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# Safety and efficacy of a carboxymethyl chitosan dermal injection device for the treatment of skin defects: a first-in-man, pilot, comparative, split-body study

*Background:* Injectable soft-tissue devices are increasingly used for improving skin defects and deficiencies related to ageing. *Objectives:* To assess the safety and efficacy of KIO015, a new injectable soft-tissue device formulated with carboxymethyl chitosan for the intradermal treatment of skin defects associated with ageing. *Materials & Methods:* Twenty-two subjects (40-65 years) were randomized to receive injections in the neckline of KIO015 and a non-cross-linked HA-based device, and were followed for up to 10 months. Injection site reactions (ISRs) and adverse events (AEs) were documented. Skin improvement was assessed instrumentally and clinically. Skin biopsies at injection zones in the lower back were taken at Day 28 for histopathology and immunohistochemistry analyses, to further assess product performance. Histomorphometric analyses on rabbits and *in vitro* assessment of KIO015 antioxidant capacity were also conducted. *Results:* KIO015 was very well tolerated. Only expected and transient ISRs were observed; mainly erythema and hematoma. No adverse local effects or foreign body granuloma were observed histologically. Both clinical and instrumental evaluations confirmed the performance of KIO015. The skin was firmer and more elastic. Skin hydration showed significant improvement three days after injection. KIO015 exhibited superior overall maintenance of skin hydration after 10 months as compared to HA. These clinical results were supported by *in vitro* trials and implantation tests in the rabbit. *Conclusion:* The results from this pilot study support the use of KIO015 as an innovative alternative to HA-based devices for intradermal treatment of skin disorders

**Key words:** ageing skin, carboxymethyl chitosan, injectable soft-tissue device, non-animal origin

Skin ageing is a multifactorial process involving the synergistic effects of intrinsic and extrinsic factors that cause structural and functional deterioration of the skin [1]. Intrinsic aging is due to genetic background, whereas extrinsic aging is attributed to the skin exposome, among which exposure to sun, pollution, and tobacco, are known to be the main triggers [2]. The major biological factors influencing skin structural damage are loss of dermal proteins (collagen, elastin), hyaluronic acid (HA) and proteoglycans, atrophy and displacement of subcutaneous fat, as well as oxidative stress, resulting in a decrease in volume and elasticity and increase in skin dryness [3].
Soft tissue injection devices (skin rejuvenation products, skin boosters) are increasingly used for improving defects and deficiencies related to ageing skin as they provide the desired aesthetic outcomes with minimal invasiveness [4]. These viscous biomaterials show hydrating capabilities and provide a temporary scaffolding effect for dermal tissue reconstruction through mechanical stimulation of fibroblasts. Therefore, they allow the restoration of a hydrated, supple, and dense skin [5]. Natural polysaccharides are particularly interesting for designing new intradermal devices since they represent structural analogues of living tissues (*i.e.* the glycosaminoglycans [GAGs]) [6]. The majority of commercially available injectable skin rejuvenation products are formulated with linear HA, and the skin boosters are formulated with cross-linked HA. Chitosan, in particular, is one of the most abundant polysaccharides (β-(1-4)-linked D glucosamine and N-acetyl-D-glucosamine) and can be extracted from various animal or non-animal sources [7]. Its derivative, carboxymethyl chitosan (CM-chitosan), is soluble at physiological pH and possesses many advantages over other polymers, especially nontoxicity [8-12], biocompatibility [13, 14], immunocompatibility, biodegradability [15-18], and antioxidant (namely free radical scavenging) capacity [19-23]. Chitosan and its derivatives have been extensively investigated for many diverse medical and cosmetic applications, including wound dressings, oral hygiene, hair care, contact lenses, drug delivery, and tissue engineering [24, 25].

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All these findings have supported the development of a new CM-chitosan-based soft tissue injection device with enhanced performance, persistence, and tolerance over other available polymers. When formulated as a viscous biomaterial and injected into the dermis, CM-chitosan is expected to exhibit hydrating capabilities, mechanical scaffolding effects, and potent antioxidant capacity, and to resorb in the dermal tissues without adverse effects.
We conducted a first-in-man pilot study to evaluate the safety and performance of a new injectable soft-tissue device, formulated with non-cross-linked CM-chitosan extracted from the edible white mushroom *Agaricus bisporus,* for the intradermal treatment of skin conditions associated with ageing.

# Materials and methods

## Ethical approval

The study was reviewed and approved by an Independent Ethics Committee (EC), on November 15, 2018 (CPP Ile-de-France X, Aulnay-sous-Bois, France). The investigation was also approved by the French competent authority (ANSM) on October 19, 2018. Informed consent was obtained from all subjects before the study procedures were conducted.

## Interventions

The investigational product KIO015 (KiOmed Pharma) is composed of 20 mg/mL CM-chitosan, 3.5% sorbitol and pH 7.2 phosphate buffer provided in a pre-filled 1-mL syringe for intradermal injection.
Commercially available Teosyal® Meso (Teoxane), composed of 15 mg/mL HA, was used in the study as a comparator and was also provided in a 1-mL syringe. For convenience, Teosyal® Meso will be referred to as HA throughout the article.
Both products were injected using a TSK Steriject needle (30G 0.3 × 13 mm) into the superficial dermis, according to the micro-papular injection technique to standardize the dosing. On each 9-cm$^2$ zone (3-cm square grid) of about 20 papules, around 25 µL was injected resulting in 0.5 mL of product per zone.

## Study design

This study was a single-centre, split-body, open study comparing the safety and efficacy of KIO015 versus HA for the correction of skin defects associated with ageing.
At the time of the screening, subjects who matched the eligibility criteria were selected. The subjects ($n = 22$) were randomized to receive two injections in the neckline, on Day (D) 84 and D112, of KIO015 and HA in two adjacent zones. The use of injectable lidocaine or any other local anaesthetic agent was prohibited during all injection procedures. For all procedures performed in the neckline, the untreated zone was positioned in the centre whereas the injection sites were randomized on the left/right sides.
Additionally, subjects in both groups had an injection in the lower back at the initial visit (D0) for histological analysis on D28: in the KIO015 group ($n = 11$), subjects received a single injection of KIO015 and an adjacent untreated zone was defined as a control, while subjects in the comparator group ($n = 11$) received injections of HA and KIO015 in two adjacent zones (one product per zone).
All the subjects were followed for 10 months in order to evaluate the safety and efficacy of the products.

## Study population

Twenty-two healthy subjects, aged between 40 and 65 years, with signs of cutaneous ageing and dry skin in the neckline (< 50 A.U with Corneometer®) were enrolled in the study.
Major exclusion criteria included: cutaneous inflammatory or infectious processes; severe, ongoing and uncontrolled diseases (*e.g*., depression, malignancy or history of malignancy, HIV, auto-immune diseases); any skin or systemic disease (acute and/or chronic) within 12 months; predisposition or known allergy to the device's components or the treatment; known severe allergies manifested by a history of anaphylaxis, or history or presence of severe multiple allergies; hypersensitivity, keloid or hypertrophic scarring; active dermal response (*e.g*., filling product injections, laser or chemical peeling procedures) within 12 months; intensive exposure to sunlight or UV rays within the last four weeks; use of topical agents (*e.g*., corticoids) within the last four weeks; anticoagulant or antiplatelet therapy within the last four weeks; systemic treatment (*e.g*. anti-inflammatory medication, immunosuppressive therapy and/or corticoids, retinoids); pregnancy or breast-feeding; and known alcohol or drug abuse.

## Safety assessments outcomes

### Injection site reactions and adverse events

The investigator examined the injection sites on the lower back and the neckline for side effects (erythema, pain, induration, swelling, lumps, hematoma, itching, and pigmentation) immediately and 3, 14 and 28 days after each injection session, as well as 3 and 6 months after the last injection. The investigator rated the local tolerance with a 4-point numerical rating scale (0 = none, 1 = mild, 2 = moderate, 3 = severe). The subjects completed a diary using the same scoring to evaluate the injection and this was used as a support by the investigator to report adverse events (AEs) and adverse device effects (ADEs), as well as their severity and relation to the investigational products.

### Histopathological analyses

Histological analysis of skin biopsies in the lower back was assessed as a secondary safety endpoint of the study. On D28, all the subjects had two 4-mm punch biopsies of 3 × 3-cm areas taken in the lower back on the sites where injections had been performed on D0. For the subjects in the KIO015 group ($n = 11$), a second biopsy was taken from an untreated skin area in each subject as control, while for the subjects in the comparator group ($n = 11$), two distinct biopsies were taken from the zone injected with HA and the zone injected with KIO015. The skin samples were fixed in formalin and sent to an independent laboratory (GREDECO) for blinded safety and efficacy analyses. The biopsies were analysed with specific stains according to in-house standard

operating procedures, and analyses of inflammatory reactions, foreign body reactions, and other local tissue effects were conducted (*supplementary table 1*).

## Assessment of efficacy

Efficacy outcome was considered as a secondary endpoint of the study and was assessed at baseline and 3, 14 and 28 days after each injection in the neckline and three and six months after the last injection (M7, M10). Outcomes were compared to HA and an untreated zone in all the subjects.

### Skin properties

Skin properties changes from baseline were instrumentally documented. Skin hydration on the epidermis (stratum corneum) and the dermis were measured with the Corneometer® CM 825 (Courage & Khazaka electronic GmbH; Köln, Germany) and the MoistureMeter®-D +S15 probe (Delfin Technologies Ltd; Kuopio, Finland), respectively.

Skin firmness and elasticity were assessed with the Cutometer® MPA 580 (Courage & Khazaka electronic GmbH; Köln, Germany) based on the calculation of {Ue, Uf} and {Ua, Ur/Ue} parameters, respectively.

### Clinical evaluation

The investigator scored skin dryness in the neckline on a scale from 0 (normal skin) to 4 (extremely dry skin). The subjects' skin improvement in the neckline was also evaluated clinically at the follow-up visits by the investigator based on the 5-point Global Aesthetic Improvement Scale (GAIS), which was scored as 1 = very much improved, 2 = much improved, 3 = improved, 4 = no change, and 5 = worse.

### Immunohistochemistry

The anti-aging effect of the products was evaluated at different skin depths according to the above-mentioned procedure by a dermatopathologist based on the following parameters: dermal neocollagenesis, dermal thickness, elastic fibres, epithelial proliferation, epidermal hydration, and collagen IV synthesis (*supplementary table 1*).

### Subjective evaluation

The investigator was asked to rate his/her level of satisfaction with the injection procedure after each injection and for each subject.

## Statistical analysis

Analysis of the safety endpoints of the clinical study was performed on the safety population, which included any subject who used the tested device. Analyses of the performance parameters were performed on the Full Analysis Set (FAS) which corresponded to any subject included in the study with at least one post-baseline value.

Adapted descriptive statistics were used to summarise quantitative data by visit point for each investigational product. A mixed ANOVA model for repeated measures (fixed factors: product, time, and product by time interaction) was fitted to raw data. An unstructured variance-covariance matrix was used to take into account the correlation between data obtained from the same subject. From this model, adjusted means (*LS-Means statement*) were used to assess the changes from baseline and perform comparisons between products. To judge the model validity, the underlying assumptions (residual normality and homoscedasticity) were analysed with a graphical representation of residuals distribution (scatter plots, histogram) and a Shapiro-Wilk test was also reported.

For comparisons that included M7 and M10, paired t-tests were carried out. The ANOVA model used for intermediate analysis was not repeated for consistency of data already analysed.

A McNemar's test was performed for GAIS analysis and a Wilcoxon signed-rank test was used for assessment of skin dryness.

All statistical tests were assessed at a 5% level of significance using a bilateral approach.

## In vitro *and* ex vivo *analyses*

Additional *in vitro* and *ex vivo* analyses were conducted separately from the study to further assess product performance.

### Radical scavenging activity

The antioxidant capacity of the investigational product, KIO015, was quantified according to the ABTS assay [26]. The commercial hydrogels, HA (Teosyal® Meso; Teoxane), A (Stylage® Hydro, Vivacy), and B (Belotero® Hydro, Merz Pharma), were used as comparators at a concentration of 4 mg/mL, while ascorbic acid at different concentrations was used as a positive control. The standard procedure consisted of determining the 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) equivalent antioxidant capacity (TEAC) after incubating the product samples in a 2,2′ Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) radical solution for one hour at room temperature. ABTS radicals were produced by mixing the ABTS stock solution with potassium persulfate and incubating the mixture in the dark at room temperature for 12-16 hours. The absorption decrease at 734 nm was monitored in time. All measurements were performed with a microplate reader Infinite M200 (Tecan) and all reagents were provided by Sigma.

In addition, the free radical scavenging protective effect of CM-chitosan on oxidative degradation of hyaluronan was evaluated by gel permeation chromatography with refractive index detector (GPC-RID) which measures the molecular weight (Mw expressed as Mpeak) of hyaluronan at 7 mg/mL at T0, after four hours and after 24 hours in the presence of 3% $H_2O_2$. The hyaluronan test sample was mixed with CM-chitosan and compared to the hyaluronan test sample alone at the two time periods. As no CM-chitosan standard was commercially available, the calibration was based on the injection of seven GPC standards of monomodal dextran with molecular weights (Mn, Mw, Mp) ranging from 5 kDa to 670 kDa. The raw data were processed using Agilent GPC Data Analysis software.

### Evaluation of histomorphology

A histological analysis was conducted on rabbits (New Zealand White; 2.9-3.4 kg) to determine dermal collagen

content following injection of KIO015 or a comparator B (Belotero® Hydro, Merz Pharma). A saline solution was also injected as a negative control. The rabbits were euthanized at 1, 4, and 12 weeks after injection and the injection sites of the devices were biopsied. A total of 10 samples per product injected and per observation time were taken (except for KIO015 after 12 weeks in which nine samples were taken). The collagen content was also measured in the untreated dermis in the negative control group biopsies at one week, in a remote area from the saline solution injection zone.
The biopsies were sent to an independent laboratory (GREDECO) for morphometric analysis of collagen content. The skin samples were stained with sirius red (*picrosirius red*) and the collagen content was quantified by computer-assisted image analysis (QImaging camera with Image Pro Plus software, lens 40). The surface area occupied by collagen was measured in the dermis at a depth between 1,300 and 1,800 μm outside the pilosebaceous unit and above the muscle fascicles.

# Results

## Subjects

A total of 39 subjects were screened, of whom 22 subjects (19 females and three males) were randomized to receive the investigational products and were assessed for safety (safety population) and efficacy (full analysis set [FAS]) (*figure 1*). Two patients prematurely dropped out of the study. One subject dropped out on D84 (before measurement and injection in the neckline) due to severe redness and telangiectasia on the neckline and was therefore excluded from the FAS population for assessment of efficacy outcome on the neckline. Another subject withdrew from the study on D112 for personal reasons. The two subjects were included in the safety analyses.
The patient ages ranged from 43 to 65 years (mean: 54.3 years), and all patients were Caucasian, had skin Phototype I-III, dry skin in the neckline (< 50 A.U with Corneometer®) and signs of cutaneous aging, including: fineness of the skin, laxity, roughness, spots, dryness, lack of elasticity/firmness /suppleness or wrinkles (*supplementary table 2*). No previous or ongoing treatment prevented subject inclusion or product injection. Injected volumes were similar between the two products.

## Safety outcome

**Injection site reactions and adverse events**
Both KIO015 and HA products induced temporary local reactions immediately after the first injection (D84) in the neckline (*supplementary table 3*). All the subjects experienced mild lumps and induration reactions after injection, regardless of the product. Erythema was observed after injection in more than 85% of the subjects with both products, while a few subjects had pain (24% with KIO015 and 5% with HA) and hematoma (10% with KIO015 and 24% with HA). Induration reactions in one subject in the comparator group persisted for three days after injection (D87). No ISRs were observed by the investigator 14 days and 28 days after the first injection in the neckline (D98 and D112, respectively), regardless of the injected product.
Similarly, all the subjects experienced lumps and induration reactions immediately after the second injection (D112) in the neckline with both KIO015 and HA. In total, 35% of the subjects had erythema with both products, and 20% had hematomas with HA. Three days after the second injection in the neckline (D115), hematoma appeared in 25% of the subjects in the KIO015 group and 45% of the subjects in the HA group, and persisted up to 14 days in one subject in the HA group. Only one subject (5%) in the HA group still experienced lumps three days after the second injection and two subjects, one in each group, had itching. No ISRs were observed at follow-up visits (D140, M7, M10) after the second injection on the neckline, regardless of the injected product. All reactions were mild in intensity, except in one subject who experienced moderate redness/erythema on the neckline immediately after the first injection of KIO015 (D84). Similar results were observed in the lower back injection zone in both groups.
ADEs were experienced by most of the subjects (KIO015: 38 ADEs in 86.4% of the subjects; HA: 51 ADEs in 81.8% of the subjects). All reported ADEs were expected ISRs of mild intensity and mainly included hematoma (KIO015: 52.6% of ADEs vs HA: 49.0% of ADEs) and erythema (KIO015: 10.5% of ADEs vs HA: 5.9% of ADEs) or redness (KIO015: 13.2% of ADEs vs HA: 11.8% of ADEs) at injection points.
The frequency and type of AEs were consistent with the population and study period. In total, 68 AEs were experienced by 22 subjects. Forty-two AEs were skin hyperpigmentation (90.9%) or skin discolouration (4.5%) issues related to the skin biopsies in the lower back and resolved spontaneously. The other AEs were related to nervous system disorders (16 AEs: headache 13.6%, migraine 9.1%, and neuralgia 4.5%), infections and infestations (five AEs: cold 13.6%, dental abscess 4.5%, pharyngitis 4.5%), gastrointestinal disorders (one AE: tooth pain 4.5%) and musculoskeletal and connective tissue disorders (three AEs: cervicalgia 9.1%, tendinitis 4.5%). Most of the AEs were mild (only one AE was of moderate-intensity) and did not require corrective treatment (61.8% of AEs). No serious adverse events (SAEs) were reported. There was no patient withdrawal related to the safety of the study device and no follow-up was necessary after the end of the study.

**Histopathology results**
Both KI0015 and HA were well tolerated. Only sparse lymphocytes without a significant inflammatory islet were observed on the stained slides, but were also observed in untreated zones in two subjects (data not shown). There was no statistically significant difference between the two groups with regards to the number of lymphocytes.
Haemorrhagic suffusions in the dermis and the dermo-hypodermic junction were observed in some subjects corresponding to the hematomas caused by the punch biopsy (data not shown). There was no correlation between the presence of lymphocytes or haemorrhagic suffusions and clinical symptoms. No foreign body granulomas were observed.

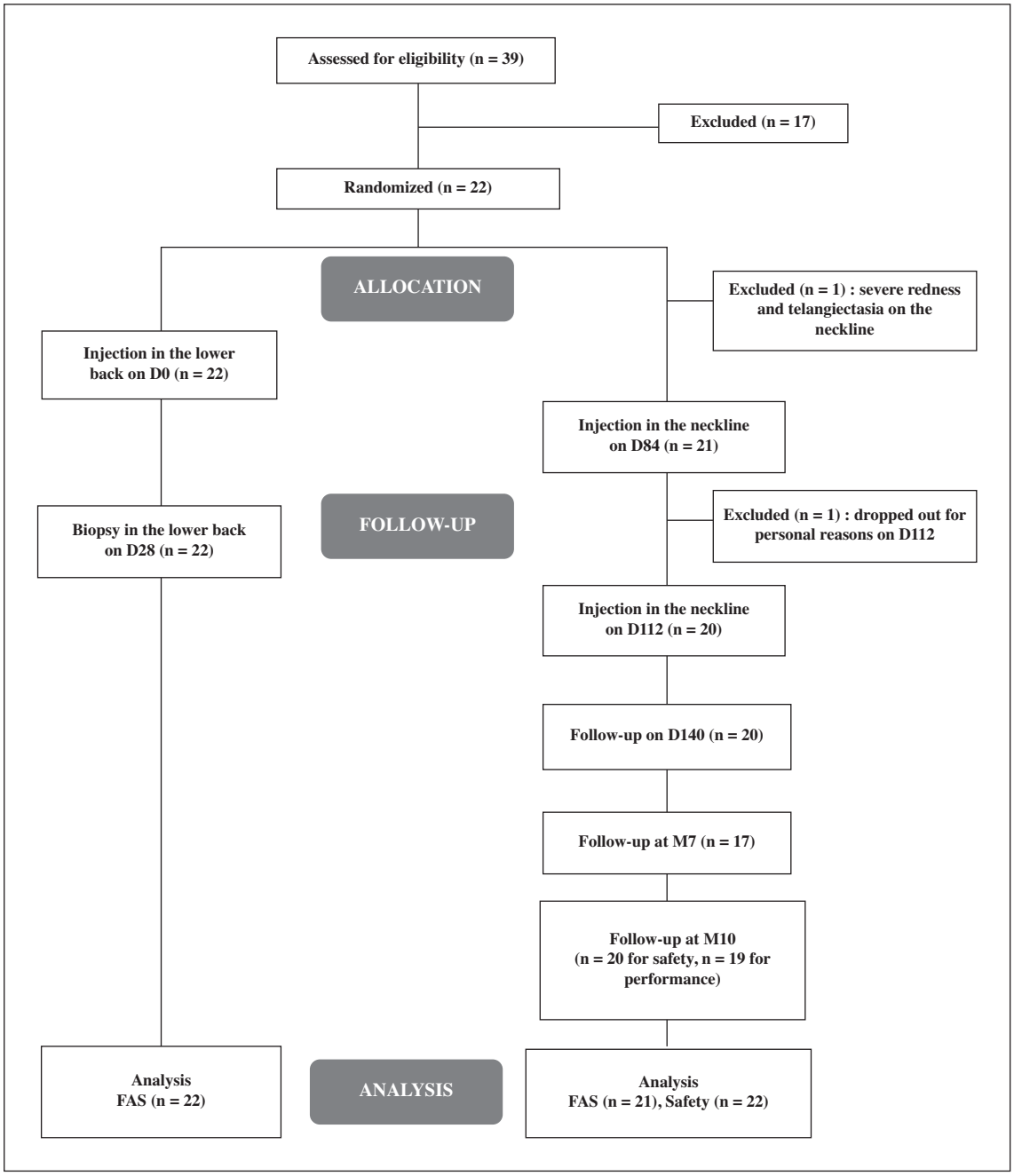


**Figure 1.** Study flow chart.
FAS: Full analysis set

## Efficacy outcomes

### Instrumental measurements of skin properties

**Skin hydration.** The mean baseline values measured by the Corneometer® and the Moisturemeter® on the neckline were similar for the two groups but lower than that for the untreated zone, which is most probably related to the difference in area between the treated and untreated zones. Change from baseline over time - taking into account the variation on the untreated zone - in the epidermis (*stratum corneum*) and dermal hydration following injection of the two products is presented in *figure 2*.

At the epidermal level (Corneometer® measurements), a significant increase in epidermal hydration rate, compared to baseline, was observed with KIO015 at each time point following injections and was sustained up to D126. There was no statistically significant difference between the two products with regards to the epidermal hydration rate, except on D112 and at M10, when the change in hydration from baseline (D84) was significantly higher with KIO015 as compared to HA, suggesting a greater persistence of epidermal hydration with KIO015. It should be mentioned that the hydration rate slightly, but significantly, increased in the untreated zone from D115 to M7 (data not shown), although

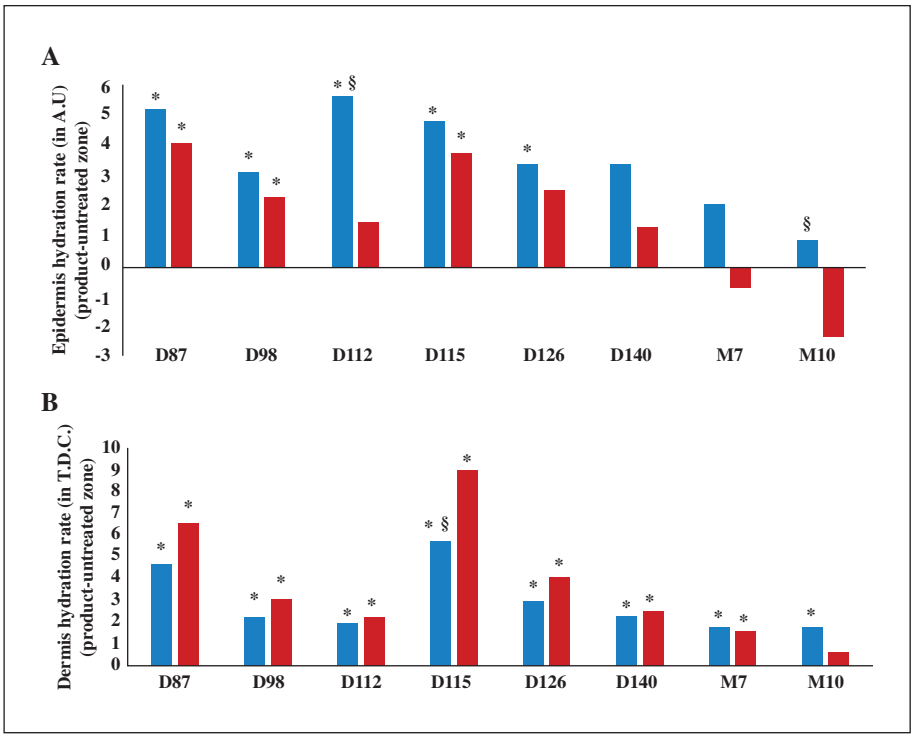


**Figure 2.** Change in epidermal (**A**) and dermal (**B**) hydration from baseline. The subjects were randomized to receive two injections in the neckline on D84 and D112 with KIO015 and HA. The Corneometer® and the Moisturemeter® were used for measurements of epidermal (*stratum corneum*) and dermal hydration, respectively. Blue: KIO015; red: HA, AU: arbitrary unit, TDC: tissue dielectric constant.
* $p < 0.05$ change from baseline at each time point relative to untreated zone.
§ $p < 0.05$ change from baseline at each time point; comparison between KIO015 and HA.

at a much lower magnitude when compared to KIO015. This variation observed in the untreated zone could be explained by the changes in environmental conditions over time which can greatly impact the epidermal hydration rate.

At the dermal level (Moisturemeter® measurements), a significant improvement in hydration was observed and persisted up to M10 with KIO015 and up to M7 with HA. There was no statistically significant difference between the two products except on D115 (three days after the second injection), when the change in dermal hydration was higher with HA. The hydration rate remained stable in the untreated zone, regardless of the time point.

**Skin elasticity and firmness.** As observed with skin hydration measurements, baseline values of skin biomechanical parameters measured by the Cutometer® were similar between the two injected zones but different from the untreated zone (*supplementary figure 1*). Variation in skin firmness parameters Ue and Uf after injection was not statistically significant, except at three days after the second injection (D115) of HA. Ua values, corresponding to skin total retraction, significantly decreased with both products three days after the second injection (on D115: $0.097 \pm 0.042$, $p = 0.0245$ with KIO015 vs $-0.099 \pm 0.040$, $p = 0.0183$ with HA). No significant difference was observed between KIO015 and the comparator with regards to Ue, Uf, and Ua values. Skin net elasticity, as measured by Ur/Ue ratio, significantly increased with KIO015 as compared to HA three days after the first injection (on D87: $+0.025 \pm 0.024$ with KIO015 vs $-0.041 \pm 0.025$ with HA).

**Clinical evaluation**

Skin dryness on all injected and untreated zones was graded by the investigator at each time point (*supplementary figure 2*). Similar mean baselines values were reported for the two injected zones and the untreated zone corresponding to slightly dry skin (score = 1). Both products resulted in a significant decrease in skin dryness three days after the first and second injection, respectively. Compared to the untreated zone, improvement in skin dryness was statistically significant only with HA, three and 14 days after the first injection. At M7, the dryness score for both products was significantly higher than that for the untreated zone, which was due to the increase in moisture measured at the *stratum corneum* level with the Corneometer® on the untreated zone. No statistical difference was observed between the two products.

GAIS scores for each product presented in *figure 3* showed a clinical improvement in the majority of the subjects with both products. No statistical difference was observed between the two products, regardless of the time point.

**Subjective evaluation**

The investigator was asked to rate the level of satisfaction with the injection procedure after each injection and for each subject. The injector was generally satisfied with both products with regards to immediate results, results after the massage, ease of injection, and ease of product

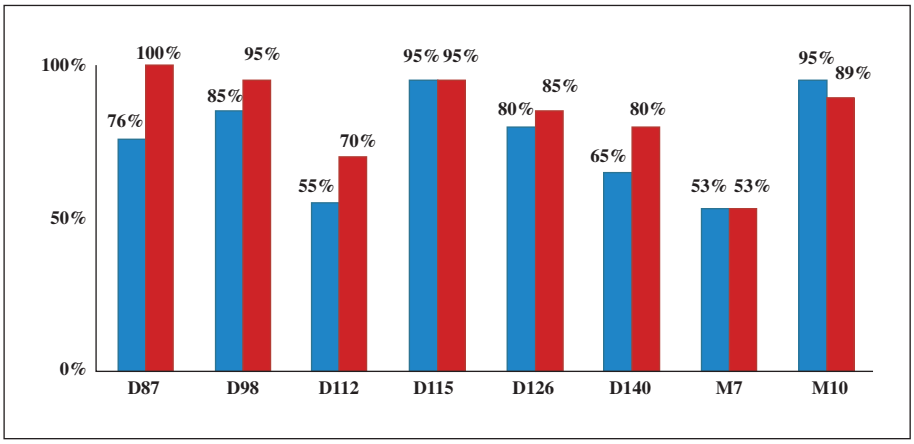


**Figure 3.** Proportion of subjects with aesthetic improvement (GAIS). Improvement of subjects' skin on the neckline was evaluated using the Global Aesthetic Improvement Scale (GAIS) and scored as 1 = very much improved, 2 = much improved, 3 = improved, 4 = no change, 5 = worse. Only percentages of subjects with improvement are presented (score: 1, 2 or 3). Blue: KIO015; red: HA.

positioning (100% "satisfied" or "very satisfied" answers with both products).

**Immunohistochemistry of skin biopsies**

Trends above 5% are discussed in the paper. The injection of KIO015 increased the number of mitotic cells in the basal layer by 5.3% ($p = 0.603$) compared to untreated skin. The injection of KIO015 also increased CD44 expression in the epidermis by 5.8% ($p = 0.401$) and collagen IV expression at the dermal-epidermal junction by 18% ($p = 0.216$), compared to the area injected with HA (*supplementary figure 3*). No difference was observed in type I and III collagen levels after injection of KIO015 compared to untreated skin and HA-injected skin.

***In vitro* radical scavenging activity.** The free radical scavenging capacity of all tested products was calculated based on a calibration curve and expressed as percent of ascorbic acid antioxidant activity. Results displayed in *figure 4* show that KIO015 was associated with the highest free radical scavenging capacity, with almost 80% ascorbic acid antioxidant activity. All comparators showed less than 50% ascorbic acid antioxidant activity.

In addition, the protective effect of CM-chitosan on hyaluronan compared with hyaluronan alone was examined *in vitro,* in which hydrogen peroxide was added to induce oxidative stress. Protection of hyaluronan was greatest with the preparation containing CM-chitosan after four hours (100% ± 1% vs 92% ± 1%) and after 24 hours (93% ± 1% vs 79% ± 6%) (*figure 5*).

## Histomorphometry in rabbits

One week (1w) after injection, the surface occupied by collagen in the deep dermis was on average 58.3% ± 4.9% (median: 60.0%) for the KIO015 group and 44.6% ± 5.3% (median: 44.5%) for the comparator group, as compared to the untreated zone (*supplementary figure 4*). Empty spaces, attributed to the presence of the polysaccharide-based injected products, were visible between the collagen bundles of the dermis. Four weeks after injection (4w), KIO015 led to an 8.8% increase ($p = 0.028$) in the surface occupied by collagen in the deep dermis vs 11.6% ($p = 0.011$) with the comparator, as compared to the untreated zone. The quantity of collagen in the dermis was preserved up to 12 weeks after injection (8.6% with KIO015, $p = 0.041$ vs 9.5% with the comparator, $p = 0.018$). No statistically significant difference was observed between the two products in terms of collagen synthesis at all time-points.

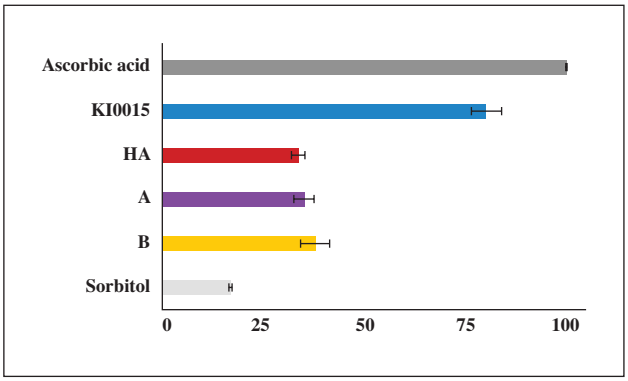


**Figure 4.** Free radical scavenging capacity of the tested injectable soft-tissue devices. Ascorbic acid (0.02 mg/mL) was used as a positive control. Free radical scavenging activity is expressed as percent of ascorbic acid antioxidant activity. HA: Teosyal® Meso (Teoxane), 4 mg/mL; A: Stylage® Hydro (Vivacy), 4 mg/mL; B: (Belotero® Hydro (Merz Pharma), 4 mg/mL.
*$p < 0.05$, comparison with ascorbic acid; § $p < 0.05$, comparison with KIO015.

# Discussion

In the present study, KIO015, injected in the neckline and the lower back of 22 healthy subjects, was very well tolerated, as observed following clinical and histological evaluations. Clinically, only usual and expected ISRs were

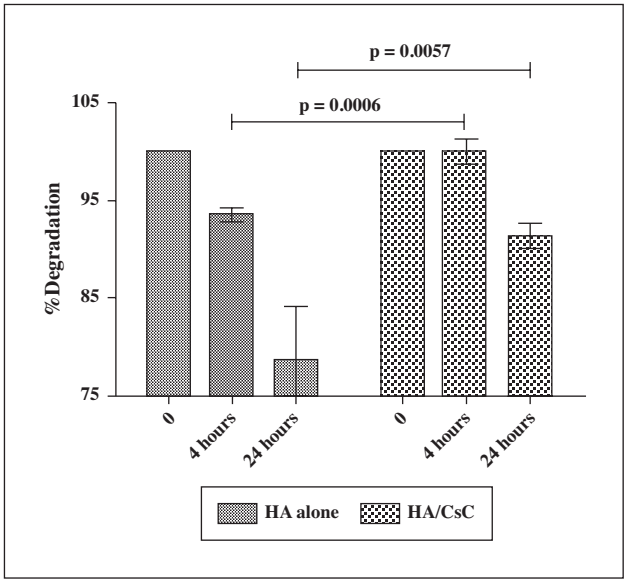


**Figure 5.** Antioxidant protection of hyaluronan.

observed, mainly erythema immediately after injection and bruising/hematoma. All ISRs were transient and resolved within 14 days after the injection. No adverse local effects or foreign body granuloma were observed histologically. The tolerance of KIO015 was similar to that of HA, with less reported ISRs and ADEs.

Both clinical and instrumental evaluations were concordant in defining the efficacy of KIO015. All the combined effects associated with the injected product, including hydration measurement and GAIS evaluation, were sustained at least for the whole study period of seven months. Skin hydration showed significant improvement three days after injection. KIO015 effects on skin hydration were comparable to that induced by the comparator HA. Six months after the last injection of KIO015, a mild but significant improvement was still observed in the dermis. For epidermal hydration, a significant difference was observed between KIO015 and HA at M10, likely related to a visible decrease in epidermal hydration with HA in the long-term, which was not observed with KIO015. KIO015 sustained epidermal and dermal hydration significantly better than HA after 10 months, suggesting superior overall maintenance of skin hydration and therefore longer-lasting effects.

The structure of the CM-chitosan backbone is similar to that of non-cross-linked HA and is characterized by the presence of numerous hydroxyl and carboxyl groups known for their hygroscopic and water retention capabilities [27, 28]. Therefore, like HA, CM-chitosan plays an important role in the hydration of the dermal extracellular space due to its ability to attract water molecules, which in turn creates appropriate physiological conditions for fibroblasts in the dermal extracellular matrix [29]. Furthermore, the formulation is also composed of ∼95% highly purified water and ∼3.5% sorbitol, which is also used with its skin moisturizing properties in several HA-based soft-tissue devices.

The instrumental measurements also highlighted immediate significant changes in skin properties three days after injection; the skin was firmer and more elastic (decrease in Ue, Uf and Ua and increase in Ur/Ue). The effects on skin elasticity were significantly higher with KIO015 than with the HA-based comparator.

Biopsy analysis performed 28 days after injection of KIO015 and HA in the lower back of the subjects did not show any significant difference, compared to untreated skin, for either product nor between the two products with regards to the number of mitotic cells in the basal layer (vs untreated skin, $p = 0.603$), CD44 expression in the epidermis (vs HA, $p = 0.401$), as well as collagen IV expression at the dermal-epidermal junction (vs HA, $p = 0.216$). This could be due to methodological considerations (*e.g.* the time point of 28 days after injection, biopsy size) and underpowered sample size (11 subjects for comparison with HA Teosyal® Meso and an untreated zone).

In the current study, KIO015 was found to be almost as effective as the positive control (ascorbic acid) in reducing free radical concentration. In addition, hyaluronan was found to be less rapidly degraded when mixed with CM-chitosan under oxidative stress, due to the intrinsic protective capacity of CM-chitosan to resist free radical oxidation. As the formation of free radicals -for instance reactive oxygen species- has emerged as a major factor for dermal tissue alteration, the use of intradermal treatments with effective antioxidant and free radical scavenging capacity is increasingly considered to treat structural skin changes [30]. Intradermal injection of CM-chitosan may, therefore, provide enhanced protection against oxidative stress in the skin, as compared to HA

The intended action of KIO015 is achieved through a viscosity-based temporary mechanical scaffolding effect in the dermal tissue. From rheological bench testing, the biomaterial formulation is a viscous fluid, soluble in physiological conditions. It exhibits the rheological behaviour of a non-Newtonian biomaterial, similar to the behaviour of non-cross-linked HA-based injectable soft-tissue devices (data not published). Upon intradermal injection, the device creates volume within dermal tissue, and exhibits temporary scaffold and tissue remodelling (tissue reconstruction), integrated progressively with gradual resorption without adverse effects. As a result of tissue scaffolding and mechanical tension on surrounding fibroblasts as well as gentle integration into dermal tissues, the dermal tissue may produce new collagen fibres, as shown for hyaluronan [31]. In the present study, the ability of the dermal tissue to produce new collagen fibres was evaluated in rabbits. Compared to the untreated dermis, the total collagen content in the dermis was increased by 9% at four weeks ($p = 0.028$) after intradermal injection of KIO015–presumably as a result of its tissue scaffolding effect, mechanical tension on surrounding fibroblasts, and gentle integration into dermal tissues. Moreover, the quantity of collagen in the dermis was preserved up to 12 weeks (+9%, $p = 0.041$, compared to the untreated dermis) after injection (at the last follow-up time point), showing that the extra collagen produced remains in the skin. The space observed between the collagen bundles due to the presence of the injected products (temporary scaffold effect) disappeared after four weeks, most probably due to product resorption and mechanical stimulation of fibroblasts.

Degradable biomaterials are preferred candidates for developing dermal injection devices in order to prevent unwanted long-term reactions. The bioresorption rate of KIO015 was evaluated *in vitro* and *in vivo* in preclinical studies (data not shown). Consistent with the conclusions of Dong *et*

*al*. 2010 and 2012 [15, 32], Chang *et al*. 2008 [13] and Liu *et al*. 2016 [16], KIO015 was found to be susceptible to enzymatic degradation and biodegradable *in vivo* with no clinical or histopathological signs of toxicity or local adverse effects, and complete resorption after four to 12 weeks.

The main limitation of the study pertains to the limited sample size which likely did not provide appropriate power for hypothesis testing for a number of the study assessments, such as analyses of subjects' biopsies. The present study was a pilot study and the promising results warrant larger clinical trials with KIO015. Despite this limitation, we believe that the combined results from instrumental, clinical, and histological evaluation provide a strong foundation to support the safety and efficacy of KIO015 as intradermal treatment for skin conditions associated with ageing.

## Conclusion

These results support the use of KIO015, a non-animal, non-cross-linked, CM-chitosan-based injectable soft-tissue device, as an innovative alternative to HA-based dermal devices with similar or superior characteristics based on *in vitro* and *in vivo* assessment, for intradermal treatment of skin disorders. ■

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## Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1684/ejd.2021.4091.

Table S1. Tissue sample investigations based on biopsies.

Table S2. Subject demographics and baseline characteristics.

Table S3. Injection site reactions (ISRs) in the neckline.

Figure S1. Mean Cutometer® values over time.

Figure S2. Skin dryness score over time.

Figure S3. Collagen IV expression in the epidermal-dermal junction in one subject.

Figure S4. Dermal collagen expression in rabbits.

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