PrimalHyal™ Ultrafiller at 0.1% for 24h before the stress induced a decrease of MMP-1 expression in epidermis and dermis (figure 17 A). These results showed PrimalHyal™ Ultrafiller protects the dermal matrix by relevant inhibition of MMP-1 expression in presence of outdoor pollutant (figure 17 A).
Regarding the MMP-9 expression, we observed a slight expression in the epidermis and dermis at basal level. After outdoor photo-pollution exposure, we showed a drastic increase of its expression in epidermis and dermis. A pre-treatment with PrimalHyal™ Ultrafiller at 0.1% promoted a relevant decrease of MMP-9 expression in the epidermis and dermis (figure 19 B). Similarly to MMP-1, these results demonstrated that PrimalHyal™ Ultrafiller protected the dermal matrix through an inhibition of expression of MMP-9 in presence of outdoor photo-pollutant (figure 17B).



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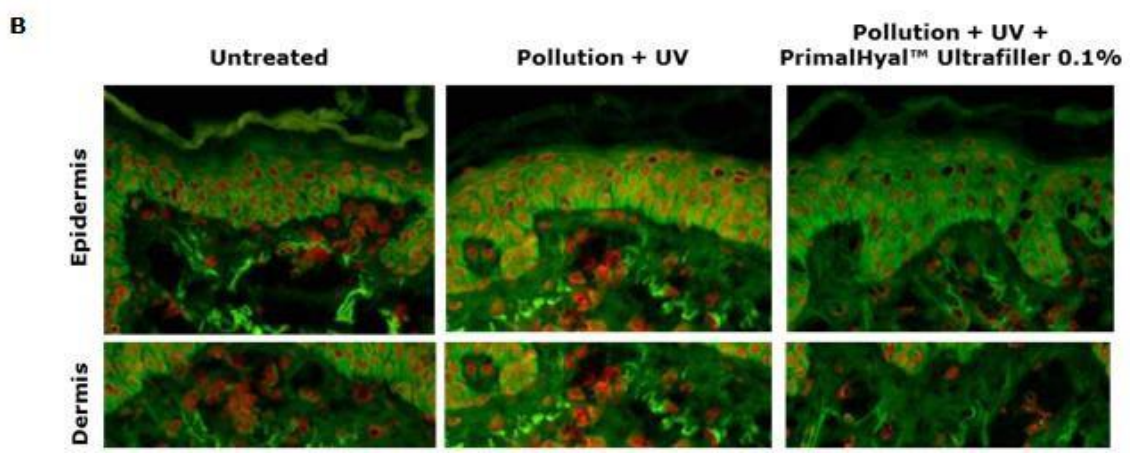

Figure 17: MMP-1 (A) and MMP-9 (B) immunostaining from skin explants exposed to outdoor photo-pollution

The quantification of MMP-1 and MMP-9 in the epidermis and dermis permit to confirm the visual effects. Indeed, we showed that the outdoor photo-pollution induced a significant increase of MMP-1 and MMP-9. Regarding the MMP-1, an over-expression of +29% and +33% were found in the epidermis and dermis respectively. The MMP-9 analysis evidenced a significant increase of its expression of +26% and +285% in the epidermis and dermis respectively (figure 18 A and B).

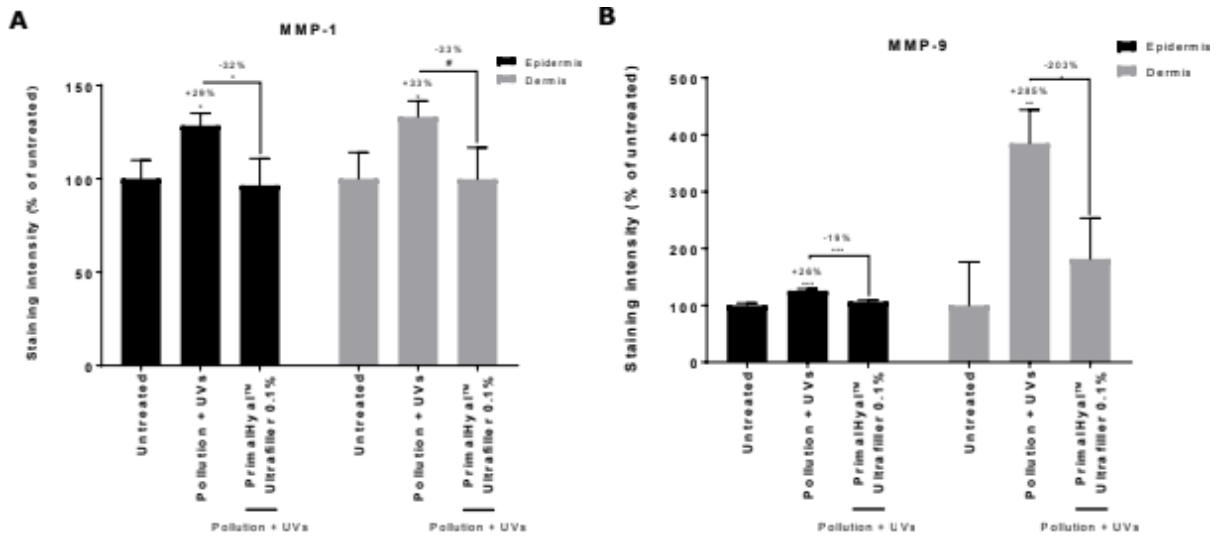

*Figure 18: Quantification of MMP-1 and MMP-9 expression from skin explants exposed to outdoor photo-pollution using imagery analyzer (Image J). Statistical analysis was performed on T student's test with * p<0.05 and *** p<0.001.*

4.3.4. Protection of Collagen I degradation *in vitro*

MMPs are enzyme involved in matrix degradation responsible for the skin aging and wrinkles formation. MMP-1 is a collagenase able to degrade the type I collagen which is the dominant collagen

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in the dermis. MMP-9 and MMP-3 are a gelatinase and Stromelysin-1 respectively which are two metalloproteinase able to cleave collagen already degraded by other MMPs.
We analyzed the impact of PrimalHyal™ Ultrafiller in the type I collagen degradation *in vitro* in order to demonstrate that the MMPs inhibition is able to lead to efficient matrix protection.
The type I collagen degradation was measured using DQ-type I collagen and its visualization by zymography *in situ*.
The results obtained with the DQ-collagen that is a type I collagen linked to a quencher which block the fluorescence emission. If the collagen is degraded the quencher is cleaved that promotes emitted fluores- cence. The emitted fluorescence is directly proportional to the type I collagen degradation.
We observed an increase of 50% of type I collagen degradation after the oxidative stress which proved active MMPs were released during the stress. When we incubated the cells with PrimalHyal™ Ultrafiller, we observed a significant decrease of collagen degradation demonstrating that the treated induced an efficient MMPs inhibition leading to a protection of type I collagen (-42% *). We didn't observe type I protection with non-acetylated hyaluronate in presence of oxidative stress as showed by the important increase of the type I collagen degradation.

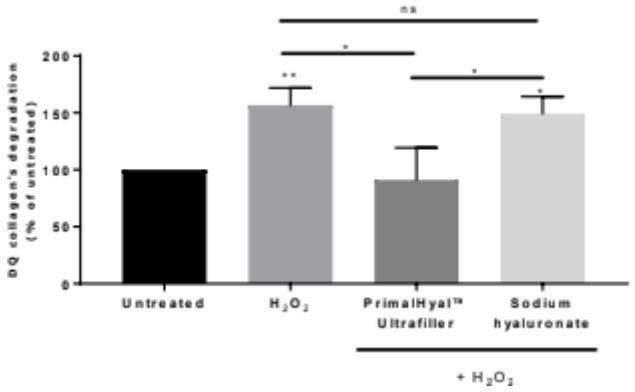

Figure 19: Analysis of collagen degradation in presence of PrimalHyal™ Ultrafiller

We used zymography *in situ* to visualize the effect on PrimalHyal™ Ultrafiller on type I degradation. The oxidative stress was performed by PMA (phorbol myristate acetate) in order to have sufficient fluorescent signal to detect it. As observed in these pictures, there is more fluorescent signal after PMA stimulation than untreated control, showing an efficient DQ-collagen's degradation with PMA. When the cells were pre-incubated with PrimalHyal™ Ultrafiller before PMA stimulation, we can observe a decrease of the fluorescent signal in comparison with PMA control, confirming the previous studies about the MMPs release inhibition leading to matrix protection. In contrast, when we incubated the cells with non-acetylated form before the PMA stimulation, we observed an increase of fluorescence around the cells that showed collagen degradation. These results confirmed that the presence of acetylation is essential for the active efficacy on matrix protection.

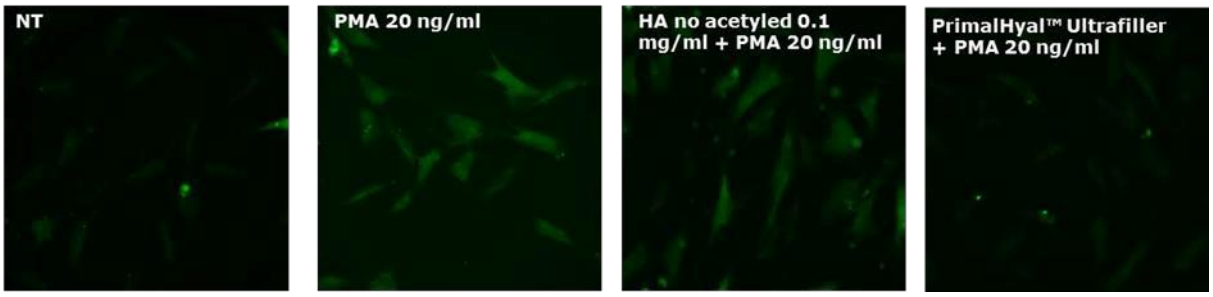

Figure 20: Visualization of collagen degradation in presence of PrimalHyal™ Ultrafiller using zymography in situ

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4.3.5. Clinical anti-aging activity

The anti-aging efficacy of sodium hyaluronate was performed on face wrinkles after a flash application or after prolonged using 1 and 2 months.

Flash anti-wrinkles effect on crow's feet and nasogenian area

In this first clinical study based on the anti-aging efficacy of PrimalHyal™ Ultrafiller, we are interested by a flash anti-wrinkles effect on crow 's feet and nasogenial area. The products were applied on hemiface at D0 and the wrinkles on crow's feet and nasogenian area were analyzed by profilometry and VISA respectively afer 6h.In this study we evaluated the flash anti-aging efficacy of PrimalHyal™ Ultrafiller at 0.2%.

- **Anti-aging effect on crow's feet after 1h and 6h**

After 1h of application, we observed a significant decrease of average lengths of furrows (0-0.5 mm) relative to D0 and Placebo in crow's feet area. Placebo didn't show relevant effect in comparison to D0 (figure 21). These results evidenced a flash anti-aging effect.

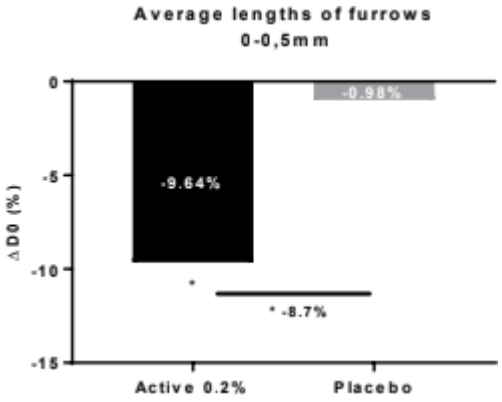

*Figure 21: Impact of PrimalHyal™ Ultrafiller at 0.2% on average lengths of furrows 0-0.5mm on crow's feet area using profilometry after 1h of application. Statistical analysis was performed by T student's test with * p<0.05.*

Average length of furrows 0-0.5 mm	Cream 213 (Placebo)			Cream 808 (PrimalHyal™ Ultrafiller 0.2%)			Student t-test versus placebo (p)
	Mean +/- SD (µm)	Δ% (mean)	Student t-test versus D0 (p)	Mean +/- SD (µm)	Δ% (mean)	Student t-test versus D0 (p)	
D0	0.25 ± 0.01			0.26 ± 0.01			ns
T1h	0.25 ± 0.02	-0.98%	ns	0.24 ± 0.03	-9.64%*	p<0.05	p<0.05

Table 4: Impact of PrimalHyal™ Ultrafiller at 0.2% on average lengths of furrows 0-0.5mm on crow's feet area using profilometry after 1h of application

After 6h of application, we observed a significant improvement relative to placebo of number of average wrinkles 0.5-2mm (-26.5% *) and total number (-6%) of wrinkles on crow's feet area treated with the product containing PrimalHyal™ Ultrafiller at 0.1%. Regarding the effect of Placebo, we showed an increase of number of average wrinkles 0.5-2 mm and total number of wrinkles

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probably reflecting the fatigue effect on the face wrinkles observed during the day (figure 22). These results showed that a cream containing 0.2% of PrimalHyal™ Ultrafiller evidenced a relevant flash anti-wrinkles effect and that maintained this all day long.

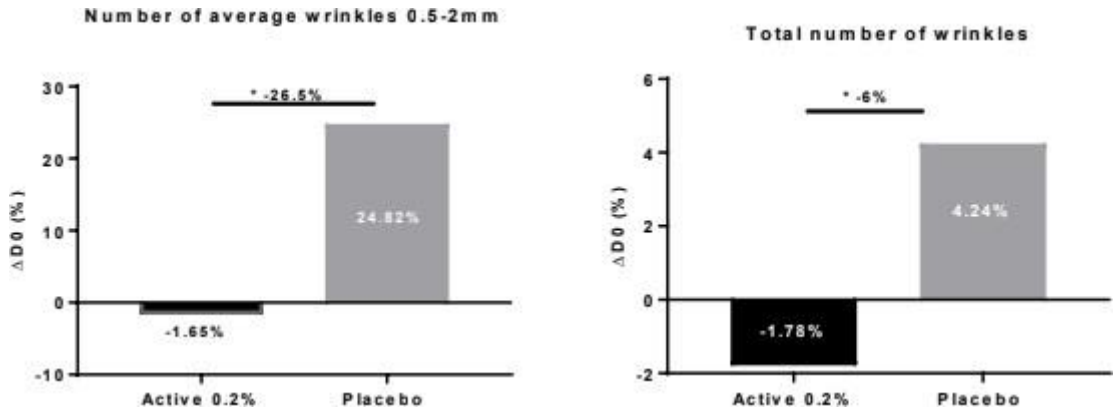

*Figure 22: Impact of PrimalHyal™ Ultrafiller at 0.2% on crow's feet using profilometry after 6h of application. Statistical analysis was performed by T student's test with * p<0.05.*

Number of average wrinkles 0.5-2mm of microrelief	Cream 213 (Placebo)			Cream 808 (PrimalHyal™ Ultrafiller)			Student t-test versus placebo (p)
	Mean +/- SD (arbitrary unit)	Δ% (mean)	Student t-test versus D0 (p)	Mean +/- SD (arbitrary unit)	Δ % (mean)	Student t-test versus D0 (p)	
D0	7 ± 0.94			6 ± 1.04			ns
T6h	8 ±5.92	24.82%	ns	6 ± 5.23	-1.65%	NS	p<0.05

Table 5: : Impact of PrimalHyal™ Ultrafiller at 0.2% on number of average wrinkles (0.5-2mm) from crow's feet area after 6h of application.

Total number of wrinkles	Cream 213 (Placebo)			Cream 808 (PrimalHyal™ Ultrafiller)			Student t-test versus placebo (p)
	Mean +/- SD (arbitrary unit)	Δ% (mean)	Student t-test versus D0 (p)	Mean +/- SD (arbitrary unit)	Δ % (mean)	Student t-test versus D0 (p)	
T0	26 ± 2.96			23 ± 2.84			NS
T6h	27 ± 12.07	4.24%	ns	23 ±12.26	-1.78%	ns	p<0.05

Table 6: Impact of PrimalHyal™ Ultrafiller at 0.2% on total number of wrinkles from crow's feet area after 6h of application.

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- **Anti-aging effect on nasogenian area after 6h**

During the flash anti-aging study performed on PrimalHyal™ Ultrafiller, we also focused on the nasogenian wrinkles after 6h of application.
After 6h of application, we observed a significant improvement of nasogenian wrinkles especially the coarse area (-8% *). This effect is signicant relative to placebo (figure 23).
These results demonstrated a flash anti-wrinkles efficacy of PrimalHyal™ Ultrafiller on the nasogenian area after only 6h of application.

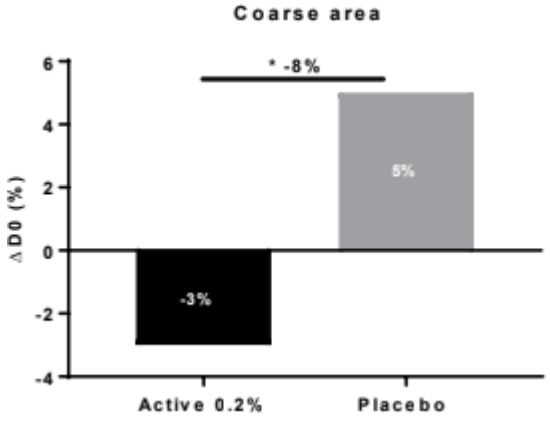

*Figure 23: Impact of PrimalHyal™ Ultrafiller at 0.2% on coarse area of nasogenian wrinkles after 6h of application after 6h of application. T student's test was used with * p<0.05.*

Coarse aera on nasogenial wrinkles	Cream 213 (Placebo)			Cream 808 (PrimalHyal™ Ultrafiller)			Student t-test versus placebo (p)
	Mean +/- SD (arbitrary unit)	Δ% (mean)	Student t-test versus D0 (p)	Mean +/- SD (arbitrary unit)	Δ % (mean)	Student t-test versus D0 (p)	
D0	34.6 ± 11.90			30.81 ±7.88			NS
T6h	36.4 ± 10.28	5%	ns	29.84 ± 8.14	-3%	ns	p<0.05

Table 7: Impact of PrimalHyal™ Ultrafiller at 0.2% on coarse area of nasogenian wrinkles after 6h of application after 6h of application.

- **Anti-aging efficacy perception by volunteers after 1h and 6h**

During this study, volunteers were submitted to a questionnaire in order to evaluate their sensorial perception of the efficacy of the product after 1h and 6h of application.
After 1h of application, the product containing PrimalHyal™ Ultrafiller at 0.2% was evaluated as more efficacy than placebo cream on each items. Indeed, 67% of volunteers find an improvement of their skin appearance with the active, 67% of them found that their crow's feet wrinkles were smoother with the active and 62% of them found their nasolabial wrinkles were smoother with the active (figure 24).

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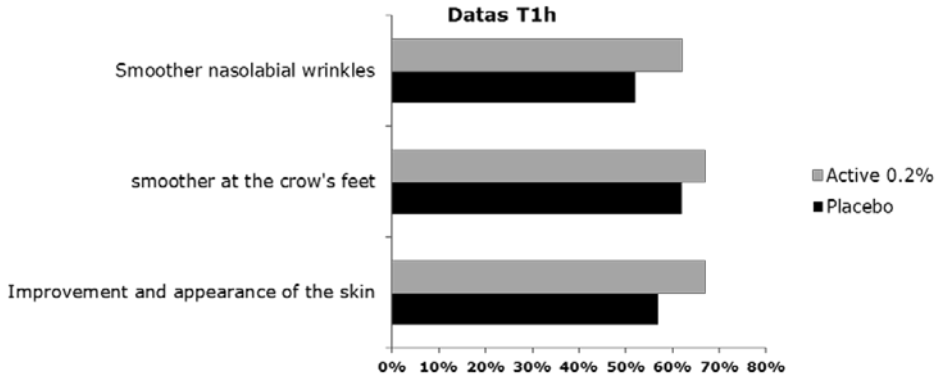

Figure 24: Self-assessment of the tested products after 1h of application

After 6h of application, the product containing PrimalHyal™ Ultrafiller at 0.2% was evaluated as more efficacy than placebo cream on each items. Indeed, 76% of volunteers find an improvement of their skin appearance with the active, 71% of them found that their crow's feet wrinkles were smoother with the active and 62% of them found their nasolabial wrinkles were smoother with the active (figure 25).

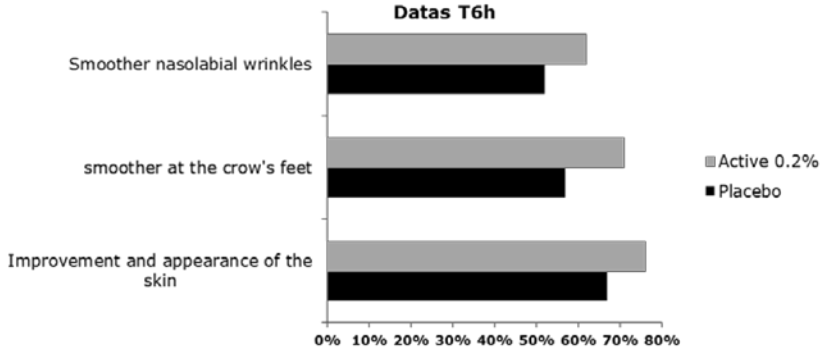

Figure 25: Self-assessment of the tested products after 6h of applucation

<u>Anti-wrinkles efficacy on nasaogenian area after month of application</u>

In the second clinical study, we evaluated the anti-aging efficacy of PrimalHyal™ Ultrafiller at 0.1% on the nasogenian wrinkles after 1 month of application in hemiface in comparison with Placebo.
We analyzed the nasogenian wrinkles by VISIA CR using specific software.
After 28 days of application, we observed a significant decrease of wrinkles from nasogenian area especial- ly a decrease of count, total area and medium line (thickness 0.34 – 0.41) parameters.
The decreasing of count, total area and medium line analysis showed a significant reduction with the active relative to D0 and placebo. These effects evidenced a decrease of -9%*, -12%* and -13%* on wrinkles count, total area and medium line respectively (figure 26).

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These results demonstrated an efficient anti-aging activity after 1 month of application.

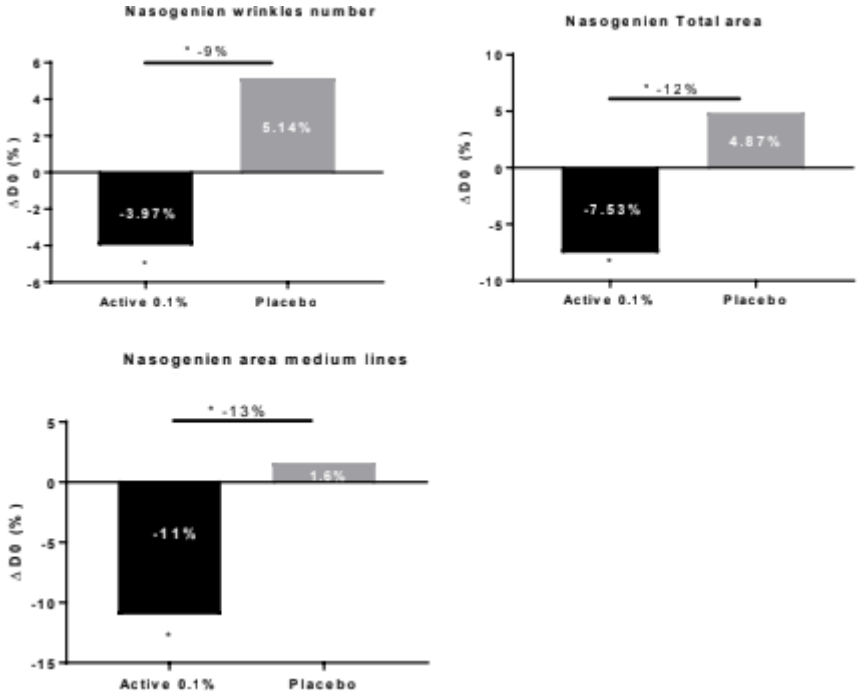

*Figure 26: Impact of PrimalHyal™ Ultrafiller at 0.1% on nasogenian wrinkles after 1 month of application. T student's test was used for the statistical analysis with * p<0.05.*

Wrinkles number	Placebo			PrimalHyal™ Ultrafiller at 0.1%			Student t-test versus placebo (p)
	Mean +/- SD (arbitrary unit)	Δ% (mean)	Student t-test versus D0 (p)	Mean +/- SD (arbitrary unit)	Δ % (mean)	Student t-test versus D0 (p)	
D0	40 ± 8.75			38 ±9.26			Ns
D28	42 ± 10.59	5.14%	ns	36 ± 7.70	-3.97%	p<0.05	p<0.05

*Table 8: Impact of PrimalHyal™ Ultrafiller at 0.1% on wrinkles number from nasogenian area after 1 month of application. T student's test was used for the statistical analysis with * p<0.05*

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Total area	Placebo			PrimalHyal™ Ultrafiller at 0.1%			Student t-test versus placebo (p)
	Mean +/- SD (arbitrary unit)	Δ% (mean)	Student t-test versus D0 (p)	Mean +/- SD (arbitrary unit)	Δ % (mean)	Student t-test versus D0 (p)	
D0	59.0 ± 13.73			56.13 ± 14.58			Ns
D28	61.9 ± 15.53	4.87%	ns	51.91 ± 11.14	-7.53%	p<0.05	p<0.05

*Table 9: Impact of PrimalHyal™ Ultrafiller at 0.1% on total wrinkles area from nasogenian area after 1 month of application. T student's test was used for the statistical analysis with * p<0.05*

Medium lines	Placebo			PrimalHyal™ Ultrafiller at 0.1%			Student t-test versus placebo (p)
	Mean +/- SD (arbitrary unit)	Δ% (mean)	Student t-test versus D0 (p)	Mean +/- SD (arbitrary unit)	Δ % (mean)	Student t-test versus D0 (p)	
D0	13.6 ± 4.00			12.76 ± 3.87			ns
D28	13.9 ± 4.07	1.6%	ns	11.35 ± 2.67	-11%	p<0.05	p<0.05

*Table 10: Impact of PrimalHyal™ Ultrafiller at 0.1% on medium lines from nasogenian area after 1 month of application. T student's test was used for the statistical analysis with * p<0.05*

We selected two volunteers in order to illustrate the results on nasogenian wrinkles analysis. We presented the pictures from D0 versus the effect after 28 days of application for the hemiface treated with the placebo followed to those treated with active.

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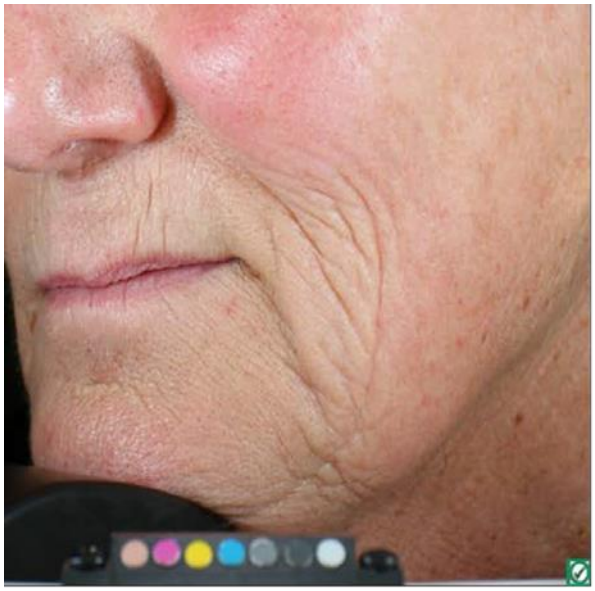
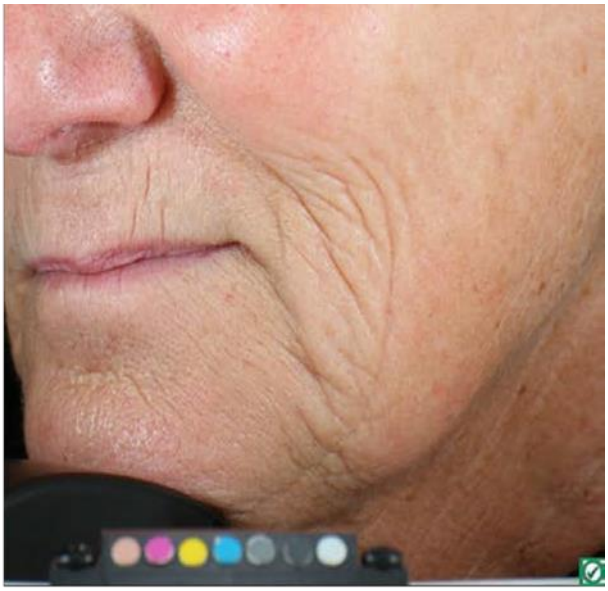
- Volunteer 1:

Placebo

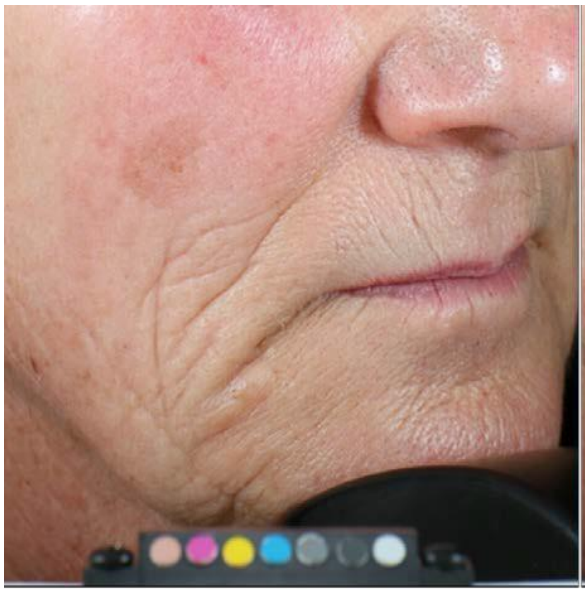


D0

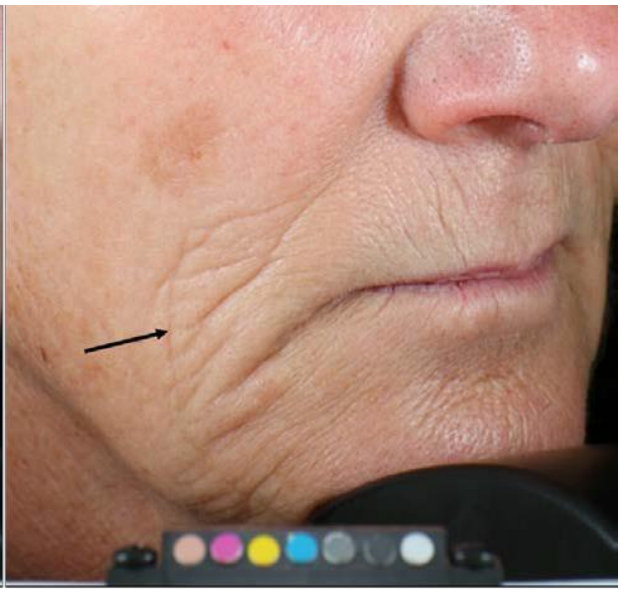


D28

PrimalHyal™ Ultrafiller



D0



D28

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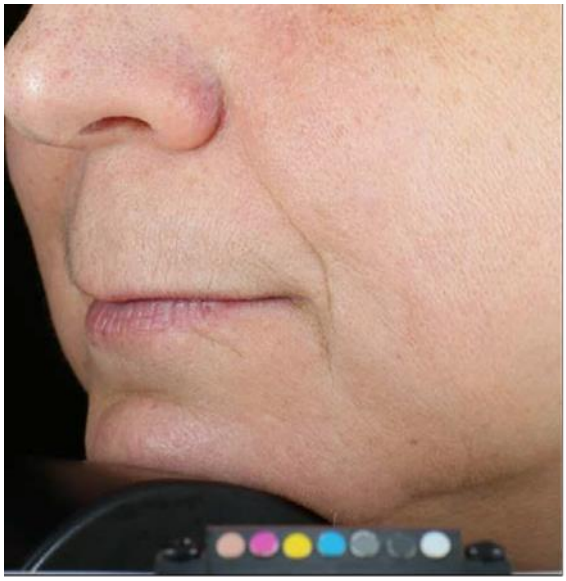
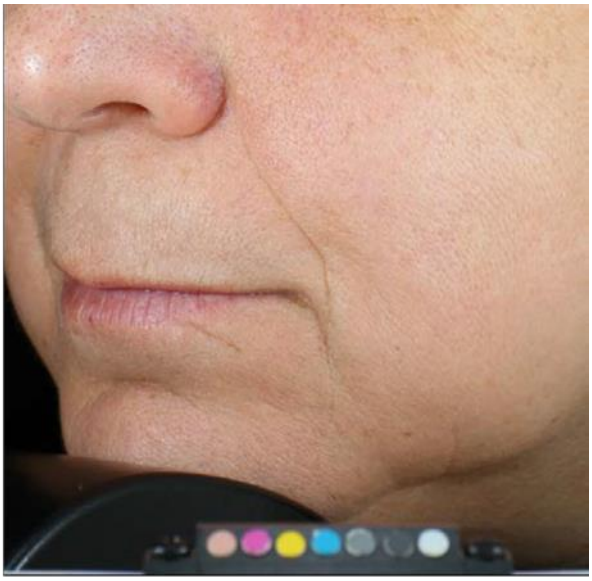
- Volunteer 2:

Placebo

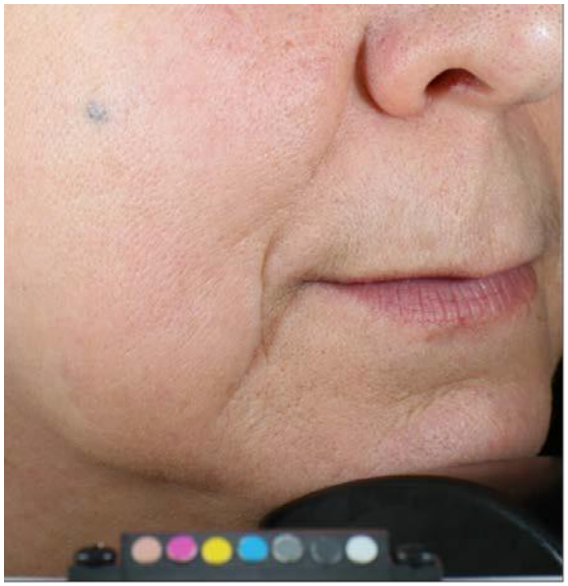


D0

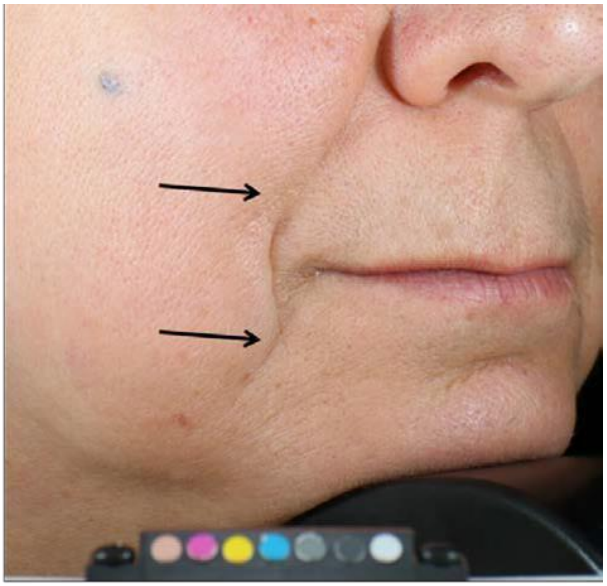


D28

PrimalHyal™ Ultrafiller



D0



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Skin texture improvement after two months of application

In third clinical study was carried out on skin texture analysis from a panel presenting visible wrinkles on face. In this study, the product containing PrimalHyal™ Ultrafiller at 0.1% was analyzed in comparison to placebo after 2 months of application in hemiface. The skin texture was analyzed at D0 and after 56 days of application by VISIA CR using specific software.

After 56 days of application, we observed a relevant improvement of skin texture with a smoothing effect of -11%% with the active at 0.1%. This effect is significant relative to D0 and Placebo (-9% *).
In constrat, the hemiface treated with placebo didn't show any improvement of skin texture after 2 months of application (Figure 27).
These results evidenced an efficient anti-aging activity after 2 months of application on the global aspect of skin with an improvement of skin texture by a smoothing effect.

*Figure 27: Impact of PrimalHyal™ Ultrafiller at 0.1% on skin texture after 2 months of application. T student's test was used for statistical analysis with *p<0.05*

Skin texture	Placebo			PrimalHyal™ Ultrafiller at 0.1%			Student t-test versus placebo (p)
	Mean +/- SD (arbitrary unit)	Δ% (mean)	Student t-test versus D0 (p)	Mean +/- SD (arbitrary unit)	Δ % (mean)	Student t-test versus D0 (p)	
D0	0.198 ± 0.082			0.207 ± 0.082			ns
D56	0.193 ± 0.079	-2%	ns	0.184 ± 0.080	-11%	p<0.05	p<0.05

*Table 11: Impact of PrimalHyal™ Ultrafiller at 0.1% on skin texture after 2 months of application. T student's test was used for statistical analysis with *p<0.05*

Anti-aging efficacy perception by volunteers after 2 months

During this study, volunteers were submitted to a questionnaire in order to evaluate their sensorial perception of the efficacy of the product after 2 months of application.
After 2 months of application, the product containing PrimalHyal™ Ultrafiller at 0.1% was evaluated as more efficacy than placebo cream on each items (17 items). Indeed, 70% of volunteers find their skin healthier-looking with the active, 77% of them found their skin smoother with the active, 70%

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of them found their skin more moisturizing with the active and 63% of them found their skin more supple and elastic with the active (figure 28).

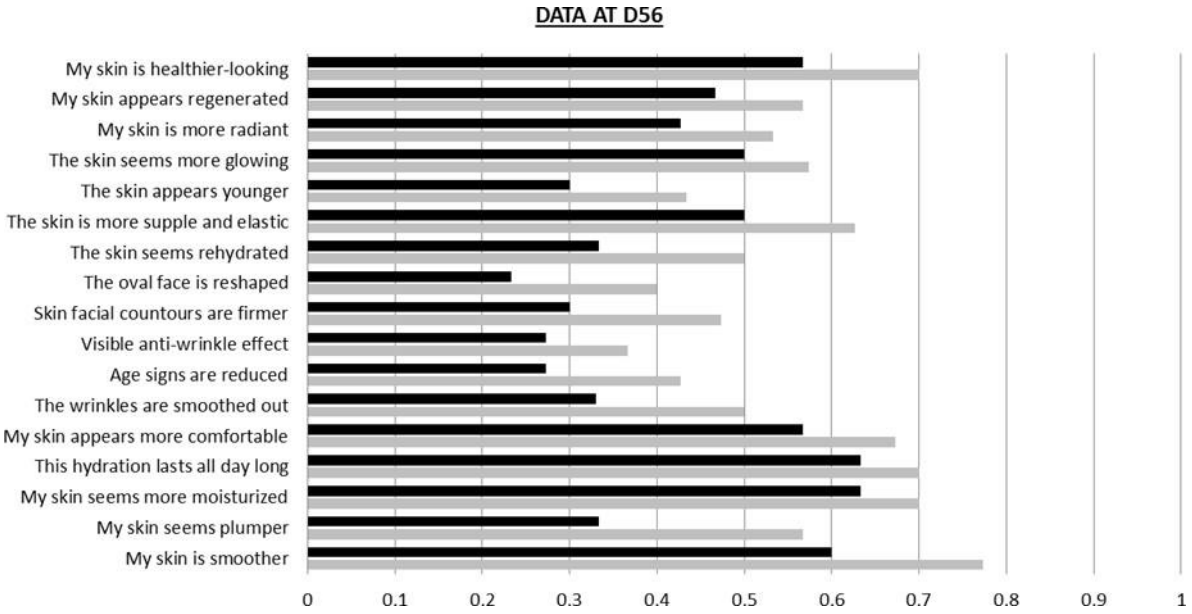

Figure 28: Self-assessment on the efficacy of the tested products after 2 months of application

4.3.6. Conclusion on anti-aging efficacy of PrimalHyal™ Ultrafiller

In this part, we demonstrated the efficacy of PrimalHyal™ Ultrafiller on anti-aging activity through an efficient matrix protection.
We evidenced at the *in vitro* and *ex vivo* level:

- Significant inhibition of **MMP-1 (-40%)** et **MMP-3 (-37%)** on **normal human fibroblasts**
- These results were confirmed using **chronological aging** *ex vivo* model showing a significant inhibition of **MMP-1 (-18%)** and **MMP-9 (-27%)** expressions at dermis level.
- Under **intrinsic aging** mediated by photo-pollution we observed an inhibition of :
  - **MMP-1 with -32% and – 33% in the epidermis and dermis** level respectively
  - **MMP-9 with -19% and -203% in the epidermis and dermis** level respectively
- These results were correlated to a significant **protection of matrix** as observed by the decrease **of type I collagen degradation (-42%)**

At the clinical level:
We demonstrated a **flash anti-aging efficacy** of the product **at 0.2%** on **crow's feet and nasogenian** areas.

- On the **crow's feet** area:
  - After **1h**: significant decrease of **average lenghts of furrows** (0-0.5 mm) **-8.7%.**
  - After **6h**: significant reduction of **number of wrinkles** (0.5-2mm) **-26.5%** and the **total wrinkles -6%.**
- On the **nasogenian** area:
  - After **6h**: a signficiant decrease of **coarse area -8%.**

After **1 month of application at 0.1%** we observed on the **nasogenian area:**

- A significant reduction of **wrinkles number (-9%), total wrinkles area (-12%) and the medium lines (-13%).**

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After **2 months of application at 0.1%** we observed on the **full face**:

- A significant improvement of **skin texture 9%.**

5. General conclusion

We demonstrated that acetylation promoted **a relevant improvement of skin penetration** and **prolong the half-life (x13)** of the molecule thank to an increase of hyaluronidase resistance to degradation.

The transcriptomic screening of PrimalHyal™ Ultrafiller performed on normal human keratinocytes and fibroblasts in comparison with non-acetylated form permit to identify two key biological pathways potentially modulate by the active:

- **Antioxidant activity** by the **over-expression of antioxidant defenses**
- **Anti-aging activity** though the capacity to **protect** the skin against **matrix degradation**

In this part we demonstrated the efficacy of PrimalHyal™ Ultrafiller on antioxidant and antipollutant defense through:
At *in vitro* level:

- Increase of **TRXR1 expression** from NHEK **up to 35%** in basal condition and under oxidative stress.
- That is correlated to efficient decrease of **ROS production** with **-30%** under oxidative stress.

At *ex vivo* level:

- An increase of **sequestosome p62 expression** on basal condition **+17%** and under photo-pollution exposure with **+40%.**
- That is correlated to a significant decrease of **MDA production -29%** confirming the anti-photopollution efficacy thank to the increase of the antioxidant defence as a **"vaccine like" effect**.

At clinical level:

- Significant decrease **of LPO production** after UV exposure with **-7%.**
- Correlated to an increase of **global antioxidant power of the skin** as observed by the **FRAP** measurement with **+20%.**

In the second part, we demonstrated the efficacy of PrimalHyal™ Ultrafiller on anti-aging activity through an efficient matrix protection.
We evidenced at the *in vitro* and *ex vivo* level:

- Significant inhibition of **MMP-1 (-40%)** et **MMP-3 (-37%)** on **normal human fibroblasts**
- These results were confirmed using **chronological aging** *ex vivo* model showing a significant inhibition of **MMP-1 (-18%)** and **MMP-9 (-27%)** expression at dermis level.
- Under **intrinsic aging** mediated by photo-pollution we observed an inhibition of :
  - **MMP-1 with -32% and – 33% in the epidermis and dermis** level respectively
  - **MMP-9 with -19% and -203% in the epidermis and dermis** level respectively
- These results were correlated to a significant **protection of matrix** as observed by the de-crease of **type I collagen degradation (-42%)**

At the clinical level:
We demonstrated **a flash anti-aging efficacy** of the product **at 0.2%** on **crow's feet and nasogenian** areas.

- On the **crow's feet** area :
  - After **1h**: significant decrease of **average lenghts of furrows** (0-0.5 mm) **-8.7%**
  - After **6h**: significant reduction of **number of wrinkles** (0.5-2mm) **-26.5%** and the **total wrinkles -6%.**

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- On the **nasogenian** area :
  - After **6h**: a significant decrease of **coarse area -8%.**

After **1 month of application at 0.1%** we observed on the **nasogenian area**:

- A significant reduction of **wrinkles number (-9%), total wrinkles area (-12%) and the medium lines (-13%).**

After **2 months of application at 0.1%** we observed on the full face:

- A significant improvement of **skin texture 9%.**

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