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BIOMOLECULES AND MATERIAL-TISSUE INTERACTIONS IN REGENERATIVE DENTISTRY

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Abbreviations

AAP	American Association of Periodontology
BMMSC	bone marrow mesenchymal stem cells
BMPs	bone morphogenetic proteins
CAD	computer-aided design
CAM	computer-aided manufacturing
DMLS	direct metal laser sintering
DLP	digital light processing
EMD	enamel matrix derivatives
FDA	Food and Drug Administration (USA)
GBR	guided bone regeneration
GTR	guided tissue regeneration
HA	hyaluronic acid
hMSCs	human mesenchymal stem cells
HMW	high-molecular weight
IL	interleukin
LAMP	lysosomal associated membrane protein
LMW	low-molecular weight
PDGF	platelet-derived growth factor
PDL	periodontal ligament
PDLSC	periodontal ligament stem cells
PGD2	prostaglandin D2
PGE2	prostaglandin E2
rh	recombinant human
SLA	stereolithography
SLM	selective laser melting
ULMW	ultra low-molecular weight

1. Introduction

1.1. Background

Periodontal disease is a major cause of tooth loss in adults, affecting a considerable proportion of the population worldwide. It has a significant impact on masticatory function and the quality of life for those affected (Ferreira et al. 2017). The most recent Global Burden of Disease Study (Disease et al. 2018) estimates that 670 to 930 million people worldwide suffer from this condition, which can vary in severity.

Periodontitis is not due to aging (Huttner et al. 2009), but increases with aging (Konig et al. 2010). In the US population, it has been shown to increase from 24.8 ± 1.9% in 30 to 34-year-old adults to 68.0 ± 2.2% in adults aged 65 years and older (Eke et al. 2015). A more widespread prevalence of periodontitis has been reported in Germany, where it interests respectively 70.9% and 87.4% of the age cohorts (Holtfreter et al. 2010). Due to the progressive aging of the global population, with the number of people over 60 being more than double by 2050 (2013, 2015), the incidence of periodontitis is expected to increase steadily (Schwendicke et al. 2018). On one side, improved oral health conditions and therapies have resulted in a decline in edentulism caused by periodontitis in various European Union countries (Sheiham and Netuveli 2002). On the other hand, this raises a greater need to target the functional and aesthetical issues linked to the alveolar bone loss around the remaining teeth (Madianos et al. 2016).

The main aim of periodontal therapies is the control of the infection by transitioning from a state of microbial dysbiosis to a beneficial subgingival microbiome (Van Dyke et al. 2020, Hajishengallis et al. 2023). Although conventional treatment measures can impede the progression of periodontitis, they cannot fully restore the tissue architecture that has been compromised by this condition (Hasturk and Kantarci 2015). In fact, after the treatment of periodontitis itself, very often, what remains are periodontitis-associated tissue defects.

Several regenerative procedures have been studied to re-establish lost or injured tissues, which combine the osteoconductive, osteogenic, or osteoinductive properties of graft materials, as highlighted by Ramseier et al. (Ramseier et al. 2012). In this context, it is essential to distinguish between the concepts of regeneration and repair. The American Association of Periodontology defines regeneration as the reproduction of a lost or damaged part, in a way that closely resembles or identical to its original form. In contrast, repair refers to the healing of a wound with tissue that does not fully restore the architecture or function of the original part (AAP). The common periodontal treatments result in tissue repair (Polimeni et al. 2006), with the junctional epithelium repositioned apically during this healing process. However, the optimal objective should be to achieve complete regeneration of the tissues, with the formation of new attachment. In fact, the significance of achieving a reliable and effective regeneration of a fully functional periodontal organ system was emphasized during the first consensus workshop on regenerative therapies for periodontal disease, which took place in 2014 (Cochran et al. 2015).

In order to achieve this, cell-based and cell-free approaches are being developed and tested (Galler et al. 2014). Cell-based clinical applications have been more complicated, partly due to ethical concerns (Volarevic et al. 2018). Meanwhile, there has been a wide use of cell-free treatments with materials that can leverage the presence of cells in the host tissues, mainly that of endogenous mesenchymal stem cells.

1.2. Aim of the study

The primary focus of this research lies in exploring the underlying biological principles of the employed regenerative materials and their role in facilitating healing processes. Through more comprehensive cytological analysis, this work aimed to gain a deeper understanding of how specific biomolecules function and contribute to the regeneration of periodontal tissues.

Additionally, this habilitation work focuses on the application of biomolecules in the diagnosis of periodontal treatment need and in monitoring the results of periodontal therapy. By studying these components, their potential can be exploited in clinical practice.

Another key objective of this research is to evaluate factors and materials that impact various aspects of periodontal and peri-implant health. Specifically, it seeks to examine how regular check-ups may influence the implant surface, as well as the compatibility of new materials for 3D printing of temporary restorations on cells of the periodontal tissues. By investigating these factors, advancements can be made in enhancing the success and predictability of periodontal and implant treatments.

In conducting the experiments, the present studies utilized cell lines isolated from intraoral tissues, that were either isolated in the laboratories of the Ludwig Maximilian University or obtained through collaborations with other research institutions. The research aimed to mimic the periodontal cellular environment, using for the different experiments, cells from all periodontal tissues: oral keratinocytes, periodontal ligament cells, mesenchymal stem cells.

The paragraphs that follow will present seven original papers that have been published in English in peer-reviewed dental journals with impact factor. They are preceded by an introduction in more detail to the respective topics.

2. Original research and its relevance to the field

2.1. Research related to biomolecules in periodontal regeneration and repair

The concepts of guided bone and tissue regeneration (GBR and GTR), which involve the use of a membrane to separate the desired tissues from unwanted cells, were initially proposed by Hurley in orthopedic research (Hurley et al. 1959). It was later discovered that the regenerative properties of this technique were due to the presence in bone of stem cells, as described by McCulloch and Till in their pioneering work in 1963 (Siminovitch et al. 1963, Becker et al. 1963).

Similarly, in dentistry, Melcher advanced the hypothesis that the nature of the newly formed tissue would be dictated by the cell type that first reaches the root surface during wound healing (Melcher 1976). Thus, the same concepts of GBT and GTR were also adopted in dentistry and the earliest reports of new attachment formation in periodontology can be traced back to the 1980s (Nyman et al. 1982, Gottlow et al. 1984). A few years later, it was suggested that this phenomenon could be attributed to what we now recognize as periodontal stem cells (PDLSC) (McCulloch et al. 1987). In addition to using autogenous grafts and graft substitutes, biologic mediators have also been utilized to achieve complete regeneration (Chambrone et al. 2022). The materials used target exactly this subpopulation of cells, stimulating cell migration, adhesion, proliferation and/or differentiation in the affected areas (Meng et al. 2022). Extensive research is currently being conducted to analyze the factors influencing the efficacy of these molecules. In fact, several factors, such as the mode of delivery, dosage, and timing of application, contribute to their overall effectiveness (Vo et al. 2012).

Among the approaches that have given the most encouraging results in periodontal regeneration, there is the application of growth factors, such as platelet-derived growth factor

(PDGF), fibroblast growth factor (Ishii et al. 2013) and other biomolecules like platelet rich fibrin (Strauss et al. 2018, Tavelli et al. 2021), bone morphogenetic proteins (BMPs) (Ern et al. 2017) and enamel matrix derivatives (EMD) (Frasheri et al. 2023a). The first part of this work is particularly oriented towards some of these biomolecules that have demonstrated promising potential in the field of periodontology, namely enamel matrix derivatives and hyaluronic acid (HA).

2.1.1. Original work: Frasheri I and Paschalidou M, Imhof T, Steinberg T, Spinell T, Hickel R, Folwaczny M. An in vitro investigation on the effects of amelogenin on Oral Keratinocytes. Dent Mat 2023 Aug 23 doi: 10.1016/j.dental.2023.08.176 Epub ahead of print. (IF 2022: 5.0)

For more than 20 years now, enamel matrix derivatives (EMD) have been investigated among other emerging biomolecules, for their regenerative capacities in vivo and in vitro (Hammarstrom 1997, Aimetti et al. 2017, Wyganowska-Swiatkowska et al. 2017, Tavelli et al. 2022). EMD have been found to stimulate the formation of new bone tissue (Heijl et al. 1997) and promote cell differentiation in bone marrow mesenchymal stem cells (BMMSC) (Cheng et al. 2021). Numerous in vitro investigations have confirmed their positive impact on mesenchymal stem cells (MSCs) (Tanimoto et al. 2012a) and periodontal ligament (PDL) cells in the periodontium (Zeldich et al. 2007, Tanimoto et al. 2012b). The clinical application of enamel matrix derivatives, as an FDA-approved commercial product, has also grown in popularity among emerging regenerative approaches. A systematic review and meta-analysis has supported their use in minimally invasive clinical procedures, for the improved recession coverage and bone fill (Estrin et al. 2022). These represent interesting results, because, without resorting to the advantages of GTR, periodontal regeneration is obtained while we would expect just repair with the formation of a long epithelial junction (Trombelli 2005).

Part of the research for this habilitation particularly targets this interesting aspect from a cytological point of view (Frasheri et al. 2023a).

The enamel matrix derivatives are a complex mixture of proteins and growth factors derived from the enamel of unerupted porcine tooth buds (Hammarstrom et al. 1997). This mixture is obtained through a specialized processing technique that isolates and concentrates these biologically active components from the enamel matrix. For this reason, the commercialized product may exhibit variations in composition between different batches (Swiderski 2019). This inconsistency poses challenges in ensuring uniformity and reliability in its performance. If the product lacks a standardized formulation, it may lead to difficulties in reproducibility and comparability of results across different users or research studies. Moreover, also the extraction of these proteins is challenging due to the need to obtain teeth at a specific developmental phase. For overcoming these, and also ethical concerns associated with animal-derived materials, the application of recombinant proteins might be beneficial.

The first part of this research specifically focuses on using a recombinant version of what constitutes the main protein of the enamel matrix derivatives, the full-length amelogenin protein. Amelogenin is a key protein found in the enamel matrix, which is responsible for the enamel production during tooth development. Self-assembling in nanospheres it participates in giving a structural organization to the enamel for the further mineralization (Fang et al. 2011). Deutsch et al. discovered its presence also in soft tissues, brain, salivary glands, periodontal ligament, etc. (Deutsch et al. 2006). The role in these tissues is not a structural role, but a functional role (Frasheri et al. 2016). The plausible signaling role of this protein is supported by evidence that various amelogenin isoforms, including the full-length protein, have been observed to interact with the cell surface receptors LAMP-1 and -3 in mouse models, indicating these receptors as potential binding sites for amelogenin (Xu et al. 2008,

Zhang et al. 2010). Regarding periodontal tissues, amelogenin has shown to induce the proliferation and migration of PDL cells (Zeichner-David et al. 2006, Li et al. 2010).

The aim of the present research was to analyze the less studied effects of amelogenin on periodontal soft tissue wound healing and, particularly, on the epithelial component in the sulcus area. For this, oral keratinocytes were isolated from healthy donors and immortalized for establishing a cell line that could be cultured and used for research purposes. The full-length amelogenin protein (Fig. 1) was produced using a genetically modified E.coli strain, ClearColi BL21. The absence of lipopolysaccharide in ClearColi BL21 allows for improved purification of recombinant proteins with reduced contamination of endotoxins, enabling better compatibility with mammalian cell cultures and downstream applications.

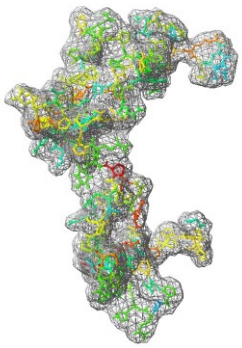

Fig. 1 *Protein structure of the full-length amelogenin protein. Copyright: Iris Frasheri*

The produced recombinant amelogenin protein was used in serial dilutions from 10-10000 ng/ml and its effect on immortalized oral keratinocytes was tested for 21 days. Cell metabolism, cell proliferation and motility were analyzed. We found that the presence of the full-length amelogenin inhibits cell motility and proliferation of keratinocytes (Frasheri et al 2023). The effects of amelogenin on keratinocytes´ migration start to be visible already after 24 hours with concentrations of as little as 1000 ng/ml. Then, at day 7, appear the effects on the metabolic activity and finally the effects on keratinocytes´ proliferation are to be seen.

Thus, this work evidenced that amelogenin helps periodontal tissue regeneration in two ways, not only directly stimulating periodontal ligament cells, but also giving them a biological advantage, by preventing keratinocytes from occupying the periodontal ligament space.

2.1.2. Original work: Frasheri I, Tsakiridou N.D, Hickel R, Folwaczny M. The molecular weight of hyaluronic acid influences metabolic activity and osteogenic differentiation of periodontal ligament cells. Clin Oral Investig. 2023 Aug 17. doi: 10.1007/s00784-023-05202-z. Epub ahead of print. PMID: 37589747. (IF 2022: 3.4)

One further biologic agent that has given interesting results in periodontal therapy is hyaluronic acid. This is a linear polysaccharide of D-glucuronic acid and *N*-acetyl-D-glucosamine (Della Sala et al. 2021). It has been found in the extracellular matrix of many connective tissues and it is also present in the periodontal ligament (Pilloni et al. 2003). The native form presents a high-molecular weight >1000kDa (HMW HA) (Albano et al. 2016). In numerous tissues there has also been identified the presence of fragments with different sizes up to fragments as small as oligosaccharides (4-mer) (Cowman 2017). In physiological conditions, these derive from de novo production or fragmentation operated by hyaluronidases during local clearance (Jiang et al. 2007, Kavasi et al. 2017). In pathological conditions there seem to be an accumulation of these small fragments with a low molecular weight, due to the fragmentation induced by reactive oxygen species (superoxide dismutase, peroxynitrite, etc.) (Kennett and Davies 2007).

Research conducted in various medical fields has demonstrated that the biological activity of hyaluronic acid strongly depends on its molecular weight (Yang et al. 2012). While both high-molecular weight (HMW) and low-molecular weight (LMW) hyaluronic acid bind to the same receptors (CD44, RHAMM, etc.), native HMW HA is involved in maintaining homeostasis, whereas LMW HA promotes angiogenesis, thereby improving wound healing (Gao et al. 2010). In a study involving rat osteoblasts, it was observed that high molecular weight hyaluronic acid

(HMW HA) with molecular weights of 900 kDa and 2300 kDa enhanced mineralization (Huang et al. 2003). Conversely, low molecular weight hyaluronic acid (LMW HA) with a molecular weight of 60 kDa did not demonstrate any effect on mineralization. This antagonistic behavior was also observed on other cell lines, including dental pulp stem cells and fibroblasts (Schmidt et al. 2020). Notably, on fibroblasts, LMW HA was found to increase the expression of the pro-inflammatory cytokine IL-6 and the chemokine IL-8 (Vistejnova et al. 2014). This supports the pro-inflammatory role of LMW HA in wound healing. On the contrary, treatment with HMW HA reduced the expression of these interleukins, suggesting an anti-inflammatory effect.

The current study aimed to investigate the impact of various molecular weights of hyaluronic acid fragments (from 4.6 up to 1510kDa) on periodontal ligament cells. Four different sizes of hyaluronic acid were used and applied at the same concentration to allow for a better comparison: high-molecular weight (HMW) hyaluronan with a mass of 1510kDa; medium-molecular weight (MMW) hyaluronan with a molecular mass of 229kDa; low-molecular weight (LMW HA) with a mass of 37kDa and ultra-low (ULMW) hyaluronan with a mass of 4.6kDa. PDLhTERTs are cells derived from the periodontal ligament that have been proven to differentiate towards different mesenchymal lineages (Docheva et al. 2010). Introducing human telomerase reverse transcriptase gene (hTERT) through retroviral infection, PDLhTERTs were immortalized for overcoming problems linked to their short in-vitro life span. Osteogenic medium alone was used as a positive control. The metabolic activity was tested. Von Kossa staining was performed to compare osteogenic differentiation and a calcium deposition assay was performed for quantifying mineralization.

The study found that all fragments of hyaluronic acid induced osteogenic differentiation in PDLhTERTs. Comparably to the osteogenic control, all tested groups presented microscopically aspects of a typical osteogenic differentiation, as evidenced by rolled aggregates and oval-shaped, cell-free areas typical of the beginning of mineralization.

Metabolic activity was observed to increase in PDLhTERTs treated with medium molecular weight hyaluronic acid during all the 21 experiment days, compared to the control. During the early stages of osteogenic differentiation, also ULMW HA seemed to induce an increase in metabolism. The groups treated with LMW HA presented until day 14 values comparable with the control, with an increase in metabolism during the last week in culture. This habilitation study also evidenced that LMW HA induced a higher calcium deposition than hyaluronan with other molecular weights.

Further research in this field is needed because, as Bhati et al. have proposed, the effectiveness of HA may also may be influenced by multiple factors, including the concentration of HA used, the method of delivery, and the severity of the disease (Bhati et al. 2022).

In conclusion, while HA shows promise as a therapeutic agent for periodontal therapy, further research is needed to fully understand its clinical efficacy and optimal use in periodontal treatment.

2.2. Research related to personalized periodontics: biomolecules in the diagnosis, monitoring and treatment of periodontal conditions

Periodontitis poses significant challenges in terms of accurate diagnosis and monitoring of its progression. The current diagnosis and prognostic indicators for periodontitis heavily rely on subjective and time-consuming clinical measurements (Stathopoulou 2015). This highlights the need for alternative approaches, such as the exploration of molecules as diagnostic and monitoring markers, to improve the accuracy and efficiency of periodontitis diagnosis and management. In recent years, there has been a growing interest in transitioning from a mass dentistry approach to personalized dentistry (Bartold and Ivanovski 2022).

This approach aims to enhance the precision and effectiveness of periodontal care. The use of biomolecules as diagnostic and monitoring markers in periodontal conditions aligns with the principles of personalized dentistry, enabling a more targeted and individualized approach. While the term "personalized dentistry" has been more commonly associated with other areas of dentistry, such as restorative and preventive care, its application in periodontology is gaining recognition (Bartold 2018, Malcangi et al. 2023). Although there is limited historical background specific to personalized dentistry in periodontology, the concept has its roots in the broader field of personalized medicine and the advancement of molecular diagnostics and targeted therapies (Collins et al. 2003).

In the field of dentistry, there has been an increasing focus on utilizing molecules as diagnostic and monitoring markers to tackle these challenges. Biomarkers, enzymes, and cytokines are among the molecules that have gained attention, as they offer the potential to enhance the early detection, assessment, and management of this oral condition (Zhang et al. 2021).

2.2.1. Original work: Frasheri I, Heym R, Ern C, Summer B, Hennessen TG, Högg C, Reichl FX, Folwaczny M. Salivary and gingival CXCL8 correlation with periodontal status, periodontal pathogens, and smoking. Oral Dis. 2022 Nov;28(8):2267-2276. doi: 10.1111/odi.13994. Epub 2021 Oct 8. PMID: 34388304. (IF 2022: 3.8)

By analyzing the presence, levels, or changes in specific molecules within the oral cavity, clinicians and researchers aim to improve the precision and effectiveness of periodontitis diagnosis, and even to foresee its manifestation, as well as to enable more tailored treatment approaches (Cafiero et al. 2021). Cytokines, which are signaling molecules involved in immune responses, have also been explored as potential markers for periodontitis.

Periodontitis is induced by oral microorganisms, which also contribute to its perpetuation, however it has been shown that the severity of the disease manifestation is critically depending on the inappropriate chemotaxis and activation of neutrophils (Herrmann and Meyle 2015, Cortes-Vieyra et al. 2016). They have been shown to mediate inflammatory tissue destruction (Rijkschroeff et al. 2018) expressing proinflammatory and anti-inflammatory cytokines. Examples include interleukin-1 beta (IL-1β), interleukin-6 (IL-6), interleukin-8 (IL-8) and tumor necrosis factor-alpha (TNF-α) (Noh et al. 2013, Pan et al. 2019). Elevated levels of these cytokines have been associated with inflammation and tissue destruction in periodontal disease (Ishimi et al. 1990, Noh et al. 2013).

This habilitation project includes a translational study involving 279 patients, examining both clinical aspects, and the chemokine interleukin-8 (also named CXCL8) in periodontitis. Increased expression of this chemokine in the gingival crevicular fluid triggers the chemotaxis of neutrophils and the production of a cascade of proinflammatory molecules, which is no longer proportional to the bacterial challenge in the affected tissues (Gamonal et al. 2001, Konopka et al. 2012). The research evidenced a strong correlation between IL-8 levels in gingival crevicular fluid and the clinical severity of periodontitis (Frasheri et al. 2022b). The evaluation of clinical severity was performed taking into consideration the new classification of periodontal disease introduced jointly by the American Academy of Periodontology and the European Federation of Periodontology (Caton et al. 2018). This habilitation project also investigated how smoking habits influence these IL-8 levels. We have found evidence that smoking induces significantly lower levels of IL-8 in both gingival crevicular fluid and saliva. This inappropriate expression of IL-8 might lead to an impaired neutrophil recruitment. Moreover, in conjunction with the smoke-induced suppression of neutrophil functions reported by Zhang (Zhang et al. 2018), this might increase susceptibility of smokers to bacterial infections. Therefore, even though smokers are at a higher risk for periodontitis

(Tomar and Asma 2000, Iwasaki et al. 2018), they exhibit lower concentrations of IL-8 in saliva and sulcus fluids. In this specific group of patients, IL-8 does not appear to have any predictive value for periodontal disease, thus it cannot be considered for diagnostic or monitoring purposes.

2.2.2. Original work: Ern C, **Frasheri I,** Berger T, Kirchner HG, Heym R, Hickel R, Folwaczny M. Effects of prostaglandin E2 and D2 on cell proliferation and osteogenic capacity of human mesenchymal stem cells. Prostaglandins Leukot Essent Fatty Acids. 2019 Dec; 151:1-7. doi: 10.1016/j.plefa.2019.09.005. Epub 2019 Sep 13. PMID: 31589940. (IF 2019: 3.084)

Prostaglandins E2 (PGE2) and D2 (PGD2) are naturally occurring lipid mediators, synthesized by the body from the fatty acid, arachidonic acid, and they are also bioactive molecules that act as local signaling molecules. They have been specifically linked to the manifestation of periodontitis-related inflammatory reactions, with PGE2 identified as a potential biomarker of the condition and proposed to correlate with disease severity (Sanchez et al. 2013).

PGE2 has been shown to induce osteoclast formation (Sakuma et al. 2000), involved in the destruction of periodontal tissue (Hienz et al. 2015). The study included in this habilitation project has identified the presence of PGE2 and PGD2 receptors also on hMSCs. Moreover, the negative effects of both these prostaglandins on osteogenic differentiation and metabolism was shown (Ern et al. 2019). This evidences that both PGE2 and PGD2 are involved in periodontitis-induced tissue damage, not only stimulating catabolic processes, but also inhibiting anabolic processes, i.e regeneration and repair of these tissues. The determination of the levels of these inflammatory markers in gingival crevicular fluid can be suggested for diagnosis and monitoring of periodontitis. These findings help as well the identification of

these inflammatory molecules as targets for a needed adjuvant therapy of periodontitis, in addition to the mechanical treatment. By considering individual variations in the production and response to prostaglandins, inhibitors of PGE2 and PGD2 could be utilized as adjuncts to mechanical periodontal treatment, enabling a more tailored approach to periodontitis management.

2.3. Research related to the influence of materials and iatrogenic interventions on periodontal/peri-implant health

2.3.1. Original work: Folwaczny M, Rudolf T, Betthäuser M., **Frasheri I.** Ultrastructural changes of smooth and rough titanium implant surfaces induced by metal and plastic periodontal probes. Clin Oral Investig. 2021 Jan; 25 (1):105-114. doi: 10.1007/s00784-020-03341-1. Epub 2020 Jun 21. Erratum in: Clin Oral Investig. 2022 Jan;26(1):1101. PMID: 32564141; PMCID: PMC8590678. (IF 2020: 3.573)

The long-term maintenance of healthy conditions in peri-implant areas poses a significant concern, being approximately half of the implants affected by mucositis (Renvert and Polyzois 2018). In this regards, annual check-ups using a periodontal probe are recommended to ensure optimal patient care (Herrera et al. 2023). As patients ideally undergo lifelong annual controls, the importance and number of our instrument-based interventions increases substantially. Metal instruments used in the treatment of peri-implantitis can alter the chemical and physical properties of the titanium surface, increasing the risk of bacterial adhesion and inflammation (Augthun et al. 1998, Cha et al. 2019). Although surface roughness is not the sole factor determining bacterial adhesion, smoother surfaces have shown a positive correlation with reduced bacterial colonization (Wu et al. 2011). The cumulative effect of using periodontal probes on implant surfaces during long-term maintenance care might also compromise the attachment of the peri-implant epithelium (Etter et al. 2002).

The in vitro study included in this habilitation work, investigated the changes in the ultrastructure of titanium implants caused by the motion of periodontal probes, considering the material of the probe and the probe's angulation as factors of interest. The metal probe caused slight increases in roughness on the smooth surface areas of the implant, while a decreased roughness was observed on the intraosseous, rough parts of the implant. The contact with plastic probes yielded smaller changes on the surfaces. However, none of these alterations reached statistical significance.

Studies have shown that rougher surfaces positively influence osteoblasts adhesion compared to smoother areas (Liu et al. 2013, Velasco-Ortega et al. 2016). Consequently, routine probing might compromise the reattachment of osteoblasts after the treatment of peri-implant defects (Folwaczny et al. 2021). In line with this, the abrasion particles caused by instrumenting the implant surface might impair its biocompatibility and interfere with the attachment of osteoblasts.

2.3.2. Original work: Frasheri I, Aumer K, Kessler A, Miosge N, Folwaczny M. Effects of resin materials dedicated for additive manufacturing of temporary dental restorations on human gingival keratinocytes. J Esthet Restor Dent. 2022 Jun 22. doi: 10.1111/jerd.12938. Epub ahead of print. PMID: 35731110. (IF 2022: 3.2)

AND

2.3.3. Original work: Folwaczny M, Ahantab R, Kessler A, Ern C, **Frasheri I.** Cytotoxicity of 3D printed resin materials for temporary restorations on human periodontal ligament (PDL-hTERT) cells. Dent Mater. 2023 May;39(5):529-537. doi: 10.1016/j.dental.2023.04.003. Epub 2023 Apr 11. PMID: 37055304. (IF 2022: 5.0)

The combination of computer-aided design (CAD) and computer-aided manufacturing (CAM) has revolutionized the production of dental restorations, allowing for the routine use of

industrially fabricated high-quality materials with standardized physical and chemical properties in both temporary and long-term treatments (Frasheri et al. 2022a). Additive manufacturing, also known as 3D printing, already introduced in the 1980s (Liu et al. 2021), offers advantages over subtractive manufacturing in the preparation of dental restorations. It enables the creation of complex shapes and requires smaller amounts of resin material (Moon et al. 2021). Many 3D printing techniques have been introduced for dental applications: stereolithography (SLA- one of the oldest techniques used), digital light processing (DLP- with faster printing speed compared to SLA), selective laser melting (SLM) or direct metal laser sintering (DMLS- which are also used for metals), filament-based 3D printing. The two articles included herein, discuss the selection of appropriate materials for the fabrication of temporary restorations in dental treatments. Resin-based materials, specifically composite resins, are commonly used for manual fabrication of temporary restorations. In particular, the traditionally used material (Luxatemp, DMG, Hamburg, Germany) for the manufacturing of temporaries is a hand-mixed product. This often creates an inhomogeneity in the material (bubbles and voids). In these studies, samples of dental resins 3D printed with DLP technology were tested and compared on one side to Luxatemp, and on the other side to a material used in subtractive manufacturing: Grandio disc (Voco, Cuxhaven, Germany). The biocompatibility of temporary materials, is a crucial factor due to the close contact between the restoration and the gingival tissue (Pituru et al. 2020). The findings of a recent study on 3D printed materials with DLP technology unequivocally demonstrated a higher concentration of residual non-polymerized monomers (Alifui-Segbaya et al. 2019). Unpolymerized monomers released from dental materials in general have been associated with cytotoxic effects, mucosal reactions, and alterations in cell proliferation and viability (Yang et al. 2018, Baldion et al. 2021).

In the studies for this habilitation project, two types of cells, oral keratinocytes and periodontal ligament (PDL) cells, were used to analyze the gingival reaction at two different tissue levels. Keratinocytes, which form the first barrier of gingival tissues, were isolated from the dental papilla and further immortalized. This has been an important step, because other keratinocyte cell lines provided by companies showed a short life-span, of just two or three days. The immortalization of these cells gave us the opportunity to prolong the cell culture for the whole duration of the planned study. The studies aimed to investigate the biological effects of four 3D printed resin materials, specifically assessing cell proliferation and the expression of proinflammatory mediators (IL-6 and IL-8). The results obtained suggest that the cytotoxicity of dental resin materials is greater for these materials used in DLP additive manufacturing compared to conventional and subtractive manufacturing. The resin materials, 3D printed with DLP technology, exhibited stronger effects on cell viability and proinflammatory response compared to other materials tested in the study. This was evident particularly for keratinocytes. The lower monomer-to-polymer conversion rate in 3D printed materials for digital light processing compared to industrial-manufactured dental resins may contribute to these observations (Frasheri et al. 2022a).

3. Discussion

Research on dental materials is transitioning from focusing solely on biocompatibility to encompassing bioactivity, where the ideal material is expected to possess not only biocompatible properties but also biomimetic and bioactive characteristics (Spagnuolo 2022). Bioactive molecules are gaining an increasing significance in dental practices for various applications ranging from preventive dentistry, where they are used in enamel remineralization (Goldberg et al. 2006, Lelli et al. 2014, Bossu et al. 2019, Grohe and Mittler 2021), to implantology, where they are applied for bone regeneration (Dang et al. 2018).

Particularly the field of periodontal research has experienced notable progress in comprehending and employing regenerative materials and techniques (Tatullo et al. 2020).

In fact, regenerative therapies have become integral to periodontology in addressing deficiencies in hard and soft tissue (Fraser et al. 2022). Scaffolds (Ivanovski et al. 2014) and grafting materials (Sheikh et al. 2017) have dominated the market in this field. In the last years, newer therapy strategies are taken more and more into account: growth factors and signaling molecules hold great promise for dental regenerative therapies (Galli et al. 2021). Enamel matrix derivatives (Grandin et al. 2012), recombinant human platelet-derived growth factor-BB (Suarez-Lopez Del Amo et al. 2015), rhBMP-2 and rhBMP-7 have already obtained FDA-approval and are being commercialized (Spiller and Vunjak-Novakovic 2015). The challenge for a widespread application is to obtain consistent outcomes by exploring various factors and approaches that might increase the success rate of these therapies. For each product, further research should focus on finding the right topical concentration, exploring interactions in the tissues as well as stability of the protein over time, which might all have an influence on the final outcome (Liu et al. 2007, Kempen et al. 2008).

In the original works (Frasheri et al. 2016, Ern et al. 2017, Frasheri et al. 2023b, Frasheri et al. 2023a) singular molecules (BMP-7, isoforms of amelogenin and hyaluronic acid) were considered, analyzing their cell-material interactions in 2D or 3D cell culture models. In addition to its positive effects as a biomolecule, favoring probing depth reduction, clinical attachment level gain and bleeding on probing reduction (Eliezer et al. 2019), HA has also been proposed for use as a natural hydrogel scaffold for gingival tissue engineering (Miranda et al. 2016). However, more studies are needed regarding the physical and mechanical properties of these hydrogels, which still need to be improved (Hutomo et al. 2023).

Biomolecules can be beneficial not only in periodontal therapy, but also diagnosis and monitoring of this condition. Personalized dental care, within the context of periodontology,

involves tailoring diagnostic and treatment approaches to individual patients based on their specific needs and characteristics. Periodontitis itself is a complex inflammatory disease primarily caused by bacteria and their metabolic byproducts, serving as the main etiological factors (Donos et al. 2020). However, the host response to these bacterial pathogens, plays a critical role in the host-bacteria interaction, influencing the progression of periodontal disease. In particular, TNF-α, IL-1α and β, IL-6, IL-8, MMPs and PGE2 are some of the inflammatory biomarkers that can be measured in the gingival crevicular exudate, which show an exacerbated reaction of the host (Aleksandrowicz et al. 2021, Frasheri et al. 2022b). Currently, there are no salivary diagnostic tests approved by the FDA for assessing the risk of periodontal disease (ADA). Additional clinical validation involving larger cohorts is necessary to enhance the reliability and effectiveness of these diagnostic approaches (Bostanci et al. 2021). Despite the limitations of the study, the original work (Frasheri et al. 2022b), showed a significant potential for gingival crevicular fluid as a source of biomarkers. IL-8 can be considered as a potential biomarker for the diagnosis and monitoring of periodontitis, with the exception of the subgroup of smokers. Notably, the biomarker values obtained from gingival crevicular fluid showed stronger correlations with the severity and activity of periodontal disease compared to saliva.

However, it's important to note that the use of molecular markers in periodontitis is still an area of ongoing research, and further studies are needed to validate their clinical utility and establish standardized protocols for their use.

As suggested by the results of the original works (Ern et al. 2017, Ern et al. 2019), it is plausible that an adjunctive therapy targeting the inhibition of the cascade of PGE2 and PGD2 could improve the individual response to periodontal treatments. Thus, besides plaque control, we have the possibility to influence the progression of periodontal disease by controlling the

host-response. The use of anti-inflammatory drugs in periodontal tissue regeneration represents an emerging area of research (Ren et al. 2023).

Important for the oral and the periodontal well-being are also the interventions that we, as dental professionals, undertake. In this regard, the materials that we choose to use for restorations play a crucial role due not only to the surface contact, but also to a deeper immunologic interaction with the tissues. New materials are coming to the market, that are being developed primarily for additive manufacturing, or 3D printing (Saratti et al. 2019, Mayer et al. 2021, Balhaddad et al. 2023, Cai et al. 2023). Since its origins in the early ´80, and particularly over the last decade, 3D printing has experienced significant development in numerous fields including manufacturing, design, medicine and education (Cui et al. 2021). This progress over the last decade coincides with the expiration of patents that previously protected many 3D printing technologies, including SLA and laser sintering (Patent nr: US5155324, US5597589, US5382308). In dentistry, 3D printing primarily focuses on the utilization of polymeric constructs due to the wide range of possible materials and the high versatility they offer, although other materials such as metals and ceramics are also utilized (Wang and Lim 2019, Jang et al. 2019). In the original works (Frasheri et al. 2022a, Folwaczny et al. 2023), we tested on gingival keratinocytes and periodontal ligament cells some of these materials for dental applications in the manufacturing of temporary restorations. Compared to traditionally used materials and to materials manufactured through a subtractive process, these materials for 3D printing with DLP technology presented negative effects. The residual monomer release might be responsible for the biological effects observed. In fact, the degree of monomer conversion obtained for restorations milled from blocks is difficult to attain with other processing methods (Berghaus et al. 2023).

Herein we analyzed the material as samples produced with DLP, but also the additive manufacturing technology plays a big role influencing the efficacy of the polymerization of

each material. On the market, there will soon be available 3D printers that make the production of restorations with every composite possible, not only those specifically produced for this particular purpose. Additionally, other production-related aspects such as surface glazing (Nam et al. 2023) could also contribute to the different monomer release.

Given the novelty of these materials, there is a scarcity of studies examining their long-term effects and implications (Balhaddad et al. 2023). Future research in developing further dental materials for additive manufacturing should also investigate how the surfaces of these materials influence cell attachment and bacterial colonization.

4. Summary

Periodontal disease is a prevalent condition affecting a substantial proportion of the global population. It has a significant impact on the quality of life and its incidence is projected to increase as the population ages.

This habilitation work focuses on various aspects of periodontal regeneration, personalized periodontics, and the influence of materials and interventions on periodontal and peri-implant health. The first part of the research explores the role of biomolecules in periodontal regeneration and repair. While common periodontal treatments result in tissue repair, the ultimate objective is achieving complete regeneration. Regenerative procedures that aim to restore lost or injured tissues in periodontal disease are being extensively studied. Two specific biomolecules, amelogenin (component of EMD) and hyaluronic acid (HA), were examined for promoting the regeneration of periodontal tissues. The studies evaluated the effects of these biomolecules on cell proliferation, migration, and differentiation, highlighting their potential in improving periodontal tissue regeneration. One study specifically focused on a recombinant version of the main protein found in EMD, amelogenin, investigating the effects of the full-length protein on periodontal wound healing and its interaction with oral

keratinocytes. The results show that amelogenin inhibits the motility and proliferation of keratinocytes, suggesting its potential in preventing the occupation of periodontal ligament space by these cells. Another study explored the influence of different molecular weights of hyaluronic acid on periodontal ligament cells. Hyaluronic acid fragments induce osteogenic differentiation in these cells, with medium molecular weight hyaluronic acid showing the most significant effects. The study highlights the importance of considering the molecular weight of hyaluronic acid in its clinical application for periodontal therapy.

The second part of the research focuses on the use of biomolecules in the diagnosis, monitoring, and treatment of periodontal conditions. It discusses the potential of cytokines, such as interleukin-8 (IL-8), as diagnostic markers for periodontitis. The study showed a strong correlation between IL-8 levels in gingival crevicular fluid and the clinical severity of periodontitis. The research also investigated the correlation between IL-8 levels and smoking habits, revealing that for this group IL-8 cannot serve as a biomarker of periodontitis. Additionally, the research explored the effects of prostaglandins E2 (PGE2) and D2 (PGD2) on cell proliferation and osteogenic capacity of human mesenchymal stem cells. It demonstrated that both PGE2 and PGD2 negatively affect osteogenic differentiation and metabolism, suggesting their involvement in periodontitis-induced tissue damage.

The third part of the research examines the influence of materials and iatrogenic interventions on periodontal and peri-implant health. One study investigated the ultrastructural changes of titanium implant surfaces caused by metal and plastic periodontal probes. Although slight changes in surface roughness were observed, they did not reach statistical significance. Further studies need to investigate how routine probing might affect the reattachment of osteoblasts after peri-implant defect treatment.

Two other studies focused on the cytotoxicity of 3D printed resin materials used for temporary dental restorations. The research evaluated the effects of these materials on human

periodontal ligament cells and gingival keratinocytes. The results indicate a higher cytotoxicity of 3D printed resin materials compared to conventional and subtractive manufacturing materials.

Overall, this research provides valuable insights into the biological principles of regenerative materials, the potential of biomolecules in periodontal therapy, the use of molecules as diagnostic markers, and the influence of materials and interventions on periodontal and peri-implant health. The findings contribute to the advancement of periodontal treatment and personalized dentistry, aiming to improve patient care and outcomes in the field of periodontology.

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