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Ultrasound and Histologic Examination after Subcutaneous Injection of Two Volumizing Hyaluronic Acid Fillers: A Preliminary Study

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Background: This study examined the influence of hyaluronic acid (HA) crosslinking technology on the ultrasound and histologic behavior of HA fillers designed for subcutaneous injection.
Methods: One subject received subcutaneous injections of 0.25 ml Cohesive Polydensified Matrix (CPM) and Vycross volumizing HA in tissue scheduled for abdominoplasty by bolus and retrograde fanning techniques. Ultrasound analyses were performed on days 0 and 8 and histologic analyses on days 0 and 21 after injection. A series of simple rheologic tests was also performed.
Results: Day 0 ultrasound images after bolus injection showed CPM and Vycross as hypoechogenic papules in the hypodermis. CPM appeared little changed after gentle massage, whereas Vycross appeared more hyperechogenic and diminished in size. Ultrasound images at day 8 were similar. On day 0, both gels appeared less hypoechogenic after retrograde fanning than after bolus injection. Vycross was interspersed with hyperechogenic areas (fibrous septa from the fat network structure) and unlike CPM became almost completely invisible after gentle massage. On day 8, CPM appeared as a hypoechogenic pool and Vycross as a long, thin rod. Day 0 histologic findings confirmed ultrasound results. Day 21 CPM histologic findings showed a discrete inflammatory reaction along the injection row after retrograde fanning. Vycross had a more pronounced inflammatory reaction, particularly after retrograde fanning, with macrophages and giant cells surrounding the implant. Rheologic tests showed CPM to have greater cohesivity and resistance to traction forces than Vycross.
Conclusions: CPM HA volumizer appears to maintain greater tissue integrity than Vycross after subcutaneous injection with less inflammatory activity. *(Plast Reconstr Surg Glob Open 2017;5:e1222; doi: 10.1097/GOX.0000000000001222; Published online 24 February 2017.)*

INTRODUCTION

For effective and lasting use, all hyaluronic acid (HA) fillers have their HA modified through crosslinking to provide higher resistance to endogenous hyaluronidase action. In addition to preventing the rapid degradation of HA, the proprietary crosslinking technique used provides the HA with specific rheologic properties and influences its suitability for different treatment indications and injection depths.[1,2]

Cohesive Polydensified Matrix (CPM) HA Volumizer Belotero Volume (Anteis S.A., Geneva, Switzerland, a wholly owned subsidiary of Merz Pharmaceuticals GmbH, Frankfurt am Main, Germany) and Vycross technology Juvéderm Voluma (Allergan, Pringy, France) use 2 different crosslinking technologies to create HA gels that are designed to be injected subcutaneously or in deeper

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soft-tissue layers to restore facial volume and recontour the aging face.[3–6] To provide effective volumizing with natural-looking results and perfect biointegration, the products must have an optimal balance of cohesivity, elasticity, plasticity, and viscosity. For Belotero, this is achieved by the exclusive use of patented crosslinking technology. CPM gel uses 1,4-butanediol diglycidyl ether to crosslink the high-molecular-weight HA strands but differs from other products in a second crosslinking step to produce a polydensified gel that combines variable densities of crosslinking in one cohesive structure. In contrast, Vycross gel is formulated with a high proportion of low-molecular-weight HA (LMW-HA) and only a small proportion of high-molecular-weight HA. The lower overall amount of HA used is reported to improve the crosslinking efficiency of the HA chains.

Both CPM and Vycross technologies have been used to create separate ranges of HA fillers, including FDA-approved products, that address different treatment indications and injection depths. Ultrasound imaging provides clear visualization of individual skin layers and allows the behavior of HA fillers to be observed at the correct injection depth for their indication and viscosity. The technology, backed-up by histologic studies, has already been used to determine how a range of HA fillers behave after injection into the superficial and mid-reticular dermis.[7–9] To the authors' knowledge, no studies have examined the ultrasound and histologic behavior of volumizing HA fillers designed for subcutaneous injection, but because of their specific viscoelastic properties, this is expected to be different from that of HA fillers designed for dermal injection.[1,2,7,8,10] The aim of this preliminary study was to compare the tissue behavior of 2 HA volumizers after subcutaneous injection with 2 different injection techniques.

METHODS

This study used 2 CE-marked HA gels with an indication for volumization of the face: Juvéderm Voluma with lidocaine (lot no. VB20888191, Allergan, Pringy, France) and Belotero Volume (lot no. 542087/1, Anteis S.A., Geneva, Switzerland). At the time of the study, CPM HA volumizer gel with lidocaine was not yet registered in Switzerland, and the tested syringe did not include lidocaine.

One volunteer female subject, 66 years old, consented to participate in this preliminary study and provided informed consent. The study was conducted in accordance with the ethical principles that had their origin in the Declaration of Helsinki. The subject was scheduled to undergo corrective abdominoplasty 21 days after the initial injection, and the HA gels were injected into the tissue that was going to be surgically removed.

Both HA gels were injected subcutaneously between the umbilicus and the pubis, at a distance of 4–6 cm from the umbilicus on days 0 and 21 under ultrasound control (**see figure, Supplemental Digital Content 1,** ***http://links.lww.com/PRSGO/A375***). No local anesthesia was used for the injections.

Each gel was injected with 2 different injection techniques. For the bolus technique, a 0.25 ml bolus of HA gel was injected using a 27G sharp needle (TSK, lot no. 130351/2018-05, TSK Laboratory, Tochigi City, Japan). For the retrograde fanning technique, 0.25 ml of each product was injected using a double retrograde fan injection technique (2 lines of 0.125 ml each) using a 27G/37mm blunt microcannula (Magic Needle, lot no. 11098/2016-05, Needle Concept, Paris, France). The right side of the abdomen was injected on day 0 (21 days before surgery) and the left side on day 21 just before the abdominoplasty. On both injection days, CPM gel was injected on the lateral sides of this area and Vycross gel on the medial sides. Sites of injection were marked with a color-coded tattoo (India ink, FMB13042B, Gémenos, France; Omnican 50 syringe with integrated 30G½ needle, B. Braun, lot no. 3F24048/2018-06; see table, **Supplemental Digital Content 2,** ***http://links.lww.com/PRSGO/A376***). Both injection sites were gently massaged after the procedure.

Ultrasound Examination

Ultrasound images were taken before and after the injection on day 0. A second ultrasound examination took place 8 days after the first injection (day 8). All images were obtained using a General Electric LogiQ *E9* with Hockey stick L8 18i (General Electric Company, Fairfield, Conn.) probe, at a frequency of 17 MHz. To obtain a better definition of the ultrasound image, a Sonar Aid Geistlich Pharma solid gel interface (lot no. 1000353, Geistlich Pharma AG, Wolhusen, Switzerland) was placed over the treated area.[7,8] The thickness of the different skin layers was measured from the ultrasound images before and after injection. The angle of needle or cannula penetration, length of insertion, and depth of injection were also measured.

Histologic Examination

On the day of surgery, before the surgical procedure, a second injection of the 2 products was performed in the same manner. In this way, the abdominoplasty yielded histology specimens at day 0 and 21 days after injection. Immediately after abdominoplasty, the areas injected with the HA volumizers were dissected from the surgically removed tissue, fixed with buffered 10% formalin, and sent to Laboratory Viollier, Geneva, Switzerland. After 24 h, the fixed tissue was embedded in paraffin, cut into 5-µm tissue sections, mounted on to slides, and stained with hematoxylin and eosin before histologic examination.

Rheologic Tests

The HA volumizers were also subjected to simple rheologic tests in our office (P.M.) for cohesivity and resistance to stretch.[11] For the cohesivity test, 0.6 ml of saline serum (NaCl 0.9%) was combined with 2 drops of a coloring agent (Ecoline no. 548 Talens blue violet, Apeldoorn, The Netherlands). To this were added 0.2 ml of the HA gel to be tested and 2 drops of ethanol 70%, and the recipient was gently shaken. The gels were observed visually and under a microscope between slides. For the resistance to stretch test, 0.2 ml of each gel was placed on a Petri dish. The gels were then pinched with an Adson's plier to draw them out, and the maximum stretch distance was measured.

RESULTS

For both CPM and Vycross gels, the angle and length of needle and cannula insertion were similar (Table 1). The very small difference in depth of injection was due to the manual technique of injection and reflects what would happen in real life.

Day 0 Ultrasound Examination

After bolus injection, CPM gel was visible in the hypodermis as a hypodense, homogeneous hypoechogenic papule (Fig. 1A). A small decrease in the size of the papule was observed after gentle massage (Fig. 1B). Vycross gel appeared as a mildly hypodense, hypoechogenic papule in the hypodermis with some hyperechogenic septa and some air bubbles (Fig. 1C). The papule appeared significantly diminished in size after similar gentle massage (Fig. 1D).

After retrograde fanning injection, CPM gel formed several large gel pools, which were less hypoechogenic than those formed after bolus injection and more heterogeneous at the injection site in the hypodermis (Fig. 2A). There was a decrease in papule size after gentle massage (Fig. 2B). The Vycross papule also appeared less hypoechogenic after retrograde fanning injection and was interspersed with areas of hyperechogenicity at the injection site in the hypodermis (Fig. 2C). It appeared greatly reduced in size after gentle massage (Fig. 2D).

Day 8 Ultrasound Examination

For both products, ultrasound images at day 8 showed almost no change compared with images taken on day 0 immediately after bolus injection and gentle massage (Fig. 3A, C).

After retrograde fanning injection, hyperechogenic areas (white on the images) were observed in the CPM injection area (Fig. 3B), which the echographist determined to be air bubbles. The disappearance and sedimentation of previous hyperechogenic elements were also observed. These may have represented red blood cells or other cells, which had diffused after the retrograde fanning injection technique. At day 8, the CPM papule was almost superimposable on the day 0 image indicating gel persistence; the slight decrease in size observed was thought to be due to resolution of injection-related swelling. The Vycross image at day 8 appeared as a long, rod-shaped collection of HA (Fig. 3D). The disappearance and sedimentation of a few hyperechogenic elements were observed but with no other significant changes compared with day 0.

Table 1. Needle and Cannula Angle of Penetration and Depth of Injection

	CPM **Needle**	CPM **Cannula**	Vycross **Needle**	Vycross **Cannula**
Angle of penetration, degrees	11	8	6	8
Length of inserted needle/ cannula, mm	10.0	20.0	10.0	20.0
Depth of injection (top of needle/ cannula), mm	2.0	3.0	2.1	2.6

Day 0 Histologic Examination

Tissue samples taken shortly after bolus injection of CPM showed small homogeneous pools of HA in the subcutis (**see figure, Supplemental Digital Content 3, *http://links.lww.com/PRSGO/A377***). After bolus injection of Vycross, small amounts of HA were observed in the hypodermic fat (**see figure, Supplemental Digital Content 3, *http://links.lww.com/PRSGO/A377***). In both CPM and Vycross tissue samples, low levels of lymphocytes and histiocytes were observed in the superficial dermis and hypodermis and around some adipocytes, probably a result of previous surgery or the tattoo ink.

After retrograde fanning injection, CPM gel was visible as small pools and thin rows of gel (**see figure, Supplemental Digital Content 4, *http://links.lww.com/PRSGO/A378***). Vycross gel was visible as small pools in the hypodermis and along the injection tract (**Fig., Supplemental Digital Content 4, *http://links.lww.com/PRSGO/A378***). In tissue samples from both products, some defects consistent with air bubbles were visible surrounded by macrophages and pericapillary lymphocytes, which were probably a consequence of the cannula penetration.

After retrograde fanning injection, a granulomatous reaction to a foreign body and birefringent particles in polarized light were observed in tissue from both gel injection sites on day 0, probably due to threads from a previous abdominoplasty (**see figure, Supplemental Digital Content 5, *http://links.lww.com/PRSGO/A388***).

Day 21 Histologic Examination

On day 21 after bolus injection, CPM gel was visible as large homogeneous pools in the fat and along the hypodermic septa without any deformation (Fig. 4A). There were no signs of any inflammatory reaction. Vycross gel was visible as large homogeneous pools. There was a discrete positive inflammatory reaction with lymphocytes and histiocytes around the dermal tattoo but no signs of an inflammatory reaction around the implant.

On day 21, after the retrograde fanning technique, small homogeneous pools of CPM gel were visible along the injection rows, within fat lobules and the hypodermis septa (Fig. 4C, D). There was also a discrete inflammatory reaction along the injection rows and around the tattoo pigment in the dermis. There was no inflammatory reaction around the implant. For Vycross gel, large HA pools were observed inside the fat lobules and along the hypodermis septa. A dissociation of the septa was also observed. Signs of a moderate inflammatory reaction were appearing with macrophages and giant cells around the Vycross implants (Fig. 4B).

Dermal Thickness

Ultrasound measurements of the different skin layers before and after injection showed that epidermal and dermal thickness were essentially the same for both

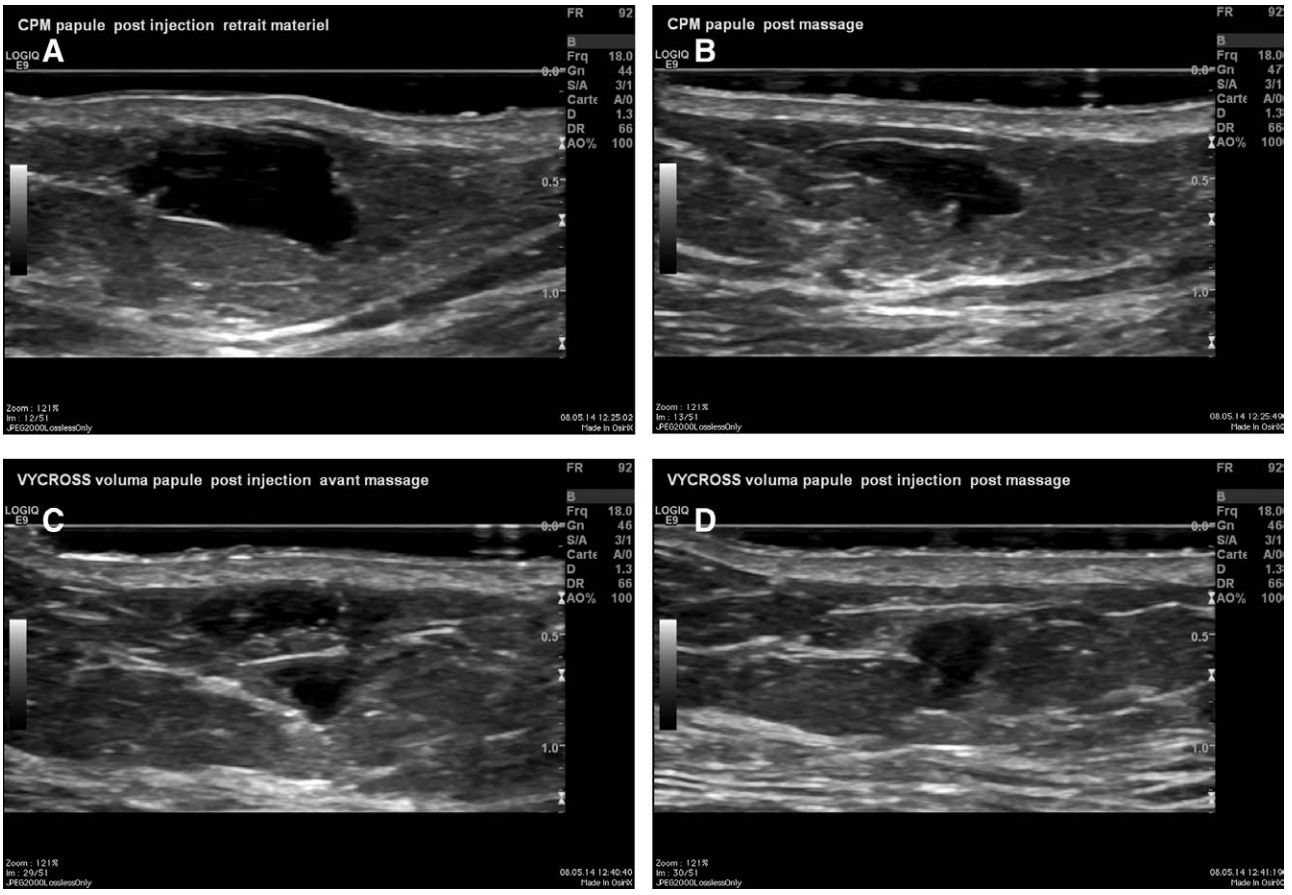

Fig. 1. Ultrasound images at day 0 of (A) CPM gel immediately after bolus injection. B, CPM gel after gentle massage. C, Vycross gel immediately after bolus injection. The papule appears to be divided into 2 by fibrous tissue from the fat compartment. In the upper part, air bubbles are visible (white spots). D, Vycross gel after gentle massage. The epidermis appears as a hyperechogenic line at the very top of the image. The dermis appears as the homogeneous, echogenic (bright) structure below this. The injected HA appears as the hypoechogenic (dark) area within the hypodermis.

products (Table 2). Immediately after CPM injection, hypodermis thickness increased more with the bolus than with the retrograde fanning cannula injection technique (Table 2). This may be explained by the fact that, with the same amount of gel, a bolus of 0.25 ml has a larger volume than 2 gel threads of 0.125 ml placed with a retrograde injection technique. For Vycross, hypodermis thickness was slightly greater after cannula injection than after bolus injection, possibly due to its tendency to break up into smaller pools and spread through the subcutaneous tissue.

The thickness of the different dermal layers immediately after injection and 21 days after injection was also measured in tissue specimens taken for histologic analysis (Table 3). In contrast to the ultrasound results, hypodermis thickness was greater after retrograde fanning than after bolus injection. This may have been due to fat distribution, with more fat in the area of the cannula injections compared with the area of bolus injections, or the slight natural asymmetry in body fat distribution. There are also likely to be differences between in situ ultrasound measurement and histologic examination of biopsy tissue.

Rheologic Tests

In the simple cohesivity test, the CPM gel remained as one cohesive strand, whereas the Vycross gel disintegrated into several smaller pieces of gel. The CPM gel was also more cohesive between Adson's pliers and could be stretched to 1.5 cm without breaking compared with 1 cm with Vycross gel.

DISCUSSION

This case report demonstrates the different in vivo behaviors of 2 HA fillers developed for volumizing indications and deep injection. Both products were injected at the same tissue depth and with the same angle of insertion. Immediately after injection, ultrasound observations showed both CPM and Vycross gels as hypoechogenic papules in the hypodermal plane. Vycross gel began to dissipate after gentle massage on day 0, and its appearance remained significantly diminished at day 8, whether injected by bolus or retrograde fanning techniques, probably due to breakdown into smaller pools and diffusion in the fatty subcutaneous tissue network. In contrast, the CPM gel papule only decreased slightly in size after gentle

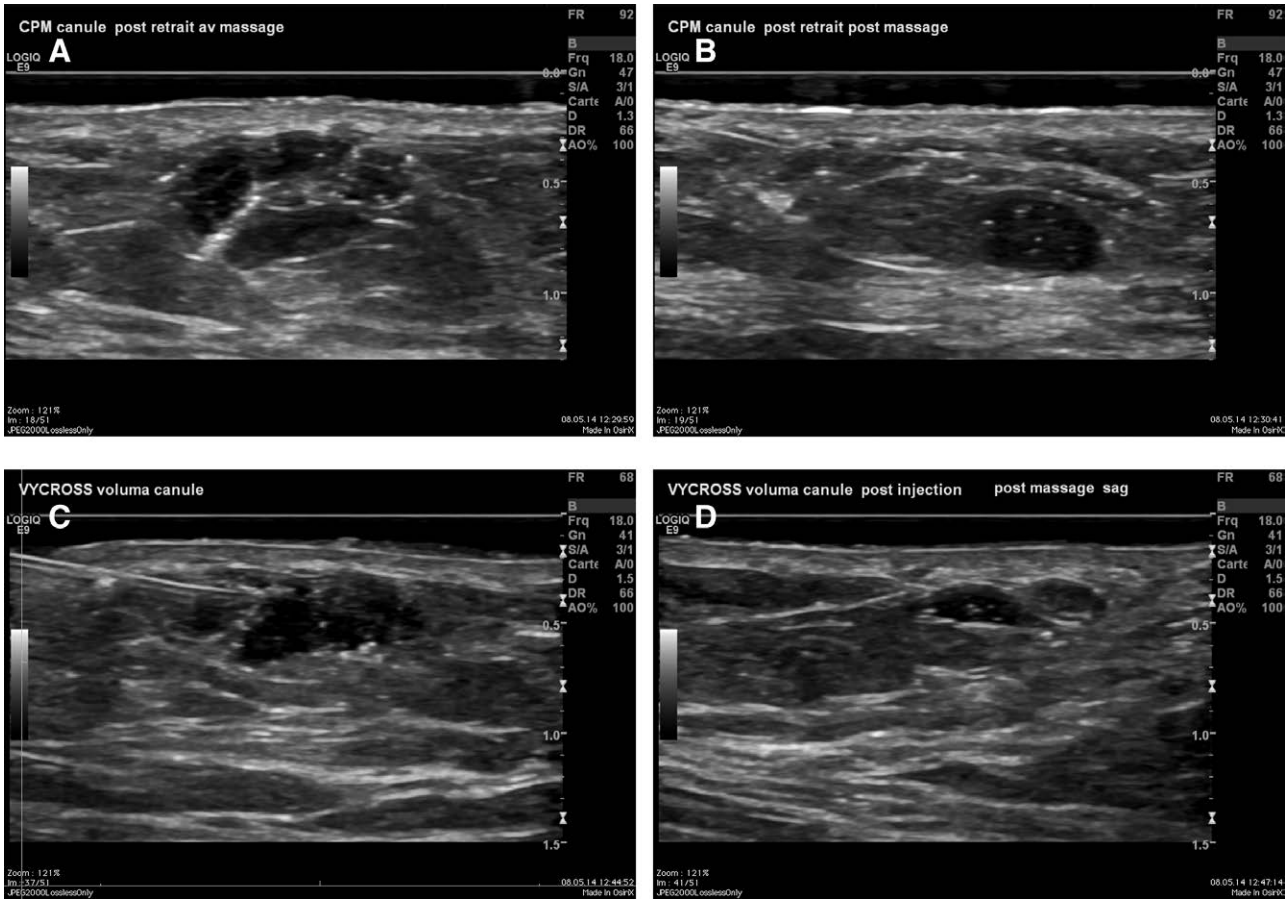

Fig. 2. Ultrasound images at day 0 of (A) CPM gel immediately after retrograde fanning cannula injection. B, CPM gel after gentle massage. C, Vycross gel immediately after retrograde fanning cannula injection. D, Vycross gel after gentle massage.

massage and remained visible at both time points whichever injection technique was used. When HA breaks up into smaller pools, the reflected ultrasound echo increases resulting in a more hyperechogenic image, which becomes indistinguishable from the surrounding tissue. The findings suggest that CPM gel is therefore more cohesive than Vycross gel, as it remains visible as a hypoechogenic area and does not break up after gentle massage. This has recently been confirmed using a standardized cohesivity assay.[12] Ultrasound observations at day 8 did not differ from those at day 0. A potential explanation for the decrease in volume of the Vycross gel after gentle massage is provided by the results of a recent article that examined the behavior of a range of HA gels to shear stress and compression.[12] The results showed that the higher the elastic modulus (E prime) of the tested HA filler, the greater the volumizing capacity, with CPM gel having an E prime value over 2.5-fold greater than that of Vycross gel.

Histologic examination of the injected tissue confirmed the ultrasound observations and provided information on inflammatory reactions taking place. Tissue taken from skin immediately after bolus injection and gentle massage showed CPM gel visible as large pools within the hypodermis. In contrast, only a few small pools of Vycross gel were visible with greater diffusion into the subcutaneous fat probably as a result of its high proportion of LMW-HA. With the cannula technique, CPM gel was visible as rows of gel along the route of cannula implantation, whereas Vycross gel was visible as small pools of gel. The data suggest that, after both bolus and retrograde fanning injection, CPM gel remains more cohesive than Vycross gel.

A low-grade inflammatory reaction at day 0 was observed after injection of both HA fillers but was too early to be a response to the fillers themselves and more likely a response to the ink tattoo used to mark the injection spot or remnants of a response to previous surgery. The granulomatous reaction to a foreign body observed in tissue from both gel injection sites on day 0 was probably due to threads from a previous abdominoplasty. Foreign body granulomatous reactions typically develop after a variable period of time ranging from 6 months to 2 years.[13]

Histologic examination of tissue taken at day 21 after bolus injection revealed a similar picture for both products, the 2 gels diffusing as large homogeneous pools within the fat and along the septa. The visible inflammatory reaction was probably due to the tattoo pigments or more likely previous abdominoplasty. After cannula implantation of CPM gel, an inflammatory reaction was visible along the cannula route of entry and surrounding

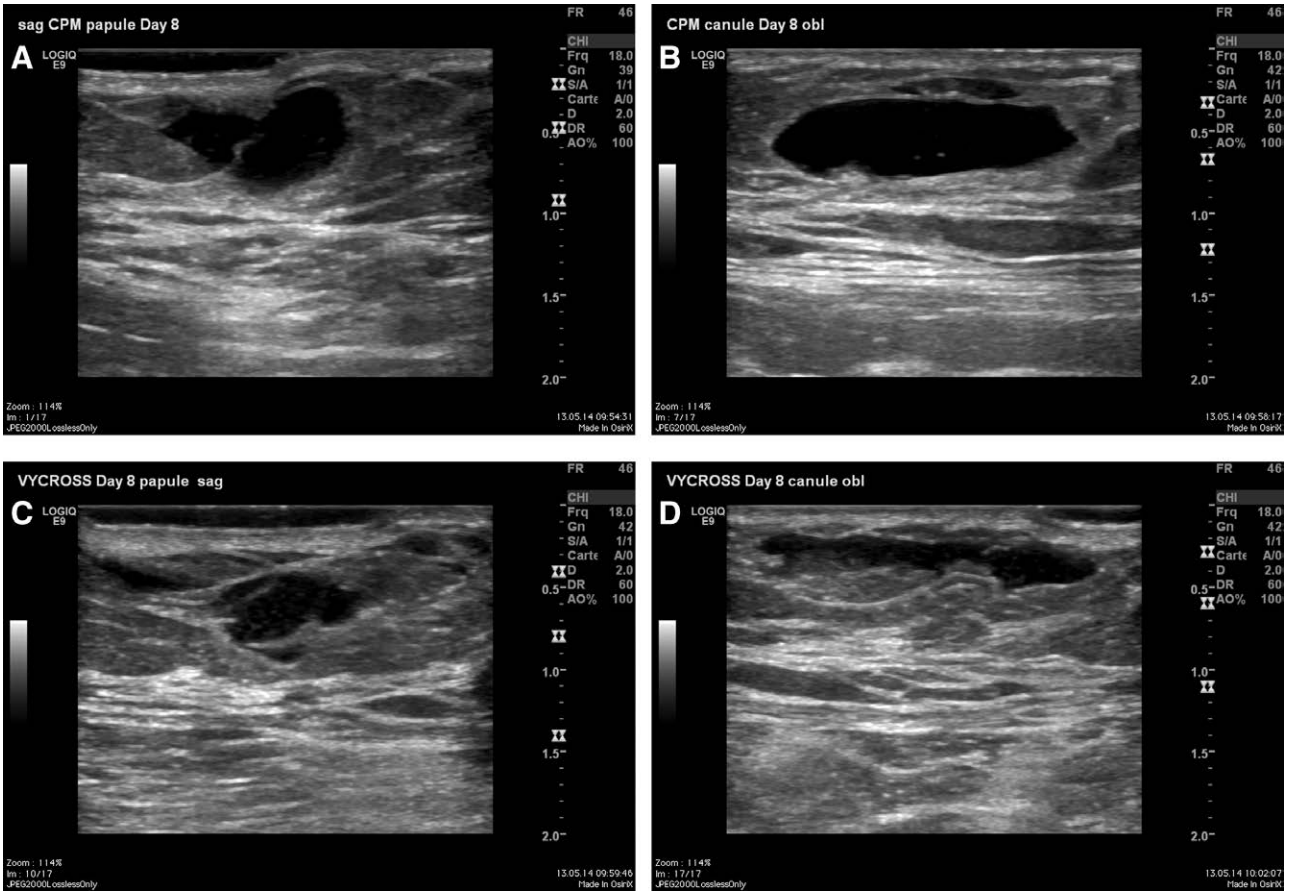

Fig. 3. Ultrasound images at day 8 of (A) CPM gel injected by bolus technique, (B) CPM gel injected by retrograde fanning cannula technique, (C) Vycross gel injected by bolus technique, and (D) Vycross gel injected by retrograde fanning cannula technique.

the pigment tattoo. At day 21, a greater and more diffuse inflammatory reaction was observed after cannula implantation of Vycross gel and the beginning of a foreign body reaction with giant cells visible around the implant and along the cannula route of entry. The greater inflammatory reaction observed after cannula injection could be a consequence of either the fanning technique, which is more irritating for the tissue in the area of injection because of the forward and backward movement of the cannula, or sampling error. Although the fillers were injected under ultrasound control, the authors acknowledge that there may have been slight differences in injection depth between the bolus and fanning injections because of asymmetry in the thickness of the hypodermis layer.

There are several explanations for the greater inflammatory reaction observed with Vycross gel. First, 2 different tattoo inks were used to mark the sites of the CPM and Vycross injections, which may have triggered inflammatory reactions of varying severity and therefore influenced the histologic results. Second, the less cohesive nature of Vycross means that more small microboluses are in contact with lymphocytes, which could trigger a greater inflammatory reaction. A third hypothesis relates to the structural composition of Vycross. LMW-HA fragments such as those found in Vycross are proinflammatory and can trigger the immune system.[14] The LMW-HA fragments may increase in concentration as the product is metabolized after injection. This effect, alone or in combination with a triggering event, may produce a vigorous inflammatory response. A recent report documents delayed-onset nodules in 23 of 2,342 (0.5%) patients treated with Vycross over 68 months, with a median time to onset of 4 months (range, 1–13 months).[15] Following the results of this preliminary report, a larger comparative study has been initiated to clarify the nature of the inflammatory reactions observed.

Each product in the CPM and Vycross range of HA fillers has been developed with a specific balance of cohesivity, viscoelasticity, and plasticity. This rheologic tailoring results in products with different degrees of lift, malleability, and fluidity. Both the CPM and Vycross volumizing agents are designed for deep injection and volume restoration and have high G prime, a measure of gel hardness and elasticity.[12] In the current study, 2 simple and reproducible rheologic tests were used to try to explain the differences in behavior of the 2 gels. They showed that the cohesivity of CPM gel was greater when subjected to the resistance to stretch and cohesivity test. The latter test, which examines the dispersion of HA gels when they come into contact with saline solution, has recently been standardized and validated in a more sophisticated laboratory test confirming its utility for assessing the cohesivity of HA gels.[16] A recent study that used this protocol confirmed the higher cohesivity of CPM,

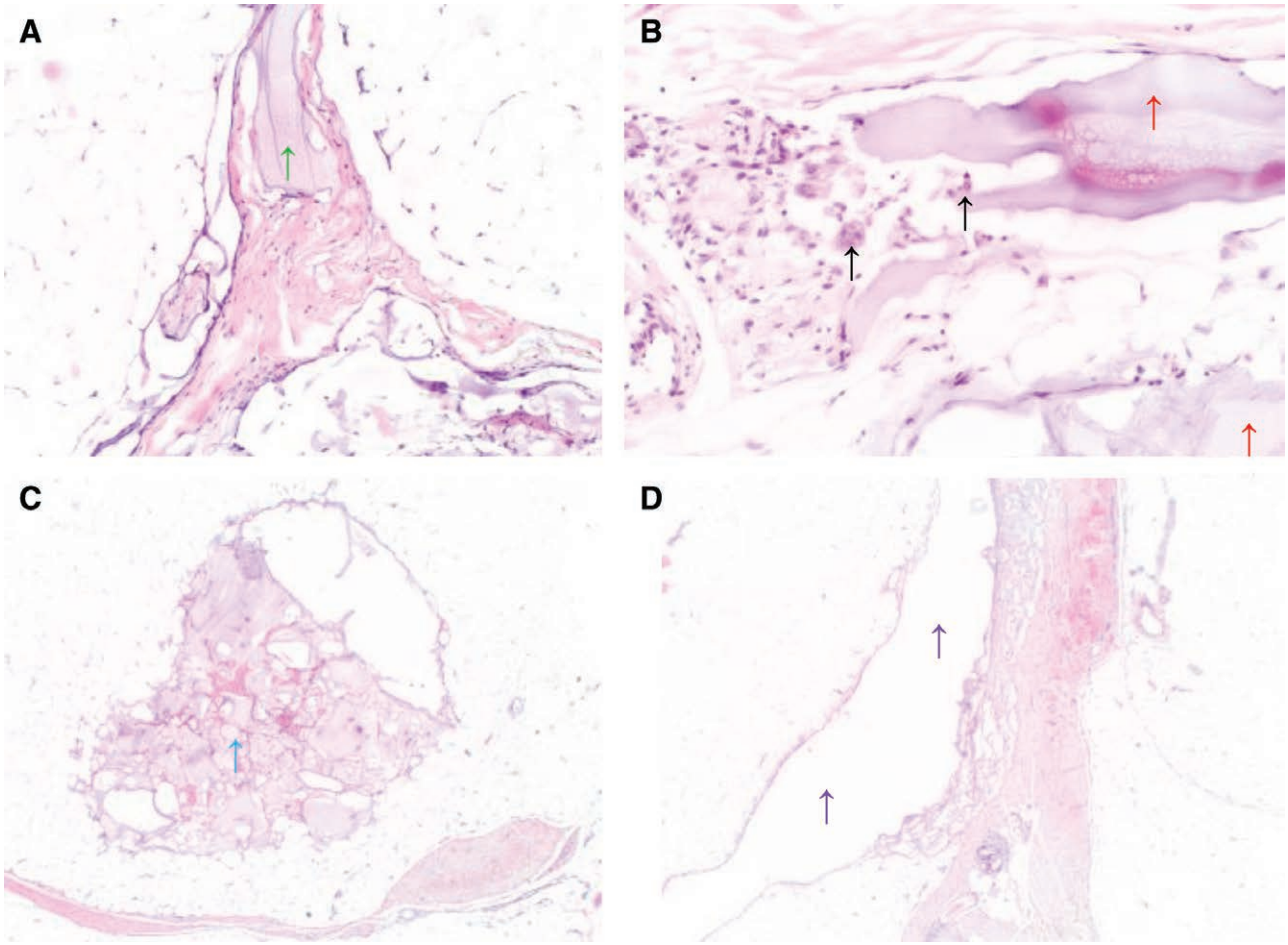

Fig. 4. Histology at day 21. A, CPM bolus technique, ×40. CPM gel is visible as small pools along the septa and inside the fat lobules (green arrows). B, Vycross (red arrows) cannula technique, ×100. Granuloma and giant cells (black arrows) are visible around the gel and there is a local fibrosis with postsurgical macrophagic granuloma. The fat lobules are split by large pools of Vycross gel. C, CPM cannula injection technique showing presence of CPM gel within the fat lobules (blue arrow), ×12.5. D, CPM cannula injection technique showing the presence of CPM gel along the cannula rows (violet arrows), ×12.5.

Table 2. Ultrasound Measurements of Skin Thickness Before and After Injection at Days 0 and 8

Skin Thickness (mm)	CPM		Vycross	
	Day 0	Day 8	Day 0	Day 8
Epidermis				
Before injection	0.30–0.40	—	0.30–0.40	—
After injection	0.30–0.40	0.30–0.40	0.30–0.40	0.30–0.40
Dermis				
Before injection	0.96–1.07	—	0.96–1.07	—
After injection	0.96–1.07	0.96–1.07	0.96–1.07	0.96–1.07
Hypodermis				
Before injection	9.80–10.30	—	9.60–10.30	—
After injection	—	—	—	—
Bolus injection technique	15.00–17.00	15.00–17.00	8.82–9.32	7.85–8.19
Cannula retrograde injection technique	11.30–12.50	11.30–12.50	9.80–10.20	9.90–12.10

which scored 3 on the standardized 5-point scale (1 non-cohesive to 5 fully cohesive) compared with 2 for Vycross.[12] This may explain the greater ability of the CPM volumizing HA to maintain its shape under gentle massage and not break up into smaller pools of gel.

To our knowledge, this is the first study to examine the behavior of 2 volumizing HA gels after injection in the hypodermis and how it varies by injection technique. The data complement findings from previous research that has used ultrasound imaging and histologic examination to determine the behavior of HA gels after injection into the different dermal layers.[7–9,17] These studies have shown that the tissue distribution and morphology of HA gels is significantly affected by their respective crosslinking technology. Further studies are now warranted to examine the tissue behavior of CPM volumizing gel with lidocaine, which was not available at the time the current study was performed.

CONCLUSIONS

Ultrasound and histologic observations confirm differences in tissue behavior after subcutaneous injection of 2

Table 3. Histologic Measurements of the Thickness of the Different Dermal Layers for Both Products and Both Injection Techniques Immediately After Injection and 21 Days Later

	CPM Day 0 Bolus	CPM Day 0 Cannula	CPM Day 21 Bolus	CPM Day 21 Cannula	Vycross Day 0 Bolus	Vycross Day 0 Cannula	Vycross Day 21 Bolus	Vycross Day 21 Cannula
Total thickness, mm	8.95	12.90	5.67	14.76	6.78	13.20	9.72	13.11
Epidermis	0.07	0.11	0.08	0.09	0.09	0.10	0.12	0.09
Papillary dermis	0.20	0.14	0.17	0.19	0.14	0.20	0.25	0.16
Reticular dermis	1.86	1.65	1.12	1.48	1.55	1.90	1.35	1.86
Hypodermis	7.00	11.00	4.30	13.00	5.00	11.00	8.00	11.00

HA volumizers. Whether injected by needle bolus or cannula retrograde fanning technique, CPM gel appeared as a homogeneous pool of gel that only slightly diminished in size after gentle massage. Vycross gel appeared more heterogeneous and was less resistant to gentle massage with a tendency to break up into smaller pools particularly after cannula injection. It was also associated with a more diffuse inflammatory reaction at day 21. The results suggest that CPM gel maintained greater integrity after subcutaneous injection and gentle massage.

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