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 ORIGINAL ARTICLE

Cell-Free Blood Cell Secretome (BCS) Counteracts Skin Aging and Restores Firmness and Elasticity

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ABSTRACT

Background: Blood Cell Secretome, BCS (also Autologous Conditioned Serum, ACS) is efficacious in treatment of musculoskeletal disorders. It contains inflammation resolving cytokines, growth factors, exosomes, and lipid mediators. Skin aging is associated with reduced TGF-ß signaling and collagen synthesis and chronic (sub-acute) inflammation, among other factors. Pre-clinically, BCS counteracts these mechanisms, suggesting it as a treatment against cutaneous aging.

Objective: This 24-week study evaluated the effects of deep dermal to immediate sub-dermal micropuncture injections of cell-free BCS in patients with age-related reduced facial skin elasticity.

Methods: In this prospective, single-armed, mono-center study, 21 women underwent 4 BCS treatment sessions over 12 weeks with follow-up at 24 weeks. The primary endpoint was skin elasticity measured by cutometry. Secondary endpoints were safety, skin hydration, and aesthetic assessments using global aesthetic improvement scale.

Results: Skin firmness increased significantly between baseline and 12 weeks ($P<0.001$) and further increased by 24 weeks ($P<0.001$). Skin tiring was congruently reduced ($P<0.001$). Skin hydration and aesthetic ratings improved significantly. No BCS-related adverse reactions occurred.

Conclusion: BCS treatment resulted in increased firmness and hydration, usually attributed to younger skin. BCS is potentially the first cell-free autologous therapy for skin rejuvenation derived from patients' own blood.

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INTRODUCTION

Longer life expectancy and the desire for a youthful appearance have led to increased demand for nonsurgical and minimally invasive skin anti-aging interventions, including micropuncture injections.[1-3] Skin aging is complex and results from the interaction of many intrinsic and extrinsic/environmental factors.[4,5] A recent expert consensus recommended a "pan-facial combined approach" combining aesthetic interventions, to target different manifestations of aging, often lead to better results than single modalities alone.[6]

Common treatments for reduced facial skin elasticity, botulinum toxin (BTX), and dermal fillers do not target the underlying mechanisms of skin aging. In contrast, regenerative medicine consists of therapies intending to replace or restore damaged tissues. Products derived from the patient themselves, such as stem cells or blood plasma, are thought to provide personalized regenerative therapies.

For example, platelet rich plasma (PRP) is produced by centrifuging patient's own blood to concentrate platelets. PRP cell therapy has shown limited efficacy in wound healing, orthopedics, and more recently in facial skin rejuvenation.[7,8] Recent publications have reported the potential of injectable growth factors and cytokines for skin rejuvenation.[9-11] In contrast to PRP, Blood Cell Secretome (BCS) is produced by incubating the patient's own blood. This process fundamentally differentiates BCS from injections such as PRP, which also contain potentially aggressive cells with innate immune functions, complement proteins, clotting factors, and additives.

BCS is efficacious in musculoskeletal disorders, including osteoarthritis, radiculitis, tendon, and muscle injury due to its inflammation resolving and regenerative/remodeling effects.[12] BCS has a unique potential to alter molecular and cellular processes underlying skin aging towards regeneration and

remodeling. Restoration of collagen is a phenotype measurable by cutometry.[13]

BCS is obtained via a blood coagulation process under physiological conditions in a specific medical device (bioreactor). BCS contains cell-derived, inflammation-resolving, and regenerative components including lipid mediators (SPM, eg, ResolvinD1 and ProtectinD1) and extracellular vesicles, such as exosomes[14] with an outstanding role in tissue regeneration. Additionally, interleukins (IL-1Ra, IL-4, IL-10) and growth factors, (transforming growth factor beta [TGFβ], insulin-like growth factor [IGF]) contribute to resolution of inflammation, modulation of immune cell functions, promotion of cell division, tissue matrix formation and acceleration of tissue regeneration and healing.[14] The auto-regenerative conditioning of BCS depends on temperature, bioreactor architecture, oxygen availability, and duration of blood incubation. In the first phase physiological coagulation (±30 minutes) leads to fibrin formation, complement activation, and release of ADP, serotonin, and histamine by platelets. In the second phase, lasting several hours, a regenerative secretome is formed. Thus, BCS enables tissue regeneration and restores dysfunctional cells, tissues and organs.[14]

The objective of this study was to quantify the effectiveness and safety of manual micropuncture injections of cell-free BCS as a regenerative treatment for skin aging with reduced facial skin elasticity in female patients. Viscoelastic properties of the skin were measured before and following treatment, as well as skin hydration and assessments of the patients' attractiveness and aesthetic satisfaction.

MATERIALS AND METHODS

This investigator-initiated, prospective, single-arm, monocenter study was conducted at the University of Hamburg (Division of Cosmetic Sciences, Hamburg, Germany) and registered with the German Clinical Trials Register (DRKS00009259). The study was conducted in accordance with the Declaration of Helsinki and International Conference on Harmonization Guidelines for Good Clinical Practice. Before the start of the study, approval of a local ethics committee was obtained, and all patients gave written, informed consent to participate.

Participants

Female patients aged 35–55 years who presented to the study center with clinical signs of reduced facial skin elasticity based on investigator evaluation, and their own dissatisfaction, were eligible for inclusion in the study.

Exclusion criteria were: pregnancy or breast feeding; severe chronic skin diseases; systemic diseases with skin involvement; acute infections; tendency to form keloids; hypertrophic scars or other healing disorders; any history of skin cancer; treatment with chemotherapy, immunosuppressive agents or immunomodulatory therapy in the last 3 months; previous treatment with laser, BTX or hyaluronic acid in the lower face; severe dieting in the last 3 months; history of bleeding disorders or treatment with anticoagulants or inhibitors of platelet aggregation; any other medical history that, in the opinion of the investigator, would make the subject unsuitable for inclusion.

Intervention

BCS was obtained from the blood of the respective patients. Approximately 10 ml of the patient's blood was drawn using Exokine® bioreactors (Orthogen AG, Düsseldorf, Germany). The blood-filled medical device was stored at 37°C for 6 hours, then centrifuged for 10 minutes at 3000 g. The resulting cell-free BCS was aspirated from the syringe and filtered through a 0.22 µM syringe tip filter (Millex GP, Merck Millipore, Tullagreen, Carrigtwohill, Cork, Ireland), aliquoted at 1 ml, and stored (at -18°C).

Patients underwent a series of 4 treatment sessions at 0, 2, 4, and 12 weeks. At each session, 2 ml of cell-free BCS was injected via a manual micro-puncturing technique,[2] deep dermally in the lower face at a predefined area (1 ml per side). Syringes of 1 ml volume with 30 G, 4 mm mesotherapy needles (RI.MOS SRL, Mirandola, Italy) were used to administer 4 superficial microinjections per cheek, each 1 cm apart following an injection grid.

Outcomes

All outcomes were measured at 0, 2, 4, 8, 12 and 24 weeks. Skin status evaluation was performed at screening.

The primary outcome was skin elasticity measured using by cutometry (Cutometer® dual MPA 580; Courage Khazaka GmbH, Cologne, Germany)[13] at 450 mbar of negative pressure. Both sides of the face were examined twice independently, the two measurements of each side were averaged, then an overall average of the whole face was calculated.

The primary efficacy criterion was inverse skin firmness (R0), representing the passive behavior of the skin to force. Skin tiring (R3), amplitude increases under repeated force, was used as a secondary efficacy criterion.

Other secondary outcomes were skin hydration, aesthetic evaluation, and safety. Skin hydration was measured using corneometry (Corneometer® CM 825; Courage Khazaka GmbH, Cologne, Germany).[15] Both sides of the lower face were examined three times independently, the three measurements of each side were averaged, then an overall average of the whole face was calculated. Aesthetic evaluation was performed using the Global Aesthetic Improvement Scale (GAIS); a qualitative, 5-point, relative-improvement scale, which has been

commonly accepted as a clinically meaningful measure.[16] The GAIS method was modified to allow evaluation of the lower face alone. A blinded investigator independently compared the patient's appearance at each visit with baseline using a high magnification photograph, whilst patients completed GAIS questionnaires at each visit. This was done for each cheek, then an average of both was calculated. Patients were also asked to rate their own attractiveness on a 4-point scale at each visit.

Safety

Adverse reactions (ARs) were documented over the whole study and classified according to seriousness, intensity, and relationship to treatment. Intensity was classified as; mild (not affecting day-to-day activities), moderate (affected day-to-day activities), or severe (day-to-day activities or work were no longer possible).

Sample Size

Effect Sizes (Cohen's d) were calculated from differences in instrumental measures between timepoints and, according to the classification suggested by Sawilowsky (extended Cohen's d)[17] to small (0.2); medium (0.5); large (0.8); very large (1.2), and huge (2.0). Effectiveness was conservatively considered clinically relevant with a mean effect size of ≥0.9. Under these conditions a minimum sample size of 18 patients would provide 80% power to show a significant therapeutic effect. A two-sided global significance level of α=0.05 was chosen.

Statistical Methods

Statistical analyses were performed with descriptive methods (frequency, mean, standard deviation) and inferential analyses using appropriate significance tests and confidence intervals. Missing values were not replaced. The statistical analysis of primary and secondary endpoints was performed with the full analysis set (FAS), because no protocol deviations or violations occurred. Statistical analyses were performed using SAS® software (version 9.4 Cary, NC).

Cutometer®, Corneometer® and patient self-assessment parameters at weeks 12 and 24 were compared with baseline using a two-sided exact Wilcoxon test for paired data. To counteract multiple comparisons in the primary outcome (independent testing of effects between baseline and 12 or 24 weeks in the parameters R0 and R3), the Bonferroni correction was used to test the individual hypothesis at α = 0.0125. GAIS ratings were tested using a two-sided Binomial test with a hypothesized probability of 'success' of 0.35 for improvement.

TABLE 1.

Summary of Baseline Characteristics of FAS Population		
Basic Demographics		**Baseline**
n	Total	21
Age	Mean (SD)	46.7 (5.9)
Gender	Female/Male	21/0
Skin Phenotype		**Baseline**
Type II	Number	11
	%	52.38
Type III	Number	7
	%	33.33
Type IV	Number	3
	%	14.29
Skin Condition		**Baseline**
Normal	Number	8
	%	38.10
Dry	Number	10
	%	47.62
Greasy	Number	3
	%	14.29
Skin Sensitivity		**Baseline**
Not-sensitive	Number	14
	%	66.67
Sensitive	Number	7
	%	33.33

SD = Standard Deviation

RESULTS

Baseline Patient Characteristics

Twenty-four female patients were screened from December 2015 to February 2016. Of these, 21 were allocated for treatment, finished the treatment schedule, and attended all visits. Baseline characteristics of these patients are summarized in Table 1. The FAS population had a mean (SD) age of 46.7 (±5.9). Skin status evaluation found 11 patients with type II skin phenotype (52%), 7 patients with type III skin phenotype (33%), and 3 patients with type IV skin phenotype (14%). Eight patients had normal skin (38%), 10 patients had dry skin (48%), and 3 patients had oily skin (14%). Seven patients had sensitive skin (33%) and 14 patients had non-sensitive skin (67%).

TABLE 2.

Summary of Change in Cutometer® Parameters vs Baseline at Weeks 12 and 24								
Cutometer Parameter	**Difference Week 0 - 12**				**Difference Week 0 - 24**			
	Mean	**SD**	***P* value**	**ES**	**Mean**	**SD**	***P* value**	**ES**
R0	0.1	0.05	<0.0001	2.00	0.18	0.04	<0.0001	4.50
R3	0.1	0.05	<0.0001	2.00	0.19	0.04	<0.0001	4.75

SD = Standard Deviation, ES = Effect Size (Cohen's d)

Skin Firmness and Tiring

Viscoelastic properties of the treated skin, measured by cutometry, significantly improved by 12 weeks and by 24 weeks of treatment with BCS (Table 2).

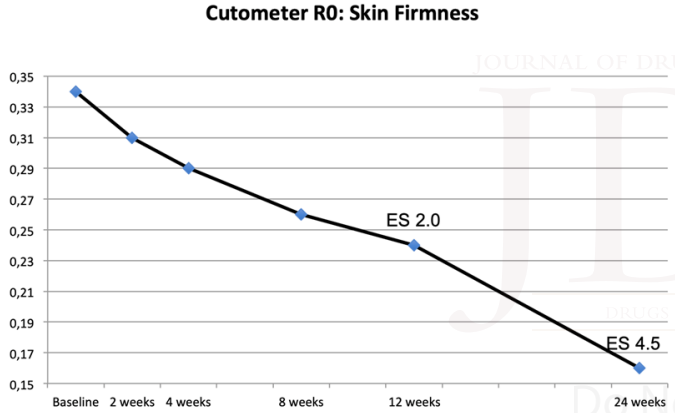
Mean (SD) inverse skin firmness decreased from 0.34 mm/s (±0.03) at baseline to 0.24 mm/s (±0.05) at 12 weeks (ES = 2.0, *P*<0.001) and to 0.16 mm/s (±0.03) at 24 weeks (Figure 1A, ES = 4.5, *P*<0.001). This represents a highly significant increase in skin firmness.

Mean (SD) skin tiring decreased from 0.39 mm/s at baseline (±0.04) to 0.29 mm/s (±0.06) at 12 weeks (ES = 2.0, *P*<0.001) and to 0.20 mm/s (±0.03) at 24 weeks (Figure 1B, ES = 4.75, *P*<0.001).

Skin Hydration

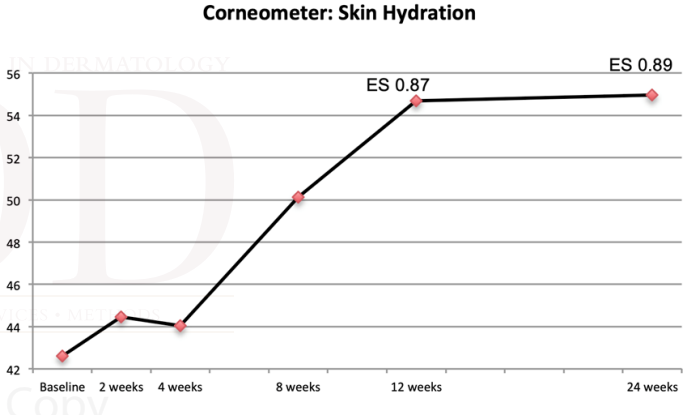
Mean (SD) skin hydration increased with treatment from 42.6 (±12.1) arb. units at baseline to 54.7 (±0.1) arb. units at 12 weeks (ES = 0.87, *P*<0.001) and to 55 (±0.14) arb. units at 24 weeks (ES = 0.89, *P*<0.001; Figure 2).

FIGURE 1A. Improvement of skin firmness recorded at each visit from baseline to week 24. Mean values for skin firmness (lower values mean higher skin firmness).



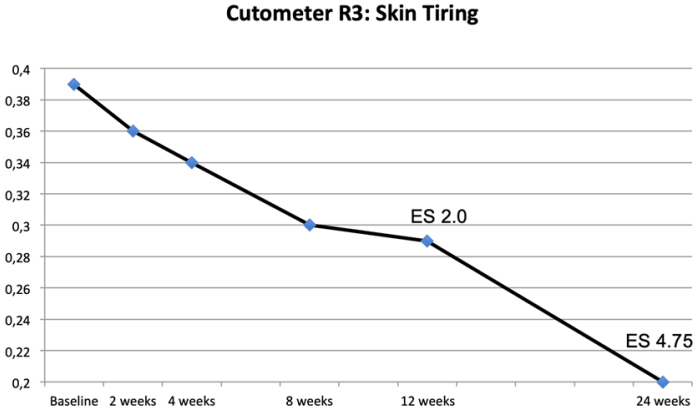
ES: Effect Size. Changes of R0 at week 12 and week 24 were statistically significant compared to baseline (*P*<0.001).

FIGURE 2. Improvement of skin hydration recorded at each visit from baseline to week 24. Mean values for skin hydration (higher values mean more skin hydration).



ES: Effect Size. Changes of Hydration at week 12 and week 24 were statistically significant compared to baseline (*P*<0.001).
Optimal skin hydration values are ~50 CM; this was reached after 8 weeks and sustained over 24 weeks.

FIGURE 1B. Improvement of skin tiring recorded at each visit from baseline to week 24. Mean values for skin tiring (lower values mean less skin tiring).



ES: Effect Size. Changes of R3 at week 12 and week 24 were statistically significant compared to baseline (*P*<0.001).

FIGURE 3. Patients' satisfaction assessed at every visit using the Global Aesthetic Improvement Scale (GAIS). GAIS scale percentages means of left and right cheek. The GAIS scale was assessed at the time points indicated and modified to assess only the lower face. Data are presented as the percentage of patients reporting improvement/strong improvement and worsening/unchanged in their appearance at each visit. Worsening was reported 2 times (4%) at 12 weeks and 1 time (2%) at 24 weeks.

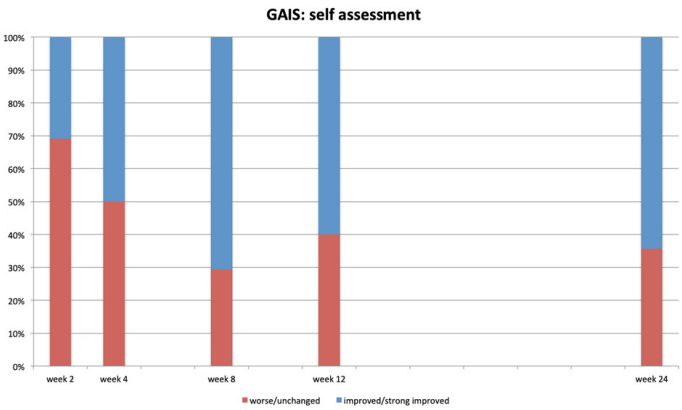


TABLE 3.

Summary of Patient Attractiveness Self-Assessment							
Attractiveness Self-Assessment		Baseline	Week 2	Week 4	Week 8	Week 12	Week 24
Total	%	100	100	100	100	100	100
More unattractive	%	4.8	0	0	0	0	0
Average attractiveness	%	71.4	66.7	76.2	81	57.1	76.2
More attractive	%	23.8	28.6	23.8	19.1	42.9	23.8
Very attractive	%	0	4.8	0	0	0	0

GAIS

Results by GAIS questionnaire for patients are summarized in Figure 3. GAIS significantly improved in patients treated with BCS compared to baseline at 8 weeks and up to 24 weeks (P<0.01). By 12 weeks 57.1% and by 24 weeks 64.3% patients reported improvement. By 12 weeks the investigator reported improvement in 59.5% and by 24 weeks in 11% of patients. Only 1 patient reported deterioration, which occurred at 12 weeks, and the investigator did not report any deterioration.

Patient Attractiveness Self-Assessment

Patients also reported their attractiveness on a 4-point scale at each visit (Table 3). At baseline 71% of patients reported their attractiveness as 'average', 24% reported appearing 'more attractive', and 5% reported appearing 'less attractive'. By 12 weeks only 57% of patients reported their attractiveness as 'average' but 43% reported appearing 'more attractive'. Finally, by 24 weeks, 76% reported appearing averagely attractive and 24% reported appearing 'more attractive'.

Safety Results

A total of 82 treatments were performed in 21 patients (in weeks 0, 2, 4, 12). There were no serious ARs and none were considered related to BCS. All ARs were considered related to the microinjection method. This included rashes/redness, swelling, bruising, and itching, as well as pain, transient small hematomas, or petechiae occurring at the injection site. Of these reactions, 51.5% were mild, 36.4% were moderate, and 12.1% were severe but did not lead to withdrawal of treatment. In 92.4% of cases, the adverse reaction did not last longer than 2 days.

DISCUSSION

Intradermal micropuncture injections of BCS significantly improved viscoelastic properties of aged skin. Skin firmness and skin tiring were both improved significantly at 12 and 24 weeks compared to baseline, with very large effect sizes. R0 represents firmness as a surrogate parameter for collagen and skin elasticity, and R3 examines the change in skin tiring (which will be reduced as firmness increases). These results indicate that BCS may have similar regenerative effects in dermal tissue and skin aging as in musculoskeletal disorders.[12,14]

Cutometer® measurements have been validated as reflecting changes in skin elasticity which are perceived visually.[18] Deterioration of these parameters accompanies changes in viscoelasticity, which are observed in older and more aged skin.[19] Accordingly, improvements in biophysical measurements were mirrored by patient and physician ratings of attractiveness, as measured by GAIS questionnaires. Most patients felt that BCS treatment improved their appearance by 12 weeks and that this was maintained at 24 weeks. This improvement was also confirmed by patient self-assessment on a 4-point scale. These ratings were based on overall attractiveness, although the treatment was administered only to a small area besides the mouth angle. This suggests a clinically relevant effect of the treatment on overall facial appearance.

BCS offers a regenerative and remodeling approach to rebuilding tissue and restoring skin elasticity. We hypothesize that the efficacy of BCS is in part mediated by growth factors it contains, especially TGF-β, which promotes collagen synthesis and homeostasis. Daily exposure to environmental stressors, including ultraviolet light, increases oxidative stress in skin.[4,20,21] This results in tissue damage and production of reactive oxygen species (ROS), also known as free radicals. ROS can be formed, eg, also by damaged mitochondria, which are a sign of senescent or dysfunctional cells. Increased ROS suppress TGF-β receptor II (TGF-β-R2) in human skin, leading to decline of collagen.[22] ROS have been shown to be decreased by BCS-injections into osteoarthritic knees.[14] TGF-β signaling regulates collagen homeostasis and has a significant impact on the aging of human skin and connective tissue.[23] Lack of TGF-β signaling appears to be a critical event in decline of dermal collagen production, with attendant skin thinning. BCS injected in tendons of horse and rat has caused significantly increased levels of collagen I which is also present in facial skin.[12] These studies and the present cutometry data suggest that BCS may induce collagen I synthesis in skin. Skin fibroblast growth factors, especially TGF-β1, enhance production of high molecular weight hyaluronic acid (HA) by upregulating HA synthase.[11,24] HA production is also upregulated in BCS treated osteoarthritic joints.[14] In addition, inflammation-resolving components including lipid mediators, cytokines, and extracellular vesicles (exosomes) known to be

present in BCS may modulate chronic age-related inflammation of the skin and support skin regeneration and remodeling.[25-27]

BCS improved skin hydration to an optimum level by 12 weeks, which was maintained at 24 weeks. This represents an excellent long-lasting improvement. Similar improvements in skin hydration have been observed directly after 8 to 12 weeks of HA treatment.[28,29] BCS contains components described to enhance HA production and decrease HA breakdown.[24] Since the effect is long-lasting it is likely that the improvements described here are triggered by a regenerative alteration in skin metabolism. In contrast, BTX has been found to decrease skin hydration,[30] which highlights an additional relative advantage of BCS.

Overall, BCS was well tolerated. There were few adverse reactions, all of which were local, transient, and the majority mild or moderate. This is in line with results observed using BCS in orthopedic indications.[12,14]

Current treatments for reduced facial skin elasticity include BTX, dermal fillers, and other autologous products such as PRP. BCS achieved a similar or better improvement as these treatments.[7,30,31] Micropuncture injections of PRP administered over 3 months improved Cutometer® measurements by a similar magnitude to 12 weeks of BCS treatment.[7] However, unlike cell-free BCS, PRP contains leukocytes and platelets, which increase tissue inflammation by multiple mechanisms.[32,33] PRP is prone to release a coagulation-linked burst of platelet borne inflammatory signals such as ATP, ADP, histamine, and Serotonin as well as complement activation.[14] This may explain the high incidence of mild-to-moderate erythema observed with PRP.[7]

Expert consensus has recommended combining multiple interventions, to target different manifestations of aging for better overall results. Based on its good safety profile and efficacy in both biophysical measures and patient satisfaction, this study indicates that BCS should be considered as a new class of skin treatment. By targeting multiple molecular mechanisms of aging and stimulating regeneration of skin tissue, BCS provides an innovative complementary or individual therapy. The components of BCS are released largely during incubation. This process may be described as an extracorporeal wound simulation using a specific bioreactor with limited incubation time under hypoxia. This leads to activation of an evolutionarily preserved program that initiates secretion of essential components, the secretome, required for healing. In an in vitro skin model, BCS upregulated important genes according to Affymetrix GeneChip analysis; S100A2, HAS3, FGF17, and CXCL8 at day 3, followed by HSPB2, FLG, FLG2, IL-37, DSC1, KRT4, and Serpine1 at day 5.[11] Gene ontology analysis for day 3 and 5 showed significant effects on skin development and keratinocyte differentiation.

Analytical data together with concurrent clinical improvements encourage the authors to hypothesize that BCS may be capable of inducing tissue macrophages to regenerative M2 polarization. In conjunction with enhanced stem cell activity macrophages, also present in skin, may induce a fundamental metabolic shift in favor of restoring skin homeostasis.[34,35]

Given the main limitations of the study being the open, non-controlled design and the low patient number, it is noteworthy that effects observed in patient and investigator GAIS assessments were all in accordance with instrumental measurements, confirming the relevance of the findings. Notably, there was some inconsistency between patients' GAIS ratings (based on their experience) and investigator GAIS ratings (based on photographs) at 24 weeks. Over extended time periods patients tend to get used to the facial improvements, therefore a subjective perception after 3 and 6 months may not be experienced as persistent. In contrast, the patients' 4-point self-assessment scores more closely resemble investigator's GAIS ratings. This inconsistency supports the use of validated biophysical measures as primary outcomes.

The sample size of this study was not calculated based on published trials. However very large effect sizes were observed for improvements in the primary efficacy parameter, indicating that Type-II error has been avoided.

CONCLUSION

Four biweekly micropuncture injections of BCS significantly improved skin firmness, elasticity, and hydration of the lower face at 12 weeks, further increasing until 24 weeks post baseline. This corresponded to improvements in attractiveness, as assessed by both the investigator and patients. An in vitro skin model has confirmed upregulation of genes relevant to skin regeneration.[11] Thus, BCS should be considered as a new class of regenerative and remodeling treatment to target underlying causes of skin-aging, for use either alone or in combination with other treatments. These results may be attributed to a fundamental shift in skin metabolism leading to a status usually attributed to younger skin: increased firmness, elasticity, and hydration.

DISCLOSURES

The sponsor provided grant to the University of Hamburg, as well as consultant fees to Professor Kerscher and Clinical Trial Support (Münster, Germany). Dr. Drabik is employed by Clinical Trial Support. Dominique Hertz-Kleptow is employed by the University of Hamburg. Dr. Kaptan is a former employee of Orthogen AG. Julio Reinecke is employed by Orthogen AG.

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