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Biophysical and biological characterization of a new line of hyaluronan-based dermal fillers: A scientific rationale to specific clinical indications

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ABSTRACT

Chemico-physical and biological characterization of hyaluronan-based dermal fillers is of key importance to differentiate between numerous available products and to optimize their use. These studies on fillers are nowadays perceived as a reliable approach to predict their performance *in vivo*. The object of this paper is a recent line of hyaluronic acid (HA)-based dermal fillers, *Aliaxin*®, available in different formulations that claim a complete facial restoration. The aim of the study is to provide biophysical and biological data that may support the clinical indications and allow to predict performance possibly with respect to similar available products. *Aliaxin*® formulations were tested for their content in soluble HA, water uptake capacity, rheological behavior, stability to enzymatic degradation, and for *in vitro* capacity to stimulate extracellular matrix components production. The formulations were found to contain a low amount of soluble HA and were equivalent to each other regarding insoluble hydrogel concentration. The different crosslinking degree declared by the producer was consistent with the trend in water uptake capacity, rigidity, viscosity. No significant differences in stability to enzymatic hydrolysis were found. *In vitro* experiments, using a full thickness skin model, showed an increase in collagen production in the dermoepidermal junction.

Results support the claims of different clinical indications, the classification of products regarding *hydro-*, *lift-action* and the specifically suggested needle *gauge* for the delivery. The biological outcomes also support products effectiveness in skin structure restoration. These data predicted a better performance regarding hydro-action, tissue integration, clinical management during delivery, and a high durability of the aesthetic effect when compared to data on marketed similar products.

1. Introduction

In the field of aesthetic medicine, the demand for safe, minimally invasive, and effective soft tissue augmentation procedures is steadily growing, and the availability of hyaluronan-based dermal fillers on the market is in constant expansion [1,2]. HA-based dermal fillers are made of hydrogel microparticles made of crosslinked HA suspended in physiological or phosphate-buffered solution, with 1,4-butandioldiglycidyl ether (BDDE) as the most commonly used crosslinker [3–7]. Even though marketed gels have much in common, cumulative clinical experience has demonstrated that they differ in final clinical behavior in a significant way [2,8–10]. As a consequence, differently from the past, physicians do not currently use a single product to treat all facial areas but select different products for distinct purposes depending on their personal clinical experience.

More recently, the evaluation of biophysical features of marketed products has proven effective in supporting clinicians' choices [10] and then scientific efforts toward dermal fillers biophysical and biochemical characterization and studies on the correlation of these data to the clinical outcomes have increased [2,3,8–15]. These studies have found that different manufacturing procedures result in different HA crosslinking degree and hydrogel particle size. Such properties, together

Abbreviations: HA, Hyaluronic acid or hyaluronan; BDDE, 1,4-butandioldiglycidyl ether; A_{EV}, *Aliaxin*® EV–Essential Volume; A_{GP}, *Aliaxin*® GP–Global Performance; A_{FL}, *Aliaxin*® FL–Lips; A_{SR}, *Aliaxin*® SR–Shape and Restore; J_{VF}, Juvederm Volift® with lidocaine; R, Restylane ®; BTH, Bovine testicular hyaluronidase; PBS, Dulbecco's phosphate-buffered saline; SEC-TDA, Size exclusion chromatography–triple detector array; COLIV, Collagen IV; COLVII, Collagen VII; HAS1, Hyaluronic acid synthase 1; AQP3, Aquaporin 3.

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with gel concentration, are responsible for different rheological behavior, water uptake (swelling), and resistance to degradation, characteristics that affect clinical performance (e.g. injectability, spreadability after injection, appropriateness of application site, lift capacity, duration of the effect, etc.) [2,3,8–15].

It is then understandable that data on biophysical and biochemical properties of fillers can be a practitioner's valuable pillars to the optimal clinical use of available products.

The current trend in soft tissue augmentation procedures is towards products for a full-face treatment: the aim is not only to fill single wrinkles or individual defects but also to obtain a global face restoration, including tissue volume, harmonization, and hydration [16–18]. Global face restoration cannot be reached by using one type of filler only and, as a consequence, companies are launching lines of filler products, specifically designed for different areas and purposes (e.g. deep dermal, lips, etc.), for a full-face approach to rejuvenation. An example of these lines is represented by *Aliaxin*® products commercialized by IBSA that consist in a range of soft tissue fillers including four formulations: *Aliaxin*®EV–Essential Volume (A_{EV}), *Aliaxin*® GP–Global Performance (A_{GP}), *Aliaxin*® FL–Lips (A_{FL}), and *Aliaxin*® SR–Shape and Restore (A_{SR}).

Specifically, A_{EV} claims a high-volumizing capacity, indicated for use at the supraperiosteal layer and deep tissue, ideal for redefining facial contour, for correcting deficits following injuries, and for volume restoration [19]. A_{GP} is indicated for deep infiltrations, for the restoration of small volumes [20] and to fill deep folds and wrinkles. A_{FL} is indicated for lips enhancement [21]. A_{SR} is indicated for more superficial layer (middle dermis), with the aim to both restore shape and re-toning [22]. A_{SR} is registered as a combination of crosslinked and linear HA that claims to produce an excellent lifting on the cutaneous wrinkles along with a bio-restructuring effect through neocollagenesis stimulation and a hydrating anti-aging action [17].

The aim of this work was to apply current expertise in dermal filler characterization to this line of HA fillers to highlight differences between each other and to other marketed products and to predict relative clinical performance. In particular, among already commercialized products, *Restylane*, the first FDA approved dermal filler and, therefore, the one with the longest clinical history and the most extensive characterization, was selected for comparison [11,23]. A more recent product belonging to the *Juvederm* line of HA-based dermal fillers was also selected considering that *this brand* products lead, as well as Restylane, the market in HA fillers. [24].

A discussion of the expected effect of the investigated biophysical parameters on fillers clinical behavior is provided. Outcomes of the work were thought to be useful for the design of novel highly performing dermal fillers for specific needs in full-face treatment.

2. Experimental methods

2.1. Biophysical characterization

2.1.1. Materials

A_{EV}, A_{GP}, A_{FL}, and A_{SR} are produced by *Rose Pharma S.A.* (Lugano, Switzerland) and distributed by *IBSA Farmaceutici Italia S.R.L.* (Lodi,Italy). They are all BDDE-crosslinked HA hydrogels. The different products of the line are claimed to contain diverse combinations of HA molecular weights: 1000 and 2000 kDa for A_{EV} and A_{GP}, 500 and 1000 kDa for A_{FL}, and a combination of three molecular weights (500, 1000, and 2000 kDa) for A_{SR}. A low content of residue BDDE (<0.1 ppm) is also declared [17,25,26]. Available information on the products as reported on their package insert (i.e. total HA concentration, duration of the effect, needle size, hydro-action, lift action, crosslinking degree, etc.) are summarized in Table 1.

JuvedermVolift® (J_{VF}) with lidocaine is produced and distributed by *Allergan S.P.A.* (Pringy, France). It is a sterile pyrogen-free physiological solution of BDDE-crosslinked hyaluronic acid (non-animal origin) [27,28].

Restylane® (R) is produced by Q-MED (Uppsala, Sweden). It is a sterile, transparent, biodegradable gel of BDDE-crosslinked hyaluronic acid of non-animal origin [23,29].

Bovine testicular hyaluronidase, BTH (EC 3.2.1.35), salt-free lyophilized powder with a specific activity of 1275 U/mg, was purchased from *Sigma–Aldrich S.R.L.* (Milan, Italy) (cat. N. H3884, lot. SLBF8562V).

Dulbecco's phosphate-buffered saline (PBS) without calcium and magnesium was purchased from *Lonza Sales Ltd.*, Switzerland (cat. N. BE17-516F, lot. N. 3 MB191).

2.1.2. Soluble fraction quantification and hydrodynamic characterization

Filler soluble fraction was quantified by diluting each filler up to 4 mg/mL in PBS (1.0 mL final volume). The resulting suspension was kept under stirring (1000 rpm) for 24 h at 37 °C. The sample was then centrifuged at 13,000 ×*g* for 5 min, and the supernatant was removed and filtered on 0.22 μm. Filtered samples were then quantitatively analyzed for the HA content by carbazole assay [11,30].

The soluble fraction was determined as

$$\text{soluble fraction}(w/w\%) = \frac{\text{HA concentration in the permeate}(\text{mg/mL})}{\text{total HA concentration}(=4\text{mg/ml})} \times 100$$

A hydrodynamic characterization of filler soluble fractions was also performed by using the Size Exclusion Chromatography–Triple Detector Array (SEC-TDA) equipment by Viscotek (*Lab Service Analytica S.R.L.*, Italy). Specifically, several aliquots of filtered samples, obtained as described above, were lyophilized and then dissolved again at concentrations suitable for the chromatographic analysis.

A detailed description of the SEC-TDA system and analysis conditions are reported elsewhere [11,31]. Sample molecular weight (M_w, M_n, M_w/M_n), molecular size (hydrodynamic radius-R_h), and intrinsic viscosity ([η]) distributions were derived.

2.1.3. Swelling studies

160 mg of each *Aliaxin*® gel corresponding to 0.160 mL gel (gel density equal to 1 g/mL) and to 0.004 g of total (insoluble + soluble) HA (HA concentration in the gel: 25 mg/mL) were incubated with PBS (1.0–1.4 mL PBS) at 37 °C. The samples were left until the equilibrium swelling was reached and then centrifuged at 13,000 ×*g* for 5 min.

Table 1
Available information on the fillers under investigation: data reported on the package insert of the products were reported. Data available for Restylane and Juvederm, used for comparison, are also indicated.

Filler	Total HA (mg/mL)	Needle size (Gauge)	Hydro-action	Lift action	Crosslinking
Aliaxin EV	25	25	1/5	5/5	5/5
Aliaxin GP	25	27	2/5	4/5	4/5
Aliaxin FL	25	27–30	3/5	4/5	3/5
Aliaxin SR	25	27	4/5	3/5	2/5
					–
Restylane	20	30 1/2	–	–	
Juvederm	17.5	30 1/2	–	–	–

After supernatant removal, the pellet was recovered and weighed (hydrated sample mass). The swelling ratio of the total HA present in the suspensions was calculated as

$$total\ HA\ swelling\ ratio\left(\frac{g}{g}\ or\ \frac{mL}{g}\right) = \frac{hydrated\ sample\ mass\ or\ volume(g\ or\ mL)}{dry\ sample\ mass\left(4\times10^{-3}g\right)} \quad (1)$$

Such values represent the mass or the volume reached by 1 g of HA (insoluble hydrogel + soluble HA, dry powder) contained in the formulation when allowed to reach the equilibrium in PBS.

Considering that all *Aliaxin*® formulations are concentrated 25 mg/mL in total HA, the maximum volume (after equilibrium swelling) for 1 mL of filler formulation can be calculated as

$$maximum\ volume\ for\ 1mL\ of\ filler(mL/mL) = \frac{gel\ volume\ at\ equilibrium(mL)}{volume\ of\ filler\ formulation(mL)} \quad (2)$$

where *gel volume at equilibrium* was calculated as:

$$gel\ volume\ at\ equilibrium = injected\ volume(mL)\times 25mg/mL \times total\ HA\ swelling\ ratio\ (mL/mg)$$

2.1.4. Rheological measurements

Rheological measurements were performed using a Physica MCR301 oscillatory rheometer (*Anton Paar*, Germany) equipped with a parallel plate geometry, 25 mm plate diameter, 1.0 mm gap, and a Peltier temperature control. Measurements were performed at 37 °C. The linear viscoelastic range (LVR), the mechanical spectra, and the complex viscosity of the materials were determined. In particular, amplitude sweep tests were performed at a constant oscillatory frequency of 1.59 Hz over a strain range of 0.01–100% in no time setting mode. The LVR was determined as the range of strain or stress values in which the storage modulus (G′) and the loss modulus (G″) remain constant.

Oscillation frequency sweep tests were then carried out over a frequency range from 0.159 to 10 Hz (a range of frequencies considered physiologically relevant for the specific application [32]), at a constant strain selected within the linear viscoelastic range (0.1%). G′ and G″ were measured and reported as a function of frequency (mechanical spectra). Complex viscosity values were registered in the frequency range exploited.

2.1.5. Extrusion force measurements

Extrusion force was measured using a material tester (model H5KT; Tinius Olsen, Ltd., Salfords, Surrey, UK) provided with a support suitable for the placement of the syringe. Fillers were extruded using the commercial syringes, through 27 G-inch needles, at a compression rate of 12 mm/min. The extrusion force represents the force needed to extrude at the fixed rate.

Measurements were performed at least in triplicate. Results were reported as means ± SD. A Student t test was used for statistical analysis and p values lower than 0.05 accounted for significant differences.

2.1.6. Evaluation of fillers stability

Resistance to enzymatic degradation was evaluated with a method fully reported elsewhere [11]. In synthesis, gels were diluted at 4 mg/mL in PBS and incubated in the presence of BTH (5 U/mL and 1.5 U/mL) at 37 °C under stirring (1000 rpm). At different incubation times (6, 16, and 72 h), the samples were withdrawn and boiled for 10 min to inactivate the enzyme and then they were centrifuged at 13,000 ×g for 5 min. The supernatant was removed, filtered on 0.22 μm, and then quantitatively analyzed for the HA content by carbazole assay [30]. The amount of soluble fraction at each incubation time was determined as described above (paragraph 2.2). The degradation was monitored by following the increase in soluble fraction amount during enzymatic incubation. In each set of experiments, a previously characterized marketed dermal filler (*Restylane*®) incubated with BTH (BTH 5 U/mL, 6 h) was employed as control [11]. Each filler was analyzed at least in triplicate. Results were reported as means ± SD. A Student t test was used for statistical analysis and p values lower than 0.05 accounted for significant differences.

2.2. Biological evaluation

2.2.1. The model

The Phenion® Full Thickness Skin Model, produced by Henkel (Düsseldorf, Germany, diameter 1.3 cm) was used for this study. In this model, epidermal keratinocytes and dermal fibroblasts (derived from biopsy material from healthy donors), under culture conditions, form a multilayered skin equivalent that resembles human skin multilayered structure and tissue functionality. Under this experimental model, the products under examination can be injected at the same concentration and mode than *in vivo*.

One of the *Aliaxin*® formulation, specifically A_{SR}, was analyzed for its effect on production and homeostasis of Collagen IV (COLIV), Collagen VII (COLVII), Hyaluronic Acid Synthase 1 (HAS1), Aquaporin 3 (AQP3), and CD44.

Upon arrival, the FT models were transferred into Petri dishes provided with metal supports and incubated (37 °C, 5% CO_2 e 90% R. H.) with 5 mL of ALI® medium. Six injections of 50 μl of A_{SR} gel in six different points of the model just below the epidermal surface in the dermal compartment were performed. The samples were then incubated for 24 h and 3 days. Non-treated FT-SKIN and FT-SKIN injected with saline solution (INJFIL, 6 injections of 50 μl) were used as controls.

2.2.2. Real-time PCR

Total RNA was extracted from the FT-skin models using RNAqueous® kit and cDNA retrotranscription was performed using the High-Capacity cDNA Reverse Transcription kit according to the manufacturer's instructions. The Applied Biosystems 7500 Fast Real-Time PCR (ABI 7500 Fast) with fluorescent-based PCR chemistry, the TaqMan assay, was used to study gene expression of significant biomarkers (Table 2). The thermal conditions were 95 °C 20 s; 40 cycles (95 °C 3 s + 60 °C 30 s). Beta-actin was used as the housekeeping gene to normalize the expression of the specific mRNA. In particular, for each sample (non-treated FT model, FT model injected with physiological solution, and FT model injected with A_{SR}), the expression of a specific mRNA was calculated as relative quantification values (RQ, fold expression normalized for the housekeeping gene), using the SDS 2.0.6 software. Gene expression values for FT-SKIN injected with the filler were finally reported as RQ values normalized compared to the controls. RQ values higher than 2 and lower than 0.5 (compared to the control RQ = 1) were considered significant for up-regulation and down-regulation, respectively.

2.2.3. Immunofluorescence staining of collagen IV

After the incubation period, the FT-Skin models were immersed in formalin 4% v/v in PBS. After deparaffination and rehydration, 8 min at 99 °C incubation in citrate buffer was performed as antigen retrieval;

Table 2
Marker genes and evaluation end points.

Marker gene		End point
Cluster differerentiation 44	CD44	Efficacy HA homeostasis
Hyaluronic acid synthase	HAS1	Efficacy HA production
Aquaporin 3	AQP3	Improved skin hydrobalance
Collagen IV	COLIVA1	Efficacy collagen production
Collagen VII	COLVIIA1	Efficacy collagen production

after that, blocking with 5% BSA in PBS for 90 min followed by incubation overnight with primary COLIV mouse monoclonal antibody (*Santa Cruz*, sc-59814) were performed. After these procedures, the sections were incubated with appropriate secondary antibody, Alexa Fluor 488-conjugated secondary antibody (goat anti-mouse; Invitrogen, A10-680) for 1 h. The nuclei were stained with Dapi. Samples were then examined under the Leica TCS SPE confocal laser scanning microscope and analyzed by confocal software (Multicolor Package, Leica).

2.2.4. Immunohistochemistry analysis

The tissues were placed on the slides, endogenous peroxidase activity was quenched. After this procedure, the tissues were covered with primary antibody COLIVA 1:100 (mouse monoclonal, *Santa Cruz*, sc-59814) for overnight incubation. In place of the secondary antibody, an enhanced HRP polymer technology was used to provide a high sensibility and specificity staining method. After hematoxylin counterstain, the sections were dehydrated and mounted to be analyzed with a Leica DM 2500 (20×) microscope. The images were acquired with a Leica DFC 320 camera.

3. Results

3.1. Biophysical characterization

3.1.1. Soluble fraction quantification and hydrodynamic characterization

Results of the analysis on the soluble fraction are summarized in Table 3. In particular, the content in soluble HA expressed as weight percentage compared to the total HA content, and the results of the hydrodynamic characterization are reported. As shown, all gels presented a soluble fraction (from 6 ± 5 to 11 ± 3 w/w %) that was not significantly different from each other. The soluble fraction consists of HA chains ranging from about 200 to about 500 kDa of weight average molar mass values and variable distribution width (M_w/M_n in the range 1.9–3.3) with A_{SR} presenting the highest molecular weight. Intrinsic viscosity values varied from 3.9 ± 0.2 dL/g for the shorter chains distribution (A_{EV}) to 6.7 ± 0.3 dL/g for the soluble part of A_{SR} sample. The minimal hydrodynamic radius was 21 ± 1 nm for A_{EV} toward 34 ± 1 nm for A_{SR}.

A similar analysis performed on J_{VF} revealed a soluble fraction equal to 25 ± 1 w/w% (data not shown). Considering that the total HA concentration is 25 mg/mL for the *Aliaxin*® formulations and 17.5 mg/mL for J_{VF}, a higher HA-insoluble hydrogel concentration was observed in *Aliaxin*® samples.

3.1.2. Filler swelling properties

The results of the swelling studies are reported in Table 4. As expected, the fillers had a high water uptake capacity. The values of total HA (insoluble + soluble) equilibrium swelling ratio indicate the volume that 1 g of the biopolymer contained in the filler could reach. 1 g of HA in A_{EV} swells up to about 111 g that means 111 mL (gel density equal to 1 g/mL). Taking into account that A_{EV} concentration is 25 mg/mL, the content of a syringe (1 mL) could expand if allowed to reach the equilibrium in the medium, to a final volume equal to 2.8 mL. According to the swelling data, the volume expansion that can be predicted for A_{GP}, A_{FL}, and A_{SR} will be higher than 3.4, 5.6, and 7.4, respectively.

Table 4
Results of the swelling studies. Total HA swelling ratio in PBS indicates the volume at equilibrium for 1 g of HA constituting the filler (insoluble + soluble dry powder). The maximum volume for 1 mL of filler is calculated basing on the total HA swelling ratio and considering that Aliaxin gels are concentrated 25 mg/mL in HA: it represents the volume that 1 mL of the filler formulation reaches when completely swollen.

Filler	Total HA swelling ratio (mL/g)	Maximum volume (after equilibrium swelling) for 1 mL of filler
Aliaxin EV	111 ± 12	2.8
Aliaxin GP	>135	>3.4
Aliaxin FL	>265	>5.6
Aliaxin SR	>295	>7.4

Only a minimum swelling extent could be determined directly for A_{GP}, A_{FL}, and A_{SR}, due to difficulties in phase separation (insoluble swollen hydrogels from the solvent).

Similar measurements, performed on J_{VF}, resulted in a total HA swelling ratio of 146 ± 13 mL/g (data not shown); HA concentration here is 17.5 mg/mL, so 1 mL of this formulation is expected to expand to a 2.6 mL final volume.

3.1.3. Rheological properties of fillers

Amplitude sweep data are shown in Fig. 1(a). In particular, values for G′, G″, and strain, measured at 1.59 s^{-1} angular frequency at 0. 2 Pa stress, are reported. All fillers showed an elastic behavior: G′ values (elastic component) exceeded G″ values (viscous component) (2 to 8-fold). A_{EV} was the most rigid one having the highest G′ value (162 ± 28 Pa), followed by A_{GP} with a G′ of 95 ± 12 Pa; A_{FL} and A_{SR} were not significantly different regarding rigidity (45 ± 5 Pa, 39 ± 2 Pa, respectively). Consistently, at the same stress applied, strain values increased from about 0.10 for A_{EV} to about 0.23–0.28 for A_{FL} and A_{SR} confirming the latter as the most deformable and the former as the less deformable one.

The mechanical spectrum for A_{EV} is shown in Fig. 1(b): as evident, G′/G″ ratio was substantially maintained for the entire frequency range examined. The same trend was observed in the other formulations (data not shown).

The J_{VF} characterization performed under the same conditions resulted in a G′ value equal to 202 ± 22 Pa and, consistently, a strain value equal to 0.08 ± 0.01% at 0.2 Pa (data not shown).

Curves of complex viscosity as a function of frequency are reported in Fig. 1 (c). As expected, viscosity decreased with frequency for all samples in the range studied. A_{FL} and A_{SR} showed the lowest and equivalent values of viscosity (14 ± 2 e 13 ± 1 Pa at 0.7 Hz), followed by A_{GP} (22 ± 1 Pa at 0.7 Hz). The most rigid gel, A_{EV}, also was the one with the highest viscosity (highest curve: 38 ± 3 Pa.s at 0.7 Hz). A complex viscosity equal to 40 ± 3 Pa at 0.7 Hz was observed with J_{VF} (data not shown).

3.1.4. Extrusion force measurements

The values of the extrusion force measured for the Aliaxin gels, evaluated in similar conditions (syringe and needle dimension), were 26 ± 3 N (A_{EV}), 19 ± 2 N (A_{GP}), 20 ± 2 N (A_{FL}), and 17 ± 2 N (A_{SR}). No significant difference in the extrusion force was found for A_{GP}, A_{FL} and A_{SR}

Table 3
Results of filler soluble fraction analyses: soluble HA content expressed as weight percentage with respect to the total HA and hydrodynamic parameters (weight average molar mass (M_w), numeric average molar mass (M_n), polydispersity index (M_w/M_n), intrinsic viscosity ([η]), and hydrodynamic radius (R_h) values). The analyses were performed at least in triplicate.

	Soluble fraction					
Filler	(w/w %)	M_w (kDa)	M_n (kDa)	M_w/M_n	[η] (dL/g)	R_h (nm)
A_{EV}	11 ± 3	196 ± 13	103 ± 31	1.9 ± 0.3	3.9 ± 0.2	21 ± 1
A_{GP}	6 ± 5	378 ± 45	194 ± 77	2.1 ± 0.6	6.2 ± 0.6	31 ± 3
A_{FL}	8 ± 5	322 ± 14	99 ± 14	3.3 ± 0.5	5.4 ± 0.2	27 ± 1
A_{SR}	7 ± 5	498 ± 29	212 ± 35	2.4 ± 0.3	6.7 ± 0.3	34 ± 1

(a)

Filler	G' (Pa)	G'' (Pa)	γ(0.2Pa) (%)
A_{EV}	162 ± 28	24 ± 3	0.10 ± 0.02
A_{GP}	95 ± 12	22 ± 2	0.16 ± 0.03
A_{FL}	45 ± 5	18 ± 2	0.23 ± 0.02
A_{SR}	39 ± 2	20 ± 1	0.28 ± 0.04

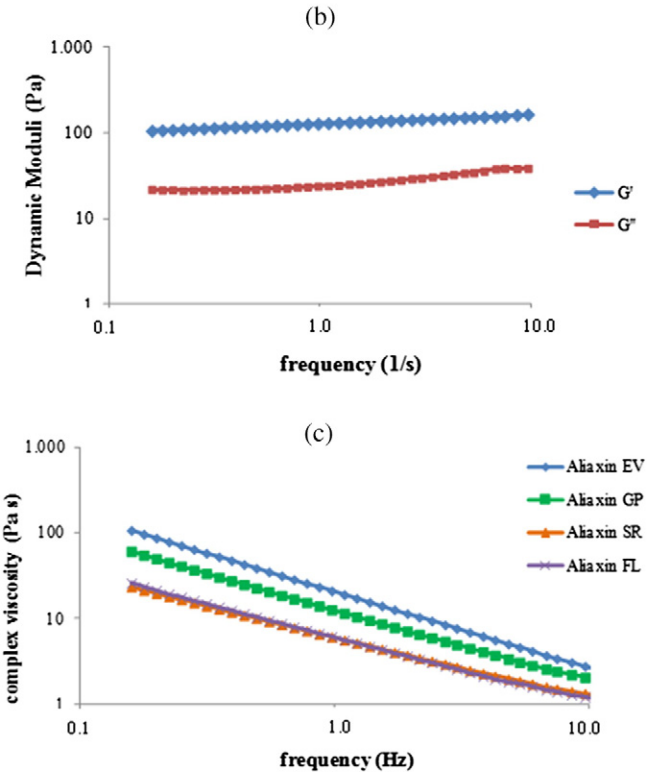


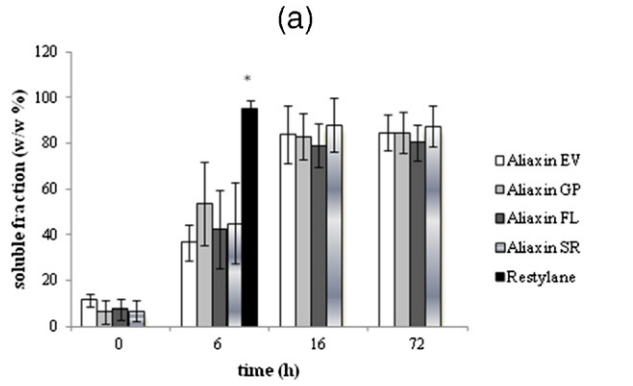
Fig. 1. (*a*) Storage modulus (G′), loss modulus (G″), and strain values (γ) measured at 1.59 Hz frequency and 0.2 Pa stress (within the linear viscoelastic range) at 37 °C. (*b*) Aliaxin EV mechanical spectrum; (c) complex viscosity values, as obtained from the frequency sweep tests, as a function of frequency.

($p > 0.05$) while A_{EV} resulted the gel requiring significantly higher force to be ejected at the same rate ($p < 0.05$).

3.1.5. Enzymatic degradation of fillers

The results of enzymatic hydrolysis studies are reported in Fig. 2. In particular, the soluble fraction amounts (w/w%) of the gels before enzymatic action ($t = 0$) and after 6, 16, and 72 h of incubation with BTH 5 U/mL are shown in Fig. 1.a. A certain extent of hydrolysis was evidenced after 6 h of incubation: the soluble fraction increased about 3- to 4-fold with respect to the starting value. All samples reached about 85% soluble fraction within 16 h of enzyme action, and this value remained the same after 3 days of incubation. At each time tested, no significant differences in sensitivity to BTH were observed among the Aliaxin formulations under these hydrolysis conditions ($p > 0.05$). The results obtained with R (6 h, BTH 5 U/mL), used as internal control, were in line with the data reported in the literature [11]. R solubilization was found significantly higher with respect to the Aliaxin gels ($p < 0.05$).

To better highlight potential differences between the Aliaxin gels, further time course experiments were run using a lower BTH concentration (1.5 U/mL). Results are reported in Fig. 2.b. As evident, in 72 h experiments, also in this case, no significant degradation could be detected for the gels except for A_{SR} for which a slight but significant increase in soluble fraction was found.



(b)

sample	soluble fraction (w/w%)	
	t =0	72h incubation with BTH 1.5U/mL
Aliaxin EV	11.4 ±2.7	12.3 ±1.4
Aliaxin GP	6.3 ±5.2	8.4 ±0.3
Aliaxin FL	7.5 ±4.5	7.1 ±0.8
Aliaxin SR	6.9 ± 4.5	15.5 ±3.3**

Fig. 2. Aliaxin gels solubilization during incubation with BTH 5 U/mL(a) and 1.5 U/mL(b). In Fig. 2.a, the soluble fraction for Restylane ® after 6 h incubation in the same conditions (internal control) is also reported. For each time tested, * indicates the sample for which the solubilization degree is significantly different with respect to the one found for the other gels. In Fig. 2.b, ** indicates the soluble fraction extent that is significantly different with respect to the value at $t = 0$.

3.2. Biological evaluation

The results of the quantitative real-time PCR analysis for the FT model injected with A_{SR} are shown in Fig. 3. In particular, RQ values for A_{SR}-treated samples were normalized for FT samples treated with physiological solution (negative control). Data analysis demonstrated a significant up-regulation ($RQ > 2$) of HAS1 ($RQ = 2.5$) 24 h after injection and of COLIV ($RQ = 2.8$) and COLVII ($RQ = 2.9$) after 3 days of incubation. No significant results were observed in the expression of AQP3 and CD44.

The results of COLIV immunolocalization obtained with confocal microscopy and immunohistochemistry are reported in Figs. 4 and 5, respectively. As expected, both techniques indicated the greater protein expression, revealed by the higher intensity of the signal, in particular in the dermal–epidermal junction area.

4. Discussion

Non-invasive aesthetic facial treatments recently evolved their target from individual wrinkle filling to a complete restoration treatment

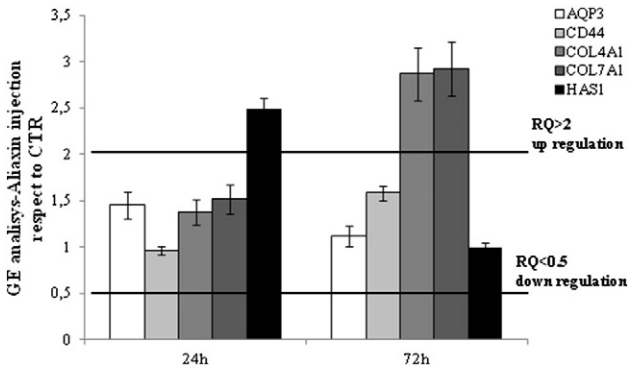


Fig. 3. Gene expression analyses (qRT-PCR) in A_{SR}-treated FT-skin model was expressed as relative quantification (RQ) respect to control.

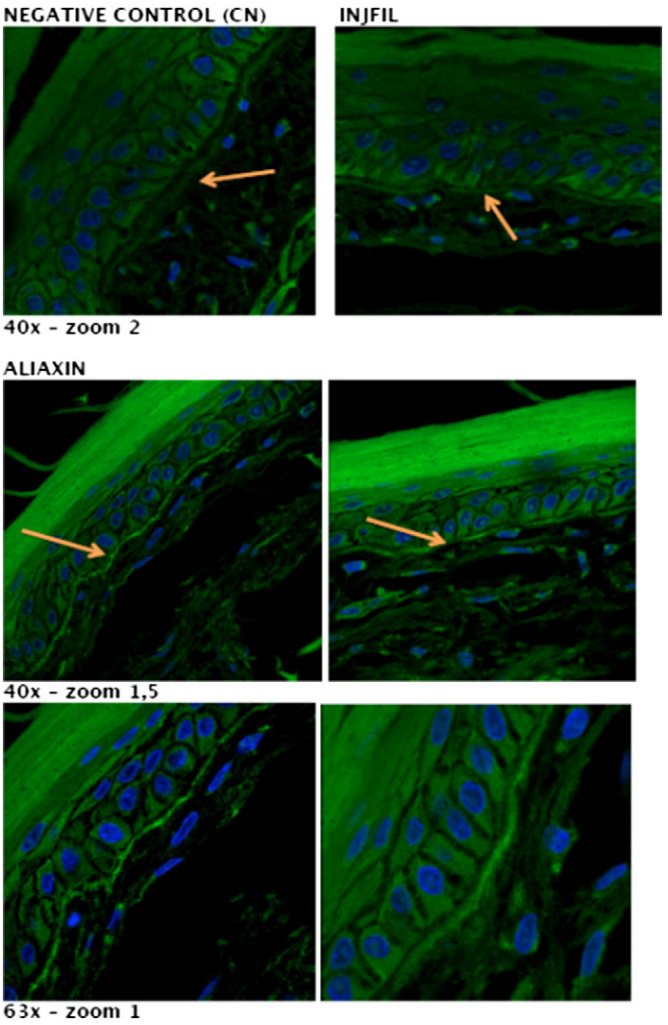


Fig. 4. Immunofluorescence staining of COLIV protein in FT-skin treated with the tested product (Aliaxin®SR) compared with controls, after 3 days of incubation (COLIV = green labeling, dermis-epidermis junction, orange arrow).

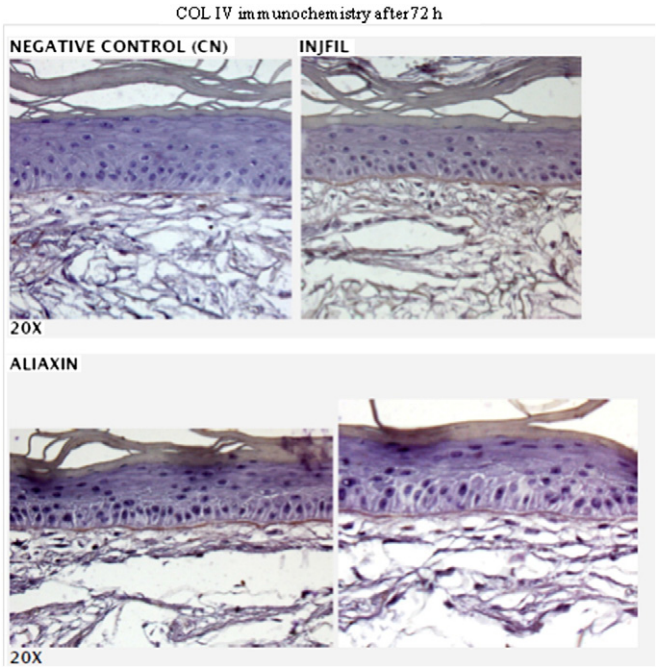


Fig. 5. Immunohistochemistry staining of COLIV protein in FT-skin treated with the tested product (Aliaxin® SR) compared with the controls. (COLIV = brown staining).

[16–18]. This modified approach prompted companies to propose a line of dermal fillers in place of a single product.

Aliaxin gels are representative of such trend. The line includes four products that are claimed to meet different needs (i.e. areas to be treated, hydro-action, lift action, etc.). Specific biophysical features (rigidity, viscosity, swelling capacity, etc.) are therefore expected to be at the basis of the diverse clinical indications. Such features are known to be mainly dependent on HA concentration in the gel and on HA crosslinking degree.

As reported in Table 1, *Aliaxin*® formulations are equally concentrated in HA (25 mg/mL) but the declared HA content refers to the total HA present in the formulation and, in addition, to the insoluble crosslinked HA, a soluble fraction of the biopolymer is usually present in this kind of products [4,11]. A quantitative analysis of the soluble fraction is important because two fillers could be equally concentrated in total HA but can have different amounts of insoluble gel, the unique responsible for the final dermal filler properties (water uptake, long-lasting effect, firmness, etc.). Results reported in Table 3 showed that all *Aliaxin*® formulations contain soluble HA in a similar amount (about 10% w/w), so they resulted equivalent regarding both total HA and insoluble hydrogel content.

HA-soluble fraction in the *Aliaxin*® gels resulted lower than the values reported in the literature for similar products (11–33% w/w) including the value found for J_{VF} [11]. These results place *Aliaxin*® among the on the market HA fillers with the highest insoluble gel concentration (e.g., about 22.5 mg/mL *vs.* about 13.4 mg/mL of R and 13.2 mg/mL of J_{VF}). It is well known that the hydro-action, the filling/volumizing action and the durability of the effect of a filler can be improved augmenting the insoluble hydrogel concentration to a certain extent, beyond which the final gel extrudability would be impaired. The high content of insoluble active HA that does not impair a proper injectability (25–30 G needles are considered suitable for the specific application) makes Aliaxin an appealing treatment.

The results of the soluble fraction analysis (Table 3) are in agreement with available information on A_{SR} soluble biopolymer amount (about 10% w/w) [17]. The similar extent of soluble fraction found in the other formulations (not declared by the manufacturers) is possibly due to the specific process of crosslinking that can leave some non-insolubilized HA and/or to the partial solubilization that usually occurs during the final thermal sterilization process. This finding is in line with data reported for previously characterized dermal fillers [11].

Since no differences in soluble HA concentrations were observed between the four Aliaxin® products, the different properties of the formulations might be related to the different crosslinking degree values (see Table 1) and, for A_{SR} only, to the addition of linear HA.

Dermal fillers *hydro-action* is strictly dependent on hydrogel water uptake capacity. As expected, all *Aliaxin*® formulations showed a great swelling extent (Table 4) inversely related to the crosslinking degree (see Table 1), having the most crosslinked gel, A_{EV}, the lowest water absorption capacity and the less crosslinked gel, A_{SR}, the highest. It is interesting to note that A_{EV} swelling degree is comparable to the one previously reported for Restylane® and to the one found for J_{VF}, while A_{GP}, A_{FL}, and especially A_{SR} presented a far more hydration capacity [11]. Swelling data are consistent with the *hydro-action* indicated in Table 1 so that A_{GP}, A_{FL}, and especially A_{SR} can be classified as fillers with a very high hydration capacity, followed by A_{EV} performing as a high hydration capacity gel similar to Restylane® and to Juvederm®Volift.

Because the swelling degree data are obtained *in vitro* in PBS, the gels expansion *in vivo* is expected to be globally lower, maintaining the ratio between the products.

Storage Modulus (G′) and viscosity (η) are the main rheological parameters affecting final gels performance regarding ease of delivering, lift/volumizing effect and dermal tissue integration pattern, and therefore, the recommended injection site.

G′ represents a measure of gel capacity to resist deformation under an applied stress such as the one occurring during extrusion and after implantation when subjected to facial movements [2,9,11,16,32]. The higher the G′ value, the higher is the resistance to deformation (the lower is the deformability). Gel deformability crucially affects the ease of delivering; in particular, the higher the deformability, the easier is the injectability: gel particles can deform under the pressure applied during extrusion and pass through the needle.

G′ values also significantly affect filler behavior after dermal tissue delivering thus suggesting the most appropriate site of application. In particular, under the stress due to facial movements, high G′ gels remain more stable in volume due to their low deformability; therefore, they are better indicated for contouring or sculpturing (volumizing) deeper areas of the face at the subcutaneous or preperiosteal layer. Low G′ gels (more deformable) are better indicated for the treatment of more superficial zones instead, where "palpability" is not desired [2,4,8].

Gels viscosity is a measure of the resistance of a fluid to deformation under shear stress like the one occurring during extrusion and, after application within the tissue: the higher is the viscosity, the higher is gel resistance to flow [2,16]. A dermal filler should have high viscosity at low shear forces and low viscosity at high shear forces. This *shear thinning* behavior indeed allows the gels to flow easily under the pressure applied during extrusion (high shear forces) and remaining in place after injection (low shear forces), avoiding the dispersion in the surrounding physiological medium [32].

Viscosity values can predict tissue integration patterns. Less viscous gels are expected to spread more in the surrounding tissue after injection (high tissue integration) resulting in a natural-looking, not "palpable" effect, suitable for the treatment of more superficial areas. High viscous gels, on the contrary, are better indicated for injections at deeper levels.

Both G′ and η of fillers depend on hydrogel concentration and crosslinking degree. Because *Aliaxin*® formulations are equivalent regarding hydrogel concentration, it is expected that rigidity and viscosity will be related to the crosslinking extent and, consistently, the results reported in Fig. 1 (a) show that rigidity increases with the crosslinking degree: $A_{EV} > A_{GP} > A_{FL} = A_{SR}$. Deformation values registered at a same applied stress (Fig. 1 (a)) confirmed the rank in rigidity: the higher the rigidity, the lower the deformation evidenced. The much higher deformability showed by A_{FL} eA_{SR} can be the reason of their better injectability than that of A_{EV} (G 27 *vs.* G 25). A_{GP} was found less firm than A_{Ev} and, consistently, a 27 G needle (in place of a 25 G one) is suggested for its application (Table 1).

Results of extrusion force measurements performed in similar conditions (syringe dimensions and needle) (paragraph 3.1.4) were consistent with the needle dimension indicated for the delivering (Table 1). The values of extrusion force found for Aliaxin gels are slightly higher than the one reported for Restylane (products compared considering the syringes and the needles supplied by the vendor) [33].

As shown in Fig. 1 (c), complex viscosity (η^*) of the *Aliaxin*® gels decreased with the oscillation frequency, indicating, as expected, the shear thinning behavior functional to the specific clinical purpose [34].

Predictably, as well as firmness (G′), complex viscosity, too, was found to increase with the crosslinking degree, and the trend was consistent with the suggested clinical indications. In particular, A_{EV}, being the most rigid and viscous gel, is expected to be the most defined in volume/less spreadable product after application and, consistently, A_{EV} formulations are indicated for the "correction of deep cutaneous depressions and volume restoration." The use of the other formulations is suggested for the "correction of medium and deep cutaneous depressions and lips treatment" instead [19].

It is worth underlying that *Aliaxin*® gels exhibit G′ and viscosity values much lower than other marketed fillers such as R. Specifically, complex viscosity values extrapolated at 0.7 Hz (the frequency typically used to compare rheological properties of injectable hydrogels) for the most and the less viscous of the *Aliaxin*® gels resulted about 15- and 4-fold lesser than that of R. Rigidity values of *Aliaxin*® resulted about 3- to about 10-fold lower than the R value [32]. Rigidity and viscosity values found for J_{VF} were comparable to the ones of the most rigid of the *Aliaxin*® formulations, and these data are coherent with the similar indications of use of the two products.

The results of rheological characterization support a better tissue integration of all *Aliaxin*® formulations compared to R; when compared to J_{VF}, A_{GP}, A_{FL} and A_{SR} are expected to perform better while A_{EV} should behave similarly.

These findings have relevant consequences even considering filler *hydro-action*. Specifically, as reported above, A_{GP}, A_{FL}, and A_{SR} showed a swelling extent (*hydro-capacity*) much higher than A_{EV} and other characterized dermal fillers (i.e. R and J_{VF}). However, an expansion after injection is desired only to a certain extent, beyond which it could not be easily managed by the clinician, and undesired palpability and formation of protrusions could occur. Due to the high deformability/low viscosity of the *Aliaxin*® formulations, they spread easily in the surrounding tissues allowing to achieve a *hydrolift*® effect much higher than the other fillers studied, without causing complications in clinical use.

Dermal filler stability to hyaluronidases action is crucial for a successful outcome: this parameter allows to predict the relative *in vivo* duration of the aesthetic effect. As shown in Fig. 2, *Aliaxin*® formulations showed quite similar sensitivity to BTH. Differences in the manufacturing process of the fillers may affect rate of enzymatic degradation. For the Aliaxin gels, the crosslinking degree and the molecular weight of the HA undergoing crosslinking are known to be different. The combined effect of these features could be responsible for the similarity in the enzymatic degradation rate registered. It has to be noted that, in comparable conditions (enzyme concentration), *Aliaxin*® products maximum extent of solubilization was lower than the extent reported for other marketed products such as Restylane® predicting potentially a longer permanence *in vivo* [11].

It is well recognized that, in addition to their *lifting*, *hydro*, and *volumizing* effects, HA-based dermal fillers stimulate the production of several components of the extracellular matrix. In particular, the overexpression of Collagen I and III has been demonstrated [35], and this effect is therefore expected also in subjects receiving *Aliaxin*® injections. COLI, III, COLIV, and COLVII are the most frequent collagen types present in the skin and the most involved in wound healing, and they are mainly present in the basement membrane that anchors the epidermis to the dermis. To the best of our knowledge to date, no data have been reported on the variation in the expression of COLIV and COLVII in skin treated with HA-based dermal fillers. Thanks to the presence of natural HA, A_{SR} is the most innovative formulation of the *Aliaxin*® line and, for this reason, was chosen to evaluate the effect on production and homeostasis of COLIV, COLVII, and HA. The early up-regulation found for HAS1 (Fig. 3.b) suggests a matrix remodeling and a dermal regeneration due to the boost in hyaluronan production. Besides, the increase in COLIV and COLVII expression observed 3 days after the injection (Fig. 3.b) suggests a late activation of the cutaneous basement membrane, which stimulates the formation of an organized and flexible cutaneous basement membrane structure, responsible for the optimal skin architecture. Boosting COLIV and COLVII, the product helps to regulate the remodeling process globally, promoting the structural organization of the full network of dermal proteins. Immunostaining analysis (Figs. 4 and 5) demonstrated that *Aliaxin*® injections induce the deposition of COLIV on the dermal–epidermal junction, thus supporting *Aliaxin*® capacity to contrast the COLIV-aging-related decrease in this area.

Recent research works allowed to hypothesize that crosslinked hyaluronic acid induces production of collagen I mainly by mechanical stretching of the dermis: it stimulates fibroblasts to produce growth factors and inhibitors of metalloproteinases of the matrix [36,37]. Considering the similarities in the biosynthetic pathways of the diverse types of collagen such mechanism could be potentially involved in the induction of collagen IV production occurring in Aliaxin-treated samples [38].

Overall, biological studies demonstrated a valuable effect of A_{SR} injections on skin structure restoration. Additionally, it is worth underlying that to date *in vivo* models have been generally used for studying the capacity of a dermal filler to promote skin regeneration [35,36,39]. Results reported here suggest that the full thickness skin model could be an attractive tool to *in vitro* assess biological effects of dermal fillers.

5. Conclusions

The current expertise on the characterization of dermal fillers has been applied to study a recently developed line of HA-based products, *Aliaxin*®. Data obtained (swelling extent, rigidity, viscosity, etc.) consistently correlated with

1) insoluble hydrogel concentration and crosslinking degree, confirming them as the key parameters to set to fine-tune biophysical features of the final formulations;
2) the clinical indications from the producers, confirming that biophysical data are a valuable guide for the clinicians in dermal fillers use.

Compared to other marketed dermal fillers, Aliaxin® gels had a higher concentration of the insoluble component and showed a very high water uptake capacity (higher hydro-action) and high durability (prolonged aesthetic effect); moreover, the lower rigidity and viscosity support an easier management while delivering.

Finally, the biological effects of A_{SR} were evaluated *in vitro*: gel ability to promote skin restoration was demonstrated.

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