Degradation pattern of a porcine collagen membrane in an in vivo model of guided bone regeneration

E. Calciolari1,2 | F. Ravanetti3 | A. Strange4 | N. Mardas2 | L. Bozec4 | A. Cacchioli3 | N. Kostomitsopoulos5 | N. Donos1,2

1Centre for Oral Clinical Research, Institute of Dentistry, Queen Mary University of London (QMUL), Barts and The London School of Medicine and Dentistry, London, UK
2Centre for Oral Immunobiology and Regenerative Medicine, Queen Mary University of London (QMUL), Barts and The London School of Medicine and Dentistry, London, UK
3Department of Veterinary Science, University of Parma, Parma, Italy
4Department of Biomaterials and Tissue Engineering, UCL Eastman Dental Institute, London, UK
5Laboratory Animal Facilities, Biomedical Research Foundation of the Academy of Athens, Athens, Greece

Correspondence
Elena Calciolari, Centre for Oral Clinical Research and Centre for Oral Immunobiology, Institute of Dentistry, Queen Mary University of London (QMUL), Barts and The London School of Medicine and Dentistry, London, UK.
Email: e.calciolari@qmul.ac.uk

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Background and Objective: Although collagen membranes have been clinically applied for guided tissue/bone regeneration for more than 30 years, their in vivo degradation pattern has never been fully clarified. A better understanding of the different stages of in vivo degradation of collagen membranes is extremely important, considering that the biology of bone regeneration requires the presence of a stable and cell/tissue-occlusive barrier during the healing stages in order to ensure a predictable result. Therefore, the aim of this study was to investigate the degradation pattern of a porcine non-cross-linked collagen membrane in an in vivo model of guided bone regeneration (GBR).

Material and Methods: Decalcified and paraffin-embedded specimens from calvarial defects of 18, 10-month-old Wistar rats were used. The defects were treated with a double layer of collagen membrane and a deproteinized bovine bone mineral particulate graft. At 7, 14 and 30 days of healing, qualitative evaluation with scanning electron microscopy and atomic force microscopy, and histomorphometric measurements were performed. Markers of collagenase activity and bone formation were investigated using an immunofluorescence technique.

Results: A significant reduction of membrane thickness was observed from 7 to 30 days of healing, which was associated with progressive loss of collagen alignment, increased collagen remodeling and progressive invasion of woven bone inside the membranes. A limited inflammatory infiltrate was observed at all time points of healing.

Conclusion: The collagen membrane investigated was biocompatible and able to promote bone regeneration. However, pronounced signs of degradation were observed starting from day 30. Since successful regeneration is obtained only when cell occlusion and space maintenance exist for the healing time needed by the bone progenitor cells to repopulate the defect, the suitability of collagen membranes in cases where long-lasting barriers are needed needs to be further reviewed.

Keywords: collagen membrane, guided bone regeneration, histomorphometry, immunohistochemistry
1 | INTRODUCTION

The use of a barrier membrane to promote the selective repopulation of a periodontal/bone defect by cells with regenerative, rather than reparative, potential has been successfully applied for more than 40 years. In particular, in guided bone regeneration (GBR), the surgical placement of a tissue-occlusive membrane has been applied for the predictable treatment of atrophic ridges and peri-implant defects, for socket preservation and for de novo bone formation in healthy and medically compromised conditions (for review see refs 6, 7). An ideal barrier for GBR should combine biocompatibility and tissue occlusiveness with clinical manageability, space maintenance and the possibility to resorb gradually over time.

Collagen forms a significant part of the body and of the connective tissue’s proteins and it is continuously remodeled by specific enzymes called collagenases. Collagen-based membranes can be obtained from human skin, bovine Achilles tendon, porcine skin, and porcine inner organs. There are several advantages of collagen-based membranes over nonresorbable membranes: a simplified one-stage surgical procedure; cost-effectiveness; decreased patient morbidity; quick resorption in the event of exposure; and improved soft-tissue healing. Nevertheless, it is difficult to predict and control the time period during which occlusive properties are maintained, and this can interfere unfavorably with the bone healing process. Another disadvantage of collagen membranes is related to their unfavorable mechanical properties, which leads to collapse into the bony defects; hence, their combination with a bone graft is recommended when clinically applied.

Although the clinical application of collagen membranes in guided bone/tissue regeneration is well established, surprisingly only a limited number of studies have investigated their resorption pattern and they have shown that the degradation of collagen membranes might start within 4 days to 6 weeks after surgical placement. However, in most of the published studies, resorption of collagen membrane was evaluated after subcutaneous implantation, which does not resemble the clinical milieu, in which the membrane is in direct contact with the pristine bone, the graft and the soft tissues within the context of a surgical osseous wound. Moreover, the available data mainly consist of qualitative histologic observations and/or the measurement of membrane thickness, with little characterization of the enzymatic degradation process.

Hence, the aim of this study was to investigate, qualitatively and quantitatively, the early and late degradation pattern of a commonly used porcine collagen type I and III membrane associated with a bovine osteoconductive graft in an in vivo model for GBR.

2 | MATERIAL AND METHODS

This study was carried out on rat samples obtained as part of a previously approved project. The protocol was approved by the Ethical Committee of the General Directorate for Agricultural Economy and Veterinary Medicine of Athens, Greece (protocol number 590), and was carried out in accordance with the EU Directive 2010/53/EU for animal experiments. The ARRIVE guidelines for reporting animal research were followed.

2.1 | Experimental GBR model

Eighteen, 10-month-old Wistar rats were used. After 2 weeks of acclimatization, the experimental GBR surgical procedure was performed, as previously described. Briefly, a standardized 5-mm-diameter critical size defect was created on each parietal bone by the use of a trephine burr. A porcine collagen membrane (Geistlich Bio-Gide®; Geistlich, Wolhusen, Switzerland) was trimmed and adapted to the intracranial part of the defects, and then a deproteinized bovine bone mineral (DBBM) graft (Geistlich Bio-Oss®; Geistlich) was loosely compacted into the defect and eventually covered by a second resorbable collagen membrane (Geistlich Bio-Gide®; Geistlich). The flap was sutured in layers (Vicryl® 5.0; Ethicon, Cincinnati, OH, USA). Six rats were randomly sacrificed at 7, 14 and 30 days of healing, at which point one defect was randomly chosen for decalcified histology and one for proteomic analysis (results described in refs 23, 24).

2.2 | Histology analysis and histomorphometric measurements

The three most central sections of each sample were stained with hematoxylin and eosin and one section was stained with Masson trichrome stain. The following evaluations were performed by a blind, previously trained examiner, using a light microscope (Olympus BX50; Olympus America, Inc., Center Valley, PA, USA) connected to a digital color camera (CoolSNAP-Pro; Media Cybernetics Inc., Marllow, Buckinghamshire, UK):

1. Qualitative descriptive histology at different magnifications (×4, ×10, ×20, ×40). One section of each sample was also stained with picrosirius red and qualitatively examined under polarized light. This stain allows aligned (red) fibers to be distinguished from nonaligned (green) collagen fibers.

2. General and specific tissue reactions in three regions of interest (one at one end, one at the opposite end and one in the middle) according to Bozkurt et al. Details of the parameters evaluated and of the scoring system are presented in Table 1.

3. Thickness of the upper and lower collagen membrane (in microns) measured with the help of the software Image-Pro Plus (Version 4.5.0; Media Cybernetics Inc.) at ×4 magnification in three areas alongside the full length of each membrane (two at the margins of the defect and one in the middle). The mean value was calculated for the extracranial and intracranial membranes. Moreover, the thickness of an intact dry collagen membrane (Geistlich Bio-Gide®; Geistlich) and of an intact collagen membrane that was processed and embedded into paraffin, were measured.
The general tissue reaction was evaluated using the following scoring system: −, not present; +, present in ≥10% of the viewed area; ++, present in ≥20% of the viewed area; and ++++, present in ≥50% of the viewed area. The number of blood vessels (and SE) in the extracranial and intracranial membranes of each sample was counted.

The specific tissue reaction was evaluated using the following scoring system: 0, no cells; 0.5, <10 cells; 1, 10–50 cells; 2, 50–90 cells; and 3, >100 cells. Mean values and SE are reported.

### 2.3 | Atomic force microscopy and scanning electron microscopy

An intact porcine collagen membrane and two randomly selected samples per healing point were qualitatively assessed with atomic force microscopy (AFM) and scanning electron microscopy (SEM). The histology sections used for AFM were first stained with picrosirius red, then imaged using light microscopy to identify red, yellow and green areas. Once the areas were identified, AFM contact imaging in air was performed using Dimension 3100 and Dimension Icon atomic force microscopes (Bruker, Santa Barbara, CA, USA) with MSNL-10 probes. The sections were cooled in the buffer until they reached room temperature, then they were blocked by immersion in phosphate-buffered saline containing 5% bovine serum albumin. Sections were then imaged using light microscopy to identify red, yellow and green areas. After a washing step, the secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) was added. Finally, the sections were stained with DAPI for 5 min and then were mounted with anti-fading medium. A standard DAPI-FITC-TRITC combination filter was used to assess positivity for the aforementioned antibodies.

A semi-quantitative scoring system was applied as follows: −, when <10% positivity was detected; +, for ≥10% positive cells; ++, for ≥25% positive cells; and ++++, for ≥50% positive cells (modified from ref. 27).

### 2.4 | Immunofluorescence analysis

The following antibodies were applied for immunofluorescence analysis on 3 randomly selected samples per group:

- MMP-1 (bs-0463R; Bioss, Boston, MA, USA), MMP-8 (bs-1913R; Bioss) and TIMP-1 (sc-6834; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) as collagenase activity markers;
- tumor necrosis factor-alpha (TNF-α) (bs-2081R; Bioss), interleukin-1β (IL-1β) (GT15102; Neuronics Antibodies, Edina, MN, USA) as inflammation markers;
- osteopontin (bs-0026R; Bioss), bone sialoprotein (bs-4729R; Bioss) and osteocalcin (bs-4917R; Bioss) as new-bone-formation markers; and
- vimentin (bs-3471R; Bioss) as a mesenchymal cell marker.

Three sections per rat were used, in order to obtain triplicates of the results. The sections were treated with boiling EDTA buffer (1 mm EDTA, pH 8.0) and maintained at 80°C for 10 min for antigen retrieval. Sections were cooled in the buffer until they reached room temperature, and then they were blocked by immersion in phosphate-buffered saline containing 5% bovine serum albumin for 30 min. Then, the sections were then incubated, overnight at 4°C, with the primary antibodies diluted 1:50 in phosphate-buffered saline containing 5% bovine serum albumin. After a washing step, the secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) was added. Finally, the sections were stained with DAPI for 5 min and then were mounted with anti-fading medium. A standard DAPI-FITC-TRITC combination filter was used to assess positivity for the aforementioned antibodies.

A semi-quantitative scoring system was applied as follows: −, when <10% positivity was detected; +, for ≥10% positive cells; ++, for ≥25% positive cells; and ++++, for ≥50% positive cells (modified from ref. 27).

### 2.5 | Statistical analysis and sample size

In light of the "reduction" principle in animal research, for this project we used samples of a previously approved study that aimed to describe histologically and at a proteomic level the process of bone regeneration in healthy and osteoporotic-like rats. Hence, sample size was not calculated based on a specific membrane-degradation parameter.

Differences in membrane thickness (measured in microns) at the different healing periods were investigated using one-way ANOVA (SPSS® software, IBM Corp. Released 2013, Version 22.0. Armonk, NY: IBM Corp.). Post-hoc Tukey’s test was applied to explore differences in means between the healing times. One week after the first evaluation, 20% of the samples were measured again and the reproducibility was tested with the Bland and Altman diagram, the calculation of the British Standards Institution repeatability coefficient and the Lin’s concordance correlation coefficient. The general and specific tissue reactions, as well as the immunofluorescence markers were graphically presented in summary tables.

### 3 | RESULTS

### 3.1 | Qualitative histology

At 7 days of healing, the margins of the defects were clearly detectable and both collagen membranes were in place and well preserved in all specimens. The penetration of body fluids had given the membranes a "spongy" aspect, with initial separation between the collagen bundles. The most prevalent cells were erythrocytes, although a few polymorphonuclear leukocytes and lymphocytes were also identified. The picrosirius...
red stain showed widespread red areas in both intracranial and extracranial membranes, with only isolated green spots amongst the red fibers, thus identifying an overall aligned collagen structure (Figure 1A, A-1).

At 14 days of healing, initial signs of breakdown of the eosinophilic collagen bundles (especially in the extracranial membrane) were identified. A limited infiltrate of polymorphonuclear leukocytes, lymphocytes and macrophages was observed throughout the whole thickness of both intracranial and extracranial membranes. The sections stained with picrosirius red showed an increasing number of green spots within the red collagen bundles (Figure 1B, B-1).

At 30 days of healing, both intracranial and extracranial membranes were infiltrated by a few pyknotic cells (mainly polymorphonuclear leukocytes and lymphocytes), and marked signs of dissolution/degradation were detectable. In particular, the eosinophilic collagen bundles were fragmented in short and scattered clusters, with new vessels penetrating them. Close to the defect margins it was also possible to identify new bone formation spreading from the margins of the defect into the membranes, thus embedding the collagen fibers. The picrosirius red stain confirmed a general loss of collagen register, with diffuse orange and green areas throughout the membranes and limited red areas (Figure 1C, C-1).

3.2 | General and specific tissue reactions

The general tissue-reaction score showed the presence of a significant hemorrhagic infiltrate at both 7 and 14 days of healing and an increasing number of blood vessels in both intracranial and extracranial membranes from 7 to 30 days of healing (Table 1). A higher number of blood vessels was observed in the extracranial membrane, particularly at day 30.

The specific tissue reaction indicated a limited inflammatory reaction, as at neither of the healing periods we identified a score higher than 0.5, which indicates an average number of cells of <10 in the regions of interest. The identified cells consisted mainly of polymorphonuclear leukocytes, lymphocytes and macrophages.

Representative histological microphotographs obtained with different histological stainings at 7, 14 and 30 days of healing are presented in Figure 2, in order to support the data presented in Table 1.

3.3 | Membrane thickness

The thickness of both intracranial and extracranial membranes was significantly reduced from 7 to 30 days of healing (Figure 3).

Although the extracranial membrane tended to present a reduced thickness in comparison with the intracranial membrane, this trend was not statistically significant. In comparison with the intact control sample processed and embedded into paraffin, both extracranial and intracranial membranes showed an increased thickness at 7 days, that decreased to approximately the same thickness of the intact control sample processed and embedded into paraffin at 14 days, and then showed a further decrease at 30 days of healing.

The reproducibility of the measurements was fairly accurate, with a non-statistically significant difference between the first and second readings of $\bar{d} = -0.005$ mm (SD = 0.014 mm) for the extracranial membrane and of $\bar{d} = -0.011$ mm (SD = 0.019 mm) for the intracranial
3.4 | Atomic force microscopy and scanning electron microscopy

At day 7 of healing, only a loose collagen network could be seen in both extracranial and intracranial membranes, when analyzed using SEM. This network was highly porous with thin fibrils, but a large infiltration of erythrocytes and limited neutrophils were present (Figure 4A). The AFM returned an irregular picture, but it was possible to identify areas where collagen bundles were still visible (Figure 4D).

At day 14 of healing, a less porous collagen network was observed, with an increase in the quantity of fibrils. Erythrocytes were still present and they were embedded within both membranes (Figure 4B). Remarkably, collagen fibrils were also organized around the graft particles inside the defect area.

At day 30 of healing, the collagen network size had increased in both membranes, with few pores observed. Infiltration of various cell types was observed, with signs of colliquiation of the collagen membranes. The British Standards Institution repeatability coefficient was 0.028 mm and 0.038 mm, respectively and the Lin’s correlation coefficient was 0.997 (95% CI: 0.991-0.999) and 0.996 (95% CI: 0.986-0.999), respectively.

FIGURE 2 | Representative histological microphotographs at 7, 14 and 30 days after surgery. (A–C) Giemsa staining of samples at the different healing periods (asterisks indicate fatty infiltrates). (D–F) Masson trichrome staining of samples at the different healing periods (arrows indicate blood vessels; arrowheads indicate extravasated erythrocytes). (G–I) Hematoxylin and eosin staining (circular arrowheads indicate inflammatory infiltrate). Scale bar = 100 microns

FIGURE 3 | Bar chart showing thickness of the extracranial membrane (EM) and intracranial membrane (IM) after 7, 14 and 30 days of healing. Thickness of an intact dry membrane (DM) and of a processed membrane (PM) are also shown. Data represent mean ± SD. *Significant difference compared with 7 days of healing. †Significant difference compared with 14 days of healing.
membranes (Figure 4C). At high magnification, it was impossible to distinguish the peculiar collagen bundle structure, but both membranes were infiltrated by areas of mineralization and what appeared as a denser tissue network.

AFM pictures at 14 and 30 days of healing did not provide good quality data, owing to the irregular surface of the samples, and it was impossible to identify collagen fibrils.

3.5 Immunofluorescence analysis

The markers of collagenase activity (MMP-1, MMP-8 and TIMP-1) showed no positivity at day 7 of healing, whilst punctiform positivity was detected at day 14, which significantly increased at day 30 (Figure 5). On average, at 14 days of healing, ≥10% of cells were positive for MMP-1 and TIMP-1 within the defect area, while ≥25% cells were positive for MMP-1 and MMP-8 and ≥10% of cells were positive for TIMP-1 within the membranes. At 30 days of healing, positivity for collagenase markers further increased within the membranes, with ≥50% of cells positive for MMP-1 and ≥25% cells positive for MMP-8 (Table 2). The positivity was mainly observed in proximity to the membrane elastic fibers.

Markers of inflammation (TNF-α and interleukin-1β) were not detected (or <10% of positive cells were detected), either in the membranes or in the defect area, at all healing periods (Table 2).

Markers of bone formation were identified starting from day 14 of healing, both in the defect and within the membranes (Figure 6). At 14 days, ≥10% of cells within the defect were positive for osteopontin and osteocalcin, while ≥25% of cells were positive for bone

FIGURE 4 (A) Section after 7 days of healing, as assessed by scanning electron microscopy (SEM). At this stage, a loose network of collagen fibers was detected, which were infiltrated mainly by erythrocytes and few polymorphonuclear leukocytes and lymphocytes. (B) Section after 14 days of healing, as assessed by SEM. A more organized network of fibers and cells was detected. (C) Section after 30 days of healing, as assessed by SEM. Signs of colliquation of the membrane (arrows) and infiltration by different cell types were observed. (D) Section after 7 days of healing, as assessed by atomic force microscopy (AFM). The image was taken in a “red” area of a section stained with picrosirius red. We can foresee the bundle structure of collagen in some areas (arrows), although overall the surface was very irregular and did not allow for good quality pictures.

FIGURE 5 (A, D) Immunofluorescent spots indicate cells positive for MMP-1 at 14 (A) and 30 (D) days. (B, E) Immunofluorescent spots indicate cells positive for MMP-8 at 14 (B) and 30 (E) days. (C, F) Immunofluorescent spots indicate cells positive for TIMP-1 at 14 and 30 days. All markers are identifiable using tetramethylrhodamine (TRITC) filter.
sialoprotein. At 30 days, ≥ 10% of cells within the defect were positive for osteopontin and ≥ 25% of cells were positive for bone sialoprotein and osteocalcin (Table 2). In the defect area, most of the fluorescent spots were identified in close proximity to the DBBM graft particles. In the membranes, the positivity started at 14 days and reached the highest scores at 30 days for all markers, when ≥ 25% of cells were positive for bone sialoprotein and osteopontin and ≥ 10% of cells were positive for osteocalcin (Table 2).

Vimentin was expressed in the defect area in ≥10% of the cells at day 14 of healing, whilst at 30 days a moderate positivity (≥10% of cells) was found in the membrane area (Table 2).

### 4 | DISCUSSION

The porcine type I and III collagen membrane adopted was biocompatible and inert, did not elicit an inflammatory or foreign body reaction, and was able to promote the bone regeneration process. Membrane integrity was well maintained during the first 14 days but, at 30 days, pronounced signs of degradation, high levels of remodeling and a significant reduction in thickness were identified. These data confirm previous findings in a similar model from Moses et al.22 that showed a significant reduction in membrane thickness from 14 to 30 days of healing, as well as a significant reduction in the total amount of collagen. Remarkably, at 30 days, bone formation markers (alkaline phosphatase, bone sialoprotein, osteopontin), a mesenchymal cell marker (vimentin) and histology features suggested that bone formation was spreading inside the membranes and embedding the fragmented collagen bundles. While this implies loss of integrity as a barrier, at the same time the membrane was able to support the bone regeneration process23 and to create an environment that promoted the deposition and mineralization of the bone matrix, with blood vessels penetrating throughout the membrane at 30 days. A recent study in a similar calvaria defect model also confirmed that, at 30 days of healing, the collagen membrane network is progressively invaded by areas of bone formation and presents a dense infiltrate of leukocytes and fibroblast-like cells.28
The trend for higher resorption of the extracranial membrane compared with the intracranial membrane observed in our study might be justified by the increased number of blood vessels detected in the extracranial barrier. The vessels originating in the overlying periosseum and inner skin might have, in fact, contributed to accelerate the resorption of the extracranial barriers. Our results are in line with previous studies reporting that the porous structure of non-cross-linked collagen membranes is suitable for the formation of transmembrane blood vessels, which may also facilitate membrane resorption.\textsuperscript{12,21,29}

Recently, Turri et al. have also shown that collagen membranes act as bioactive compartments rather than passive barriers, as they are involved in attracting cells into the wound area, which secrete signals for bone regeneration and remodeling, and they promote the expression of chemotactic factors, thus modulating the overall osteogenic process.\textsuperscript{30}

Throughout the SEM images taken of the collagen membrane, a clear pattern of healing could be observed from 7 to 30 days. First, the collagen network increased in size, with individual fibrils being harder to see as the network grew and the porosity reduced. The increase in the amount of collagen could be linked to the recovery and maturation of the wound area, as this occurred in parallel with the progressive increase in the amount of bone formed and maturation of the newly formed bone (histomorphometric data presented in ref. 23). Second, the apparent cell make-up in the network changed over time. Initially, there was a large contribution from erythrocytes, but as the healing progressed, larger numbers of leukocytes, eosinophils and neutrophils were detected at the healing site.

The collagen remodeling was confirmed by the immunodetection of MMP-1 and MMP-8. The remodeling process is necessary for the wound-healing process. Leukocytes, particularly macrophages and neutrophils, are major sources of MMP.\textsuperscript{31,32} Other cells involved in the postacute stages of wound healing can also contribute to MMP release, such as fibroblasts and endothelial cells.\textsuperscript{33} Most MMPs are not constitutively expressed in normal tissues, but inflammatory cytokines, such as IL-1 and TNF-\(\alpha\), or growth factors, such as TGF-\(\beta\), and noxious stimuli are required to initiate the transcription.\textsuperscript{34–36} In this study, the high positivity for MMP-1, MMP-8 and TIMP-1 found at the later healing periods was not associated with a high inflammatory infiltrate, and the IL-1\(\beta\) and TNF-\(\alpha\) markers did not return significant positivity (or a positivity <10%) at all healing times. The lack of (or low) positivity for IL-1\(\beta\) and TNF-\(\alpha\) might be related to sample processing. It is well known that decalcification and processing of samples are antigen masking\textsuperscript{37} and that different antigens may show immunoreactivity differences in relation to time and preservation of the samples,\textsuperscript{38} so it is possible that a weak positivity for inflammatory markers might have been concealed, or at least reduced. Previous studies have shown that osteoblasts and osteocytes express MMPs and TIMP-1 in the course of appositional bone formation,\textsuperscript{39} so our data on the expression of MMPs at 14 and 30 days also suggest a maturing osseous wound.

As there was no control group without bone graft particles, we do not know if the graft influenced collagen barrier degradation in terms of MMP expression. Previously, Elgali et al.\textsuperscript{40} tested the early bone healing events and the cellular activities in response to a combination of GBR membrane and different bone substitute materials. No data on MMP activity were reported, but the study showed that the defects treated with a collagen membrane and the defects treated with a combination of collagen membrane and deproteinized bovine bone had similar expression of osteoclasts and inflammatory markers. Only at 12 hours it was possible to detect a temporary higher expression of RANKL in the defects covered only by the membrane and a higher expression of TNF-\(\alpha\) in the defects in which the graft was also applied. However, at later healing points no significant differences were observed.

To the best of our knowledge, this is the first study that has thoroughly evaluated the degradation of a collagen membrane using qualitative histology, qualitative SEM and AFM evaluations, antigen–antibody characterizations and histomorphometry in an in vivo model that is a close approximation of the clinical scenario in which this membrane is commonly applied and in combination with a particulate bone graft. Previously, Rothamel et al.\textsuperscript{18} studied the degradation of two collagen membranes associated with different bovine bone grafts. The authors only performed qualitative histologic and SEM observations, but their results are in line with ours in terms of low inflammatory reaction, early vascularization and limited cell proliferation.

DBBM has been successfully applied in combination with membranes in numerous human bone regeneration and augmentation studies.\textsuperscript{3,41–44} The use of DBBM does not enhance per se the capacity of the membrane to promote bone formation, but it demonstrates osteoconductive properties and mechanically supports/provides space maintenance to the barrier against its collapse into the defect.\textsuperscript{45,46} In the current project, the marked positivity to osteopontin, bone sialoprotein and osteocalcin around the DBBM particles corroborates the osteoconductivity of this graft.

In clinical practice, successful regeneration is obtained only when cell occlusion and space maintenance exist for the healing time needed by the progenitor cells to repopulate the defect. While in periodontal regeneration a period of 6–8 weeks might be sufficient,\textsuperscript{47} a longer period of up to 6 months has been advocated for bone regeneration.\textsuperscript{48,49} Hence, future studies considering longer healing periods are warranted to test the degradation process of collagen membranes in GBR models.

Nevertheless, good long-term clinical results have been documented when collagen membranes without a prolonged barrier function were combined with autologous bone chips and DBBM for contour augmentation around chemically modified, sandblasted and acid-etched implants.\textsuperscript{50} Therefore, the use of the porcine collagen membrane tested in this study in GBR needs to be reviewed in light of the new implant surfaces and biomaterials adopted and in relation to the clinical application (GBR around implants, for socket preservation, or for the regeneration of atrophic ridges).

In order to increase the stability of collagen membranes and delay their degradation, different approaches have been proposed, including cross-linking the collagen,\textsuperscript{51} applying a double layer of membranes,\textsuperscript{12} using tetracycline impregnation\textsuperscript{52,53} and systemically administering tetracycline.\textsuperscript{54} By modifying surface properties, particle size, porosity and the release of ions, future studies should aim at manufacturing
an ideal immune-mediated collagen membrane that promotes anti-inflammatory M2 macrophages and the secretion of regenerative cytokines, whilst at the same time prevents the migration of undesired soft tissues into the osseous wound.55

The combined approach proposed in the current project to assess membrane degradation (quantitative and qualitative histology, immunofluorescence, SEM/AFM) allowed a thorough in vivo characterization of collagen degradation and might be successfully used in the future to test the behavior of other barrier membranes (eg cross-linked collagen membranes). Longer healing times are advised in future studies, in consideration of the long-lasting barrier effect advocated for GBR.

Finally, this study investigated barrier degradation during an uneventful healing process. However, in clinical practice it is not uncommon to observe membrane exposure,56 especially during the early healing stages. As the membrane becomes exposed to the oral environment, bacterial enzymatic products and saliva enzymes will trigger faster degradation of the biomaterial, with the possibility that bacterial colonization of the membrane may compromise the final regeneration outcome.57–61 In the future, it would be interesting to apply the methodologies developed in this study to investigate also how the exposure to the oral environment might influence membrane properties and degradation.

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ORCID

E. Calciolari http://orcid.org/0000-0001-8781-1997
N. Donos http://orcid.org/0000-0002-4117-9073

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