INTRODUCTION

Modern dental treatment concepts sometimes involve soft tissue augmentation procedures around teeth or implants, which can be divided into two categories: methods to generate and/or widen keratinized mucosa (KM) and methods to increase soft tissue volume and/or thickness (Bassetti, Stahli, Bassetti, & Sculean, 2016; Thoma, Buranawat, Hammerle, Held, & Jung, 2014). Soft tissue thickening is often required to cover recession and to prepare a site for implant or prosthetics, for ridge preservation procedures, and for soft tissue contouring around dental implants. In clinical practice, the subepithelial connective tissue graft (SCTG) is regarded as the gold standard for such procedures (Jung, Siegenthaler, & Hammerle, 2004; Schwarz et al., 2014; Sculean et al., 2015; Thoma et al., 2014; Zuhr, Baumer, & Hurzeler, 2014). However, autologous tissue grafting is associated with postoperative patient morbidities and operative
risks, motivating investigations of potential alternatives, such as porcine-derived 3D collagen-based matrices (Bassetti et al., 2016; Ghanati et al., 2011; Nocini et al., 2014; Sanz, Lorenzo, Aranda, Martin, & Orsini, 2009; Schmitt et al., 2013).

Clinical and preclinical studies report the successful use of various collagen matrices for root coverage and soft tissue thickening procedures (Jepsen et al., 2013; Schwarz et al., 2014; Sculean et al., 2015; Thoma et al., 2011, 2016). Our research group previously demonstrated that the integration process for both porcine collagen matrix (CM) and SCTG is associated with substantial volume loss in the first month of healing (Schmitt et al., 2016). Another preclinical histological study investigated the degradation and integration of a 3D collagen matrix, showing that after degradation the matrix is replaced by newly formed connective tissue (Rothamel et al., 2014). However, massive volume changes after grafting suggest that most of the matrix is simply degraded during integration, leading to outcomes almost comparable to the pre-graft situation (Schmitt et al., 2016). It remains unclear exactly what happens during integration after grafting with SCTGs or CMs, and how tissue turnover processes are correlated with new tissue formation and graft degradation. Therefore, more 3D and histological data after soft tissue thickening are needed to understand these biological processes and make treatment outcomes more predictable.

In the present study, soft tissue thickening in a dog model was performed with the aim of quantitatively and qualitatively comparing ten-month histological outcomes with a native porcine collagen matrix (CM) versus the SCTG. Thereby, the three-dimensional measurements of soft tissue alterations after gingiva thickening published in the previous paper are substantiated by the histological outcomes. To exclude tissue changes of the surrounding hard and/or soft tissues, the soft tissue volume augmentation was simulated by thickening attached periodontal soft tissues in a preclinical dog model, as described in our previous study.

2 | MATERIALS AND METHODS

2.1 | Study characteristics and outcome variables

A preclinical study in healthy beagle dogs was performed, investigating histological and immunohistological changes over a 10-month examination period (May 2014–January 2015). The three-dimensional soft tissue changes from this study have been previously published (Schmitt et al., 2016). Each animal received the natural porcine 3D collagen matrix (CM, mucoderm®, Botiss Biomaterials GmbH) as test group and an SCTG (control group) from the palate, at the buccal gingiva of the upper canines (region of interest; ROI) using randomised split-mouth allocation.

The primary outcome variable was as follows:

Histomorphometrically measured connective tissue thickness (CTT) in mm within the ROI at 10 months after gingiva thickening.

The secondary outcome variables were as follows:

Clinical Relevance

Scientific rationale for the study: Soft tissue thickening in the oral cavity is usually performed with autologous subepithelial connective tissue grafts (SCTG). To avoid autologous grafting, native collagen matrices (CM) are discussed as alternative. Comparable data concerning soft tissue thickness gain and soft tissue quality after augmentation are scarce.

Principal findings: Soft tissue thickening around teeth resulted in a higher significant CTT in the SCTG groups versus the CM group. Tissue quality was comparable between the CM and the SCTG groups.

Practical implications: The SCTG performs superior compared to the CM for gingiva thickening. The tested collagen matrix could serve as an alternative to the SCTG for soft tissue thickening in terms of tissue quality.

1. Descriptive histological analyses of the ROI, and
2. Immunohistological quantification of VEGF and collagen I expression in the ROI to qualify the augmented soft tissues and compare outcomes between groups. With the VEGF quantification, differences between groups concerning tissue vascularization should be detected (e.g. due to ongoing integration/ degradation processes or inflammation). By quantifying the collagen I expression, inter-group differences of the collagen density should be detected. Both for VEGF and collagen I, it was hypothesized that the degree of expression would not differ between groups.

This study was approved by the Pest County Government Department for Food Safety and Animal Health, Hungary (number PEI/001/961-2/2013). The manuscript was prepared in accordance with the Animal Research: Reporting In Vivo Experiments (ARRIVE) Guidelines Checklist (Kilkenny, Browne, Cuthill, Emerson, & Altman, 2010).

2.2 | Animal care and surgical procedure

This study included eight healthy female beagle dogs that were at least 12–18 months of age. Surgeries were performed after a 1-month acclimatization period. Details concerning animal care; baseline measurements; and specific treatments before, during and after surgery have been previously described (Schmitt et al., 2016).

The two treatments (CM and SCTG) were allocated to either the right or left upper canine by simple randomization. Local anaesthesia (Ultracain® DS forte; adrenaline 1:100,000; Sanofi-Aventis GmbH) was administered at the buccal aspect of the canine. Then, a mesial vertical subgingival incision was made, and the keratinized part of the buccal soft tissues was tunnelled in a mesiodistal direction with a split-thickness flap. The tunnel was performed in an extent that...
the soft tissue graft could be inserted stable without the need of any further fixation. After rehydration with sterile saline solution, the resulting tunnel was augmented with either CM or an SCTG (including the periosteum) that had been harvested from the palate (Schmitt et al., 2016). After local anaesthesia of the plate, a paramarginal palatal submucosal incision was made from P1 to P4 and a split-thickness flap sharply dissected towards the midline of the palate. The SCTG was harvested with the periosteum and the wound bed immediately treated with local haemostatic measures such as electrocoagulation and suturing with resorbable sutures (Vicryl 5.0; Ethicon GmbH). The graft size was standardized in both groups: 20 mm × 10 mm × 1.2–1.7 mm in the CM group prior rehydrating (thickness ranging from 1.2 to 1.7 mm as it is commercially available) and 20 mm × 10 mm × 1.5–2 mm in the SCTG group. After graft insertion, the mesial vertical incision was closed using resorbable sutures (Vicryl 5.0; Ethicon GmbH & Co KG). After ten months, the animals were sacrificed as previously described (Schmitt et al., 2016).

2.3 | Specimen preparation

Jaws were first sectioned using a precision saw (EXAKT Advanced Technologies GmbH) to obtain small samples from the augmented ROI. These samples were then fixed by immersion in formalin solution, dehydrated in alcohol, and embedded in Technovit 9100 New
The embedded specimens were bisected through the longitudinal axis of the canine in a bucco-palatal direction. Half of each specimen was ground for histological examination using the technique described by Donath et al. (Donath, 1985; Donath & Breuner, 1982), while the other half was sliced into thin sections for analyses.

2.4 | Histology and primary outcome measurements (CTT)

Half of each embedded section was reduced to 25–30 µm using a grinding unit (EXAKT), then polished, stirred continuously for 5 min in 10% H₂O₂, rinsed under cold running water, dried and stained for 15 min with toluidine blue O solution (Sigma-Aldrich Chemie GmbH). Sections were coated with Technovit 9100 New and then light cured for 8 min. Digitized histological data were then analysed using ZEN 2011 software (Carl Zeiss Microscopy GmbH). Tissue thicknesses in the augmented ROIs were evaluated in five regions per sample. Region definition and the measuring process are explained in Figure 1.

2.5 | Descriptive histological analyses and immunohistology

The other half of each embedded section was fixed in a sledge microtome (Leica RM 2165; Leica Microsystems) and sliced into multiple 2- to 4-µm-thick sections. These sections were processed and dried for 12 hr at 57°C on microscope slides. After appropriate pre-treatment, Cason staining was performed to enable descriptive histological analyses focusing on the connective tissue portions of the augmented ROIs.

For immunohistochemical analyses, sections were stained for VEGF and collagen I expressions using the avidin–biotin complex method, with an automated staining machine (Dako Autostainer Plus, Dako). Sections were next subjected to chromogen treatment (Dako REAL™ Chromogen Red 1–3; Dako) and then counterstained with haematoxylin (Automation Hematoxylin, Code 53301; Hämalaur, Dako), followed by fixation with Aquatex® (Merck KGaA).

Histological data from Cason and immunohistological staining were digitized applying the "whole-slide imaging method" with the Zeiss MiRAX MIDI Scanner (Carl Zeiss MicroImaging GmbH) and were transferred to MRXS files. Then, all slices were analysed using the Panoramic Viewer 1.15.2 (3DHISTECH Ltd.).

Collagen I and VEGF expressions were quantitatively analysed in the connective tissue portions of five areas corresponding to the regions defined for thickness measurements (Figure 1). Collagen I was analysed in a 586 µm × 428 µm region, under 20× magnification. The percentage of coloured area within the total image area was determined using Bioquant Osteo® 2011 (BIOQUANT Image Analysis Corporation). VEGF was analysed in a 293 µm × 214 µm region, under 40× magnification. Selected VEGF regions were subjected to cell counting, and the percentage of VEGF-positive cells was determined using the program ImageJ 1.46r (Wayne Rasband, National Institutes of Health, USA).

2.6 | Statistical analyses

The sample size of 8 beagles was selected using an equivalence test of means based on the increase of gingival thickness after 10 months, as previously explained (Schmitt et al., 2016). Results were entered into Excel 2016 (Microsoft Corp.), and data were analysed using SPSS version 23.0 for Windows (IBM SPSS Software). Descriptive statistics included the frequency (absolute and relative abundances; %), arithmetic mean and standard deviation. For inter-group comparisons of all test parameters (CTT, VEGF expression and collagen I expression), a nonparametric Mann–Whitney U test was used to determine statistical significance, defined as p < .05.

3 | RESULTS

3.1 | Connective tissue thickness (CTT)

Table 1 displays the tissue thickness measurements for all tissue portions in the augmented regions. Mean CTT (region 1–5) significantly

| Table 1 | Thickness measurements of various tissue portions and of connective tissue in regions 1–5 for the subepithelial connective tissue graft and collagen matrix groups |

| Group                  | Oral epithelium | Connective tissue, p = .008 SCTG versus CM | Buccal alveolar bone | Periodontal ligament |
|------------------------|-----------------|--------------------------------------------|----------------------|----------------------|
| SCTG                   | 0.31 ± 0.08     | 1.32 ± 0.44                                | 0.42 ± 0.13          | 0.36 ± 0.09          |
| CM                     | 0.34 ± 0.12     | 1.06 ± 0.27                                | 0.36 ± 0.18          | 0.32 ± 0.07          |

Note: Mean and standard deviation (SD) is given for each parameter. The p value is given for statistically significant differences (p < .05).

Abbreviations: CM, collagen matrix; SCTG, subepithelial connective tissue graft.
differed between the SCTG group (1.32 mm ± 0.44 mm) and the CM group (1.06 mm ± 0.27 mm; \( p = .008 \)).

### 3.2 Descriptive histological outcomes

Figure 2 shows representative Cason-stained sections for both groups. In all cases, the augmented region (connective tissue) and the nearby periodontal structures, including the oral epithelium, sulcular epithelium, junctional epithelium, alveolar buccal bone and periodontal ligament, were observed. These surrounding structures showed typical anatomical characteristics with no visible differences between both groups and thus are not described in detail.

In both groups, the characterization of the augmented regions after 10 months of healing revealed mature connective tissue that was rich in blood vessels and cells, predominantly fibroblasts spreading within the collagen structures. Towards the epithelium, the connective tissue showed papillary indentations due to the rete peg formation spreading into the connective tissue. These indentations appeared to be slimmer and showed deeper penetration into the epithelium in the SCTG group than the CM group. Both groups exhibited connective tissue directly bound to the periosteum towards the bone, lymphocyte accumulation near the junctional epithelium, and a large proportion of connective tissue characterized by mature collagen fibres. Moreover, both groups showed a visible and prominent system of coordinated fibres of the dentogingival complex, with densely packed clearly arranged fibres—most prominently including the dentoperiosteal fibres, followed by the dentogingival and alveologingival fibres (Figure 2).

### 3.3 Collagen I and VEGF expressions

Table 2 presents all parameters in detail, and Figure 3 includes representative images. Collagen I and VEGF expressions in the augmented regions 1–5 and in total did not significantly differ between the CM
TABLE 2  Collagen I and VEGF Expressions in the Augmented Regions of Connective Tissue for the Subepithelial Connective Tissue Graft (SCTG) and Collagen Matrix (CM) Groups

| Group | Collagen I Expression (%; mean ± SD) | Region 1 | Region 2 | Region 3 | Region 4 | Region 5 | Total   |
|-------|-----------------------------------|----------|----------|----------|----------|----------|---------|
| SCTG  |                                   | 30.36 ± 9.23 | 32.49 ± 5.19 | 30.11 ± 6.93 | 35.60 ± 6.27 | 34.65 ± 7.53 | 32.64 ± 7.09 |
| CM    |                                   | 29.87 ± 9.13 | 29.83 ± 10.09 | 32.62 ± 6.57 | 29.33 ± 7.66 | 31.18 ± 6.78 | 30.57 ± 7.83 |

| Group | VEGF Expression (%; mean ± SD) | Region 1 | Region 2 | Region 3 | Region 4 | Region 5 | Total   |
|-------|---------------------------------|----------|----------|----------|----------|----------|---------|
| SCTG  |                                 | 40.29 ± 8.88 | 38.52 ± 9.37 | 36.11 ± 7.29 | 40.60 ± 5.83 | 39.76 ± 5.51 | 39.06 ± 7.27 |
| CM    |                                 | 33.50 ± 9.05 | 33.56 ± 6.55 | 40.23 ± 11.28 | 38.86 ± 8.84 | 39.60 ± 12.74 | 37.15 ± 9.80 |

Note: Mean and standard deviation (SD) is given for each parameter. Inter-group comparisons revealed no statistically significant differences.

FIGURE 3  (a, c) Representative images showing immunohistochemical staining for collagen I in connective tissue sections (the augmented region) from the CM group (a) and the SCTG group (c). Magnification, 20×. Red indicates collagen I staining. (b, d) Collagen I expression for the corresponding image excerpts from the CM group (b) and the SCTG group (d) was quantified via an automated selection process of the positive stained sections (green parts) using the Bioquant Osteo® program. (e, f) Representative images showing immunohistochemical staining for VEGF in tissue sections from the CM group (e) and the SCTG group (f). The arrows indicate VEGF-expressing cells.
group (collagen I, 30.57% ± 7.83%; VEGF, 37.15% ± 9.80%) and the SCTG group (collagen I, 32.64% ± 7.09%; SCTG: 39.06% ± 7.27%; p > .005).

4 | DISCUSSION

In the present study, attached periodontal soft tissues were augmented to simulate mucosal soft tissue thickening procedures in a dog model. The aim was to histologically and immunohistologically characterize the augmented connective tissue at 10 months after grafting, and to compare the results with a native porcine collagen matrix (CM) versus an autologous free subepithelial connective tissue graft (SCTG). To confirm the 3D data generated in the previously published part of this study, the CTT was measured and the augmented soft tissue quality assessed via descriptive histology and immunohistological quantification of collagen I and VEGF expressions.

Results showed that the mean CTT was significantly higher in the SCTG group (1.32 mm ± 0.44 mm) than the CM group (1.06 mm ± 0.27 mm; p = .008). Descriptive histological analyses revealed a mature connective tissue in both groups and comparable collagen I and VEGF expressions in the augmented regions.

The present experimental set-up was not designed to replicate a specific clinical treatment scenario. Rather, it was intended to enable measurement of soft tissue volume and thickness alterations after gingiva thickening via superimposed virtual data of dental casts as previously explained. This was considered to be the most accurate experimental scenario for this purpose, as it excluded factors that can influence tissue volume alterations in the ROI and mask the actual effect of the soft tissue augmentation procedure (i.e. bony alterations by subperiosteal matrix placement and changes of chronic alveolar ridge defects) as it is known from previous studies by Thoma et al. (2011, 2010). The previously reported 3D tissue measurements revealed that only 27.08% and 11.03% of the initial augmented soft tissue volume remained after 10 months of healing in the SCTG and CM groups, respectively. In terms of thickness, this reflected maximum soft tissue thickness increases of 0.66 mm ± 0.29 mm (SCTG) and 0.79 mm ± 0.37 mm (CM), and mean soft tissue thickness increases of 0.13 mm ± 0.26 mm (SCTG) and 0.01 mm ± 0.26 mm (CM), with no significant between-group differences (Schmitt et al., 2016).

A prior preclinical study reports that the CM used in our present study shows fast tissue integration and vascularization, with almost no signs of ingrowing inflammatory cells and a resorption time of approximately 8–12 weeks (Rothamel et al., 2014). This fast tissue turnover and matrix resorption may explain the tremendous volume loss during the first 3 months of healing after soft tissue thickening (Schmitt et al., 2016). It is possible that most of the matrix is simply degraded, with only a small proportion leading to new connective tissue formation and subsequent gain of mucosal soft tissue thickness. The CM group showed 10-month maximum and mean soft tissue thickness increases of 0.79 mm ± 0.37 mm and 0.01 mm ± 0.26 mm, respectively. Interestingly, autologous SCTG integration also led to significant decreases in volume and thickness, showing insignificant superiority compared to CM. The SCTG group showed 10-month maximum and mean soft tissue thickness increases of 0.66 mm ± 0.29 mm and 0.13 mm ± 0.26 mm, respectively (Schmitt et al., 2016). Considering the tissue thickness increase measured in the 3D study and the absolute mean CTT histomorphometrically measured in the present study groups (SCTG: 1.32 mm ± 0.44 mm; CM: 1.06 mm ± 0.27 mm), one can conclude that the procedure achieved a thickness gain of only a few hundred micrometre, yielding a CTT of almost the same thickness as prior to surgery. Notably, the CTT measurements were significantly superior with SCTG versus CM. Therefore, SCTG use should be prioritized over CM use in soft tissue thickening procedures until further data, and thus, reliable evidence is available.

The literature does not describe any comparable outcomes for soft tissue thickening using the specific collagen matrix from our study. However, preclinical and clinical studies have investigated other collagen-based matrices for oral soft tissue thickening (Thoma et al., 2011; Thoma, Naenni, Benic, Hammerle, & Jung, 2017; Zeltner, Jung, Hammerle, Husler, & Thoma, 2017). This collagen matrix is a volume-stable cross-linked porcine collagen matrix (VCMX, Fibrogide, Geistlich Pharma AG) (Thoma et al., 2011, 2017; Zeltner et al., 2017). Cross-linking generates more stable collagen matrices that exhibit slower biological degradation (Rothamel et al., 2014; Thoma, Villar, Cochran, Hammerle, & Jung, 2012), which should enhance connective tissue formation compared to non-cross-linked collagen matrices. However, cross-linking can also favour inflammatory and foreign body reactions, potentially leading to wound healing complications and poorer clinical outcomes (Annen, Ramel, Hammerle, & Jung, 2011; Becker et al., 2009).

The presently used matrix (VCMX) has been preclinically tested for soft tissue volume augmentations in chronic ridge defects (Thoma et al., 2011), and after immediate implant placement with simultaneous guided bone regeneration (GBR) (Thoma et al., 2017). Regarding the biological response to the VCMX, histological outcomes show slight-to-moderate (but clinically insignificant) infiltration with inflammatory cells during the first 2 months. When used for tissue thickening in chronic ridge defects, the histomorphometrically measured soft tissue thickness at 84 days after augmentation was 4.0 mm ± 0.9 mm with VCMX versus 5.4 mm ± 0.5 mm with SCTG (Thoma et al., 2011). In the case of soft tissue thickening after immediate implant placement and GBR, the greatest mucosal thickness after 6 months was 0.8 mm ± 0.3 mm with VCMX versus 0.7 mm ± 0.2 mm with SCTG (p = .754) (Thoma et al., 2017). These previous results indicate much greater tissue thickness increases with VCMX than achieved with the native non-cross-linked collagen matrix used in our study (Thoma et al., 2011). However, all of these studies reported rather heterogeneous results from using collagen-based matrices for soft tissue thickening, making it difficult to compare the results between studies (Schmitt et al., 2016; Thoma et al., 2011, 2017). The available data clearly suggest that the differences are not solely attributable to the utilized collagen matrix, but are rather affected by the following...
factors: (a) the matrix itself (native collagen or cross-linked collagen), (b) the defect model or experimental clinical scenario (soft tissue thickening at teeth, in chronic ridge defect, or after immediate implant placement and GBR), (c) the follow-up duration (3–10 months), (d) the matrix placement (subperiosteal or epiperiosteal), (e) the matrix thickness and folded or unfolded use, and (f) the methodologies for histomorphometrically measuring the thicknesses (Schmitt et al., 2016; Thoma et al., 2011, 2017).

The experimental design applied in our study ensures that the observed tissue alterations were solely attributed to the connective tissue’s biological responses to the inserted soft tissue grafts, without the influence of any confounders (Schmitt et al., 2016). There are no prior data available regarding soft tissue quality characterization based on quantification of collagen I and VEGF expressions in the augmented zone (connective tissue), making it impossible to compare our present study outcomes to prior research.

The role of VEGF in wound healing is in stimulation of angiogenesis (Bao et al., 2009). This implies that the VEGF expression in the early healing phase after augmentation is higher. However, it is known from VEGF that the expression is also upregulated in any kind of hypervascularization processes in human connective tissue (Ferrara, 2001; Josko, Gwozdż, Jedrzejowska-Szypulka, & Hendryk, 2000). This can be present in (a) ongoing integration/vascularization processes of tissue transplants, (b) the compensation of tissue hypoxia and (c) hypervascularization processes due to infections, tissue trauma or neoplasia. In this study, it is hypothesized that comparable levels of VEGF expressions mean a comparable tissue quality after augmentation with the absence of inflammation or any kind of tissue turnover processes due to the ongoing integration of the used tissue transplants ten months after augmentation.

Collagen is the most abundant protein in the various connective tissues of the human body. Specifically, collagen I is predominant in the repair of human connective tissues and scar tissue formation (Chattopadhyay & Raines, 2014; Olczyk, Mencner, & Komosinska-Vassev, 2014). The comparable levels of collagen I between groups after 10 months of healing mean a comparable degree of collagen I density and scar tissue formation in the augmented zone. Therefore, the present findings suggested that after a healing period of 10 months, the connective tissues augmented by CM and SCTG are equal in tissue quality.

The present animal studied had several limitations. Notably, relatively few animals were treated, which may have influenced the outcomes of the statistical comparisons between groups. Additionally, since multiple sacrifice time-points were not implemented for histological evaluations during the early healing phase, one cannot biologically explain the integration of soft tissue grafts and the circumstances that may have led to the tremendous loss of volume after augmentation. Moreover, a negative control was not included and no baseline data are available to compare connective tissue thickness measurements of the augmented sites with measurements of untreated sites. However, the previously reported 3D data indicate that there would be a tissue thickness increase after 10 months, which would be directly attributed to the connective tissue thickness increase. At the end, it should be emphasized that the results of this research should be interpreted with caution and only limited conclusion can be drawn due to the mentioned circumstances.

5 | CONCLUSION

The 10-month histomorphometric outcomes in our present study indicated that the investigated porcine collagen matrix was statistically inferior for soft tissue thickening procedures of the gingiva relative to the gold standard of care: the SCTG. However, the connective tissues augmented by CM and SCTG showed comparable quality. Further research should focus on the soft tissue graft integration process to elucidate the circumstances associated with soft tissue dimension alterations, and to make treatment outcomes more predictable.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest related to this study.

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