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Contents

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All articles in their final version - completed with name, surname, affiliation, address, phone number and e-mail address of the author (s) - must be sent in word format to the Editorial Committee at the following e-mail address: aemj@aestheticmedicinejournal.org. Manuscripts must be written in English, and authors are urged to aim for clarity, brevity, and accuracy of information and language. All manuscripts must include a structured abstract. Authors whose first language is not English should have their manuscripts checked for grammar and stylistic accuracy by a native English speaker.

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LARGE INCREASES IN AMERICANS' CONSUMPTION OF sugar-sweetened beverages (SSB) have been a topic of concern. Between 1977 and 2002, the intake of "caloric" beverages doubled in the United States, with most recent data showing that children and adults in the United States consume about 172 and 175 kcal daily, respectively, from SSB,[1] it is estimated that SSB account for about 10% of total energy intake in adults[2,3]. High intake of SSB has....

References Section Example

References

1. Duffey KJ. Popkin BM. Shifts in patterns and consumptions of beverages between 1965 and 2002. *Obesity.* 2007:15(11):2739-2747.
2. Nielsen SJ. Popkin BM. Changes in beverage intake between 1977 and 2001. *Am J Prev Med.* 2004;27(3):205-210.
3. Drewnowski A. Bellisle F. Liquid calories, sugar, and body weight. *Am J Clin Nutr.* 2007;85(3):651-661.

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Original Article

Dermaroller devices and Topical 5-Fluorouracil: a therapeutic modality for recalcitrant Acral Vitiligo

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The running head: Acral Vitiligo

Names of the institutions at which the research was conducted: Department of Dermatology and Venereology, Faculty of Medicine, Suez Canal University, Ismailia, Egypt

Abstract

Background: Acral Vitiligo (AV) is a chronic condition that is recalcitrant to treatment.
Aim: to evaluate the efficacy and safety of combining dermaroller devices (DD) and 5Fluorouracil (5FU) in treating AV.
Methods: this study included 66 adult patients with AV who were randomly assigned to 3 groups. Treatment was delivered for group I by 5FU, DD for group II, and DD for group III, followed by 5FU for a maximum period of 3 months and a 3-month follow-up. Lesions were then evaluated both qualitatively and quantitatively.
Results: the overall qualitative response to treatment was significantly higher using the combined treatment. Results with G3 and 4 were not statistically significant in the periungual and dorsum of hands and feet areas at the end of the 6th month. Pain was tolerable during sessions or at sites of 5FU application.
Conclusion: despite the tolerability and safety of micro-needling with the dermaroller device technique with 5-Fluorouracil, the results were not promising.

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Introduction

Acral vitiligo (AV) is a chronic condition that is recalcitrant to treatment[1,2]. Many causes contribute towards the development of this resistance. Factors include paucity of the pilosebaceous apparatus, melanocyte stem cell reservoirs, melanocyte, baseline epidermal stem cell factor, stem cell factor (SCF), c-kit, MHCII expression and a lower density of Langerhans cells (LC)[1,3,4]. Some patients with AV are resistant to medical treatment alone and many other surgical modalities can be used either alone or adjuvant to medical treatment for the repigmentation of lesions in stable AV.

Objective

This is a single blind, comparative, randomized controlled study to investigate the efficacy and safety of dermaroller devices (DD) and 5Fluorouracil (5FU) in treating AV.

Methods

Study Design and Patient Population

Approval for this pilot, parallel-group, randomized controlled trial was obtained from the institutional review board and ethical committee of the Suez Canal University Faculty of Medicine, Ismailia, Egypt. It was conducted over 12 months in accordance with the guidelines of the Declaration of Helsinki. Patients with approved informed consent were recruited from the outpatient clinic of the dermatology department of the Hospital of Suez Canal University. Eligible patients were stable recalcitrant AV for at least 2 years and had no evidence of any systemic or dermatological disease. Pregnant women, lactating women, and patients who were receiving treatment for AV or oral retinoid within 6 months, were excluded from the study. Seventy patients were eligible for participation and 66 completed the study. Before the study, a complete medical history was obtained from each patient and all patients underwent a general physical examination. They were randomly assigned to 1 of 3 groups described below.

Group 1 (5FU)

Twenty-four patients applied 5FU 5% once daily to the lesions for 7 days per month for 3 consecutive months [in a ratio of 4 pea-sized amounts per whole face].

Group 2 (dermaroller devices)

Treatment was then delivered to lesions of AV in 23 patients using twice weekly 1 mm dermaroller device sessions **(Microneedle Therapy SystemTM and MTS RollerTM manufactured by Clinical Resolution Laboratory, Inc.Beverly Hills, California 902210)**. After sterilization with 70% alcohol, rolling is done 15-20 times in horizontal, vertical, and oblique directions, within 15 to 20 min, depending on the extent of the area to be treated, until punctiform bleeding occurs. Needling device (the dermaroller) movement was from the dark periphery towards the white centre or from a dark spot (color island) towards depigmented areas. Needle pricks sometimes led to the oozing of a small drop of blood, which was managed by physical pressure, but never resulted in frank bleeding. No dressings were needed in any cases. Successive sessions were repeated up to 3 months later.

Group 3 (dermaroller and 5FU)

As in group two, dermaroller 1mm devices were delivered to 25 patients with AV. 5FU 5% and applied to the abraded area on days of the procedure, for 3 months.

Assessment Methods

Assessment of the results achieved by three methods was based on: an Investigator's global assessment (IGA), vitiligo disease activity score (VIDA score), and subjective evaluation of tolerance (pain). At each bi-weekly visit, digital photos were taken of the tested target area at standardized distance and lighting conditions.

1 - Assessment of treatment effectiveness

3 professional independent observers examined the clinical photos at 0 and 3 months at the institution, using a Sony digital camera (14.1 mega pixels). The investigator's global assessment (IGA) consists of a 5 grade scale of repigmentation as follows:[G0, none; G1, < 25% (poor); G2, 25%–49% (fair); G3, 50%– 74% (good), G4, ≥ 75% (excellent)].

An IGA score of 3 or 4 with at least 50% repigmentation was the desirable response and considered the criterion for success5 For statistical evaluation purposes, quantitative evaluation of the response was performed in a numerical percentage6.

2 - Assessment of treatment safety

Tolerance (pain) was evaluated subjectively using a five-point scale (1=none, 2=slight, 3=moderate, 4=severe, 5=intolerable) and patient's satisfaction was evaluated on a 5 point scale (1=not satisfied, 2=slightly satisfied, 3=satisfied, 4=very satisfied, 5=extremely satisfied), and their willingness to repeat the procedure. Side effects were also assessed during visits.

3 - Assessment of disease activity

Disease activity was scored using the "vitiligo disease activity score (VIDA)" before and after 3 months of treatment. The VIDA is a six-point scale for assessing vitiligo activity based on the individual's own opinion of disease activity regarding the expansion of existing lesions or appearance of new lesions, perceived during a period ranging from less than 6 weeks to one year, as shown in table7. Surgery for vitiligo is only suggested for patients with VIDA scores of 1 or 0.

Statistical Analysis

All statistical analysis was performed using SPSS software, version 21 (SPSS, Inc, an IBM Company, Chicago, Illinois). Statistical significance in a two-sided test was defined as $P < .05$.

Results

Demographic Findings

Sixty-six patients with AV completed the entire study [resistant to topical and systemic steroids, phototherapy and photochemotherapy], with an additional 3 months of follow-up. Six patients were lost to follow-up.

The mean age of study completers in groups I, II and III was (37.5 ±9.6, 32.2 ± 8.3 and 35.4 ± 8.7, respectively). The prevailing skin phototype in all groups was type III. 82.7%, 67.8%, and 73.6% of patients in group I, II and III, respectively belonged to this phototype. There was no significant difference between the three groups in terms of a family history of vitiligo. The mean age of the onset of lesions was (27.9±0.5, 29.9±2.5 and 33.1 ±0.6 years) in group I, II, and III, respectively. The mean duration of the disease was (5.1 ± 3.8, 4.6±1.7 and 5.8 ± 4.7 years) in group I, II, and III, respectively. Re-epithelization and inflammatory reaction time among patients was 5.9 ±3.7 and ranged from 2 to 7 days.

Grading assessment with G1, G2, G3 and G4 was used to classify lesion repigmentation. The overall qualitative response to treatment was significantly higher using the combination treatment. This was expressed at a rate of repigmentation [G2, G3 and G4] for all three groups (*Table 1*). The mean number of lesions to achieve G3 and G4 repigmentation in the dorsum of hands among responders in group III was not significantly higher than those in group I and II ($P > 0.05$).

At the end of the 6th month, no statistically significant results with G3 and 4 were recorded in the dorsum of hands and feet. Regarding the fingers and toes, a response was identified in the 2nd month but the difference in results between the two groups was not statistically significant. No G3 and G4 results were found in the periungual area during the entire treatment period in any of the groups. (*Tables 2-5*).

Patients in all groups experienced erythema and tanning of normal skin, which returned to normal within a few days to weeks. This occurred in 21.2%, 28.2% and 34.3% of subjects in group I, II and III, respectively. However, this difference was statistically insignificant (p=0.759). This was accepted by patients.

Assessment of disease stability

The VIDA score in all patients were zero before and after the end of treatment and follow-up period, which indicates the stability of the disease [no progression of old lesions, no new lesions and absence of Koebner phenomenon].

Assessment of treatment safety

All patients in group II and III reported no (41.1%) to slight (11.2%) pain during the session.

Discussion

The combined medical and surgical treatment of recalcitrant vitiligo lesions has been investigated in many studies[6,8-20]. The combination of 5FU with other strategies can treat vitiligo lesions, decrease resistance, and increase patient compliance. Therefore, this study was designed to compare the efficacy and safety of combination treatment with a dermaroller device plus 5FU cream in treating resistant AV. This study showed that the mean duration of re-epithelization and inflammatory reaction time among patients was 5.9 ±3.7 and ranged between 2 and 7 days. This was consistent with the findings of Anbar et al.[6,13]. Conversely, the combination of dermabrasion (by sandpaper or dermabrader) with 5- FU cream revealed longer re-epithelization time (3-4 weeks) after 7-10 days of application of 5-FU cream[16,18]. The overall qualitative response to treatment in this study was statistically higher in the combination treatment group. In group III, 49 lesions (14 %) showed G3 (good) and G4 (excellent) re- pigmentation versus 2 lesions (0.8%) in group I and 13 lesions (4.3%) in group II. This response was statistically different from other groups, which indicated that the combination treatment was a successful technique in treating acral vitiligo. These results were in line with other previous studies[6,8-20]; however, high responses were achieved in non-acral vitiligo. Regarding the periungual area of fingers and toes in the 6 months study period, there was a statistically non-significant difference between the groups, in line with Asker E et al. 2019[18]. Therefore, even melanocyte transfer causes a poor response, reliant upon other factors. A comparison of therapeutic efficacy of the three treatment groups on a monthly basis according to distribution on hands and feet, showed that the mean number of lesions which achieved G3 and G4 on the dorsum of hands, fingers and in the feet along the 6 months of study was not significantly different among patients ($P > 0.05$). Our results did not align with those of Mina, 2018.

The therapeutic effect of the dermaroller device plus 5-fluorouracil cream may be explained by several mechanisms: (1) the inflammatory response resulting from the treatment, such as the local release of leukotrienes C4 and D4, presumably creates a multicellular infiltrate in which melanocyte proliferation and migration is promoted[6]. (2) After epidermal needling, 5-FU penetrates easily, deeply, stimulates proliferation and migration of the inactive melanocytes at the outer root sheath of the hair follicle. This appears clinically as perifollicular pigmentation, which gradually enlarges to cover the affected area[21]. (3) 5FU produces colonization of melanocytes in the vitiliginous epidermis by stimulating the division of epidermal melanocytes from the surrounding pigmented skin and reinforcing their migration toward the affected areas for 2-3 mm after epithelialization of the epidermis[22]. (4) 5-FU competes with deoxyuridine and its derivatives for the enzyme thymidylate synthetase, and damages some inhibitory agent or cells within the epidermis or dermis that may be responsible for the destruction of pigment cells producing vitiligo[11,22]. (5) 5FU increases the level of melanocyte stimulating hormone, the direct stimulation of melanocytes, increased number of melanosomes in the keratinocytes, and the activation of melanocytes. However, hyperpigmentation is a known side effect of 5FU observed during the treatment of skin tumors and psoriasis[23]. (6) The dermaroller induces the mechanical transfer of melanocyte from the pigmented to non-pigmented area[10]. In our study, the mode of repigmentation in vitiligo lesions was either follicular (small, brown, perifollicular macules, which then enlarged

and coalesced) or by extension of pigment from the edges by a few millimeters (perilesional hyperpigmentation). In the periungual region, where no hair follicles are present, melanocyte reactivation is expected from adjacent skin only. Repigmentation from the periphery will yield different degrees of response according to the distance between the healthy edges. This points to the imperativeness of early treatment of vitiligo lesions with scant or no hair[6]. Side effects of applied treatment in all groups showed a statistically non-significant difference. These side effects were transient and rapidly disappeared[13-14,16].

Conclusion

In spite of the tolerability and safety of micro-needling with dermaroller device technique with 5- Fluorouracil, the results were not promising.

Conflict of interest

No conflict of interest.

	Topical 5FU (n = 249)		Dermaroller (n= 301)		Dermaroller and Topical 5FU (n = 350)		ANOVA
	No. of lesions	%	No. of lesions	%	No. of lesions	%	
G0 (0%)	**215**	86.4 %	233	77.4%	201	57.4 %	0.7
1 (< 25%) or poor	**21**	8.4 %	34	11.3%	50	14.3 %	0.08
(25 – 49%) or fair	**11**	4.4 %	21	7%	50	14.3 %	0.01*
(50 – 74%) or good	**1**	0.4 %	8	2.7%	27	7.7 %	0.006*
4 (≥ 75) or excellent	**1**	0.4 %	5	1.6%	22	6.3 %	0.03*

*Statistically significant at P < 0.05

Table 1 *- Overall qualitative response to therapy.*

		Topical 5FU cream			Dermaroller			Topical 5FU cream			Anova*
		No of lesion	Mean ± SD	Range	No of lesion	Mean ± SD	Range	No of lesion	Mean ± SD	Range	
Treatment	1st month	0	0±0	0	0	0±0	0	0	0±0	0	**
	2nd month	1	0.09 ± 0.3	0 - 1	0	0±0	0	0	0±0	0	0.3
	3rd month	4	0.36 ± 1.2	0 - 4	2	0.21 ± 1.1	0 - 3	0	0±0	0	0.3
Follow up	4th month	8	0.72 ± 1.5	0 - 5	6	0.62 ± 2.5	0 - 5	010	0.90 ± 0.8	0 - 3	0.7
	5th month	12	1.09 ± 1.5	0 - 5	11	1.89 ± 2.5	0 - 5	14	1.27 ± 1.4	0 - 5	0.7
	6th month	13	0.18 ± 0.4	0 - 6	11	1.89 ± 2.5	0 - 5	16	1.45 ± 1.5	0 - 5	0.7
Treatment	1st month	0	0±0	0	0	0±0	0	0	0±0	0	**
	2nd month	0	0±0	0	0	0±0	0	0	0±0	0	**
	3rd month	0	0±0	0	0	0±0	0	0	0±0	0	**
Follow up	4th month	1	0.09 ± 0.3	0 - 1	1	0.09 ± 0.3	0 - 1	0	0±0	0	0.3
	5th month	4	0.3 ± 0.6	0 - 2	3	0.4 ± 0.5	0 - 1	12	1.09 ± 1.4	0 - 4	0.1
	6th month	7	0.67 ± 1.0	0 - 3	6	0.54 ± 0.9	0 - 2	25	2.27 ± 2.7	0 - 7	0.09
Treatment	1st month	0	0	0	0	0±0	0	0	0±0	0	**
	2nd month	0	0	0	0	0±0	0	0	0±0	0	**
	3rd month	0	0	0	0	0±0	0	0	0±0	0	**
Follow up	4th month	0	0	0	0	0±0	0	4	0.36 ± 0.8	0 - 2	0.1
	5th month	1	0.09 ± 0.3	0 - 1	0	0±0	0	8	0.72 ± 1.1	0 - 3	0.1
	6th month	1	0.09 ± 0.3	0 - 1	1	0.09 ± 0.3	0 - 1	9	0.81 ± 1.2	0 - 3	0.08

*Statistically significant at P < 0.05
**no statistical values when standard deviation equals zero

Table 2 *- Number of vitiligo lesions achieved G1 regimentation in the hands and feet.*

		Topical 5FU cream			Dermaroller			Dermaroller plus Topical 5FU cream only			p- value*
		No of lesion	Mean ± SD	Range	No of lesion	Mean ± SD	Range	No of lesion	Mean ± SD	Range	
Treatment	1st month	0	0±0	0	0	0±0	0	0	0±0	0	**
	2nd month	0	0±0	0	0	0±0	0	0	0±0	0	**
	3rd month	0	0±0	0	0	0±0	0	0	0±0	0	**
Follow up	4th month	1	0.27 ± 0.4	0 - 1	1	0.98 ± 0.4	0 - 3	23	2.09 ± 1.9	0 - 5	0.01
	5th month	5	0.36 ± 0.6	0	0	1.31 ± 0.6	0 - 3	37	3.36 ± 3.1	0 - 9	0.01
	6th month	0	0.45 ± 0.6	0	0	1.35 ± 0.5	0 - 4	46	4.18 ± 3.9	0 - 11	0.01
Treatment	1st month	0	0	0	0	0	0	0	0±0	0	**
	2nd month	0	0	0	0	0	0	0	0±0	0	**
	3rd month	0	0	0 - 1	7	0	0	0	0±0	0	**
Follow up	4th month	3	0	0 - 2	8	0	0	0	0±0	0	**
	5th month	4	0.09 ± 0.3	0 - 2	9	0.09 ± 0.3	0 - 1	4	0.236 ± 0.5	0 -1	0.1
	6th month	5	0.45 ± 0.8	0 - 2	1	0.09 ± 0.3	0 - 1	5	0.45 ± 0.6	0 -2	1.0
Treatment	1st month	0	0	0	0	0	0	0	0±0	0	**
	2nd month	0	0	0	0	0	0	0	0±0	0	**
	3rd month	0	0	0	0	0	0	0	0±0	0	**
Follow up	4th month	0	0	0	0	0	0	0	0±0	0	**
	5th month	1	0.09 ± 0.3	0 - 1	0	0	0	0	0±0	0	0.3
	6th month	1	0.09 ± 0.3	0 - 1	0	0	0	0	0±0	0	0.3

Table 3 *- Number of vitiligo lesions achieved G2 regimentation in the hands and feet.*

*Statistically significant at P < 0.05
**no statistical values when standard deviation is equal to zero

		Topical 5FU cream			Dermaroller			Topical 5FU cream			Anova*
		No of lesion	Mean ± SD	Range	No of lesion	Mean ± SD	Range	No of lesion	Mean ± SD	Range	
Treatment	1st month	0	0±0	0	0	0±0	0	0	0±0	0	**
	2nd month	0	0±0	0	0	0±0	0	0	0±0	0	**
	3rd month	0	0±0	0	0	0±0	0	0	0±0	0	**
Follow up	4th month	0	0±0	0	1	0.09 ± 0.3	0 - 1	7	0.63 ± 0.8	0–2	0.02
	5th month	1	0.09 ± 0.3	0 - 1	1	0.09 ± 0.3	0 - 1	18	1.63 ± 1.7	0 –5	0.01
	6th month	1	0.09 ± 0.3	0 - 1	2	0.19 ± 0.3	0 - 1	22	2.00 ± 1.6	0 -5	0.004
Treatment	1st month	0	0±0	0	0	0±0	0	0	0±0	0	**
	2nd month	0	0±0	0	0	0±0	0	0	0±0	0	**
	3rd month	0	0±0	0	0	0±0	0	0	0±0	0	**
Follow up	4th month	0	0±0	0	0	0±0	0	0	0±0	0	**
	5th month	0	0±0	0	0	0±0	0	3	0.27± 0.4	0-1	0.08
	6th month	0	0±0	0	0	0±0	0	5	0.45 ± 0.9	0 -3	0.13
Treatment	1st month	0	0±0	0	0	0±0	0	0	0±0	0	**
	2nd month	0	0±0	0	0	0±0	0	0	0±0	0	**
	3rd month	0	0±0	0	0	0±0	0	0	0±0	0	**
Follow up	4th month	0	0±0	0	0	0±0	0	0	0±0	0	**
	5th month	0	0±0	0	0	0±0	0	0	0±0	0	**
	6th month	0	0±0	0	0	0±0	0	0	0±0	0	**

Table 4 *- Number of vitiligo lesions achieved G2 regimentation in the hands and feet.*

*Statistically significant at P < 0.05
**no statistical values when standard deviation is equal to zero

		Topical 5FU cream			Dermaroller			Topical 5FU cream			Anova*
		No of lesion	Mean ± SD	Range	No of lesion	Mean ± SD	Range	No of lesion	Mean ± SD	Range	
Treatment	1st month	0	0±0	0	0	0±0	0	0	0±0	0	**
	2nd month	0	0±0	0	0	0±0	0	0	0±0	0	**
	3rd month	0	0±0	0	0	0±0	0	0	0±0	0	**
Follow up	4th month	0	0±0	0	1	0.09 ± 0.3	0-1	11	1.00 ± 1.4	0–4	0.04
	5th month	1	0.09 ± 0.3	0 - 1	1	0.09 ± 0.3	0 -1	21	1.90 ± 2.0	0 –6	0.01
	6th month	1	0.09 ± 0.3	0 - 1	1	0.09 ± 0.3	0 -1	28	2.54 ± 2.8	0 -9	0.01
Treatment	1st month	0	0±0	0	0	0±0	0	0	0±0	0	**
	2nd month	0	0±0	0	0	0±0	0	0	0±0	0	**
	3rd month	0	0±0	0	0	0±0	0	0	0±0	0	**
Follow up	4th month	0	0±0	0	0	0±0	0	0	0±0	0	**
	5th month	0	0±0	0	0	0±0	0	0	0±0	0	**
	6th month	0	0±0	0	0	0±0	0	0	0±0	0	**
Treatment	1st month	0	0±0	0	0	0±0	0	0	0±0	0	**
	2nd month	0	0±0	0	0	0±0	0	0	0±0	0	**
	3rd month	0	0±0	0	0	0±0	0	0	0±0	0	**
Follow up	4th month	0	0±0	0	0	0±0	0	0	0±0	0	**
	5th month	0	0±0	0	0	0±0	0	0	0±0	0	**
	6th month	0	0±0	0	0	0±0	0	0	0±0	0	**

Table 5 - *Number of vitiligo lesions achieved G4 regimentation in the hands and feet.*

*Statistically significant at P < 0.05
**no statistical values when standard deviation is equal to zero

		Topical 5FU cream		Dermaroller		Topical 5FU cream		p- value*
		No of lesion %	Mean ± SD	Range	No of lesion %	Mean ± SD	Range	
Dorsum	G0	30 (22.2%)	2.72 ± 1.6	0 - 5	86 (81.2 %)	7.81 ± 7.7	0 - 20	0.04
	G1	16 (11.9 %)	1.45 ± 1.5	0 - 5	13 (12.3 %)	1.18 ± 1.7	0 - 6	0.7
	G2	45 (33.3 %)	4.09 ± 3.7	0 - 10	5 (4.7 %)	0.45 ± 0.68	0 - 2	0.01
	G3	22 (16.3 %)	2.00 ± 1.06	0 - 5	1 (0.9 %)	0.09 ± 0.3	0 - 1	0.004
	G4	22 (16.3 %)	2.00 ± 2.6	0 - 9	1 (0.9 %)	0.09 ± 0.3	0 - 1	0.04
Fingers and toes	G0	91 (72.3 %)	8.27 ± 6.1	0 - 20	70 (85.4 %)	6.36 ± 4.8	0 - 16	0.43
	G1	25 (19.9 %)	2.27 ± 2.7	0 - 7	7 (8.5 %)	0.63 ± 1	0 - 3	0.92
	G2	5 (3.9 %)	0.45 ± 0.6	0 - 2	5 (6.1 %)	0.45 ± 0.8	0 - 2	1
	G3	5 (3.9 %)	0.45 ± 0.9	0 - 3	0 (0 %)	0 ± 0	0 - 0	0.13
	G4	0	0 ±0	0 - 0	0 (0 %)	0 ± 0	0 - 0	**
Periungal area	G0	80 (89.8 %)	7.27 ± 6.2	0 - 18	59 (96.8 %)	5.36 ± 6	0 - 20	0.47
	G1	9 (10.2 %)	0.81 ± 1.2	0 - 3	1 (1.6 %)	0.09 ± 0.3	0 - 1	0.08
	G2	0 (0 %)	0 ± 0	0 - 0	1 (1.6 %)	0.09 ± 0.3	0 - 1	0.34
	G3	0 (0 %)	0 ± 0	0 - 0	0 (0 %)	0 ± 0	0 - 0	**
	G4	0 (0 %)	0 ± 0	0 - 0	0 (0 %)	0 ± 0	0 - 0	**

Table 6 - *Number of vitiligo lesions achieved G4 regimentation in the hands and feet.*

*Statistically significant at P < 0.05
**no statistical values when standard deviation equals zero

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aesthetic medicine

Original Article

Serum Vitamin D and C-Reactive Protein levels in patients with chronic spontaneous urticaria

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Short title: Serum Vitamin D and C-RP in chronic spontaneous urticaria

Abstract

Background: Chronic Spontaneous Urticaria (CSU) is a disease with multifactorial etiology, associated with autoimmune and inflammatory phenomena. Decreased vitamin D levels are known to be associated with autoimmune and atopic diseases.

Aim: to assess the serum levels of vitamin D and C-Reactive Protein (C-RP) in chronic urticaria patients, compare them with age and sex matched controls and to determine the occurrence of chronic autoimmune urticaria using Autologous Serum Skin Test (ASST).

Methods: this hospital-based case-control study involved 116 patients (58 each case and controls). Detailed history, clinical examination and baseline investigations were carried out. Serum vitamin D, C-RP, Thyroid Peroxidase antibody (TPO-Ab), Thyroglobulin antibody (TgAb) levels were analyzed and ASST was done for cases only.

Results: mean serum vitamin D levels were significantly lower in CSU cases (12.93±5.66 ng/ml), compared to controls (23.31±6.13 ng/ml, $p<0.001$). Mean C-RP levels were significantly higher in cases (4.02±6.83 mg/l). However, no correlation between serum C-RP and vitamin D levels was found in CSU patients. ASST was positive in 50% of CSU patients, of which 31% patients had significantly higher rates of TPO-Ab and TgAb levels. The median duration of CSU was longer in ASST positive (36 months) compared to negative patients (8 months).

Conclusions: in the study population, low vitamin D is associated with CSU but not associated with its severity. C-RP levels were significantly higher, but there was no correlation with CSU severity. Our study found a 50% ASST-positivity rate, suggesting that ASST could be a simple screening test for autoimmune urticaria in resource-poor settings.

Keywords

C-Reactive Protein, chronic spontaneous urticaria, skin test, thyroid diseases, thyroglobulin, vitamin D

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Introduction

Urticaria is a common, distressing disorder clinically affecting the skin with the appearance of transient, pruriginous erythematous or edematous lesions, with or without angioedema. While acute urticaria is a self-limiting disorder with a rapid onset which disappears within six weeks, Chronic Urticaria is present most days of the week and lasts for over six weeks up to several years. According to the GA2LEN/EAACI/WAO/EDF (joint initiative of the Dermatology Section of the European Academy of Allergology and Clinical Immunology (EAACI), the EU-founded network of excellence, the Global Allergy and Asthma European Network (GA²LEN), the European Dermatology Forum (EDF) and the World Allergy Organization (WAO) guidelines, Chronic Idiopathic Urticaria (CIU) or Chronic Spontaneous Urticaria (CSU) consists of cases where no triggering factor is found to be responsible for the spontaneous appearance of wheals[1].

The sera of roughly around 30-50% of patients with chronic urticaria have been shown to cause a pink wheal when intradermally injected into the patient's own skin. This subgroup is referred to as Chronic Autoimmune Urticaria (CAU)[2].

Another theory for the disorder suggests an autoimmune association; this view stems from the understanding that thyroid dysfunction and thyroid autoantibodies are more common among CSU patients. Based on this, there was a search for autoantibodies as well as other immune biomarkers potentially causing increased histamine release[3]. Some reviews have suggested that most patients with chronic urticaria can be divided into CIU (55%) and CAU (45%), the latter having autoantibodies against mast cell FceR1α receptor Immunoglobulin E (Ig E). The Autologous Serum Skin Test (ASST) is an intradermal test where patients are injected with their own serum. It is considered positive if induced wheal diameter is equal to or more than 1.5 mm of saline induced wheal response[4]. ASST is considered one of the best clinical tests for the detection of autoimmune urticaria, due to its simplicity and cost.

Vitamin D deficiency is a wide-spread clinical issue. Though commonly associated with diseases affecting joints, muscles and bones, vitamin D may also have immunomodulatory effects in chronic urticaria and is reported to be instrumental in the pathogenesis of urticaria[5,6].

There have been reports of urticaria patients improving after vitamin D supplementation[7]. CSU is considered to be a mast cell-mediated inflammatory disease. C-reactive protein (C-RP) is a laboratory marker for inflammation and elevated levels of C-RP have been reported in patients with CSU. C-RP has also been considered as a biomarker of CSU activity or severity[8]. Given the limited nature of literature from India on vitamin D status in urticaria patients and on the role of C-RP as an inflammatory marker, this study aimed to analyze the relationship between serum vitamin D levels, C-RP and chronic urticaria.

We also aimed to assess the autoimmune nature of urticaria, using the simple, autologous serum skin test.

Patients and Methods

This hospital-based case-control study involving 116 subjects was conducted in the outpatient department of Dermatology, Venereology and Leprosy, Justice K. S. Hegde Charitable Hospital, Mangaluru (India) from October 2016 to March 2018. Fifty-eight patients with a history-based and clinical diagnosis of chronic spontaneous urticaria along with fifty-eight age and gender-matched controls were enrolled after obtaining ethical clearance from the Institutional Ethical Committee.

All chronic spontaneous urticaria patients aged 18 years and above of either gender who consented to participating in the study were included. The inclusion criteria for controls required age and gender-matched individuals without chronic urticaria, with normal clinical parameters, sourced from applicants for various health check-up packages available in our hospital. Patients who did not consent to participating in the study, and those under the age of 18 years, pregnant and lactating women, individuals with acute urticaria, secondary causes such as physical urticaria, a history of systemic corticosteroids in the past 6 weeks and other systemic illness requiring steroid treatment, as well as those with malignancy and chronic medical illness, were excluded from this study. After fulfilling the selection criteria, all subjects were counseled and informed consent was obtained. For each patient, a detailed medical history and a questionnaire based information regarding the characteristics of the disease were collected. A detailed physical examination was carried out.

The Urticarial Activity Score (UAS) according to EAACI/GA2LEN/EDF/WAO guidelines was assessed in each patient, based on the daily intensity of pruritus and the number of hives per day. Pruritus was scored ranging from 0-none to 3-severe, and the number of hives were graded as None = 0 point, <10 = 1 point,10-50 = 2 points, >50 per day = 3 points.

These were summed up to create a daily UAS ranging from 0-6 points per day and daily UAS scores were summed over seven days to create the UAS7 (0-42 scale). According to the sum of the UAS7 in one week, the patients were subdivided into three subgroups: mild (7-15), moderate (16-27), severe (28-42).

Baseline investigations were carried out, including a full blood count, platelet count, urine analysis, ESR, serum glucose, hepatic function test and renal function test. A thyroid function test was performed following the chemiluminescence protocol. Serum vitamin D-25(OH)D concentration was estimated with the use of an automated direct electrochemiluminescence immunoassay (Roche Diagnostics, Mannheim, Germany). Levels of vitamin D were divided as follows; 30-70 ng/ml (normal), 20-29 ng/ml (insufficiency) and <20 ng/ml(deficiency). Serum C-RP concentration was measured using the nephelometric method (MISPA-i2). Elevated serum C-RP was defined as >6 mg/l. Serum levels of Thyroid Peroxidase antibody (TPO-Ab) and Thyroglobulin Antibodies (TgAb) were estimated by fully automated chemiluminescence immunoassay. TPO-Ab was considered raised if >34 IU/ml and TgAb raised if >115 IU/ml.

Statistical Analysis

The collected data was analyzed using SPSS version-17. Descriptive data were presented in the form of frequency percentage for categorical variables and in the form of mean, median and standard deviation for continuous variables. The Chi-square test, student's unpaired t-test, Fisher's exact test, Spearman's rank correlation coefficient, Mann-Whitney U test and Wilcoxon W tests were used. P <0.05 was considered as significant.

Results

The youngest study participant was 18 years of age and the oldest was 65 years of age. The average age in cases and controls was 38.5±11.97 years and 38.12±12.15 years, respectively. However, the majority of subjects (39, 33.6%) belonged to the 35-45 years age group. Additionally, among the fifty-eight individuals (age and gender- matched cases and controls), 25 (43.1%) were males and 33 (56.9%) were females, with a male to female ratio of 1:1.36 (*Table 1*). The mean UAS score in the study population was 21.74 ± 9.04, which was estimated according to EAACI/GA2LEN/EDF guidelines. The majority (44.8%) of subjects had moderately severe urticaria, severe in 15 patients (25.9%) (*Figure 1*). Out of the 58 chronic urticaria patients in this study, wheals occurred in 52 (89.65%) of patients. Urticaria and angioedema occurred together in 6 (10.34%) of patients. A positive correlation between UAS7 and disease duration was found (r=0.508, p <0.001) (*Figure 1*). High C-RP levels were found in ten patients and one control in this study. The difference was found to be statistically significant (p=0.004) (*Table 2*). There was no correlation between UAS and C-RP levels (r =0.26, p=0.046) (*Figure 2*). Serum vitamin D levels were lower than 30 ng/mL in 57 (98%) of patients with chronic urticaria and 53 (91%) of controls. A deficient level of vitamin D was seen in 52 (89%) cases and 19 (32%) control subjects. Mean vitamin D levels in cases and controls were 12.93±5.66 and 23.30±6.13 (p<0.001), respectively. There was no significant association of serum vitamin D level with the severity of chronic urticaria (p=0.524) (*Table 1* and *Figure 3*). Also, there was no correlation between serum concentrations of C-RP and serum levels of Vitamin D in the study group (r=-.118, p=.380) (*Figure 4*).
ASST was performed on 58 patients, 29 of which (50%) were ASST positive. There was a slightly higher ASST positivity rate among women (17 out of 29, 51.5%, p=0.791), compared to men. Median disease duration was found to be longer in the ASST positive group (median 36 months (IQR 12, 48)) compared to in the ASST negative group (median 8 months (IQR 5, 24) and was found to be statistically different (p<0.001) (*Table 2*). There was a statistically significant association between ASST and UAS7 (p<0.001). There was no difference in serum vitamin D levels in both groups (13.51±6.24 in the ASST positive group vs. 12.34±5.05 (p=0.138). In our study, thyroid antibodies, TgAb Ab TPO Ab were found to be positive among 15 (25.9%) and 17(29.3%) of CSU patients, respectively (*Table 1*). The associations between ASST and both thyroid antibodies (TPO Ab and TG Ab) were found to be statistically significant (p= 0.001) (*Table 3*).

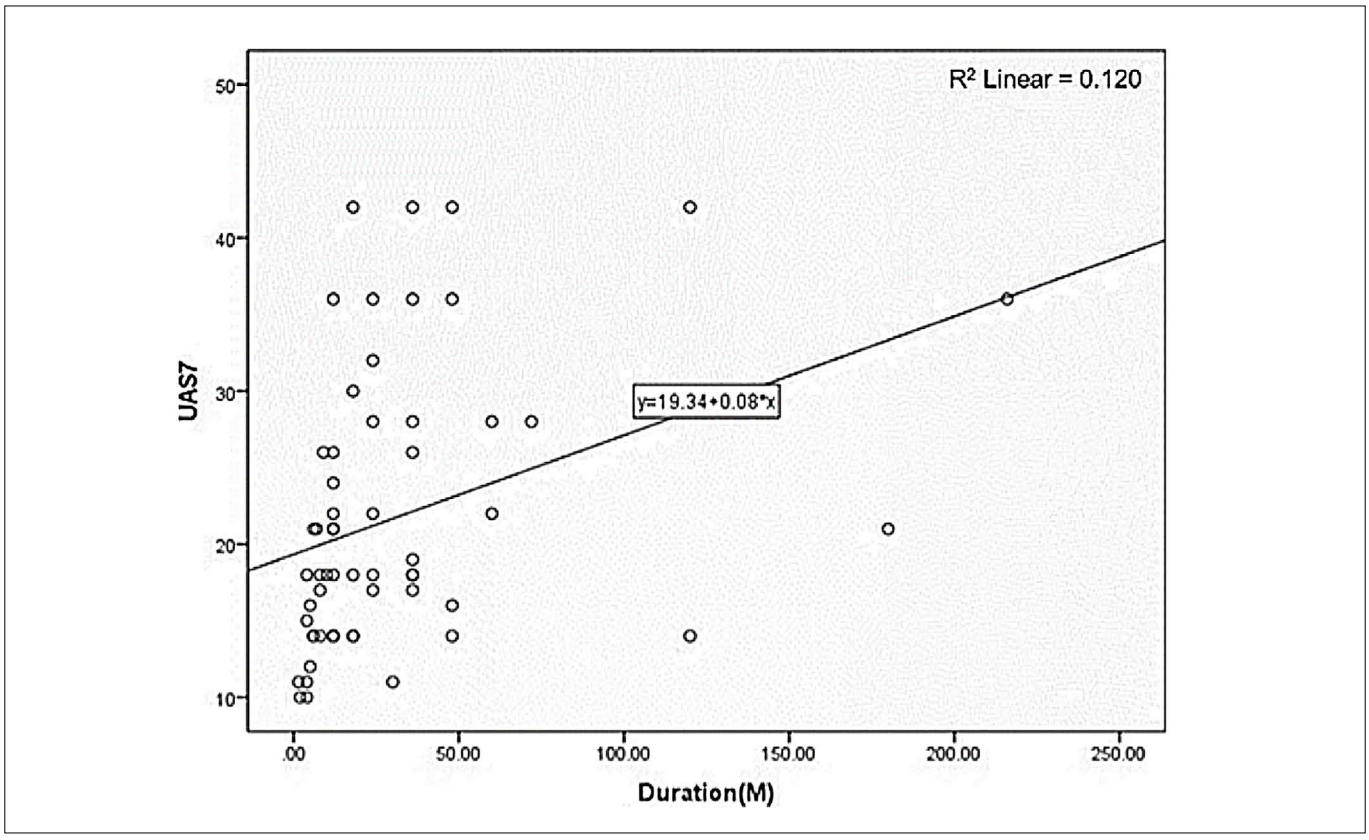

Figure 1 *- Correlation between disease duration and Urticarial Activity Severity Score.*

Parameter	Cases (N =58)	Controls (N = 58)	p value
Age in years (Mean ± SD)	38.5 ± 11.97	38.12 ± 12.15	0.865
Gender [N (%)]			
Male	25(43.1%)	25(43.1%)	1.0
Female	33(56.9%)	33 (56.9%)	
Duration of CSU [N (%)]			
<1 year	27 (46.5)	NA	
1-2 years	11 (19)	NA	
>2 years	20 (34.5)	NA	
Urticarial Activity Severity Score [N (%)]			
Mild (7-15)	17 (29.3)	NA	
Moderate (16-27)	26 (44.8)	NA	
Severe (28-42)	15 (25.9)	NA	
Positive family history	8 (13.8%)	1 (1.72%)	0.015
C-Reactive Protein (C-RP)			
Normal	48(82.8%)	57(98.3%)	0.004
High	10(17.2%)	1(1.7%)	
Serum Vitamin D level			
Normal (30-70 ng/ml)	1 (1.72%)	5 (8.6%)	<0.001
Insufficient (20-29 ng/ml)	5 (8.62%)	34 (58.6%)	
Deficiency (<20 ng/ml)	52 (89.66%)	19 (32.8%)	
Mean Vitamin D level (Mean ± SD)	12.93±5.66	23.30 ± 6.13	<0.001
Thyroid status and antibodies			
Abnormal TFT [N (%)]	4 (6.9)	1 (1.72)	
Normal TFT [N (%)]	54(93.1%)	57(98.28)	
TgAb			
positive	15	1	<0.001
negative	43	57	
TPO Ab			
positive	17	1	0.001
negative	41	57	

Table 1 *- Epidemiologic, clinical and laboratory characteristics of study subjects. p<0.05 is considered significant.*

ASST		Median	IQR	Mann - Whitney U	Wilcoxon W	Z	P
Duration	Positive	36.000	12, 48	169.500	604.500	-3.923	<0.001
	Negative	8.000	5, 24				
CRP	Positive	2.2000	1.13,5.50	320.500	755.500	-1.562	0.118
	Negative	1.0700	0.54,4.50				

Table 2 *- Association of ASST with CSU duration. p<0.05 is considered significant.*

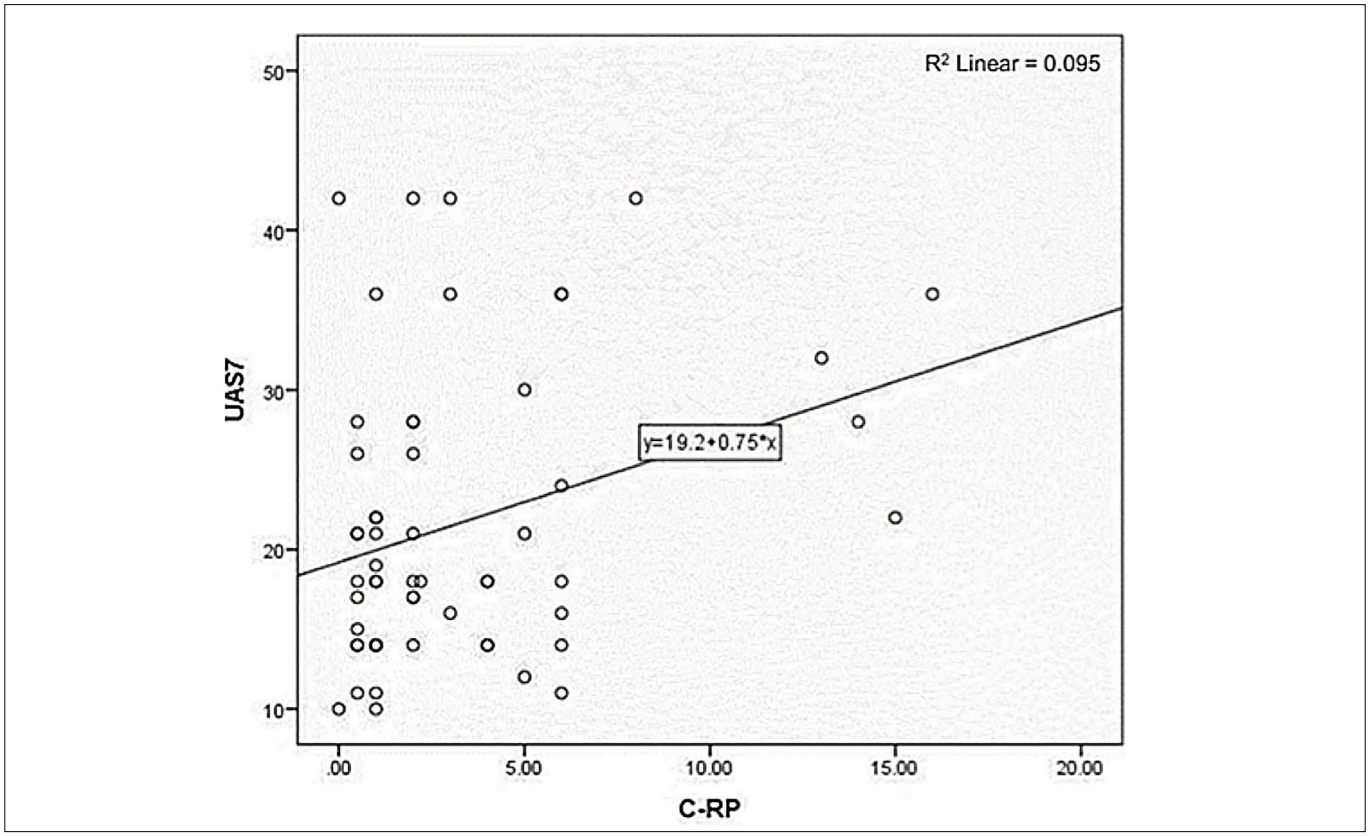

Figure 2 - Association of C-RP levels with severity of chronic spontaneous urticaria.

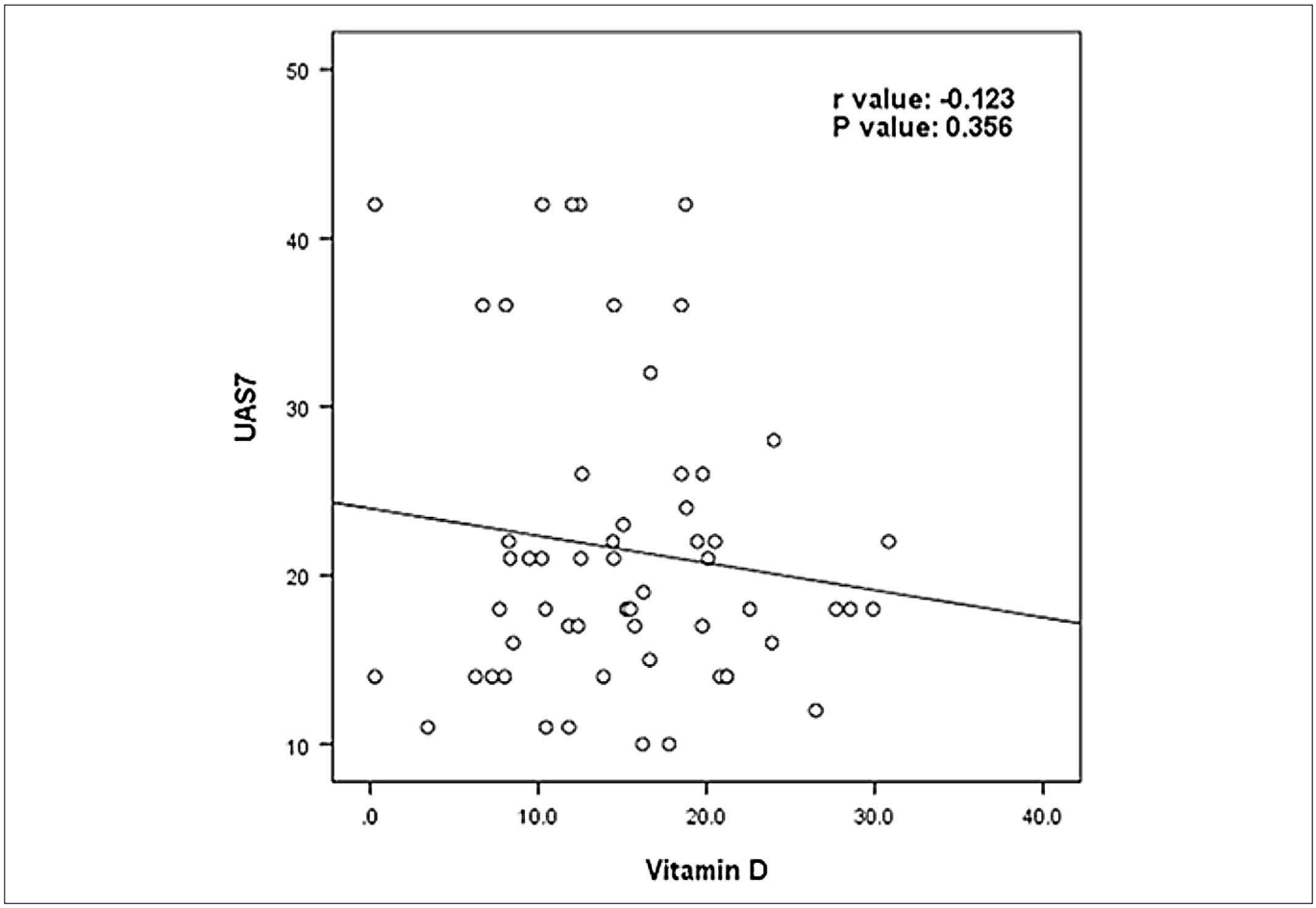

Figure 3 - Absence of any correlation between UAS7 and vitamin D in CSU patients.

TgAb		ASST		Total
		Negative	Positive	
Negative	Count	27	16	43
	%	93.1%	55.2%	74.1%
Positive	Count	2	13	15
	%	6.9%	44.8%	25.9%
Chi square value= 10.881, p =0.001 (sig)				

TgAb		ASST		Total
		Negative	Positive	
Negative	Count	28	13	41
	%	96.60%	44.80%	70.70%
Positive	Count	1	16	17
	%	3.40%	55.20%	29.30%
Chi square value= 18.723, p =0.001 (sig)				

Table 3 *- Association between ASST and TgAb or TPO-Ab.*

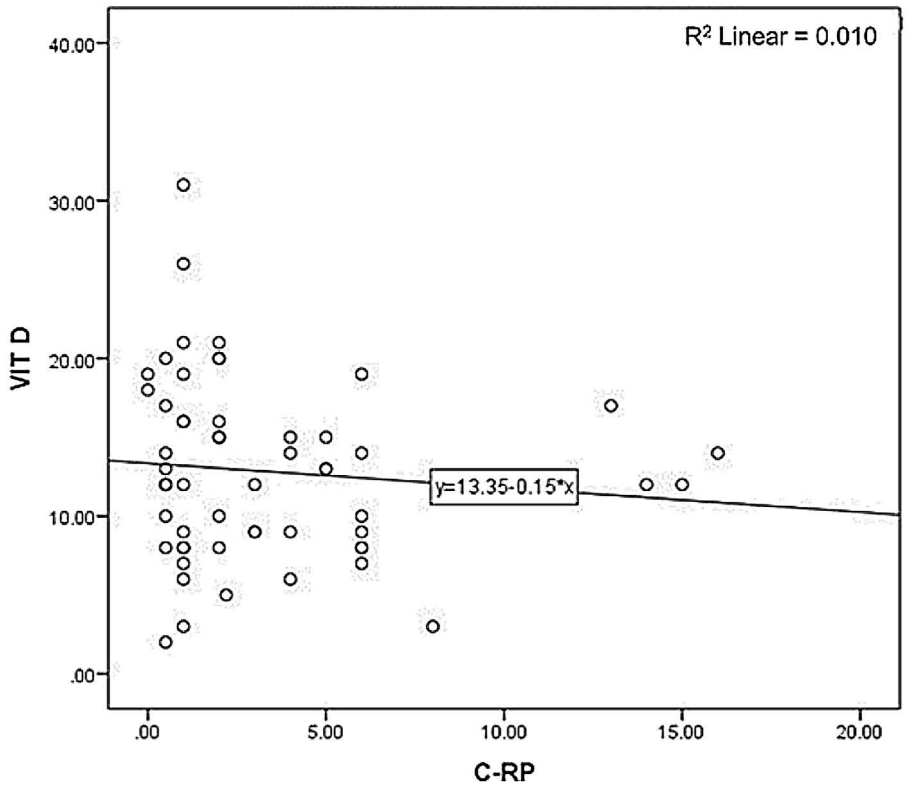

Figure 4 *- Absence of any correlation between C-RP and Vitamin D in CSU patients.*

Discussion

Scientific evidence on the underlying pathophysiological mechanisms of chronic spontaneous urticaria is still evolving. In addition to inflammation and autoimmunity, vitamin D deficiency is proposed as being associated with CSU by some researchers. Vitamin D deficiency is a major public health problem worldwide in all age groups and India is no exception[9]. In this study, serum vitamin D concentration was significantly lower in CSU patients compared to in controls. A study from southern India with a comparable study population also reported significantly lower mean vitamin D levels in CSU compared to control group (12.7 ± 2.7 vs.24.3 ± 13.5 ng/mL, P < 0.0001). It observed higher serum levels of IL-17 and TGF-β1, negatively correlated with vitamin D serum levels. Researchers of this study opined that low vitamin D levels might be the result of systemic inflammation in chronic urticaria and that suggesting reduced vitamin D as the cause for chronic urticaria is implausible, as 50-90% of the Indian population may be vitamin deficient[10]. Abdel-Rehim et al.[11] found significantly low vitamin D levels in CSU. They also noted a negative correlation between IgE and vitamin D. An insignificant correlation was found with age and duration of CSU. However, the study was limited due to the small sample size of 22 cases and 22 controls. Information on vitamin D levels and CSU from various studies is provided in *Table 4*. In this study there was no significant association/correlation between serum vitamin D concentration and disease severity as indicated by the urticarial activity score. This, along with significant low levels in controls, makes the role of vitamin D alone in the pathogenesis of CSU debatable. The therapeutic benefit of vitamin D in CSU may be due to its immunomodulatory effect. Recently there has been a lot of interest in the role of T regulatory cells in this respect[12]. C-RP is an acute phase reactant in the body and it has been listed as one of the first line investigations in the latest guidelines on urticaria management[13]. Our study found the mean C-RP level in cases was significantly higher compared to controls (4.02±6.825 vs 1.08±1.24, P=0.004).

In this study, autoimmunity was assessed using ASST. Positive ASST indicates the presence of functional antibodies. It is a simple and feasible in-vivo test used to measure the histamine releasing capacity of basophils. The prevalence of ASST positivity in chronic urticaria varies from 26.7% to 58% in various studies[14-17]. In this study with the CSU group, 29 (50%) patients tested positive for ASST. There was no difference in vitamin D levels in the ASST positive and ASST negative groups. Our findings were similar to those of the study by Thorp et al.[18]. However, Chandrasekhar et al.[6] found that the mean 25(OH)D level was significantly lower in the ASST positive group and was also correlated with disease severity, unlike in our study. We observed that the mean disease duration in the ASST positive group was 42.52± 44.004 months compared to 18.66 ± 32.623 months in the ASST negative group (p=0.015). This was similar to the studies by Lunge et al.[19] and Bajaj et al.[15] This discrepancy may be due to the autoimmune process in the pathogenesis of autoimmune urticaria, which tends to be refractory to standard treatment.

This study also investigated the association between ASST and anti-thyroid antibodies (TPO Ab and TG Ab), which was found to be statistically significant (p=0.001): nine patients with positive ASST (31%) showed both elevated levels of TPO Ab and TG Ab. Only 4/29 (13.7%) ASST positive patients had deranged thyroid antibody tests. ASST is capable of detecting thyroid autoimmunity based on serum TPO-Ab estimation[19]. With regards to the most prevalent type of thyroid antibody, our study showed a slightly higher prevalence of TPO-Ab than TgAb which is in line with the results of Aamir et al[20]. Our study showed ASST positivity was not found to be significantly associated with thyroid disease (p=0.112). Given that the majority of our CSU patients were euthyroid, it remains to be seen whether these thyroid antibodies have the pathogenic potential to cause CSU or whether they are merely bystanders, and this requires further research.

The key limitation of the study is a higher probability of chance findings, especially in the subgroup analysis, as the sample size was not designed for subgroup analysis. Hence subgroup analysis findings should be evaluated by further large-scale studies. This study was conducted throughout the year and seasonal changes, which may cause variations in vitamin D levels, were not considered. Furthermore, there was no therapeutic trial with oral supplementation of vitamin D for study subjects; antibodies for the diagnosis of other autoimmune diseases were not estimated either.

Study by	Place of study	No. of Cases	No. of Controls	Mean± SD (ng/ml)	p value
Chandrasekar et al[10]	India	45	45	12.7 ± 2.7 vs. 24.3 ± 13.5	p < 0.0001
Thorp et al[18]	US	50	50	29.4±13.4 vs 39.6±14.7	p = 0.016
Movahedi et al[21]	Iran	114	187	14.9±1.8 vs 22.6±1.6	p = 0.005
Woo et al[22]	Korea	72	72	11.86±7.16 vs 20.77±9.74	p <0.001
Present study	India	58	58	12.93±5.66 vs 23.31±6.13	p<0.001

Table 4 *- Vitamin D status and CSU in various studies.*

Conclusions

The findings of our study indicate an association of low vitamin D with CSU but not with its severity. C-RP levels were significantly higher among the CSU group of patients but there was no correlation with severity. There was a ASST positivity rate of 50% in our study and ASST was significantly associated with thyroid antibodies. We feel ASST could be used as a simple screening test for autoimmune urticaria in resource-poor settings without the need for expensive antibody testing. Further studies are required to 1) to check for various antibodies present in the serum of CSU patient and their association with disease severity and ASST and 2) to ascertain the effect of vitamin D supplementation in vitamin D deficient CSU patients.

Disclosure

All the authors declare that there is no conflict of interests in the subject of study and the source of any financial or material support.

Conflict of interest

None.

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aesthetic medicine

Original Article

Botulin toxin purity: the basis of the conscious choice of patients

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Short title: Choosing botulinumtoxin purity

Abstract
Botulin toxin is an established treatment for smoothing out dynamic facial wrinkles. It is widely used in the treatment of facial lines, mostly frontal, glabellar and crow's feet lines.
Our study aims to show that the purity of an injectable neurotoxin is a quality desired by patients and may determine their preferences.
We interviewed 4029 European women in Germany, Great Britain, Italy and Russia.
70% were already "users" of the botulin toxin for aesthetic purposes and 30% were "intenders", i.e. women intending to use the toxin in the next 12 months.
A large number of them had already undergone 2-4 treatments in the last couple of years and declared their willingness to be treated again in the next 12 months.
Despite this, only a few were aware of the differences between various neurotoxins and their purity.
Patients rely on their physicians, friends, relatives and websites.
They would appreciate more information on product composition, short and long term efficacy and costs.
Our study shows that only 21% of patients were aware of the real differences between various botulin toxins. Some (54%) were aware that immune responses to the toxin may reduce its clinical efficacy and that this can be influenced by its purity. When adequately informed, up to 86% of patients declared to be interested in purity. We conclude with the finding that 82% of patients considered toxin purity a determining factor when choosing treatment.

Keywords
Botulinum toxin, immune response, ageing process

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Introduction

Three different botulin toxins have been available in Europe over the last 10 years: incobotulinum, onabotulinum and abobotulinum[1,2].
They show comparable efficacy in the treatment of lines in the upper part of the face, with dose ratios of 1:1 between incobotulinum and onabotulinum and of 1:2,5 between inco/onabotulinum and abobotulinum[3-8].
Treatments have been extended to other parts of the face, i.e. the median/lower and inferior, with good results, also in combination with fillers.
Patients rely on their physicians when selecting a product, but during preliminary encounters they are not informed on two fundamental aspects of the toxin: its immunogenicity, i.e. how the immune response of the host may inactivate it, and the purity of the molecule to be used.
The different purity levels of various botulin toxins has been shown[9]: some contain nothing but the toxin (incobotulinum), while others contain accessory molecules (onabotulinim and abobotulinum)[10] which may induce an immune response[11-16]. Our study aims to elucidate to what extent the purity of these molecules determines patient choices.

Materials and methods

We interviewed 4029 women in four European countries (Germany, Great Britain, Italy and Russia) over the phone by means of a questionnaire requiring approximately 15 min (*Figure 1*) of their time.

Seventy percent of women declared themselves to be USERS, i.e. they had been treated at least 3 times with botulin toxin in the last 2 years, while 30% could be considered INTENDERS, i.e. patients who had been treated only once in the previous two years or intended to undergo such treatment in the following year.

Results

3083 patients (*Figure 2*) had been treated with a botulin toxin, starting at an average age of 34 years. 35% of them had undergone two treatments in the last three years, 24% three treatments and 19% four treatments, while 95% had undergone treatment at least once in the last year. Frontal, glabellar and crow's feet lines were the most frequently treated areas, respectively in 65%, 45% and 45% of cases, much more than for nasolabial folds (29%) and lips (26%). The majority of patients (86%, *Figure 3*) were highly satisfied with botulin toxin treatment and in 66% of the cases results were seen in the first seven days following treatment.
Based on these satisfactory results, 88% of the patients declared they would undergo further treatment in the next 10 years. Patients were also asked to disclose their main sources of information, which they considered most reliable, and their main unanswered questions (*Figure 4*). Patients' physicians were the main source of information (65% of cases), followed by the web (48%) friends or relatives (48%), social media and brochures (32% and 27%). However, doctors and friends were considered more reliable than social media and brochures, TV programs, websites or newspapers.

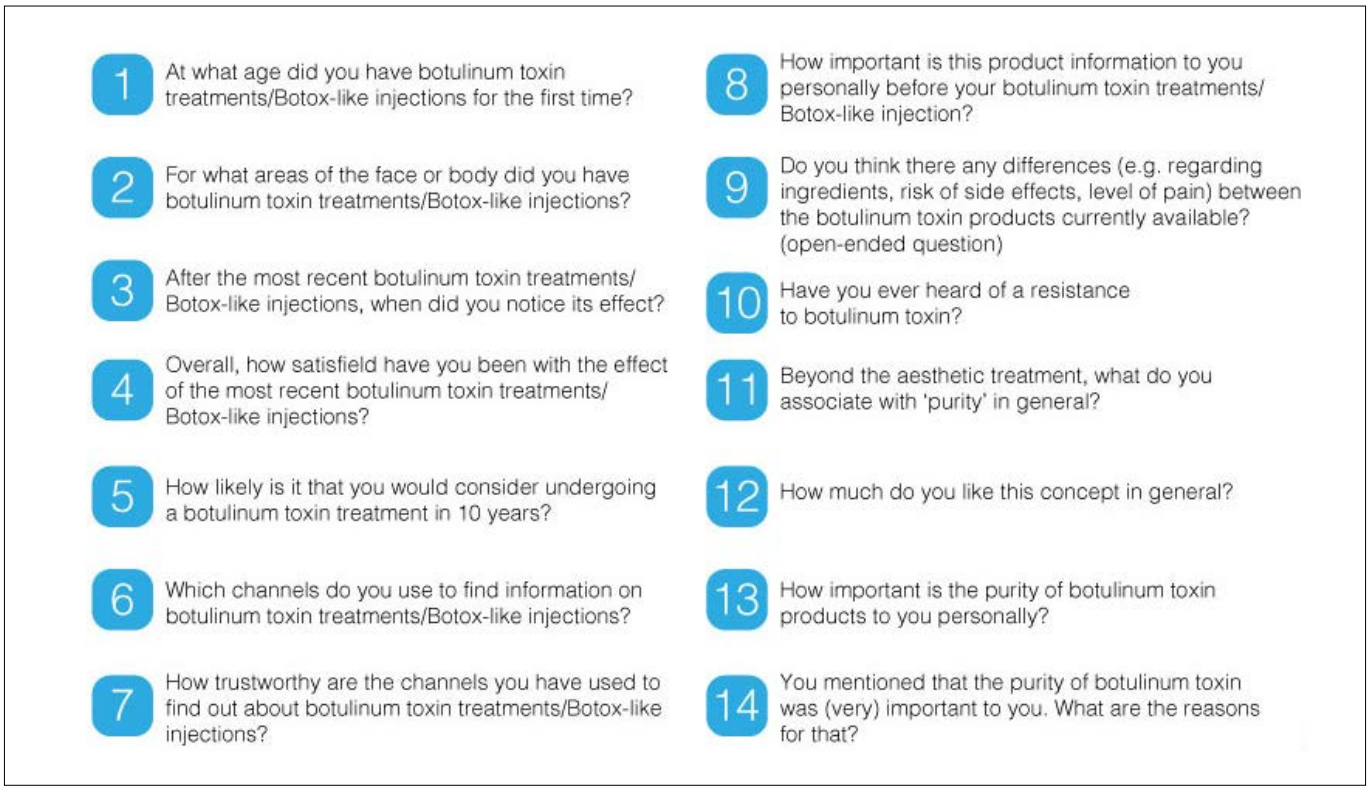

Figure 1 *- Questionnaire submitted by patients.*

The interview then focused on the patient's interest in and knowledge of various products (*Figure 5*). Results indicate that patients are interested in the duration of the effects, authority approvals, the active molecule and costs. They are less interested in the specific brand, production site origin, pain and onset of effect.

Figure 6 shows that 56% of patients are not aware of differences between different toxins and while remaining ones realize that there are differences, only 21% actually know what they are.

Patients were asked if they had knowledge of possible resistance to botulin toxin (*Figure 7*). Answers differed according to country. In Italy, 56% of interviewed patients were aware, compared to just 42% in Germany.

Patients were then informed about product purity, for botulin toxin in particular, and on implications:

- Botulin toxin is used to treat facial lines and its efficacy vanishes between 4 and 6 months after treatment, thus all patients will undergo several treatments per year and throughout their life
- Most toxins contain complexing proteins which are not necessary for therapeutic effects but can elicit immune responses
- Such immune responses are more likely to develop after repeated treatment and may interfere with clinical efficacy
- Thus, products containing less complexing proteins are less likely to induce the production of antibodies and lose effectiveness over time

Figure 8 shows the extent to which patients understood and acknowledged such principles. 82% of patients agreed and understood why purity is a highly desirable property. *Figure 9* shows how patients evaluated the importance of purity on a scale from 1 to 5.

It was considered a very important factor by 86% of patients.

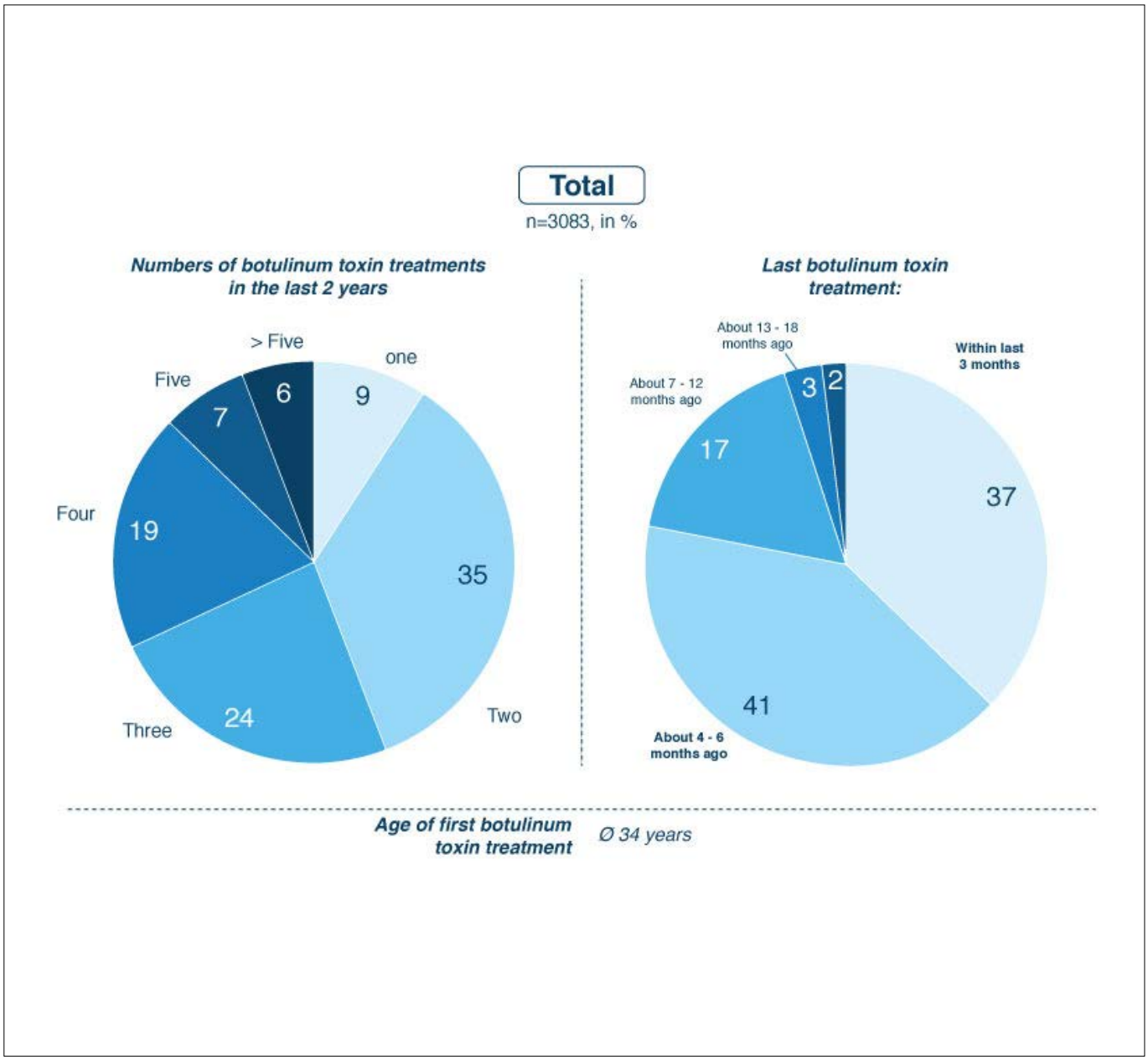

Figure 2 *- Numbers of treatments during the last 3 years in 3083 patients (first Botulinum treatment: mean 34 years-old).*

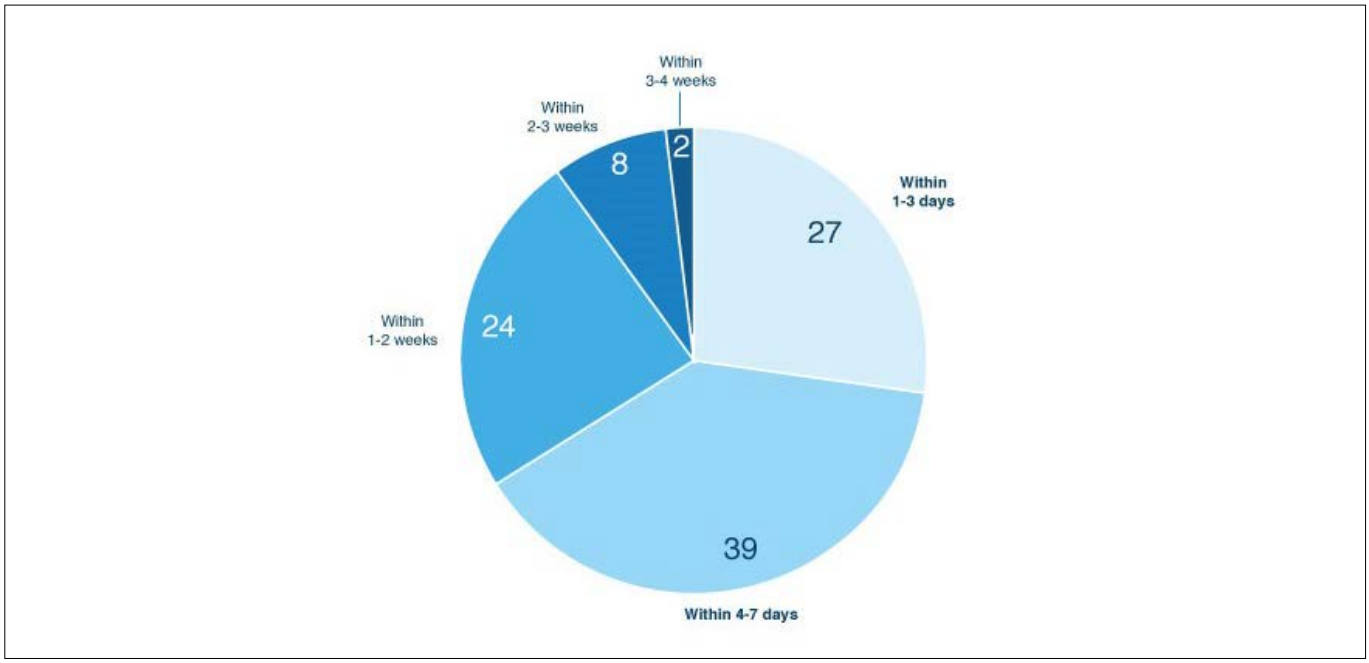

Figure 3 *- Highlight of botulinum toxin effect, patients%.*

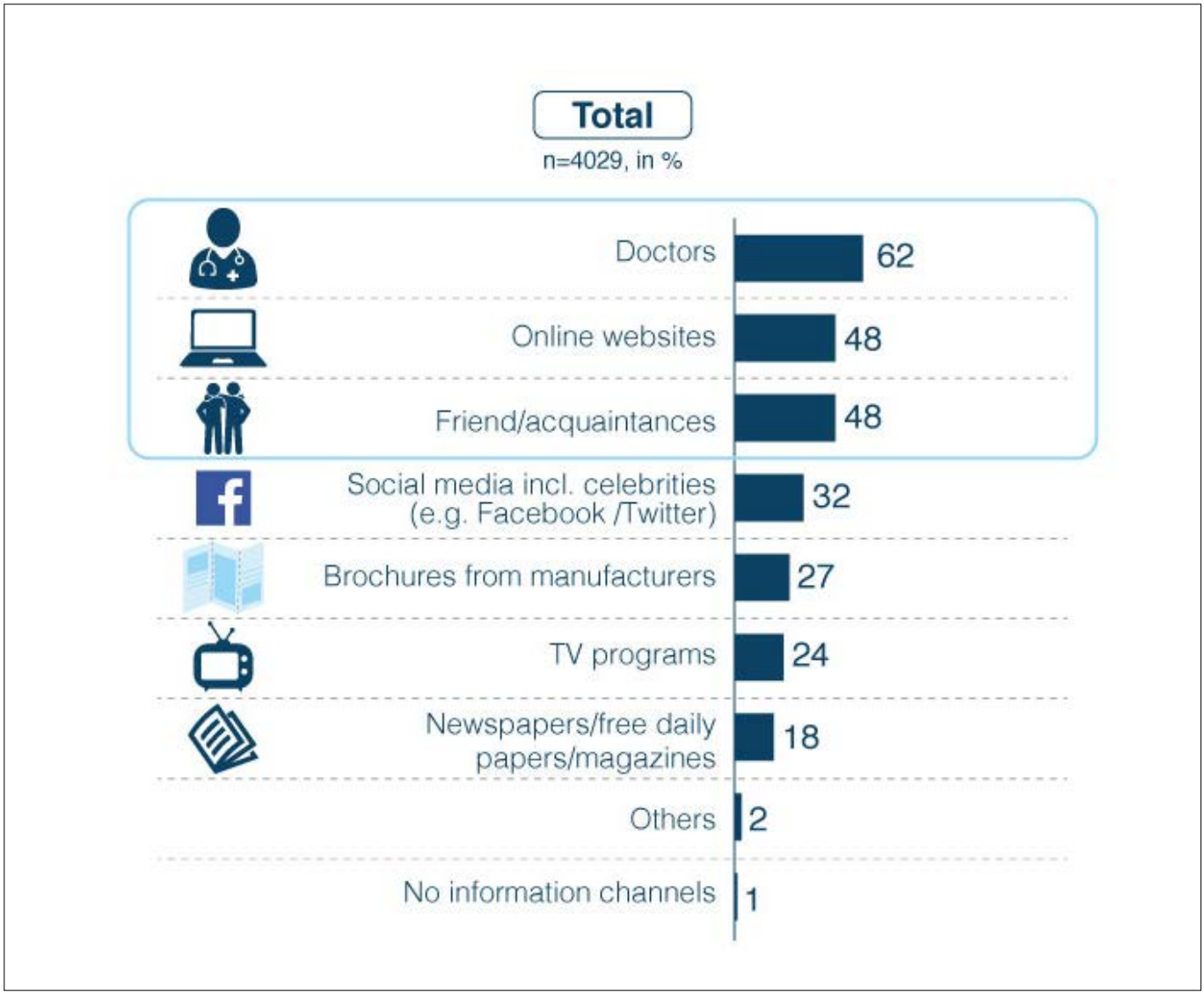

Figure 4 *- Percentage evaluation information request by patients.*

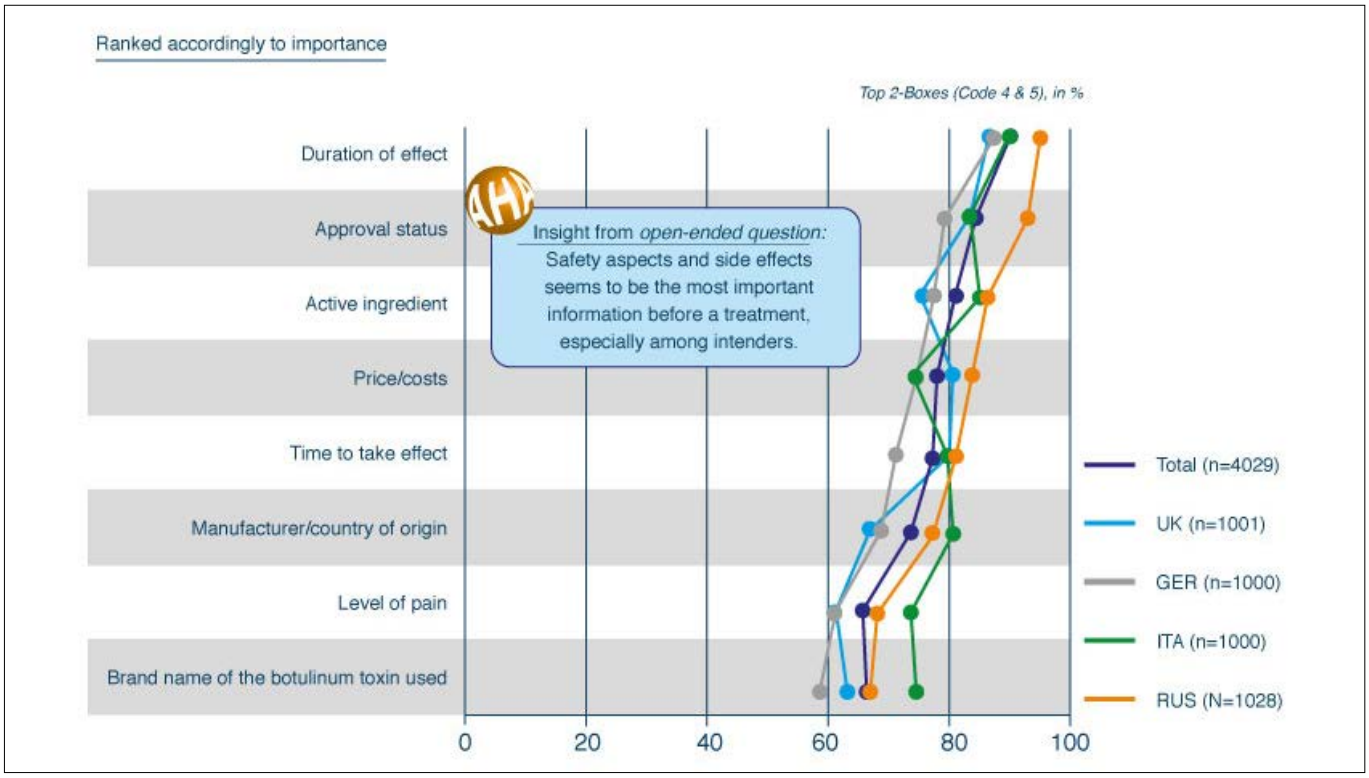

Figure 5 *- Most important factors for patients in product comparison.*

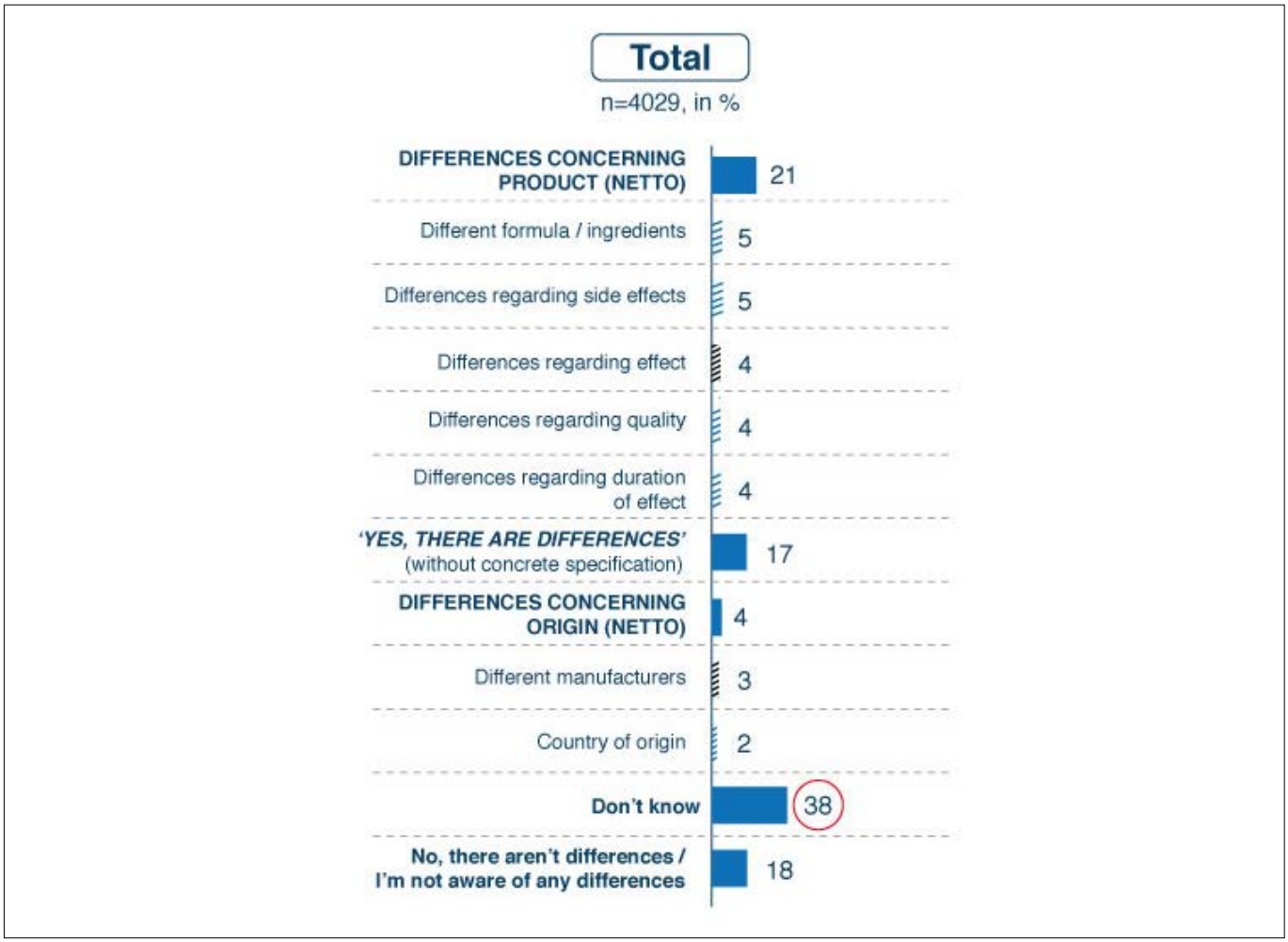

Figure 6 *- Patients' knowledge of toxin differences.*

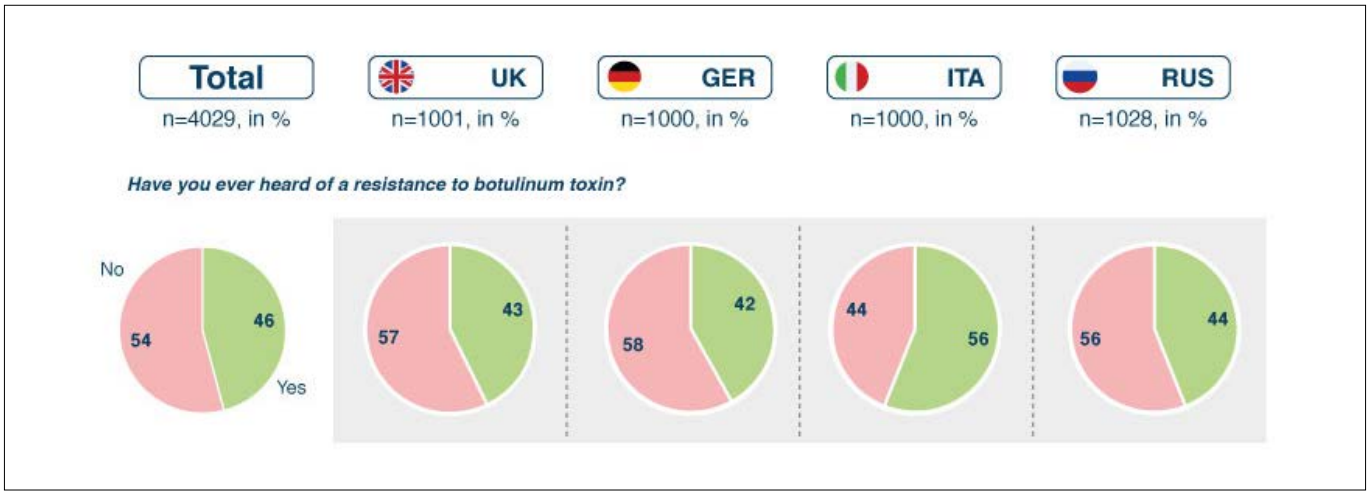

Figure 7 - *Awareness of botulinum toxin resistance.*

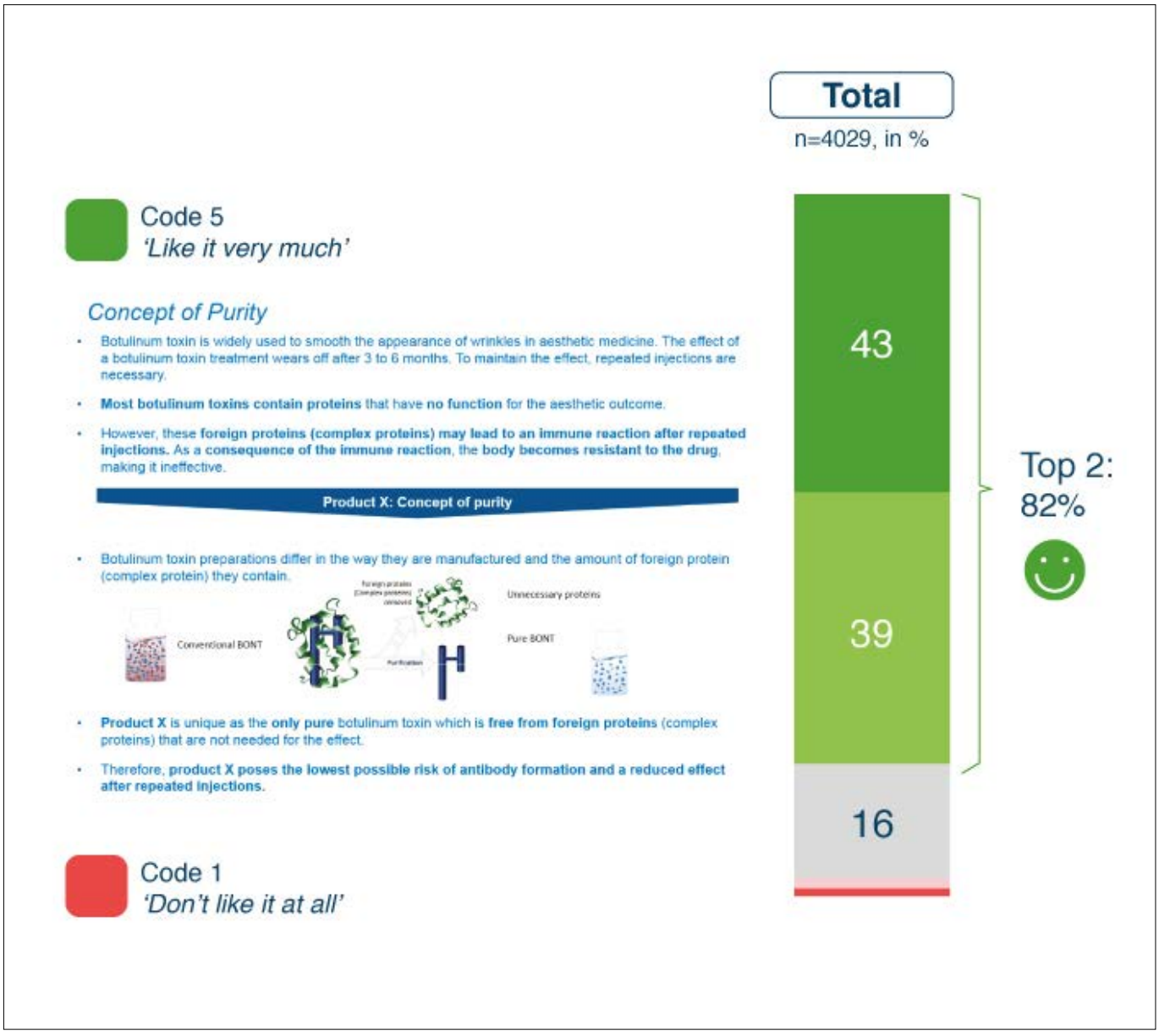

Figure 8 - *From 1 to 5 evaluation - 82% of patients like purity.*

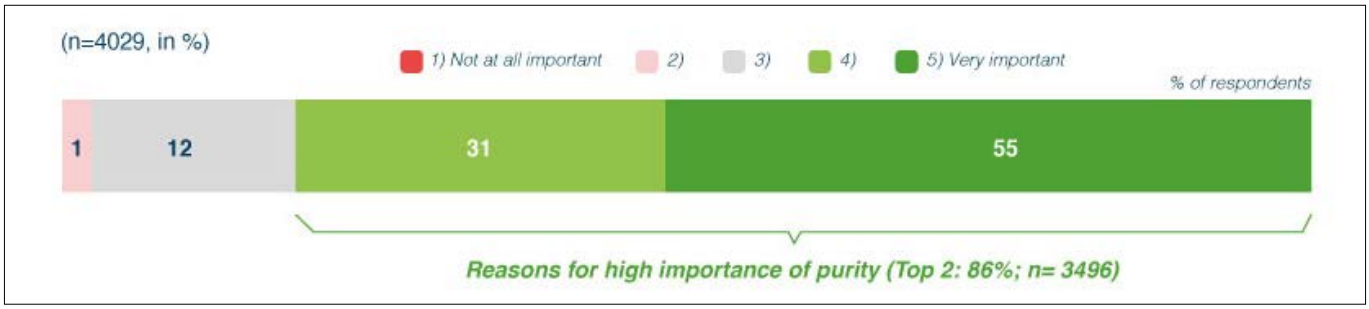

Figure 9 *- Importance of purity for patients.*

Discussion

It is known that three different toxins are available on the market: incobotulin, onabotulin and abobotulin.
Their efficacy on facial lines has long been proven and their activity is overimposable, as shown by various studies. Clinically, the dose ratios is 1:1 for inco and onabotulin and 1:2.5 for inco/ona and abobotulin.
The main difference lies in the molecule formula. Incobotulin is a 150kD protein and contains botulin toxin only, whereas abobotulin and onabotulin also contain accessory proteins. These proteins play no role in the activity of the botulin toxin but may induce immune responses and the formation of antibodies, which may reduce toxin activity[17-20].
In this study we interviewed 4029 patients; 70% were users and 30% intenders, i.e. persons who might have received treatment in the previous three years or intend to undergo treatment in the following year.
We know that the majority of treatment is applied to frontal, glabellar and crow's feet lines, much more frequently than to lips or nasolabial folds. Therefore botulin toxin is important for facial rejuvenation.
We focused on botulin toxin since 35% of those interviewed had undergone at least two treatments in the previous two years, 24% three and 19% four. The vast majority of patients reported being satisfied already 15 days after receiving the drug, due to a visible effect on line reduction, muscle relaxation and wrinkle distension.
The study indicates that patients wish to be informed, and are informed mainly by their doctors (62%) friends and relatives (48%) and through the web (48%). Other sources of information include social media, brochures, TV programs and newspapers.
The search for more knowledge is strictly related to the reliability of sources and patients realize that physicians, friends and relatives, are clearly more reliable when seeking advice on the duration of effects, product certifications, rapidity of onset and costs.
Trust is such that 56% of patients are not aware of the fact that botulin toxin differs and only 21% know that the composition of the products also differs.
The possible onset of clinical resistance to the toxin is not clear to patients (54% are not aware of such a possibility). This could either be due to a lack of information or to a lack of attention to such explanations.
Nonetheless, the answers to the survey indicate that patients may be willing to be informed on product purity. When patients are correctly informed on how the purity of various products may determine a reduced response due to the presence of antibodies, 82% showed an interest in such aspects and this may determine product choice in 86% of cases.

Conclusions

We know that there is increasing demand for botulin toxin treatment and that its efficacy is highly appreciated. Patients are rather unaware of qualitative differences between the products they are using, of differences in purity, and of possible side effects, such as the appearance of antibodies, which may reduce treatment efficacy. Patients are informed by their doctors, friends and relatives, who are also considered the most reliable sources. Patients showed an interest in product purity and this could determine their choices.

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Conflict of interest

There is no conflict of interest, and no funds were received for this article.

Disclosure

KOL of Merz, I have received no funds for the article.

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aesthetic medicine

Review

Pharmacokinetic properties of botulinum neurotoxin agents in Aesthetic Medicine

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Abstract
The pharmacokinetic profile of BoNT/A-based agents directly affects treatment doses and regimens, their safety, action onset rate and duration. However, complex BoNT interaction with nerve terminals, in minimal yet highly biologically active concentrations, presents considerable challenges when studying various aspects of BoNT/A pharmacological action. This review discusses major pharmacokinetic parameters for botulinum neurotoxin type-A (BoNT/A) metabolic transformations: distribution, diffusion, migration, neutralization and excretion as well as specifics for post-BoNT/A neuromuscular transmission restoration and factors influencing it.

Keywords
Botulinum toxin type A (BoNT/A), BoNT/A diffusion, BoNT/A distribution, nerve sprouting, BoNT/A degradation

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Introduction

Botulinum neurotoxin type-A (BoNT/A)-based agents have radically transformed the management of various neurologic pathologies and truly revolutionized aesthetic medicine while the list of disorders where they are proving their usefulness and effectiveness is continuously expanding. However, our knowledge of such treatment pharmacodynamics and pharmacokinetics remains incomplete and fragmentary. Not least due to the unprecedented complexity of studies in the field since not only therapeutic but even toxic BoNT/A doses are extremely small, all too often making the application of conventional pharmacological analytical techniques impossible.

Complex process of spatial distribution in tissues, receptor acceptance and conformational and metabolic transformations start at the very moment BoNT/A solution leaves the needle lumen. This process is defined with three constituents: drug solution distribution, BoNT/A molecule diffusion from injected solution and migration to remote body regions.

Spread

Solution distribution means rapid physical toxin translocation from the initial injection site. The distribution area and rate are defined by a wide range of variables depending on injection technical features, introduced solution volume and its conformance with target tissue capacity[1] as well as injection site anatomical and physiological features. In particular, BoNT/A solution spread is influenced by muscle fibre architecture, its spatial and functional bonds with surrounding tissues[2] as well as the presence of intermuscular fascia and connective tissue partitions[1]. Many researchers have noted that the spread of solution depends extensively on the following injection technical features - needle calibre, injection speed, course and depth as well as the way total agent dose for one muscle is distributed among injection points[3]. Finally, BoNT/A solution spread may also depend on other, additional factors – target tissue injury due to thick needle use, hematoma at the injection site, applied pressure, tissue massage, thermal impact, etc.[1,4].

Diffusion

BoNT/A molecule diffusion is relatively slow and dispersion takes place in a matter of dozens of minutes to beyond the initial injection site[5] when toxin molecules move passively from higher concentration areas to lower concentration ones until equilibrium is reached. Terms of BoNT/A diffusion and distribution are frequently interchanged, resulting in terminological confusion.

The most significant factors affecting BoNT/A molecule diffusion area are concentration and dose, i.e. the number of molecules to be introduced in a specific volume. The Borodic et al. (1994) studies on rabbits showed that when small doses are administered (1 IU), the diffusion gradient collapsed to 15-30 mm, whereas with a dose of 5-10 IU, BoNT/A diffused along the entire muscle, with no evident end point[6]. Moreover, BoNT/A molecules are able to penetrate through muscular fascia into adjusted muscles, thus weakening them[1,7].

Therefore, high doses entail higher risk for adverse events[7]. Larger doses, if distributed as small portions per target muscle, may prevent this[1,6,8].

Unlike concentration, vehicle concentration and dose affects agent distribution rather than diffusion. Hsu et al. (2004)[9] showed that when the same BoNT/A dose (5 U) is injected into forehead muscle, a 5-fold vehicle volume resulted in myorelaxation area expansion of about 50%. The myorelaxation area expanded due to enhanced solution distribution, while decreased toxin diffusion was not of significant value.

Do BoNT/A-based agents differ in terms of distribution and diffusion features? In their studies de Almeida and De Boulle (2007) compared the diffusion properties of OnaBoNT-A vs. AboBoNT-A after intradermal forehead injections of equal agent volumes with dose ratios of 1:2.5; 1:3 and 1:4, respectively[10]. The authors concluded that AboBoNT-A diffusion is higher, however in their critical essay Pickett et al. (2008) stated that the expanded anhydrosis area is a result of varying doses being injected rather than of different diffusion properties of agents studied[11].

Regarding adverse event risks that may be related to the higher diffusion capacity of one agent compared to another, the recently published cross-sectional analysis of the US Food and Drug Administration (FDA) reports on adverse events after BoNT/A-based therapies in aesthetic medicine patients from January 2014 until September 2019[12] showed that out of all documented 29471 BoNT/A-related adverse events in aesthetic medicine procedures for patients with eyebrow/eyelid ptosis amounted to 1783 cases (6.1%). Such events developed more frequently with OnaBoNT-A (6.4%) vs. AboBoNT-A (4.2%) or IncoBoNT-A (5.7%)[12]. Thus, the reporting rate for said adverse event does not indicate higher risks for lower molecular weight agents and depends mainly on injection protocol deviations. BoNT/A-based agent reconstitution was found to result in virtually momentary neurotoxin complex dissociation on active neurotoxin and complexing proteins, giving rise to discussions on diffusion differences among BoNT/A-based agents[3,11]. Therefore, there are no difference in agents with different neurotoxin complex size (OnaBoNT-A и AboBoNT-A) or those containing purified neurotoxin only (IncoBoNT-A), and dose titration can easily overcome any differences related to therapeutics and their diffusion profiles[13].

Retrograde axonal transport and systemic effects

When a very high BoNT/A dose is administered instantaneously, the third toxin distribution mechanism might be involved - it migrates to remote body regions. Many authors consider the presumable advance of BoNT/A light chain along nerve fibbers as retrograde axonal transport[5,8]. This phenomenon may explain systemic and distant effects which develop after local BoNT/A injection[8]. On the other hand, it is not always possible to discern differences between local and systemic BoNT/A distribution on a clinical basis. For example, headache and dysphagia can be signs of both systemic botulism and local post-injection toxin spread in the neck region[12].

Haematogenic distribution followed by distant effects is possible if significant toxin amounts enter systemic circulation. This may occur only in cases of very high doses accidentally injected into a blood vessel[8].

BoNT/A pharmacokinetics and distribution studies with radioactive label I125 after single injection in rats and rabbits showed that almost the entire toxin amount remains locally at the injection site[14], confirming its local action on cholinergic nerve terminals[15].
Already in the 70's several animal studies[16] proved retrograde BoNT/A transport. In addition to central effects, retrograde axonal transport can lie behind BoNT/A action on antagonistic or contralateral muscles, as confirmed by electrophysiological studies[17-19].
Clinical observations discussing systemic BoNT/A effects are related to application in neurological settings and for hyperhidrosis treatment, involving high and ultra-high doses[19-21]. When administered in doses recommended for aesthetic medicine, BoNT/A only acts locally[13].

Time to onset of response and duration of effect after BoNT/A injection

Time to onset of response and its duration are key factors determining patient satisfaction with treatment[22,23]. However, extended latent period to first signs of muscular relaxation (several hours to several days) and gradual paralysis development impedes the exact identification of BoNT/A effect onset (*Figure 1*). Factors acting upon time to onset of response and its duration are as follows: anatomy features and functional status of the toxin-injected muscle[22,25,26], BoNT/A injection precisely next to motor end-plates[4], patient's clinical parameters, injected agent dose and properties[22,24,25].

Multiple clinical studies indicate that relaxation rate of various mimic muscles in the same patient is different[22]. It might be related to physiologic status dissimilarities in different muscles. Ones which contract intensely[4,26] or are hypertonic[25] have been found to bind BoNT/A significantly more actively, facilitating muscular relaxation and extending the duration of the effect[4,26].
Therefore, the recommendation for active muscular contractions several minutes post-injection has its rationale. Current publications are quite contradictory regarding the effect development rate and post-injection duration for various BoNT/A-based agents. Some authors claim that relaxation appears faster and lasts longer for ABOBoNT-A[22], while others opt for INCOBoNT-A[24]. However, the majority of independent researchers tends to believe that there is no significant difference in effect development rates and duration between treatments and that all have a well-established action duration of weeks to months, while some therapeutic effects may remain for up to 1 year[25]. Whatever commercially available agent is used, the development rate for neuromuscular block and its duration are determined by the amount of BoNT/A injected: the higher the dose, the shorter the time interval for muscle relaxation. Predicted BoNT/A effect duration is governed by four major constants: BoNT elimination rate from the circulatory system, L-chain elimination rate from nerve endings, time needed for intact substrate synthesis (SNAP-25 for BoNT/A or synaptobrevin 2 for BoNT/B) and sprouting activity (*Figure 2*).

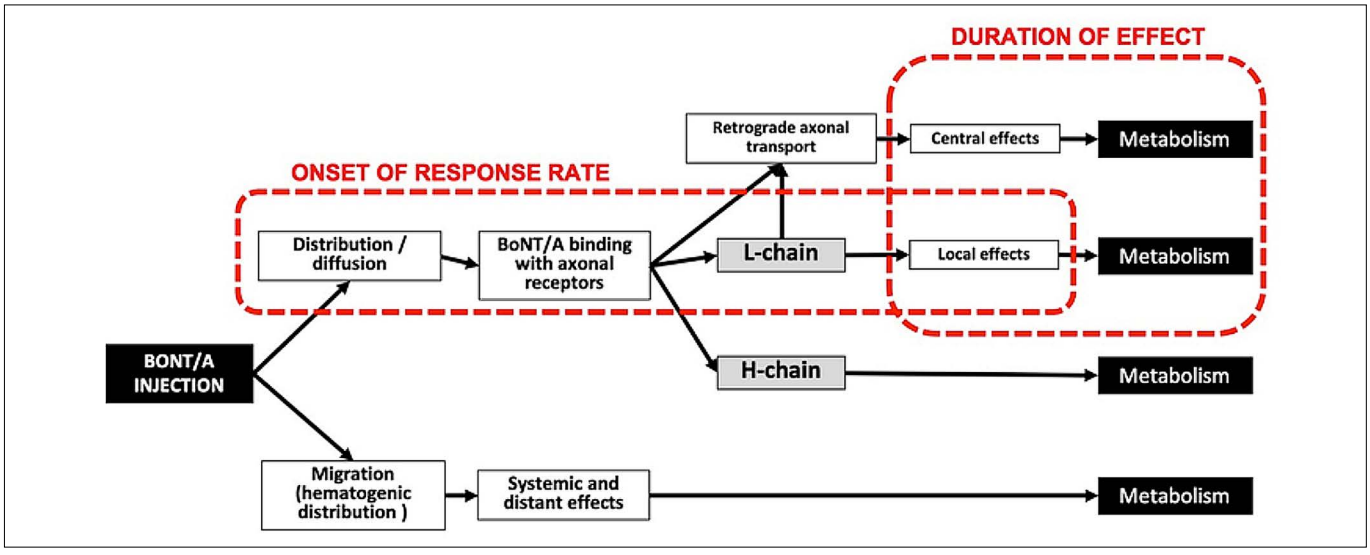

Figure 1 *- Key pharmacokinetics factors determining the onset of response rate and duration of the effect of BoNT-A*

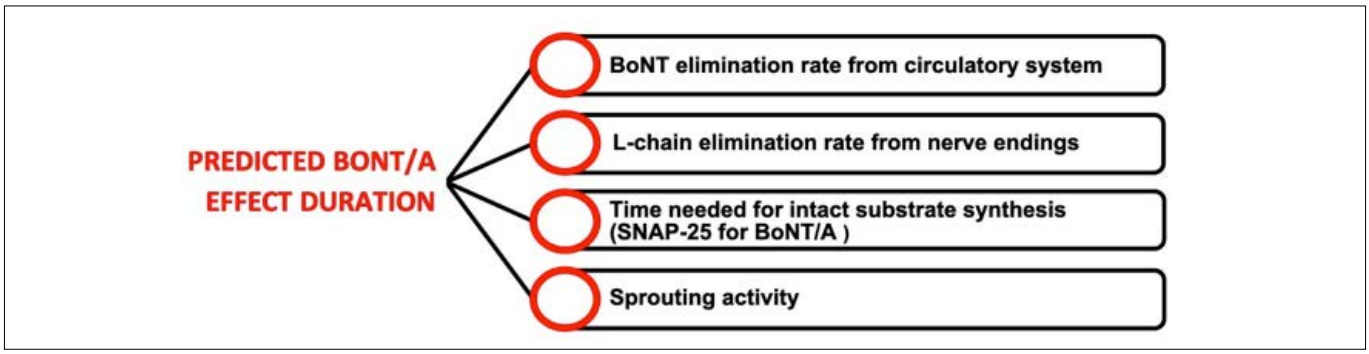

Figure 2 *- Factors determining BoNT/A effect duration.*

As previously mentioned, intramuscular use and recommended doses result in local toxin binding with virtually none of it in systemic blood flow[13]. Nevertheless, to understand BoNT/A pharmacokinetics, all possible metabolic options should be considered at whole-body level. Current data show that once the toxin enters the systemic blood flow it remains there as an intact structure and active form until it reaches target cells or is eliminated[15,27]. There is no evidence for toxin uptake and accumulation in blood cells or its proteolytic cleavage, since the toxin remains in circulation, structurally and functionally intact[27].
Another potential way to eliminate the toxin might be its excretion with urine. However, the molecular weight limit for renal filtration is about 50-70 kDa, whereas the molecular weight of an entire neurotoxin molecule is 150 kDa. Therefore, it cannot be excreted by healthy kidneys. Finally, the third and most likely mechanism of metabolism and elimination is liver biotransformation. Experimental data do not confirm any considerable liver and spleen involvement in BoNT/A biotransformation[27]. Therefore, in essence the whole botulinum neurotoxin molecule is not metabolized at body level, rather it is rapidly eliminated from circulation, binding to receptors in neuromuscular junction regions. After intramuscular injection this happens even faster and in a more complete way. So, its journey after binding with receptors is of significantly greater interest.

Intracellular metabolism for light and heavy botulinum neurotoxin chains

Upon entering axons, BoNT/A breaks into H- and L-chains. Experiments showed BoNT effect duration is only determined with particularities of L-chain intracellular behaviour and metabolism[28]. Immediately after entering cytosol BoNT/A LC binds with the internal plasmalemma surface[29], fixed next to its substrate. On the one hand it provides more effective SNAP-25 protein cleavage and on the other hand, it decreases LC availability to proteolytic degradation systems in cytosol which in turn helps it to remain enzymatically active for several months after entering the cell. The main mechanism for L-chain metabolism is ubiquitin-proteasome system in cytosol. Various BoNT serotypes have different toxic action durations on neurons, ranging from several days to several months[30]. Currently it can be stated with confidence that effect duration for one or another serotype is determined to a large extent by proteasome-related light chain degradation features[31]. Topmost proteolytic activity duration for BoNT of serotype A vs. other serotypes is determined by its capacity to avoid ubiquitin-proteasome degradation for a long time[31].
The duration of the effect of various serotypes is also affected by differences in their proteolytic effects. BoNT/A LC was found to cleave only 9 amino acids from SNAP-25. It is of crucial significance, since minor SNAP-25 molecule damage does not disturb its binding to other SNARE-complex proteins but works as a blocking component for “neuroexocytosis nanomachinery” and prevents intracellular proteases from metabolizing damaged proteins[29].
Some researchers claim that after being internalized into neurons, BoNT/A HC undergoes endocytosis in autophagosomes, but part of it remains localized in endosomes and does not participate in the mechanism of BoNT / A cytosol toxicity. Some fractions of BoNT / A HC are sent to lysosomes for degradation[32].

Reinnervation

Sprouting, i.e. the formation of new terminals, is commonly considered as the main mechanism for neuromuscular conductivity restoration[33].
Currently some researchers doubt that sprouting plays a leading role in functional neuronal restoration[25,34], since neuromediator release restoration in areas of new terminal area vs. initial terminals takes place in roughly the same length of time; moreover, over 80% of the entire acetylcholine amount is released exactly from initial terminals[34]. Are botulinum neurotoxins neurotoxic? *In vitro* experiments the toxicity of BoNT of serotypes C[35] and E[36]. However, botulinum neurotoxin concentrations significantly exceeding the ones causing botulism were applied. For BoNT/A cytotoxicity was not documented, even in experimental settings using both cell cultures and electrophysiological studies in healthy volunteers[37]. Besides, vast experience of therapeutic application of agents containing BoNT of serotypes A and B for various indications shows no signs of neuronal damage even in cases of regular use over many years[3,38,39]. It appears that neuromuscular conductivity may be restored time and time again without any NMJ function loss or disturbance, justifying the safe use of repeated BoNT/A injections for treating humans.

Conclusion

Processes discussed herein reflecting the pharmacological features of BoNT/A- based agents are of huge clinical significance since they enable the specification of patient management and treatment regimens, safety and effectiveness. Quick and specific BoNT/A binding to its receptors, reversible paralytic effects indicate high safety of such agents in cosmetology if dosed as recommended and for approved indications. Studies on fine mechanisms fundamental for BoNT/A metabolism and neuromuscular transmission restoration open up new frontiers in the search for means to managing these processes. BoNT clinical application has huge potential for implementation in multiple fields of medicine as we gain new knowledge on its mechanisms of action and pharmacological features.

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aesthetic medicine

Review

Dermal fibroblasts and keratinocytes under normal and aging conditions

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Abstract
Fibroblasts and keratinocytes represent the main structural elements of the skin. The main types of their functional activity are the synthesis of the intercellular matrix, cytokines, chemokines, growth factors and other biologically active molecules, as well as participation in the reactions of native immunity and supporting the activity of adaptive immunity cells. The interaction between fibroblasts and keratinocytes mutually modifies their functional, immunobiological and synthetic activity. Aging results in a significant decrease of cells number and capacity. Accumulate senescent cells, especially fibroblasts, receive the senescence associated secretory phenotype (SASP) and already act as senescent secreted cells (SMS), producing a large number of cytokines, the spectrum of which is very different from that of presenescent cells. The action of these substances contributes to the development of chronic inflammatory processes and the deepening of involutional skin changes. Research into the outlined processes supports development of new skin disease treatment methods and enhances knowledge of aging mechanisms.

Keywords
Dermal fibroblasts, keratinocytes, stem cells, senescent secretomas

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Origin and properties of dermal fibroblasts

The effective impulse for studying the nature of dermal fibroblasts was the initiation of ideas about multipotent (otherwise, stem) mesenchymal stromal cells, which are traditionally abbreviated as MSCs or MMSCS. According to the International Classification, these cells are of bone marrow origin, have fibroblast-like morphology, are adhesive to plastic and form fibroblast colonies in vitro, are obligately positive for CD44, CD73, CD90, CD105 and negative for endothelial (CD31) and hematopoietic (CD45 and CD14) markers. They are also obligately differentiated in osteogenic, adipogenic, and chondrogenic directions[1]. The possibility of MSC differentiation and transdifferentiation into other cell types is currently under study.

Based on experimental studies of bone marrow, adipose MSCs, and fibroblasts of various localizations, it is now assumed that dermal fibroblasts mainly originate from bone marrow MSCs. After differentiation of MSCs, they retain their main phenotype (CD73+, $CD90^+$, $CD105^+$), which is also inherent in bone marrow and adipose MSCs[2-6]. Dermal fibroblasts are also capable of linear differentiation in osteogenic, adipogenic, and chondrogenic directions, similar characteristic of MSCs. In wounds, they also differentiate into myofibroblasts, just like MSCs[7]. However, this characteristic and their colony-creating ability are much less pronounced than in MSCs[8,9]. In addition, less pronounced telomerase expression is found in the dermal fibroblast population[10]. Some authors have noticed significant differences in the properties of MSCs and fibroblasts and other indicators, such as culture, phenotype, and differentiation[11-13]. Therefore, the question of including dermal fibroblasts in the cell variety of MSCs has not yet been ultimately resolved, but it is important that both cell types are located side by side in the dermis and function together.

Fibroblasts and MScs constitutively produce most of the cytokines inherent in stromal cells affecting the differentiation, proliferation, migration, and functional activity of hematopoietic cells: monocyte colony stimulating factor (m-CSF), tyrosine kinase receptor (Flt-3L), stem cell factor (SCF), IL-7, monocyte chemoattractant protein (MCP1-), IL-8, IL-11, IL-12, il-14, IL-15. Stimulated MSCs can produce leukemia cell inhibition factor (LIF), granulocyte colony stimulating factor (CSF-G), granulocyte macrophage colony stimulating factor (CSF-GM), IL-10, transforming growth factor beta (Tgfb), insulin-like growth factor (IGF-1), vascular endothelial growth factors (VEGF-D, VEGF-A), IL-8, basic fibroblast growth factor (bFGF), nerve growth factor (NGF), angiogenin, leptin, and some other biologically active substances and factors[14]. This list is being gradually updated.

In addition, there is strong evidence that MSCs and their stromal progeny play a fundamental role in lymphocytopoiesis and participate in positive thymocyte selection, while immature T cells exhibit membrane affinity for MSCs. The development of B lymphocytes also requires close interaction of immature B and stromal cells, especially with the $CXCL12^+$ CAR-subpopulation- of MSCs. In this case, the pre-B cell receptor (pre-BCR) and its stromal ligand Galectin-1 play an important role. Research gives an overall regulatory effect evaluation of MSCs on $CD4^+$- and $CD8^+$-T cells, B-cells, NK cells, dendritic cells of monocyte origin (DCs), and neutrophils. The effect of dermal fibroblasts on lymphocyte differentiation is usually less than that of MSCs[15]. Dermal fibroblasts produce an extracellular matrix: collagen types I, III-VII, fibronectin, fibrillin, thrombospondin, laminin, tenascin, glucosaminoglycans (versikan, decorin, heparan sulfate, chondroitin sulfate and hyaluronic acid), elastin, and matrix metalloproteinases. Collagen and elastin create the fiber scaffold, while glycosaminoglycans and fibronectin form the diffuse cementing component of the Matrix. Dermal fibroblast properties are widely used in bioengineering technologies[16].

The activity of dermal fibroblasts is largely regulated by cytokines. Cell proliferation is stimulated by IL-1A, tumor necrosis factor (TNFa), and lymphotoxin. Interferons cause an antiproliferative effect. IL-1A also stimulates the production of collagen, especially Type III; TNFa suppresses collagen synthesis, but stimulates collagenase activity. Cytokines released into the intercellular space have autocrine and paracrine effects, which is one of the main mechanisms of stimulating the action of transplanted fibroblasts. The autocrine effect is manifested by a number of growth factors. Thus, connective tissue growth factor induces the production of Tgfb, responsible for the chemotaxis of fibroblasts and their production of collagen and fibronectin. Paracrine activity of fibroblasts can be observed by stimulating the formation of blood and lymphatic vessels due to the secretion of endothelial growth factors VEGF-A, -B, -C, -D. Carrying out cocultivation of endothelial cells and dermal fibroblasts on collagen gel, endothelial cells were activated with the increased expression of matrix metalloproteinase MMP-1. As a result, endothelial cells were destroyed around the collagen field and formed capillary-kind arm-like structures[17].

Fibroblasts are located in different parts of the body and the seat may influence some of their properties, determined by the level of tissue genes expression[18,19]. HOX gene expression, which begins in embryogenesis, determines positional affiliation, leading to site-specific cell differentiation and tissue morphogenesis. The analysis of fibroblast gene expression from different anatomical parts of the body showed that their diversity is to some extent related to the localization site. For example, Hoxb genes (HOXB2, HOXB4, HOXB5, HOXB6, HOXB72, HOXB9) are expressed in dermal body samples, while HOXD4 and HOXD8 are found mainly in body and leg samples. HOXA13, which regulates the development of elements during embryogenesis, is expressed exclusively in fibroblasts derived from distal areas of the body. This kind of orientational mechanism available in fibroblasts provides information about the location of tissues of the body and the implementation of specific functions that they perform in this area. Transcriptome analysis of dermal MSCs showed that they significantly increased the expression of genes responsible for interaction with the epithelium, as in thymic MSCs[20].

Dermal fibroblasts make up a heterogeneous population of cells and accordingly, their localization in the structures of the dermis. Two subpopulations of

fibroblasts are localized in the papillary and reticular dermis, and they have different properties[21]. The third subpopulation is associated with hair follicles. These cells originate from the neural crest. They are located in the dermal follicle papilla and throughout the vagina[22-24]. Papillary fibroblasts are localized close to the surface of the dermis and can promote epithelial-mesenchymal interactions, as well as the delivery of soluble molecules to the epidermis. The division rate of papillary fibroblasts is faster than that of reticular fibroblasts, and in vitro, they form a denser monolayer, since they have an insufficiently effective mechanism of contact inhibition[21].

Different matrix molecules are found in the reticular, papillary, and hair follicles and the same ones are often found in various amounts (*Table 1*).

Why the molecular composition of the extracellular matrix is so different in different parts of the dermis is not entirely clear. This cannot be explained solely by the secretory activity of fibroblasts in these regions because in culture, the differences in synthesis are not so great. Apparently, their interaction with other types of cells is of great importance in the synthetic activity of fibroblasts.

Keratinocytes and epithelial stem cells

Keratinocytes create the first protective barrier of the skin and originate from epithelial stem cells[26]. The proportion of epidermal stem cells (SCE) in the skin is less than one percent[27]. ScES are heterogeneous in various indicators, including life expectancy. ScES are closely adjacent to the basement membrane, which provides them with spatial orientation and correct passage of morphogenetic processes with the polarization of keratinocytes. Intermediate transient cells originate from SCE, which still have time to go through several cell cycles in the basal layer. In general, the skin epithelium renewal rate is quite fast, with full regeneration over 4-5 weeks.

In the epidermis, special structural and functional units (SFO) are distinguished, which include cells that proliferate and differentiate. In the basal layer there are proliferating cells, and above them there is a cord containing differentiative cells. This conventional structure is defined as a proliferative unit. It consists of about 10 cells. One of them is SCE, another is Langerhans dendritic interepithelial cell, 6-7 proliferating keratinocytes, and the rest are postmitotic cells. The mechanism of SFO formation is debatable. The most common point of view is that the formation of SFO occurs due to self-organization as a result of intercellular interactions, as evidenced by the ability of keratinocytes to form clusters similar to SFO in vitro. This point of view is also supported by the formation of SFO in the epidermis of the pre-cultured epithelium transplanted onto wounds. The life span of epidermal scES is not long and in mice, scES division can occur about 100 times in the space of 3 years. However, when mice were given a serial, up to 5 times, skin transplant of young animals so that the total life span of the skin was up to 7 years, the graft remained viable and skin flap condition remained quite satisfactory. Therefore, these data indicate that the life span of epidermis SCE of these flaps is significantly longer than the life span of the animal[28].

In cell culture, keratinocytes form clones with an opposite proliferative potency. Paraclones contain cells with low potential, yielding no more than 15 generations. Holoclones have maximum proliferative activity. In 12 days, they pass about 100 generations and produce 20-50 thousand basal keratinocytes. Holoclones derived from hair follicle cells have the greatest proliferative potential. SCE expresses specific proteins of intermediate filaments: keratins 15 and 19 and protein p63, which is also characteristic of some transient cells. Glyco - and mineralcorticoid receptors play an important role in the functioning of keratinocytes. Cells losing them cause deterioration in the structural and functional properties

Matrix component	Papillary dermis	Reticular dermis	Hair follicular
Collagens I and III	Close interaction of type III and I	Loose interaction of type III and I	Present
Collagen IV	Present in basal membrane	Absent	Present dermal papilla
Collagen VI	Present at dermal - epidermal junction (DEJ)	Low presence	Present in dermal membranes
Collagen XII	Present	Low-to-absent	Strong expression around follicular membrane
Collagen XIV	Low-to-absent	Present	Low expression
Collagen XVI	Present in DEJ-region	Absent	Not known
Tenastin-C	Present in DEJ-region	Absent	Present in dermal membranes and papilla
Tenastin -X	Loose in DEJ- region	Present	Not associated
Versican	Diffuse in DEJ- region, Present in matrix fibers	Present bonded with elastic fibers	Present in dermal papilla
Decorin	Present	Present	Not known

Table 1 *- Distribution of certain molecules of the extracellular matrix in skin compartments[25].*

of skin[29]. Keratinocyte proliferation, differentiation and their expression of barrier proteins largely depend on the interaction of genes in the OVOL1-OVOL2 axis[30].
The study demonstrated the morphological heterogeneity of keratinocytes[31]. In the interfollicular epidermis, one subpopulation is located on the shallow epidermal ridge and is characterized by a high content of tonofilaments in the cytoplasm and a rather broken border between the dermis and epidermis. The most important function of these cells is the ability to ensure the attachment of the epidermis to dermis. Basal keratinocytes of the second subpopulation are located on the tops of deep ridges and are distinguished by a flat surface, rounded shape, a large nuclear- cytoplasmic ratio and a "primitive" cytoplasm. Unlike weakly melanized keratinocytes with a truncated basal surface, they are strongly melanized. According to the dominating point of view, SKEs are located on the tops of deep ridges.
As regards stem cells, hair follicles attract much attention. Sufficient evidence has been collected to indicate they contain SCE[32]. This idea can be supported by convincing data of the regeneration process in the interfollicular epidermis in low-severity skin wounds, due to the proliferation of keratinocytes of hair follicles. It is believed that normally the participation of hair follicle SCE is not necessary for maintaining the interfollicular epidermis. The upstream migration of SCE along with regeneration of the interfollicular epidermal layer is stimulated by damage to epidermis. SCEs are localized in the upper part of the hair follicle in the bulge area, at the muscle lifting and hair insertion site[33]. Bulge-located cells proliferate much less frequently compared to matrix cells. Moreover, it is calculated that 95% of all rat follicular clonogenic cells are concentrated around the bulge, 5% are contained in the hair bulb. The multipotence of SCE from the bulge region is demonstrated in in vitro cloning. They can make the start to all cells of the hair follicle, sebaceous gland and interfollicular epidermis. The most recognized marker of bulge region cells is considered to be the membrane glycoprotein CD200[34].
Apart from their barrier function, mature postmitotic keratinocytes protect the body from infection, as appropriate for innate immune cells. For this purpose, they have a corresponding receptor apparatus in the form of several types of pattern recognition receptors (PRR), including Toll-like receptors (TLR). As a result of their activation, pro-inflammatory cytokines, chemokines, and antimicrobial peptides are produced. Strong activation of keratinocyte TLR leads to the synthesis of interferon type I and the polarization of Th1 reactions[35].
Constitutively, keratinocytes synthesize numerous cytokines and chemokines, creating a wide range for interaction with fibroblasts and other cells in the epidermis. They produce IL-1α и IL-1β, IL3, IL4, IL6, IL7, IL8, IL10, IL12, IL15, IL16, IL18, IL20, IFN, Ifnb, IFN, TNPA, Tgfb, CXCL1 and CXCL8, as well as β-defensins and cataliciding[36].

Interaction of dermal fibroblasts and keratinocytes

The interaction of dermal fibroblasts and keratinocytes is the most important homeostatic mechanism in the skin that ensures the implementation of its inherent functions. Paracrine action is the basis of intercellular interaction. It is mainly provided by the secretion of keratinocyte growth factor (KGF), epidermal growth factor (EGF), CSF-GM, IL-6, and FGF by fibroblasts[37].
In culture, keratinocytes can form a thin epidermal layer. However, without fibroblasts, keratinocytes die as a result of apoptosis in two weeks. When keratinocytes are cultured on a collagen gel together with fibroblasts, the latter stimulate the proliferation of keratinocytes and contribute to the stratification of the epidermis into basal, spiny, granular and horny layers. In culture, irradiated fibroblasts were also found to support the growth of adult keratinocytes; keratinocyte growth factor was then identified, which is produced exclusively by mesenchymal cells. Fibroblasts produce a number of other factors that regulate keratinocyte proliferation and play their role in wound healing: CSF-GM, KGF-2, hepatocyte growth factor (HGF/SF), EGF, and IL-1. Fibroblast-secreting factors effectively modulate keratinocyte activity. In turn, IL-1A secreted by keratinocytes significantly increases the synthesis of cytokines, KGF and other fibroblasts. The co-cultivation of fibroblasts and keratinocytes modifies the activity of both types of cells. Keratinocytes induce TGF expression by dermal fibroblasts; the latter also regulate the production of laminin and Type VII collagen by keratinocytes via Tgfb. Epithelial differentiation is enhanced in vitro in the presence of MSCs[38].
The interaction between fibroblasts and keratinocytes is observed in the course of basement membrane formation, arranged by cells in such a way so that they collectively produce membrane building substances. In this case, types IV and III collagen, laminin-1, nizogen, and cytokines produced by fibroblasts stimulate keratinocyte synthesis of basement membrane components[19].
Studies have also considered the kinetics of basement membrane formation in organ culture in the presence or absence of fibroblasts. In the latter case, the production of collagen types IV and III and laminin-1 by self-cultured keratinocytes was either significantly delayed or absent, indicating the need for fibroblasts to induce the synthesis of these molecules by keratinocytes. On the other hand, in the presence of keratinocytes in fibroblasts, an increase in the level of Type III collagen mRNA was noted. Not all dermal fibroblasts interact equally effectively with keratinocytes in the construction of the basement membrane. It was demonstrated that wound-derived myofibroblasts did not retain keratinocytes like normal dermal fibroblasts. Moreover, papillary dermal fibroblasts caused the formation of the basement membrane faster in the presence of reticular fibroblasts[39], thus indicating that both cell types have different properties and alternative interactive abilities. Fibroblast-keratinocyte interaction is crucial in skin repair after damage[40,41]; the epidermal response to fibroblast signaling molecules depends on the ratio of active factors. This is especially true for KGF-1 and CSF-GM. Meanwhile, papillary and reticular fibroblasts differ greatly in the intensity of secretion of these substances. The CSF-GM/KGF-1 secretion ratio is higher in papillary fibroblasts than in reticular fibroblasts[19]. Phenotypic differences in skin fibroblasts may also be related to the characteristics of the response to external signals and the modulation of groups of genes that are regulated by AP-1 transcription factors[42].
Fibroblasts play an important role in the processes of epithelialization. The interaction process between

fibroblasts with keratinocytes begins at the first stage of regeneration, resulting in cell migration from the wound edge along the wound bed perpendicular to its edges, with cell proliferation at or near the wound edge and reproduction of the newly formed epithelium migrating to the wound center. The result is the formation of an epithelium with characteristic features attributable to a normal epithelium of this area of the skin. Fibroblasts are among the first to synthesize regulatory factors KGF/FGF7, which bind to the receptor on keratinocytes. In cases of extensive skin damage, fibroblasts differentiate into myofibroblasts characterized by the altered synthesis of fibronectin and glycosaminoglycans, increased synthesis of Tgfb-1 and 2, Type I collagen, and the IGF-II/manooze-6- phosphate receptor. However, the activity of these cells in scar tissue causes dystrophic keratinocytes changes[43]. Fibroblasts obtained *in vitro* form the structural basis for epithelialization and growth, stimulating the proliferation of the patient's own fibroblasts and keratinocytes[44].

Aging and dermal fibroblasts

The number of fibroblasts and their synthetic activity significantly decreases with age. A decreased number of young and functionally mature fibroblasts in ageing skin are primarily associated with the age-related accumulation of senescent fibroblasts, with reduced cell resistance to activation and pro-apoptotic signals. The total number of different types of immune cells in the dermis is also significantly reduced. Only the number of mast cells increases with age, which contributes to the development of immuno-inflammatory processes. Involutional skin changes affect all skin compartments, including derivative structures. At the same time, pronounced dystrophic and destructive changes are observed in the epidermis, and the border between the epidermis and dermis is a pathological straight band. Fibroblasts with weak synthetic activity and destructive changes are the dominating cells of aging dermis; they contain lipofuxin and fat in their cytoplasm. Decreased turgor and elasticity of skin, the development of wrinkles, pigmentation and other manifestations of aging is caused by the decomposition of the intercellular matrix as a result of violation of the functioning conditions of fibroblasts and keratinocytes, with inhibition of their remodeling activity[45].

In fibroblast cultures from elderly donors, cells are represented by larger and mature elements. It is possible to observe their faster aging, with a significant inhibition of proliferative activity. However, a certain proportion of dermal fibroblasts do not lose their ability to divide, even after the age of 60, and as shown above, primary cultures obtained even from 95-year-olds contained about 14% of proliferating fibroblasts. Data show that the dermis always contains immature fibroblast precursors, possibly dermal MSCs; due to the clonogenic potential, it is possible to obtain a sufficient number of active cells in early passages in people at any age. At the same time, some studies report a decrease in the effectiveness of fibroblast autotransplantation in people over 65 y.o., despite the fact that fibroblasts of elderly donors preserve a share of their proliferative potential and the ability to moderately produce Type I collagen. Data on the effectiveness of cloning in an aging organism, depending on the age factor, have large discrepancies. It has also been shown that the life span of fibroblasts in culture does not correlate with the donor's age, which is probably due to the positive selection in the culture of young and active fibroblasts and the negative selection of senescent cells, or due to a deficiency in the culture of intercellular matrix molecules that have a fibroblast-stimulating effect. Thus it was shown that as a result of the cultivation of dermal fibroblasts from old mice in the presence of a Collagen Complex from the tails of adult mice for 5 consecutive passages, the cell growth rate significantly increased and spontaneous fibroblast death decreased. Significant demethylation was detected in the promoter regions of their cell cycle-related genes. The efficiency of reprogramming into induced pluripotent cells (iPS) was significantly higher when working with cells of old animals cultured with a collagen complex than in control fibroblasts of old mice. In collagen-activated fibroblasts, the expression of ink4a/Arf and p53 loci genes involved in antiproliferative processes was also reduced, and the activation process of old cells was mediated by the α2β1 integrin- dependent Bmi-1 pathway[46]. The results suggest that effective approaches can be developed for positive gene-directed effects on senescent fibroblasts.

Cellular senescence greatly contributes to the development of involutional skin changes, with the induction of immuno-inflammatory processes. Currently, it is considered that an antitumor mechanism stops the growth of old (senescent) cells that have damage at both genetic and epigenetic levels, thus preventing malignant transformation. However, available research suggests that senescent cells acquire abnormal secretory activity and can thus cause such changes in the tissue microenvironment that it can lose control of cellular behavior. Senescence is associated with the secretory SASP phenotype[47,48]. Despite the cessation of division, senescent cells remain metabolically active, expressing and secreting protein molecules. Such a cellular phenotype is defined as senescence-messaging secretome (SMS). SMS is formed by almost all evaluated cells, including human fibroblasts and epithelial cells[48].

The biggest differences in molecular features between presenescent and senescent cells are related to cell cycle genes, metabolism, and genes encoding secretory proteins. Secretomes produce increased amounts of cytokines, chemokines, growth factors and inflammatory factors, soluble receptors and ligands, non-protein factors and extracellular matrix proteins. These factors affect the cells microenvironment, activate various surface receptors and transmit signals that can lead to pathology. SASP proteases can cause shedding of membrane proteins, with the appearance of soluble membrane receptors, inactivate signaling molecules, and interfere with the formation of the extracellular matrix.

Cellular senescence also occurs *in vitro* culture in response to extracellular or intracellular stress. The senescent program stops the cell cycle, which prevents the spread of lesions to subsequent cell generations and prevents the development of potential malignant transformation. Senescent cells accumulate throughout life. They are found mainly in long-term inflammatory tissues. Cellular senescence is triggered by excessive stress, which induces telomeric dysfunction resulting

from repeated cell divisions (reactive senescence), mitochondrial insufficiency, changes induced by oxidative stress, significant and unrepaired DNA damage and chromatin breaks (genotoxic stress) and the expression of certain oncogenes (oncogenically induced senescence)[49]. One of the mechanisms of senescence of dermal fibroblasts may be a change in the degree of DNA methylation[50] and desialization of CD44 with an increase in sialidase activity, which blocks cell differentiation and contributes to the appearance of cosmetic abnormalities in elderly people[51]. DNA damage, with an increased number of somatic mutations, occurs with age in fibroblasts as a result of exposure to UV and endogenous factors on skin[52]. Keratinocytes are also involved in stress-induced senescence. It was found that they are able to independently synthesize catecholamines and steroid hormones[53,54]. Stress contributes to the process of senescence of dermal papillary fibroblasts and a pronounced violation of epithelial-mesenchymal interaction on hair follicle territory[55]. The secretion of IL-6 and IL-1 by senescent cells is particularly worthy of mention. The most important cytokine of SASP is considered to be the pleiotropic pro-inflammatory cytokine IL-6. Its action is associated with DNA damage and senescence induction of fibroblasts, keratinocytes, melanocytes, and monocytes[48]. IL-1 is also more actively produced by senescent cells. Both IL-1A and IL-1B are produced in large quantities by senescent fibroblasts and endothelial cells. These cytokines act on surrounding cells via surface IL-1 receptors and are triggers for nuclear factor kappa B (NF-kB) and AP-1. Senescent fibroblasts, including dermal ones, carry increased secretion of MCP (CCL-7), I-309 (CCL-1), granulocyte chemoattractant protein (GCP or CXCL-6), epithelial neutrophil-activating reptide-78 (ENL-78 or CXCL-5), CXCL-4, and CXCL-12 (SDF-1). Senescent fibroblasts, epithelial and endothelial cells secrete a large number of almost all IGF-bound proteins (IGFBPs), including IGFBP-2, -3, -4, -5 and -6 and their regulators IGFBP - rP1 and rP2 [also known as connective tissue growth factor (CTGF)]. Senescent fibroblasts also produce an increased amount of pro - inflammatory cytokines-CSF-GM and CSF-G[48]. Osteoprotegerin, a modified TNFa receptor found in significant amounts in the extracellular environment of fibroblasts, is also secreted. The overproduction of prostaglandin E2 (PGE-2) and Cox-2, an enzyme responsible for prostaglandin synthesis, has been noted[56]. Data on increased levels of senescent fibroblast secretion of MMP Family enzymes are important. These include stromelysin-1 and -2 (MMP-3 and -10, respectively) and collagenase (MMP- 1). Sometimes MMP - 1 and MMP-3 produced by senescent cells can affect SASP factors; they inactivate MCP-1, -2, and -4, as well as IL-8; chemokines of the CXCL/CCL family can be neutralized by MMP-9, -2, -7[57]. It should be noted that SASP is not a sign of an overall increase in the secretion of all cytokines. Thus, in senescent fibroblast secretomas, the production of anti-inflammatory cytokines IL- 4, -10, and -11 does not change. Also, secretion levels of factors necessary for cell differentiation and proliferation does not change: fractalkin (CX3CL-1), GCP-2, glucocorticoid-induced tumor necrosis factor receptor (GITR), platelet growth factor (PDGF-BB)[48]. Senescent fibroblasts are able to mobilize lymphocytes and monocytes from blood to the sites of tumors. Proinflammatory cytokines of SASP fibroblasts attract t-Th2 lymphocytes associated with malignant growth and regulatory T cells to tumors. It is known that other cells of the innate and adaptive immune system, in particular, natural killer cells, neutrophils, eosinophils, dendritic cells, B - and T cells of various subpopulations, can also be mobilized by cytokines produced by senescent fibroblasts[58]. Dermal fibroblasts and endothelial senescent cells produce 50-fold amounts of plasminogen as well as increased amounts of protease inhibitors (PAI-1 and -2). Data obtained in the study of Werner's premature aging syndrome, indicate that as a result of senescence, fibroblasts increase fibronectin production[59]. Senescent cells demonstrate increased production of nitric oxide and reactive oxygen species as a result of the inhibition of inducible nitric oxide synthetase, endothelial nitric oxide synthetase, and superoxide dismutase. It is known that these substances affect cell differentiation, accelerate aging and associated degenerative processes[60]. Therefore, based on the above data, it can be concluded that skin aging is caused by cellular decomposition, characterized by a decrease in the number of cells of various skin types, primarily fibroblasts, with normal functional activity and the accumulation of senescent cells, in which functional activity, especially of a secretory nature, changes so much that these cells stop proliferating, acquire signs of SASP and begin to produce a large number of biologically active factors, the spectrum of which deviates significantly from the norm, resulting in immune inflammation and a cascade of events leading to deep involutional changes. The study of these processes is promising for the development of new methods for treating skin diseases and an enhanced understanding of aging mechanisms.

Abbreviations

MSC (MMSC)	· multipotent mesenchymal stromal cells
ESC	· epidermal stem cells
SFU	· structure-functional unit
INFA	· interferon
AP-1	· activating protein-1
CAR-cells	· CXCL12-abundent reticular cells
CCL	· CC chemokine ligand
CSF-G	· granulocyte colony stimulating factor
CSF-GM	· granulocyte-macrophage colony stimulating factor
CXCL	· CXC chemokine ligand
DEJ	· dermal-epidermal junction
EGF	· epidermal growth factor
FGF	· fibroblast growth factor

GCP · granulocyte chemotactic protein (CXCL6)

IGF · insulin-like growth factor

KGF · keratinocyte growth factor

LIGHT · Living Intervention and Guidance for Healthier Teens

MCP · monocyte chemoattractant protein

MMP · matrix metallopeptidase

TGFβ · transforming growth factor

TNFα · tumor necrosis factor

SASP · enescence associated secretory phenotype

SMS · senescence messaging secretome

TGFβ · transforming growth factor

TLR · toll-like receptor

VEGF · vascular endothelial growth factor

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Case report

The neck doesn't lie - Tixel®: the safe and low-risk option for the treatment of wrinkles in the neck area: case report

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Abstract

Background & goals: wrinkles around the neck and décolleté are difficult to treat due to their inadequate response to most anti-aging treatments. This case report shows the use of a thermomechanical device (Tixel®, Novoxel®) to improve the complexion and the transdermal delivery of skin-active substances.
Patients & methods: a 61-year-old woman with skin type II, sun-damaged, poikiloderma skin changes and vitiligo was treated with a fractionated thermomechanical device (a protrusion of 500 µm and a pulse duration of 10 ms) for a total of two treatments (with 3 weeks between each session) after 3 weeks each. After the treatment sessions, 60% snail extract serum was applied on the skin.
Results: already after one application there was an improvement in skin condition as regards wrinkles and blotchiness/sagging in the neck area. Some depigmented areas, vitiligo patches, were also activated.
Conclusions: anti-aging and rejuvenation users know about the difficulties in treating the neck and décolleté. This alternative treatment method shows an impressive response of the treated area/areas to be treated. As a side effect, it was also shown that skin cells in the area of the vitiligo sites were activated. This should be discussed through further case reports in medicine and aesthetics and supported by studies, to encourage the more frequent use of thermomechanical infiltration, if appropriate.

Keywords

Wrinkles, Tixel, anti-aging, thermomechanical drug delivery

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Introduction

The NECK doesn't lie! Sagging and the formation of wrinkles on the neck are tell-tale signs of ageing, like the rings on a tree. The common and frequent use of botulinum toxin and dermal fillers on the face makes neck irregularities even more noticeable. Rejuvenation therapies in the neck area aim to improve unattractive and age-related characteristics that result from the aging process just like on the face. Often, practitioners and patients focus on facial rejuvenation without paying attention to the fact that the neck should be part of a holistic treatment concept. Commonly used aesthetic methods for neck rejuvenation include surgery, laser therapy or injection treatment. The goal is smooth-looking, radiant skin and the healthy redistribution of soft tissue volume. Individual patient characteristics are inherent challenges that can limit the effect of improvement. To this end, aesthetic experts are always looking for improvements to therapy options. However, numerous therapeutic methods for rejuvenating the neck show a particular limitation in use and results.
Another goal in the treatment of skin is the precise and efficient introduction of therapeutic agents for better resorption.

Case report

The article presents the case of a 61-year-old female patient with vitiligo (without affected areas in the treatment area, only two depigmented areas adjacent to it) as the only pre-existing condition (*Figure 1*).
Topical anaesthesia containing lidocaine was applied to the neck area and towards the cleavage. The patient's skin areas were treated using Tixel® technology (Novoxel Ltd., Israel), a device that combines thermal energy with movement.
The tip was heated to 400° C and a single pulse delivery to the skin was set to last 10 ms. A protrusion depth (so-called projection, i.e. the distance over which the heated tip of the handpiece is moved) of 500 µm was set. A total of 244 individual pulses were delivered to the patient's skin. Immediately after the skin treatment, a 60% snail slime serum was applied topically on the areas. Post procedure and follow-up care treatment was recommended with the same topical 60% snail extract serum, twice daily for the following 3 weeks. The patient was also prescribed a broad spectrum sun protection factor SPF 50 for the following 3 months.

Results

Two treatment sessions were held three weeks apart. Treatment was carried out using Tixel® (with the following settings: protrusion of 500 µm and a pulse duration of 10 ms). A 60% snail extract serum was then applied on the skin. The patient was treated without persistent side effects. The patient only reported overheating and a slight burning sensation during treatment, as well as mild erythema and slight crusting in the treatment area for about 7 days after treatment. The patient did not report pain during or after treatment. There was no downtime. Sun protection was strongly required and is recommended as part of special aftercare for the treated area.
After the first treatment, an improvement in the treated area with regard to the appearance of wrinkles and better skin structures/flatter skin surface was observed (*Figure 2*). There was also an unspecific mild erythema in the area of the vitiligo patches on both sides of the neck above the collarbones. It remains to be seen whether an additional improvement in the depigmented vitiligo sites will be achieved.

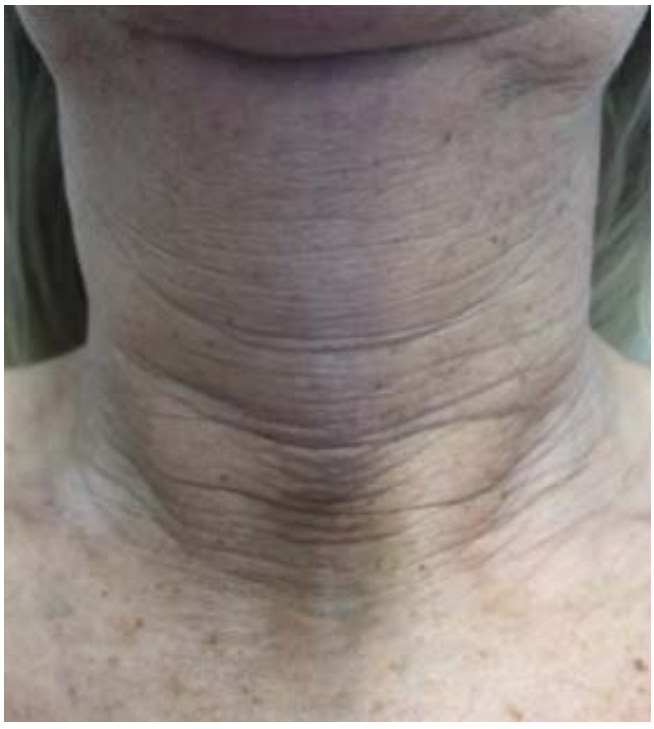

Figure 1 *- Before treatment.*

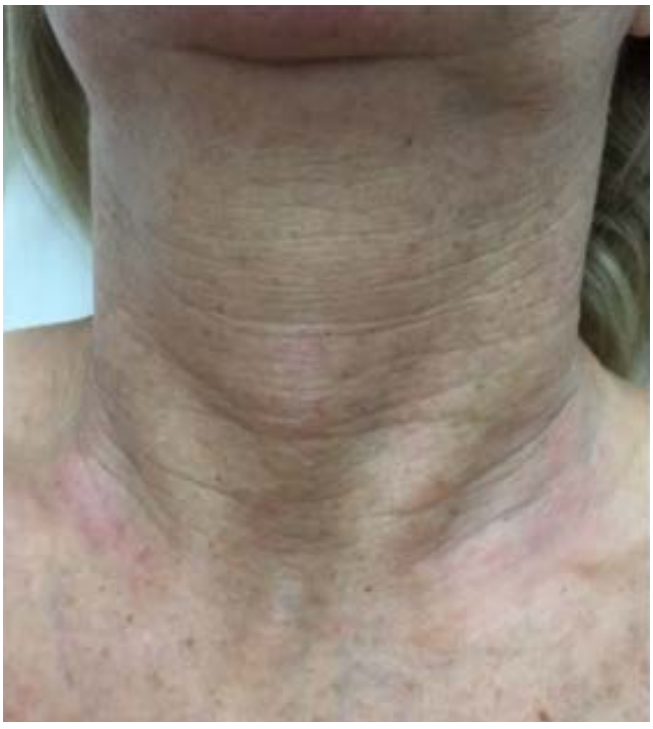

Figure 2 *- 6 weeks after treatment.*

Tixel® - Thermomechanical Fractional Injury (TMFI) Technology

Tixel® (Novoxel®, Israel) is a thermomechanical system developed for fractional treatment. The system consists of a titanium tip that heats to 400 ˚C. The tip is advanced until it comes into contact with skin. The tip has an ablative effect on the skin due to physical contact and heat transfer to the superficial layers of the skin[1-3].
The system is designed for the treatment of soft tissue through direct heat conduction and enables rapid water evaporation, with little thermal damage to the surrounding tissue. The system consists of a handpiece that is connected to a console. The handpiece has a therapeutic element, the "tip", which is attached to the distal section. The tip consists of a gold-plated copper base and a thin-walled cover made from titanium alloy (*Figure 3*).

The handpiece

The handpiece is equipped with a precise movement system, which is based on a linear motor with low inertia and a DSP movement controller (digital signal processing). The design of the system enables precise transfer for the duration of skin contact.

The Tip

The tip consists of a 1 cm^2 area with an arrangement of 81 (9 x 9) square pyramids (*Figures 3* and *4*). The pyramids are 1.25 mm high and have a flat rectangular tip of approximately 0.01 mm^2. The blunt tip of the pyramid enables effective heat transfer and prevents mechanical piercing of the skin.
The tip backplane is attached to a ceramic heater that is kept at a temperature of 400 ˚C during treatment. The heating process enables effective self-sterilization before and during treatment, which significantly reduces the risk of cross-contamination. The tip is safely retracted to its original position when the handpiece is not activated. When the handpiece is activated, the linear motor quickly advances the tip that briefly comes into contact with the tissue and then pulls it back. In this way, heat energy is transferred to the skin, creating micropores. This is done by the evaporation of water, without damaging the tissue. Pulse duration, i.e. the contact time between tip and skin, is between 5 and 18 milliseconds[1-3].
A second system parameter is tip protrusion. Protrusion is defined as the distance the tip travels from the distal edge of the handpiece (which also acts as a distance meter) to the tissue (Figure 3). The protrusion setting is measured in micrometers (µm) and is intended to ensure good heat coupling between the tip and the tissue, especially in relatively "flexible" areas such as the neck. Heat coupling or thermal resistance are influenced by two factors: heat conduction from the tip to the skin at the contact points, and heat resistance due to trapped air between the tip and skin. Higher protrusion rates increase the contact area between the tip and skin and reduce air pockets.
Improved coupling results in lower heat resistance between the tip and tissue, for a more significant dermal effect A well-planned setting of pulse duration and protrusion contributes to the desired thermal effect, followed by a successful clinical result[1-3].

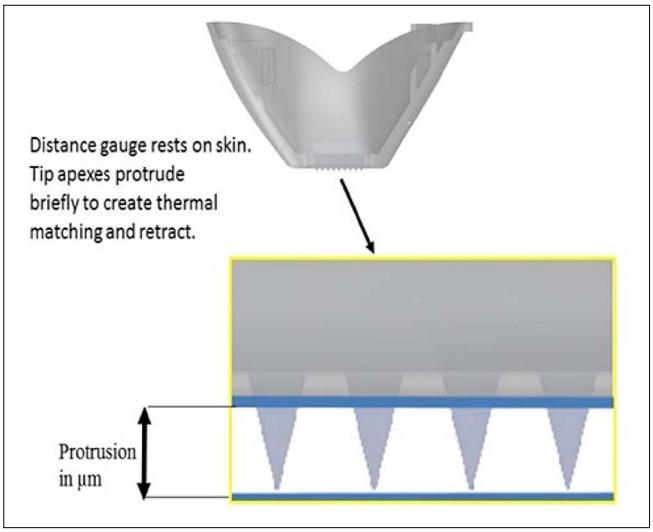

Figure 3 *- Tixel® - the tip and representation of the protrusion.*

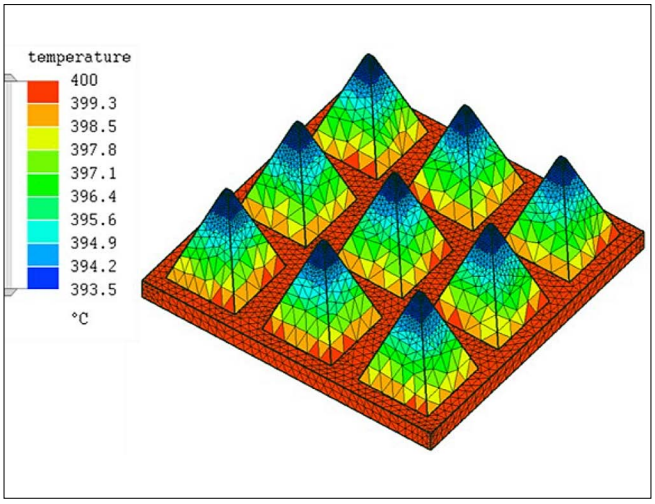

Figure 4 *- Arrangement of the pyramids on the tip and the temperature levels.*

Discussion

Harmonious and minimally invasive rejuvenation of the face / neck complex is the goal in anti-aging medicine. This includes more than just the usual treatments in the facial area using botulinum toxin and fillers. Other areas of the body, such as the back of the hand, as well as the neck and décolleté, are increasingly finding their way into the focus of interest. Until recently however, no reliable or standardized treatment method existed, especially for such deficient areas of the skin. The Tixel® thermomechanical system constitutes a reliable option with few side effects. Its effectiveness has already been proven in studies here[4,5]. This treatment is associated with both minor side effects and low downtime[6]. This method of treatment was shown not only in aesthetic medicine, but also in dermatology, as a therapeutic option[5,6]. Furthermore, we were able to determine an additional effect on the depigmented vitiligo sites as an accompanying effect. It remains to be seen whether this will have a soothing or even healing effect. Further studies are certainly necessary here.

Tixel® is an alternative, safe, well tolerated, low-risk and easy-to-use therapy option for the user[4,5].
In short, treatment with Tixel® has many advantages. This treatment method extends our therapy options and is characterised by a mild but effective method. This alternative treatment method should be discussed in medicine for more frequent use. Further studies with a larger number of patients are necessary to assess its potential more precisely.

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aesthetic medicine

Narrative Review

Polynucleotides Highly Purified Technology and the face skin, a history of innovative skin priming

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Skin tonicity and roughness control are especially crucial in the perioral, periocular, and cheeks and eyelid areas as well as the décolleté, including the neck. Crow's feet and generally all wrinkling of facial senescent skin is almost entirely the result of dermal changes, associated with the depletion and fragmentation of elastin and collagen fibres and increasing scarcity of fibroblasts[1].

Many aesthetic medicine treatments may enhance the appearance of face skin without general anaesthesia and surgery. Some examples are chemical peeling, fillers, skin boosters, botox injections, radiofrequency, laser, and microdermabrasion treatments. The preliminary study of the patient's skin and personalized treatment protocols based on a combination of different procedures allow for the best rejuvenation outcomes. Today it might be reasonable to consider highly purified, natural-origin polynucleotides (PN-HPT™, Polynucleotide Highly Purified Technology) as the critical foundation of a wealth of aesthetic treatments.

PN-HPT™ improve dermal trophism and overall skin turgidity; moreover, acting as re-activating "primers" of dermal tissues, PN-HPT™ can improve the outcomes of other skin rejuvenation techniques. PN-HPT™ are indeed extensively used to prime the skin before aesthetic treatments with fillers and polydixanone thread lifts; PN- HPT™ may also be important before radiofrequency and laser treatments because better aesthetic results are achieved in hydrated and metabolically active tissues[1,2].

Products containing long-chain, highly concentrated polynucleotides of high molecular weight combine the filling efficacy of conventional linear HA-based products with powerful regenerating efficacy on the dehydrated wrinkles of sagging and aging face skin[2].

Several Class III CE 0373 PN-HPT™-based medical devices for intradermal infiltration are commercially available in Italy, Europe and on extra-European markets, as single-agent formulations[(a)] or co-formulated with hyaluronic acid[(b)], for the face, neck, décolleté, and other critical face areas such as perioral and periocular skin, the cheeks and eyelids.

As extensively described in this narrative review, with special reference to the previous "Introduction to Polynucleotides Highly Purified Technology" section, intradermally infiltrated PN-HPT™ increase skin turgidity, hydration and elasticity by promoting the activity of dermal fibroblasts and the *de novo* regeneration of autologous glycosaminoglycans, fibril proteins, and glycoproteins (*Figure 1*).

PN-HPT™ may be of especially great benefit to areas like the neck and the décolleté, where the aesthetic medicine specialist does not have many alternatives and skin ageing is often more severe than in face skin.

As regards periocular skin and eyelids, both aesthetically sensitive areas due to locally thin skin, PN-HPT™ may once again act as re-activating "primers" of dermal tissues before and in concomitance with other treatments.

Thanks to such "priming", specifically formulated PN-HPT™ formulations may enhance tissue tightening and improve the efficacy of other widely used skin tightening and rejuvenating techniques widely used in these areas, like botox infiltrations or laser and plasma treatments. Low-concentration PN-HPT™ formulations have the additional benefit of avoiding the subjectively unpleasant persistence of wheals for some hours, as may happen with more concentrated PN-HPT™ formulations with or without HA[1-4].

(a) Plinest® (PN-HPT™ intradermic gel, 20 mg/mL, 2 mL pre-filled syringes), Plinest® fast (PN-HPT™, 7.5 mg/mL, 2 mL pre-filled syringes), Plinest® Eye (PN-HPT™ for eye contour, 2 mL pre-filled syringes) - Mastelli Srl, Sanremo (Italy)

(b) Newest® (PN-HPT™ and HA, both at a concentration of 10 mg/mL, and mannitol 200 mM per liter, 2 mL pre-filled syringes) - Mastelli Srl, Sanremo (Italy)

The prolonged isosmotic hydration of the dermal matrix contributes to preserve the ideal conditions for fibroblast metabolic activation over time in face skin[6,8,9]. The text box outlines two representative examples of available clinical findings that illustrate the boosting power of PN-HPT™ on face skin.

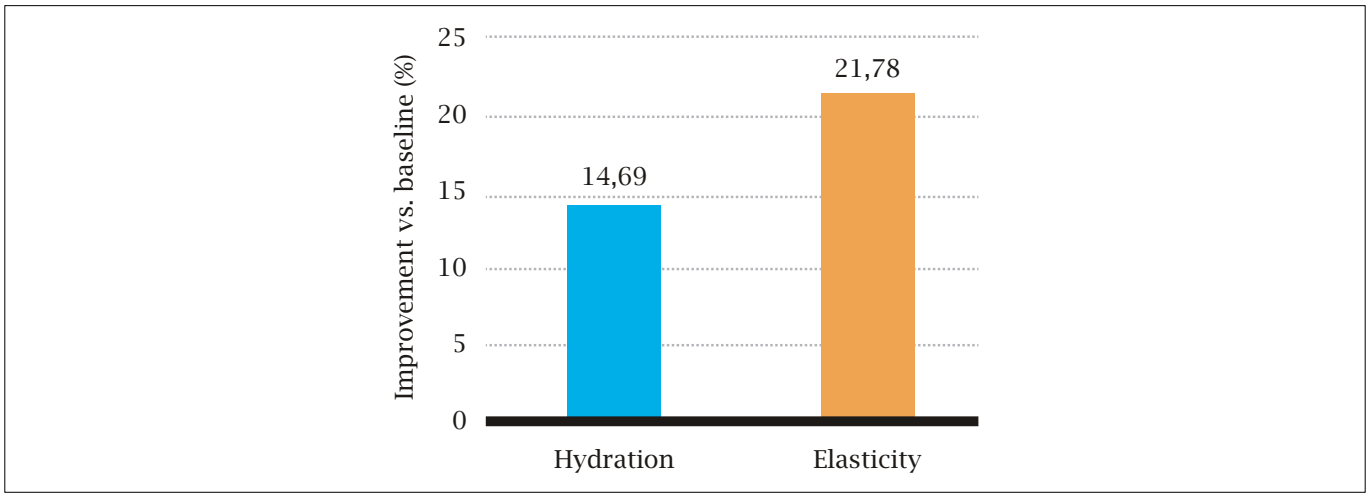

Figure 1 *- Skin hydration and elasticity: percent improvement after intradermal infiltration of PN-HPT™ in 143 subjects of both sexes (Plinest®, Mastelli Srl, Sanremo; serial or micro wheals technique, retrograde linear injections or cross-link technique), 3-4 sessions every 1 to 2 weeks, evaluation one month after the last infiltration[1].*

■ Overall clinical improvements due to orthodermy and reductions of superficial fine wrinkles (*Figure 2a*) can be expected in more than 90% of individuals after PN- HPT™ intradermal infiltrations (40 mg/2 mL, 30G-needle Plinest® pre-filled syringes; 3 or 4 infiltration sessions according to the type of skin). The evidence is not only subjective in treated subjects, but it is also objectively supported by the quantitative outcomes of analyses with the camera-associated ANTERA® 3D CS skin imaging devices (*Figure 2b*)[5]. As shown in a two-year study in 148 patients of both sexes (134 females and 14 males), with ages ranging from 32 to 75 years, the increase in face skin turgidity in the days immediately following infiltration is associated with readily appreciable skin texture improvements. The best clinical results are appreciated after about one month after the last treatment (91% of "improved" global assessment by the Investigator, along with a 21.8% increase in hydration and improved elasticity (*Figure 3*)[1]. Thin periocular skin is an area where PN-HPT™ appear to offer distinctive aesthetic outcomes with a specific 15 mg in 2 mL protocol (e.g., Plinest® Eye, Mastelli Srl)[6,7]. *Figure 4* illustrates a study based on such a protocol (micro-wheal technique). Tightening of the periocular skin translated into the disappearance of the most severely sagging areas[6].

■ If the aesthetic choice falls on a PN-HPT™ + hyaluronic combination (e.g., Newest®, Mastelli Srl), the infiltration technique may leverage the benefits of handy, thin and flexible cannulas (diameter 27 G, length 37 mm) which minimize problems associated with standard needles (e.g., diameter 30 G, length 13 mm-discomfort, bruises, especially in the periocular and perilabial areas, the need to repeatedly reposition the needle to treat extensive skin areas, slower recovery, less uniform technique[7].

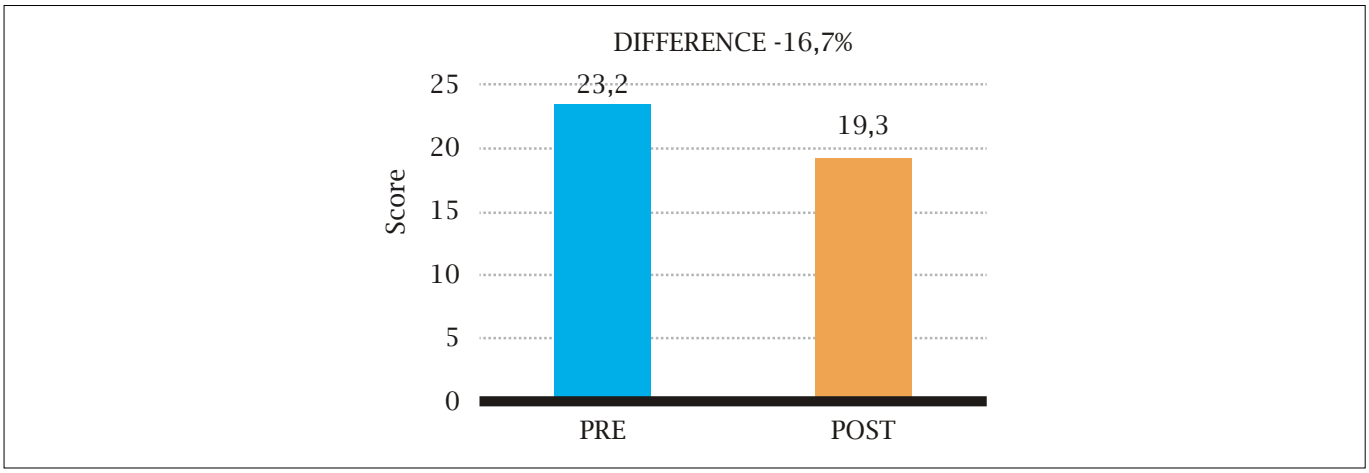

Figure 2a *- Roughness (%) of less than 2.5 mm in the lateral dimension: baseline (PRE) and after 30 days of follow-up with 3 to 4 PN-HPT™ (Plinest®) infiltration sessions (POST)[5].*

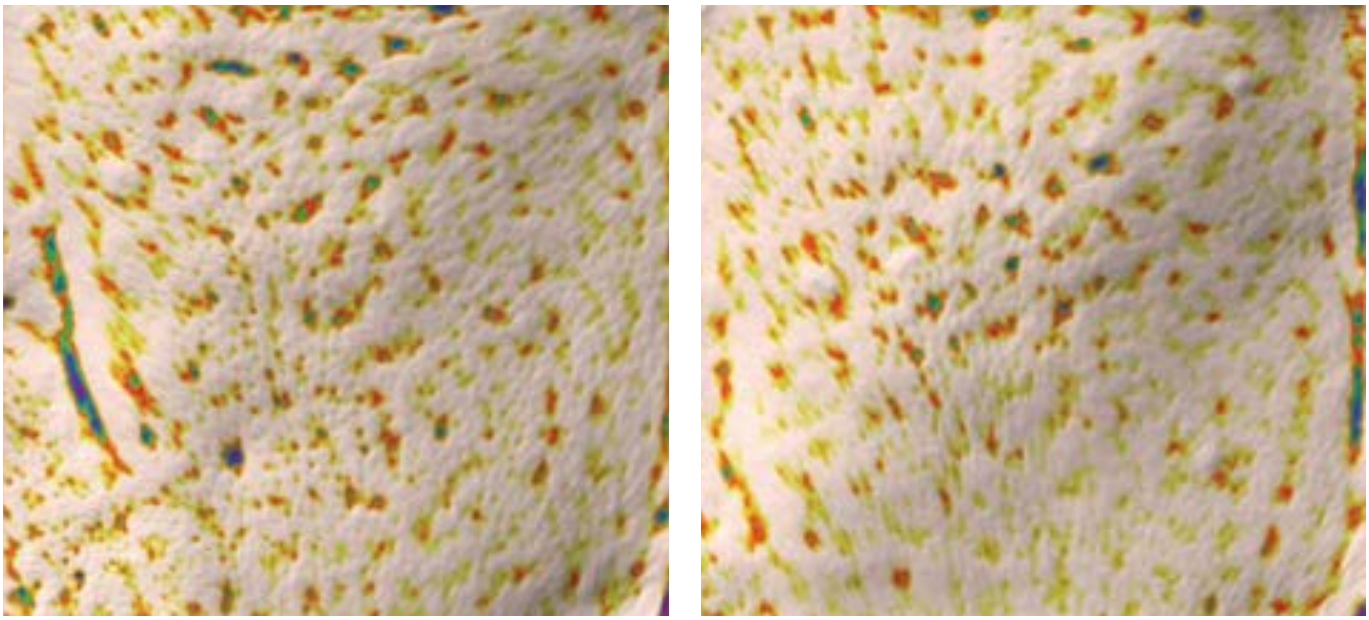

Figure 2b *- Objective improvement of skin texture in the treated face area (camera-associated ANTERA® 3D CS skin imaging device): baseline (left) and after 30 days of follow-up with 3-4 PN-HPT™ (Plinest®) infiltration sessions (right)[5].*

Figure 3 *- Left: before neck treatment with long-chain PN-HPT™ (Plinest®, Mastelli Srl), right: one month after four PN-HPT™ sessions: reduction of fine wrinkles and improvement of tonicity and overall skin appearance[1].*

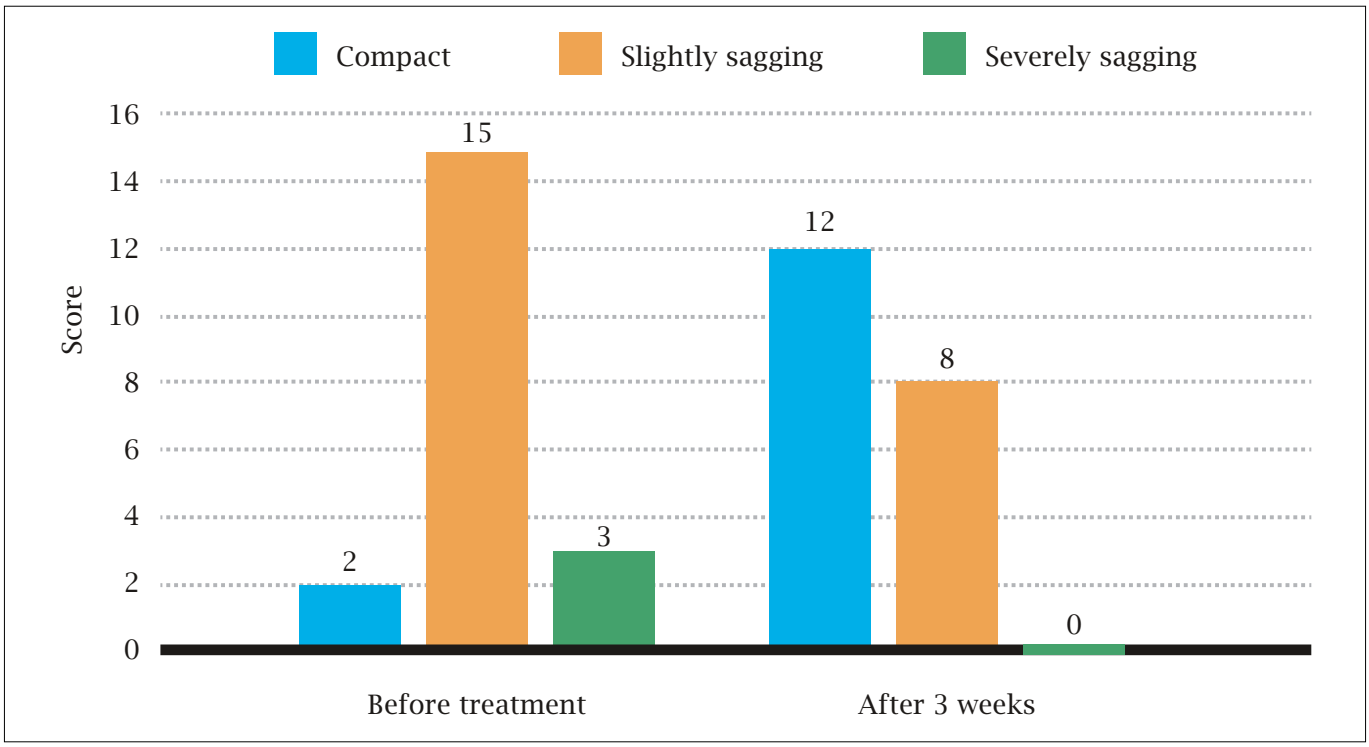

Figure 4 *- Quality of the periocular skin texture after 3-4 sessions of treatment with Plinest® Eye (Mastelli Srl): outcomes after one month (absolute count of favourable aesthetic self-assessment by 20 subjects)[6].*

Suggested PN-HPT™ infiltration technique in face skin biorevitalization

Based on clinical experiences, a standard treatment protocol for skin face biorevitalization could be as herein described, associated with the at-home application of PN-HPT™-based creams/gels/food supplements, as strongly advised. As always (for comparison see also the "Introduction to Polynucleotides Highly Purified Technology" section), the first period of PN-HPT™ infiltrations (micro-wheal technique, either with a linear or reticular distribution of intradermal infiltrations) could be followed either by further PN-HPT™ infiltrations without concomitant hyaluronic acid or by combined PN-HPT™ + hyaluronic acid infiltrations, in agreement with the "PN-HPT™ priming" strategy.
Intradermal infiltrations should be performed with very thin needles (30 G). The following techniques are advised: micro-wheal technique spaced 0.5-1 cm or retrograde linear infiltrations (ideal for filling superficial and/or medium dermis wrinkles, Langer lines or in large skin areas such as cheeks or nasolabial folds) or cross-link (reticular) technique (net of linear intersecting infiltrations, ideal for widely distributing PN-HPT™over large skin areas, such as the cheeks). The infiltration technique may leverage the benefits of thin

flexible cannulas (diameter 27 G, length 37 mm) that minimize the problems of standard needles-discomfort, bruises (with special reference to the periocular and perilabial areas), repeated needle repositioning to treat extensive skin areas, slow recovery, and less uniform technique[7].

Neck and décolleté biorevitalization - PN-HPT™ or PN-HPT™ + HA, one session every 2-3 weeks for a total of 3-4 infiltrations. Maintenance long-term treatment: repeat at-home treatment with a PN-HPT™-based gel every 1-3 months, strongly advised.

Eyelid biorevitalization - PN-HPT™ in specific formulations for thin, sensitive areas like Plinest® *fast* (15 mg in 2 mL), 1 session every week or every two weeks for a total of 3-4 infiltrations. Maintenance long-term treatment: repeat every 1 to 3 months. At-home periocular treatment with a nucleotides-based gel is strongly advised.

Face and perioral biorevitalization - PN-HPT™ (40 mg in 2 mL) or PN-HPT™+HA (20 mg + 20 mg in 2ml): one session every 2-3 weeks for 3-4 infiltrations. Maintenance long-term treatment: PN-HPT™ or PN-HPT™ + hyaluronic acid infiltrations every one or two months. At-home PN-HPT™- based gels/creams/food supplement are strongly advised.

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aesthetic medicine

Narrative Review

Polynucleotides Highly Purified Technology, the new class of body skin biorevitalizing agents

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Skin laxity, loss of elasticity, depleted dermal collagen and elastin fibres, epidermal and dermal thinning all characterise skin ageing. Wrinkling and a rough-textured appearance also mark the action of both intrinsic and extrinsic ageing factors in the skin. The face, neck, chin, décolleté, abdomen, medial surfaces of the arms and thighs are the most severely affected areas[1]. Oxidative stress, DNA damage and telomere shortening by ultraviolet (UV) radiation, as well as oxygen free radicals, cause important skin ageing mechanisms. Similarly, Advanced Glycation End products (AGE), mainly affecting long-lived proteins in the dermal matrix and the cytoskeleton, also lead to tissue stiffening, loss of elasticity and chronic low-grade inflammation ("inflammaging") (*Figure 1*)[1].
Non-surgical techniques for body skin tightening are common in aesthetic medicine, demand from patients is on the rise. Skin biorevitalization is a technique that aims to restructure the skin by leveraging the biologic effects of numerous active ingredients that are variably used, such as hyaluronic acid, vitamins, amino acids, antioxidants, mannitol, and collagen[2]. In 2007, the intradermal infiltration of polynucleotides extracted from trout gonads was first reported to enhance the viability of human skin fibroblasts, leading to the effective remodelling of the fibrillary and amorphous matrix[3,4]. Highly purified, natural-origin polynucleotides are also identified by the acronym PN-HPT™ (Polynucleotides Highly Purified Technology). Regeneration of several skin fractions, including collagen, elastin fibrils and glycosaminoglycans, has since been documented[3,4], leading to the extensive use of PN-HPT™ in aesthetic medicine for skin rejuvenation and skin tightening[5]. PN-HPT™ may be considered precursors of a novel class of biorevitalizing body skin agents. Similar considerations apply to unsightly dermal scarring caused by stretch marks that develop in up to 90% of primigravidae. In striae rubrae, the earliest stages in the path to mature stretch marks (striae albae), disruption of the elastic fibre network is associated with inflammatory changes like infiltration of perivascular lymphocytes, dilated dermal venules and oedema, all of them often quite prominent[6]. Overall, skin atrophy, loss of rete ridges and vascularity, densely packed, thin and horizontal collagen bundles are the histological markers of the final, atrophic stages of stretch mark scarring[6,7]. Based on morphologic findings, the primary goals of treatment should be to reduce inflammatory redness, swelling, and irritation in striae rubrae, and to increase collagen and elastin fibre production in striae albae. Infiltrative Here too, PN-HPT™ appears to be an ideal option, alone or in combination with other techniques, e.g., chemical peeling, needling or laser treatments and NAFL (Non-Ablative Fractional Laser). PN-HPT™ are commercially available as Class III CE 0373 medical devices and nutritional supplements specifically formulated for body skin biorevitalization[(a)], ideally to prime the dermis and the skin before leveraging the combined action of PN-HPT™ with other aesthetic treatment techniques[(b)].

(a) Plinest® Body and Plinest® One (PN-HPT™ intradermal gel, 8 mg/mL, 4 mL vials), Plinest® care food supplement (nucleotides with folic acid as pro-trophic agents and vitamin C, vitamin E, zinc and coenzyme Q as anti-oxidants), Plinest® care oil (spray dry oil with vitamin E and emollient and elasticizing vegetal oils, Mastelli Srl, Sanremo, Italy)

(b) Newest® (PN-HPT™ and HA, both at a concentration of 10 mg/mL, and mannitol 200 mM/L, 2 mL pre- filled syringes), Newest® ONE (PN-HPT™ plus HA intradermal gel, 8 mg/4mL vials), Mastelli Srl, Sanremo (Italy)

Several studies suggest that PN-HPT™ allows for rapid and efficient skin biorevitalization and the prevention of hypertrophic and keloid-like scarring (see the "Introduction to Polynucleotides Highly Purified Technology" section). The text boxes at the end of the section illustrate the outcomes of a study about upper skin laxity managed with intradermal biorevitalization with a PN- HPT™ gel (first box) and of two pilot studies regarding the challenging management of devious scarring (second box)[8-10] as examples of an extensive body of literature on body skin rejuvenation[9,11].
PN-HPT™ may be infiltrated in all skin areas; the arms, thighs, breasts and knees are most frequently treated. Rejuvenating treatment of the hands is also important, due to their continuous exposure to the environment and the resulting, frequent signs of ageing. Rejuvenating treatments with PN-HPT™ could also be useful for restoring the tonicity of tissues after dieting, weight loss, and pregnancies.

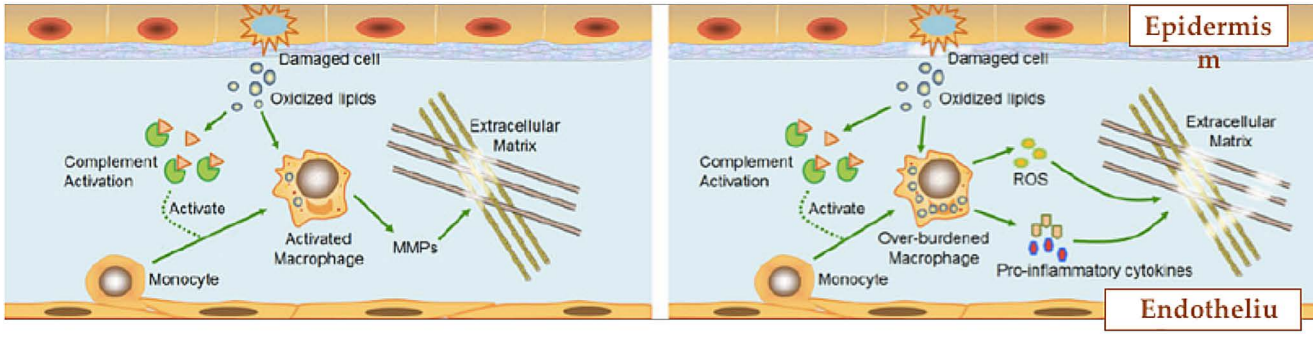

Figure 1 *- Model of epidermal and dermal "inflammaging". Oxidative stress in the epidermis, due to UV radiation and aggressive environmental pollutants, damages cells and cell epitopes and oxidizes membrane lipids, leading to the activation of macrophages and matrix metalloproteinases (MMPs) and depletion of the extracellular matrix. Overburdened with oxidized lipids, macrophages release pro-inflammatory cytokines and further reactive oxygen species that trigger chronic low- level inflammation. Modified from[1].*

■ Prospective cohort of 10 female patients (age range 40-60 years old), who underwent 8 sessions of mesotherapy (needle: 30G/4 mm) in the upper arms region every 7 days with a PN-HPT™ gel (prefilled Plinest® syringe), administered dose: 5 mL). Evaluation of efficacy (before and 8 weeks after the end of the mesotherapy cycle) with the skin imaging quantitative tridimensional evaluation of skin texture ad roughness (analysis with Antera® 3D CS skin imaging device of high-resolution digital photographs) and Global Aesthetic Improvement Scale (GAIS).
The quantitative analysis of the Antera® 3D CS images showed an improvement of fine rythides in superficial skin texture (< 1mm) of 19%, while the GAIS score improved up to ≥ 1 (improved) in 54% of the enrolled patients, up to ≥ 2 (much improved) in 28% of patients, and up to ≥ 3 (very much improved) in 18%. All skin texture improvements were reached without side effects such as erythema, oedema or nodules[8].

■ Prospective 16-women cohort with striae distensae, aged between 16 and 60 years. A session of standard skin peeling with salicylic acid, pyruvic acid, and retinoic acid was followed, after 7 and 14 days, by two sessions of intradermal PN-HPT™ infiltration (one 2-mL prefilled Plinest® syringe per session). The at-home supportive treatment was based on Plinest® care, two applications per day in the morning and the evening. All systemic treatment with corticosteroids or retinoids was carefully avoided. The illustrated cycle was repeated three times, with a total of 3 peeling sessions (spaced about 3 weeks) and 6 PN-HPT™ infiltrations.
The cumulative length of all treatment cycles was about 3-4 months; after the baseline evaluation (T1), two assessments were carried out at the third peeling session (T2) and 4 weeks after the last PN-HPT™ infiltration (T follow-up). *Figure 2* illustrates the study outcomes[9].

■ Eighteen individualized, mature striae albae (3 women) randomized to one of three treatment options: 1st group, PN-HPT™ intradermal infiltrations (Plinest® One, 8 mg per 4 ml solution; 0.5 ml per cm2 corresponding to 1 mg polynucleotides per cm2; eight infiltrations over 11 weeks; Group B); PN-HPT™ infiltrations combined with three CO2 laser sessions; Group C); no treatment (controls, Group A). Two stretch marks from each woman were randomly treated with each treatment option. Tridimensional quantitative assessments of the striae albae width and wrinkling were collected before the first treatment session and at the end of the post-treatment 3-week follow-up period with a camera-equipped Antera® 3D CS device. *Figure 3* illustrates the study outcomes[10].

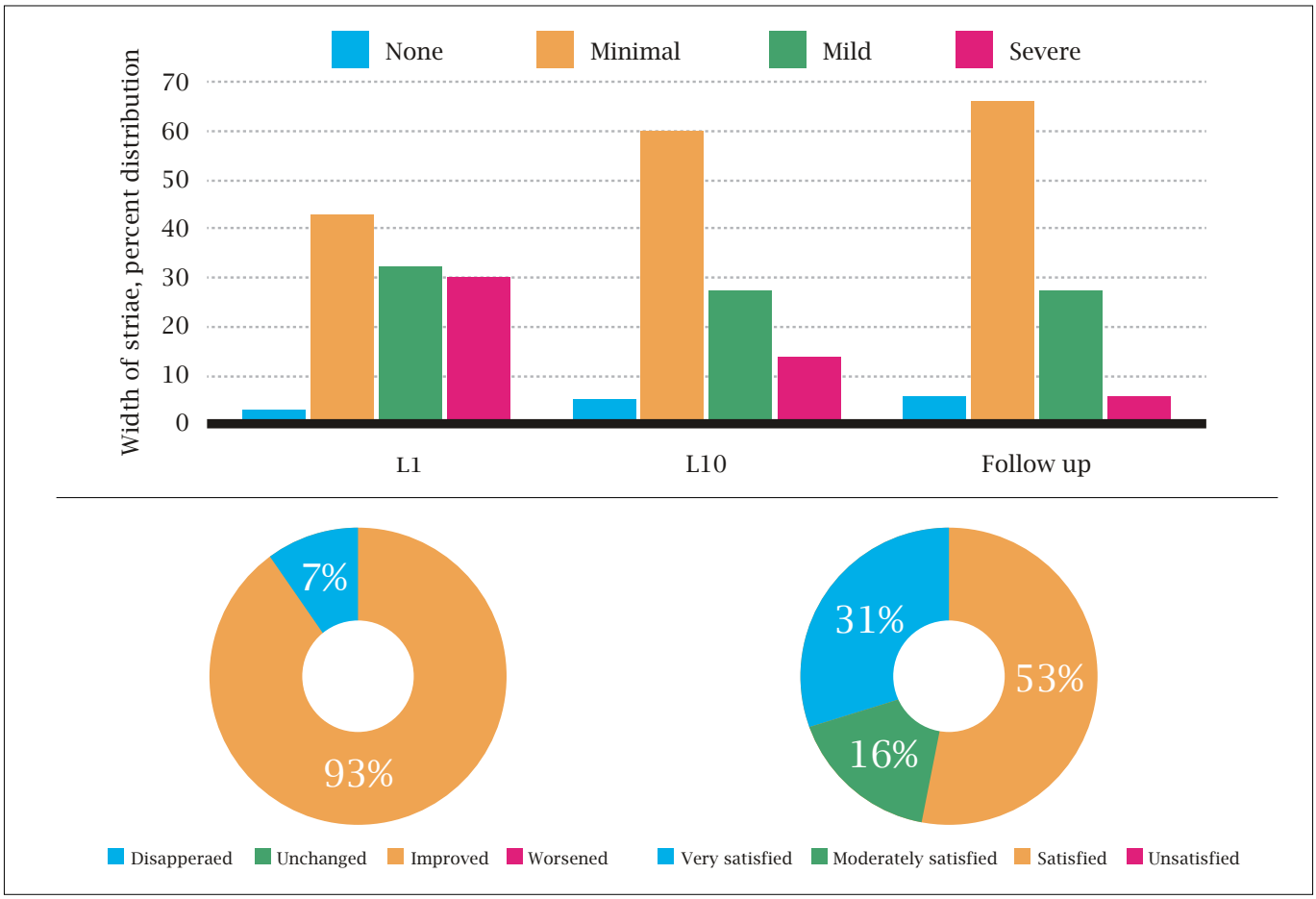

Figure 2 *- Upper graph: maximum width (%) of striae distensae at each assessment point (none, 0 mm; minimal, 1-3 mm; mild, 3-6 mm; severe, >6 mm). Mid graph: 4-score objective final global assessment (investigator). Lower graph: 4-score subjective final global assessment (treated women)[9].*

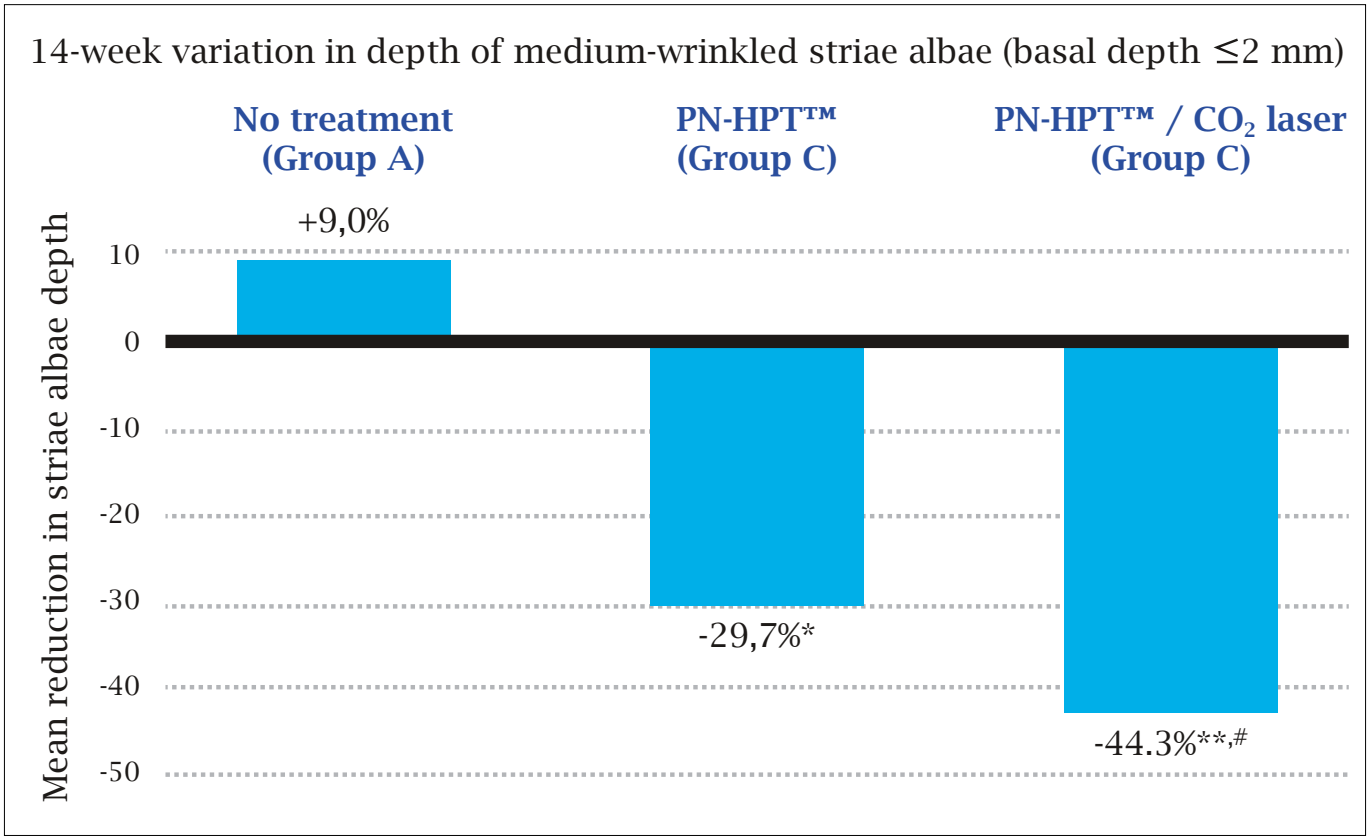

Figure 3 *- Mean percent changes in the depth of thin and medium-wrinkled mature striae albae (depth before treatment, respectively, ≤1 and ≤2 mm) at the end of the follow-up period after 11 weeks of dermal PN-HPT™ infiltrations alone (Group C) or the PN- HPT™ / CO_2 laser combination (Group B); evaluations with an Antera 3D® CS camera- equipped device. * p <0.05 and ** p <0.01 vs. basal evaluation; # p <0.05 vs. monotherapy with PN-HPT™ infiltrations*[10].

Suggested PN-HPTTM infiltration technique in total body skin biorevitalization

Based on clinical experience, a standard treatment protocol could be proposed as follows, possibly with some individual variations and other supportive treatments, e.g., chemical peel, derma roller, laser, radiofrequency treatments, platelet-enriched plasma, at-home applications of nucleotides as nutritional supplements. The first period of PN-HPT™ intradermal infiltrations (linear or micro- wheal technique, 30G needle) could be followed by either further PN-HPT™ infiltrations or by combined PN-HPT™ + hyaluronic acid infiltrations within an overall "PN-HPT™ priming" strategy.

First period: 1 session per week for 1 month (4 infiltrations).

Second period: 1 session every 2-3 weeks for 2 months (3-4 infiltrations).

Maintenance: every one or two months.

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18 - 19 February - Online Event
2nd Scientific Congress of Aesthetic and Anti-Aging Medicine
Scientific Association of Aesthetic Medicine of Peru
President: I. Ogata
Email: info@asocime.com.pe
Web: http://asocime.com.pe/congreso-cientifico-de-medicina-estetica/

14 - 21 February - Online Event
36th SEME National Congress
Spanish Society of Aesthetic Medicine
President: P. Vega
Email: seme2021@pacifico-meetings.com
Web: https://www.seme2021.org/index.php/es/

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Argentinian Society of Aesthetic Medicine SOARME
President: R. Pinto
Email: info@soarme.com
Web: https://www.soarme.com

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Congress of the Belgian Society of Aesthetic Medicine SBME
Radisson Blue Hotel
President: J. Hebrant
Email: info@sbme-bveg.be
Web: http://sbmebveg.be/en/

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Italian Society of Aesthetic Medicine
Rome Cavalieri Congress Center
President: E. Bartoletti
E-mail: congresso@lamedicinaestetica.it
Web: www.lamedicinaestetica.it

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17th Congress of the Swiss Society of Aesthetic Medicine
Hotel Suisse Majestic, Montreux
President: V. Parzin
Email: info@ssme.ch
Web: www.ssme.ch

19 - 20 June - Opatija (Croatia)
3rd Croatian Congress of Aesthetic and Anti-Aging Congress
Aesthetic Medicine Psychophysical Harmony
Croatian Society of Aesthetic Medicine
Hotel Milenij
President: E. Bunar
Email: congress@huem.eu
Web: www.huem.eu

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French Society of Aesthetic Medicine
Palais des Congrès de Paris
President: J.J. Legrand
Email: info@sfme.info
Web: www.sfme.info

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Pepsi Center, WTC Mexico City
President: B. Miller
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