Water absorption and 3D expansion of different injectable hyaluronic acids

Patrícia Paulino
Faculdade de Medicina Dentária da Universidade de Lisboa
Virgínia Santos
Faculdade de Medicina Dentária da Universidade de Lisboa
Neusa Silva
Unidade de Investigação em Ciências Orais e Biomédicas (UICOB), Faculdade de Medicina Dentária da Universidade de Lisboa
António Mata
Unidade de Investigação em Ciências Orais e Biomédicas (UICOB), Faculdade de Medicina Dentária da Universidade de Lisboa
Mariana Brito da Cruz (mariana.cruz@campus.ul.pt)
Unidade de Investigação em Ciências Orais e Biomédicas (UICOB), Faculdade de Medicina Dentária da Universidade de Lisboa

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Abstract

Hyaluronic acid's water absorption and expansion capacities define a filler's ability to lift the tissues. Therefore, studying these properties is essential to better understand filler's clinical performance. The aim of this study was to evaluate and compare water absorption and expansion (bidimensional and three-dimensional) of five Fillmed fillers (Universal, Fine Lines, Volume, Lips and Lips Soft). Water absorption was measured through swelling ratio 24 hours post-hydration. For two-dimensional expansion, samples were analysed quantitatively and qualitatively, using calibrated photographs, before and two hours after hydration. Three-dimensional volume was evaluated before and immediately after injecting the fillers, and 30 minutes, 2 hours and 24 hours post-injection in ex vivo pig skin. The tissue was scanned with the 3Shape TRIOS scanner and resulting STL files were compared. Group comparisons were analyzed with the one-way ANOVA test, and a p-value $\leq$ 0,05 was established. Lips showed a statistically higher swelling ratio than other fillers ($p < 0,05$). Fine Lines had the lowest swelling ratio, even if only statistically significant when compared to Universal ($p = 0,021$). Fine Lines had a significantly higher initial bidimensional width than all fillers ($p < 0,05$) except Lips Soft. 24 hours post-injection, Fine Lines had the highest three-dimensional volume, which was statistically higher than Volume's ($p = 0,049$). All fillers absorbed water and expanded, with Fine Lines tending to have the highest three-dimensional expansion, despite its lowest viscosity and water absorption. Further studies with larger sample sizes are needed to investigate the influence of other properties over water absorption and expansion.

1. INTRODUCTION

Hyaluronic acid (HA) is the most abundant glycosaminoglycan in the human dermis, whose main functions are to draw water into the tissues, volumizing and giving structure to the skin [2, 26, 37, 39]. It's made up of molecules of D-glucuronic acid and N-acetylglucosamine, linked by β-(1–4) and β-(1–3) glycosides, with a natural life of 24h to 48h, since in its non-crosslinked state it is quickly degraded by hyaluronidase [2, 21, 26, 27, 33, 34, 39].

It was discovered in 1934 by Karl Meyer and John Palmer, who isolated it from bovine vitreous humour [2, 5]. HA has been used for several purposes like wound treatment, ophthalmic surgery, drug delivery and aesthetic treatment [2, 11, 39]. There are currently many different HA dermal fillers, with none of them being universally fitting for every situation [2, 5, 9, 21]. Clinicians should thus be familiarized with HA's rheological and physicochemical properties, as they influence clinical performance [5, 6, 12, 27, 34].

One of hyaluronic acid's essential property is its hydrophilia, which determines its capacity to absorb water and expand and influences the filler's lifting capacity [2, 20, 30, 37]. Water absorption and lifting capacity depend on HA's cohesivity, elastic modulus (G') [30], cross-linking (higher cross-linking density means lower chain flexibility and lower capacity to absorb water) [8–10, 21, 26, 34, 39], and HA concentration (the higher it is, the more a filler will absorb water and volumize the tissues) [10, 21, 32, 34, 37].

The swelling ratio represents the gel's ability to absorb water while remaining in one single *in vitro* phase and determines a gel's hydration level [8–10, 32]. When the swelling ratio is 1, the gel is at equilibrium, and as it gets higher, the further away from equilibrium is the gel [9, 13, 36]. Fillers that are bellow equilibrium will swell more post-injection, as they can absorb water from the surrounding tissues [10, 21, 32, 34, 37].

Most water absorption capacity studies are related to different biomaterials, and not always specifically to HA fillers [8–11, 15–17, 19, 20, 28, 29, 31, 32, 35, 38]. To the best of our knowledge, there are no studies evaluating and comparing neither water absorption and expansion properties, nor the indications of Fillmed's ART FILLER® products, which makes it necessary to deepen our knowledge in this area.

This study aims to evaluate and compare water absorption of the fillers from the ART FILLER® collection, and filler expansion, both two-dimensional (2D) and three-dimensional (3D). The null hypotheses are, respectively, that there are neither any differences between all tested fillers on their water absorption capacity, nor regarding their expansion capacity.

2. MATERIALS AND METHODS

An *in vitro* and *ex vivo* experimental study was conducted in Faculdade de Medicina Dentária da Universidade de Lisboa. As is shown in Fig. 1, five different fillers commercialized by Fillmed in their ART FILLER® collection were used: Universal, Fine Lines, Lips, Volume and Lips Soft (Paris, France: Laboratoires FILLMED; 1978). The Universal filler was used as a control group in all experiments aside from 3D volume evaluation. The sample size for filler expansion was set in the form of a pilot study, as no previous studies conducted the same protocol.

2.1 Water absorption

Ten filler samples for each study group were evaluated. Water absorption method was based on previous ones, involving the swelling ratio [8–10, 32, 22, 25]. 0,1 mL of each sample were added to 10 previously weighed Eppendorf tubes per group. All tubes were weighed again at this point. Then, 0,3 mL of PBS were added to each sample. Each tube was placed in a ZX Classic Vortex Mixer from VELP Scientifica® (Usmate Velate, Italy: VELP Scientifica; 1983) for 5 seconds at 16 rpm x 100 rotation. The samples absorbed PBS for 24 hours inside a Memmert® TV30U laboratory oven (Büchenbach, Germany: Memmert; 1947) at 37^{o} C to mimic physiological temperature. After this, the solutions were centrifuged for 5 minutes at 3500 rpm using a Costar® 10 Mini Microcentrifugue (New York, USA: Corning Inc.; 1851), so all gel particles would deposit at the bottom of the tubes. Supernatant was poured out, and the Eppendorf tubes with the fully swollen gel were weighed a final time. All weight measurements were performed with a METTLER TOLEDO® AB54 analytical balance (Ohio, USA: METTLER TOLEDO; 1945).

Swelling ratio (g/g) was calculated through the division W_w/W_0 [22], in which W_0 (g) corresponds to the initial weight of the fillers and W_w (g) to that of the fully swollen gel.

2.2 Filler expansion

2.2.1 Two-dimensional volume evaluation

Six filler samples for each study group were evaluated qualitatively and quantitatively. 0,05 mL of each filler were placed in a line on top of microscope coverslips. Using a metallic ruler placed by the coverslips, an initial photograph was taken and a measurement corresponding to line width was recorded by a calibrated operator. All coverslips were then stored inside Petri dishes, and 0,15 mL of PBS were added to each sample. The samples were left for 2 hours, after which each Petri dish was tilted gently so excess PBS would drain off the coverslips and be absorbed by filter paper. Using the same previous ruler, new photographs were taken so that a qualitative comparison could be established. Fiji ImageJ software was used to measure the fillers' initial width through the photographs taken before adding PBS. Three pairs of points were chosen in each filler line and the distance between them was measured in cm.

2.2.2 Three-dimensional volume evaluation

One sample from each study group was used, as well as *ex vivo* pig skin obtained from a local slaughterhouse, since porcine models show tissue similarities to humans, regarding histology, anatomy and physiology [7, 14, 29]. The skin sample was stored fully submerged in Dulbecco's Modified Eagle Medium (DMEM) at 25ºC to preserve cell viability.

0,3 mL of each filler were injected in 13 mm lines (corresponding to needle size) into the pig skin. The end of each line was sutured to serve as a reference point for future comparison in the STL files (Fig. 2). A sixth line was established where no filler was injected, to serve as a control for skin deformation. The pig skin was scanned with the 3Shape TRIOS intra-oral scanner (Copenhagen, Denmark: 3Shape; 2000) before injecting the fillers (t0), immediately after injecting the fillers (t1), and then 30 minutes (t2), 2 hours (t3) and 24 hours after (t4).

STL files were matched and compared (as is shown in Fig. 3) to measure the vertical volume changes (mm) that occurred throughout time, based on three different points selected from each line. Each filler's total percentual 3D expansion was calculated by subtracting the average vertical volume increase at t1 (when compared to t0) from the average vertical volume increase at t4 (when compared to t0).

2.3 Statistical analysis

Statistical analysis was performed with the 28th version of IBM® SPSS® Statistics. The Shapiro-Wilk normality test and the Levene test were applied to all experiments. As homogeneity of variance was present and samples were proved to follow a normal distribution, the one-way ANOVA test was applied to identify differences between expansion and water absorption capacity among fillers. Pairwise comparisons were analyzed with the Tukey post-hoc test. Differences were considered statistically significant at $p\text{-value} \leq 0,05$. Results were reported in the form of mean ± SD.

3. RESULTS

3.1 Water absorption

Figure 4 shows the initial and final weight of each filler. The Lips filler showed the highest final weight (W_w= 0,2579 ± 0,1661 g), followed by the control group, Universal (W_w= 0,2433 ± 0,1927 g), Lips Soft (W_w= 0,2416 ± 0,2004 g), Volume (W_w= 0,2315 ± 0,2496 g), and lastly Fine Lines (W_w= 0,2018 ± 0,1552 g).

The results of swelling ratio (Sw) are present in Fig. 5. Once more, the Lips filler's water absorption was the highest (Sw = 2,6158 ± 0,1605), followed by Universal (Sw = 2,3062 ± 0,1644), Lips Soft (Sw = 2,2678 ± 0,1598), Volume (Sw = 2,2074 ± 0,2945), and lastly Fine Lines (Sw = 2,0262 ± 0,1651). The Lips filler showed a statistically significant higher swelling ratio when compared to all other groups ($p < 0,05$). Additionally, Universal showed a statistically significant higher swelling ratio when compared to Fine Lines ($p = 0,021$).

3.2 Filler expansion

3.2.1 Two-dimensional volume evaluation

A qualitative comparison of bidimensional expansion, corresponding to increase in width, was obtained by observing the photographs in Fig. 6, which shows one sample of each filler. Fine Lines appears to be most fluid in both times, and the one with the highest width, showing a noticeable expansion, apparently higher than the other fillers. Lips Soft and Universal also seem to be more fluid, showing a similar width in both times, while Lips and Volume appear to be more viscous. Volume and Lips also have smaller widths, with Volume having the smallest, both before and after adding PBS. Still, all fillers lost some of their consistency and expanded sideways once two hours had passed.

Figure 7 shows the initial width of each filler. Fine Lines had the highest width (0,426 ± 0,041 cm), followed by Lips Soft (0,374 ± 0,028 cm), Universal (0,366 ± 0,038 cm), Lips (0,352 ± 0,024 cm), and lastly, Volume (0,303 ± 0,027 cm). Fine Lines showed a statistically significant higher width when compared to Universal ($p = 0,027$), Lips ($p = 0,004$) and Volume ($p < 0,001$). Volume also showed a statistically significant lower width when compared to Universal ($p = 0,018$) and Lips Soft ($p = 0,007$).

3.2.2 Three-dimensional volume evaluation

Figure 8 shows the changes in vertical volume throughout time of all fillers aside from Lips Soft, since we could not obtain results regarding this filler, due to sample contamination. Immediately post-injection (t1), both Fine Lines and Lips showed statistically significant higher volume when compared to Universal and Volume ($p < 0,05$). Two hours after the injection (t3), Volume had statistically significant lower volume when compared to Universal ($p = 0,046$) and Fine Lines ($p = 0,048$). 24 hours after injecting the fillers (t4), Fine Lines had a statistically significant higher volume when compared to Volume ($p = 0,049$).

The overall percentual expansion each filler underwent, meaning the percentual volume increase measured vertically from t1 to t4, is show in Fig. 9. Fine Lines expanded the most (92 ± 43%), followed by

Universal (79 ± 39%), Lips (44 ± 41%) and Volume (39 ± 13%). No statistically significant differences were found.

4. DISCUSSION

Aesthetic demand is growing rapidly, with more people showing great concern regarding facial aging. Hyaluronic acid fillers are thus an essential tool that must be thoroughly studied. Nowadays there are few comparative studies regarding these fillers' behaviour found in literature, although it's necessary for clinicians to familiarize themselves with their properties, reaching further than the manufacturers' indications.

The ART FILLER® line is a group of fillers produced with Tri-hyal technology, meaning they have long chain, very-long chain, and free non-crosslinked HA in their composition [1, 3]. The Universal filler was chosen as the control group, as it is the most used one out of the five and the most versatile.

As is shown in Fig. 4, all fillers had an increase in weight after 24 hours, meaning neither of the products were saturated prior to their use. All fillers absorbed the maximum amount of water they could, since after 24 hours there was an excess PBS inside the Eppendorf tubes [25].

According to previous studies that compared the swelling ratios of different HA fillers, those with lower cross-linking density and G' were capable of absorbing more water and expand, even though expansion wasn't a direct variable and was instead deduced from water absorption [8–10, 22–24, 26].

However, in this study, the Lips filler showed the highest swelling ratio (Sw= 2,6158), even though, according to the manufacturer, the filler with the lowest cross-linking density and G' is actually Fine Lines, which absorbed the least amount of water (Sw=2,0262). Lips Soft and Universal, which have the same indications, showed the most similar swelling ratios (Sw=2,2678 and Sw=2,3062, respectively), and they also present the same cross-linking degree and the most similar G', even though there is a difference in price, with 1 mL of Lips Soft being around 20€ more expensive than 1 mL of Universal. The properties of each filler as stated by the manufacturer are shown in table 1.

Still, aforementioned studies used different fillers and protocols from the ones used in the present study, meaning we cannot establish a straightforward comparison between them. Furthermore, hyaluronic acid's properties act synergistically, influencing one another, meaning there are plenty more factors to take into consideration, such as cohesivity, the process used to hydrate the gel and HA concentration [9, 10, 21, 30, 37].

In fact, fillers with lower HA concentration are supposed to absorb less water [23, 26, 37], which is in line with what happened regarding the Fine Lines filler. Despite having the lowest cross-linking degree and G', this is the filler with the lowest HA concentration, and the one that showed the lowest swelling ratio, which suggests the possibility that HA concentration might have more influence over water absorption than other properties. Aside from Fine Lines, all other fillers have the same HA concentration, and out of these, Volume is the one with both the highest cross-linking degree and G’, and the one with the lowest swelling ratio, which is in agreement with literature. However, the same can’t be said for Lips, as it absorbed the most water, whilst not having the lowest cross-linking degree or G’. This suggests that there might be another property influencing these results, that would allow the Lips filler to absorb more water and be more efficient in the treatment of deeper perioral wrinkles and the restoration of larger lip volumes, as the manufacturer’s indications advocate.

Rather than measuring filler expansion caused by water absorption in the 2D evaluation, an increase in width was determined instead, as height changes weren’t evaluated, and without surrounding tissue to sustain the fillers, they ended up quickly losing their consistency, and spreading out sideways on the coverslips. The observed increase in width is possibly caused by other properties.

When looking at Fig. 6, we can see that Volume had both the lowest initial width and the lowest width increase after adding PBS, which can be explained by the fact that this filler has the highest cohesivity according to Fillmed. Cohesivity corresponds to internal adhesion forces of a filler [30], therefore it makes sense that this filler would retain its shape and spread out less. Fine Lines, on the other hand, seemed to generally show the highest initial width and ensuing increase, while also being the least cohesive filler, which causes it to be more malleable and easily deformed [30].

Besides cohesivity, other properties like cross-linking degree or the amount of uncrosslinked HA, can also be responsible for the different initial line widths observed in Figs. 6 and 7, since lower cross-linking degrees and higher amounts of uncrosslinked HA increase fluidity [4, 13, 21, 34]. This can help explain why Volume had the lowest initial width, given its highest cross-linking degree and least amount of free HA, which would reduce its fluidity and make it spread out less on the coverslip. In fact, Volume, alongside Lips, seems to be one of the more viscous fillers. Fine Lines is the opposite, showing the largest initial width and the highest amount of free HA (alongside Lips Soft) and lowest cross-linking degree, while seemingly being the most fluid filler.

For 3D evaluation, pig skin was stored in DMEM at 25ºC, which allows the maintenance of its cellular viability up until 18 hours [14]. Still, an additional scanning at 24h was performed, to see whether there were any further volume changes.

As is shown in Fig. 9, all fillers expanded after being injected into the pig skin. This helps reenforce that clinicians should be careful when planning how much filler to inject into patients’ skin, and that an under-filling may be beneficial [24], since it’s expected that the initial volume will increase for at least up until 24 hours after the injection.

The Lips filler absorbed the most water and revealed high viscosity, which translated to an overall 3D expansion of 44%. As is shown in Fig. 9, this was the second lowest total expansion observed after Volume (39%), which was the most viscous and cohesive of the fillers. On the other hand, Fine Lines tended to absorb less water, while being the most fluid and least cohesive of the fillers, which translated to a total expansion of 92%. The Universal filler, which was highly fluid and had the second highest swelling ratio, also had the second highest total expansion (79%).

Expansion or lifting capacity, like all HA characteristics, is influenced by many other properties. A stronger gel, with higher G’, cross-linking degree and viscosity, is expected to better resist deformation and lift the tissues [8, 13, 18]. Fillers with higher HA concentration and ratio of crosslinked HA/uncrosslinked HA similarly allow for bigger expansion [24]. Since lifting capacity correlates to how much projection fillers can induce, it would be expected that a filler meant to be applied in deeper planes of the skin and restore bigger volumes (such as Volume) should expand more, and a filler meant to be applied more superficially and correct more delicate fold and wrinkles (such as Fine Lines) should expand less [18].

Our results are not in accordance with this, since Volume, which expanded the least, was the most viscous filler, while also having the highest G’, cross-linking degree and crosslinked HA/uncrosslinked HA ratio. Fine Lines, which showed both the highest total expansion and the highest volume immediately after injection, was the most fluid, while simultaneously having the lowest G’, cross-linking degree, HA concentration and crosslinked HA/uncrosslinked HA ratio.

The impact cohesivity may have on fillers’ capacity to expand is still debatable, since there isn’t a standardized protocol to measure it [10, 13]. Nonetheless, some studies suggest that more cohesive fillers will have a greater capacity to volumize the tissues and show bigger initial vertical projection, since they resist surrounding tissue pressure more easily [10, 13, 27, 30]. We aren’t able to confirm this based on our results, since it was the least cohesive filler (Fine Lines) that showed the biggest capacity to expand vertically.

Some papers mention that higher water absorption translates to a higher potential for volume increase [18, 24], but such association was not perceived in this study, as Fine Lines expanded the most, even though it absorbed the least amount of water.

The discordance between our 3D results and the data present in literature could exist due to the small sample size, scanning errors caused by skin deformation or different injection depths (filler injection in a superficial plane proved to be challenging, due to pig skin’s high thickness and strength), or the influence of other physicochemical and rheological properties and tissue-related factors, such as surrounding tissue structure and consistency (muscle tensile strength can act as a constricting barrier to filler expansion), tissue fluid balance and pH value [22, 24, 25].

Based on our results, since water absorption was determined from swelling ratio which corresponds to an increase in filler weight [22], and a filler’s lifting capacity depends on its ability to resist surrounding tissue pressure [30], we could hypothesise that fillers which absorb more water and become heavier, can’t resist as easily to tissue pressure due to their weight, thus having a lower projection. This would help explain why fillers with higher swelling ratio values had lower 3D volume values. Besides, even though these results seem to not be in accordance with literature, it’s important to remember that the chemistry of these fillers is extremely complex, and that all their properties are interconnected, making it difficult to specify exactly which ones caused Fine Lines to expand the most and Volume the least. To justify and validate these results, and achieve statistical significance, future studies with a bigger sample and the inclusion of other fillers are necessary.

It´s also important to further investigate HA fillers' volume changes in the skin throughout time and clarify their connection with water absorption, and how each HA property can influence it, along with expansion capacity. Additionally, *in vivo* filler performance should be evaluated and compared to *in vitro* results regarding HA rheological and physicochemical properties.

5. CONCLUSION

The present study intended to analyse two properties of HA fillers from the ART FILLER® line: water absorption and expansion capacity. Lips was capable of absorbing significantly more water than all other fillers, and Fine Lines showed a tendency to absorb the least amount of water. In 2D evaluation, Volume was the most viscous, with the smallest initial line width, while Fine Lines was the most fluid. All evaluated fillers showed an increase in 3D volume up until 24 hours post-injection. Fine Lines showed a tendency to expand the most overall, while Volume showed a tendency to expand the least.

Future studies should seek to further understand how, and which properties influence one another, and especially water absorption and expansion. A larger sample is required to achieve statistical significance when it comes to volume evaluation.

Declaration

Competing Interests: The authors declare that they have no conflict of interest.

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Table

Table 1 is available in the Supplementary Files section.

Figures

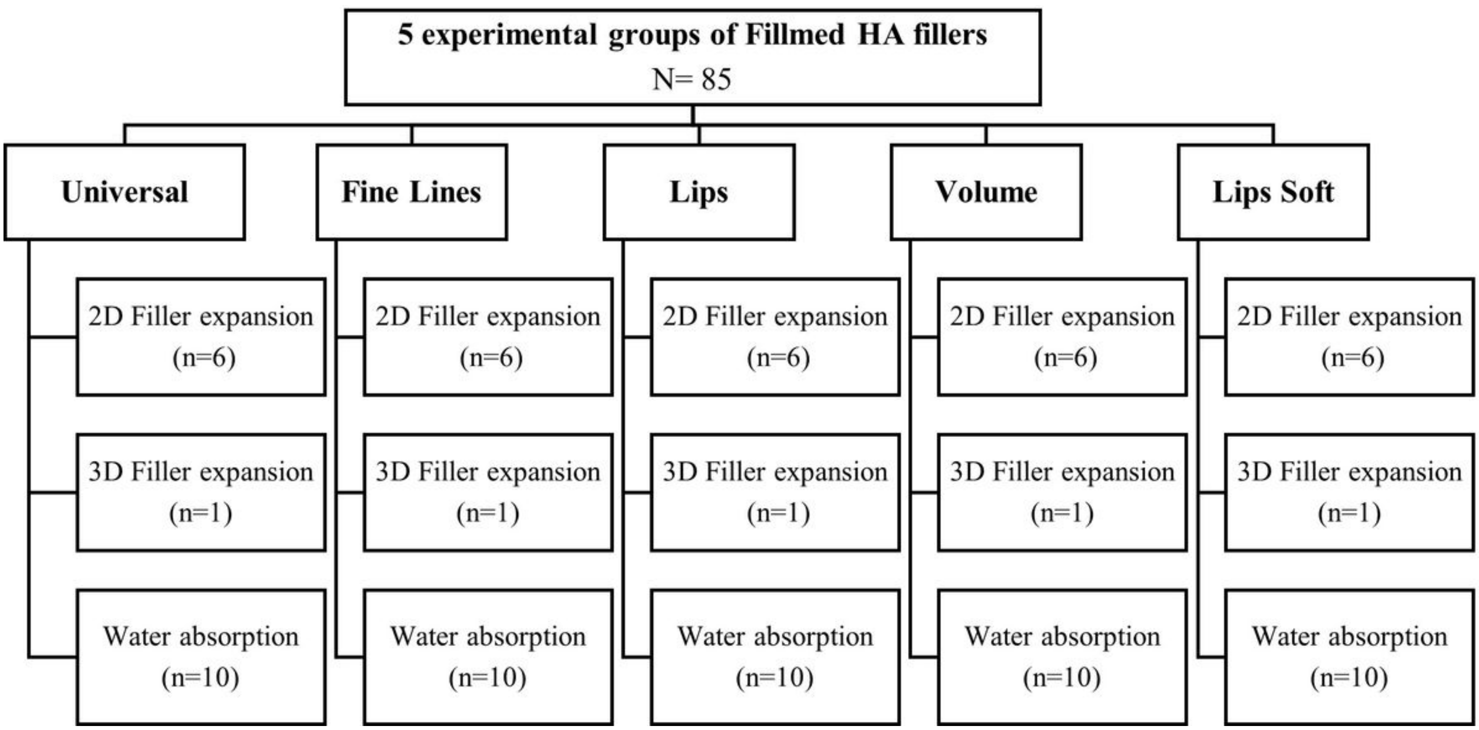

Figure 1

Illustrative schematic of study design

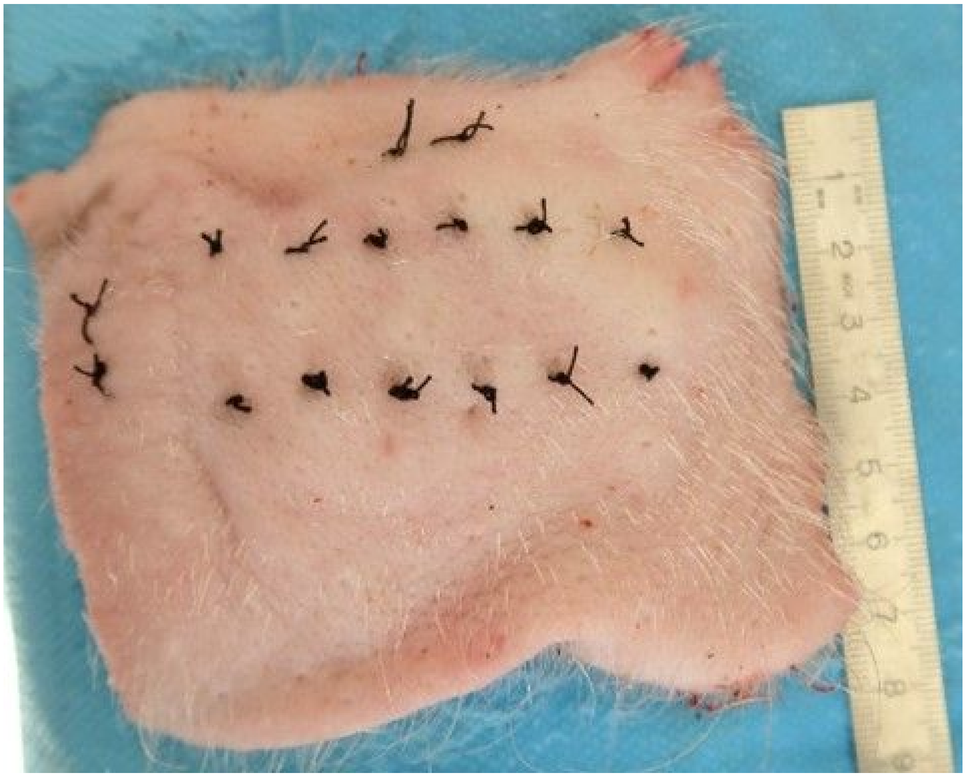

Figure 2

Skin sample

Figure 3

Exemplificative images of matching and measurement of STL files obtained with the 3Shape intra-oral scanner

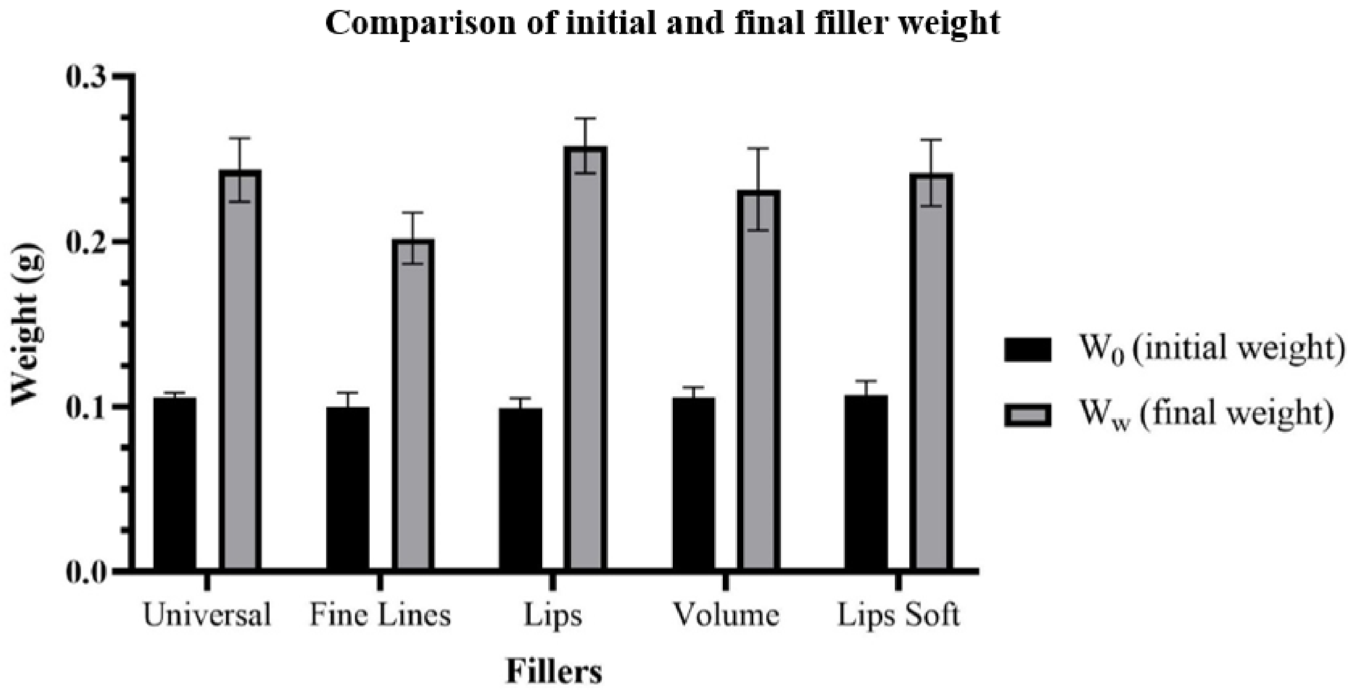

Figure 4

Graphical representation of the differences in initial and final filler weight for each filler

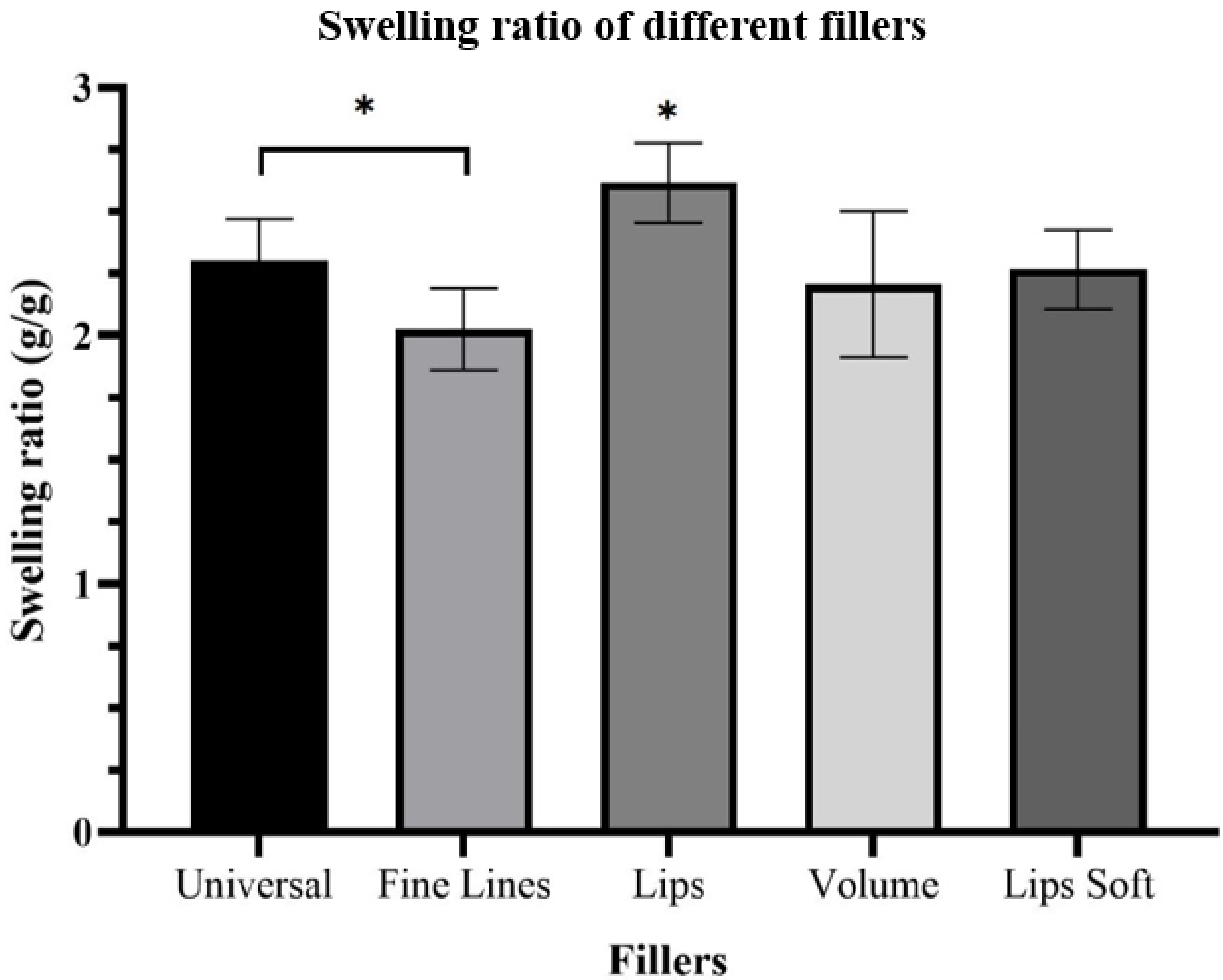

Figure 5

Graphical representation of the swelling ratio means of different fillers. Level of significance: * $p \leq 0,05$

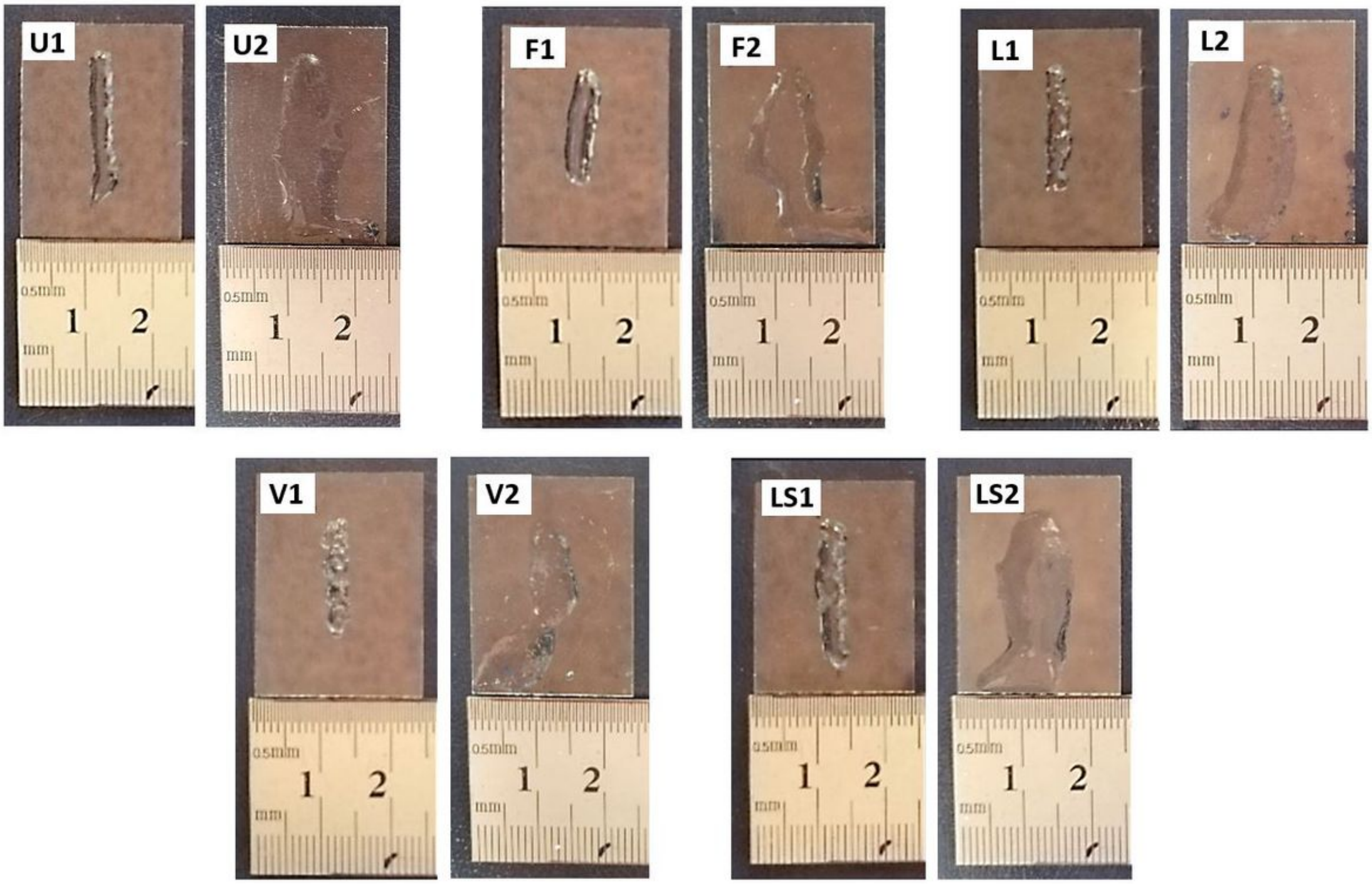

Figure 6

Exemplificative photographs showing a side-by-side comparison of filler line width before (photos on the left - 1) and two hours after adding PBS (photos on the right - 2). U= Universal, F= Fine Lines, L= Lips, V= Volume, LS= Lips

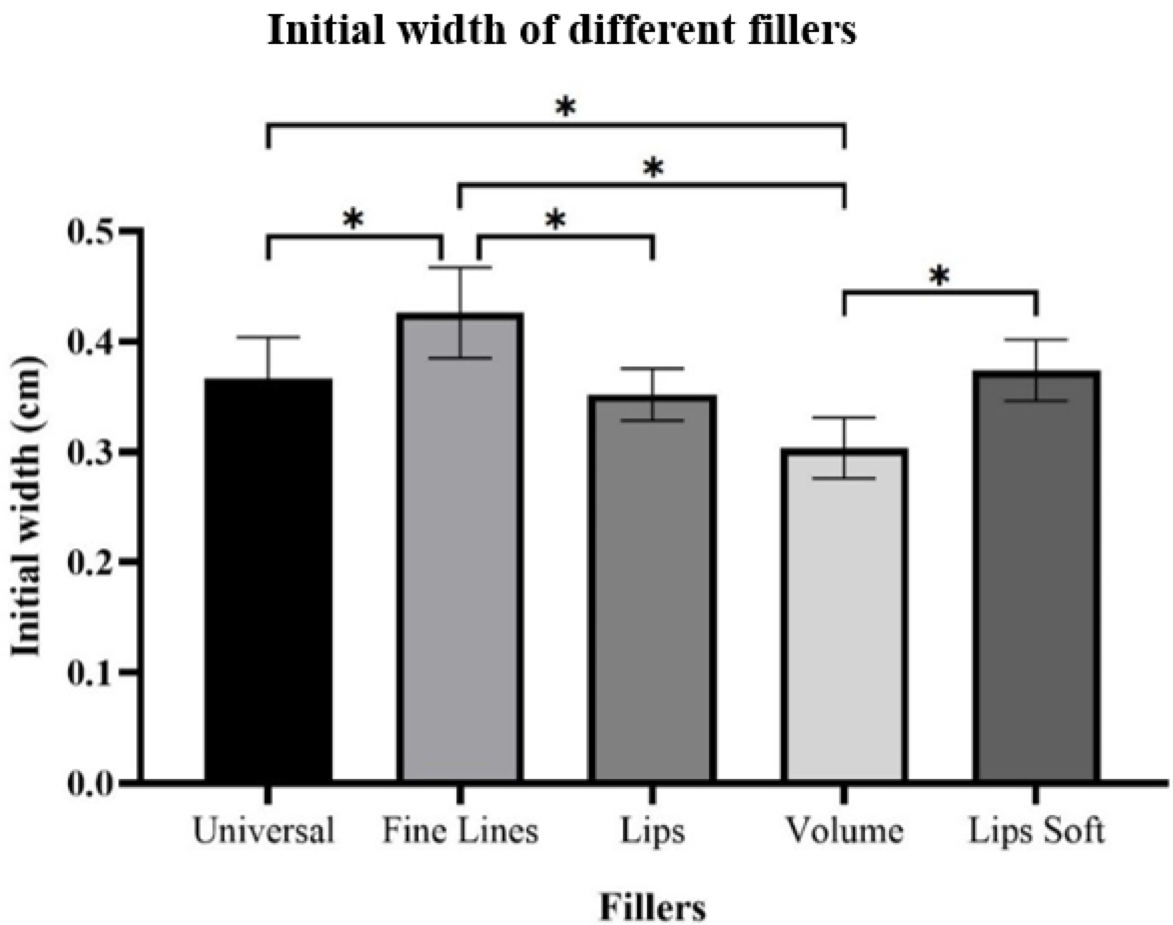

Figure 7

Graphical representation of the initial width of different fillers. Level of significance: * $p \leq 0,05$

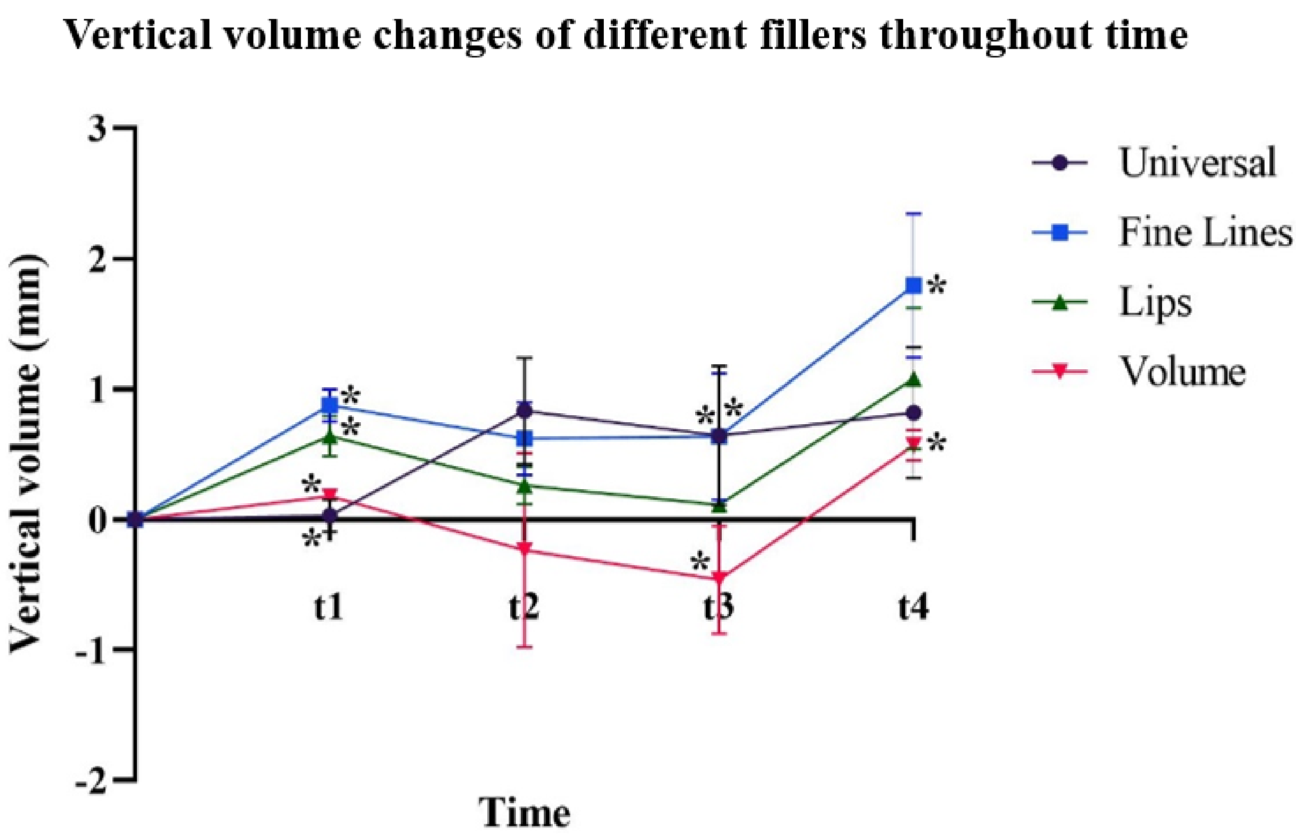

Figure 8

Graphical representation of the volume changes of different fillers throughout time. Level of significance: * p ≤ 0,05

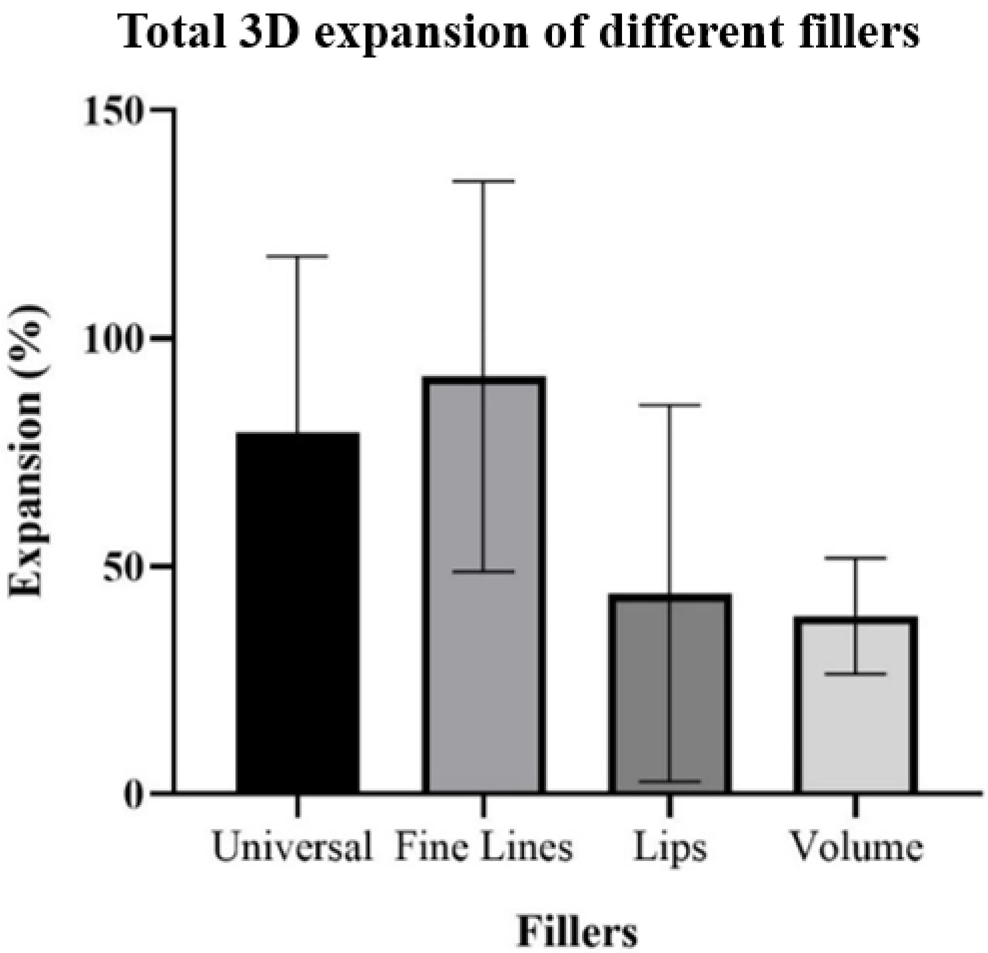

Figure 9

Graphical representation of the total volume expansion of different fillers after 24 hours

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Table1.jpg