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Systematic Review

Twenty years of enamel matrix derivative: the past, the present and the future

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Abstract

Bacground: On June 5th, 2015 at Europerio 8, a group of leading experts were gathered to discuss what has now been 20 years of documented evidence supporting the clinical use of enamel matrix derivative (EMD). Original experiments led by Lars Hammarström demonstrated that enamel matrix proteins could serve as key regenerative proteins capable of promoting periodontal regeneration including new cementum, with functionally oriented inserting new periodontal ligament fibres, and new alveolar bone formation. This pioneering work and vision by Lars Hammarström has paved the way to an enormous amount of publications related to its biological basis and clinical use. Twenty years later, it is clear that all these studies have greatly contributed to our understanding of how biologics can act as mediators for periodontal regeneration and have provided additional clinical means to support tissue regeneration of the periodontium.

Aims: This review article aims to: (1) provide the biological background necessary to understand the rational for the use of EMD for periodontal regeneration, (2) present animal and human histological evidence of periodontal regeneration following EMD application, (3) provide clinically relevant indications for the use of EMD and (4) discuss future avenues of research including key early findings leading to the development of Osteogain, a new carrier system for EMD specifically developed with better protein adsorption to bone grafting materials.

Conflict of interest and source of funding statement

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This manuscript is dedicated to Professor Lars Hammarström, to honour his landmark and pioneering work in discovering the regenerative capacities of enamel matrix proteins.

Key words: EMD; Emdogain; enamel matrix derivative; enamel matrix proteins; intrabony defect; Osteogain; periodontal regeneration

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Over 20 years ago, a team of researchers in Sweden including Lars Hammarström, Sven Lindskog and Leif Blomloff found that enamel matrix proteins (EMPs) could be utilized as a biological agent capable of periodontal regeneration (Hammarström et al. 1991, 1992, 1995). These reports originated from previous studies 15 years earlier by Lindskog et al. and Slavkin et al. reported that certain EMPs (which until then were considered enamel-specific proteins) were deposited on the surface of developing tooth roots prior to cementum formation (Fig. 1) and may play a possible role in cementogenesis (Lindskog 1981a, b, Lindskog & Hammarström 1981, Slavkin et al. 1989). These observations led to the hypothesis that EMPs may play an integral role in the future differentiation of periodontal tissues prior to cementum formation, and has been the basis of a number of biological and clinical studies thereafter demonstrating that EMPs are proteins secreted by Hertwig's epithelial root sheet capable of promoting periodontal regeneration (Gestrelius et al. 1997a,b, Hammarström et al. 1997, Heijl 1997, Zetterstrom et al. 1997). The purified fraction derived from the enamel layer of developing porcine teeth was given the working name enamel matrix derivative (EMD) and has been the basis of numerous publications investigating its future use in periodontal regeneration.

The major components of EMD are amelogenins, a family of hydrophobic proteins that account for more than 90% of the total protein content derived from different splice variants and post-secretory regulation, all controlled from the expression of a single gene (Lyngstadaas et al. 2009). These proteins self-assemble into supramolecular aggregates that form an insoluble extracellular matrix and function to control the ultrastructural organization of the developing enamel crystallites (Lyngstadaas et al. 2009). Other proteins found in the enamel matrix include enamelin, ameloblastin (also called amelin or sheathlin), amelotin, apin and various proteinases (Bartlett et al. 2006, Margolis et al. 2006). Although these proteins are expressed in less quantities, further investigation has confirmed their valuable roles in various aspects of periodontal regeneration discussed later in this article.

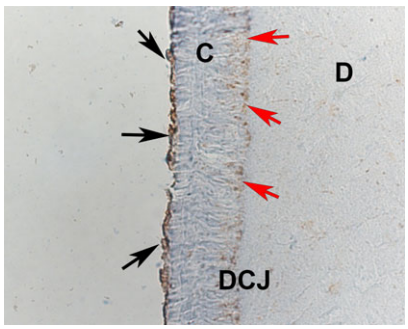

Fig. 1. Histological section depicting enamel matrix proteins localized at the dentinocemental junction (DCJ). Results from the early 1990s demonstrated that enamel matrix proteins (which until then were considered enamel-specific proteins) were found also localized at the DCJ were the hypothesis for numerous subsequent investigations characterizing the role of EMPs in periodontal tissue differentiation (C = cementum, D = dentin, DCJ = dentinocemental junction, black arrow = EMD deposited on the root surface (ex vivo experiment), red arrow = enamel matrix proteins found localized at the DCJ).

The aim of this review article is to provide the reader with four important aspects concerning integral research avenues on EMD over the past 20 years. First, a biological background is provided to fully comprehend the rational for utilizing EMD in periodontal regeneration by summarizing the in vitro research that has characterized the numerous individual roles of EMPs for cells derived from both soft and hard tissues. Thereafter, studies based on animal and human histology analysing periodontal regeneration following application with EMD are discussed. The third aim of this article is to provide the clinician-scientist a summary of the clinical trials utilizing EMD for a number of regenerative procedures, while, at the same time, provide a summary of evidence-based indications for EMD in clinical practice. Lastly, the article will discuss future avenues of research including the five key early studies leading to the development of Osteogain, a new product incorporating EMD with better physicochemical properties for improved protein adsorption of EMPs to bone grafting materials.

Biology of Periodontal Regeneration with Enamel Matrix Proteins

The aim of the first section of this review article is to summarize the

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cell biological data largely but not exclusively originating from numerous in vitro studies, where cells were exposed to EMPs and to link these to their potential beneficial effect on periodontal wound healing and regeneration. For those highly interested in this topic, the authors kindly point the reader to a number of review articles covering the diverse roles of EMPs (Zeichner-David 2001, Bosshardt 2008, Gibson 2008, Lyngstadaas et al. 2009, Miron et al. 2014b). Within the context of this manuscript, the effects of different EMP extracts, recombinant EMPs or EMD are summarized from in vitro studies on various cell types including epithelial cells, gingival fibroblasts, periodontal ligament fibroblasts, cementoblasts, osteoblasts and bacteria. It has been demonstrated that EMD exerts a significant influence on cell behaviour of many cell types by mediating cell attachment, spreading, proliferation, differentiation and survival, as well as expression of transcription factors, growth factors, cytokines, extracellular matrix constituents and other molecules involved in the regulation of bone remodelling (Bosshardt 2008). Furthermore, EMD has been shown to play a significant role in wound healing favouring soft tissue regeneration and angiogenic activity (Fig. 2) (Miron et al. 2014b). Due to the overall size of the current manuscript, the in vitro section has been added as a Supporting Information to the current article.

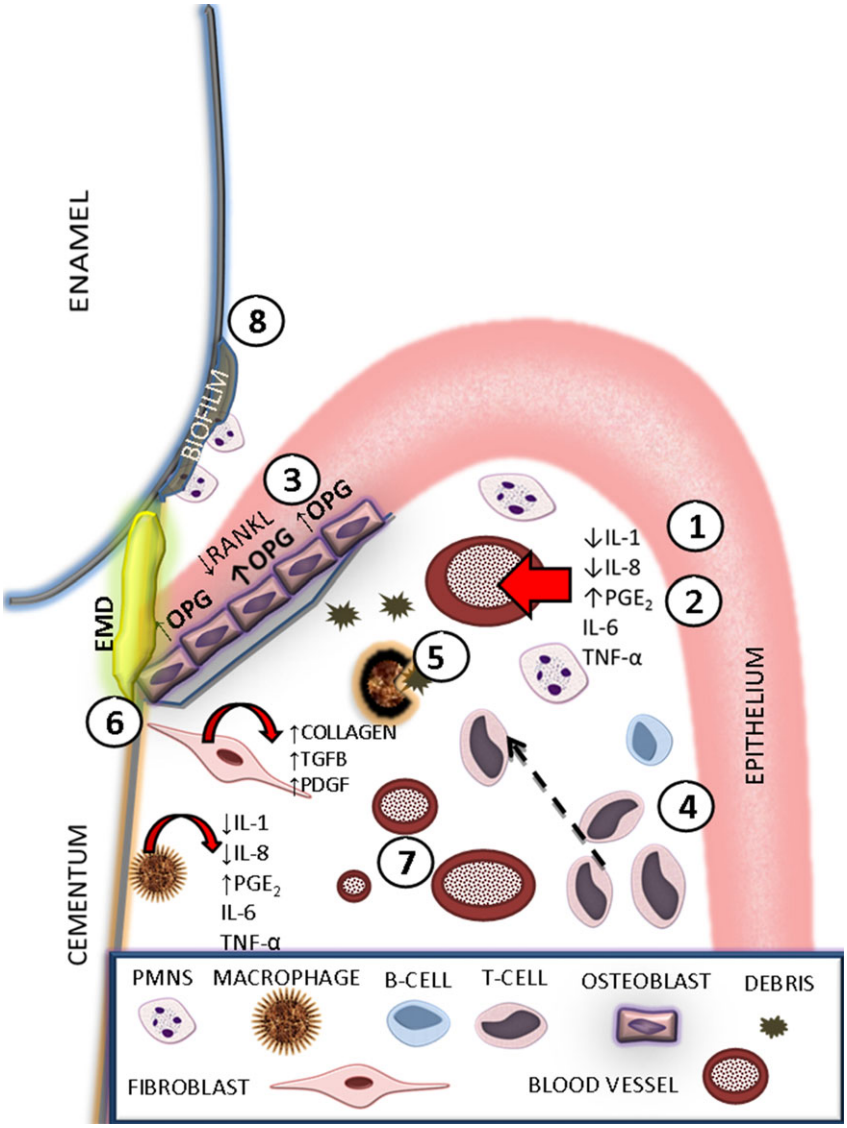

Fig. 2. Diagram depicting inflammation-modifying changes induced by enamel matrix derivative. Following application of EMD, decreased production of IL1β and IL8 (1) and increased levels of PGE_2 (2) are observed with little differences in TNF-alpha expression. EMD also substantially changes the OPG/RANKL balance by increasing OPG and decreasing RANKL levels, resulting in diminished osteoclast formation/activity (3). EMD also increases the proliferation and migration of T-lymphocytes (4), which enable tissue debridement by macrophages (5). Furthermore, EMD promotes mesenchymal cell differentiation into hard tissue-forming cells and also improves PDL cell regeneration (6). Microvascular cell differentiation and angiogenesis are improved following EMD application (7) and studies demonstrate that EMD also lowers bacterial numbers (8), resulting in a reduced inflammatory state (reprint from the Journal of Periodontal Research with permission).

Animal and Human Histological Evidence of Periodontal Regeneration with the Use of EMD

Approximately two decades ago, the first animal model investigating EMD as an adjunctive agent to periodontal surgery, involved surgically created recession defects treated with either coronally advanced flap (CAF) alone or in combination with EMD (Hammarström et al. 1997). Following an 8-week healing period, the histological evaluation revealed formation of acellular cementum, periodontal ligament and alveolar bone in all defects treated with EMD. In control samples (CAF alone), defects presented a long junctional epithelium onto the exposed root surface, and only very limited periodontal regeneration was observed. Following these original findings, subsequent animal experiments have evaluated the healing of different types of induced periodontal defects treated with EMD or guided tissue regeneration (GTR) (i.e. fenestrations, dehiscence-type, recessions, intrabony and furcation defects) (Sculean et al. 2000b,c, Cochran et al. 2003, Donos et al. 2003, Sallum et al. 2003, 2004, Regazzini et al. 2004, Sakallioglu et al. 2004, Nemcovsky et al. 2006, Ivanovic et al. 2014). It was reported in these studies that application of EMD resulted in substantially larger amounts new cementum, periodontal

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ligament and bone formation when compared to controls (i.e. flap surgery alone). Moreover, these studies revealed that the amount and quality of the newly formed periodontal tissues were comparable between EMD and GTR.

Human histological studies were subsequently performed to provide further evidence for periodontal regeneration in intrabony and recession defects, thus corroborating the findings from animals (Heijl 1997, Mellonig 1999, Sculean et al. 1999, 2000a, Rasperini et al. 2000, Yukna & Mellonig 2000, Carnio et al. 2002, McGuire & Cochran 2003, Majzoub et al. 2005). Yukna and Mellonig evaluated histologically 10 intrabony defects around teeth with advanced adult periodontitis that were treatment planned for extraction (Yukna & Mellonig 2000). Following treatment with EMD, biweekly to monthly recalls were made until removal of small block section biopsies at about 6 months showed evidence of periodontal regeneration (new cementum, new bone, and new periodontal ligament) in three specimens, new attachment (connective tissue attachment/adhesion only) in three specimens and a long junctional epithelium in four specimens. No evidence of root resorption, ankylosis or untoward inflammation was found (Yukna & Mellonig 2000). In another human histological study of 14 periodontitis patients, each of them contributing with one deep intrabony defect around teeth scheduled for extraction were treated with either EMD or a synthetic bioabsorbable membrane (Sculean et al. 1999). The results revealed that at 6 months following reconstructive surgery using either EMD or GTR, substantial clinical improvements (i.e. reduction of PD and gain of CAL) occurred. In both groups, the clinical improvements were characterized histologically by a new connective tissue attachment, and to a varying extent, new bone (Sculean et al. 1999). In the cases treated with EMD, the new connective tissue attachment was followed by substantial bone re-growth in only two cases while in four specimens, bone regeneration was either minimal (0.5 mm) and confined to the apical part of the defects or the reformed connective tissue attachment was not accompanied by any signs of bone regeneration. On the other hand, in all cases treated with GTR, the new connective tissue attachment was followed by bone formation (Sculean et al. 1999). The substantial amount of newly formed bone following GTR application was explained by the lack of membrane exposure and subsequent bacterial caused infection, well-known factors that influence the healing process (Tonetti et al. 1996, Sanz et al. 2004). In summary, these results demonstrate that it is possible to achieve periodontal regeneration, but this does not occur in all cases; other factors are also important such as wound integrity, infection, patient age and systemic conditions (Tonetti et al. 1996, Sanz et al. 2004, Jepsen et al. 2008).

Since EMD is applied in a gel formulation, a relevant question has been whether the EMD proteins would remain adsorbed to the root surface following regenerative surgery, or whether they would leak out from the site after flap closure. By using an anti-EMD antibody, it has been demonstrated in human tooth biopsies that EMD remains on the root surface for up to 4 weeks (Sculean et al. 2002c, 2003b,c). Furthermore, it was noted that after a period of only 2 to 6 weeks following application of EMD, newly formed periodontal tissues were seen deposited on the treated root surfaces that appeared thick, collagenous and devoid of extrinsic fibres (Sculean et al. 2002c, 2003b,c). Histological analysis revealed the presence of an electron-dense, organic material in the collagenous matrix indicating that at least partial mineralization had occurred following application with EMD (Bosshardt et al. 2005, 2006). Taken together, these results have confirmed that EMD application onto debrided root surfaces is capable of inducing a cascade of biological events leading to *de novo* formation of cementum and stimulates matrix deposition on old native cementum. In context, the wound maturation process after EMD application in a periodontal wound may take up to 6 months post-surgery.

Clinical Applications of EMD

The regeneration of lost periodontium remains the ultimate goal in periodontal regenerative therapy. A large number of techniques, including – but not limited to – root surface modification, bone and bone substitute grafting, GTR, biological mediators, and combination thereof have been employed to fulfil true periodontal regeneration. For each of the above-mentioned techniques, limitations and complications have been associated with their use, and it may thus not be surprising that the search for the ideal biomaterial capable of true periodontal regeneration continues. Over the years, the use of biologics (growth factors) has become more prominent in daily practice. A plethora of documented research from in vitro, in vivo and clinical trials is now available for enamel matrix proteins that now spans over two decades. In this section, we briefly summarize 20 years of clinical research and provide an evidenced-based flow chart for relevant clinical indications for the use of EMD either alone or in combination with a bone grafting material or barrier membrane.

Safety of EMD

We start by describing the accumulated evidence for EMD used in a clinical setting regarding patient safety. It is important to note that amelogenins are a highly conserved gene across a variety of species including porcine and human. For these reasons, incompatibility or allergic reactions after treatment with EMD have not been reported in any clinical trial that were the direct result of EMD (Zetterstrom et al. 1997, Petinaki et al. 1998, Nikolopoulos et al. 2002, Froum et al. 2004). Following a multicentre study evaluating the potential for sensitization following two applications of EMD, 376 patients in 11 university-based programmes and five private practices were treated with open flap debridement, root conditioning and application of EMD. No complications were reported resulting from the application of EMD. The results from this study further showed that treatment of intrabony defects with EMD resulted in a significant reduction in probing depths (PDs) and gain in clinical attachment level (CAL) (Froum et al. 2004). Following these preliminary human studies, the use

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of EMD has now been utilized for the treatment of a variety of defects in over 60 randomized clinical trials and over 1 million patients worldwide. No patient allergic reaction or adverse event has been reported over this 20 year period.

Effects on early wound healing

As mentioned previously, certain studies have attempted to characterize the early wound healing capabilities of EMD in a clinical setting (Wennström & Lindhe 2002, Hagenaars et al. 2004, Tonetti et al. 2004). In a double-masked, split-mouth, placebo-controlled, randomized study, 28 patients with moderately advanced chronic periodontitis received scaling and root planning, followed by application of EDTA and treatment with EMD *versus* a PGA carrier (Wennström & Lindhe 2002). After periods of 1, 2 and 3 weeks, all sites were re-examined including a visual analogue scale to score the degree of post-treatment discomfort. The results demonstrate that topically applied EMD had a positive effect on the early periodontal soft tissue wounds as determined by the proportion of patients reporting a VAS score $\leq$20. Tonetti et al. (2004) also evaluated the healing, post-operative morbidity and patient perception of outcomes following regenerative therapy of deep intrabony defects. In this study, papilla preservation flaps were used to obtain access and primary closure. After debridement and root conditioning, EMD was applied in the test subjects and omitted in the controls. Healing was monitored 1, 2, 3, 4, 6 and 12 weeks after surgery. During the first 12 weeks of healing, supracrestal soft tissue density was evaluated with a computer-assisted densitometric image analysis system using underexposed radiographs taken on 34 patients. Patient perceptions were also evaluated with a questionnaire immediately after the procedure, at suture removal 1 week later and at 1 year (Tonetti et al. 2004). It was found that up to 6 weeks post-operatively, soft tissue densities were significantly higher in subjects treated with EMD with respect to controls. One year after completion of the surgery, patients reported high levels of satisfaction with the outcomes. These findings indicate earlier gains in soft-tissue density following application of EMD (Tonetti et al. 2004). A third study on the healing of soft tissue wounds following periodontal surgery failed to demonstrate a statistically significant positive effect following treatment with EMD (Hagenaars et al. 2004).

A recent study in the rat model has shown that EMD improves oral mucosa incisional wound healing by promoting formation of blood vessels and collagen fibres in the connective tissue. EMD treatment increased significantly the number of blood vessels and the collagen content. EMD also enhanced (by 20–40%) the expression of transforming growth factor (TGF) β1 and TGFβ2, vascular endothelial growth factor (vEGF), interleukin-1β (IL-1β), matrix metalloproteinase-1 (MMP-1), versican and fibronectin (Maymon-Gil et al. 2016). Therefore, it remains difficult to draw conclusions on the wound healing properties from clinical studies performed in dentistry on soft tissue healing as most of the common parameters used in dentistry involve hard tissue healing. However, the available literature on Xelma® (EMD formulation for the treatment of hard-to-heal wounds as previously described) along with in vitro studies strongly suggests that EMD may additionally improve soft tissue wound healing, although this may be difficult to be evaluated quantitatively in a clinical setting (Vowden et al. 2006, 2007a,b, Hampton et al. 2007, Huldt-Nystrom et al. 2008, Romanelli 2008, Romanelli et al. 2008, Bond et al. 2009, Chadwick & Acton 2009).

Clinical outcomes following non-surgical periodontal therapy

To date, only two randomized, placebo-controlled clinical studies have evaluated the effects of EMD as adjunct to non-surgical periodontal therapy (SRP) (Gutierrez et al. 2003, Mombelli et al. 2005). In both studies, EMD failed to show any beneficial effect. Therefore, it is recommended that EMD is combined with surgical periodontal therapy and a treatment guideline will be later provided highlighting the clinical indications supporting regenerative periodontal therapy with enamel matrix proteins.

Clinical outcomes in intrabony defects using EMD alone

Heijl et al. published the first multicenter, randomized, placebo-controlled study evaluating the effectiveness of EMD for the treatment of intrabony defects. In that study, contra-laterally located intrabony defects were treated with either open flap debridement (OFD) alone or with additional application of EMD (Heijl et al. 1997). Following 36 months of healing, the results demonstrated that EMD significantly improved CAL gains and pocket depths. It was also concluded from radiographic analysis that a progressive bone gain following application with EMD amounted to 2.6 mm (66% fill) at the end of the evaluation period when compared to control defects, which showed no significant bone gain (Heijl et al. 1997). A subsequent controlled clinical study further showed that OFD in combination with EMD led to a three times greater defect fill when compared to OFD alone (Froum et al. 2001). Furthermore, additional benefits following regenerative procedures demonstrated that EMD led to significantly higher soft tissue density in three clinical studies (Trombelli et al. 2002, Yilmaz et al. 2003, Jentsch & Purschwitz 2008). Tonetti et al. investigated the use of EMD in regenerative therapy of deep intrabony defects in 172 patients with advanced chronic periodontitis in 12 centres (Tonetti et al. 2002). All patients had at least one intrabony defect of > or =3 mm. The surgical procedures included access for root instrumentation using either the simplified or the modified papilla preservation flap in order to obtain optimal tissue adaptation and primary closure. After debridement, roots were conditioned for 2 min with a gel containing 24% EDTA followed by application of EMD in the test subjects, whereas omitted in the controls. On average, the test defects gained 3.1 ± 1.5 mm of CAL, while the control defects yielded a significantly lower CAL gain of 2.5 ± 1.5 mm (Tonetti et al. 2002). Pocket reduction was also

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significantly higher in the EMD group (3.9 ± 1.7 mm) when compared to the controls (3.3 ± 1.7 mm). The results of this trial indicated that regenerative periodontal surgery with EMD offers an additional benefit in terms of CAL gains, PPD reductions and predictability of outcomes with respect to papilla preservation flaps alone (Tonetti et al. 2002). On the other hand, one randomized, double-masked, placebo-controlled clinical trial failed to demonstrate any advantage for treatment of EMD when compared to placebo for the treatment of intrabony defects (Rosing et al. 2005). In 2009, Esposito et al. demonstrated in a Cochrane database systematic review that the use of EMD alone after 1 year significantly improved probing attachment levels (1.1 mm) and PPD reduction (0.9 mm) when compared to a placebo or control (Esposito et al. 2009). However, the high degree of heterogeneity observed among trials suggests that results should be interpreted with caution (Esposito et al. 2009).

Clinical outcomes in intrabony defects using EMD or GTR

Another series of experiments focused primarily on comparing the use of EMD to GTR using either non-resorbable or bioabsorbable membranes (Pontoriero et al. 1999). The results from these studies demonstrated that the use of EMD or GTR led to significantly comparable results and that both treatments led to substantially higher CAL gains and defect fill when compared to OFD alone for the treatment of single intrabony defects (Heijl et al. 1997, Pontoriero et al. 1999, Okuda et al. 2000, Silvestri et al. 2000, Froum et al. 2001, Sculean et al. 2001b, Tonetti et al. 2002, Zucchelli et al. 2002). Furthermore, the use of EMD in combination with antibiotics or root conditioning agents was investigated. It was found that the use of EMD in combination with postoperative administration of an antibiotic regimen (i.e. amoxicillin and metronidazole (Sculean et al. 2001a,b) or doxycycline (Eickholz et al. 2014)), a selective cyclooxygenase-2 inhibitor, or EDTA root conditioning did not additionally enhance periodontal regeneration (Sculean et al. 2001a, 2003a, 2006, Parashis et al. 2006, Eickholz et al. 2014).

Interestingly, a new series of studies have now reported that the effects of EMD may be maximized when minimally invasive surgical techniques (MIST) are applied, thus improving initial wound stability while minimizing patient morbidity (Cortellini & Tonetti 2007, Cortellini et al. 2008, Harrel et al. 2010). Although these authors show that MIST alone provides similar results to MIST plus EMD, these concepts have been the basis of more focused research in recent years and future investigation aims to predictably restore lost periodontal tissues via minimally invasive surgeries as discussed later in this article. Although promising, further evaluation in large-scale, multicentre-controlled clinical trials are still necessary.

In summary, the results comparing EMD and GTR did not show significantly different results in the majority of reports concerning treatment of single intrabony defects (Pontoriero et al. 1999, Okuda et al. 2000, Silvestri et al. 2000, 2003, Sculean et al. 2001b, Zucchelli et al. 2002, Esposito et al. 2009). Reports from a prospective multicenter, randomized, controlled clinical trial has shown that treatment with GTR using a bioabsorbable membrane typically demonstrated surgical complications, mostly membrane exposure, whereas those treated with EMD displayed fewer complications (Sanz et al. 2004). These data indicate that although the use of EMD is generally characterized by improved periodontal regeneration with or without membrane use, the findings from a number of clinical studies have demonstrated that anatomical factors such as defect configuration seem to play an important role in EMD-induced periodontal regeneration. This concept is further discussed within the subsection on clinical indications for EMD.

Clinical outcome in intrabony defects using combinations of EMD with barrier membranes or grafting materials

Although numerous clinical studies have provided evidence for substantial clinical and radiographic improvements following application of EMD alone (Fig. 3), concerns regarding the viscous nature of EMD which may not be sufficient to prevent a flap collapse and maintain space for periodontal regeneration have been raised (Polimeni et al. 2004, Siciliano et al. 2011). In order to overcome this potential limitation and improve clinical results, various combinations of EMD with barrier membranes and/or grafting materials have been tested (Trombelli & Farina 2008).

Over 15 years ago, original studies had assessed the treatment of single intrabony defects following treatment with EMD, GTR or a combination of both (Sculean et al. 2001b). Although the results demonstrated that all three regenerative procedures resulted in a significantly higher improvement of the clinical parameters compared to the conventional flap surgery, no additional benefit could be observed for the combined treatment of EMD + GTR. Comparable results were also reported by other groups, thus indicating that, for treatment of single self-contained intrabony defects, the additional use of a barrier membrane in combination with EMD alone led to no additional improvements when compared to EMD alone, or to GTR alone (Minabe et al. 2002, Sipos et al. 2005).

For these reasons, more research was then performed combining EMD with a bone grafting material. A detailed review article is referenced for this combination evaluating all in vitro, in vivo and randomized clinical trials (Miron et al. 2014c). Within the context of this article, a brief overview from clinical trials investigating the use of EMD plus a bone grafting material is discussed.

A recent systematic review and meta-analysis on 12 studies reporting on 434 patients found that the combination of bone grafting material + EMD led to statistically significant better outcomes (Matarasso et al. 2015). In that study, the mean CAL gain amounted to 3.76 ± 1.07 mm following treatment with EMD + bone graft and to 3.32 ± 1.04 mm following treatment with EMD alone. Mean PD reduction measured 4.22 ± 1.20 mm at

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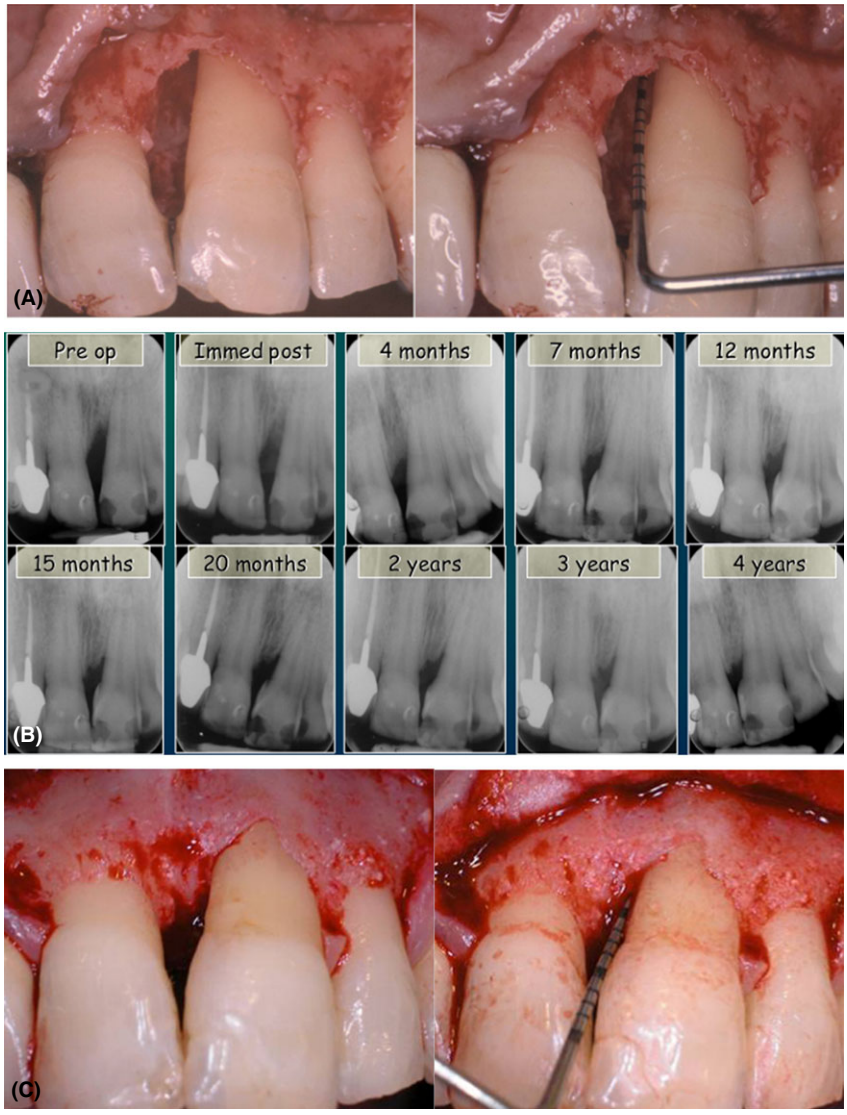

Fig. 3. (A) Pre-operative clinical images depicting a deep intrabony defect. (B) X-rays demonstrating excellent defect fill following treatment with EMD combined with a bone graft. (C) Four year outcome following intrabony defect regeneration with EMD.

sites treated with EMD and bone graft and yielded 4.12 ± 1.07 at sites treated with EMD alone. Interesting to note however is that while the combination of some bone grafting materials with EMD seems to favour periodontal regeneration, many other studies show no additional benefit when compared to bone grafting material alone or to EMD alone (Lekovic et al. 2000, Velasquez-Plata et al. 2002, Zucchelli et al. 2003, Gurinsky et al. 2004, Kuru et al. 2006, Guida et al. 2007, Trombelli & Farina 2008).

To date, only two clinical studies have reported the combination of EMD with autogenous bone (AB) (Guida et al. 2007, Yilmaz et al. 2010). In a parallel study of 28 intra-osseous lesions, the combination of EMD with AB did not offer a statistically significant advantage when compared to EMD alone (Guida et al. 2007). In a second study evaluating two- and three-wall intrabony defects, the effects of EMD with AB led to statistically significant differences (Yilmaz et al. 2010) (Table 1). Similarly, the combination of EMD with bone allografts has been investigated in five clinical studies (Gurinsky et al. 2004, Hoidal et al. 2008, Aspriello et al. 2011, Jaiswal & Deo 2013, Ogihara & Tarnow 2014). In general, the effects of EMD demonstrated a significant advantage in three clinical studies when compared to either demineralized freeze-dried bone allograft (DFDBA) alone or EMD alone, however, no differences in mean PD or mean CAL changes were observed in two other studies (Table 1).

The combination of EMD with a natural bone mineral (NBM, also known as bovine-derived xenograft (BDX), deproteinized bovine bone mineral (DBBM) or Bio-Oss®) has been investigated in five clinical studies (Table 1). In one study evaluating intrabony defects > 6 mm, the combination of EMD with NBM led to statistically improved mean PD and CAL changes after 6 months of healing (Lekovic et al. 2000). In the remaining four studies, variability was observed between treatment with EMD + NBM when compared to either EMD alone or NBM alone in the reported studies (Scheyer et al. 2002, Sculean et al. 2002b, Velasquez-Plata et al. 2002, Zucchelli et al. 2003). Zuchelli et al. found that after a 12 month healing period, the combination of EMD + DBBM led to significantly higher CAL gain (Table 1). In a recent study by Farina et al., 24 periodontal intra-osseous defects were accessed with a buccal single flap approach (SFA) and treated with enamel matrix derivative (EMD) or EMD + NBM according to the operator's discretion (Farina et al. 2014). Both EMD with or without NBM were clinically effective in the treatment of periodontal intra-osseous defects accessed with a buccal SFA. The adjunctive use of NBM in predominantly one-wall defects seemed to compensate, at least in part, the unfavourable osseous characteristics on the outcomes of the procedure (Farina et al. 2014).

The combination of EMD with synthetic bone grafting materials has for the most part demonstrated no advantage for the combination approach (Table 1) (Sculean et al. 2002a, 2005, Kuru et al. 2006, Jepsen et al. 2008, Meyle et al. 2011, De Leonardis & Paolantonio 2013, Peres et al. 2013). In general, Kuru et al. and De Leonardis et al. have demonstrated a significant advantage in mean PD and mean CAL gains

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Table 1. List of randomized clinical trials comparing the combination of EMD + bone grafting material to either EMD alone or bone grafting material alone.

Author and Year	Study design and Patient number	Defects	Healing period	Treatment groups	Mean PD change (mm)	*P* value	Mean CAL change (mm)	*P* value
Guida, 2007	Parallel : 27 patients	28 intra-osseous lesions ≥ 4 mm	6–12 months	EMD	5.1–5.6	n.s.	4.4–4.6	n.s.
				EMD + AB	4.6–5.1		4.4–4.9	
Yilmaz, 2010	Parallel : 40 patients	40 intrabony defects ≥ 3 mm	12 months	EMD	4.6	<0.01	3.4	<0.01
				EMD + AB	5.6		4.2	
Gurinsky, 2004	Parallel : 40 patients	67 intrabony defects ≥ 3 mm	6 months	EMD	4.0	n.s.	3.2	n.s.
				EMD + DFDBA	3.6		3.0	
Hoidal, 2008	Parallel : 32 patients	41 intrabony defects ≥ 3 mm	6 months	DFDBA	2.45	n.s.	1.63	n.s.
				DFDBA + EMD	2.56		1.47	
Aspriello, 2011	Parallel : 56 patients	56 intra-osseous defects	12 months	DFDBA	4.0	<0.05	3.25	<0.05
				DFDBA + EMD	5.0		4.0	
Jaiswal, 2013	Parallel : 30 patients	30 class II furcation defects	12 months	DFDBA + GTR	0.81	<0.05	0.85	<0.01
				DFDBA + GTR + EMD	1.74		2.12	
Ogihara, 2014	Parallel: 69 patients	69 intrabony defects	12–36 months	EMD	3.26–3.09	<0.05	3.04–3.00	<0.05
				EMD + DFDBA	3.7–3.74		3.52–3.61	
				EMD + FDBA	4.38–4.43		4.14–4.19	
Lekovic, 2000	Split mouth : 21 patients	42 intrabony defects	6 months	EMD	1.91	<0.05	1.72	<0.05
				EMD + NBM	3.43		3.13	
Velasquez-Plata, 2002	Split mouth : 16 patients	32 intrabony defects ≥ 3 mm	6–8 months	EMD	3.8	n.s.	2.9	n.s.
				EMD + NBM	4.0		3.4	
Sculean, 2002	Parallel : 24 patients	24 intrabony defects ≥ 4 mm	12 months	BDX	6.5	n.s.	4.9	n.s.
				BDX + EMD	5.7		4.7	
Scheyer, 2002	Split mouth : 17 patients	34 intrabony defects ≥ 3 mm	6 months	BDX	3.9	n.s.	3.7	n.s.
				BDX + EMD	4.2		3.8	
Zucchelli, 2003	Parallel : 60 patients	60 intrabony defects > 3 mm	12 months	EMD	5.8	n.s.	5.8	<0.01
				EMD + NBM	6.2		4.9	
Sculean, 2002	Parallel : 28 patients	28 intrabony defects ≥ 3 mm	12 months	BG	4.22	n.s.	3.07	n.s.
				EMD + BG	4.15		3.22	
Sculean, 2005	Parallel : 30 patients	30 intrabony defects ≥ 3 mm	12 months	EMD	4.5	n.s.	3.9	n.s.
				EMD + BG	4.2		3.2	
Kuru, 2006	Parallel : 23 patients	23 intrabony defects ≥ 4 mm	8 months	EMD	5.03	<0.05	4.06	<0.05
				EMD + BG	5.73		5.17	
Bokan, 2006	Parallel : 56 patients	56 intrabony defects ≥ 3 mm	12 months	EMD	3.9	n.s.	3.7	n.s.
				EMD + β-TCP	4.1		4.0	
Jepsen, 2008	Parallel : 73 patients	73 intrabony defects ≥ 4 mm	6 months	EMD	2.55	n.s.	1.83	n.s.
				EMD + BCP	1.93		1.31	
Meyle, 2011	Parallel : 73 patients	73 intrabony defect ≥4 mm	12 months	EMD	2.9	n.s.	1.9	n.s.
				EMD + BC	2.8		1.7	
De Leonardis, 2013	Parallel : 34 patients	34 intrabony defects ≥3 mm	12–24 months	EMD	3.51–3.76	<0.001	2.73–2.95	<0.001
				EMD + HA/β-TCP	4.00–4.25		3.47–3.63	

for the combination approach (Kuru et al. 2006, De Leonardis & Paolantonio 2013), however, the remaining five studies have shown no clear advantage (Sculean et al. 2002a, 2005, Jepsen et al. 2008, Meyle et al. 2011, Peres et al. 2013). Longer term follow-ups have confirmed the lack of an added benefit of a synthetic bone substitute (Pietruska et al. 2012, Hoffmann et al. 2015). Therefore, the results from a number of clinical trials demonstrate a large variability between both the bone grafting groups utilized (autograft *versus* allograft *versus* xenograft *versus* alloplast) as well as within individual groups (Table 1). Possible reasons for this variability will be discussed later in the future perspectives section of this review article. More recently, it has been demonstrated that the combination of a graft biomaterial in association with biological agents, including EMD, may reduce the post-surgery recession following treatment of deep intra-osseous defects accessed with the single flap approach (Farina et al. 2015).

Clinical outcomes in recession defects using EMD alone or as adjunct to soft tissue grafting

The use of EMD has been investigated in several controlled clinical studies for the treatment of buccal Miller class I and II gingival recessions by means of coronally advanced flap (CAF). In the majority of cases, the additional use of EMD led to more formation of keratinized tissue and long-term stability of the results compared to CAF alone (Hagewald et al. 2002, Cueva et al. 2004, Spahr et al. 2005, Castellanos et al. 2006, Pilloni et al. 2006, Cairo et al. 2008, 2014) (Fig. 4). One randomized controlled clinical study comparing treatment of Miller class I and II recessions demonstrated that after a healing period of 2 years, complete root coverage could be maintained in 53% in patients treated with EMD *versus* 23% in the control group (Spahr et al. 2005). Comparable results were reported from various other groups for the treatment of either Miller class I or class 2 recession defects with topical application of EMD leading to better results (Cueva et al. 2004, Castellanos et al. 2006, Pilloni et al. 2006, Cairo et al. 2008). Another study has compared the use of EMD to a connective tissue graft (CTG) for the treatment of buccal Miller class I and II recessions with CAF (McGuire & Nunn 2003). The results from that study demonstrated very similar results after 1 year for mean root coverage. A recent consensus conference concluded that at single recessions, the addition of autologous CTG or EMD under CAF improves complete root coverage and may be considered the procedure of choice at maxillary anterior and premolar teeth (Tonetti & Jepsen 2014).

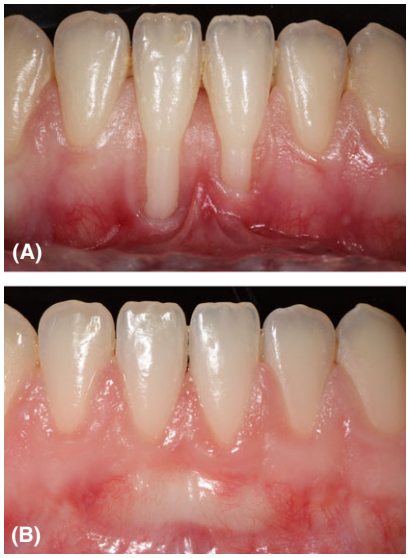

Fig. 4. (A) Baseline photograph illustrating Multiple Miller Class II recessions. (B) Two year outcome following recession coverage with EMD and connective tissue graft.

Histological evaluation of human biopsies in recession defects was then performed to analyse periodontal regeneration (Heijl 1997, McGuire & Cochran 2003). It was found that the application of EMD during conjunction with CAF resulted in enhanced formation of root cementum, periodontal ligament and alveolar bone while treatment with a CAF and a connective graft or CAF alone (McGuire & Cochran 2003) was characterized by a long junctional epithelium and even signs of root resorption. Comparable results were reported in a multicenter, controlled clinical trial (Rasperini et al. 2011). More recently, Roman et al. evaluated whether the combination of EMD with a subepithelial connective tissue graft (SCTG) plus CAF would further improve the treatment outcomes of Miller class I and II gingival recessions in 42 patients (Roman et al. 2013). Both treatments, STCG plus EMD and SCTG alone, resulted in a significantly higher than baseline final mean root coverage (2.91 ± 0.95 mm and 2.91 ± 1.29 mm, respectively) and in a high mean percentage of root coverage ($82.25 \pm 22.20\%$ and $89.75 \pm 17.33\%$, respectively) 1 year after surgery, however, differences between the two techniques were not statistically significant. Cordaro et al. (2012) compared, in a split-mouth design, CAF with or without EMD for coverage of multiple gingival recession defects with follow-up at 6- and 24 months. Clinical measurements (recession length, keratinized tissue, probing depth and clinical attachment level) were assessed at baseline and 6 and 24 months after surgery by a blinded examiner. At the 6-month evaluation, both treatment procedures displayed good results with significant root coverage gain (CAF, $80.7\% \pm 20\%$; CAF + EMD, $82.8\% \pm 14\%$). No significant difference was found between groups (Cordaro et al. 2012).

Thus, the accumulated evidence from these studies suggests that the use of EMD for the treatment of gingival recessions utilized alone is capable of enhancing regeneration and improves soft tissue height/thickness, while the combination with SCTG may further support recession coverage; however, this approach presents great variability in the clinical parameters analysed (Henriques et al. 2010, Rasperini et al. 2011).

Clinical outcomes with EMD in furcation defects

The data on the efficacy of the use of EMD in the regenerative therapy of furcation defects are still limited (Sanz et al. 2015). When investigating the adjunctive use of enamel matrix derivative with open flap debridement in 10 patients with 20 Class II furcation defects on contralateral molars by re-entry after 6 months, a significantly enhanced

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horizontal resolution (reduction of 2 mm in the enamel matrix derivative *versus* 0.8 mm in the open flap debridement group) of the bony defects was found in enamel matrix derivative-treated furcations (Chitsazi et al. 2007). In a multicentre, randomized, controlled, split-mouth, clinical trial of mandibular buccal class II furcation defects, a total of 45 patients with 90 comparable defects on contra-lateral molars were treated with either EMD or GTR (Jepsen et al. 2004, Meyle et al. 2004, Hoffmann et al. 2006). At 8 and 14 months, both treatment modalities led to significant clinical improvements. The EMD group showed significantly better results with regard to the primary outcome reduction in horizontal furcation depth as assessed during a 14 months re-entry procedure. Enamel matrix derivative demonstrated a mean reduction in horizontal probing bone level of 2.6 + /- 1.8 mm, and the guided tissue regeneration-treated sites showed a horizontal probing bone level reduction of 1.9 + /- 1.4 mm. Furthermore, with regard to patient-centred outcomes, post-operative wound healing as assessed by questionnaires on pain and swelling was superior following EMD application.

In proximal class II furcation defects, the use of EMD led to a higher conversion rate into class I when compared to OFD alone although complete furcation closure was only rarely found (Casarin et al. 2010). In another trial on the treatment of proximal class II furcation defects, the effects of OFD + hydroxyapatite (HA)/β-tricalcium phosphate (β-TCP) filling, or OFD + HA/β-TCP + EMD were evaluated (Peres et al. 2013). No significant difference was reported between treatment modalities 6 months after therapy (Peres et al. 2013). In summary, the limited data on the effects of EMD in regenerative furcation therapy is encouraging, however, more evidence from further well-controlled studies is clearly needed. A clinical treatment guideline has been added to Appendix S2 (Fig. 5).

Future Perspectives

Although EMD has been utilized for a variety of clinical applications over the past 20 years, research concerning its clinical use as well as basic research to further understand its properties and biological effects are still ongoing. This section is divided into the following six subsections: (1) future use of EMD in minimally invasive surgeries, (2) use of EMD for the treatment of supra-alveolar-type defects, (3) possible use of EMD for the treatment of peri-implantitis and mucosal recessions around implants, (4) characteristics of various fractions of EMD, (5) development of Osteogain, a new product incorporating EMD with better physicochemical properties for bone grafting material adsorption and (6) final remarks.

Future use of EMD in minimally invasive surgeries

In the great majority of studies, EMD was applied in reconstructive surgery using a conventional flap design prepared by means of intrasulcular incisions. More recently, a few studies have investigated its use in conjunction with minimally invasive surgery. Although minimally invasive surgical techniques cannot be utilized in all cases, clinical outcomes in certain cases appear to be associated with reduced morbidity of the patient

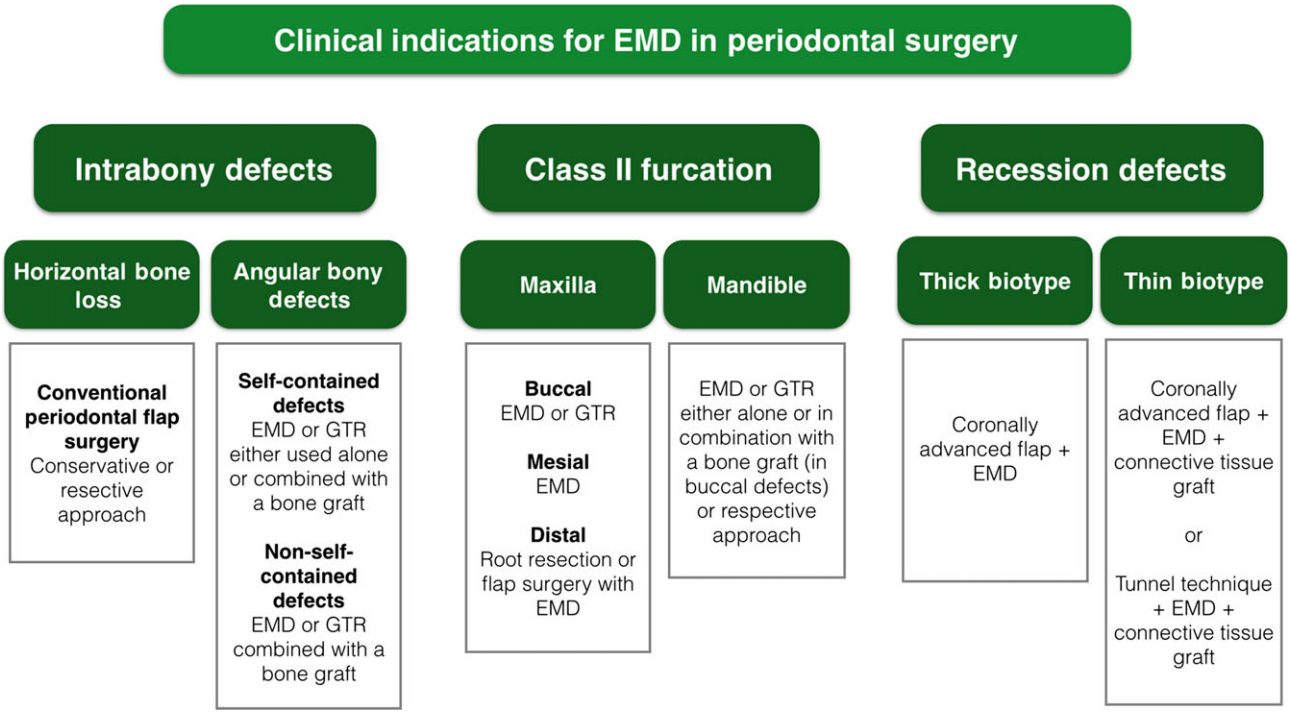

Fig. 5. Flow Chart – clinical indications for the use of EMD in periodontal surgery. Intrabony defect, furcation defect and recession defect regeneration have all demonstrated long-term clinical improvements following treatment with EMD in certain clinical indications.

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post-operatively (Cortellini 2012, Cortellini & Tonetti 2015). Both MIST and modified MIST (M-MIST) techniques (Cortellini 2014) present great opportunities for future research in conjunction with EMD for a variety of clinical situations. Future randomized, multicentre clinical trials are necessary to further investigate the clinical benefit of utilizing EMD during MIST techniques.

Uses of EMD for the treatment of supra-alveolar-type defects with access flap surgery

A very limited amount of research has investigated the use of EMD for the treatment of supra-alveolar-type defects. In a pilot by Jentsch & Purschwitz (2008), 39 subjects were either treated with access flap surgery + EMD *versus* access flap surgery alone. In that study, it was found that significantly higher attachment gain and PD reduction was observed for the test group when compared to controls (Jentsch & Purschwitz 2008). The data suggest a significant clinical benefit for additionally combining access flap surgery with EMD for the treatment of supra-alveolar-type defects, especially in deeper pockets (Jentsch & Purschwitz 2008). Furthermore, in 2013, Di Tullio et al. found similar results by treating 54 patients with either simplified papilla preservation flap technique (SPPF) + EMD *versus* SPPF alone (Di Tullio et al. 2013). After 1 year, the test group showed significantly greater PD reduction, and AL gain compared to the control group (Di Tullio et al. 2013). Thus, in the light of these two studies and the limited clinical data, it may be suggested to combine either access flap surgery or SPPF with EMD to further improve the regenerative outcomes following periodontal therapy for the treatment of supra-alveolar-type defects.

Graziani et al. (2014) investigated in a systematic review and meta-analysis the effects of EMD on additional clinical benefits in residual periodontal pockets associated with suprabony defects. The adjunctive mean benefit of EMD was: 1.2 mm for CAL gain [confidence interval (CI): (0.9, 1.4), $p < 0.00001$, $I(2) = 66\%$], 1.2 mm for the PPD reduction (CI: [0.8, 1.5], $p < 0.0001$, $I(2) = 0\%$), −0.5 mm for the REC increase (CI: [−0.8, −0.2], $p = 0.003$, $I(2) = 0\%$). Although no differences were noted in tooth survival, EMD application resulted in clinical and radiographic additional benefits compared to OFD alone (Graziani et al. 2014). Future research on this topic is however still necessary to fully characterize the additional benefit of EMD for the treatment of such defects.

Possible use of EMD for the treatment of peri-implantitis and mucosal recessions around implants

The wound healing properties of EMD, along with its effect on new bone formation have been the basis for investigating the treatment of peri-implantitis and mucosal recessions around implants. In a report of 51 cases with 3–7.5 year follow-up, Froum et al. demonstrated that implants showing PDs ≥ 6 mm, and bone loss ≥4 mm could be successfully regenerated using a combination approach including surface decontamination, use of EMD, a combination of PDGF with anorganic bovine bone or mineralized freeze-dried bone, and coverage with a collagen membrane or a subepithelial connective tissue graft. The rationale for combining PDGF with EMD was derived from an in vitro study conducted by Chong et al. (2006) showed that the combination of PDGF + EMD led to greater cell

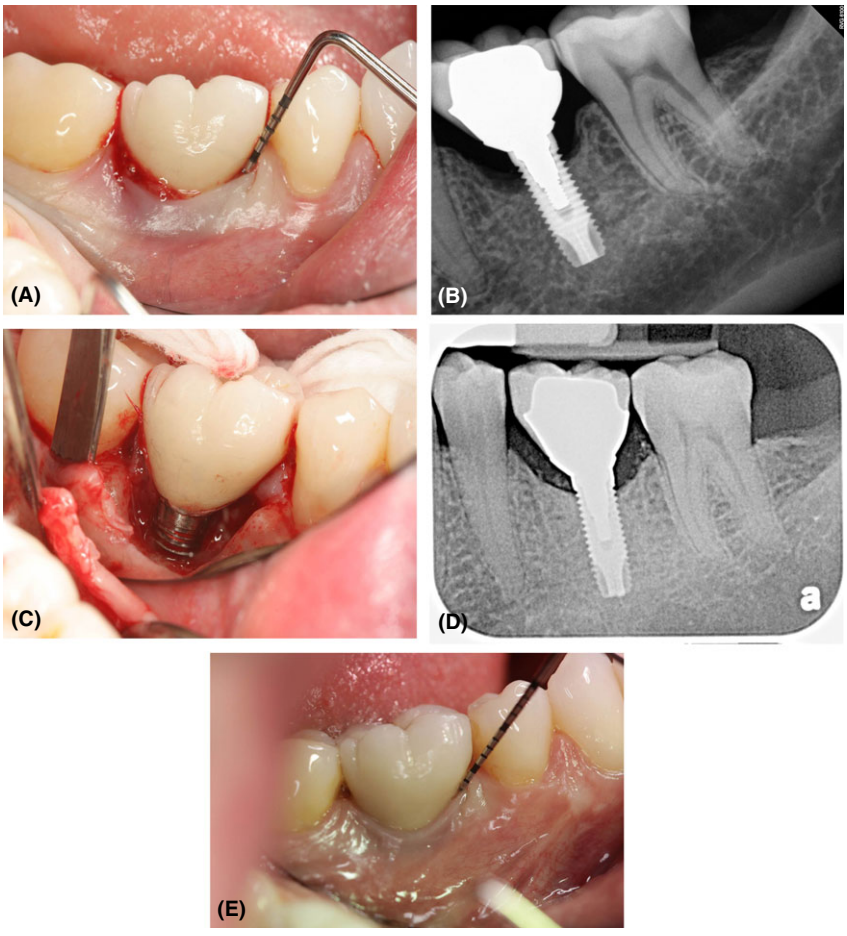

Fig. 6. Treatment of Peri-implantitis utilizing a combination therapy approach with EMD. (A) A 41-year-old, healthy female presented with peri-implantitis around implant #19i characterized by bleeding on probing, 8 mm probing depths and loss of bone. (B) Pre-surgical X-ray indicating bone loss. (C) Flap reflection prior to surface decontamination showing 7 mm of interproximal bone loss on the mesial and distal aspects of the implant, (D) Radiograph 4 years following treatment showing fill of the defect. (E) Clinical photo, 4 year post-op, showing the deepest probing depth of 3 mm with a complete absence of bleeding on probing.

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proliferation and in vitro wound fille rates when compared to either utilized alone. Patients were divided into two groups as follows: (1) the greatest defect depth was visible on radiographs and (2) the greatest loss of bone was on the facial or oral aspect of the implant. The results from that study demonstrated that probing depth reductions were 5.4 and 5.1 mm with bone level gain 3.75 mm and 3.0 mm in groups 1 and 2 respectively (Fig. 6). No implant in either group was lost or demonstrated reduced bone height throughout the duration of the study. Although a variety of control groups were lacking in this study, the regenerative approach utilized by these authors for the treatment of peri-implantitis appears to be encouraging (Froum et al. 2012). Further research is necessary to confirm the beneficial effect of EMD in the treatment of peri-implantitis lesions as it is difficult to assess the single role of each of the individual regenerative approaches utilized by these investigators.

Characteristics of various fractions of EMD

Although a great deal of information has been learned over the years about EMD, the mechanisms by which individual proteins found in EMD are capable of mediating cell and tissue responses remains unclear. The results from numerous investigations now demonstrate that individual fractions and protein components found in EMD are responsible for different cellular and tissue effects of EMD and likely account for the observed clinical results when utilizing EMD therapeutically (Appendix S3).

Development of Osteogain, a new carrier system for EMD

The clinical combination of EMD with a bone grafting material has been one of the most widely used biomaterial combinations utilized for the treatment of intrabony defects. While the majority of studies combining EMD with a membrane do not lead to additional improvements, the use of EMD with a bone grafting material has demonstrated additional clinical advantages (Table 1). While a recent systematic review and meta-analysis found that the combination of bone grafting material + EMD led to statistically significant better outcomes, large variability between studies were also reported (Lekovic et al. 2000, Velasquez-Plata et al. 2002, Zucchelli et al. 2003, Gurinsky et al. 2004, Kuru et al. 2006, Guida et al. 2007, Trombelli & Farina 2008). In vitro results have also indicated variability in gene expression when primary human osteoblasts and PDL cells were cultured on various bone grafting materials in vitro with or without EMD thus raising the concern that protein function, stability or adsorption may be responsible factors in the gel-delivery system currently utilized for EMD (Miron et al. 2012, 2013, 2014a).

Recently, the adsorption of amelogenins to bone grafting materials under various conditions was investigated (Miron et al. 2015a). These results confirm that large variability existed between the adsorption of amelogenins to different bone grafting material. More importantly, it was found that the commercially available EMD-gel (Emdogain) adsorbed significantly less protein when compared to a liquid formulation of EMD. These preliminary findings led to a series of five subsequent studies over the past 3 years during the developmental phases of Osteogain, a new product incorporating EMD with better physicochemical properties specifically designed for combining EMD with bone grafting materials (Miron et al. 2015a,b, 2016, Wen et al. 2015, Zhang et al. 2015). Future work in this area is ongoing.

Final remarks

It remains hard to believe that over 20 years have now passed since enamel matrix derivative was first introduced as a regenerative agent for periodontal tissues. Equally as surprising, it remains one of the only biomaterials still available for clinical use capable of histologically demonstrating true periodontal regeneration with new cementum formation, periodontal ligament and alveolar bone along with inserting Sharpeys fibres spanning the periodontal apparatus. It is clear that over the years, we have learned a great deal regarding the biological roles of specific enamel matrix proteins and future investigation is constantly underway to further characterize their effects on cell and tissue behaviour. It also becomes clinically important to further investigate the use of EMD in both carrier systems described to determine if regenerative outcomes can be even further improved by slight modifications in EMD-carrier systems or through minimally invasive surgeries. During these 20 years, over 900 publications documenting the use of EMD for a variety of in vitro and in vivo studies as well as numerous clinical trials. EMD has remained one of the gold standards for periodontal regeneration using biologics and it remains of interest to discover how the next 20 years of intensive research will further improve EMD clinical outcomes.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Scanning electron micrograph of an osteoblast seeded on a deproteinized bovine bone mineral following coating with EMD.
Appendix S1. Biology of Periodontal Regeneration with Enamel Matrix Proteins.
Appendix S2. Clinical Treatment Guidelines for EMD.
Appendix S3. Characterization of various fractions of EMD.

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Clinical Relevance

Scientific rationale for the study: Provide an extensive summary of the research performed on enamel matrix derivative (EMD) over the past 20 years.
Principal findings: Over the past 20 years, in vitro, in vivo and clinical studies have shown the ability for EMD to improve both soft and hard tissue formation leading to periodontal regeneration.
Practical implications: The results from this review article demonstrate the safety and efficacy of enamel matrix proteins for the regeneration of periodontal defects and provides future research avenues currently being investigated utilizing EMD.

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