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Managing Pathologic Scars by Injecting Auto-Cross-linked Hyaluronic Acid: A Preliminary Prospective Clinical Study

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Abstract

Background Pathological scars are unattractive and may significantly impair the patient's quality of life. Current treatments provide inconsistent results, and none may be regarded as definitive. Recently, an auto-cross-linked hyaluronic acid (HA) formulation, IAL-SYSTEM ACP, featuring a long residence time and an enhanced safety profile, has been successfully used to prevent surgical adhesions, treat tendon lesions and rejuvenate the face and the décolletage. This study aims to preliminarily investigate whether IAL-SYSTEM ACP may also be effective in treating pathological scars resulting from burns, trauma or iatrogenic causes.

Methods Patients presenting one pathological scar were prospectively recruited and treated with two IAL-SYSTEM ACP injections carried out two weeks apart. Scar improvement was measured comparing the patient and observer scar assessment scale (POSAS) scores collected before treatment (T0) and 90 days after the second injection (T90) using nonparametric tests. The effect of age and scar type over score variation was investigated through quantile regression analysis.

Results Forty-one patients, 10 women and 31 men (median age, 34 years) were recruited. No patient dropped out, and no significant adverse event was observed. At T90, the median observer total score decreased by 11 units (-77.5%) and the median patient total score decreased by 15 units (-73.7%). The difference was significant ($p < 0.001$) in both cases. Traumatic injuries and young patient's age were the most significant predictors of a positive treatment outcome.

Conclusions Treatment of pathological scars by two IAL-SYSTEM ACP injections, carried out two weeks apart, may provide significant clinical benefits. These findings should be the subject of further investigations.

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Introduction

When damaged skin heals, fibroproliferative disorders may lead to the insurgence of keloids and hypertrophic scars [1]. These are different lesions [2–4]. Keloids, sometimes referred to as benign tumors, are elevated fibrous scars that extend beyond the borders of the original wound, do not regress, and usually recur after excision. Conversely, hypertrophic scars do not grow beyond the boundaries of the original wound and usually regress over time. Histological assessment shows that hyalinized collagen bundles are thick in keloids and absent or fewer in hypertrophic scars [5]. Normal wound healing is characterized by an

inflammatory, a proliferative and a remodeling phase [6], the three exhibiting coordinated and partially overlapping cellular activities [7, 8]. Scar formation marks the end of wound healing. Here, the action of fibroblasts, myofibroblasts and growth factors on extracellular matrix (ECM) deposition is crucial, with both hypertrophic scars and keloids resulting from abnormal responses to dermal injury that lead to excessive collagen deposition [9].

Hyaluronic acid (HA) plays a key role throughout the entire wound healing process [10]. As the tissue is wounded, HA levels at the lesion site first increase rapidly and then decrease as HA undergoes degradation by hyaluronidases [11]. Platelets contain large amounts of HA that is released during the formation of the initial clot: This leads to leukocytes recruitment from the blood and bone marrow via CD44 receptors, starting the inflammation stage and leading to the formation of the initial edema. Further, HA orchestrates the differentiation of a certain population of dermal fibroblasts into stress fiber-expressing precursors of myofibroblasts: Approximately 4 days after wounding, myofibroblasts appear in the wound [12] and exert their contractile forces by focal adhesion, linking their intracellular cytoskeleton to the ECM. This stage is followed by the deposition of granulation tissue, rich in HA and CD44 [13]. The final stage is characterized by the invasion and proliferation of fibroblasts that begin to lay collagen, with HA specifically stimulating the production of type III collagen [14]. HA also regulates keratinocyte proliferation during the re-epithelialization phase [15]. Any imbalance in the action of metalloproteases (MMP) and tissue inhibitors of metalloproteases (TIMP) may disrupt the remodeling phase; this leads to a significantly lower HA content in keloids than in ordinary scars [16, 17] and a still lower content of HA in hypertrophic scars [18].

At present, no single treatment acting on the pathophysiologic mechanism of pathological scar exists, and many strategies have been attempted to reduce the risk of pathological scars formation. Experimental and clinical approaches include surgery [19], corticosteroid injection [20, 21], radiotherapy [22], compression therapy [23, 24], therapy with triamcinolone [25], 5-fluorouracil [26, 27], bleomycin [28], verapamil [29], laser therapy [30–32], combination therapy [33–35], stem cell therapy [36–38] and fat tissue grafting [39, 40]. However, results reported are inconsistent. A possible adjuvant treatment might consist of infiltrating the scar with HA, under the rationale that replenishing HA might facilitate those processes that lead to the physiological deposition of a healthy ECM. Indeed, HA infiltrations were shown to prevent adhesions following knee surgery in a rabbit model [41] and, when used following tendon surgeries or to treat chronic tendinopathies, HA injections resulted in reduced formation of scars and granulation tissue, less adhesions and gliding

resistance and improved tissue healing [42]. When infiltrating HA, a longer residence time of active, non-degraded HA at the injection site may provide enhanced clinical benefits because of the prolonged HA effect on the undergoing regenerative processes. The half-life of HA may be prolonged by subjecting the molecule to cross-linking reactions, which may also improve the rheology and water-retaining property of the final formulation [43, 44]. Recently, an auto-cross-linked HA-injectable formulation, IAL-SYSTEM ACP (Fidia Farmaceutici S.p.A., Abano Terme, Italy), having an increased half-life and an enhanced safety profile, has been introduced. IAL-SYSTEM ACP slowly hydrolyzes into native HA providing a long-lasting action on fibroblasts, which are stimulated to produce new collagen [45]. IAL-SYSTEM ACP was shown to accelerate tendon healing in an experimental study on flexor tendon injury [46] and to reduce adhesions after abdominal [47], pelvic [48] and nerve surgery [49, 50], and after tenolysis of flexor tendons of the hand [51]. IAL-SYSTEM ACP has also been successfully used in skin rejuvenation and skin blemish treatment [52, 53]. Given its stimulating and healing-accelerating effects, IAL-SYSTEM ACP might also provide beneficial effects in the treatment of pathologic scars. Accordingly, the present prospective pilot study aimed to provide a preliminary assessment of the effectiveness and tolerability of IAL-SYSTEM ACP when used for that purpose.

Materials and Methods

This was an open label, investigator-initiated pilot prospective study carried out at a single center (Department of Reconstructive Plastic Surgery, Hand Surgery, AOU 'Ospedali Riuniti,' Ancona, Italy). The investigation was performed in compliance with the Declaration of Helsinki and the Guidelines for Good Clinical Practice. All subjects provided their informed consent.

Subjects were recruited according to the following inclusion criteria: age between 30 and 65 years, having a II to IV skin phototype and presenting at least one hypertrophic scar caused by trauma, burn or iatrogenic (surgical) injury, anywhere on the body. Subjects also had to be willing to perform all study procedures and to undergo follow-up visits without being subjected to any additional treatment. Subjects were excluded if they suffered from any autoimmune or systemic disease or from any neoplasm; if they were undergoing any therapy for their scar or had received any other treatment within the previous 2 years; or if subjected to any additional treatment during the follow-up period. Patients were also excluded if pregnant or breast-feeding, if undergoing any treatment with

corticosteroids as well as if smoking more than 10 cigarettes a day.

IAL-SYSTEM ACP (Fidia Farmaceutici S.p.A., Abano Terme, Italy) is a highly viscous gel obtained by condensation of hyaluronic acid, a main component of human connective tissue and of epithelial and mesothelial tissues. The product is a steam-sterilized, 2% auto-cross-linked HA-injectable water-based solution. Being HA auto-cross-linked, IAL-SYSTEM ACP does not contain residual chemicals, thus enhancing the safety profile. After injection, IAL-SYSTEM ACP is completely degraded to CO_2 and H_2O [54]. During its degradation, fragments of HA at different molecular weights are released, that stimulate the proliferation of cutaneous fibroblasts and keratinocytes [55]. Further, IAL-SYSTEM ACP exhibits longer residence time than the corresponding non-cross-linked formulation [52]. Thanks to its prolonged residence in the application site, it slowly releases hyaluronic acid that, due to its natural hydrating properties, improves and maintains turgidity and skin elasticity for a longer time with respect to hyaluronic acid-based preparations.

Patients underwent two treatment sessions at a two-week (15 days) interval. This protocol was carried out taking into account the in vivo and in vitro studies of Alessandrini et al. [52, 53] about the use of IAL-SYSTEM ACP for skin rejuvenation treatments. No anesthesia was performed. Following skin disinfection, the pathological scar was infiltrated with IAL-SYSTEM ACP into the deep and the intralesional dermal planes through a 12.7-mm-long 30G needle. This procedure was repeated until the scar was completely infiltrated, using at most two 1-ml IAL-SYSTEM ACP syringes. For each patient, we administered a sufficient volume of ACP up to obtain a transitory ischemia of the entry scar point out by the transition to a white color of the lesion. Moreover, we injected the same amount of ACP in the papillar dermal level (Fig. 1C). The maximum volume injected of ACP for each treatment was 2 ml. All patients were prescribed to apply SPF50 + sunscreen for 7–10 days post-treatment. The infiltration was performed by a single physician, who was not involved in the post-clinical patient evaluation. Patients were prescribed no further medications and were followed up for 24 months after the intervention. The physician performing the treatment took photographs of the scars before the first treatment session (T0) and 3 months after the last session (T90) after placing the patient in the same position, using a single camera with the same settings, under consistent lighting conditions.

Two independent plastic surgeons, blinded to the treatment, assessed the scars for vascularity, color (pigmentation), thickness, relief, and elasticity (pliability) through the patient and observer scar assessment scale (POSAS) [56, 57] before the first treatment session (T0) and

3 months after the last treatment (T90). The POSAS questionnaire allows assessing the quality of a scar using an observer and a patient scale and has been shown to be an effective tool for evaluating both linear scars and burn scars with good internal consistency and agreement [56, 57]. The observer scale of the POSAS consists of six items (vascularity, pigmentation, thickness, relief, pliability, and surface area). All items are scored on a scale ranging from 1 ('like normal skin') to 10 ('worst scar imaginable'). The sum of the six items results in a total score of the POSAS Observer scale. Category boxes are added for each item. Furthermore, an overall opinion is scored on a scale ranging from 1 to 10. All parameters should preferably be compared to normal skin on a comparable anatomic location. The patient scale of the POSAS consists of six questions, to be scored from 1 to 10, two concerning pain and itching (1, not present; 10, very much) and four concerning skin appearance (color, thickness, stiffness, irregularity) again scored from 1 to 10 (1, as normal skin; 10, very different). Patients were also asked to compare their scar to normal skin again on a 1 to 10 scale (1, as normal skin; 10, very different). A copy of the POSAS questionnaire is provided as supplementary material.

Statistical Analysis

Application of the Shapiro–Wilk test showed that data were not normally distributed; accordingly, all statistical analyses were carried out according to a nonparametric approach. The median and the first and third quartiles were calculated to describe quantitative variables and absolute and relative frequencies to describe qualitative ones. To investigate the effectiveness of IAL-SYSTEM ACP, the total POSAS observer and patient score absolute variations between T0 and T90 were calculated, as well as the corresponding median values and their 95% confidence interval (95% CI). Median values were then compared using the Wilcoxon signed-rank test. Further, the percentage variation between T0 and T90 was calculated for each item of the Observer scale, and differences in pigmentation, thickness, relief and pliability between T0 and T90 were investigated by means of the Friedman's test.

Quantile regression analysis was carried out to investigate factors that may influence the absolute variation of the patient and observer scales total scores, when they were regarded as dependent variables. Quantile regression does not require any assumption on the distribution of the variables, and it allows to estimate the effect of the independent variable on the quantile-specific distribution of the dependent variable. The analysis concerned the 25th, 50th, and 75th percentile of the two dependent variables. Independent variables selected were all the demographic and

Fig. 1 A traumatic pathological scar in a woman aged 43; **a** and **b** before treatment; **c** and **d** during the first IAL-SYSTEM ACP injection; **e** and **f** 90 days after the second IAL-SYSTEM ACP injection

clinical characteristics, and the final models were the most parsimonious ones, including those covariates that most contributed to the goodness of fit.

Finally, to investigate whether the patients and the physicians provided a significantly different assessment of the scar variation over time, absolute variations of the observer and patient total scores were compared using the Wilcoxon signed-rank test.

Results of the quantile regression analysis were expressed as point and interval estimates of regression coefficients; regression coefficients were considered statistically significant when the 95% CIs did not include 0. All other statistical tests were regarded as significant if $p < 0.05$. Statistical analyses were performed using a standard statistical software (R, version 3.4.3, R Core Team [58]).

Results

Forty-one Caucasian patients, 10 women and 31 men, were enrolled in the study between March 2017 and March 2018. The last follow-up visit was carried out in July 2018. The patients' median age was 34 years (first–third quartiles: 33–49). Main demographic and clinical patients'

characteristics are summarized in Table 1. Each patient presented one scar. Scars had different etiologies, characteristics, sizes, and sites, with nine (22.0%) scars being iatrogenic, 13 (31.7%) traumatic and 19 (46.3%) caused by burns. After IAL-SYSTEM ACP injections, a significant improvement in appearance and texture of pathological scars could be observed at T90 compared to baseline conditions. Scar bulk and scar height reduced, and a textural improvement could be observed. Skin pigmentation, pliability and overall appearance improved, and scar-related symptoms such as itching and burning disappeared. Twelve out of 41 patients (29.3%) stated that they were satisfied, and 29 (70.7%) were very satisfied. Representative cases are shown in Figs. 1, 2 and 3.

Subjects reported no pain during or following the injection. Slight erythema and edema were observed in two patients (4.9%) a few hours after each treatment session. They resolved spontaneously in a few hours. All patients completed the study, and no other complications were observed.

POSAS patient and observer total scores are shown in Table 2, together with their absolute variation from T0 to T90; both scores showed a significant reduction at the T90 follow-up control visit ($p < 0.001$ in both cases): The median observer total score decreased by 11 units

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Table 1 Main patients' demographic and clinical characteristics

Variable	N
Patients [n]	41
Gender, men [n (%)]	31 (75.6)
Gender, women [n (%)]	10 (24.4)
Age, years [median (1st–3rd quartiles)]	34 (33–49)
Smoking habits, yes [n (%)]	4 (9.8)
Smoking habits, yes [n (%)]	37 (90.2)
Scar type [n (%)]	
Iatrogenic	9 (22.0)
Traumatic	13 (31.7)
Burn	19 (46.3)
Time from scar development, months [median (1st–3rd quartiles)]	20 (14–29)

Fig. 2 A traumatic pathological scar in a woman aged 52; **a** before treatment; **b** during the first IAL-SYSTEM ACP injection; **c** 90 days after the second IAL-SYSTEM ACP injection
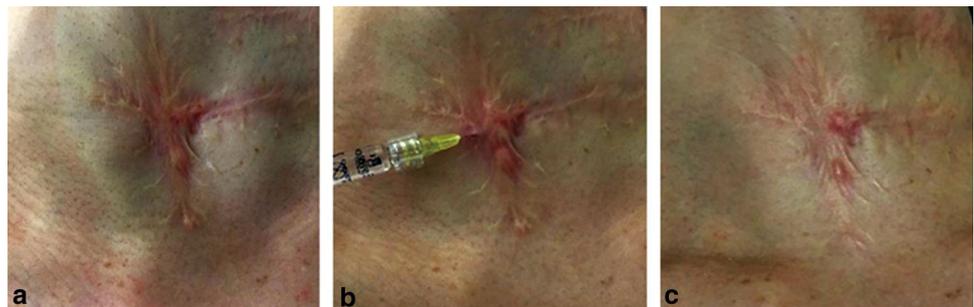
Fig. 3 A pathological scar caused by a burn in a 37-year-old man; **a** before treatment; **b** during the first IAL-SYSTEM ACP injection; **c** 90 days after the second IAL-SYSTEM ACP injection

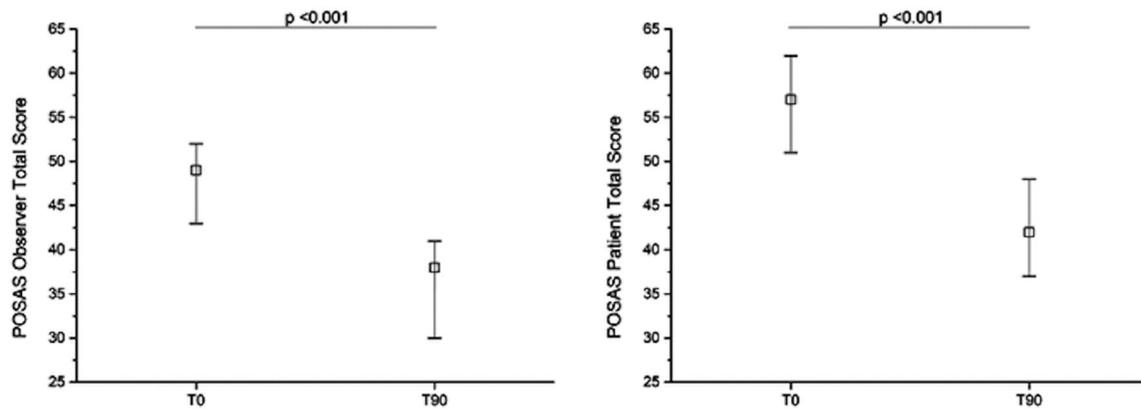**Table 2** Variation of POSAS patient and observer total scores between T0 and T90

POSAS total score	T0		T90		T0–T90		p
	Median (1st–3rd quartile)	95% IC	Median (1st–3rd quartile)	95% IC	Median (1st–3rd quartile)	95% IC	
Observer	49 (43–52)	46.8–51.2	38 (30–41)	35.3–40.7	11 (9–14)	9.8–12.2	< 0.001
Patient	57 (51–62)	54.3–59.7	42 (37–48)	39.3–44.7	15 (8–20)	12–18	< 0.001

p values refer to Wilcoxon signed-rank test

($-$ 77.5%), whereas the median Patient total score decreased by 15 units ($-$ 73.7%) (Fig. 4). Results concerning the single items of the POSAS Observer scale are shown in Table 3. All scores decreased between T0 and T90, the greatest reduction being observed in thickness,

relief and pliability. However, no significant difference was found for pigmentation, thickness, relief and pliability percentage scores reduction ($p = 0.56$).

Results of the quantile regression analysis are shown in Table 4a, b). Greater POSAS observer scale total scores

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Fig. 4 Variation of POSAS observer (left) and patient (right) total scores (squares, medians; bars, first and third quartiles). The reduction of the total score is significant ($p < 0.001$) in both cases

Table 3 Variation of the POSAS observer single item scores between T0 and T90

| Items | Percentage variation T0–T90 | |
	Median (1st–3rd quartile)	95 %CI
Vascularity	16.7 (0–40)	6.8–26.5
Pigmentation	20 (14.3–33.3)	15.3–24.7
Thickness	25 (12.5–37.5)	18.8–31.2
Relief	25 (12.5–28.6)	21–29
Pliability	25 (12.5–44.4)	17.1–32.9
Surface area	16.7 (0–25)	10.5–22.8
Overall opinion	20 (12.5–28.6)	16–24

absolute variations (75th percentile) were significantly more associated with iatrogenic scars than to traumatic ones. Moreover, age negatively significantly affected the absolute variation of the Observer total score in both the 50th and 75th percentile distribution, while the higher absolute variation (75th percentile) was significantly associated with higher basal values of the variable (Table 4a). Concerning the patient total score, both the 50th and 75th percentiles of its absolute variation were significantly more associated with iatrogenic scars than with traumatic ones, while only the 50th percentile absolute variation was significantly more associated with iatrogenic than with burned ones (Table 4b). The absolute variation of the score (25th, 50th and 75th percentiles) was significantly positively associated with the basal values of the variable and significantly negatively associated with age (25th and 50th percentiles of the distribution). Observer and Patient total score variations were found to be not significantly different ($p > 0.05$) indicating that both the patients and the physicians provided a similar overall assessment of the scar improvement over time (Table 5).

Discussion

Hypertrophic scarring in humans is a common and serious problem following deep dermal injuries including burns, abrasions and deep donor site excisions. These scars typically present as raised, red, itchy, firm lesions. They result in both cosmetic deformities and functional impairment that can have a devastating impact on the quality of life for those affected [1, 59, 60]. Physical symptoms may include itching, stiffness, scar contracture, tenderness and pain [61, 62]. Hypertrophic scars are characterized by enhanced growth factor signaling, excessive proliferation activity and altered ECM production, including increased collagen and decreased HA deposition [63–65]. Replenishing HA by external administration is thought to represent a beneficial therapy for the prevention of scar formation [66]. A study by Hoffmann and colleagues [67] demonstrated that exogenous HA can normalize some of the characteristic features of pathological fibroblasts such as hyperproliferation activity, growth factor production and extracellular matrix deposition. Accordingly, the present preliminary study explored the effect of a new auto-cross-linked HA formulation, IAL-SYSTEM ACP, featuring an increased tissue residence time, on formation and healing of several types of hypertrophic scars in the clinical setting. Mainly, results of the present study show that exogenous administration by infiltration of IAL-SYSTEM ACP accelerates the improvement of the overall scar appearance, while not causing any significant complications. These findings are consistent with those of previous investigations, showing IAL-SYSTEM ACP was effective in preventing postsurgical adhesions after different kind of surgeries [47–49], in promoting healing of primary flexor tendon injury [46] and preventing adhesions after tenolysis of flexor tendon in zone 2 of the hand [51]. Results of the present study are also consistent with those observed when IAL-SYSTEM

Table 4 Results of the quantile regression analysis concerning the POSAS

Variables	Coefficient	95% CI
(a) Observer scale		
25th percentile		
Scar type (traumatic vs iatrogenic)	2.32	− 6.27; 10
Scar type (burn vs iatrogenic)	3.56	− 5.50; 10.86
Total score T0	− 0.02	− 0.35; 0.39
Age (years)	0.04	− 0.12; 0.07
50th percentile		
Scar type (traumatic vs iatrogenic)	− 2.48	− 2.82; 1.31
Scar type (burn vs iatrogenic)	0.26	− 4.06; 1.23
Total score T0	0.04	− 0.19; 0.04
Age (years)	− 0.15	− 0.15; − 0.07*
75th percentile		
Scar type (traumatic vs iatrogenic)	− 2.87	− 3.66; − 0.21*
Scar type (burn vs iatrogenic)	0.03	− 1.94; 0.80
Total score T0	0.05	0.03; 0.11*
Age (years)	− 0.16	− 0.16; − 0.08*
(b) Patient scale		
25th percentile		
Scar type (traumatic vs iatrogenic)	− 3.23	− 12.56; 7.88
Scar type (burn vs iatrogenic)	− 4.59	− 9.19; 8.58
Total score T0	0.56	0.32; 1.27*
Age (years)	− 0.10	− 0.35; − 0.01*
50th percentile		
Scar type (traumatic vs iatrogenic)	− 6.48	− 8.30; − 4.57*
Scar type (burn vs iatrogenic)	− 1.38	− 10.10; − 0.71*
Total score T0	0.88	0.35; 1.22*
Age (years)	− 0.48	− 0.48; − 0.19*
75th percentile		
Scar type (traumatic vs iatrogenic)	− 4.99	− 28.72; − 4.96*
Scar type (burn vs iatrogenic)	− 0.81	− 34.16; 0.27
Total score T0	0.86	0.84; 1.00*
Age (years)	− 0.40	− 0.48; 0.03

*Marks significance

ACP was used effectively as a biorevitalizing gel for facial and décolletage rejuvenation [52, 53]. All these studies, together with the paper of Soranzo and colleagues [68], have shown that IAL-SYSTEM ACP is characterized by prolonged residence time due to particular chemical structure. Cross-linking is a method of increasing hyaluronic acid residence time and viscosity. The particularity is also due to the absence of synthetic molecule insertion as it happens for other hyaluronic acids in which synthetic molecules create bridges between the hyaluronic acid chains. This particularity increases the safety profile of IAL-SYSTEM ACP. The present study describes the first

clinical results concerning treatment of scars using IAL-SYSTEM ACP, indicating that its use is significantly associated with positive, regenerative changes in scar tissue in the epidermis and dermis, leading to significant clinical benefit. IAL-SYSTEM ACP was well tolerated, and its use appeared to be completely safe. The adverse events reported were usually slight bruising and redness associated with intradermal injections. The lack of foreign body or other severe reactions might be due to the enhanced safety profile of HA-ACP that, being auto-cross-linked (that is, cross-linked without using any additional chemical reagent), may cause less irritation, sensitization or foreign body reactions. Results of the present study concerning safety of IAL-SYSTEM ACP are therefore consistent with previous clinical reports concerning its use in the clinical setting. In the whole, results of this study indicate infiltrative therapy of pathological scars using IAL-SYSTEM ACP is safe and effective, allowing to achieve an average 25% reduction of the POSAS score. In particular, the evaluation of redness and vascularity is significant only on the younger scars. Because of that the number of scars in which the evaluation of redness and vascularization are significant quite low in the respect of the entire sample, so the parameter's improvement is still more significant. Results of this study suggest that IAL-SYSTEM ACP injection may have contributed to normalize pathological scar hyperproliferation activity and disruption of extracellular matrix deposition, as is expected according to the known roles of HA in modulating ECM deposition and published results concerning IAL-SYSTEM ACP; the actual mechanism of action of IAL-SYSTEM ACP on pathological scars, yet, should be clarified through appropriate studies including histological qualitative and quantitative assessments. The principal limitations of this study are its small sample size and the lack of a control group as well as the variability in type, morphology, and size of scars being treated. Further, this study did not analyze the separate effect of the two treatment sessions and therefore does not provide any information about the effectiveness of the treatment when a single injection is carried out. Further controlled prospective studies on a larger number of subjects, over a longer follow-up period and using a proper control group should be carried out to confirm the present findings, possibly assessing also other IAL-SYSTEM ACP injection regimens.

Conclusion

Treatment of pathological scars by IAL-SYSTEM ACP administration according to a two-injection protocol, with single injections carried out two weeks apart, may provide significant objective and subjective clinical benefits, while

Table 5 Results of the significance concerning the POSAS

Statistic	T90–T0 (score operator)	T90–T0 (score patient)
Mean	− 10.78	− 15.83
Dv Std	3.45	10.87
Kurtosis (Pearson)	0.25	0.063
Calculated Shapiro–Wilk statistic, W	0.9097	0.8777
Critical value of W (5% significance level)	0.94582	0.94099
V	861	859
V (normalized)	5.594	5.559
Expected value	430.500	430.500
Variance (V)	5921.500	5942.125
p value (bilateral)	< 0.0001	< 0.0001
Alfa	0.05	0.05

being safe and quite well tolerated, possibly by normalizing pathological scar hyperproliferation activity and disruption of extracellular matrix deposition.

Non-pathological scars improve their appearance over time, but everyone also knows that these improvements are very slow and not so significant; meanwhile, in this study the scar's improvements were quick (last evaluation after 90 days) and statistically significant. Not only in more than 50% of the patients we treat, a hypertrophic scar usually does not improve during the time. Despite this, we believe that this preliminary study is just a sign that must be confirmed by a future multicenter study with a more homogeneous study cohort. This preliminary study is the first investigating safety and performance of IAL-SYSTEM ACP in treating pathological scars; its findings should be the subject of further investigation through prospective, controlled clinical studies on a greater number of subjects.

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical Approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed Consent All subjects provide their informed consent.

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