

Generation of Site-Appropriate Tissue by a Living Cellular Sheet in the Treatment of Mucogingival Defects

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Background: Generation of site-appropriate tissue in the oral cavity includes the restoration of the correct anatomic type, amount, and distribution of the tissue. This study is a post hoc analysis of data collected during previously published results from two randomized clinical trials of a living cellular sheet (LCS; allogenic cultured keratinocytes and fibroblasts in bovine collagen) versus a free gingival graft (FGG), evaluating their ability to augment keratinized tissue or gingiva.

Methods: Post hoc histologic and clinical (photographic) comparisons of the outcomes of treatment were performed on histologic and photographic data gathered in the two randomized clinical trials.

Results: Histologic findings showed that LCS-treated sites resembled gingiva rather than alveolar mucosa. Photographic analysis indicated that LCS treatment resulted in more site-appropriate tissue than FGG in terms of tissue color, with adjacent untreated tissue, absence of scar formation or keloid-like appearance, and mucogingival junction alignment.

Conclusion: Treatment of mucogingival defects with LCS resulted in the generation of tissue that is more site appropriate than tissue transplanted from the palate. *J Periodontol* 2014;85:e57-e64.

KEY WORDS

Gingival recession; periodontics; regenerative medicine; tissue engineering.

The optimal goal for treating oral mucosal defects is to restore function while preserving esthetic appearance. Current options are limited and rely predominantly on grafting techniques; i.e., free gingival graft (FGG), subepithelial connective tissue grafts, and rotated pedicle and papillae flaps.^{1,2} Unfortunately, FGG techniques cause patient morbidity as a result of graft harvesting, and they may not restore tissue that is as esthetically pleasing as native tissue.^{1,2} Previous research has shown that gingival and palatal grafts retain their tissue characteristics after transplantation to an ectopic site.³⁻⁵ Because of these limitations, alternative or adjunctive treatments, such as dermal substitutes, growth factors, and other biomimetics, are being considered.⁵⁻⁷ The goal of many of these technologies is to repair mucogingival tissue and to restore function and esthetics in a site-appropriate manner, while reducing patient morbidity.⁵⁻⁷

Although this living cellular sheet (LCS) has been used for >14 years to treat patients with cutaneous chronic wounds and has also been evaluated in patients with acute cutaneous wounds, its application in oral soft-tissue therapy is relatively recent.^{2,8-12} LCS is composed of living allogenic human cells, bovine collagen, and human

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Table 1.

Differentiating Characteristics of Alveolar Mucosa, Palatal Mucosa, and Gingiva³⁻⁵ in LCS-Treated Sites

Component	Alveolar Mucosa	Palatal Mucosa	Gingiva
Papillae and rete ridges	Wide, short	Shorter, more slender (compared with gingiva)	Long, slender
Elastin fibers	Numerous, evenly distributed	Delicate fibers, evenly distributed, fewer than alveolar mucosa	Some, in connection with blood vessels
Collagen	Wavy, coarse, and loose density	Dense	Dense
Keratinized epithelium	Absent	Present	Present

extracellular proteins. The LCS does not engraft but produces a wide array of growth factors and cytokines¹³⁻¹⁶ that are thought to improve the course of wound healing and tissue regeneration by influencing, transiently and locally, the way that the patient's own cells differentiate into site-appropriate tissue.² Two randomized, within-patient controlled clinical trials have demonstrated that LCS can stimulate the patient's own cells to predictably generate a clinically significant amount of keratinized tissue (KT) and attached gingiva (AG) surrounding teeth that do not require root coverage.^{2,12} A hallmark of tissue regeneration is the formation of site-appropriate tissue (e.g., correct anatomic type, amount, and distribution).¹⁷ In addition to demonstrating the generation of KT, results from these trials showed that tissue generated at LCS-treated sites was superior ($P < 0.001$) to FGG-treated sites in terms of color and texture match with adjacent, untreated tissue.¹²

In addition to differences in gross clinical morphology, histology may be used to differentiate gingiva, palatal mucosa, and alveolar mucosa (Table 1).³⁻⁵ Hematoxylin and eosin staining can be used to evaluate the presence and formation of rete ridges, whereas additional histologic stains may be used to evaluate the arrangement and distribution of collagen, elastin, and reticulin. The specific aim of the present post hoc analysis is to evaluate whether changes in histology and extracellular protein expression correlated with the observed clinical differences in appearance between FGG- and LCS-treated sites from the two clinical trials.^{2,12}

MATERIALS AND METHODS

Study Design

The results reported here are based on post hoc analyses of data from the previously published pilot and pivotal trials evaluating the efficacy and safety of LCS for the treatment of mucogingival defects not

requiring root coverage.^{2,12} Both trials were open-label, randomized, within-patient controlled and approved by recognized institutional review boards (pilot study:² Western Institutional Review Board; pivotal trial:¹² Western Institutional Review Board, University of Michigan Medical School Institutional Review Board, and The University of Texas Health Science Center–San Antonio). Eligible participants were at least 18 years of age and had at least two non-adjacent teeth in contralateral quadrants of the same jaw with ≤ 1 mm AG that required soft-tissue grafting without the need for root coverage. After surgical creation of a partial-thickness wound bed, LCS^{††} (test) was applied to one site, and autologous FGG (control) was applied to the contralateral site. The primary clinical outcome measures of these trials were the amount of AG (pilot) or KT (pivotal) at 6 months, which are common endpoints in trials of FGG. Investigators in both trials also evaluated the color and texture of each treated site compared with the untreated tissue immediately adjacent to the treated site.^{2,12}

Histologic Evaluations of Biopsy Samples From the Pilot Study

To more thoroughly evaluate the quality of the newly generated tissue in LCS-treated sites and the grafted tissue in FGG-treated sites, the histologic characteristics of the treated sites were evaluated and compared within patient to baseline and between groups at 6 months. Three-millimeter diameter punch biopsies were obtained from seven participants in the pilot study. Biopsies were taken at baseline during preparation of the treatment site and at 6 months from the LCS- and FGG-treated sites. Baseline biopsies were taken at the location of the mucogingival junction (MGJ) in which very little AG was present (i.e., < 1 mm); therefore, much of

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the tissue at baseline was representative of alveolar mucosa. After histologic processing, masked evaluation and scoring was performed by an oral and maxillofacial pathologist (S-BW). Additional details of the scoring are described in supplementary Table 1 in the online *Journal of Periodontology*. Paired (within-patient) observations were made on slides prepared with biopsies taken at baseline and 6 months. Categorical data were cross-tabulated according to treatment group, and comparisons between treatment groups were made using a paired discordance test. Summary statistics were generated for quantitative values, and differences between LCS- and FGG-treated sites were assessed using paired *t* tests.

Independent Esthetic Assessment of Photographic Images From the Pivotal Study

During the pivotal study, clinical assessment of gingival tissue color and texture was made by investigators or examiners at each of the trial sites. Photographs were taken with consistent magnification (1:1.5 for global photographs and 1:1 for close-up photographs) at baseline, 1 and 4 weeks, and 3 and 6 months.¹² To confirm the outcome of the clinical color and texture evaluation performed during the pivotal trial, a post-study, independent photographic review was performed using the 6-month photographs. Three independent, calibrated reviewers (two general dentists and one periodontist; Drs. Peter Arsenault, Luis Del Castillo, and

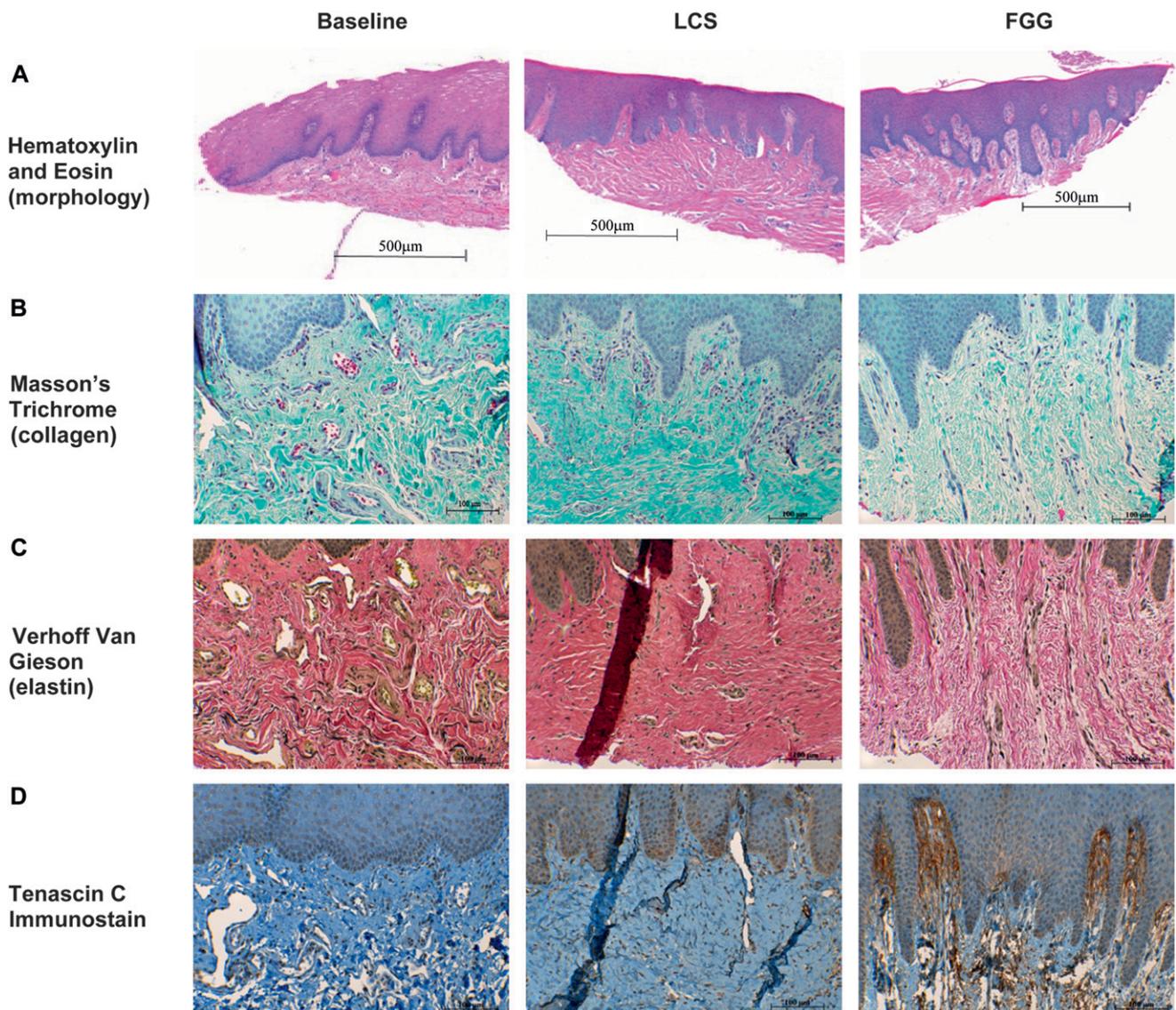


Figure 1. **A through D)** Representative histologic images of biopsies obtained at baseline or 6 months (LCS or FGG) from a subset of patients (n = 7) enrolled in the pilot study. Indicator bars in the images show the scale for each row of photographs. Scale bar = 100 µm.

Table 2.
Changes in Histologic Scores at Individual Sites From Baseline to 6 Months (pilot)

Characteristic	LCS (n = 7)	FGG (n = 7)
	n	n
Rete ridge formation		
Decreased	1	0
Remained the same	3	6
Increased	3	1
Inflammation		
Decreased	0	1
Remained the same	3	5
Increased	4	1
Reticulin		
Decreased	2	0
Remained the same	5	7
Increased	0	0
Elastin*		
Decreased	4	3
Remained the same	1	1
Increased	1	2
Tenascin-C		
Decreased	2	2
Remained the same	4	2
Increased	1	3
Fibronectin		
Decreased	0	1
Remained the same	7	4
Increased	0	2

* One participant had a missing LCS value for baseline elastin; the control side had a score of 1.

Maria E. Gonzalez Del Castillo, Tufts University School of Dental Medicine, Boston, Massachusetts) compared each treatment site (LCS or FGG) with the adjacent, untreated tissue in the 6-month photographs in a masked manner using predefined, clinically relevant criteria as described previously by Cairo et al.¹⁸ From the pivotal study, only participants not designated as training cases (n = 85) were used in the post hoc analyses.¹²

All photographs included in the independent assessment were annotated to adequately identify the study tooth and all treated teeth adjacent to the study tooth. In the study by Cairo et al., five variables and an associated scoring system were recommended for consideration for esthetic evaluations after the treatment of gingival recession defects.¹⁸ Four of the five recommended variables (color, soft-tissue texture, marginal tissue contour, and mucogingival alignment) were chosen for inclusion in the independent assessment. The fifth variable, percentage of root coverage, is not a study endpoint and is therefore not included in this esthetics assessment. Because the scoring system¹⁸ proposed is based on the inclusion and weighting of all five variables, it is not used in this independent review. Before conducting the independent assessment, the reviewers were familiarized with the Cairo et al. article.¹⁸ To ensure satisfactory calibration of the reviewers to the esthetic variables, their individual assessments of photographs 1 through 8 were discussed after the presentation of each photograph. The results of each reviewer's assessment of photographs 1 through 8 did not change based on the discussion that followed; these data were used in the analysis of the results of the photograph assessment.

Table 3.
Changes in Histologic Metrics From Baseline to 6 Months (pilot)

Tissue Component	LCS (n = 7)	FGG (n = 7)	Paired Difference* (n = 7)	P Value†
% of dense collagen,‡ mean change (SD)	28.6 (24.1)	0.0 (24.5)	26.0 (39.1)	0.2114
% of collagen‡ parallel to epithelium, mean change (SD)	-34.2 (38.3)	-39.3 (36.3)	-1.7 (29.4)	0.8951
% of thick collagen fibers,§ mean change (SD)	4.2 (29.1)	-8.6 (42.5)	24.2 (40.1)	0.1995
Number of blood vessels, mean change (SD)	-14.2 (9.7)	-18.0 (16.2)	5.0 (14.6)	0.4878
Number of myofibroblasts, mean change (SD)	-14.7 (5.8)	-12.8 (15.9)	0.0 (15.7)	1.000

SD = standard deviation.
* LCS - FGG (within patient pair).
† Paired t test, P value for treatment differences.
‡ LCS: n = 7; FGG: n = 5; paired difference: n = 5 (two FGG biopsies unusable).
§ LCS: n = 6; FGG: n = 7; paired difference: n = 6 (one LCS biopsy unusable).
|| Number per four high-power fields.
¶ LCS: n = 6; FGG: n = 6; paired difference: n = 5.

These criteria included color (more, equal, or less red), scar formation or keloid-like appearance (presence or absence), MGJ alignment (MGJ aligned/not aligned with the MGJ of adjacent teeth), and marginal tissue contour (does/does not follow the cemento-enamel junction [CEJ]).¹⁸ In the present study, root coverage was not assessed because the pivotal study only included participants for whom achieving root coverage was not desired.¹²

κ statistics were used to test the agreement of each assessed esthetic criteria among reviewers of the photographs. The percentage of exact agreement was added post hoc because reviewer responses were not well distributed among the potential responses, and small marginal totals can make κ statistics somewhat unstable. The McNemar marginal homogeneity test was used post hoc to evaluate the superiority of LCS compared with FGG for each reviewer. Results from each independent photograph reviewer ($n = 3$) were evaluated separately.

RESULTS

Histologic Evaluations of Biopsy Samples From the Pilot Study

At 6 months, rete ridges in both groups were well formed with thin parakeratinization, (Fig. 1A). A mild increase in inflammation from baseline was observed at four of seven LCS-treated sites (change in score from 0 to 1+) and one of seven FGG-treated

sites (change in score from 1+ to 2+; Table 2), but this was within baseline variability and was not considered clinically significant (zero of seven LCS-treated sites and three of seven FGG-treated sites showed inflammation at baseline). Neither group contained substantial numbers of myofibroblasts at baseline as detected by smooth muscle actin antibody (data not shown). Both groups showed a tendency toward decreased vascularity (reduced number of blood vessels) at 6 months compared with baseline (Table 3). Both groups also showed similar amounts of reduction in the percentage of collagen parallel to the epithelium and in the number of myofibroblasts present compared with baseline (Table 3).

Analysis of all treated sites revealed that the percentage of dense collagen in LCS-treated sites was increased at 6 months compared with baseline (+28.6%). No change (0.0%) was observed in FGG-treated sites (Fig. 1B; Table 3). There was a higher percentage of dense collagen in LCS-treated sites compared with FGG-treated sites at 6 months (62.8% versus 48.3%, respectively, complete data not shown; Fig. 1B). The percentage of parallel collagen was reduced at 6 months compared with baseline in both groups (−34.2% LCS, −39.3% FGG) and was similar between groups at 6 months, as shown by the large paired difference in Table 3. The percentage of thick collagen fibers at 6 months was slightly increased from baseline in LCS-treated sites (+4.2%) and reduced from baseline in FGG-treated sites (−8.6%; Table 3).

There was no significant presence of reticulin fibers in either baseline or 6-month biopsies in either treatment group (data not shown). There was a tendency toward a decreased quantity of elastin fibers in both treatment groups at 6 months (Fig. 1C; Table 2), and both treatment groups had relatively low levels of elastin fibers, consistent with palatal mucosa and gingiva⁵ (Fig. 1C). Changes from baseline to 6 months in tenascin staining were variable in both groups (Fig. 1D; Table 2). At 6 months, tenascin staining was absent or low in LCS-treated sites and variable but overall more intensely stained in FGG-treated sites (Fig. 1D). There were no differences from baseline to 6 months in

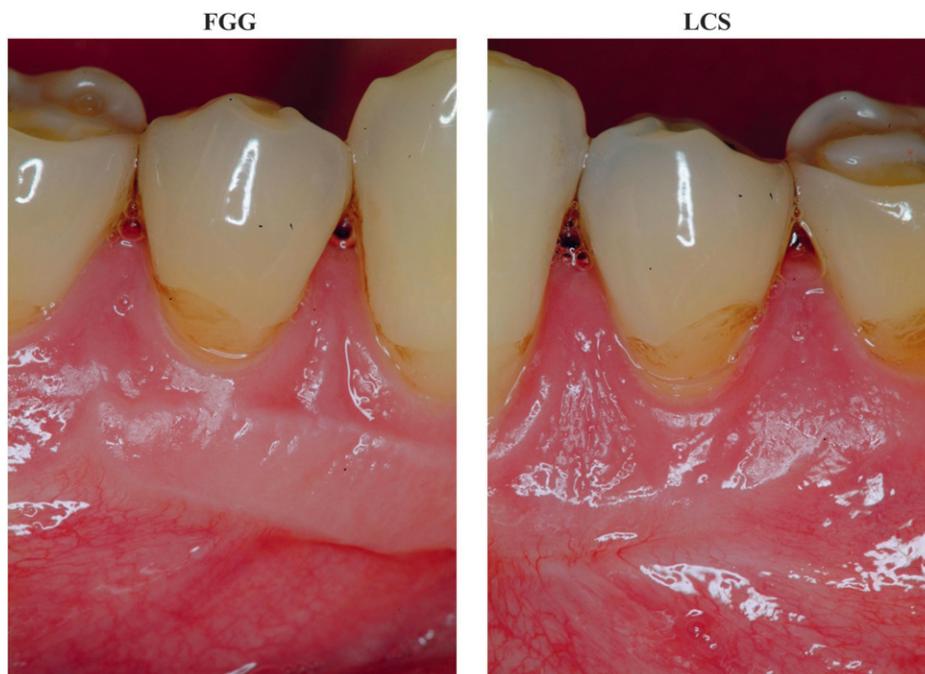


Figure 2.

Representative photographs of contralateral FGG-treated and LCS-treated sites in the same patient at 6 months.

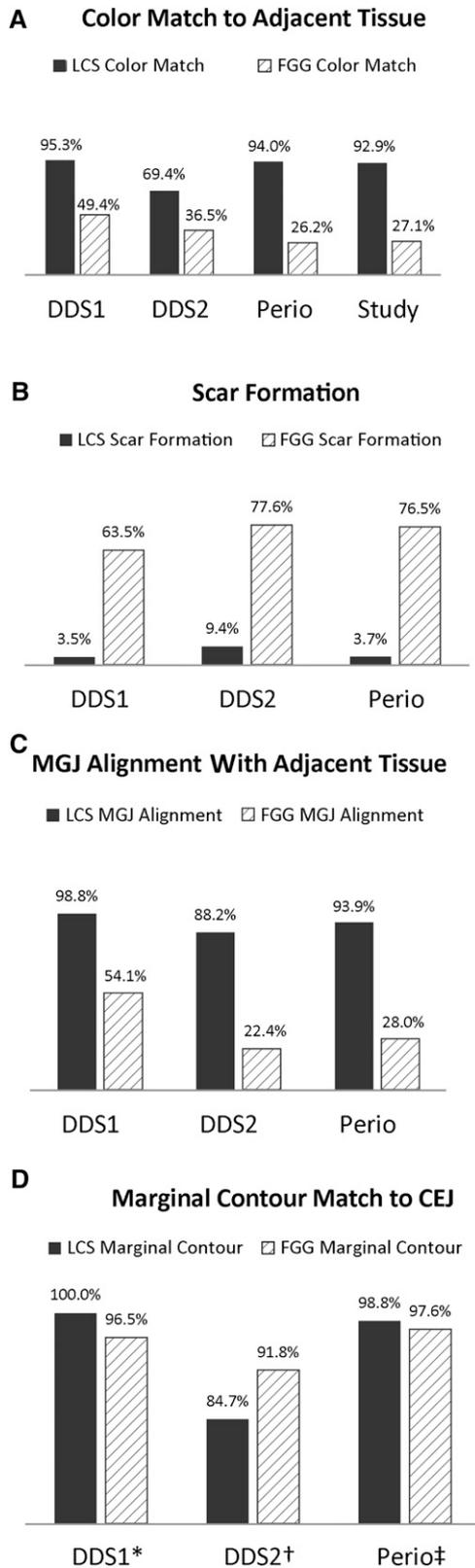


Figure 3. A) Percentage of LCS and FGG that achieved color match to the adjacent tissue. The data from the pivotal study are shown along with that of the independent examiners. Note the high level of agreement between the masked independent periodontist and the masked study examiners.

fibronectin staining in LCS-treated sites, with some variability in FGG-treated sites (Table 2). Little or no fibronectin staining was observed at baseline or 6 months in both treatment groups (data not shown).

Independent Esthetic Assessment of Photographic Images From the Pivotal Study

An independent masked assessment of photographs taken at the 6-month visit (Fig. 2) was conducted to confirm gingival tissue color assessments performed by the pivotal study investigators as well as to assess scar formation or keloid-like appearance, MGJ alignment, and marginal tissue contour. The number of participants in which the LCS-treated site achieved color comparable with the adjacent untreated tissue (n = 40, 21, 19, and 23 for each pairing) was significantly greater than the number of participants in which the FGG-treated site achieved color comparable (n = 2, 10, 3, and 0 for each pairing) with the adjacent untreated tissue (P ≤ 0.0001 by McNemar paired comparison test). This confirms observations of the pivotal study investigators (P ≤ 0.0001, respectively, for both the study investigators and for all three independent photograph reviewers; Fig. 3A). LCS-treated areas were superior to FGG-treated areas in terms of color, regardless of reviewer variability. The κ statistic for the agreement between the periodontist independent reviewer and the pivotal study investigators was 0.702, indicating good agreement. In contrast, the κ statistic values for the remaining comparisons between the independent photograph examiners (general dentists) and pivotal study examiners (0.491 and 0.401, respectively) suggested only moderate agreement (see supplementary Table 2 in the online *Journal of Periodontology*; data was filed by the manufacturer^{§§}). Exact agreement across the independent photograph reviewers ranged from 72.9% to 85.3%. Although exact agreement between the pivotal study investigators and the independent photograph reviewers ranged from 67.2% to 85.3%, with the periodontist showing the highest agreement

B) Percentage of LCS- and FGG-treated sites judged to show scar formation. **C)** Percentage of LCS- and FGG-treated sites that showed MGJ alignment with adjacent untreated tissue. **D)** Percentage of treated sites in which the marginal soft-tissue contour matched the contour of the CEJ of the treated tooth. P ≤ 0.001 for the comparisons in A through C. In panel D, P could not be determined for DDS1; P ≤ 0.1336 for DDS2; P ≤ 0.5637 for Perio. DDS1* and DDS2† = general dentists who served as masked independent photographic evaluators in this post hoc analysis; Perio‡ = periodontist who served as the masked independent specialist photographic evaluator in this post hoc analysis; STUDY = masked examiners in the original study.

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(see supplementary Table 2 in the online *Journal of Periodontology*).

The study investigators and the independent photograph reviewers interpreted the clinical results for lack of scar formation or keloid-like appearance and MGJ alignment as clinically significant in favor of LCS. For each of these parameters, a significance of $P \leq 0.0001$ was attained (Figs. 3B and 3C). No statistical difference was discerned between treatments regarding marginal tissue contour (Fig. 3D). Exact agreement and κ statistics for the agreement in assessments among reviewers are presented in Table 2. LCS-treated areas were superior to FGG-treated areas in terms of lack of scar formation or keloid-like appearance and MGJ alignment, regardless of reviewer variability.

DISCUSSION

The histologic and esthetic aspects of the tissue generated at LCS-treated sites and the tissue at FGG-treated sites in the pilot and pivotal clinical trials of LCS are evaluated in this post hoc study.^{2,12} The histologic results reported here demonstrate that tissue generated with LCS treatment appeared to be site appropriate for gingiva and was different from both alveolar mucosa (i.e., baseline biopsies) and tissue at the FGG-treated sites. Moreover, the independent photographic analysis of the treated areas suggests that LCS treatment resulted in the formation of more site-appropriate tissue versus FGG in terms of tissue color match with adjacent untreated tissue, absence of scar formation or keloid-like appearance, and MGJ alignment. Differences in the clinical appearance of the LCS-treated sites versus FGG-treated sites may be related to subtle differences in the appearance of collagen (denser in LCS) and the presence of tenascin (less in LCS) in the histologic analysis. Although there was an increase in the percentage of dense collagen in LCS-treated sites, the shape and distribution of collagen were not similar to the hyalinized collagen seen in keloid scars and in the healing described for treatment with acellular dermal matrix (ADM).⁵ These results are consistent with the generation of gingiva-like tissue at LCS-treated sites. Tenascin, which is expressed during fetal development and wound healing and has a role in active tissue modeling or remodeling,¹⁹ has also been shown to be expressed in palatal tissue in the pig.²⁰ This protein was absent in most LCS-treated sites (six of seven) and present in most FGG-treated sites (six of seven) at 6 months. A mild increase in the presence of scattered chronic inflammatory cells was observed in some LCS-treated and FGG-treated sites at 6 months, but the increase was not considered clinically significant.

Previous trials have shown that tissues (e.g., FGG) grafted onto ectopic oral sites retain the tissue characteristics of their origin,³⁻⁵ which may affect the function and/or esthetics of the grafted site. To date, efforts to generate mucogingival tissue in a site-appropriate manner have been disappointing. Wei et al.²¹ conducted a study comparing the effectiveness of ADM and FGG for increasing the width of AG. The results suggested that tissue formed at the ADM-treated site did not parallel any known mucosa and was more similar to scar tissue. In contrast, the tissue formed at the FGG-treated site was more similar to donor palatal mucosa than to the adjacent gingiva, particularly in terms of rete ridge formation.⁵ Unfortunately, some trials have thoroughly examined both the histologic and esthetic aspects of newly generated tissue to make an informed determination of the site appropriateness of the tissue.

There were several limitations of the histologic and esthetic assessments. The histologic analysis was undertaken post hoc in an attempt to find a histologic correlate for the differences in clinical appearance between LCS- and FGG-treated sites. The histologic analyses examined a relatively small number of participants ($n = 7$) and were not powered for robust statistical analyses. The esthetic assessments may have been limited by the nature of the photographic review (as opposed to direct assessment), as well as by varying degrees of consistency in the photographs (e.g., lighting, focus, global versus close-up views).

CONCLUSIONS

Both the histologic and esthetic assessments provide evidence supporting the generation of site-appropriate oral soft tissue with LCS treatment. Taken with the results of the pilot and pivotal trials, the dimension and quality of the tissue generated by LCS is sufficient to maintain health and provide acceptable esthetics in place of FGG in gingival augmentation procedures.^{2,16}

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