Peri-implantitis
Treatment and effects of enamel matrix derivative

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With love to my wonderful family

“The gull sees farthest who flies highest”

Jonathan Livingston Seagull by Richard Bach
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Abstract

Biological complications affecting osseointegrated dental implants are a growing treatment problem in clinical practice. Since the number of implant carriers has increased in recent decades, this is an urgent topic in dentistry. Peri-implantitis, inflammatory degradation of the implant-supporting jawbone, affects approximately 20% of all implant carriers and approximately 10% of all implants.

Implant surfaces are colonised by microbes that may cause an inflammatory process in the soft tissue around the implant. In some sensitive individuals, the inflammatory response leads to disturbed jawbone remodelling, with increased recruitment and activity of bone-resorbing osteoclasts, which could ultimately lead to implant loss. The corresponding degradation of the bone supporting the teeth is denoted as periodontitis. The current view is that factors such as pro-inflammatory cytokines and prostaglandins, produced by leukocytes and cells of mesenchymal origin in the inflamed connective tissue, are responsible for local osteoclast recruitment and activation. Pro-inflammatory factors and tissue degradation products will leak into the exudate in the peri-implant sulci and the gingival pockets around the teeth. Analysis of the exudate could be of use for predicting and monitoring peri-implantitis, as well as identifying new targets for treatment.

The standard treatment for peri-implantitis is surgery in combination with mechanical cleaning of the implant surface and optimisation of oral hygiene, with the goal of achieving infection control and pocket reduction. This treatment has a moderate effect on healing of the peri-implantitis lesion around the dental implant. The use of adjunctive bone grafts, membranes and antimicrobials has thus far not been shown to achieve a more successful outcome. Adjunctive treatment with enamel matrix derivative (EMD) during regenerative periodontal surgery contributes to wound healing and increased tissue support, but the adjunctive effect of EMD during surgical treatment of peri-implantitis remains unknown.

The overall aim of this thesis was to investigate the outcome of a regenerative surgical treatment approach with and without adjunctive EMD treatment from the short- and long-term perspectives and to increase our knowledge of microbial flora and biomarkers in the peri-implant sulci before and after treatment. Furthermore, an additional aim of this work was to investigate whether EMD could directly affect osteoclast formation and activity.

We performed a randomised controlled clinical trial of a surgical intervention for peri-implantitis with and without EMD. In multivariate modelling, an increased
marginal bone level at the implant site 12 months after surgery was significantly associated with EMD, the number of osseous walls in the peri-implant bone defect and a gram-positive/aerobic microbial flora, whereas a reduced bone level was associated with a gram-negative/anaerobic microbial flora and the presence of bleeding and pus, with a cross-validated predictive capacity ($Q^2$) of 36.4%. Similar trends were observed for bone level, pocket depth, plaque, pus and bleeding, but these associations were statistically non-significant in the univariate analysis. Five years after treatment, no significant differences in bone level changes were observed between groups, but fewer implants were lost to follow-up due to reinfections in the EMD-treated group.

We used mass spectrometry to analyse the protein content in peri-implant crevicular fluid (PICF) before and up to 12 months after treatment. The total protein amount and diversity displayed decreasing trends 3, 6 and 12 months after treatment. Multivariate analysis of the PICF protein content revealed two major groups, cluster 2 and cluster 3, of which cluster 2 was associated with an increased risk of implant loss. EMD treatment was associated with cluster 3, which was in turn associated with increased implant survival.

To test whether EMD affects osteoclast formation or bone resorption, we added purified EMD to RANKL-stimulated mouse bone marrow macrophage cultures in plastic dishes and counted the number of osteoclasts. We also cultured the cells on bone slices and measured the secretion of TRAP5b and the release of CTX-1 into the culture medium as biomarkers of osteoclast numbers and bone resorption, respectively, but no effect of EMD was observed.

In conclusion, adjunctive EMD during surgical treatment of peri-implantitis changed the microbial flora to a less pathogenic microbiota, and similar changes in the inflammatory protein profile of PICF were observed; these effects were associated with implant survival. However, the trend toward a positive healing response after EMD treatment was not associated with a significant radiographic bone gain in this study and needs to be further explored. In addition, our finding that EMD did not affect osteoclast formation or bone resorption in vitro indicates that the effect of EMD on bone regeneration, as seen in periodontitis treatment, does not seem to depend on a direct inhibitory effect on osteoclast formation or bone resorption.
Populärvetenskaplig sammanfattning


Syftet med den här avhandlingen var att undersöka resultatet av regenerativ kirurgisk behandling av periimplantit med eller utan EMD, både på kort och på
längre sikt. Samtidigt ville vi söka kunskap om den bakteriella mikrofloran och biomarkörer i det periimplantära exsudatet före och efter behandling. Dessutom var syftet att undersöka om EMD har en direkt inverkan på osteoklaster och bennedbrytning.

Effekten av den kirurgiska behandlingen var kliniskt positiv med en fickförlustning efter behandling, både med och utan EMD. Benförlust var associerat med EMD tillsammans bendefektens form och en bakterieflora som förknippas med tandhälsa. Benförlust var associerad med inflammation och varbildning vid implantatet samt en mer sjukdomsframkallande bakterieflora. Fem år efter behandling var det ingen skillnad i kliniskt resultat med eller utan EMD-behandling, men med EMD-behandling sågs en tendens att färre implantat förlorades till följd av återkommande infektion.

Proteininnehållet i exsudat vid periimplantit, före och efter behandling, identifierades med masspektrometri. Antalet proteiner minskade efter behandling och två dominerande grupper av proteiner framträdde, där grupp 2 var associerad till risk för implantatförlust och grupp 3 till implantatöverlevnad, vilken i sin tur också var kopplad till behandling med EMD.

För att testa om EMD påverkar osteoklastbildning och bennedbrytning, tillsattes EMD i olika koncentrationer till osteoklaster odlade på plastskivor och sedan räknades antalet bildade osteoklaster. Osteoklaster odlades också på benskivor, i närvaro och frånvaro av EMD. I cellodlingsmediet mättes nivåerna av proteinet TRAP5b, en markör för antalet osteoklaster och CTX-1, en markör för bennedbrytning. Analyserna visar att EMD varken påverkar osteoklastbildningen eller bennedbrytningen.

Sammanfattningsvis visar studierna att tillsats av EMD, vid kirurgisk behandling av periimplantit, förändrade mikrofloran till mer hälsobefrämjande bakterier, vilket också avspeglades i en minskad proteinmängd och ändrad proteinprofil i implantatexsudaten. Den förändrade profilen, tycktes vara förknippad med längre implantatöverlevnad och EMD behandling. EMD påverkar varken bildning av osteoklaster eller bennedbrytning, vilket tyder på att den benvävnadsnybildning som ses vid behandling av tänder, förmödligl inte är kopplad till en direkt hämnande effekt på osteoklaster och benresorption.
List of publications

This thesis is based on the following papers, which will be referred to by their roman numerals (I-IV):


IV. Lindquist S., **Isehed C**, Lie A., Lundberg P., 2018. Enamel matrix derivative does not affect osteoclast formation or bone resorption in mouse bone marrow macrophage cultures. Manuscript submitted to journal.
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ALP</td>
<td>Alkaline phosphatase</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BCA</td>
<td>bicinchoninic acid</td>
</tr>
<tr>
<td>BIC</td>
<td>bone to implant contact</td>
</tr>
<tr>
<td>BMM</td>
<td>Bone marrow macrophage</td>
</tr>
<tr>
<td>BMP</td>
<td>bone morphogenetic protein</td>
</tr>
<tr>
<td>BMU</td>
<td>bone multicellular unit/ bone metabolic unit</td>
</tr>
<tr>
<td>BOP</td>
<td>bleeding on probing</td>
</tr>
<tr>
<td>BSP</td>
<td>bone sialoprotein</td>
</tr>
<tr>
<td>c-Fms</td>
<td>colony-stimulating factor 1 receptor</td>
</tr>
<tr>
<td>CTX</td>
<td>carboxy terminal crosslinked telopeptides of type 1 collagen</td>
</tr>
<tr>
<td>D₃</td>
<td>1,23(OH)₂ vitamin D₃</td>
</tr>
<tr>
<td>DBBM</td>
<td>deproteinized bovine bone mineral</td>
</tr>
<tr>
<td>DFDBA</td>
<td>demineralized freeze-dried bone allograft</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EMD</td>
<td>enamel matrix derivate</td>
</tr>
<tr>
<td>EMP</td>
<td>enamel matrix proteins</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbant assay</td>
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<tr>
<td>FDR</td>
<td>false discovery rate</td>
</tr>
<tr>
<td>FGF</td>
<td>fibroblastic growth factors</td>
</tr>
<tr>
<td>FMBS</td>
<td>full mouth bleeding score</td>
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<tr>
<td>FMPS</td>
<td>full mouth plaque score</td>
</tr>
<tr>
<td>GTR</td>
<td>guided tissue regeneration</td>
</tr>
<tr>
<td>GBR</td>
<td>guided bone regeneration</td>
</tr>
<tr>
<td>HbA1c</td>
<td>glycated hemoglobin</td>
</tr>
<tr>
<td>HOMIM</td>
<td>human oral microbe identification microarray</td>
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<tr>
<td>HSC</td>
<td>hematopoietic stem cell</td>
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</table>
ICC  intra-class correlation
IFN  interferon  IFN-γ
IGF  insulin-like growth factor
IL  interleukin
ILC  innate lymphoid cell
ICT  inflammatory connective tissue lesion
ICTP  C-telopeptide crosslinked pyridinoline of type I collagen
KM  keratinized mucosa
LPS  lipopolysackarides
LRAP  leucine-rich amelogenin peptide
MCP-1  monocyte attracting protein 1
M-CSF  macrophage colony-stimulating factor
MIP-1α  macrophage inflammatory protein 1 alpha
MMP  matrix metalloproteinase
MPO  myeloperoxidase
MSC  mesenchymal stem cell
NFATc1  nuclear factor of activated T-cells, cytoplasmic 1
NF-κB  nuclear factor kappa B
OB  osteoblast
OC  osteoclast
OCN  osteocalcin
OPG  osteoprotegerin
OPN  osteopontin
PAMPS  pathogen-associated molecular patterns
P1CP  C-telopeptide of type I collagen
P1NP  N-telopeptide of type I procollagen
PBS  phosphate buffered saline
PCA  principle component analysis
PCR  polymerase chain reaction
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>PD</td>
<td>pocket depth</td>
</tr>
<tr>
<td>PDGF</td>
<td>platelet derived growth factor</td>
</tr>
<tr>
<td>PDL</td>
<td>periodontal ligament</td>
</tr>
<tr>
<td>PGA</td>
<td>propylene glycol alginate</td>
</tr>
<tr>
<td>PGE-2</td>
<td>prostaglandin E2</td>
</tr>
<tr>
<td>PICF</td>
<td>peri-implant crevicular fluid</td>
</tr>
<tr>
<td>PISF</td>
<td>peri-implant sulcus fluid</td>
</tr>
<tr>
<td>PMN-cells</td>
<td>polymorph nuclear cells / neutrophil granulocytes</td>
</tr>
<tr>
<td>PLS</td>
<td>partial least squares</td>
</tr>
<tr>
<td>PTH</td>
<td>parathyroid hormone</td>
</tr>
<tr>
<td>RANK</td>
<td>Receptor activator of nuclear factor kappa B</td>
</tr>
<tr>
<td>RANKL</td>
<td>Receptor activator of nuclear factor kappa B ligand</td>
</tr>
<tr>
<td>RCT</td>
<td>randomised controlled trial</td>
</tr>
<tr>
<td>REC</td>
<td>recession</td>
</tr>
<tr>
<td>Runx2</td>
<td>runt-related transcription factor 2</td>
</tr>
<tr>
<td>S1P</td>
<td>sphingosine-1-phosphate</td>
</tr>
<tr>
<td>TGF</td>
<td>transforming growth factor</td>
</tr>
<tr>
<td>TIMP</td>
<td>tissue inhibitor of metalloproteinases</td>
</tr>
<tr>
<td>TLR</td>
<td>toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>TRAP</td>
<td>tartrate-resistant acid phosphatase</td>
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Introduction

Osseointegration and dental implants

Osseointegration
The term osseointegration was introduced by Per-Ingvar Brånemark in 1977 (Branemark et al. 1977). In the 1950s, Brånemark discovered a close structural and functional contact between bone and an implant made of titanium; biocompatibility allowed the bone cells to integrate the implant into the osseous tissue. This concept was tested in animal studies, and in 1965, the first dental implants were placed in humans. A detailed light microscopic exploration of osseointegration facilitated the introduction of osseointegrated dental implants in dentistry (Linder et al. 1983). Clinical experiences and implant survival studies from 15 years of treating patients with osseointegrated dental implants showed that 89% of implants in the maxillary bone were stable, and 100% of implants in the mandible were stable after 5 to 9 years (Adell et al. 1981).

Dental implants
Dental implants are currently a standard treatment for replacing missing teeth, and titanium implants are also used to anchor orthopaedic and facial prostheses. Dental implants made of titanium with threads are inserted into the bone during a surgical procedure (Adell et al. 1981). After a healing period, the implant is osseointegrated and ready for loading and function as a dental unit. Biological complications, such as biofilm-induced inflammatory bone loss, that affect osseointegrated dental implants have become more prevalent with the increasing number of implant carriers, which is a challenge in clinical practice.

Peri-implant structures and peri-implant health

Peri-implant structures
The implant is osseointegrated into the jawbone by means of the implant surface in contact with the bone, and in contrast to a tooth, the implant lacks a periodontal ligament, which connect the tooth root surface with the bone tissue. The lack of periodontal ligament means absence of blood vessels and nerve fibres in absolute proximity of the implant surface. The healthy peri-implant mucosa surrounding the supracrestal part of the implant includes connective tissue covered by a keratinised (masticatory mucosa) or non-keratinised (lining mucosa) mucosa, which is 3 to 4 mm high on average. The peri-implant mucosa is weakly adhered to the implant surface, with a very thin junctional epithelium (2 mm). The collagen fibres in the supracrestal connective tissue portion run parallel to the implant surface, in contrast to the more robust gingival adhesions,
which include collagen fibres that enter the tooth root cementum. This important structural barrier that the periodontal ligament creates around the teeth functionally limits and protects against microbial invasion. At an implant site, however, the inflammatory infiltrate may penetrate closer to the bone without separation due to the supracrestal layer of collagen fibres (Lindhe et al. 1992). In addition, implants lack vascular structures close to the surface, which may impair nutrition and delay the host response (Araujo and Lindhe 2018; Berglundh et al. 1992; Tomasi et al. 2014) (Figure 1a+b).

**Peri-implant health**
Clinically, healthy peri-implant tissue presents without inflammation in the soft tissue surrounding the implant and are characterised by the absence of redness, bleeding on probing, swelling and suppuration. Furthermore, no progressive bone loss should be visible on radiographs. The pocket depth on probing depends on the position of the implant and the soft tissue volume (Araujo and Lindhe 2018; Cochran et al. 2009; Lindquist et al. 1996).

![Figure 1a) Structures of healthy tissue around a tooth and an implant. b) Structures of an inflammatory lesion around a tooth and an implant. Copyright Renvert-Giovannoli Peri-implantitis, Quintessence. International, 2012, with permission.](image)

**Peri-implant diseases**

**Definition and diagnosis**
Peri-implant diseases are inflammatory conditions affecting the peri-implant tissue and are induced by a commensal bacterial biofilm.
**Peri-implant mucositis**
Peri-implant mucositis is defined as inflammation of the peri-implant mucosa around an osseointegrated implant without loss of the supporting bone. Clinical signs of peri-implant inflammation are bleeding and/or suppuration on probing, with or without increased probing depths (Heitz-Mayfield and Salvi 2018; Lindhe and Meyle 2008).

**Peri-implantitis**
The term peri-implantitis was introduced in 1987 and was previously described as biologic complications or progressive bone loss around the implant (Berglundh et al. 2002; Mombelli et al. 1987).

Peri-implantitis is defined as a pathological condition characterised by inflammation in the peri-implant mucosa/connective tissue and progressive loss of the supporting bone around a dental implant. Clinical signs of peri-implantitis are bleeding and/or suppuration on probing, increasing probing depths and/or recession of the mucosal margin, in addition to radiographic bone loss compared to previous examinations. When a previous radiograph is not available, the following is indicative for the diagnosis of peri-implantitis: bone loss >3 mm in combination with bleeding on probing and pocket depth >6 mm (Lindhe and Meyle 2008; Renvert et al. 2018a; Schwarz et al. 2018).

**Epidemiology**
At the subject level, the prevalence of peri-implantitis is estimated to be approximately 20%, and the prevalence of peri-implant mucositis is 45%. At the implant level, the corresponding prevalence values are 10% and almost 30% respectively (Derks and Tomasi 2015; Lee et al. 2017; Mombelli et al. 2012). In a Swedish population of 588 patients receiving dental implants, the prevalence after 9 years was 14.5% for severe peri-implantitis, which was defined as bleeding and/or suppuration on probing and bone loss >2 mm (Derks et al. 2016). The onset of peri-implantitis may occur early, during the second or third year after implant placement, and the progression may be non-linear, i.e., accelerated and varied (Derks et al. 2016). Systematic reviews and meta-analyses of epidemiological reports concluded that the heterogeneity in case definitions influences prevalence estimates. In summary, peri-implant diseases are common, and the prevalence of peri-implantitis seems to be increasing over time.

**Aetio-pathology**
Peri-implantitis and periodontitis share common aetiological factors, as both conditions involve biofilm-induced inflammation in the soft tissue that subsequently triggers a host response, with possible tissue degradation (Heitz-Mayfield and Lang 2010). Peri-implant inflammation is initiated by the
accumulated bacterial biofilm. The development of disease was initially studied in an experimental gingivitis model in animals (dogs) and humans, and a cause-effect relationship between de novo plaque formation and peri-implant mucositis was observed (Berglundh et al. 1992; Pontoriero et al. 1994). Histopathological, the early biofilm-induced inflammatory host response in mucositis is comparable to that in gingivitis, but the lesion of the inflammatory connective tissue (ICT) is larger and extends apically to the junctional epithelium. The established biofilm results in a more pronounced host response, and the extension of the ICT lesion is even larger in size than that in gingivitis, with increased amounts of inflammatory cells in peri-implant mucositis (Ericsson et al. 1992). The inflammation is reversible after biofilm removal, and no difference between implant systems has been observed (Abrahamsson et al. 1998; Pontoriero et al. 1994; Salvi et al. 2012).

Peri-implantitis lesions investigated in experimental ligature-induced peri-implantitis in a dog model presented more aggressive tissue degradation at the implant site than teeth with periodontitis. A larger inflammatory infiltrate extending close to the crestal bone and more bone-resorbing osteoclasts were observed at the implant site. Spontaneous progression after ligature removal varied with different implant surfaces (Carcuac et al. 2013; Lindhe et al. 1992).

Peri-implant inflammation develops when microbes activate the host innate and adaptive immune responses. Several cell types, such as epithelial cells, fibroblasts, stromal cells, endothelial cells and osteoblasts, release pro-inflammatory mediators, such as cytokines and chemokines, to recruit leukocytes. Leukocytes are recruited from blood vessels and tissues. Human biopsies revealed that the proportion of vascular structures was smaller within the peri-implant ICT lesion and greater lateral to the peri-implant ICT than reported for periodontitis lesions. Neutrophil granulocytes (polymorphonuclear neutrophils (PMNs)) and monocytes/macrophages are prevalent close to the sulcus epithelium in the peri-vascular area and in the centre of the ICT. Although the ICT lesion is dominated by T and B lymphocytes and plasma cells, the inflammatory lesion has a more acute character in peri-implantitis than in chronic periodontitis, with a larger proportion of PMNs and macrophages (Berglundh et al. 2011; Carcuac and Berglundh 2014; Gualini and Berglundh 2003).

**Risk factors and risk indicators**
Peri-implantitis has mostly been investigated in observational, cross-sectional or retrospective studies, and these study designs reflect risk indicators. True risk factors for a disease are identified by prospective, longitudinal studies in humans, which are rare in this field (Heitz-Mayfield 2008).
Poor plaque control and lack of regular supportive therapy

Optimal plaque control and oral health have been identified as prerequisites for successful implant therapy, and poor oral hygiene is a strong risk factor for developing peri-implantitis (Ferreira et al. 2006; Lindquist et al. 1997). Poor plaque control and a lack of regular supportive therapy constitute risk factors with evidence from two longitudinal studies and a number of cross-sectional reports. The importance of plaque control in the prevention of peri-implant mucositis and subsequent peri-implantitis was highlighted in these studies. A 5-year follow-up revealed a lower incidence of peri-implantitis with regular supportive care (18%) than without (44%) (Costa et al. 2012). Similar findings were obtained in a 10-year study, in which the group of patients in a regular maintenance programme developed peri-implantitis less frequently (27%) than the patients who missed follow-up visits (44%) (Roccuzzo et al. 2012).

History of periodontitis

There is strong evidence from longitudinal and cross-sectional studies that a history of periodontitis constitutes a risk factor for peri-implantitis. Two 10-year longitudinal studies correlated peri-implantitis development with periodontitis (Karoussis et al. 2003; Roccuzzo et al. 2012). In a 9-year follow-up of 588 patients who received implants, an odds ratio of 4 was reported for patients with periodontitis (Derks et al. 2016). Periodontitis patients with residual pockets (PPD ≥ 5 mm) presented a significant risk for the development of peri-implantitis and implant loss, but with regular supportive treatment, the prevalence of peri-implantitis was reduced (Pjetursson et al. 2012).

Genetic traits

Genetic trait studies are scarce, and the overall evidence is limited and inconclusive. The most thoroughly investigated genetic factor is interleukin (IL)-1 composite gene polymorphism (Schwarz et al. 2018). IL-1-α, IL-1β and their natural specific inhibitor IL-1 receptor antagonist (IL-1ra/IL-1RN) play key roles in the regulation of the inflammatory response in periodontal tissues. These genes may also influence susceptibility to peri-implantitis development in periodontitis patients, even if the inflammatory condition is under control at the time of implant placement. IL-1ra gene polymorphism was proposed as a risk factor for peri-implantitis (Laine et al. 2006). Two of the three studies that evaluated peri-implantitis in relation to the IL-1 genotype indicated that IL-1RN/IL-1α/IL-1β gene polymorphisms were correlated with increased peri-implant tissue infection and destruction (Dereka et al. 2012). The IL-1 genotype in combination with smoking was observed to affect implant failure, i.e., implant loss or biologic complications at the implant site (Feloutzis et al. 2003) (Gruica et al. 2004; Jansson et al. 2005). The ability to predict disease with a genetic susceptibility test for peri-implantitis and severe periodontitis has been
questioned (Greenstein and Hart 2002). Future prospective studies that include a large number of subjects are needed to confirm the results.

**Systemic conditions**

Systemic conditions associated with peri-implantitis are rarely studied; therefore, information is scarce, and studies have implicated cardiovascular disease, osteoporosis, osteopenia, thyroid disease, hepatitis, body mass index, radiation and chemotherapy with no conclusive evidence (Schwarz et al. 2018). Although diabetes in patients with uncontrolled blood sugar levels has been identified as a risk factor for periodontitis, there was no association between diabetes and peri-implantitis in most of the available cross-sectional studies using patient-reported data. In 2 of 3 studies based on fasting blood sugar levels or medication (Ferreira et al. 2006) and increased levels of glycated haemoglobin (HbA1c) (Tawil et al. 2008), an association between diabetes and peri-implantitis was found (Schwarz et al. 2018).

**Smoking**

The evidence for smoking as a risk factor is inconclusive, although smoking is strongly associated with periodontitis, marginal bone loss and tooth loss (Axelsson et al. 1998; Bergstrom 2003). One 10-year cohort study reported that peri-implantitis developed for 28% of all implants in smokers, while the corresponding incidence was 6% of all implants in non-smokers (Karoussis et al. 2003); this finding was consistent with cross-sectional studies (Rinke et al. 2011; Roos-Jansaker et al. 2006; Schwarz et al. 2017). A systematic review of prospective and retrospective studies indicated an enhanced risk of biologic complications among smokers; similarly, a meta-analysis indicated an enhanced risk of implant failure among smokers (Strietzel et al. 2007). However, there are also many opposing reports on smoking, and additional studies are needed that control for confounders, e.g., history of periodontitis and categorisation of smokers and non-smokers, as well as information bias (Schwarz et al. 2018). However, smoking can be regarded as a risk indicator for peri-implantitis.

**Alcohol consumption**

Alcohol consumption was investigated in one prospective study, which showed that an intake of more than 10 mg of alcohol per day was related to peri-implant bone loss, as well as tobacco use, plaque and inflammation (Galindo-Moreno et al. 2005).

**Keratinised mucosa**

Compared to sites with a keratinised mucosa of greater than 2 mm, sites with a keratinised mucosa of less than 2 mm are associated with plaque accumulation followed by peri-implant inflammation in the soft tissue, but no change in marginal bone level has been verified. The evidence is weak that the absence of a
keratinised mucosa is a risk factor for peri-implantitis, but this factor may negatively affect self-performed oral hygiene (Roccuzzo et al. 2016).

**Excess cement and over-contoured supra-structures**
Potential risk indicators that are indirectly related to plaque accumulation have been proposed, *e.g.*, constructions with excess cement may result in a higher prevalence of peri-implantitis than screw-retained constructions (Becker and Kaldahl 1981; Staubli et al. 2017); similar results were reported for over-contoured crowns and supra-structures (Becker and Kaldahl 1981). Recently, the a wide emergence profile in the restoration contour was reported to cause an unhealthy state (Katafuchi et al. 2018).

**Implant-related factors**
Titanium particles have recently been implicated as a potential risk indicator due to new reports that titanium particles from the implant surface may be present in the tissues after implant placement and may enhance infection-induced inflammation and activate macrophages (Pettersson et al. 2017b; Pettersson et al. 2018). Nevertheless, this area must be explored in future research, as should the impact of implant surface roughness on peri-implantitis pathogenesis and progression (Albouy et al. 2009; Carcuac et al. 2013). Implant surface characteristics vary in terms of topography, surface roughness and chemical composition, *e.g.*, turned, titanium plasma sprayed (TPS), blasted, anodised, blasted and acid-etched. Today, most implants have a moderately rough surface ($S_a$ between 1.0 and 2.0 $\mu$m); compared to smoother (turned $S_a$+0.5 $\mu$m) or rougher surfaces, such surfaces show improved bone responses during initial healing after implant placement (Albrektsson and Wennerberg 2004). Marginal bone loss was found to be greater when implants with rough surfaces were used (Baelum and Ellegaard 2004), and turned surfaces generally demonstrated the smallest marginal bone loss (Wennerberg et al. 2018). In a randomised controlled trial (RCT) comparing turned and rough surface implants with a 3-year follow-up, the frequency of peri-implantitis was higher for the rougher surface (Astrand et al. 2004). In contrast, a prospective RCT with strict supportive therapy reported similar bone level changes for turned and moderately rough surface implants (Wennstrom et al. 2004).

**Overload**
Clinical signs of occlusal overload, such as abutment fracture, loss of retention and/or signs of abrasive forces on supra-structures, seem to be an indirect but potential risk, with a wide range of odds ratios (2-19). However, the evidence is limited concerning overload and its influence on the onset or progression of peri-implantitis (Klinge and Meyle 2012; Schwarz et al. 2018). In a dog model, occlusal overload did not induce marginal bone loss in implants with a healthy mucosa (Heitz-Mayfield et al. 2004).
Iatrogenic factors
The proposed potential risk of iatrogenic factors for the initiation and progression of peri-implantitis caused by implant mal-positioning, surgical trauma, and inadequate restoration abutment seating has not yet been clearly investigated (Lang and Berglundh 2011).

Peri-implant microbiota

Microbiological analysis
Most of the information on the microbiota in peri-implantitis is based on culture methods and experiences from patients with periodontitis. The use of molecular techniques and metagenomics in microbiological analyses expands our knowledge of the microbial diversity in sub-mucosal biofilms. Polymerase chain reaction (PCR) analysis was the first molecular method introduced 20-30 years ago, enabling small amounts of DNA from biofilm samples to be replicated enzymatically in an exponential manner. The next method was the DNA-DNA hybridisation “checkerboard”, a method based on preselected DNA probes for specific bacterial taxa, with DNA samples hybridised against whole genomic DNA probes on a single support membrane. Open-ended approaches have recently discovered previously unsuspected or unknown bacteria in addition to those discovered through pathogen-targeted methods. Single gene-based amplification of 16S rRNA initiated metagenomics, and whole-genome shotgun sequencing covered the DNA more comprehensively via high-throughput DNA sequencing. However, investigating virulence in cultivable organisms is still important (Charalampakis and Belibasakis 2015).

Periodontal pathogens and opportunistic infections

Biofilm formation and the transition to peri-implantitis
On the surface of a tooth or an artificial material, a biofilm will inevitably form. Early microbial culturing on an implant revealed similarities between periodontal and peri-implant disease (Mombelli et al. 1987). The peri-implant microbiota resembled that on the teeth, and it was postulated that the oral microflora and especially neighbouring teeth may act as a reservoir for the early biofilm that builds up on implants (De Boever and De Boever 2006; Heitz-Mayfield and Lang 2010; Mombelli et al. 1995; Papaioannou et al. 1996; van Winkelhoff et al. 2000).

Biofilm, i.e., plaque, formation starts when the implant surface becomes covered by an acquired pellicle layer, consisting of saliva proteins, glycoproteins and lipids, which triggers early microbial colonisation. Bacterial colonisation begins 30 minutes after implant insertion, and the initial sub-mucosal microbiota is still
a part of the mixed biofilm on the implant surface after several months (De Boever and De Boever 2006; Furst et al. 2007; Quirynen et al. 2005; Salvi et al. 2008). In healthy patients, the peri-implant microflora consists mainly of gram-positive cocci, non-motile bacilli and a limited number of gram-negative anaerobic species (Lafaurie et al. 2017; Mombelli et al. 1987; Rakic et al. 2016).

The transition to peri-implant mucositis is associated with an increased presence of cocci, motile bacilli and spirochetes, comparable to that found in patients with gingivitis (Pontoriero et al. 1994; Zheng et al. 2015; Zitzmann et al. 2001). Early colonisers, such as Streptococcus and Actinomyces species (Li et al. 2004; Nakazato et al. 1989), create optimal conditions for late colonisers, such as Fusobacterium and Prevotella species (Heuer et al. 2011; van Brakel et al. 2011). The transition to peri-implantitis, which occurs as a result of deepened pockets and subsequent dysbiotic conditions, is followed by the emergence of gram-negative motile and anaerobic species, which are commonly found in periodontitis (Mombelli and Decaillet 2011; Mombelli et al. 1987).

**Opportunistic and keystone pathogens**
The “ecological plaque hypothesis” describes a polymicrobial disturbance in the balance between the commensal microbiota and the host response. A shift in composition occurs when it becomes possible for certain bacteria to over-grow, enhance their virulence properties and subsequently act as opportunistic pathogens. Enhanced virulence and the ability to alter gene expression are supported by the well-organised biofilm structure and a type of cell-to-cell communication called quorum sensing. Dental or implant plaque is a “smart” and well-equipped biofilm (Marsh 2004). Periodontal pathogens have various virulence factors that may subvert the host response, and dysbiotic disease may develop (Lamont and Hajishengallis 2015).

In the “keystone hypothesis”, Porphyromonas gingivalis, as an opportunistic dysbiotic pathogen, both over-activates and undermines the inflammatory response and increases pro-inflammatory cytokine signalling, inducing tissue degradation, which may progress to peri-implant disease with aggressive tissue degradation. Hence, the findings from a mouse model could be of interest in providing an aetiologic perspective on progressive tissue destruction in peri-implantitis (Hajishengallis et al. 2012).

In partially edentulous individuals, larger amounts of Porphyromonas gingivalis, Tannerella forsythia and Treponema denticola have been found in peri-implantitis lesions than in healthy peri-implant pockets (Hultin et al. 2002; Shibli et al. 2008). Prevotella intermedia, Campylobacter rectus and Staphylococcus warneri were also associated with peri-implant inflammation ten years after implant placement (Eick et al. 2015). The microorganisms that
have been identified in peri-implantitis but are less common in periodontitis are *Staphylococcus aureus, Staphylococcus epidermidis, Enterobacter aerogenes, Enterobacter cloace, Escherichia coli, Helicobacter pylori and Pseudomonas* spp. (Belibasakis 2014; Charalampakis et al. 2012;Leonhardt et al. 2003;Leonhardt et al. 2002;Leonhardt et al. 1999;Persson and Renvert 2014;Renvert et al. 2007). Aerobic gram-negative bacilli (AGNB) in peri-implantitis lesions include lactose-fermenting bacteria of the *Enterobacteriaceae* (i.e., *Escherichia coli, Enterobacter, Klebsiella*, and *Citrobacter*) and non-enteric rods that do not ferment lactose (i.e., *Pseudomonas aeruginosa* and *Acinetobacter baumannii*) (Charalampakis et al. 2012; Leonhardt et al. 1999). The facultative anaerobic species *Aggregatibacter actinomycetemcomitans* has also been detected by culture at peri-implantitis sites (Leonhardt et al. 2003; van Winkelhoff et al. 2000). *Candida* species and Epstein-Barr virus (EBV) have been identified in peri-implantitis lesions and may enhance pathogenesis (Alcoforado et al. 1991; Canullo et al. 2018; Leonhardt et al. 1999; Verdugo et al. 2015).

**Bacterial colonisation**

In contrast to earlier research, emerging evidence from metagenomic analysis suggests that periodontitis and peri-implantitis could be microbiologically distinct from each other, with a less diverse microbiota in both peri-implant health and disease than in periodontal lesions. Recently, in metagenomic analysis, peri-implant disease was associated with lower levels of *Prevotella*, non-mutans *Streptococcus, Lactobacillus, Selemonas, Leptotrichia*, and *Actinomyces* and higher levels of *Peptococcus*, *Mycoplasma*, *Eubacterium*, *Campylobacter*, and *Butyrovibrio* (Dabdoub et al. 2013; Koyanagi et al. 2013; Kumar et al. 2012;Maruyama et al. 2014). The prevalence of asaccharolytic anaerobes, such as *Eubacterium nodatum, Eubacterium brachy, Eubacterium sphenum, Filifactor alocis* and *Slackia exigiu* may also suggest a non-perio-pathogen infection (Tamura et al. 2013).

The influence of differences in surface characteristics, such as morphology, material, roughness and free energy, on bacterial colonisation, both among implants and in comparison to teeth, needs to be clarified. More biofilm forms on a rough surface, and mechanical removal will be less effective when grooves and pits protect the bacteria on an implant surface (Alhag et al. 2008). A threshold roughness of less than 0.2 µm (minimal roughness) did not affect bacterial adhesion and colonisation significantly (Bollen et al. 1996).

*In summary*, the evidence presented to date shows that healthy implants are colonised by aerobic gram-positive cocci, with low bacterial counts, and that the transition to peri-implantitis is associated with increasing bacterial counts and more anaerobic gram-negative rods. Metagenomics revealed a more heterogeneous but less complex microbial infection than previously discovered.
using culture-based and preselected DNA methods. Uncultivated species have also been associated with peri-implant disease; however, the results are inconclusive (Charalampakis and Belibasakis 2015).

**Peri-implant inflammatory response**

*Host response*
Complex biofilms of commensal bacteria and opportunistic pathogens release antigens and products recognised by Toll-like receptors (TLRs) on host cells, which initiate an inflammatory response (Yucel-Lindberg and Bage 2013). Microbial endotoxins (*e.g.*, LPS), exotoxins (*e.g.*, leukotoxins) and enzymes (*e.g.*, proteinases) rupture the barrier sulcus epithelium, and resident cells, such as epithelial cells, recognise bacterial cells and peptides as pathogen-associated molecular patterns (PAMPs) (Takeuchi and Akira 2010), subsequently triggering epithelial cells to release pro-inflammatory cytokines and enzymes (*e.g.*, interleukin-1 (IL-1), IL-8, prostaglandin E2 (PGE2), tumour necrosis factor-α (TNF-α), and matrix metalloproteinases (MMPs)). A cascade of events activates resident cells in the tissue (*e.g.*, mast cells, endothelial cells, fibroblasts and macrophages), which are also triggered by bacterial components to release, *e.g.*, IL-1, TNF-α and vasoactive amines that activate the innate immune response (Kornman et al. 1997; Yucel-Lindberg and Bage 2013).

Increased numbers of leukocytes and PMNs are recruited and transmigrate through the epithelium to the peri-implant sulcus. PMNs phagocytose microbes and secrete proteolytic enzymes (reactive oxygen species), pro-inflammatory mediators and antimicrobial peptides, contributing to local inflammation, tissue degradation and antimicrobial defence. PMNs occur not only in the pocket epithelium and adjacent connective tissue areas but also in the peri-vasculature in more central areas of the ICT (Berglundh et al. 2004; Carcuac and Berglundh 2014; Gualini and Berglundh 2003; Meyle et al. 2017).

In the tissue, fibroblasts enhance tissue degradation (MMP-1 and MMP-3) and chemotactic signalling (IL-6, IL-8, PGE2, IL-33 and IL 34) (Baek et al. 2013; Beklen and Tsao us Memet 2014; Belibasakis and Guggenheim 2011; Belibasakis et al. 2005; Bostrom and Lundberg 2013; Takashiba et al. 2003). Chemotactic signalling via the gradient of inflammatory cytokines/chemokines and antigens/microbes promotes the recruitment and activation of other leukocytes, such as monocytes/macrophages. Macrophages are activated through antigen non-specific innate defence mechanisms to phagocytose microbes and release inflammatory cytokines (*e.g.*, IL-1 and TNF-α), subsequently recruiting more PMNs and monocytes. The overall inflammatory response results in extracellular matrix degradation. In some individuals with innate susceptibility and/or specific
environmental factors, macrophages and mast cells fail to clear the infection and resolve inflammation. The impaired host response may lead to monocyte/macrophage differentiation to osteoclasts and the activation of bone resorption (Di Benedetto et al. 2013; Freire and Van Dyke 2013).

Activation of the adaptive immune response by chemotactic signals recruits T lymphocytes. Antigen-presenting cells, macrophages and dendritic cells, present antigen to T lymphocytes, which in turn guide plasma cells to produce specific antibodies, mainly immunoglobulins (IgGs). Plasma cells dominate the ICT around the implant adjacent to the crestal bone (Berglundh et al. 2004; Carcuac and Berghlund 2014; Gualini and Berghlund 2003). Hence, antigen-presenting cells are the link between the innate and adaptive immune responses (Martinez and Gordon 2014).

Under healthy conditions, homeostasis is maintained, with low levels of bacteria and immune activation. This balance may be disturbed by increased microbial challenge and/or a compromised host response. In disease-susceptible individuals, inflammatory peri-implant mucositis may progress to peri-implantitis with irreversible loss of bone support. Eventually, in this cascade of released proteins and peptides, continuous leakage into the peri-implant crevice/pocket will occur. Investigating these biomarkers may provide some insight into this complex biological process (Bostanci and Belibasakis 2018; Holmlund et al. 2004).

**PICF**

The peri-implant crevice/sulcus is subjected to a constant fluid exudate called the PICF, which is a protein-poor serum transudate in healthy individuals but an accumulating inflammatory exudate in disease; this inflammatory exudate includes cellular components (i.e., exfoliated epithelial cells, bacteria, and immune cells), an increased amount of major plasma proteins and immunologically active contents (e.g., bacterial enzymes and endotoxins), and host-derived pro-inflammatory cytokines, chemokines, and degradation products (Bostanci and Belibasakis 2018). Hence, PICF may be considered a reservoir of molecular information in the search for key inflammatory factors and a potential diagnostic tool, which may help to predict, diagnose and monitor peri-implant disease. Clinical assessments of bleeding on probing and pocket depth are not always sufficient to predict future stability around implants (Sanz and Chapple 2012), and PICF has been validated as a valuable diagnostic tool (Basegmez et al. 2012; Fonseca et al. 2014).

Traditionally used assays for the identification and quantification of molecules include biochemical or antibody-based methods, such as sandwich enzyme-
linked immunosorbent assay (ELISA) or immunoblotting, which focus on a single or limited number of candidate molecules. To form a picture of the full range of possible proteins, multiplex protein arrays and mass spectrometry-based analysis were introduced, with the aim of qualitatively and quantitatively investigating proteomics. High-throughput assays and publicly available databases of the proteins encoded and expressed by every human gene are emerging methods for assessing the global protein content (Bostanci and Belibasakis 2018).

The collection of PICF is non-invasive with a filter paper adapted to the peri-implant sulcus; thus, repeated samples can be obtained (Panagakos et al. 1996) (Figure 2). The limited volume of fluid harvested from this site is a challenge. The sample is subjected to an elution process in buffer to remove larger cellular components by centrifugation and filtration and to unmask low-abundance proteins among other highly abundant proteins, such as albumin and IgG, but the elution process may also result in the removal of proteins (i.e., co-deletion).

Crucial factors for future reliable PICF analysis are subject selection, definitions associated with peri-implantitis diagnosis, PICF sampling methods (e.g., the number and severity of sites and the sampling time) and sample handling, as well as the sensitivity and specificity of the assay used.

Figure 2a) Bone loss in peri-implantitis. b) Inflammatory peri-implant lesion with pus. c) Collection of bacterial samples. d) PICF sampling.

**Biomarkers**

Biomarkers that mirror the inflammatory state of the tissues may help distinguish between normal and pathological processes. Biomarkers of peri-implantitis are primarily associated with inflammation and bone destruction, modulating
inflammatory intensity and disease progression (Dursun and Tozum 2016). The most studied pro-inflammatory biomarkers are IL-1β and TNF-α, which have been suggested to be responsible for the early differentiation of peri-implant mucositis or peri-implantitis. IL-1β regulates the degradation of extracellular matrix components of the plasminogen system and collagenase activity during inflammation and wound healing (Casado et al. 2013; Kao et al. 1995). The majority of studies investigating IL-1β reported higher levels of IL-1β in peri-implantitis tissues than in healthy tissue (Duarte et al. 2009). TNF-α is also significantly associated with peri-implantitis (Ata-Ali et al. 2015; Darabi et al. 2013; de Mendonca et al. 2009; Petkovic et al. 2010; Renvert et al. 2015). TNF-α induces fibroblast apoptosis and reduces the repair capacity of the peri-implant tissue. Interestingly, it was possible to revert TNF-α levels with mechanical therapy (Duarte et al. 2009). IL-1β and TNF-α are both involved in osteoclast formation and bone resorption. These two pro-inflammatory cytokines are suggested to be the most promising markers in the PICF for differentiating between healthy peri-implant tissue and peri-implantitis tissue (Casado et al. 2013; Li and Wang 2014; Petkovic et al. 2010). MMPs, enzymes that degrade various extracellular matrix proteins, are positively correlated with inflammatory conditions around implants (Klinge et al. 2002; Wang et al. 2015). The antimicrobial enzyme myeloperoxidase (MPO), which is derived from leukocytes, was found in higher amounts in PICF from peri-implantitis sites, but the relationship of this factor with the degree of peri-implant inflammation was not confirmed (Guncu et al. 2008; Tozum et al. 2007).

When comparing healthy and diseased implant sites, no conclusive evidence has been found regarding the levels of pro-inflammatory cytokines, such as IL-6, IL-12, and IL-17, and the levels of the anti-inflammatory cytokines IL-4 and IL-10, the chemokine IL-8 and osteoclast activating receptor activator of nuclear factor kappa B (RANKL) (Duarte et al. 2016). Some studies reported no difference in IL-1β (Hultin 2002) or prostaglandin E2 (PGE2) levels (Lachmann et al. 2007) between healthy and diseased implant sites. In addition, there were no significant changes in the total amounts of TNF-α, IL-1β and transforming growth factor-β2 (TGF-β2) in PICF from healthy implant sites exposed to de novo plaque accumulation (Schierano et al. 2008).

Contradictory results have been obtained from studies on PICF levels of osteoprotegerin (OPG)/RANKL (Arikan et al. 2008; Rakic et al. 2013); although the OPG/RANKL ratio improved with mechanical treatment of peri-implantitis (Duarte et al. 2009), the available information is inconclusive (Dursun and Tozum 2016). However, the common factor in the immune-inflammatory process that leads to bone resorption appears to be the osteoclastogenic factor RANKL.
Studies of bone turnover biomarkers in the PICF are scarce and not clearly elucidated; even though the level of crosslinking of the bone degradation product C-telopeptide pyridinoline of type I collagen (ICTP) was higher at peri-implantitis sites than at healthy sites, there was no difference in the levels of carboxy-terminal-crosslinked type I collagen (CTX-1) (Akman et al. 2018; Arikan et al. 2011). Cathepsin K, an enzyme related to bone resorption, was positively correlated with PICF volume in peri-implant alveolar bone loss (Yamalik et al. 2011). Two other enzymes related to bone turnover, alkaline phosphatase (ALP) and elastase, were detected in greater amounts at peri-implantitis sites than at healthy sites (Plagnat et al. 2002). The presence of the bone formation marker osteocalcin (OCN) (Murata et al. 2002), which is a calcium-binding protein, in PICF, has not been elucidated, and there are no studies on the bone metabolic N-terminal propeptide of type 1 procollagen (P1NP) or the carboxy-terminal propeptide of type 1 procollagen (P1CP) in PICF. Nevertheless, in most studies, comparisons between healthy and peri-implantitis sites were based on data from different patients in cross-sectional studies (Dursun and Tozum 2016).

Three interventional trials investigated PICF before and after surgical treatment of peri-implantitis and found significant reductions in TNF-α levels three and 12 months after open flap debridement (de Mendonca et al. 2009); in addition, the OPG/RANKL ratio was higher for healthy sites than for untreated diseased sites (Duarte et al. 2009). A follow-up 12 months after surgery with adjunctive titanium granules found no changes in the levels of OCN, osteopontin (OPN), parathyroid hormone (PTH), TNF-α or insulin but significant reductions in the total protein levels of MMP-8, IL-6, OPG, leptin and adiponectin (Wohlfahrt et al. 2014).

**Bone tissue**

Bone is a dynamic tissue subjected to continuous turnover processes that are very complex and involve a wide variety of intricate bioactive signalling pathways and cellular interactions.

**Bone structure, modelling and remodelling**

**Bone structure**

Bone tissue is primarily composed of calcified extracellular matrix, which is mineralised by calcium and phosphate as hydroxyapatite Ca₃[PO₄]₂Ca[OH]₂, and a smaller number of cells. The organic part of bone includes 90% collagen type 1 and 10% non-collagenous proteins, lipids and glycosaminoglycans (Clarke 2008).

The bone structure is built up by cortical and cancellous/trabecular parts. Cortical bone forms the outer shell of bones, is heavily mineralised, and represents
approximately 80% of the skeleton. Cortical bone has a dense and ordered intracortical structure, with concentric layers of bone matrix surrounding Haversian’s canals, which contains capillaries and nerve fibres. The inner core is filled with cancellous/trabecular bone, an irregular and light network, embedded in the bone marrow. The structural organisation of cortical and trabecular bone contributes to resistance to torque, compression and shear stress. The jawbone provides support to the teeth, may adapt to occlusal changes and orthodontic forces and may integrate the implant into the osseous tissue. The bone marrow harbours the haematopoietic stem cells from which blood and immune cells and bone-resorbing cells are derived (Clarke 2008). The bone marrow is a source of various bioactive molecules involved in local and systemic metabolic activities, such as calcium and phosphate homeostasis, kidney function and energy metabolism (Fukumoto and Martin 2009).

**Bone modelling and remodelling**

Bone tissue continuously undergoes modelling and remodelling processes (Hattner et al. 1965). Bone modelling adapts the structure to facilitate bone shape and size changes and allow physiological growth and adaptation to mechanical loading. Bone remodelling is a turnover process in response to mechanical stress, which replaces old bone with new bone, repairs bone microdamage and maintains calcium homeostasis. Systemic and local molecules regulate the balance between bone resorption and formation. The coupling phenomenon, in which remodelling takes place at “bone multicellular units” (BMUs), is controlled by multiple extracellular and intracellular signalling networks (Martin and Seeman 2008; Parfitt 1982). The remodelling cycle is a continuous process that normally reconstitutes 10% of the skeleton per year (Manolagas 2000). Bone cells have distinct functions in bone remodelling, strongly regulating each other by cell-to-cell communication. A monolayer of bone-lining cells covers all inactive bone surfaces. In response to distinct signals, osteoclasts are permitted to resorb bone, or osteoblasts form new compact bone and bone matrix, a well-organised network that includes entrapped mechano-sensing osteocytes. Bone-resorbing osteoclasts and bone-forming osteoblasts are only found at active remodelling sites on the bone surface (Seeman 2009).

**Osteoblasts and bone formation**

**Osteoblasts**

Osteoblasts are responsible for the production of the bone extracellular matrix and mineralisation, and osteoblasts simultaneously play a key role in the bone resorption process (Rodan and Martin 1982). Osteoblasts are derived from mesenchymal stem cells, and their differentiation involves a variety of hormones
and a large number of growth factors and cytokines. Osteoblastogenesis depends on runt-related transcription factor 2 (Runx-2). The downstream regulator of Runx-2 activity is Osterix (Komori et al. 1997; Otto et al. 1997). Phenotypic osteoblasts are able to synthesise and mineralise bone tissue. Osteoblasts are equipped with receptors for the calcium-regulating hormones parathyroid hormone (PTH) and 1,25(OH)₂ vitamin D₃ (D₃) (Lerner 2004; Vaes 1988). These systemic hormones stimulate bone resorption through osteoblast expression of macrophage colony-stimulating factor (M-CSF), RANKL and OPG to regulate bone remodelling (Silva and Bilezikian 2015), whereas the hormone oestrogen induces osteoblastic OPG expression (Hofbauer et al. 1999). In addition, the nervous system may cause delayed stimulation of bone resorption via stromal cells/osteoblasts (Lerner and Persson 2008; Lundberg et al. 1999).

**Osteocytes**

Osteocytes are entrapped osteoblasts with stellate-shaped bodies attending the lacuna, with long processes extending through a network in contact with neighbouring osteocytes or osteoblasts (Manolagas 2000; Parfitt 1994). Osteocytes are sensitive to both chemical and mechanical signals and regulate bone remodelling through cell-to-cell interactions between osteoblasts and osteoclasts (Schaffler et al. 2014). Osteocytes express receptors for PTH and vitamin D₃ and are proposed to be the major contributor of RANKL for promoting osteoclastogenesis during remodelling (Nakashima et al. 2011; Xiong et al. 2015). Additionally, osteocyte synthesis of sclerostin, a negative regulator of bone formation, limits bone resorption (Winkler et al. 2003).

**Bone formation**

Osteoblasts form tight junctions with adjacent cells, and groups of 100-400 osteoblasts per BMU produce a highly organised organic matrix called the osteoid. The formation of the osteoid on the non-resorbing surfaces involves the bone matrix and enzymes associated with mineralisation (Lian and Stein 1995). This complex mixture of a variety of proteins is mainly composed of collagen type I, glycoproteins (e.g., fibronectin, osteonectin, OPN and bone sialoprotein), γ-carboxyglutamic acid-containing proteins (e.g., OCN and collagen), enzymes associated with bone mineralisation (e.g., ALP, collagenases, and proteinases), proteoglycans (e.g., biglycan, decorin, and hyaluronan), proteolipids and a number of growth factors (e.g., fibroblastic growth factors, insulin-like growth factors, and transforming growth factor (TGF-β)), and bone morphogenetic proteins (BMPs). High levels of ALP and OCN in the serum may be useful as indicators of bone formation activity. The osteoid mineralises with the deposition of hydroxyapatite (Lerner 2004).
Osteoclasts and bone resorption

Osteoclasts

Bone resorption is exclusively performed by osteoclasts. Osteoclast progenitor cells are closely related to macrophages and dendritic cells in the immune system. The proliferation and differentiation of mononuclear precursor cells to active osteoclasts is regulated by a variety of systemic and local factors and complex intracellular signalling pathways (Boyle et al. 2003). Both progenitor and mature multinucleated osteoclasts express receptors for the calcium-regulating hormone calcitonin and show positive staining for the tartrate-resistant acid phosphatase (TRAP). Osteoclast progenitor cells differentiate in the bone marrow and are present in blood recruited to remodelling sites. Osteoclasts form multinucleated giant cells by fusion of mononucleated osteoclast progenitor cells derived from haematopoietic tissue (Suda et al. 1999), and this process occurs in close relation to the mineralised bone; hence, multinucleated osteoclasts never appear in the circulation. The proliferation and further differentiation of osteoclast progenitor cells to multinucleated osteoclasts, or osteoclastogenesis, requires two essential cytokines, M-CSF and RANKL (Felix et al. 1994; Tanaka et al. 1993). Osteoblasts and many other cell types produce M-CSF, which acts on the specific membrane receptor c-fms on mononucleated progenitor osteoclasts; the osteoclast progenitor cells then expresses RANK, a transmembrane protein that binds to RANKL, a surface-bound protein on osteoblasts. This cell-to-cell contact is decisive for the fusion of mononuclear progenitor cells to form multinucleated osteoclasts and for the activation of osteoclast migration and bone resorption (Boyce 2013; Jimi et al. 1999; Levaot et al. 2015). RANKL, which also exists in a soluble form, and RANK are both members of the TNF receptor superfamily that are specific for osteoclast differentiation but are also important in interactions between dendritic and T lymphocytes. The RANKL/RANK interaction also triggers several receptors, cellular responses, and intracellular signalling pathways and activates the key osteoclastogenetic transcription factor NFATc1. NFATc1 promotes the expression of osteoclast-specific genes (e.g., TRAP, β3 integrins, calcitonin receptors and cathepsin K). RANKL/RANK signalling activates osteoclasts to resorb bone and decreases osteoclast apoptosis. The inhibition of osteoclastogenesis follows when osteoblasts synthesise OPG, a soluble decoy receptor that can block RANKL from binding to RANK (Boyce 2013). Hence, the presence of osteoblasts is a prerequisite for regulating osteoclast activity. Furthermore, osteoclasts may regulate bone formation by secreting sphingosine 1-phosphate (S1P), which seems to recruit osteoblast progenitor cells to sites of bone resorption and stimulate their differentiation (Raggatt and Partridge 2010).
Bone resorption

Osteoclasts are highly motile cells that move across the bone surface and resorb relatively large areas of bone. Attracting chemokines influence osteoclast migration and resorption (Boyce 2013). Bone resorption comprises osteoclasts dissolving the mineral crystals and then enzymatically degrading the organic tissue (Reinholt et al. 1990b). Bone resorption requires the adhesion of multinucleated osteoclasts to the bone matrix. Adhesion occurs when the osteoclast recognises the resorption site and its membrane receptors, especially 3 integrins, bind to sites on bone matrix proteins (e.g., OPN, vitronectin, bone sialoprotein and collagen) (Nesbitt et al. 1993; Reinholt et al. 1990a), forming the sealing zone (Salo et al. 1996). The sealing zone defines the area under the osteoclast where resorption takes place, named Howship’s lacuna. The formation of a ruffled border membrane as the resorbing organ of the osteoclast increases the surface area in contact with the matrix in the resorption lacuna. Hydrochloric acid dissolves the hydroxyapatite mineral phase, and proteolytic degradation by metalloproteinases and proteolytic enzymes (MMPs, cathepsin K and TRAP) dissolves the organic matrix (Lakkakorpi and Vaananen 1996; Teitelbaum 2000). Digested matrix proteins are cleared by endocytosis through the ruffled border, transported across the cytoplasm, and released by exocytosis into the extracellular space, a process named transcytosis (Boyce et al. 2012). These proteins also diffuse into the bloodstream and can be detected in the serum and urine. Some components, namely, the collagen fragments P1NP and P1CP, may function as a measure of bone resorption (Vasikaran et al. 2011).

Inflammatory disturbance of bone remodelling

Disturbed bone remodelling occurs when the balance between bone resorption and formation compromises bone homeostasis, as observed with ageing and in some local and systemic inflammatory conditions (e.g., osteoporosis, periodontitis, osteoarthritis, rheumatoid arthritis and tumours metastasising to bone), which are considered bone metabolic disorders (Agrawal et al. 2011).

The immune system responds to bacterial challenge by mobilising immune cells from the innate and adaptive immune systems to the local lesion, inducing an inflammatory response (Yucel-Lindberg and Bage 2013). The interplay between bone and immune cells is intricate because they have common progenitor cells in the bone marrow and share a number of cell surface receptors, cytokines, signalling pathways and transcription factors, which affect haematopoiesis, local immune responses and bone cell function (Walsh et al. 2006). The uncoupled bone remodelling observed in inflammation-induced bone loss is a conglomeration of events that is not fully understood and has not been fully explored in the context of peri-implantitis.
Several molecules produced in inflammatory reactions by infiltrating leukocytes or resident cells can regulate bone resorption and bone formation both in vivo and in vitro. Evidence of crosstalk between inflammatory cells and bone cells was first observed in 1972 (Horton et al. 1972), and the first cytokine associated with osteoclast activity was IL-1β (Dewhirst et al. 1985). Many cytokines, prostaglandins and certain growth factors have been shown to affect osteoclast and osteoblast differentiation (Braun and Zwerina 2011; Souza and Lerner 2013).

Under inflammatory pathological conditions and during the subsequent activation of the immune response, pro-inflammatory cytokines produced by activated immune cells (e.g., neutrophils, macrophages, T lymphocytes and dendritic cells) disturb the balance between bone-forming osteoblasts and bone-resorbing osteoclasts, often dominated by bone resorption. Immune cells produce a large variety of cytokines that are important regulators of osteoclast and osteoblast function. The pro-inflammatory cytokines suggested to promote osteoclast formation are e.g., TNF-α, INF-γ, IL-1β, IL-6, IL-8, IL-11, IL-12, IL-17, Il-20, IL-23, IL-32, and IL-34, the inhibitory anti-inflammatory cytokines are IL-4, IL-7, IL-10, IL-12, IL-13, IL-18, IL-27 and IL-33, and the chemotactic cytokines are IL-8, monocyte attractant protein-1 (MCP-1), and macrophage inflammatory protein-1α (MIP-1α). Several of these cytokines are suggested to be important mediators in the pathogenesis of peri-implantitis (Javed et al. 2011; Li and Wang 2014; Souza and Lerner 2013). An imbalance between pro-inflammatory and anti-inflammatory cytokines may impede the resolution of inflammation. Cytokines indirectly regulate cell-to-cell interactions, modulate the expression of RANKL and RANK on osteoblasts and osteoclasts, respectively, and influence intracellular signalling via RANK, with subsequent effects on bone remodelling. The major mechanism of the RANKL/RANK/OPG axis is decisive in the interactions between immune cells and bone cells in inflammatory conditions associated with bone loss. It has been reported that activated neutrophils from inflammatory sites express high levels of RANKL (Furuya et al. 2013) and upregulate RANKL after stimulation by LPS (Chakravarti et al. 2009). Furthermore, activated T cells produce RANKL and various cytokines, with a variety of effects on both osteoclastic and osteoblastic cells, depending on the bone environment and paracrine crosstalk (Walsh et al. 2006).

Among local tissue cells, fibroblasts may also influence peri-implantitis due to stimulation of tissue matrix degradation. Cytokines produced by infiltrates can enhance the inflammatory nature of the cells in a self-feeding loop (Bordin 2009). Inflammatory mediators and matrix metalloproteinases (e.g., MMP-7 and MMP-8) are upregulated in peri-implantitis fibroblasts (Irshad 2013). Periodontitis fibroblasts appear to produce OPG in response to LPS and IL-1, limiting osteoclast activity. However, gingival fibroblasts also enhance the production of inflammatory cytokines and indirectly influence RANKL-mediated
osteoclastogenesis. Thus, fibroblasts may participate in the regulation of bone cell activity (Hienz et al. 2015). The most commonly reported cytokine associated with peri-implantitis, IL-β, is secreted locally by macrophage/monocytes and dendritic cells in response to peri-implant infection and upregulates inflammatory reactions in concert with other factors associated with the local immune response. IL-β controls extracellular matrix degradation (Hannocks et al. 1992) and bone metabolism (Watrous and Andrews 1989). Sclerostin, a negative regulator of bone formation expressed by osteocytes in the bone matrix, appears to be upregulated by pro-inflammatory cytokines (Vincent et al. 2009). Sclerostin has been noted to be important in bone loss and was observed as a biomarker in peri-implantitis (Rakic et al. 2014); it has also been reported in excessive bone loading (Spatz et al. 2013).

Other studies that focused on peri-implantitis revealed interesting findings involving the immune system and the activation of bone resorption. TLRs act as membrane sensors for a variety of bacterial PAMPs, including adhesins, DNA and LPS (Akira et al. 2001; Koide et al. 2010). Immune cells may be activated by TLR2 and enhance the expression of RANKL in osteoblasts, which activate osteoclasts to perform bone resorption (Kassem et al. 2015). LPS directly triggers osteoblasts to express M-CSF and RANKL, which activate osteoclast differentiation and activity (Koide et al. 2010; Teng 2006). Another example of an inflammation-inducing exposure that may occur at the implant site was recently presented in an in vitro study in which the peri-implant tissue was shown to harbour titanium particles; titanium ions may activate inflammasomes in macrophages to release IL-1β, especially when exposed to microbial LPS (Pettersson et al. 2017a).

Numerous studies and results have corroborated immune-inflammatory-induced osteoclastogenesis as the central pathological process in peri-implantitis. Inflammatory disturbances in bone remodelling associated with uncoupled bone resorption and bone formation result in peri-implant alveolar bone loss. Hence, peri-implant bone loss appears to be driven directly and indirectly by mechanisms mediated by immune cells interacting with bone cells (Lang and Berglundh 2011).

**Interventions for peri-implantitis**

**Treatment and adjunctive methods**

The primary goal for intervention in peri-implantitis is infection control and achieving an inflammation-free state. The strategies for prevention and treatment were initially adopted from periodontal therapies, as the pathogenesis pathways seemed to be comparable. Evidence on the efficacy of nonsurgical and surgical therapies for peri-implantitis is limited.
An early stepwise approach called “Cumulative Interceptive Supportive Therapy” (CIST) was recommended by Lang and Mombelli in the 1990s. In response to signs of peri-implant inflammation, the first step was removal of the biofilm and improved oral hygiene, and the second step included additional local antiseptics. In patients with remaining inflammatory deep pockets in connection with radiographic bone loss, local antibiotics were recommended as the third step, always in combination with mechanical implant cleaning/debridement. As a last step, if the bone destruction progressed significantly, resective or reconstructive surgery was proposed in combination with adjunctive systemic antibiotic treatment preceded by microbial testing (Mombelli and Lang 1998).

**Prevention and nonsurgical debridement**

The initial step in CIST is still recommended for patients with signs of peri-implant mucositis. Supportive therapy, including nonsurgical mechanical implant cleaning, with or without antiseptic rinsing, and optimised self-performed oral hygiene reverses mucositis and prevents peri-implantitis (Heitz-Mayfield et al. 2011; Renvert and Polyzois 2018). Effective plaque control and supra-structures that permit cleaning with interdental brushes is crucial. Removal of the bacterial biofilm and calculus from the implant surface with mechanical debridement can control peri-implant mucositis effectively, and air polishing does not seem to be more effective (Ji et al. 2014). Adjunctive use of chlorhexidine with self-administered irrigation seems to be more effective than rinsing alone (Felo et al. 1997). However, the use of chlorhexidine gel (0.05%) on a manual tooth brush did not appear to have an adjunctive effect (Heitz-Mayfield et al. 2011).

Nonsurgical therapies for peri-implantitis lesions, including mechanical debridement, laser and/or photodynamic therapy, and adjunctive local antimicrobial or systemic antibiotic treatment, may lead to an improved clinical state, but these approaches are often insufficient to achieve resolution of the peri-implantitis lesion (Bassetti et al. 2014; Mombelli and Lang 1992; Persson et al. 2011; Renvert et al. 2008; Renvert and Polyzois 2018; Renvert et al. 2009b; Sahm et al. 2011; Schwarz et al. 2005).

**Surgical intervention**

In most cases, peri-implantitis lesions require flap surgery to access sub-mucosal intra-bony defects for removal of the inflammatory tissue and debridement of the implant surface. Access flap surgery seems to be more effective in achieving clinical attachment gains than nonsurgical therapies (Esposito et al. 2012) (Figure 3a+b). Decontamination of the implant surface by mechanical debridement and cleaning with gauze and saline is no less effective than the use of other techniques, e.g., air-abrasive, laser and photodynamic techniques. The application of different chemical agents to decontaminate the implant surface
(hydrogen peroxide, citric acid, sodium chloride, chloramines, tetracycline hydrochloride and chlorhexidine gluconate) did not reveal any single method to be more successful than the others (Carcuac et al. 2016; Claffey et al. 2008; Schou et al. 2003). Modifying the implant surface, with the elimination of supra-bony threads, implantoplasty, to reduce the retention of plaque and facilitate cleaning has been tested. Implantoplasty as an adjunctive intervention was reported to influence positively on implant survival (Romeo et al. 2005) and clinical outcomes after one year (Schwarz et al. 2011). A recent report of titanium-forming metal-protein aggregates with pro-inflammatory effects indicates that the dissemination of titanium particles should be considered (Pettersson et al. 2017a; Pettersson et al. 2017b). Another more common resective therapy is re-contouring of the crestal bone, osseous resection, to reduce the intra-bony defect (Berglundh et al. 2018; Charalampakis et al. 2011; Lagervall and Jansson 2013; Serino and Turri 2011), which is a radical adjunctive treatment that results in reduced pocket depth, but this technique is not always possible and may compromise the aesthetic outcome. Further, severe peri-implantitis, with a bone loss more than one third of the implant length, was found to impair treatment success (Charalampakis et al. 2011; Lagervall and Jansson 2013).

Reconstructive or regenerative interventions
The second goal in the treatment of peri-implantitis is to achieve bone regrowth into the intra-bony defects close to the implant surface. Animal studies demonstrated that re-osseointegration may occur on a previously contaminated implant surface (Renvert et al. 2009a). Histological evidence from humans is scarce, which makes it difficult to draw conclusions about regenerative efficacy and the potential for re-osseointegration of a previously decontaminated implant surface, although radiographic bone fill may occur (Renvert and Polyzois 2018; Wohlfahrt et al. 2011).

Different surgical methods have been used to promote bone fill of peri-implant bone defects with a non-submerged approach. Reconstructive methods have been performed using adjunctive autologous bone or bone substitutes, such as xenografts, alloplastic grafts or synthetic grafts (e.g., deproteinised freeze-dried bovine bone mineral (DBBM), algae-derived minerals, porous titanium granules, and nanocrystalline hydroxyapatite), as scaffolds for bone formation (Aghazadeh et al. 2012; Leonhardt et al. 2003; Renvert et al. 2018b; Roos-Jansaker et al. 2007; Schwarz et al. 2006; Wohlfahrt et al. 2012). The most tested approach is bovine bone mineral in combination with decontamination of the surface, often combined with a collagen membrane and antibiotics. From the short-term perspective of a year, complete resolution was possible, but successful treatment of peri-implantitis occurred in less than 50% of patients (Aghazadeh et al. 2012; Carcuac et al. 2015b; Renvert et al. 2018b).
Regenerative methods aim to recreate lost tissues by stimulating resident cells. EMD and platelet-derived growth factor (PDGF) have not been tested as single therapies, but in two studies, these adjuncts were applied in combined therapies, and promising results were reported for PDGF plus EMD and inorganic bovine bone, mineralised freeze-dried bone covered with a collagen membrane, or EMD and deproteinised bovine bone mineral with 10% collagen and doxycycline powder (Froum et al. 2012; Mercado et al. 2018).

The configuration of the bone defect, including the number of walls and the position of the implant in the jaw bone, seems to influence the predictability of the treatment outcome (Schwarz et al. 2010). It is also challenging to achieve a clean implant surface after biofilm contamination and retain long-term management to support resolution (Esposito et al. 2012; Khoshkam et al. 2016).

**Explantation**

The most radical treatment is to remove the implant completely. This approach might be the only option when no other methods seem to work, and the inflammatory process continues, with progression of bone loss around the implant.

**Supportive therapy**

Regular supportive therapy to maintain peri-implant mucosal health, with good oral hygiene and examinations to detect and address possible recurrence of peri-implant inflammation, is essential for long-term management (Costa et al. 2012; Roccuzzo et al. 2012). The mean maintenance therapy interval was demonstrated to influence the prevention of peri-implant disease (Monje et al. 2016). The probability of achieving a stable peri-implant condition with no bone loss seems to be higher when the pockets are shallow and free from inflammation (Berglundh et al. 2018; Carcuac et al. 2015b). In patients with a history of periodontitis and less strict maintenance, the frequency of bone fill was less than 50% (Lagervall and Jansson 2013).

**Evaluation of treatments**

When evaluating the treatment outcome, the circumstances of patient selection and the clinical setting may affect the external validity of efficacy studies (e.g., the probability of intervention under optimal conditions, such as treatment by specialists in university clinics or treatment in general practice). To reveal the ideal intervention for peri-implantitis, RCTs are needed. Today, there is limited information from RCTs concerning the treatment of peri-implantitis; although the tested treatments may work, no reliable evidence has yet been presented regarding which is the most effective intervention for treating peri-implantitis (Aghazadeh et al. 2012; Carcuac et al. 2015b; Esposito et al. 2012; Renvert et al. 2018b).
In summary, evidence on the treatment of peri-implantitis is limited but suggests that if early diagnosis and immediate intervention are performed, treatment may be successful. In addition, surgical therapy is superior to a nonsurgical approach, and plaque control and regular supportive maintenance, especially in susceptible patients, are crucial for preventing the recurrence of peri-implant disease.

Effects of antibiotics in the treatment of peri-implantitis
The rationale for using adjunctive antibiotics in the treatment of peri-implantitis is to achieve infection control. No evidence-based antibiotic regime has been established, and evaluations of the adjunctive effect of antibiotics in the treatment of peri-implantitis are scarce (Renvert et al. 2018a; van Winkelhoff 2012).

Adjunctive local antibiotic treatment
As an adjunct to nonsurgical mechanical debridement for peri-implantitis, locally distributed antibiotics, such as minocycline-containing microspheres or a slow-release doxycycline-containing preparation, were evaluated in RCTs, with clinical benefits up to 12 months. However, complete resolution was not obtained, and mechanical nonsurgical therapy with an adjunctive local antibiotic was regarded as insufficient for treating peri-implantitis (Renvert et al. 2008; Renvert and Polyzois 2018).

Adjunctive systemic antibiotic treatment
In addition to surgical treatment combined with systemic antibiotics, metronidazole and amoxicillin were proposed for the treatment of peri-implantitis (Heitz-Mayfield et al. 2012; Mombelli 2002). Adjunctive systemic antibiotics with various drugs and doses may contribute to a short-term improvement but do not seem to have any long-term effect, as indicated by both RCTs and prospective cohort studies (Carcuac et al. 2017b; Heitz-Mayfield et al. 2016). There are a few clinical studies on peri-implantitis and only one report with clinical and microbiological data for a 5-year follow-up (Leonhardt et al. 2003; van Winkelhoff 2012). Extensive surgical treatment, combined with adjunctive systemic antibiotics and local antimicrobial rinsing, was provided, and 47% of the subjects harbourred perio-pathogens after 5 years. Despite an extended antibiotic regimen for 4 weeks, the microbiological profile did not improve. Retreatment was necessary, and implants were lost, even though the antibiotic susceptibility test was used to individualise the choice of antibiotic (Leonhardt et al. 2003). The difficulties in eliminating the pathogens from a rough surface are an important topic since research recently pointed out that treatments for peri-implantitis are less successful in patients with modified implants, and the adjunctive effect of antibiotics in these cases also seems to be limited (Berglundh et al. 2018; Carcuac et al. 2015a; Charalampakis et al. 2014).
Superinfections, antibiotic resistance and adverse effects

Opportunistic peri-pathogens, such as Escherichia coli, Escherichia cloacae, Aggregatibacter actinomycetemcomitans, and Porphyromonas gingivalis, can reappear after some time, and extended antibiotic regimens will probably not prevent specific overgrowth (Leonhardt et al. 2003; Mombelli and Lang 1992). Systemically delivered antibiotics may induce overgrowth of opportunistic pathogens (Rashid et al. 2012; Sullivan et al. 2001). A normal symbiotic ecosystem may be converted to a dysbiotic condition by Staphylococcus aureus or EBV, which negatively influences the local innate immune response, allowing superinfecting bacteria and yeast to flourish (Verdugo et al. 2016). Superinfections, possibly induced and aggravated by antibiotics, may be responsible for rapidly progressive bone loss in peri-implantitis patients (Rams et al. 2014; van Winkelhoff 2012). Antibiotic resistance has been detected in approximately 70% of microbes in peri-implantitis subjects (Rams et al. 2014), and a single dose of amoxicillin may induce resistance in oral microbes (Khalil et al. 2016). Furthermore, adverse effects, such as gastrointestinal effects, were reported in 25% of the study population following combined metronidazole and amoxicillin treatment (Heitz-Mayfield et al. 2012), and hypersensitivity reactions are a well-known risk of antibiotic administration.

Long-term success and implant loss

The definition of implant success as the absence of mucosal inflammation, a pocket depth of 5 mm or less and a stable marginal bone level at the implant site was recently accepted. However, clinical or radiographic measures may not be sufficient for predicting a stable result after peri-implantitis therapy (Heitz-Mayfield and Lang 2010; Sanz and Chapple 2012). Positive healing outcomes, reported as treatment success, success rates, complete resolution or no sign of inflammation and no progressive bone loss, have been reported for approximately 50% of patients 3 to 7 years after surgical therapy with systemic antibiotics (Berglundh et al. 2018; Carcuac et al. 2017a; Heitz-Mayfield et al. 2016; Leonhardt et al. 2003; Roccuzzo et al. 2017; Roos-Jansaker et al. 2014).

Long-term success following peri-implantitis treatment seems to be insufficient, as disease resolution is inadequate, with recurrence after therapy (Charalampakis et al. 2012; Khoshkam et al. 2013; Lagervall and Jansson 2013).

Long-standing peri-implant disease may result in extensive bone loss or loss of osseointegration, requiring explantation of the implant. Information on implant survival or loss after peri-implantitis interventions is limited. In a 10-year follow-up of long-term management, with supportive therapy after implant placement and active treatment in response to signs of peri-implantitis, the rate of implant loss was approximately 10% in moderate to severe periodontally compromised
patients (Roccuzzo et al. 2012). After treatment of peri-implantitis, including surgical osseous re-contouring therapy with adjunctive antibiotics in the majority of patients, followed by supportive therapy, an implant loss or explantation rate of 8% 2-10 years after treatment was reported; in an RCT, this figure was 11% after 3 years (Berglundh et al. 2018; Carcuac et al. 2017b). An early 5-year follow-up resulted in an implant loss rate of almost 27%, although repeated treatments were performed when needed (Leonhardt et al. 2003).

Figure. 3a) Surgical treatment. b) Exploration. c) Application of EMD. d) EMD gel in syringe.

EMD

**Enamel matrix proteins and EMD**

EMD refers to a purified acid extract of naturally occurring enamel matrix proteins (EMPs), which form an insoluble matrix under physiological conditions. EMPs are synthesised during amelogenesis by ameloblasts in Hertwig’s epithelial root sheath during tooth formation, regulating enamel structure and the formation of roots, including periodontal ligament formation and the surrounding tissues. EMPs play a crucial role in the differentiation of progenitor cells into cementoblasts that specifically produce the acellular extrinsic fibre cementum. Early research demonstrated that the properties of EMD allow it to constitute a barrier, decreasing the speed of regrowth in the gingival epithelium and connective tissue and favouring periodontal ligament cells (Gestrelius et al. 1997; Hammarstrom 1997; Hammarstrom et al. 1997; Lyngstadaas et al. 2001). This knowledge led to studies on periodontal regeneration following EMD application (Heden et al. 1999; Heijl et al. 1997; Sculean et al. 2008a; Sculean et al. 1999a).

The commercially available product Emdogain® contains hydrophobic, purified amelogenins (EMD) and a vehicle of propylene glycol alginate (PGA), a gel product extracted from porcine tooth buds (Figure 3c+d). EMD consists primarily of amelogenins (90%) and a group of non-amelogenins, including
ameloblastin (sheathlin, amelin), amelotin, enamelin, tuftelin, and proteolytic enzymes (Bartlett et al. 2006). New applications for amelogenins are being explored in the field of bone regeneration, implantology, traumatology and wound care (Lyngstadaas et al. 2009).

**Periodontal regenerative effects**

A large body of information and evidence is available regarding the ability of EMD to support wound healing and new periodontal tissue formation. EMD affects many different cell types and functions: gene expression, protein production, proliferation and survival, migration, adhesion and chemotaxis. The differentiation of various cell types locally, mesenchymal and haematopoetic cells, is affected and often enhanced by EMD, especially periodontal ligament and osteoblastic cell types. EMD permits mesenchymal cell differentiation into hard tissue-forming cells and stimulates angiogenesis; however, it has a cytostatic effect on epithelial cells (Bosshardt 2008; Hammarström 1997; Hammarström et al. 1997; Miron et al. 2016a). In clinical use, when compared to placebo or control, EMD, as adjunct to surgical therapy for periodontal intra-bony defects, showed significant improvements in probing attachment level (mean difference 1.1 mm, 95% CI 0.61-1.55) and pocket depth reduction (0.9 mm, 95% CI 0.44-1.31). No adverse reactions were reported (Esposito et al. 2009; Heden et al. 1999; Heijl et al. 1997; Sculean et al. 1999b; Sculean et al. 2008b).

**Antimicrobial effects**

Both EMD and the vehicle PGA contribute to the reported antimicrobial effects (Newman et al. 2003; Olitzky 1965; Walter et al. 2006), in vitro and in vivo. The antimicrobial effect in vitro showed that EMD with PGA and PGA alone had similar inhibitory effects on the growth of the gram-negative periodontal pathogens *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, and *Prevotella intermedia* (Spahr et al. 2002). An ex vivo study on dental plaque showed that 54% of the bacteria were viable with EMD, but EMD with PGA or PGA alone reduced the bacterial viability to 20%; the viability was 32% with chlorhexidine and 77% with the saline negative control (Sculean et al. 2001). The antimicrobial effect on established supragingival biofilms in healthy individuals followed the same pattern (Arweiler et al. 2002). Compared to mechanical debridement alone, treatment of peri-implant mucositis with mechanical debridement combined with EMD significantly decreased the counts of *Porphyromonas gingivalis* at the 3-month follow-up. This antimicrobial effect was similar to that observed for antibiotic micro-spherical mini-cycline at treated sites. Additionally, clinically significant reductions in pocket depth and bleeding on probing were obtained (Faramarzi et al. 2015).
**Wound healing effects**

EMD healing properties in periodontal tissues have been presented in clinical reports on surgical techniques for treating recession, with favourable outcomes, such as increased keratinised tissues, increased root coverage and regenerative effects, with less post-operative morbidity (Tonetti et al. 2004). Inflammatory and healing responses are affected by EMD via effects on monocytes, macrophages, lymphocytes, neutrophils, fibroblasts and endothelial cells. *In vitro* studies of human cells revealed that EMD promotes decreased levels of inflammatory mediators, such as IL-1β, IL-8, and possibly TNF-α (Myhre et al. 2006; Nokhbehsaim et al. 2011), and increased levels of PGE2 (Dean et al. 2002; Sato et al. 2008). *In vivo*, accelerated wound healing as an effect of EMD was suggested when biomarkers such as TIMP, MMP-1, and MMP-8 decreased (Okuda et al. 2001) and TGF-β1 increased (Agrali et al. 2016) in gingival crevicular fluid (GCF) after regenerative surgical treatment of infra-bony defects in the teeth; however, information on the effects of EMD on GCF biomarkers is scarce.

**Osteogenetic effects**

EMD affects osteogenic cell proliferation, including progenitor cells, and these effects decrease with increasing cell differentiation and maturation (Grandin et al. 2012; Miron et al. 2016b). The majority of studies on EMD and bone remodelling suggest that EMD inhibits bone resorption by increasing osteoblast expression of OPG and decreasing RANKL expression by approximately 50% (Galli et al. 2006; Qu et al. 2011; Takayanagi et al. 2006). The effect on osteoclast formation was studied *in vitro* using a purified EMD fraction or Emdogain, and the treatment was concluded to induce osteoclast formation in mouse bone marrow cells via the RANK-RANKL interaction (Gruber et al. 2014; Otsuka et al. 2005). In another *in vitro* study a purified EMD fraction enhanced the osteoclast formation and bone resorption in monocytic cell line RAW 264.7 (Itoh et al. 2006).

Interestingly, reports in amelogenin knockout mice, which lack the main enamel matrix protein, revealed increased RANKL, the presence of osteoclasts and cementum resorption (Hatakeyama et al. 2003). Furthermore, osteoinductive activity of an amelogenin degradation product, leucin-rich amelogenin peptide (LRAP), comparable to that of the osteogenic factor BMP-2 was observed when mineral matrix formation increased in response to LRAP in wild-type and amelogenin knockout embryonic stem cells (Warotayanont et al. 2008). Moreover, sheath proteins, as ameloblastin degradation products, stimulated the *in vivo* cementum formation, bone growth and craniofacial bone formation (Fukae et al. 2006; Grandin et al. 2012; Tamburstuen et al. 2010).
Cell growth and bone-to-implant contact (BIC) have been investigated in a few studies. In culture, EMD enhanced the proliferation and viability of human osteoblast-like cells on titanium discs in a concentration-dependent manner (Schwarz et al. 2004). Human MG-63 osteoblasts cultured on titanium surfaces demonstrated a seven-fold increase in OPG expression in response to EMD, but no changes in RANKL were observed (Qu et al. 2011). In vivo studies on the healing of bone defects around implants placed in the femurs of rats treated with EMD or PGA showed contradictory bone formation results after four to six weeks (Franke Stenport and Johansson 2003; Shimizu-Ishiura et al. 2002). An in vivo dog model was used to investigate EMD and BIC for treating bone defects at titanium implants with guided tissue regeneration using EMD, guided bone regeneration (GBR) using a membrane or the combination of EMD+GBR. After a healing period of 3 months, no significant differences among the groups were observed (EMD 18.0%, GBR 24.4%, EMD+GBR 36.1%, and control 16.9%). The new bone area represented approximately 50% of the surface in both EMD- and GBR-treated implants. These results indicated a positive response with both techniques, but no significant difference was observed compared to controls, and the authors concluded that EMD may be involved in bone healing even in the absence of periodontal ligament cells (Casati et al. 2002).

In summary, the healing capacity of the EMD response in vivo may depend on synergistic effects among the various proteins and cells involved and may have a time-dependent effect. The limitation of biofilm growth may favour resolution of inflammation and wound healing. In addition, EMD is probably osteopromotive but not osteoinductive (Bosshardt 2008; Grandin et al. 2012; Miron et al. 2016b).
Aims

The overall aim of this thesis was to investigate the outcome of a regenerative surgical treatment approach with and without adjunctive EMD from the short- and long-term perspectives and to gain knowledge about the microbial flora and potential biomarkers in peri-implant sulci before and after treatment. Furthermore, an additional aim was to investigate whether EMD could directly affect osteoclast formation and activity.

- To compare clinical and radiographic outcomes and analyse effects on the microbiota after surgical treatment of peri-implantitis, alone or in combination with EMD. Further, we explored the relative importance of these variables for radiographic bone gain or bone loss at the implant site (paper I).

- To clinically and radiographically evaluate the long-term effects of surgical treatment of peri-implantitis with or without EMD and explore these variables in association with implant survival (paper II).

- To investigate protein expression in the PICF from the active peri-implantitis site and the protein variations after treatment with or without adjunctive EMD. Moreover, to explore whether the observed protein patterns could be related to covariations in clinical and radiographical outcomes, as well as adjunctive EMD treatment and implant survival (paper III).

- To investigate whether osteoclast formation and bone resorption are directly affected by EMD in vitro (paper IV).
Materials and Methods

Randomised clinical study
This thesis is mainly based on 29 patients who were recruited for a prospective, randomised, double-blinded, longitudinal, parallel-armed intervention trial.

Clinical, radiographic, and microbial results from the intervention trial were reported after 12 months (paper I), and clinical and radiographic follow-up were repeated at 36 and 60 months (Paper II). To identify and monitor changes in specific health- or disease-promoting proteins in diseased and treated implants, samples of PICF were collected before and after intervention during the first 12 months (paper III). Finally, an in vitro study was performed to investigate the potential effects of EMD on bone-degrading osteoclasts (paper IV). This section is an overview of the materials and methods. Details are reported in each paper.

Ethical considerations
The Regional Ethical Review Board in Lund, Sweden (Ref. no. 605/2007) and the Regional Ethical Review Board in Uppsala, Sweden (Ref. no. 2016/379), approved studies I-III. The intervention trial was conducted according to Good Clinical Practice Requirements, the ethical principles for medical research involving human subjects in the Declaration of Helsinki, a policy of the World Medical Association, and the Swedish law on personal data. CONSORT guidelines for clinical studies were considered. The intervention trial is registered at ClinicalTrials.gov NCT02500654.

Mandatory information concerning status, suggested therapy plans and optional treatments, potential risks and complications, preliminary prognosis and study information were provided both orally and in writing. The research subjects/patients agreed and signed written consent documents (papers I-III). These studies and results were independent of influence from financial sources/supporters.

Patient selection, group allocation and study design
All research subjects were patients who were referred to the Department of Periodontology, Clinic in Specialised Dentistry, Public Dental Care, Gävle, Sweden, and consecutively recruited to the study between 2008 and 2012. The inclusion criteria were peri-implantitis defined as a peri-implant pocket $\geq 5$ mm combined with bleeding and/or pus on probing at an implant with angular peri-implant bone loss $\geq 3$ mm measured on radiographs. All implant systems were accepted. The exclusion criteria were uncontrolled diabetes (HbA1c $> 63$ mmol/mol), intake of antibiotics or anti-inflammatory medication during the
past 3 months or use of drugs with known relevant side effects, such as gingival hyperplasia.

Twenty-nine patients were included, and each patient had one implant with bone loss $\geq 3$ mm. Two patients with less radiographic bone loss were excluded. The patients were from different parts of Region Gävleborg, with a median age of approximately 80 years when peri-implantitis was diagnosed. The patients were randomly allocated to surgical treatment with or without adjunctive application of EMD. The number of treated implants was 15 in the EMD group and 14 in the non-EMD group at baseline.

**Randomisation**

In this RCT, the randomisation was pre-defined by a computer-aided randomisation based on boxes (of four) and performed by a statistician. Information about the intervention, adjunctive EMD or non-EMD, was concealed in closed envelopes, which were marked with the subject research ID number, and not disclosed to the surgeon (CI) until the moment of application. The research subjects (patients) and the examiners (dental hygienists or radiology specialists) were blinded to the performed intervention.

**Clinical examination and data collection**

**Paper I:** Clinical measurements of full-mouth bleeding score (FMBS), full-mouth plaque score (FMPS), plaque, BOP, pus, and recession (REC) at the implant were performed before treatment at baseline and 3, 6, 9 and 12 months after surgical treatment. The implanted screw-retained bridges were removed at baseline and at the 12-month examination before registration of PD. One experienced dental hygienist (AB) performed all the measurements in the Department of Periodontology. To evaluate the reliability of the measurements, intra-observer agreement (AB) was estimated.

The number of osseous walls in the intra-bony defects was registered during the surgery as DefectM. Bacterial biofilm samples were collected at the implant site with the deepest pocket depth prior to surgical treatment and after 2 weeks, 3 months, 6 months, and 12 months.

**Paper II:** Clinical measurements at the 3- and 5-year follow-ups were performed either by a dental hygienist or a dentist in the Department of Periodontology or by the patient’s dentist or dental hygienist. FMBS, FMPS, the occurrence of plaque, BOP, pus and PD at the implant and implant loss were registered.

**Paper III:** Samples of PICF were collected before surgical treatment and after 3, 6 and 12 months. Clinical and radiographic data from studies I and II were related to the protein amount and diversity.
Radiographic examination
Peri-apical radiographs were collected at the baseline examination and at follow-ups 1, 3 and 5 years after treatment. Peri-implant defects ≥3 mm on radiographs were evaluated for the marginal bone level at the implant by a specialist in oral radiology (BS). The reliability, as indicated by intra-observer agreement (ICC), was evaluated in papers I and II. The visibility of the implant threads on one or both sides was analysed.

A full-mouth radiographic examination of patients with remaining teeth (23 of the 29 patients) was performed to measure the marginal bone support at the teeth.

Intervention
Patients with existing periodontal infections were treated prior to the peri-implant surgery with mechanical debridement and oral hygiene to achieve infection control. All surgeries were performed by one surgeon (CI). Bridges were removed, and incisions around the implant were extended to ensure access to remove the inflammatory granulation tissue and clean the implant surface. Calculus and bacterial biofilms were removed with an ultrasonic device and titanium scalers. Next, cleaning with cotton gauze and rinsing with sodium chloride solution were performed. Finally, before closing the flap, randomisation was revealed by opening the envelope, and either EMD or no EMD was applied. After suturing, the bridges were replaced. No mechanical cleaning of the treated site and rinsing with antimicrobial chlorhexidine gluconate solution, 2 mg/ml, for 6 weeks was recommended. Post-surgery supportive care was given frequently until a 12-month examination by dental hygienists in the Department of Periodontology. After 3 and 5 years, examinations and maintenance were performed on a regular basis 2–4 times per year.

Microbiota analysis
Sampling
Sub-mucosal bacterial biofilm samples were collected at the implant site with the deepest pocket depth immediately prior to surgical treatment and after 2 weeks, 3 months, 6 months, and 12 months. Sterilised paper points were inserted into the peri-implant pocket for 30 seconds and then stored at -20°C until DNA extraction.
Sample preparation and Human Oral Microbe Identification Microarray (HOMIM) analysis

DNA was extracted from the biofilm samples using the Gen Elute Bacterial Genomic DNA kit (Sigma Aldrich, St. Louis, MO) according to the manufacturer’s instructions. Evaluation of the final quality and quantity of the DNA was performed with a Nanodrop 1000 spectrophotometer in the Department of Molecular Periodontology, Institute of Odontology, Umeå University. The prepared DNA samples were sent for microbiota characterisation by the HOMIM at the Forsyth Research Institute in Boston, MA (http://mim.forsyth.org/homim.html). At the time of the analysis, the HOMIM consisted of 422 oligonucleotide probes targeting more than 300 oral bacterial taxa. The lower limit of detection of the hybridisation signals was $10^4$ cells.

Proteomic analysis

Sampling PICF

PICF was collected immediately prior to the surgical treatment and after 3 months, 6 months and 12 months. One paper strip, 1.5x3 mm, of Whatman® filter paper, was placed in the peri-implant mucosal crevice at the implant site with the deepest peri-implant pocket for 30 seconds. The samples were immediately eluted and stored at -80°C. All samples were transported to the laboratory on dry ice.

LC-MS

The proteome of PICF samples was analysed with liquid chromatography-mass spectrometry (LC-MS). Briefly, 100-µL PICF samples were extracted, cleaned and concentrated, and 5 µL of eluate was passed through an electrospray ionisation (ESI)-equipped mass spectrometer.

Bioinformatics

Identification of proteins of interest and their functions and interactions was performed on a computer or via computer simulation in silico. The data were processed by ProteinLynx Global SERVER v.3.0.3, and the resulting spectra were searched against the UniProt Human databank. A minimum of three fragments were required, with peptide and fragment tolerances of 10 and 25 ppm, respectively, and a false discovery rate (FDR)<0.05. Protein functions were assessed using Database for Annotation, Visualisation and Integrated Discovery (DAVID) Bioinformatics Resource 6.8, and protein-protein interaction networks were assessed using the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database.
Osteoclast formation and bone resorption in vitro

Ethical considerations
Animal care and experiments were approved under and conducted in accordance with the regulations of the Local Animal-Ethical Committee at Umeå University (Ref. no. A6-17) (paper IV).

EMD and purification
Emdogain®, a commercial product, includes both EMD and PGA gel, and the latter component causes a low pH, which disturbs bone marrow macrophage (BMM) proliferation. Thus, Emdogain® 0.3 ml was dialyzed against phosphate-buffered saline (PBS) to remove the PGA gel. A final volume of 3.7 ml was reached after approximately 12x dilution to a solution of 2.5 mg/ml.

Mice
Male CsA mice, 5-9 weeks old, from an inbred colony in the Department of Molecular Periodontology were used for the experiments.

BMMs
Bone marrow cells were harvested from the tibia and femur. The bone was aseptically dissected free from the soft tissue in PBS. The cartilage bone ends were cut off, and the bone marrow cells were flushed out with a sterile syringe and alpha minimal essential medium (α-MEM) supplemented with 10% foetal bovine serum (FBS) and antibiotics (benzylpenicillin, gentamycin sulphate, and streptomycin), hereafter referred to as culture medium. Erythrocytes were lysed in ice-cold sterile water (Figure 4a-d). The remaining cells were collected and placed on Corning Suspension Culture Dishes with culture medium and additional M-CSF to stimulate proliferation of the monocyte/macrophage lineage. After incubation at 37°C with 5% CO₂ for 72 hours, the non-adherent cells were removed, and the adherent macrophages were collected and cultured for further osteoclast experiments.

Osteoclast formation and activity
The BMMs were seeded in culture medium, and M-CSF (30 ng/ml) and different concentrations of RANKL (2 or 4 ng/ml) were added to stimulate osteoclast formation. Purified EMD at three different concentrations (1, 10, and 100 µg/ml) was added to the cultures. The culture medium was changed after 72 hours, and the experiment was discontinued after 100 hours. The medium was collected after 72 and 100 hours and analysed for TRAP5b with ELISA. The cells were fixed and stained for TRAP, and photomicrographs were collected.
Osteoclast function and bone resorption

The effect of EMD on osteoclast function was analysed using bone slices from cows that were placed in microtiter wells. BMMs were seeded in culture medium, and osteoclast formation was stimulated with M-CSF (30 ng/ml) and RANKL (2 or 4 ng/ml). Purified EMD (100 µg/ml) was added to the cultures. The culture medium of the bone slices was changed on days 3 and 6, and the experiment was discontinued on day 9. The culture medium was collected on days 6 and 9 and analysed for TRAP5b and CTX-1 (Figure 4 c-d).

TRAP staining, cell counting and resorption assessment

The cells were fixed and stained for TRAP. TRAP staining of cells on plastic and on bone slices was performed using a kit with leucocyte acid phosphatase, according to the protocol provided by the manufacturer. TRAP-positive cells with a minimum of three nuclei were considered to be osteoclasts. The number of osteoclasts on plastic slices was counted using a light microscope (Olympus, BX41).

ELISA

A commercially available ELISA kit was used to assess the amount of TRAP5b and CTX-1 in the collected medium. Samples were added to polystyrene microwells pre-coated with analyte-specific capture antibodies prior to the addition of an enzyme-conjugated antibody that also bound to the analyte of interest. The addition of an enzyme substrate resulted in a colour change; the intensity of this colour change correlated with the protein concentration and was analysed using a spectrophotometer.

Statistical analysis

The RCT analysis was by per-protocol, and drop outs were excluded from further clinical and radiographic analysis in the follow-up (papers I, II, III), while implant survival was carried forward to the 5-year follow-up (paper III). The bacterial data were retained in the analysis throughout the study (paper I). The statistical power calculation in the RCT was based on a 0.5 mm difference in probing pocket depth between groups and a standard deviation of 0.5 mm with a power of 80%. The power was calculated for 16 in each group with $\alpha = 0.05$. Each paper includes details concerning data handling and statistical analysis. Intra-class correlation (ICC) was performed for pocket depth and radiographic assessments (papers I, II and III).

A non-parametric approach was used in all papers. Differences between groups were analysed using Fisher’s exact test or the Chi squared test for categorical
variables, and the Mann-Whitney U-test, the exact sig (2-tailed) test between groups and Wilcoxon’s Signed Rank test for related samples for continuous variables in papers I-II. The number of TRAP-positive, multinucleated cells and the concentrations of TRAP5b and CTX-1 in the culture media were compared with the Kruskal-Wallis exact test, and the experiments were repeated at least twice with similar results. In case of an overall significant difference, pairwise Mann-Whitney U tests were subsequently performed. Adjusted p-values for multiple testing were obtained with the Bonferroni test (paper IV). P-values <0.05 were considered statistically significant (papers I, II, III and IV). Monte Carlo simulation with 10 000 replicates was used for variables with more than two categories. Bacterial taxa were dichotomised (HOMIM score ≥ 1 or =0, respectively), and bacterial taxa with a p-value ≤ 0.01 were considered significant (paper I).

A multivariate model of principle component analysis (PCA) was used to analyse the separation of subjects by bone gain or no bone gain. In the search for variables associated with bone gain or bone loss, analysis with multivariate projection to latent structures, i.e., partial least squares (PLS) regression was performed. This linear regression model was used to find hidden variables that may explain a positive change in bone levels (bone gain) and is a suitable model when there are more variables than observations. Variables with variable importance in the projection (VIP) >1.5 were considered influential (paper I). The PLS regression was also used to search for variables associated with implant survival in months as the dependent variable and VIP>1.0 is indicated. The variables utilised for PLS regression were auto-scaled and logarithmically transformed as needed to improve normality (paper II).

To identify the primary clustering of subjects by the presence of proteins, PCA was used to visualise hierarchical clustering. Further, to identify variables/factors explaining the variations between subjects, orthogonal partial least square discriminant analysis (OPLS-DA) regression was performed. All variables utilised for PCA and OPLS-DA regression included only qualitative protein data (0/1). VIP>1.8 were considered influential. Q² was presented as an estimation of the predictive ability of the model. The generalised estimating equation (GEE) method was used to examine longitudinal data (paper III). The Kaplan-Meier method was used to visualise implant survival over time (paper II).
Results and discussion

Clinical, radiographic, microbial and long-term evaluations (papers I and II)

The major platform for this research project was the following question: “Does adjunctive EMD in surgical treatment of peri-implantitis have any effect on radiographic bone level changes, clinical healing, and microbial flora?” The aim was to investigate the effect of EMD, as a single adjunctive treatment for open flap debridement, in an RCT.

The results from the 12-month evaluation, after the surgical intervention and following supportive treatment, showed improved clinical parameters in both groups, with reductions in bleeding on probing and pus and a significant pocket depth reduction of 2-3 mm, which were similar to the levels reported in the majority of previous studies (Daugela et al. 2016). However, there was no significant difference in the clinical variables between the groups during the follow-up period. This finding might be explained by the re-colonisation of the implant surface 6 to 12 months post-surgery, as reported in other studies that performed microbial evaluation (Leonhardt et al. 2003; van Winkelhoff et al. 2000); this re-colonisation might compromise treatment success (Sanz and Chapple 2012), which is often reported as less than 50% (Aghazadeh et al. 2012; Heitz-Mayfield et al. 2012; Renvert et al. 2018b).

The median radiographic bone level change of approximately +1 mm for the EMD-treated implants was limited, with no significant between-group differences. When multivariate PCA was performed, a clustering of subjects by bone gain or no bone gain emerged. This significant positive association between EMD and bone level gain might indicate an initial wound healing and regenerative effect in the treatment of peri-implantitis and is consistent with the effect observed after EMD treatment of periodontitis (Bosshardt 2008; Esposito et al. 2009; Sculean et al. 2008b). Bone gain was associated with EMD treatment in a PLS model, together with an increased number of osseous walls in the peri-implant defect and a gram-positive/aerobic microbial flora, while bone loss was associated with gram-negative/anaerobic microbial flora, repeated bleeding on probing and pus. The multivariate PLS model suggested a rather strong association, with a cross-validated predictive capacity (Q^2) of 36.4%.

The microbial flora before surgery included 106 unique species/phylotypes in at least two samples. Unrestricted detection of several hundred species or more is possible by metagenomics, showing a great diversity between samples (Koyanagi et al. 2013; Maruyama et al. 2014), but most studies have reported a restricted
number of species identified with molecular methods (Charalampakis and Belibasakis 2015). In this trial, the microbial flora decreased significantly after surgical treatment in both groups. Furthermore, in the EMD-treated implant lesions, a significant shift to a more gram-positive/aerobic microbiota was observed in the short term. However, at the 12-month follow-up, the microbial flora returned almost to baseline levels (Figure 4). A similar pattern with reduced Porphyromonas gingivalis and bleeding on probing after treatment was observed for nonsurgical debridement with adjunctive EMD treatment (Faramarzi et al. 2015). The antimicrobial effect of EMD suggests that extended EMD treatment at post-surgery follow-ups might be beneficial for preventing the recurrence of peri-implant inflammation and improving soft tissue healing. The potential effect of EMD on bone gain might reflect diminished bone resorption as a result of the initially reduced gram-negative/anaerobic microbial colonisation and inflammation at implant surfaces and/or a direct effect of EMD on bone cells.

The microbial flora in the samples were consistent with the bacteria observed in periodontitis. Two distinct bacterial profiles have previously been found at healthy sites, with mainly gram-positive cocci, non-motile bacilli and a limited number of gram-negative/anaerobic species, and at peri-implantitis sites, with gram-negative, black-pigmented, motile and anaerobic species that are commonly found in periodontitis (Charalampakis et al. 2012; Leonhardt et al. 2003; Mombelli et al. 1987). A more opportunistic flora, with Staphylococcus
spp., enterics, yeast and Candida spp., has also been reported in peri-implantitis (Leonhardt et al. 1999). The less pathogenic microbial flora balances the microbial flora present after EMD treatment.

Oral hygiene measured by FMPS and local plaque at the implant site showed a decrease in plaque prevalence from baseline but seemed to increase at the end of the follow-up period in some individuals. Although the supra-structures were adjusted to facilitate self-performed oral hygiene, the perfect plaque control that is crucial to successful implant therapy (Pontoriero et al. 1994; Serino and Strom 2009; Serino et al. 2015) and peri-implant health (Rocuzzo 2012, Costa 2012) was not always maintained.

RCTs on treatments for peri-implantitis are sparse, and there is no clinical study on EMD as a single adjunct. A recent RCT on surgical intervention comparing adjunctive treatment with or without bovine xenograft combined with systemic antibiotics found limited bone level changes in favour of xenograft at the 12-month follow-up. The treated intra-bony defects included 3 and 4 walls (Renvert et al. 2018b), which was associated with bone gain at the implant site in our analysis. It has been suggested that the circumferential intra-bony defect may have the most promising impact on the clinical outcome of surgical regenerative therapy of peri-implantitis lesions (Schwarz et al. 2010).

To further explore the long-term results of adjunctive EMD treatment in terms of clinical, radiographic and implant survival outcomes, 3- and 5-year follow-up was performed (Figure 5). Implant survival after 3 years was 100% in the EMD-treated implant group and 83% in the non-EMD group, and at the 5-year follow-up, implant survival was 85% and 75% in the EMD and non-EMD groups, respectively. This finding is similar to the implant survival of 89% reported in an RCT with a 3-year follow-up after peri-implantitis treatment (Carcuac et al. 2017a), and rates of 71% to 73% have been reported in retrospective studies and case series (Lagervall and Jansson 2013; Leonhardt et al. 2003).

Notably, fewer implants were lost in the EMD-treated group, and further implant survival analysis with Kaplan-Meier plot revealed prolonged survival of EMD-treated implants. The loss of two implants in the EMD group occurred after three years, and in the non-EMD group, three implants were lost at 1 year, before 3 and 4 years. Successful treatment with no further need for treatment due to reinfections or removal of the implant occurred in almost 70% of the EMD-treated patients and in 40% of the non-EMD patients. Treatment success rates between 33 and 58% 3 to 5 years following surgical therapy with systemic antibiotics have been reported, although these reports were based on varying definitions of treatment success (Berglundh et al. 2018; Carcuac et al. 2017a; Heitz-Mayfield et al. 2016; Leonhardt et al. 2003; Roos-Jansaker et al. 2014).
In the search for variables that may explain the variations in implant survival time, PLS regression revealed that implants with the longest survival clustered apart from those with shorter survival time, forming two groups. The variables that significantly influenced longer survival time in this trial were positive bone level changes at the implant site and EMD treatment, while shorter survival time was influenced by pocket depth at the 1-year follow-up, bone level measurements at the 3- and 5-year follow-up, plaque at the 3-year follow-up and the number of cigarettes at the 1- and 3-year follow-up. The VIP score in this analysis indicated that an early sign of a positive bone level change and EMD treatment might be associated with implant survival, while shorter survival may be associated with signs of re-infection, such as plaque, pus and decreased bone levels, together with smoking. Known risk factors of peri-implantitis are poor plaque control and a lack of regular supportive therapy (Costa et al. 2012; Roccuzzo et al. 2012). Fifty percent of the subjects in our trial reported a history of periodontitis, which may indicate susceptibility to developing inflammation-induced jawbone loss. The association between peri-implantitis and smoking remains unclear, but there is an enhanced risk of biologic complications among smokers and an enhanced risk of implant failure (Strietzel et al. 2007), which might have an impact on the resolution of peri-implantitis after treatment. The number of cigarettes decreased in both groups until the end of the follow-up period, due to smoking cessation and the effect of lost implants/subjects. No significant differences between groups were observed from baseline to 5-year follow-up. In contrast, medication for coronary heart disease was significant in the EMD-treated group at baseline, and the influence of such medication on peri-implantitis has been discussed, but no association between CHD or its medication and peri-implantitis has been confirmed; this topic is rarely investigated (Koldsland et al. 2011; Schwarz et al. 2018). Uncontrolled diabetes was a criterion for exclusion in this trial since this systemic condition is associated with periodontitis, and it is unclear whether diabetes is associated with peri-implantitis.

In summary, adjunctive EMD might change the microbial flora, which may promote regeneration and implant survival; however, this issue needs to be further explored.

Figure 5. Radiographs. a) Before treatment. b) 1 year. c) 3-year. d) 5-year follow-up.
IN study III, we aimed to explore the PICF protein content with a hypothesis-free approach, analysing the global proteome using mass spectrometric analysis techniques. By monitoring implant loss in relation to proteomic PICF profiles at baseline and 3, 6 and 12 months after surgery, we tried to explore the protein patterns associated with active peri-implantitis.

Investigation of the peri-implant proteome before and after treatment of peri-implantitis has not been performed previously. Regarding EMD treatment in cases of periodontitis, there is no corresponding proteomic analysis (Bostanci and Belibasakis 2018; Dursun and Tozum 2016). PICF analysis following peri-implantitis treatment is scarce (de Mendonca et al. 2009; Duarte et al. 2009; Wohlfahrt et al. 2014). The majority of studies investigated PICF at healthy or diseased peri-implant sites to identify key inflammatory factors with single-candidate protein immunoassays, such as ELISA, or multiplexing methods involving immunobeads. We screened the proteome using liquid chromatography-tandem mass spectrometry (LC/MS). A total of 486 unique human proteins were identified in 99 PICF samples, and the number of proteins was 42 (32-52) for all time points and samples. A proteomic reference of GCF identified 432 human proteins at healthy and diseased sites (Baliban et al. 2012) and 275 proteins from healthy tooth sites with LC/MS (Tsuchida et al. 2012).

The key finding was the clustering of subjects based on their PICF proteomic profiles from 3 to 12 months after surgery. Two major clusters were explored, and the PICF protein profile differed significantly. Cluster 2 was related to implant loss, whereas cluster 3 was related to implant survival and EMD treatment after 5 years. Belonging to cluster 2 had an odds ratio for implant loss of 7.9 (95% confidence interval 1.8 to 35.9). Cluster 2 was also associated with increased protein concentration, protein diversity, BOP and radiographic bone loss but not PD ≥ 5 mm or the presence of pus. The lack of an association with pocket depth may be due to the large variation in peri-implant mucosal thickness, which depends on the placement of the implant and the relation to anatomical structures (Fuchigami 2017). This inconsistency calls into question the reliability of PD assessment as a diagnostic variable.

PICF volume and protein concentration are known to increase with increasing inflammation (Bostanci and Belibasakis 2018; Griffiths et al. 1992; Loe and Holm-Pedersen 1965), and the diversity of proteins might explain the differentiation between the two clusters by implant loss or survival. Adjunctive EMD treatment was significantly associated with reduced protein diversity. Interestingly, in the EMD-treated subjects, 40 proteins were reduced, of which
62.5% overlapped with increased proteins associated with implant loss; in addition, 72.5% of the increased proteins associated with cluster 2. The antibacterial effect of EMD might also indirectly reduce the release of inflammatory cytokines, as the regenerative effect and wound healing of EMD is well known (Miron et al. 2016a). The PICF proteins associated with both cluster 2 and the implant loss were also associated with a wide range of biological functions, such as stress response, wound healing, cell death/apoptosis, inflammatory response, and cell activation. This result strengthens the current hypothesis that an inflammatory process driven by bacterial biofilm formation and the host immune response may lead to implant loss (Heitz-Mayfield and Lang 2010). Furthermore, the qualitative proteomic investigation indicated the possibility of changing the proteome with adjunctive EMD in surgical therapy. However, biomarkers linked to the PICF in peri-implantitis, such as IL-1β and TNF-α (Dursun and Tozum 2016), were not detected through this LC-MS proteomic approach. The potential reasons could be methodological, including low concentration and/or high turnover of unique proteins, LC-MS pre-processing, sensitivity and influential protein abundancy (Bostanci and Belibasakis 2018), but this issue needs to be further evaluated.

Crucial factors for future reliable PICF analysis are subject selection, peri-implantitis diagnosis definitions, PICF sampling methods (e.g., the number and severity of sites and the sampling time) and sample handling, as well as the sensitivity/specificity of the assay used. Another factor to consider is that biomarkers that remain in the tissue environment for various lengths of time during different stages of inflammatory events also vary simultaneously in the PICF, giving an instant view of a single PICF collection time point (Dursun and Tozum 2016).

In summary, two different human protein expression responses emerged after surgery in peri-implantitis patients: one response (cluster 2) was associated with implant loss, bone loss and bleeding upon probing, whereas the other response (cluster 3) was associated with implant survival and EMD treatment. However, whether these effects are direct or indirect requires further exploration.

**Effects of EMD on osteoclast formation and bone resorption (paper IV)**

In our clinical peri-implantitis study, a positive bone gain and implant survival trend emerged in response to EMD treatment. We hypothesised that bone gain, in addition to the documented wound healing and osteopromotive effect of EMD, could also in part include decreased osteoclast formation and/or activity. Thus,
we added Emdogain® to RANKL-stimulated primary mouse BMM cultures in an attempt to study its effects on osteoclast formation. However, the pH in the culture media dropped when we added Emdogain®, and the cells detached. Therefore, we removed the acidic PGA gel from Emdogain® and added the purified EMD to RANKL-stimulated BMM cultures. EMD did not affect the number of osteoclasts formed on bone slices or the collagen fragment (CTX) levels in the culture medium in these experiments.

In contrast to our results, two other studies have shown that EMD can promote osteoclast formation in crude mouse bone marrow cell (BMC) cultures (Gruber et al. 2014; Otsuka et al. 2005). BMCs contain a heterogeneous mix of cells of haematopoietic and mesenchymal origin; therefore, it is not clear which cell types have a molecular effect. However, Otsuka and collaborators showed that a specific fraction of EMD increased RANKL expression in osteoblasts and that even greater numbers of osteoclasts were developed if EMD-treated BMCs were cocultured with osteoblasts. This finding indirectly shows that EMD probably induces the formation of osteoclasts in BMC cultures through increased RANKL expression in cells of mesenchymal origin (Otsuka et al. 2005). Gruber and collaborators added the complete Emdogain® product to crude BMC cultures and reported increased osteoclast formation, possibly mediated via transforming growth factor-beta receptor type 1 (TGF-bR1) kinase signalling (Gruber et al. 2014). It is not clear which cells in the BMC culture that mediated the osteoclastogenic effect, and it is surprising that the cells survived the acidic PGA gel.

We used pure primary osteoclast cultures, and the lack of an effect of EMD on osteoclast formation is not directly comparable to the results obtained from crude BMC cultures. The latter cultures include cells of mesenchymal origin, such as bone marrow stromal cells, and other haematopoietic cells, such as bone marrow monocytes, e. g., lymphocytes, that could influence osteoclast formation. We only found one report on the effects of EMD on osteoclastogenesis in pure osteoclast cultures. A study by Itoh et al. showed that a purified EMD fraction increased osteoclast formation in cultures of the monocyte/macrophage cell line RAW 264 (Itoh et al. 2006). The EMD fraction increased the expression of RANK protein in RAW 264.7 cells, and the authors proposed that EMD enhances osteoclast formation and bone resorption by increasing the number of cells that undergo RANKL-mediated terminal differentiation to mature osteoclasts as a result of not stimulating the proliferation of the monocyte/macrophage lineage (Itoh et al. 2006). Immortalised cells, such as RAW cells, do have features that differ from those of primary cells in culture. Thus, it is not surprising that the effects of EMD on osteoclastogenesis differ between our study and the study performed by Itoh.
et al. Another difference is that we used the entire purified protein content of Emdogain®, while Itoh et al. used a specific EMD fraction (Itoh et al. 2006).

When we added EMD to RANKL-stimulated BMMs on natural bone slices, we did not detect any change in bone resorption compared to those in RANKL-stimulated BMMs alone. Itoh et al. studied the osteoclastic resorption capacity of RAW 264.7 cells cultured on synthetic calcium phosphate thin films. Using the latter artificial substrate, they showed increased calcium release in RAW cells stimulated by RANKL and EMD compared to that in RAW cells stimulated by RANKL alone. Because the authors also show an effect of EMD on osteoclast numbers, it is expected that there would be a larger resorption area in the substrates (Itoh et al. 2006). Thus, the indirect effect of EMD on osteoclast formation is unclear.

In summary, our results show that EMD did not influence primary osteoclast formation or bone resorption in vitro. These findings suggest that the regenerative effect of EMD observed after clinical use is probably not due to a direct effect on osteoclast formation or bone resorption.
Conclusions

The observations from the RCT presented in this thesis, indicate that adjunctive EMD may support a less pathogenic microbial flora and a protein profile associated with implant survival, providing information for the expanding field of PICF proteomics. The finding, that EMD is associated with increased radiographic marginal bone levels and possible prolonged implant survival, indicate a trend toward a positive healing response with EMD. Furthermore, these results suggest that the effect of EMD on osteoclasts is indirect. In summary, the findings supporting our conclusions are as follows:

• Adjunctive EMD in surgical treatment of peri-implantitis changed the peri-implant microbial flora, with a reduction in gram-negative/anaerobic bacteria and an increase in gram-positive/aerobic bacteria. Associations among EMD treatment, gram-positive/aerobic bacteria, the number of osseous walls in the bone defect and radiographic bone gain were observed.

• Adjunctive EMD is one factor that might be positively associated with implant survival up to 5 years after treatment. Within the limit of the study, the multivariate analyses demonstrated that prolonged survival was associated with EMD and radiographic bone gain (paper II).

• Two different clusters of PICF protein expression after surgery in peri-implantitis patients emerged: one cluster was associated with an increased risk of implant loss, bone loss and bleeding upon probing, whereas the other cluster was associated with implant survival and EMD treatment and related to reduced protein diversity (paper III).

• Pure EMD did not to directly affect osteoclast formation nor bone resorption in vitro (paper IV).
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