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Case Report

A Case Series Report on Superficial Application of Polydensified Cohesive Matrix Hyaluronic Acid Through Biopsies

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Abstract: The skin comprises three main layers: epidermis, dermis, and hypodermis. The dermis is formed by connective tissue with an extracellular matrix composed of glycosaminoglycans and collagen fibers, providing skin resistance. During aging, the loss of the skin's biomechanical properties results in sagging. Exogenous hyaluronic acid is highly used as a filler. However, few studies using biopsies have demonstrated its action as a dermal remodeler and collagen stimulator in superficial applications and using polidensified cohesive gel. Methods: Skin tissues obtained from biopsies in the patient's arms were evaluated for histological, immunohistochemical, and biochemical analyses, in addition to clinical assessments by skin ultrasound. Biopsies were performed at time zero, three, and six months after intradermal injection of hyaluronic acid with a polydensified cohesive matrix in four women of different age groups. Results: The individual results showed hyaluronic acid synthesis, an increase in type I and III collagen, and a thickening of the dermal layer after the treatment. Conclusion: In four thirty-five to sixty-year-old patients, we observed the effectiveness of using polydensified cohesive matrix hyaluronic acid as a collagen stimulator, thickening the dermis and stimulating endogenous hyaluronic acid synthesis. This study highlights the importance of individual analysis of the variables studied.

Keywords: hyaluronic acid; neocollagenesis; dermal remodeling

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1. Introduction

The skin covers the surface of the entire body and consists of the epidermis (epithelial tissue), the dermis (connective tissue), and the hypodermis, which is composed of fat cells [1]. Unlike other tissues, which are essentially cellular, the main component of connective tissue is the extracellular matrix (ECM), a set of molecules consisting of different combinations of fibrous proteins and hydrophilic and sticky macromolecules [2].

This variety of molecules controls cell growth. It fills spaces between cells and fibers, acting as a lubricant and barrier. Fibroblasts are the main cells in connective tissue. They promote mechanical resistance by synthesizing fibers and maintain tissue homeostasis by remodeling the extracellular matrix. This occurs through the formation of glycosaminoglycans, proteoglycans, and multi-adhesive glycoproteins [3].

Chronological aging causes the dermis to thin. This is due to biochemical and structural changes in collagen, elastic fibers, and the matrix. Collagen levels fall as collagenase

rises, and elastogenesis and fiber distribution suffer. Glycosaminoglycans also decline. Throughout adulthood, collagen content drops by 1% a year, with fibers becoming disorganized and granular. Elastic fibers are lost [4,5]. We also observe an unregulated balance between the skin and the fibroblast, responsible for synthesizing and organizing the extracellular matrix (ECM), which loses its mechanoregulatory mechanism, the 'scaffold' [6].

In women, the reduction in dermal collagen content is also related to the reduction in serum estrogen levels, further increasing skin extensibility and reducing elasticity, with skin atrophy, dryness, and poor healing observed [7]. Such changes are more related to the climacteric than just chronological aging, which explains the worsening of sagging during this period [8].

In addition to hormonal changes, factors such as UV and infrared radiation, inadequate eating habits, and pollution accelerate skin cell senescence, reducing the synthesis of type I pro-collagen and increasing collagenase levels. The greater amount of collagen degradation products negatively regulates neocollagenesis [9]. Thus, changes in the mechanical properties of the skin during adult life include the progressive loss of elasticity consistent with the gradual loss of the fiber network and the prolongation of the time needed for the skin to return to its original state after clamping, which characterizes skin flaccidity [10]. A decline in glycosaminoglycan levels in the skin during the aging process also results in the formation of fine wrinkles, which in turn give rise to the appearance of cracked skin [4].

Many treatments for skin aging aim to increase collagen production, such as fractional CO2 laser, micro-focused ultrasound, and other technologies, such as chemical peels, intradermotherapy, microneedling with drug delivery, and applications of biostimulatory products such as calcium hydroxyapatite, polycaprolactone, and poly-L-lactic acid [11,12].

Exogenous hyaluronic acid is also a well-established tool in the field of skin rejuvenation. It has been employed as a filler for volumizing, for rehydration as a skin booster, and also improves skin quality, with effects that can be observed for longer than recommended by various industries [1].

Endogenous hyaluronic acid (HA) is a glycosaminoglycan in the extracellular matrix (ECM). This biomacromolecule comprises repeated disaccharides of glucuronic acid and N-acetylglucosamine. It is crucial to the extracellular matrix (ECM) of many connective tissues, including skin, cartilage, and synovial fluids. Its chemical structure and physical properties are responsible for its diverse biological functions, such as skin hydration, joint lubrication, and participation in the healing process and remodeling of the ECM [7–13].

HA also influences biological processes like cell activity, inflammation, and fibrosis. The distinction between regeneration and scarring hinges on its availability. It plays a role in tissue depending on the stage of inflammation of the ECM [14].

Exogenous hyaluronic acid is a biocompatible molecule with minimal adverse immune response, suitable for aesthetic and therapeutic use, including in patients with autoimmune conditions. Its application results in a natural aesthetic appearance, in addition to having the advantage of presenting reversible results using the hyaluronidase enzyme [15,16].

Its viscosity depends on the gel particle size, units per volume, and cross-linking. Cross-linking affects stability, viscosity, and durability. The different concentrations and types of cross-linking define their physicochemical, static, and dynamic rheological properties, determining their presentations and their use for each specific purpose, as well as the region to be treated [17,18]. The hyaluronic acid products available in the pharmaceutical industry are also distinguished by the substance used in the cross-linking process. These include divinylsulphone, diepoxyoctane (DEO), and butanediol diglycidyl ether (BDDE) [19].

We aimed to evaluate the use of hyaluronic acid with a polydensified cohesive matrix as an inducer of collagen production and dermal remodeling in clinical and pathological

examinations after intradermal application in four patients of different age groups. Skin biopsies were taken prior to application (control) and at three and six months after the product's use. A single-phase gel comprising a cohesive polydensified matrix (Belotero®soft line from Merz Pharmaceuticals, Frankfurt, Germany) was employed. The gel incorporates CPM (Cohesive Polydensified Matrix) technology, with a concentration of 20 mg/mL and a cross-linking level of 1+/4+, based on a scale of 1 to 4 degrees of intensity.

The technology in question, for its potential for greater stability, could display specific characteristics for the effects we intend to study, such as neocollagenesis and improved dosage of glycosaminoglycans, which will be elaborated on in detail later.

2. Materials and Methods

It is a longitudinal, uncontrolled clinical trial. This study was approved by the Human Ethics Committee of Centro Universitário Faculdade de Medicina do ABC under number 3,850,307.

2.1. Casuistry

We selected four female patients, one representative of each age group separated by decades: A.C., 35 years old; L.H., 44 years old; S.N., 50 years old; and R.D., 60 years old. Criteria were established for choosing patients based on physical characteristics, such as the level of photoaging compatible with their age according to the examiner's clinical assessment and lifestyle and behavioral habits, determined after completing a questionnaire. The aim was to ensure they were similar in these aspects so that the only significant variable for comparison was age.

Inclusion criteria: patients were selected according to nutritional, physical, and behavioral standards, including a stable BMI between 20 and 25, sun exposure, adherence to treatment, and practice of physical activities. We used the International Physical Activity Questionnaire (IPAQ) classification to evaluate this last criterion. Patients who consented to the protocol and signed the Informed Consent Form (ICF) were considered active. They were informed about the purpose of the research during the initial visit when the ICF was delivered.

Exclusion criteria: Patients not meeting the criteria were excluded. Exclusion factors included pregnancy, lactation, tobacco use, autoimmune disease, coagulation disorder, anticoagulant drug use, anti-inflammatory drug use, or treatment of chronic disease, infectious disease, or systemic or dermatological disease. Patients with a history of allergies or any other conditions that could affect participation in the study were also excluded. Additionally, those who declined to sign the Informed Consent Form (ICF) were not included.

2.2. Methods

Tissue samples: We delimited a region on the inner face of the arm, starting from the calculated central point, and demarcated an area of 2 × 2 cm, as shown in Figure 1A.

This region was chosen because it is a non-apparent area on the body, avoiding aesthetic compromise. The 2 × 2 cm area was subdivided into 4 quadrants of 1 × 1 cm, thus determining quadrants I to IV (Figure 1B).

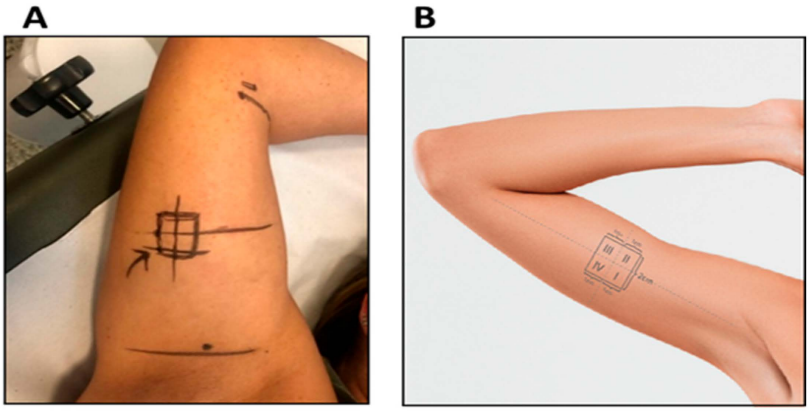


Figure 1. (**A**). Delimitation of the arm region for hyaluronic acid injection and skin biopsies. A. Central area of the inner side of the arm delimited for the procedures. The arrow indicates the quadrant I (first biopsy before applying HA) Figure 1 (**B**). Schematic representation of the four quadrants. I, area of control biopsy. II, III, and IV areas in which HA was applied.

Initially, a biopsy was performed in the lower medial quadrant, defined as quadrant I (QI), and hyaluronic acid was applied using CPM technology (concentration 20 mg/mL, crosslinking level +/4+) in the other quadrants (QII, QIII, QIV). The dose used was 0.2 mL in each quadrant, totaling 0.6 mL in each patient. Three months after the HA injection, a biopsy was performed in the medial upper quadrant (QII), and six months after the hyaluronic acid application, a biopsy was obtained in the lateral upper quadrant (QIII). No biopsy was performed in the lower lateral quadrant (QIV). The same procedures were performed on four female patients of different ages. Skin fragments were obtained from 4 mm punch biopsies during the pre-application of hyaluronic acid (control) periods and after 3 and 6 months of hyaluronic acid application. The skin fragments obtained from each quadrant were immediately stored in vials containing 10% buffered formaldehyde solution for histological, pathological, and immunohistochemical analyses and in acetone solution to determine the concentration of hyaluronic acid.

2.2.1. Pathological Analysis

Skin biopsies were divided into two portions, each 2 mm. Tissue samples from four patients were fixed in formalin and embedded in paraffin. Five micrometres thick sections were stained with hematoxylin and eosin (H&E), Masson's, and picrosirius. The slides were scanned with an Aperio CS2 (4X and 40X) by Leica Group (St. Gallen, Switzerland) resulting in digital files. Slides were analyzed with ImageScope™ Angle Two Version 2.0 Software Aperio (St. Gallen, Switzerland). Two pathologists reviewed the H&E-stained slides. The epidermis and dermis were assessed based on the control tissues and tissues affected by hyaluronic acid (HA). Subsequently, staining was conducted to assess inflammation and neovessels and to evaluate the papillary and reticular dermis. Finally, picrosirius staining was performed to ascertain the deposition of collagen types.

2.2.2. Histomorphometric Analysis

Ana Maria Amaral, a pathologist PhD in Health Sciences from UNIFESP, presented the observed changes in the form of crosses, according to the degree of intensity of the expression; which iindicate their absence or normalcy (0), mild (+), moderate (++), and intense (+++). The cross-analysis was transformed into absolute numbers for better visualization. The control was 1, mild 2, moderate 3, and intense 4. This allowed the presentation of pathological results in graphs. The present study employs a semi-quantitative approach.

2.2.3. Immunohistochemistry Analysis

The primary antibodies were Rabbit Anti-Collagen I (ab254113) and Mouse Anti-Collagen III (ab6310) (Abcam Inc., Cambridge, UK). Immunolabeling was conducted using the LSAB2+ System-HRP kit (Dako North America, Inc., Santa Clara, CA, USA) and DAB+ Substrate Chromogen System (Dako North America, Inc., Santa Clara, CA, USA). The slides were analyzed using a Nikon microscope to identify the best areas for immunostaining. The immunostaining was quantified using digital image analysis [20]. Photomicrographs of 640 × 480 pixels were obtained from non-coinciding fields under an optical microscope with a Nikon Coolpix 4300® digital camera (Bangkok, Thailand). The images were analyzed using ImageJ® (Softium Informática®, São Paulo, Brazil), with results in micrometers (µm). The collagen immunostaining values are the result of an evaluation of eight regions of each slide, expressed in terms of intensity (itE).

2.2.4. Hyaluronic Acid Dosage

The hyaluronic acid assay uses an ELISA-like fluorescence test [21]. The wells were coated with hyaluronic acid-binding protein, and 100 µL of hyaluronic acid standard solution was added. A standard curve was constructed using Tris-HCl 0.05 M, pH 7.75 with 1% albumin. The homogenate samples were diluted 1:1000 after proteolysis. An aliquot of the diluted sample was incubated for 12 h at 4 °C and then washed three times. An aliquot of the probe (biotin-conjugated binding protein) at 1 mg/mL was added at 100 µL, diluted 1:10,000 in the same buffer. The protein forms a complex with hyaluronic acid. The plate was shaken for two hours and washed nine times. A 1:10,000 dilution of europium-labeled streptavidin was added to each well. Streptavidin binds strongly to biotin-conjugated hyaluronic acid. The plate was shaken and washed nine times. The europium-streptavidin complex was removed by adding 280 µL/well and stirring for five minutes. Free europium was quantified using a fluorometer (Victor 2, Turku, Finland). Fluorescence intensity is proportional to hyaluronic acid concentration. Data were processed with MultiCalc (PerkinElmer). Hyaluronic acid was quantified per ng/mL and corrected per µg total protein from skin tissue.

2.2.5. Ultrasound Evaluation

One year after the procedure, each patient had a skin ultrasound. This examination provided a precise clinical assessment of changes in dermal thickness, allowing for a comprehensive understanding of hyaluronic acid's long-term effects on the skin. Furthermore, it allowed for a direct comparison with the adjacent area. A year after hyaluronic acid injections, skin ultrasound exams were conducted at Voluta Diagnose in Campinas, Brazil. 18 MHz high-frequency probe used. Dr. André Munhoz conducted the examinations on the four patients in the study. The parameters examined included the potential observation of hyaluronic acid gel and the thickness of the skin in the treated area, as compared to the control skin. The images were jointly analyzed by the research doctor and radiologist.

2.2.6. Statistical Analysis

Statistical analysis and graphs were performed using the GraphPad Prism 5® program (GraphPad® Prism Software Inc., San Diego, CA, USA). Values were expressed as the mean and standard deviation with a significance level of 95% ($p \leq 0.05$). The test applied was unpaired one-way ANOVA, followed by the Kruskal–Wallis post-test.

3. Results

Initially, all skin samples collected during the study were stained with hematoxylin and eosin. Qualitative analysis of H&E revealed infiltration of lymphomononuclear cells

(lymphocytes and monocytes) after three months of treatment (hyaluronic acid injection) in patients A.C., 35 years old, and S.N., 50 years old, indicating an inflammatory process in their skin. There was also neovascularization three months after treatment only in the skin of patient A.C., 35 years old (Figure 2).

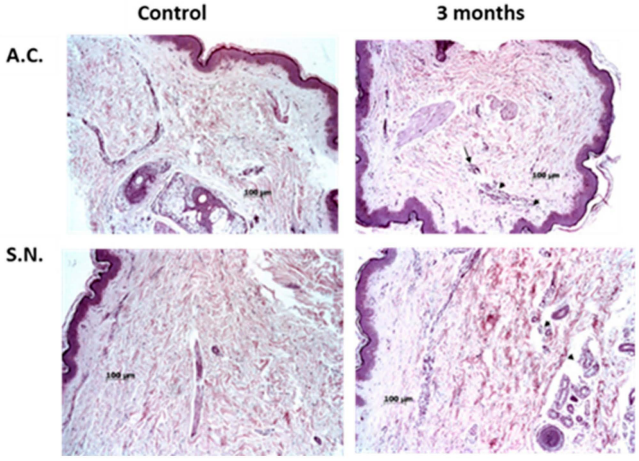


Figure 2. Hematoxylin and eosin staining. The analysis shows inflammation and neovascularization. The Inflammatory infiltrate is highlighted with an arrowhead, and the neovessels are highlighted with an arrow. Patients A.C. and S.N. are 35 and 50 years old, respectively.

However, no inflammatory process was observed in the tissue analyses of the other two patients, L.H., 44 years old, and R.D., 60 years old.

The analyses performed by pathologists, after a thorough examination, describe a significant thickening of the dermis in all skin tissues analyzed three months after hyaluronic acid injection. This thoroughness in our research process instills confidence in the results. Masson's Trichrome staining analysis also shows a substantial increase in elastic fibers in the dermis after treatment with hyaluronic acid, further confirming our findings (Figure 3).

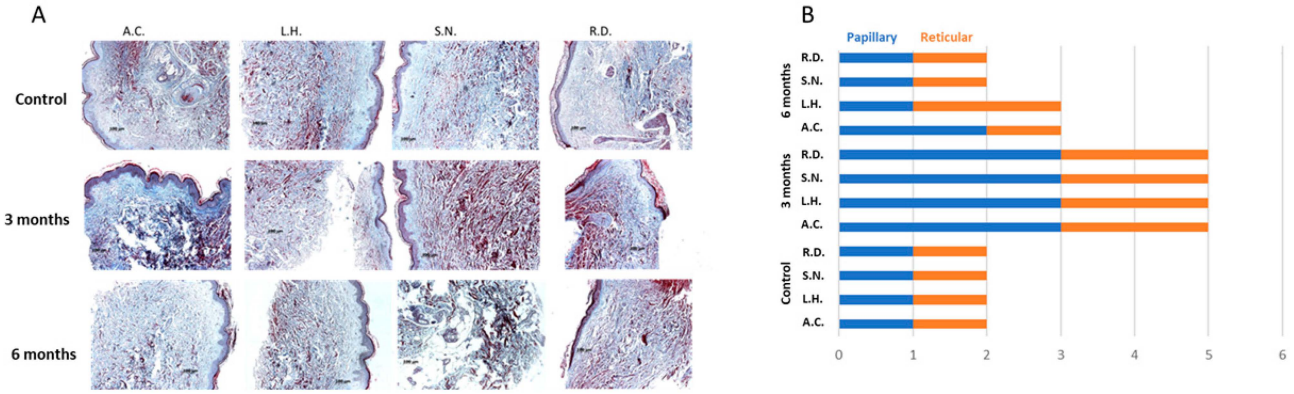


Figure 3. Masson's Trichrome Staining. (**A**). All biopsy samples collected from the four patients at time zero, before treatment (Control), and three and six months after HA injection (three months) and (three months) were subjected to this staining. (**B**). Semiquantitative representation of the analyses of tissues stained with Masson's trichrome. Thickening of the Papillary Dermis (Papillary) and Reticular Dermis (Reticular) 3 months after HA injection. A.C. 35 yr, L.H. 44 yr, S.N. 50 yr, R.D. 60 yr.

To summarize the findings with Masson's Trichrome staining, a semi-quantitative analysis was performed. This clearly shows an increase in the thickness of the papillary dermis (score 3) and reticular dermis (score 2) three months after the application of hyaluronic acid in the skin of four patients of different age groups compared to the control groups (score 1), as shown in Figure 3B. However, six months after treatment with hyaluronic acid,

an increase in the thickening of the papillary dermis was observed only in patient L.H., aged 44.

Picrosirius staining, analyzed under polarized light microscopy, allows the identification of type I collagen fibers (red) and type III collagen fibers (green). In the skin, type I collagen represents approximately 85% of the total collagen, while type III collagen represents the remaining 15%. Type III collagen is found together with type I collagen, and a yellow color can be observed due to the overlap between the two (Figure 4).

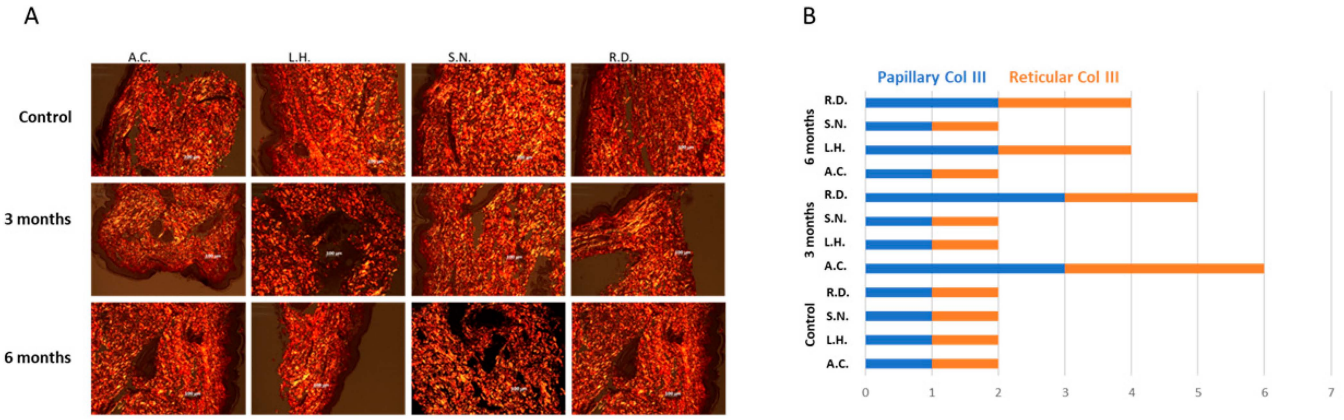


Figure 4. Picrosirius staining analyzed under a microscope with polarized light. (**A**). Type I collagen fibers are highlighted in red, and type III collagen fibers are highlighted in yellow. (**B**). Analysis of type III collagen using Picrosirius staining. Observed changes in type III collagen concentrations in the papillary dermis and reticular dermis at three and six months of treatment compared to controls (time zero). A.C. 35 yr, L.H. 44 yr, S.N. 50 yr, R.D. 60 yr.

There was no significant change in the amount of type I collagen in all samples evaluated by the pathologists; however, an increase in type III collagen was observed after three months of treatment in patients A.C. and R.D., aged 35 and 60 years, respectively, persisting in patient R.D. for up to six months. In patient L.H., aged 44 years, type III collagen increased six months after injection with hyaluronic acid (Figure 4).

Therefore, there was an increase in type III collagen in the skin of three patients who underwent hyaluronic acid injection at the different times studied after treatment with hyaluronic acid.

We analyzed the types of collagen I and III using immunohistochemistry studies that identified each collagen with specific antibodies, as described in detail in the methods.

The immunohistochemistry reactions performed by detection with specific antibodies for collagens I and III in the skin biopsy samples were subsequently analyzed for quantitative determination of the expression of these molecules. To evaluate the intensity of the reaction (ItE), with a brownish coloration, ImageJ® software was used, and the values define the amount of collagen by intensity of labeling.

Figure 5 represents immunohistochemistry reactions after specific labeling for collagen I and collagen III of skin samples obtained at time zero (controls), before the application of hyaluronic acid, and six months after treatment in the 60-year-old patient R.D. However, it is important to highlight that this analysis was performed for all samples collected in the study.

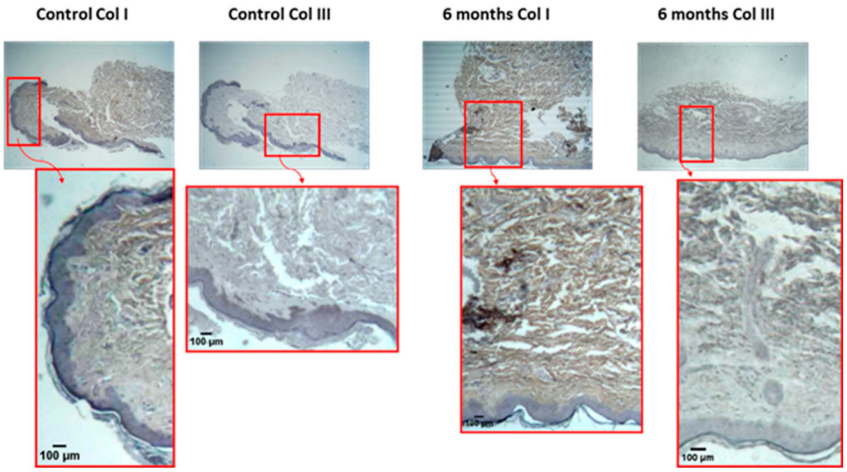


Figure 5. Images obtained by immunohistochemical reactions to identify collagen I (Col I) and collagen III (Col III). The slide represents a sample obtained from patient R.D., 60 years old, before treatment (control) and after hyaluronic acid injection (6 months). The upper panel identifies a larger area of the slide, while the lower panel identifies the amplification of a specific area of such slide.

Figure 6 shows that six months after the injection of hyaluronic acid, there was a significant increase in collagen I in patients L.H., 44 years old, and R.D., 60 years old. However, no change was observed in the quantification of collagen I after treatment with hyaluronic acid for patient L.H., 44 years old, while there was even a small decrease in collagen I in patient S.N., 50 years old.

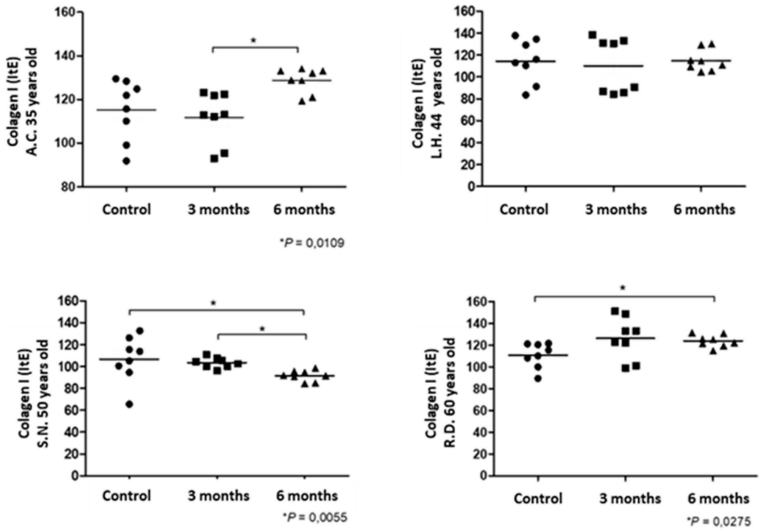


Figure 6. Quantification of collagen I. Values represent collagen I protein expression, determined by immunohistochemistry using a specific antibody. Each value was obtained by evaluating eight fields on the same slide, thus avoiding bias. Statistical analysis was performed by one-way ANOVA with Kruskal–Wallis post-test, considering significant values at $p < 0.05$.

Similarly, we evaluated the expression of type III collagen separately in each patient. We observed that patient A.C., aged 35, had a significant increase in collagen III after three months of treatment, while patient R.D., aged 60, showed an increase in this collagen at three months and six months after the injection of hyaluronic acid (Figure 7).

We can also highlight that despite the decrease in collagen III in patient L.H., aged 44, at three months, the values returned to high levels six months after the injection of hyaluronic acid, as shown in Figure 7.

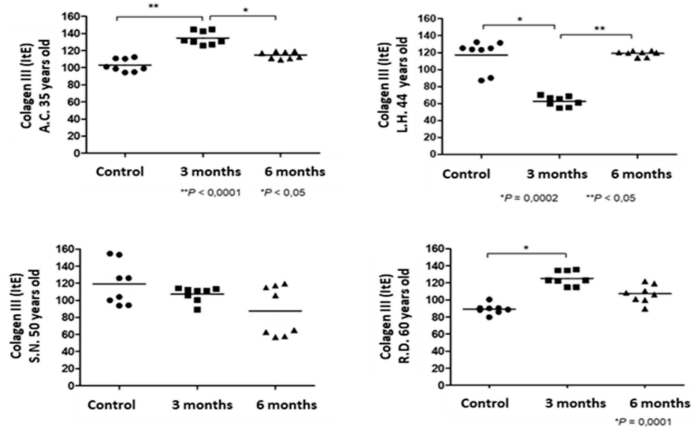


Figure 7. Quantification of collagen III. Values represent protein expression of collagen III. Each value was obtained by determining the intensity and color intensity of the reaction on the same slide to avoid bias. Statistical analysis was performed by one-way ANOVA with Kruskal–Wallis post-test, with a significant value at $p < 0.05$.

Since type III collagen is considered to be present mainly in remodeling situations, we can suggest that six months after the injection of hyaluronic acid, the synthesis of type III collagen still increased. The Col I/Col III ratio is one way to assess skin firmness; a value lower than 1 indicates sagging tissue [22].

Considering the ratio between collagen I/collagen III at time zero, before injection with hyaluronic acid, as 100%, we observed an increase in this ratio of 0.7% and 2.7%, respectively, at three and six months after treatment with hyaluronic acid, indicating that the treatment was effective (Figure 8).

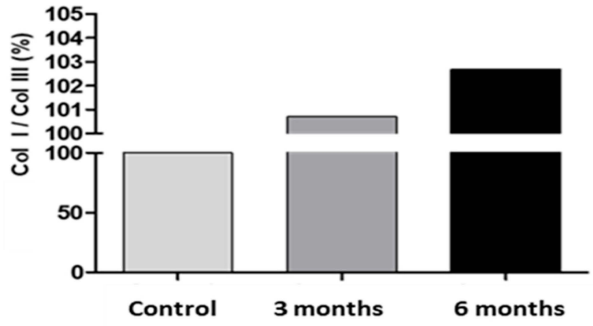


Figure 8. Relationship between type I collagen and type III collagen. The values express the percentage obtained by the relationship between collagen I and collagen III at the different times of the study, considering 100% as the ratio obtained at time zero before the injection of hyaluronic acid (Control). The graph also expresses the percentage obtained by the Col I/Col III ratio after 3 months and 6 months of hyaluronic acid injection.

Additionally, we were interested in determining the amount of hyaluronic acid present in the skin of all patients submitted to the study at time zero and after the injection of hyaluronic acid since this molecule, like collagen, as already mentioned in the Introduction, is also reduced throughout the aging process and plays an essential role in the structuring of the entire ECM.

Figure 9 shows that the amount of hyaluronic acid in patients' skin is quite similar before the start of treatment and three months after the injection of hyaluronic acid, regardless of age group. However, we observed a significant increase in the amount of hyaluronic acid in the skin samples of all patients six months after treatment. The increase in the synthesis of hyaluronic acid six months after the start of treatment represents a very positive factor since hyaluronic acid, in addition to promoting hydration, organizes the collagen fibers in

the extracellular matrix, which can contribute to improving the elasticity and firmness of the skin after treatment.

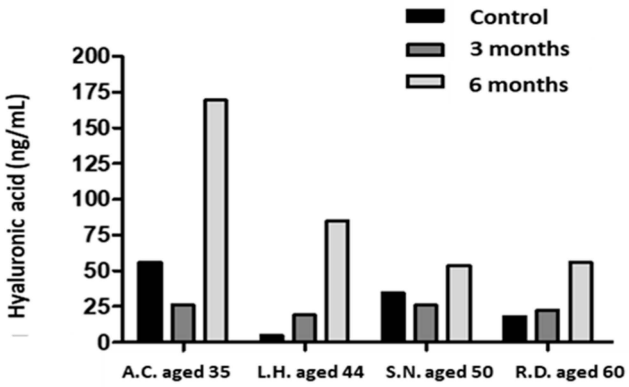
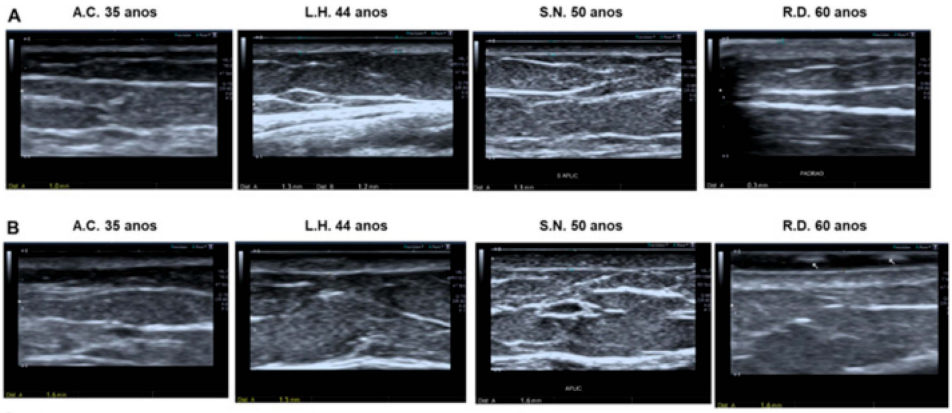


Figure 9. Hyaluronic acid measurement in patients' skin before and after hyaluronic acid injection. Quantification was performed using ELISA. The amount of total protein was accounted for in each sample, with control samples taken prior to treatment and samples taken three and six months after hyaluronic acid injection for subsequent measurements. The values express the amount of hyaluronic acid in ng/mL, and the assay was performed in quadruplicate.

To elucidate the clinical improvement of the skin, as well as to confirm the increase in dermal thickness observed in the histological examination (Masson's Trichrome staining), one year after the hyaluronic acid injection, the patients underwent an imaging exam, in which a cutaneous ultrasound was performed on each patient in the area subjected to the hyaluronic acid injection, as well as on the skin region not subjected to treatment and immediately next to the injection region. The same radiologist performed the ultrasound in all four cases studied. The data obtained by the skin ultrasound exam prove an increase in the thickness of the epidermis/dermis layer in all patients one year after the hyaluronic acid injection, which contributes to clinically proving the effective result of such treatment (Figure 10).



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Pacientes	US de Pele controle	US Pele após 12 meses	Porcentagem de aumento da espessura da pele
A.C. 35 anos	1,0 mm	1,6 mm	60%
L.H. 44 anos	1,3 – 1,2 mm	1,5 mm	20%
S.N. 50 anos	1,1 mm	1,6 mm	45%
R.D. 60 anos	0,3 mm	1,6 mm	433%

Figure 10. Skin ultrasound. (**A**). Image of an untreated control area adjacent to the studied region, with measurements of the dermis-epidermis of the 4 patients according to their ages. (**B**). Image of the quadrant in which HA was applied, with measurements of the dermis and epidermis of the 4 patients according to their ages. (**C**). Table demonstrating the increase in dermis-epidermis thickness in the treated region after 12 months of the study, with the respective percentage of increase for each patient, compared with the adjacent region (control).

4. Discussion

The imbalance in skin regulation generated by the aging process, especially during a woman's life, in which hormonal factors related to the climacteric period play a major role, leads to a number of findings, as previously mentioned. These include a reduction in the inflammatory response, which results in delayed epidermal renewal and connective tissue regeneration. This, in turn, explains the reduced inflammatory response observed in the more mature groups compared to the younger group A (aged 35 years). In patient A.C., aged 35 years, a greater inflammatory infiltrate and neovessel formation were observed by H&E staining [5,14,18].

In the present study, the infiltrate observed in patients A.C., 35 years old, and S.N., 50 years old, by H&E staining was composed of lymphomononuclear cells, chronic and compatible with what was expected for the time of the second biopsy (3 months after the application of hyaluronic acid). This infiltrate was different from that observed by Flynn and collaborators in an emblematic study, which used biphasic gels and biopsies taken 14 days after the application of the gel. In that study, the appearance of eosinophilic infiltrate characteristic of foreign body-type inflammatory reactions was noted, along with the formation of granulomas, an undesirable effect in aesthetic treatments [23]. These findings were confirmed by cutaneous ultrasound in a later study by Micheels [24].

Masson's trichrome stain revealed dermal thickening in the papillary and reticular layers in all patients at three months post-application of hyaluronic acid. This finding suggests probable remodeling and redensification of the extracellular matrix (ECM). These effects were maintained at six months, with notable thickening of the papillary dermis observed in patient A.C., a 35-year-old, and the reticular dermis in patient L.H., a 44-year-old, which indicates a more prolonged effect in both cases.

The ultrasound examination of the skin 12 months after the application of hyaluronic acid corroborates the macroscopic data regarding the thickening of the treated area, as observed through microscopic analysis using Masson's trichrome staining in all patients in the study. This reinforces the permanence of the positive effect one year after the injection of hyaluronic acid.

These findings can be explained by the characteristics of the hyaluronic acid used in the treatment, which has the property of tissue integration and spreading when mixed with the reticular dermis, as well as its ability to preserve the integrity of dermal cells and the extracellular matrix, combined with the fact that it promotes a homogeneous effect on the skin [23–25]. Our results also confirm another experiment by Micheels et al. [26], who tested the bio-integration of three different types of hyaluronic acid and found that tissue integration was related to the viscoelastic properties of the product used with single-phase cohesive hyaluronic acids with polydensity showing greater homogeneity when mixed with the reticular dermis [26].

Analyses using Picrosirius stain showed an increase in type III collagen in the dermis at three and six months in three of four patients. The data show that hyaluronic acid leads to collagen formation. This effect was sustained at six months in patients L.H. (44) and R.D. (60), proving that the oldest age group exhibited enhanced neocollagenesis following treatment.

It has been demonstrated that the ratio of collagen I to III is diminished in aged skin [27]. Consequently, the ratio between the quantities of these collagens can be regarded as a significant indicator in evaluating rejuvenation treatments, along with the absolute numbers of the isolated concentrations of each collagen type [22,28].

We emphasize that although patient S.N., aged 50, unlike the other three patients who obtained an increase in collagen, showed an increase in the type I collagen to type III collagen ratio, proven by the measurements performed in the quantification of collagens through

immunohistochemistry after the application of hyaluronic acid at 6 months, compared to time zero before the application (control), reinforcing the effect of collagen remodeling after treatment with CPM technology hyaluronic acid in superficial applications.

Therefore, the effect we observed was more related to a dermal rearrangement by the proliferation of ECM and collagen production in a more beneficial way regarding rejuvenation than the inflammatory process observed during wound healing.

In 2007, Wang et al. [29] were pioneers in discussing the properties of hyaluronic acid in collagen production. A University of Michigan study analyzed neocollagenesis in skin biopsies taken four and thirteen weeks after cross-linked hyaluronic acid (NASHA) treatment. The study revealed an increase in type I collagen and partial restoration of extracellular matrix components. Wang proposes that hyaluronic acid can restore the mechanical stimulation of fibroblasts lost over time, and this was reiterated by many authors, such as Gout et al., in 2017 [30]. HA may facilitate the restoration of mechanosensitive pathways. However, the use of biphasic hyaluronic acid in this study may have interfered with the results due to its ability to absorb water and promote an intense inflammatory reaction [29].

In Korea, a study investigated the potential efficacy of a combination treatment comprising hyaluronic acid and botulinum toxin in 102 patients with acne scars. Patients were evaluated using computational topographic analysis to assess roughness and punctual depth. The GAIS was used to evaluate the results of the treatment over a period of between one and six months. Six patients had facelift surgery one month and one year after S-HA injections. The objective was to evaluate the depth of injection, material longevity, and extent of neocollagenesis and neoelastinogenesis. The histopathological study confirmed precise dermal placement, demonstrated neocollagenesis and neoelastinogenesis, and showed hyaluronic acid particles in the skin after one year [31]. Nevertheless, the study employed Restylane® Skinboosters™ Vital, a gel with notable rehydration capabilities.

In 2023, Shin [9–32] proposed that the distension promoted by hyaluronic acid favors the stimulation of senescent fibroblasts through mechanical activation, resulting in signal transduction and cell modulation.

Hyaluronic acid stretches the dermis, improving the extracellular matrix (ECM). This stimulates fibroblasts by activating the TGF-β pathway and producing type I collagen. Furthermore, hyaluronic acid exerts a direct effect on fibroblasts via their hyaluronan receptors (CD44 and CD168), stimulating their migration and proliferation [9,33].

We can therefore consider, since we used a gel with a low crosslink and concentration, that the neocollagenesis we observed is not related to the properties previously described in the work carried out by Wang et al. but rather to more recent studies into cell modulation [32].

However, the present study demonstrated that hyaluronic acid, when applied superficially to the dermis, was effective in promoting neocollagenesis in all patients, as evidenced by the analyses of the three histological stains. This suggests that the collagen formation observed is likely due to the product's adaptation to the skin, resulting in dermal rearrangement, increased collagen, and probable elastogenesis. These changes contribute to enhanced skin firmness and the prevention of sagging rather than being solely dependent on the inflammatory reaction in the tissues themselves.

The hyaluronic acid dosage in the skin of all patients demonstrated a notable increase in concentration at the six-month mark, when its concentration peaked, higher than at three months, and in relation to the initial baseline of the study. It can be inferred from these results that the elevated hyaluronic acid concentration in the skin is due to endogenous production by the patient's own dermal and epidermal cells following treatment.

It has been observed that the bio-integration capacity of the product under study, coupled with the fact that it is an intrinsic component of the skin, justifies the restoration of cellular physiological mechanisms lost during the aging process in terms of neocollagenesis. Furthermore, the application of hyaluronic acid has been shown to promote the synthesis of this glycosaminoglycan in the treated skin [34–36].

An additional hypothesis for the observed increase in expression is that hyaluronic acid acts as a potent modulator of growth factor signaling, thereby stimulating the synthesis of this compound [37].

Replenishing the ECM would result in not only dermal hydration but also in volumization and neocollagenesis through skin redensification and re-establishment of support by components of the fundamental substance.

This study also reinforces the presence of neocollagenesis and neoelastinogenesis after the initial intervention, as well as emphasizing the importance of establishing new theories on cell–matrix interactions. It is clear to us that it is important to continue this work with a more significant sample to confirm our preliminary results, especially in patients in the 60-year age group.

5. Conclusions

The findings of this study suggest that polydensified matrix hyaluronic acid is an effective collagen biostimulator, resulting in an increase in the ratio of collagen I to collagen III, dermal thickening, and stimulation of endogenous hyaluronic acid synthesis, with such intradermal effects being proven in four patients in the 35 to 60 age group.

Despite being known that hyaluronic acid can cause disruption of the regulation of metabolism and regeneration of cellular structures of the dermis, in the present data, a thickening and increase in elastic fibers in the dermis were observed after treatment with hyaluronic acid.

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Data Availability Statement: Present data and methods were described in sufficient detail in the manuscript so that other researchers can replicate the present work. Raw data is unavailable due to patients' privacy confidentiality.

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