DClinDent Periodontology Year 3

Dissertation

# Analysis of Exosomes and molecular biomarkers in saliva and the gingival crevicular fluid in patients with periodontal disease.

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## Abstract

**Aim**: The aim of this pilot study is to evaluate if biomarker signature(s) of exosomal miRNAs, IL-1β and IL-6 isolated from GCF and saliva exist and can be used as feasible biomarkers of periodontal treatment.

**Methods**: In this pilot study, 12 randomly selected patients were included. The patients were divided in three groups (4 patients per group) and would receive either a repeated non-surgical periodontal treatment (NSPT) or two different types of periodontal surgery techniques- Resective (RPFO) or a Conservative flap (Simplified Papillae Preservation Flap (SPPF))

At baseline and prior to any hygiene instructions given, or commencement of their periodontal treatment, saliva and gingival crevicular fluid (GCF) were collected. The samples were immediately stored at -80°C and processed when required for analysis. GCF samples were processed with a centrifugation protocol for exosomes and microRNA (miRNA) extraction and analysis. Saliva samples were also processed for exosome isolation and miRNA extraction using the same centrifugation protocol. Also, the saliva supernatants of these samples were analysed using enzyme-linked immunosorbent assay method (ELISA) for detection of IL-1β and IL-6 cytokines. Data was analysed and linked to the clinical parameters; dental plaque, periodontal probing depth (PPD), gingival recession (REC), bleeding on probing (BoP) and clinical attachment loss (CAL) obtained at baseline for these patients.

**Results**: Nanoparticle tracking analysis showed that extracellular vesicles isolated from GCF showed multiple peaks, indicating the presence of vesicles of different sizes and low concentration.

Processing of saliva produced vesicles with a homogeneous size (close to that reported for exosomes) and a relatively good concentration. However, quality control analysis revealed that neither GCF nor saliva produced enough amounts of good quality RNA for miRNA expression analysis. Analysis of saliva samples allowed detection of IL-1β, (but not IL-6) across the three different groups of patients' samples. Furthermore, the level of IL-1β was not significantly different in the three patients' groups and did not correlate with any clinical parameters that define periodontitis.

**Conclusions**: At the protein level, at least by analysis of IL-1β expression (a cytokine closely associated with periodontitis), the randomly selected samples from patients at baseline time point, suggests a homogenous set of samples that would allow detection of molecular changes related to treatment intervention. Our results suggest sampling, storing and preservation

protocols used in this study, might be inadequate for the investigation and evaluation of RNA related biomarker signatures from saliva and GCF samples.

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## List of Abbreviations

| Abbreviation | Definition |
|---|---|
| AGO | Argonaut Proteins |
| AIDS | Acquired Immunodeficiency Syndrome |
| BoP | Bleeding on Probing |
| CAL | Clinical Attachment Loss |
| COVID-19 | Coronavirus Disease 2019 |
| DNA | Deoxyribonucleic Acid |
| EFP | European Federation of Periodontology |
| ELISA | Enzyme-Linked Immunosorbent Assay |
| EVs | Extracellular Vesicles |
| GCF | Gingival Crevicular Fluid |
| IL | Interleukin |
| miRNA | Micro – Ribonucleic Acid |
| MMP | Matrix Metalloproteinase |
| mRNA | Messenger Ribonucleic Acid |
| NSPT | Non-Surgical Periodontal Treatment |
| NTA | Nanoparticle Tracking Analysis |
| O/H | Oral Hygiene |
| PBMCs | Peripheral blood mononuclear cells |
| PBS | Phosphate Buffered Saline |
| PDL | Periodontal ligament |
| *P.g* | *Porphyromonas gingivalis* |
| PMPR | Professional Mechanical Plaque Removal |
| Pol I or II | Polymerase I or II |
| PPD | Periodontal or Pocket Probing Depth |
| Pre-miRNA | Precursor – miRNA |
| QMUL | Queen Mary University of London |
| RIN | RNA Integrity Number |
| RPFO | Resective Periodontal Flap Surgery |
| RT | Room Temperature |
| SD | Subgingival Debridement (non-surgical) |
| SN | Supernatant |
| SPC | Supportive Periodontal Care |
| SPPF | Simplified Papilla Preservation Flap |
| Streptavidin-HRP | Streptavidin - Horseradish Peroxidase |
| TNF-α | Tumour Necrosis Factor - α |

## Introduction

According to the most recent classification, periodontitis is defined as a "*chronic multifactorial inflammatory disease associated with dysbiotic plaque biofilm and characterized by progressive destruction of the tooth-supporting apparatus*", resulting in tooth loss (Papapanou *et al.*, 2018). The tissues affected by the disease are the gingival epithelium and the gingival connective tissue, the cementum, the periodontal ligament (PDL) and the alveolar bone of teeth. During disease, these tissues, present all the characteristics of a chronic disease, which are degradation, scaring and irreparable tissue break-down (Meyle and Chapple, 2015). The diagnosis of the disease is based on its hallmark characteristic, which is the clinical attachment loss (CAL). In particular, periodontitis is diagnosed when interproximal CAL of ≥2mm, or ≥3mm at any other given sites, to a minimum of 2 non-adjacent teeth is present (Papapanou *et al.*, 2018). Furthermore, the number and proportions of teeth with probing pocket depths above specific thresholds (commonly >4 mm with BOP and ≥6 mm), the number of teeth lost consequently to periodontitis, the number of teeth with intrabony lesions, and the number of teeth with furcation lesions are all significant descriptors of periodontitis. This needs to be confirmed radiographically, at the sites where the loss of bone is clinically noticeable. Additionally, the clinical diagnosis is supplemented with the inclusion of the bleeding on probing (BoP) sites, the pocket probing depths (PPD) and of teeth presenting CAL of ≥3mm and ≥5mm (Papapanou *et al.*, 2018). Furthermore, the severity and complexity are recorded by staging periodontitis, numerically from I to IV, and the risk factors along with the rate of progression are expressed through grading, from A to C (Papapanou *et al.*, 2018).

This latest classification divides the periodontal disease according to its pathophysiology in three distinguishable forms: i) the necrotizing type of periodontitis, ii) periodontitis as a manifestation of systemic diseases and iii) periodontitis. The last form corresponds to the chronic and aggressive periodontal diseases as described by the previous Classification in 1999 (Armitage, 1999).

## Periodontal Treatment

The current concept of addressing and treating periodontitis is mainly based on the principle of controlling the risk factors associated with periodontal disease as well as biofilm control, which is the main causative factor of the disease. The dental biofilm, if not regularly removed, will consequently induce gingivitis (Loe, Theilade and Jensen, 1965), and in susceptible individuals progress to periodontitis (Murakami *et al.*, 2018). Dental biofilm removal is therefore of paramount importance in the prevention and treatment of periodontal disease (Chapple *et al.*, 2015). However, this refers to the part of alleviating the causative factor, when addressing and treating periodontitis. The ideal objective of periodontal treatment is the

healing, restitution and regeneration of the lesion caused by periodontitis, and to consecutively restore the function of teeth and mouth (Karring *et al.*, 1993).

Within this context, in the latest guidelines for the treatment of Stage I-III Periodontitis from the European Federation of Periodontology - EFP (Sanz *et al.*, 2020), periodontal treatment comprises of four distinct therapeutic phases: i) the first step addresses risk factors, brings the oral hygiene (O/H) to adequate levels and imply professional mechanical plaque removal (PRMP); ii) the second step proceeds to the non-surgical mechanical debridement of the involved teeth through prophylactic mechanical plaque removal (PMPR); iii) the third step addresses the residual pockets of >6mm, aiming at gaining access to subgingival areas through repeated instrumentation or surgery; iv) the fourth step includes the supportive periodontal care (SPC) where patients receive supporting periodontal therapy which comprises of possible re-diagnosis and treatment of re-infected sites, reinforcement of O/H and healthy lifestyle changes.

The overall endpoints of the above-mentioned steps are the resolution of the periodontal inflammation expressed by bleeding on probing, closure of the periodontal pockets (ideally ≤4mm), gain of clinical attachment, periodontal stability and restoration of the lost architecture and function of the periodontal apparatus.

## Periodontal disease treatment steps

The therapeutic phases that are clinically employed for the treatment of periodontal disease have been proposed from the EFP (Sanz *et al.*, 2020) as S-3 clinical guidelines for the treatment of periodontitis stages I, II and III, comprising of four steps in therapy.

Non-surgical periodontal therapy (Step1 & Step2). The non-surgical periodontal treatment (NSPT) option that is provided to the 1$^{st}$ and 2$^{nd}$ step of the S-3 clinical guidelines for the treatment of periodontal disease. More specifically it includes the improvement of oral hygiene +/- professional mechanical plaque removal (PMPR), behavioural changes from the patient and control of risk factors related to periodontitis (Step 1) and the root surface instrumentation aiming at the removal of the dental biofilm and supra/sub-gingival calculus.

The NSPT treatment of periodontitis is the first line of treatment offered to patients when addressing periodontitis (Sanz *et al.*, 2020). It is the treatment which aims at rendering the dental root's environment free from the causative factor of periodontitis - the dental biofilm, and remove one of its most important retentive factors: the calculus (Timmerman and van der Weijden, 2006). It is considered a key step in the periodontal therapy that can achieve a mean pocket closure of 74% and bleeding on probing reduction of 62%, and is effective irrespectively the way of its delivery, by hand or ultrasonic-sonic instrumentation, in one or multiple sessions

of treatment (Suvan *et al.*, 2020). It is also noted that its impact is mostly beneficial in deeper periodontal pockets (≥7mm) than in the shallower ones (4-6mm), which are 2.5mm and 1.5mm of attachment gain, respectively. Suvan clearly states that this treatment modality alone, is possible to achieve endpoints in periodontal therapy (in terms of pocket closure), to such level, where further alternative treatment can be deemed unnecessary. Evidence supports that PMPR can limit the incidence of tooth loss (Trombelli, Franceschetti and Farina, 2015). However, repeated instrumentation (PMPR) for a prolonged period with these techniques (hand curettes and /or ultrasonic scalers), may cause some degree of root structure loss (Zappa *et al.*, 1991).

Following the completion of the NSPT, there is a stage where the results of the treatment are evaluated. This assessment is completed once the periodontal tissues have healed adequately, typically at approximately 12 weeks, and the purpose of it is to define the improvement or not of periodontitis. This is based on to what level, the endpoints of therapy have met, which are the closure of periodontal pockets to ≤4mm - absence of PPD ≥4mm with bleeding on probing, and absence of periodontal pockets of ≥6mm (Sanz *et al.*, 2020; West *et al.*, 2021). The therapy is considered at this point successful, and the patient should be placed in a supportive periodontal treatment routine, to preserve and maintain the periodontal healthy status achieved. When this is not the case (pockets of 4 mm or more exhibiting bleeding on probing, or presence of deep periodontal pockets ≥6mm), the 3$^{rd}$ step in therapy should be implemented, which offers two main options depending on the clinical outcomes and particularities found on evaluation: i) either repeat the NSPT, or ii) offer periodontal surgical options. As per the S-3 guidelines, the surgical options can be simple flap access, regenerative or resective periodontal surgery, all aiming at eliminating the non-responding periodontal sites following NSPT.

Periodontal surgery (Step 3): Throughout the years the periodontal flaps have developed from resective flaps to more conservative approaches which today are considered as the standard of care due to the improved clinical outcomes they present (Graziani *et al.*, 2018). Periodontal resective surgery includes a number of surgical techniques, comprising the resection of the soft tissues, and can include alveolar bone modification (Lindhe, 2015). It is interesting to mention that resective surgery is the less conservative of surgical therapeutic approaches, and compared to access flap techniques appears to be more effective in terms of probing pocket depth and clinical attachment levels (Polak *et al.*, 2020). Resective techniques aiming at reducing the pocket depth through removal of the soft and hard periodontal tissues, by altering the tissue morphology that enhances good oral hygiene levels. The method of osteoplasty is performed simultaneously to correct and re-establish bone morphology, allowing a more ‘positive architecture’ (Carnevale and Kaldahl, 2000). As periodontal tissues

are removed with this modality, an impact within the expression of specific healing patterns is expected, and in general to be reflected in the level of healing molecular cues.

The conservative flap is a group of flap techniques used in periodontal surgery aiming at preserving the papillary tissues. Some of the most commonly used flaps are the simplified papilla preservation flap, modified papilla preservation technique and the minimally invasive surgical technique (Cortellini, Prato and Tonetti, 1999; Cortellini, Prato and Tonetti, 1995; Cortellini and Tonetti, 2007). As a concept, it emerged in the mid 1980's (Takei *et al.*, 1985), and was thoroughly tested and proposed in the 1990's. The complete preservation of gingival soft tissues aiming at primary closure on top of the applied regenerative materials and substances, was the target of the surgical procedures developing in the emergent era of periodontal regeneration, particularly during the critical early stages of healing (Cortellini, Prato and Tonetti, 1999; Harrel, 2015). These clinical advancements in flap design and handling have revolutionised surgery, allowing interdental wound failure to be reduced to less than 30% of all patients treated. This particular technique allows a faster re-vascularisation in the healing process (Retzepi, Tonetti and Donos, 2007b) when compared with more resective techniques. Based on this evidence, it is reasonable to expect for healing cues to emerge in the histological and molecular level, which will lead the healing pattern and provide a specific molecular signature characterising the conservative flap technique. Within this frame of investigation, biomarkers of periodontal disease have been studied following periodontal treatment, with results that are yet not conclusive (Zekeridou *et al.*, 2017; Holmlund, Hänström and Lerner, 2004). However, research advocates for a certain level of molecular activity following surgical and non-surgical periodontal treatment, which seems to be fairly complex and requires further investigation.

Each of the above treatment modalities (NSPT and step 3-conservative approach) is expected/ speculated to have some differences on the type of wound healing that follows. The focus of this study is to define the particular traits and the expression of molecular markers that define the patients' response to treatment. Particularly, how this is exhibited within the expression of extracellular vesicles. These vesicles, as part of the broader category of microvesicles (MVs), and specifically exosomes, have attracted the attention as biological particles presenting potentials in diagnosis, prevention, and treatment for various medical conditions (Wang *et al.*, 2017). These are considered to contain biomarkers and as such, are expected to have the potential to provide additional information over standard clinical indices (Han, Bartold and Ivanovski, 2022).

A challenge of periodontitis that clinicians routinely encounter, is that its detection takes place after a noticeable amount of periodontal tissue destruction has occurred, notably CAL and

alveolar bone recession. However, these clinical parameters cannot ascertain the current status of the disease, and although they provide information on the state of tissue destruction, they cannot with certainty predict the disease, nor determine the particular prognosis of the affected teeth in an accurate fashion. Therefore, the ability to detect the tendency and predisposition of patients to develop periodontitis, even before the expression of the above-mentioned clinical symptoms, would change the clinical practicing quite radically.

The element of prediction and determination of the clinical condition in periodontitis, is a concept which has given rise to clinical indexes, prognostication and maintenance systems (Matuliene *et al.*, 2008; McGuire and Nunn, 1996; Lang, Suvan and Tonetti, 2015; Löe, 1967; Lang *et al.*, 1986). Within their limitations, all these systems have provided the grounds to diagnose and foresee the outcome of periodontitis. However, these are in constant reviewing and have failed to give clinical accuracy for the purpose they have been proposed. Clinical and biological manifestations which can predict a specific clinical condition, which otherwise is difficult to observe, are generally called biomarkers (Aronson and Ferner, 2017), and are able to define a pathological or healthy condition. Biomarkers can be molecular, cellular, tissue or organ specific Within this context, biomarkers of periodontal disease are expected to predict disease progression (Buzalaf *et al.*, 2020). Ideally, the characteristics of periodontal biomarkers should (i) have the ability to diagnose the disease, (2) inform on its severity, (iii) change with periodontal treatment and (iv) be able to reflect its prognosis. In the present study, the biomarkers used are in the molecular level, and more specifically proteins and exosomal micro-RNA (miRNA).

MiRNA have been extensively studied exhibiting remarkable results and are expected to link the specific periodontal treatment modalities with clinical results. Exosomes and their content, recently have an emerging role as promising diagnostic tools and their role in periodontitis will be entertained in this study. In **Table1**, a small fraction of exosome biomarkers for periodontitis are summarized.

| | Salivary biomarkers | Type of biomarker |
|---|---|---|
| **Periodontitis** | CD9, CD81 | Membrane proteins |
| | Histone H2A type 1 | Histone (related to genome in eukaryotic cells) |
| | Ig kappa chain V-I region AU | Protein (antibody) |
| | hsa-miR-140-5p, hsa-miR-146a-5p, hsa-miR-628-5p | Micro RNAs |

**Table 1 Saliva Exosomal Biomarkers in Periodontitis.** Some Exosomal biomarkers for periodontitis (Xing, Han and Li, 2020) are displayed. These can be miRNAs, membrane proteins, histones and antibody proteins.

Exosome-based diagnosis causes little discomfort and is widely available in a variety of physiological fluids. Exosomal cargo diversity, which gives several diagnostic indicators, may improve diagnostic sensitivity and specificity (Han, Bartold and Ivanovski, 2022).

## Exosomes

Chargaff and West in 1946 were the first to describe exosomes, being part of the sediment following centrifugation of blood plasma, as ‘corpuscles’ (Chargaff and West, 1946). Later, Wolf in his study, referred to them as “platelet dust”, reducing their importance to platelet debris (Wolf, 1967). In contrast to their initial portrayal as lesser-important particles, the latest research made them emerge as an important class of cell-to-cell communicators, shed by all cell types. Exosomes are part of a wider family of vesicles secreted by cells, called Extracellular Vesicles (EVs). There are two more types of EVs, distinguished as per their size and synthesis route: microvesicles and apoptotic bodies. Of these, exosomes present the particularity of being created by a process aiming at a cargo for a specific purpose, in contrast to the rest of EVs which are directly released by the plasma (Xing, Han and Li, 2020). Their specifically designed cargo is distinguishing them as intercellular communicators with a specific aim, able to alter the function of the targeted cells.

A more extensive portrayal of exosomes was made in the early 1980s in the field of biology by Harding (Harding and Stahl, 1983) and Johnstone (Johnstone, Adam and Pan, 1984) helped the understanding of their function. These cell-secreted vesicles, can be distinguished and qualify as ‘exosomes’, when filling a range of criteria in structure and morphology: they need to be surrounded by a phospholipid bilayer membrane, have a small diameter (between

50 to 100nm), present a cup shape (in electron microscopy) and their density to be between 1.13 and 1.19 g/mL, depending on the cell of origin (Raposo *et al.*, 1996). Exosomes have a hydrophilic content comprising mainly of mRNA, miRNA, DNA, lipids and proteins (van Niel *et al.*, 2006; Théry *et al.*, 2018; Akhtar *et al.*, 2016). Exosomes have the ability to cross epithelial barriers via transcytosis and transfer RNAs of various systemic conditions, such as neurodegenerative disorders, into saliva (Schneider and Simons, 2013). Depiction of their synthesis pathway and content are described in **Figure 1**.

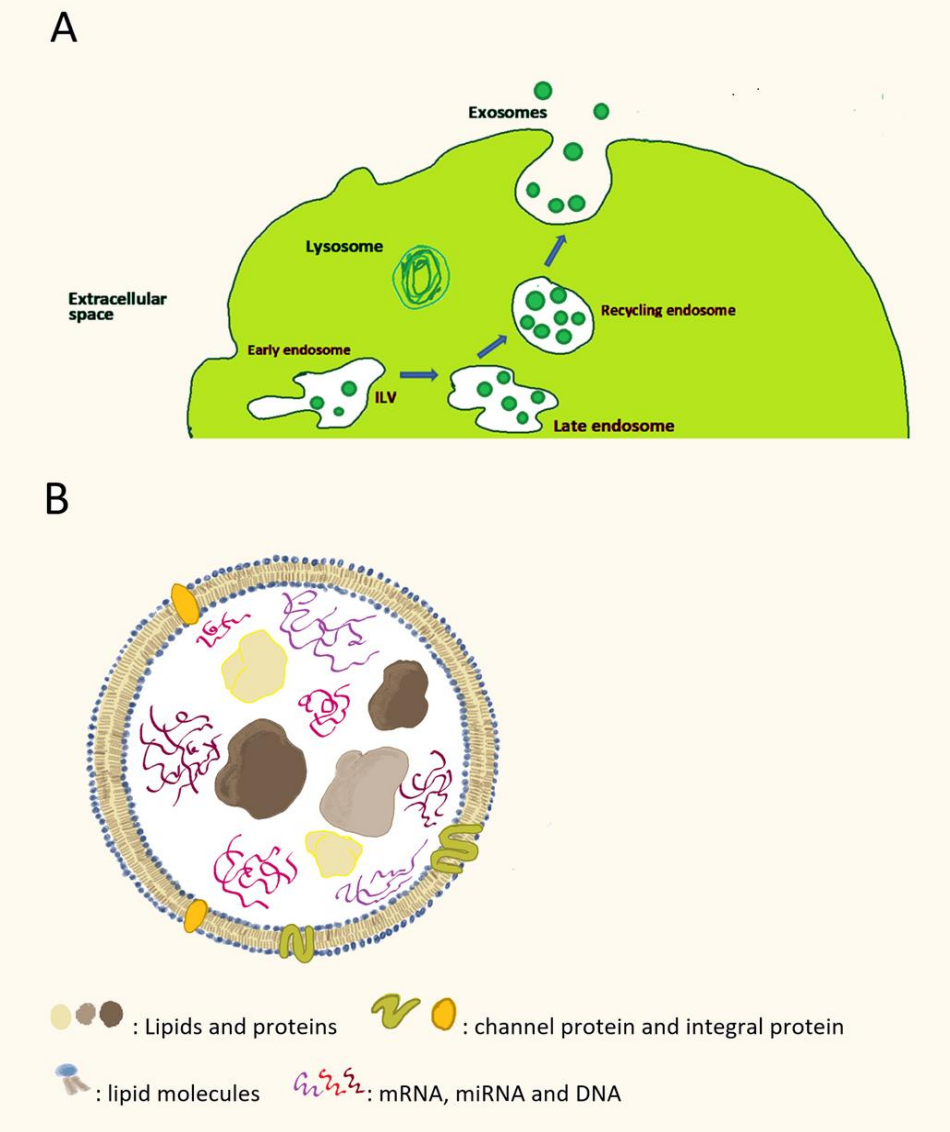



**Figure 1 Illustration of Exosome Biogenesis (A) and content (B).** Exosomes are part of a wider family of extracellular vesicles which further includes the group of (i) the shedding microvesicles and (ii) the relatively larger apoptotic bodies. The biogenesis of exosomes (Boriachek *et al.*, 2018; Yu, Odenthal and Fries, 2016) starts inside endosomes, which are membrane-bound compartments inside eukaryotic cells and comprise of three distinct stages as they evolve: early endosomes, late endosomes, and recycling endosomes **(A)**. Each one of these reflect their maturation level. At first, an inward budding of the endosomal membrane will give rise to intraluminal vesicles (ILV). The late endosomes (mature stage) can either fuse with lysosomes and become degraded via hydrolysis, or fuse with the plasma membrane and release their ILVs into the extracellular space. These ILVs, are considered exosomes. Cargo sorting of exosomes is cell type specific and influenced by cellular conditions. The biogenesis mechanism provides them with a particular biochemical composition, as already mentioned. **(B)** Illustration of an exosome depicting its main characteristics. A bilipid membrane of endocytic origin enclosing the cargo designated by the cell of origin. The cargo consists of DNA, miRNA, miRNA, lipids, and proteins, specifically chosen for the alterations of the targeted cell.

Exosomes are part of a wider family of extracellular vesicles which further includes the group of (i) the shedding microvesicles and (ii) the relatively larger apoptotic bodies. Their particularity lies on their specifically designed cargo sorting, which is cell type specific and influenced by cellular conditions. The biogenesis mechanism provides them with a particular biochemical composition, and their function and content are influenced by molecular signals received by the cell of origin from the surrounding tissues (Villarroya-Beltri *et al.*, 2014). The trans-Golgi network is closely related to their biogenesis, and Rab family proteins (membrane proteins involved in the regulation of exosome secretion), are key regulators of the process (Villarroya-Beltri *et al.*, 2014; Boriachek *et al.*, 2018). Exosomes are enriched in cholesterol, sphingomyelin, saturated phosphatidylcholine, and phosphatidylethanolamine as compared to the plasma membrane of their original cell (Villarroya-Beltri *et al.*, 2014).

Exosomes play a major role in extracellular communication, and through their content, they affect and regulate biological functions. Exosomes have been reported playing their regulatory and intracellular communication role in physiological processes such as the immune response, tissue repair, stem cell maintenance and metabolism, as well as in pathological processes such as inflammatory, cardiovascular and neurodegeneration diseases, systemic diseases and cancer (Perocheau *et al.*, 2021). They target neighbouring cells and tissues, as well as those of areas distant to their cell of origin (Boriachek *et al.*, 2018; Yu, Odenthal and Fries, 2016). The plethora of information obtained led to the creation of databases that provide catalogues of the extracellular vesicle content in different cell types: interesting information can be found in *Vesiclepedia, EVpedia* and *ExoCarta* where a multitude of studies on a variety of species can be found.

Research of exosomes is a rising trend in periodontology. It is orientated in investigation of the exosomal presence in oral fluids and periodontal tissue to better understand their local function and role. Immune response, periodontal inflammatory response, periodontal tissue destruction, periodontal treatment response, tissue healing, periodontal regeneration and dental implants are some of the areas of interest so far investigated (Xu *et al.*, 2020; Wu *et al.*, 2019; Doyle and Wang, 2019; Huynh *et al.*, 2016; Nakao *et al.*, 2021; Zhang *et al.*, 2021). The knowledge platform has given the grounds to clinical implementations in the periodontal treatment, such as periodontal regeneration (Elashiry *et al.*, 2020; Gegout *et al.*, 2021), by using them with specifically designed or altered cargo. Taking in consideration the early stages of implementing these as part of periodontal treatment, there is some promising options in the future of exosomes in periodontal clinical applications. Studies have shown that use of exosomes isolated form mesenchymal stem cells originating from the periodontal ligament, have shown that periodontal regeneration was noticeable in treatment of intrabony defects (Chew *et al.*, 2019), and have the potential to inhibit alveolar bone loss (Nakao *et al.*, 2021).

Due to their small size, the plethora and multiple functions these present in terms of gene silencing, the research continuously allows their better understanding, increasing the possibility for future applications in the clinical field and academic research.

**MicroRNA**

miRNAs are small non-coding RNA molecules of about 22 nucleotides which can affect gene expression at the post-transcriptional level by directing the RNA-induced silencing complex to their target messenger RNAs. They were discovered in the early 1990s by Lee (Lee, Feinbaum and Ambros, 1993) and are part of the exosome's content. miRNAs have a major role in regulation of gene expression and can also activate translation or regulate transcription of them. Furthermore, they have been recognised as messengers mediating cell-cell communication (O'Brien *et al.*, 2018). Their main way of control gene expression Is via targeting messenger RNAs (mRNAs) on which they can bind and either cause decay, or cleavage (O'Brien *et al.*, 2018), ultimately leading to the incapacity of the mRNA to further transcript. There is also the speculation of miRNAs having the ability to act as a gene regulators in the cell nucleus as well (Nishi *et al.*, 2013).

Their biogenesis takes place in the nucleus and the cytoplasm. Animal miRNAs are first processed in the nucleus. miRNAs responsible genes are located in intergenic regions, whereas a few were annotated in intronic regions (Wahid *et al.*, 2010). RNA polymerase II (Pol II) is mainly responsible for the transcription of miRNA genes, and some are transcribed by RNA polymerase III (Pol III). This will create after a few steps and the help of RNA polymerase III (PoL III), the first premature sequence, in the form of a hairpin loop, the pre-miRNA. At this stage, miRNA can be as long as 150 nucleotides. This will be transferred to the cytoplasm with the help of Exportin-5 (a protein of the family of karyopherins, responsible for transferring molecules in and out of the nucleus) (Murchison and Hannon, 2004). In the cytoplasm, with the help of RNase III enzyme Dicer, it will be processed and take its final shape as a miRNA. Its length now is close to 22 nucleotides. At this stage, it can circulate in the cytoplasm or outside of it as circular miRNA or extracellular miRNA (Sohel, 2016). Turnover and decay of mature miRNA takes place with the help of Argonaute (AGO) proteins. Antagomirs which

target and mark miRNAs, Uridilation, to finally be degraded by ribonucleases (Zhang *et al.*, 2012). The biogenesis and final degradation can be seen in **Figure 2**.

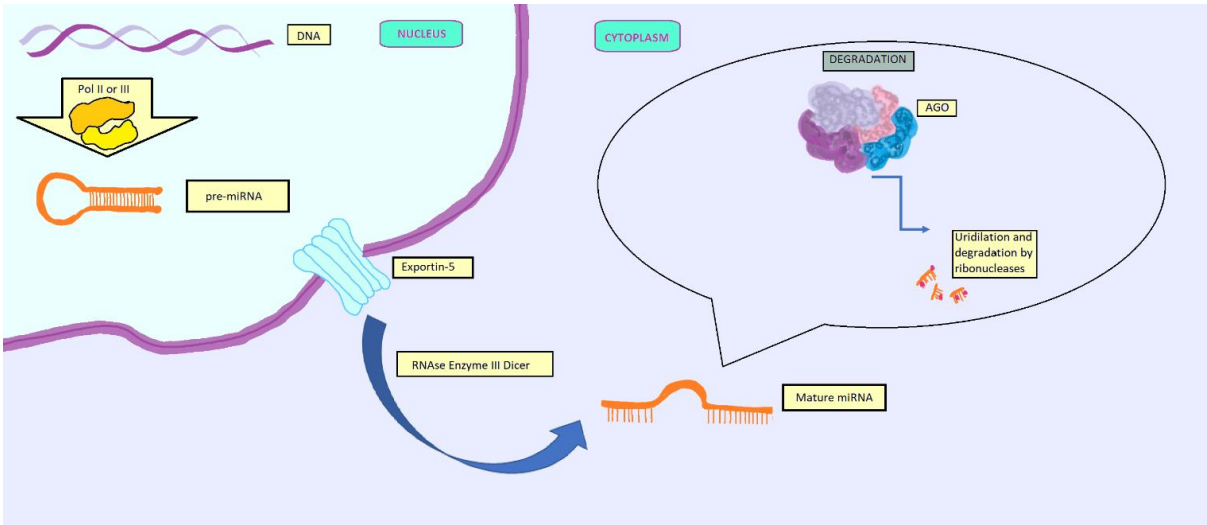



**Figure 2 Biogenesis and degradation of miRNA.** The illustration depicts the main stages of biogenesis and degradation of miRNA. First, in the nucleus, a specific region in the DNA sequence (intergenic or introgenic) will be transcribed by Polymerase II or III to the very first form of premature miRNA. It will have the shape of a hairpin and its size will be no longer than 150 nucleotides. The next step will be for this premature miRNA to be transferred to the cytoplasm of the cell, with the help of a karyopherin, the Exportin-5, through the nucleus's membrane. In the cytoplasm, after a number of complex changes and cleavage with the help of RNase III enzyme Dicer, the miRNA finalises in shape and form. The degradation and decay of miRNA will be completed with the help of Antagomirs, specialised in breaking down the sequel of nucleotides of miRNA, and ribonucleases will finally complete the procedure.

The human genome may encode over 2300 miRNAs (Alles *et al.*, 2019). These findings have shed further light in fields that are of upmost importance in every clinical discipline. It seems that they are strongly associated with the features and mechanisms of the immune system, innate as well as adaptive. Both are thoroughly investigated to further understand their function as well as possible therapeutic applications. The research around them is relatively new and in early stages. However, quite promising for future clinical application in diseases that either are fatal or chronic with serious implications (Chaput and Théry, 2011; Rupaimoole and Slack, 2017), and provide major advances in their therapy.

miRNAs can reflect in a specific time, the condition of tissues they originate in terms of physiology and pathology, and this is applicable in the field of periodontology (Hadavand and Hasni, 2019). In the periodontal field, intensive research has shed light on the pathways miRNAs are involved in various biological functions. In a recent review, Santonocito (Santonocito *et al.*, 2021) highlights the way miRNAs are involved in various cellular

processes. miRNA-584 is induced by *Porphyromonas gingivalis (P.g.)*, one of the most known periodontopathic bacteria, through the effect of the bacterium's lipopolysaccharides, causing upregulation of the interleukin IL-8 to finally induce an antimicrobial effect from the host. Leukocyte migration from the blood vessels, is indirectly controlled by the miRNA-31 and miRNA-17-3p. An example of miRNA getting involved in periodontal immune response is miRNA146, which seem to have multiple effects. On one hand, by blocking the signalling process, it favours the bacteria's actions against their host; and on the other, is being associated with reduced numbers of cytokines like TNF-α, IL-1β, hence related to prevention of periodontal tissue degradation. Schmalz (Schmalz *et al.*, 2016), presented some of the most studied miRNAs. miR-146a and miR-155 are the most noticeable ones, which seem to be targeted by multiple studies, due to their abundance in periodontal inflamed tissues, and their likely link to the presence of bacterial lipopolysaccharides.

miRNA research revealed expression in several types of cells and tissues, undertaking a variety of roles. Any abnormality in their expression is implicated in disease states. miRNA is a non-coding RNA that have as function RNA silencing and hence, the regulation and control of gene expression via a post-transcriptional way (post-transcriptional inhibition of gene expression).

Depending on the manner the targeted genes are silenced, the endpoints can be upregulation or downregulation of the genes and the biological functions related to them. Furthermore, the same miRNA can up- or downregulate the expression of genes and not be restricted in only one way of regulation, and also target multiple genes simultaneously (Irwandi and Vacharaksa, 2016; Santonocito *et al.*, 2021). miRNAs can be upper or downregulated by the presence of pathogens such *P. G.,* putting in action the host's immune pathways (Olsen, Singhrao and Osmundsen, 2017). It is thought that they can be used as predictive tools for periodontal disease and healing process of periodontal therapies (Luan *et al.*, 2018). In this study, miRNAs area considered good candidates for diagnostic purposes, as a reasonable option, due to their constant presence in oral fluids, and by comparison to studies made in the field of cancer where they are studied for the same purpose. He also sees two major advantages miRNA present: being the local produce of periodontal tissues and their easy sampling and collection, from GCF and saliva. From the two oral fluids, Luan et al., favours GCF, as being anatomically closer to the periodontal tissues.

Due to their ability to be in detectable levels in bodily fluids (Denzer *et al.*, 2000), they are harvested and studied for the above-mentioned purposes from patient's saliva and gingival crevicular fluid-GCF (Han *et al.*, 2018; Michael *et al.*, 2010). This is making miRNAs very valuable for the diagnosis of systemic diseases, including oral conditions, suggesting a role

as biomarkers and their potential for future use in routine diagnosis. Studies have brought to light and identified a large number of miRNA and their involvement in a multitude of biological functions relating to the periodontal tissues, as aforementioned. These are related to immunity, metabolism, homeostasis, oxidative stress, wound healing, tissue regeneration and all biological functions known in healthy and pathological periodontal conditions (Olsen, Singhrao and Osmundsen, 2017; Santonocito *et al.*, 2021; Amaral *et al.*, 2019; Motedayyen *et al.*, 2015; Fujimori *et al.*, 2019; Guo *et al.*, 2019; Al-Rawi *et al.*, 2020; Simões *et al.*, 2019). So far, research have come finally to propose periodontal diagnosis related to miRNAs, direct from gingival tissue (Jin *et al.*, 2020), or salivary miRNAs (Al-Rawi *et al.*, 2020), quite promising for the future. **Table 2**. presents well-characterised miRNAs related to periodontitis, and their target gene functions.

| miRNAs | Up/ down regulation | Targeted gene function |
|---|---|---|
| hsa-miR-1274b, hsa-let-7b-5p, hsa-miR-24-3p, hsa-miR-19b-3p, hsa-miR-720, hsa-miR-126-3p, hsa-miR-17-3p and hsa-miR-21-3p | Upregulation | Cell differentiation and osteogenic differentiation capacity of stem cells |
| miRNA-146a<br><br>miRNA146a – miRNA155 | Downregulation<br><br>Upregulation | Downregulation of interleukin-1 receptor–associated kinase 1 and TNF receptor–associated factor 6<br><br>Oxidative stress by targeting superoxide dismutase (SOD) |
| hsa-miR-381-3p, miR-181b, miR-19b, miR-23a, miR-30a, miR-let7a and miR-301a | Upregulation | Increased immune response |
| miRNA-218 | Upregulation | Regulation of matrix metalloproteinase-9 (Mmp9) suppress the degradation of Collagen types I and IV and dentin sialoprotein (DSP), attenuate osteoclast formation, and inhibit the secretion of proinflammatory cytokines. |
| miRNA 155, 146a/b and 203 | Upregulation | CD56+ NK cells, whole blood, CD4+ T cells, CD8+ T cells, interleukin1 and 8 regulation (chemotaxis, proliferation, production) with simultaneous positive and negative regulation. |
| miR-103 1/2, miR-107, miR-646, miR-503, miR-126, miR-424, miR-15b, miR-497, miR-15a, miR-195, miR-16-1/2, miR-126, miR-221/222, miR-17-92, miR-92a, miR-20a, miR17-5p, miR-23-27, Hsa-miR-223-3p<br><br>Hsa-miR-124-3p | Upregulation<br><br>Downregulation | Extensive role in angiogenesis |
| miRNAs | Up/ down regulation | Targeted gene function |

**Table 2 miRNAs and regulation of targeted genes and effect on cellular function.**
(Olsen, Singhrao and Osmundsen, 2017; Santonocito *et al.*, 2021; Amaral *et al.*, 2019; Motedayyen *et al.*, 2015; Fujimori *et al.*, 2019; Guo *et al.*, 2019; Al-Rawi *et al.*, 2020; Simões *et al.*, 2019).

## Saliva

Saliva is the fluid secreted by three paired major salivary glands (the parotid, submandibular and sublingual) and the minor salivary glands spread in the oral mucosa. When referring to saliva, it is implied that the above secretions are mixed with the gingival crevicular fluid (GCF), known under the term of whole saliva, which coats the hard and soft tissues of the oral cavity (Dawes, 2012). Saliva is a body fluid with many and important functions which mainly comprise of lubrication, ion reservoir, buffering by balancing the oral cavity's pH, assists in speech, swallowing and air flow, cleansing food and microorganisms, have antimicrobial functions, agglutination, pellicle formation on teeth's surface, participates in digestion, taste, excretion of substances and body water balance (Dawes, 2012). It is composed mainly of water (at least 99.5%), inorganic and organic constituents and its composition depends on various factors which have major (degree of hydration, body position, exposure to light, previous stimulation, biological rhythms such as circadian and circannual rhythms) or minor (gender, age, body weight, gland size, psychic effects such as thoughts or sight of food, and functional stimulation) influence on it. It also contains desquamated epithelial cells, bacteria, leucocytes (mainly from the gingival crevice), and possibly food residues, blood, and viruses. It is flowing from the salivary ducts of the salivary glands and gingival sulcus into the oral cavity, as forementioned, with a mean flow rate of 0.3-7ml/min, as unstimulated or stimulated whole saliva respectively. Apart from being the main source of glycoproteins for the oral microbiota to attach to the oral cavity's tissues (mainly teeth) and main medium of nutrients, it also has a determining effect on its composition and survival through complex mechanisms (Dawes, 2012; Marsh *et al.*, 2016).

Also, salivary secretions being the main sources of calcium and phosphate in the oral cavity, are the reason calculus is formed on teeth and hard surfaces, which can be supra or subgingival, and both are the result of mineralisation of the local dental plaque.

The organic components of saliva comprise of proteins, peptidases, enzymes, immunoglobulins, amino acids and lipids. Proteins play a considerable role in the oral cavity primarily. Their functions include antimicrobial systems, lubrication, water retention, controlling of endogenous and exogenous micro-organisms, protection of soft and hard tissues, and plaque acid production. Some of the most important antimicrobial proteins are lysozyme, lactoferrin, peroxidases, agglutinins, amylase, histidines and cystatins. Immunoglobulins are also found in saliva as part of the immune response, and are IgA, IgG and IgM, which are

secreted by the large salivary glands, and also by the oral epithelium, mainly from plasma cells (Carpenter, 2013; Dawes, 2012).

One of the major roles of saliva in the oral cavity, is the active participation in wound healing. The wound healing process is very similar to cutaneous and oral wounds; however, it is much more accelerated, and leaves further less scars in the oral cavity. The difference between the two tissues, is the presence of saliva, which provides in one hand a highly humid environment, and on the other be the median carrier of a multitude of components, which actively participate in the process of healing. Brandt (Brand, Ligtenberg and Veerman, 2014), presented some of the components of saliva, such as the tissue factor, epidermal growth factor and protease inhibitor, which step in the procedures of blood clotting, epithelial cells proliferation and inhibition of tissue degrading enzymes (like elastase and trypsin) respectively, in such a way that accelerates the healing process. More salivary components are contributing to this direction, along with the specific characteristics of the oral environment (as the humidity, temperature, rich in nutrients saliva) and with their synergistic action, make the oral environment extremely effective in wound healing, when compared to the cutaneous one.

In research, saliva can be collected either as a whole, or directly from the individual's major salivary glands, depending on the purpose of its analysis, and can be stimulated or non-stimulated. Whole saliva is superior and clinically more meaningful when assessing overall salivary gland function. For this purpose, the existing methods proposed are the: 1. Draining method, where saliva drips off from the lower lip in a collection vessel, 2. Spitting method, where saliva is spitted every 60 sec in a collection vessel after accumulating in the floor of the mouth 3. Suction method, where saliva is aspirated by a saliva aspirator and the 4. Swab method, where a pre-weighed swab, cotton roll, or gauze is used to collect (absorb) saliva (Navazesh, 1993). Immediate freezing following collection of the samples in low temperatures (-80°C) is necessary to ensure their long-term protection and conservation, especially in longitudinal studies. Due to multiple factors affecting the qualitative and quantitative properties of it, it is necessary to standardise the method of collection throughout the study. Saliva has the advantage of easy and painless collection, which both patients and clinicians prefer.

**Salivary proteins**

The total composition of proteins in stimulated and unstimulated hole saliva ranges between 1910 and 1640 (mg/ L). The main body of protein secretion derives from the 3 major salivary glands: the parotid, sublingual and submandibular glands (Dawes, 2012). These proteins aim at supporting the main functions of saliva and are considered intrinsic proteins. Saliva can also carry proteins produced in organs distant and/ or differentiated from the oral cavity, called

extrinsic proteins (Saitou *et al.*, 2020), making saliva a very diverse bodily fluid. However, Saitou concluded that the exact organ of origin of these proteins is still unclear, and further investigation is necessary on the homeostasis of salivary glands, as well as on how the salivary proteins are impacted and their final composition in saliva is obtained.

In pathological oral conditions (systematic or dental), saliva is expected to be the carrier of molecular cues, exhibited by the pathological sites, in particular proteins, which are considered as elements of biomarker signature analysis (Jaedicke, Preshaw and Taylor, 2016; Zhang, Xiao and Wong, 2009). Some of the most studied proteins are the ones involved in the immune response such as interleukins, which are among the first signal molecules to participate in the regulation of immune responses and inflammatory reactions. Interleukins are a group of cytokines, by which the acquired and adaptive immune responses largely are orchestrated. In periodontitis, some of the most studied inflammation inducing interleukins (IL) are the IL-1β and IL-6.

IL-1β, is a proinflammatory cytokine, produced by a plethora of cells, but mainly by monocytes, macrophages, B-cells, dendritic cells, epithelial and endothelial cells. In higher levels than the normal ones, it is mediating the inflammatory response, work as a leukocytic endogenous mediator (attracts leukocytes), causes raise of temperature (pyrogenic properties), and in prolonged inflammatory periods exacerbates damage and acute tissue injury (Ren and Torres, 2009; Lopez-Castejon and Brough, 2011). In more details, in untreated periodontitis, IL-1β is also produced by osteoblasts and mediates stimulation of adhesion molecule and chemokine expression, stimulates $PGE_2$ production, enhances osteoclast formation, induces matrix metalloproteinase (MMP) expression and stimulates apoptosis of matrix producing cells, such as fibroblasts (Graves and Cochran, 2003). Through these mechanisms, the periodontal inflammation becomes a chronic condition, the alveolar bone and connective tissue experience extensive loss and breakdown, and the periodontium presents a limited repair ability.

IL-1β is a subtype of IL-1, and directly involved in the extracellular matrix degradation and alveolar bone resorption in periodontitis. Kornman in his study (Kornman *et al.*, 1997) reported that this subtype, is causing increase levels of IL-1, and as a result, periodontal tissue degradation. The impact of IL-1β, well studied for its impact in periodontitis patients exhibiting active disease, seem to also have impact in the therapy. The study of de Sanctis and Zucchelli (De Sanctis and Zucchelli, 2000), evaluated the impact of IL-1 in periodontally treated patients with regeneration therapy for interproximal angular bony defects, concluding there is a 10 times higher fold for CAL loss ≥2mm following 1 year of regenerative surgery. The impact of IL-1β on the periodontal tissues is well studied, and its presence in periodontal patients have

a central role in the progression of the disease, being a hallmark of great importance and its early detection, seem to be crucial for the prevention and management of periodontitis patient.

IL-6 is also one of the first cytokines responding to trauma and infection, produced by cells such as macrophages, neutrophils, keratinocytes, fibroblasts, and endothelial cells. Its synthesis follows a stimulus from infection or trauma. The biologic properties of IL-6 are not only limited into inducing an inflammatory response (pro-inflammatory activity), but also periodontal tissue destruction, anti-inflammatory activity and grown-factor properties (Nibali *et al.*, 2012). Furthermore, IL-6, except of being so diverse in terms of biologic activity and implications in the periodontal inflammatory pathway, it is expressed mainly in relation to the acute phase of periodontitis and is found to be of decreased levels in patients with chronic periodontitis, comparable to the levels of healthy subjects. It is also important to point out, that IL-6 is found in saliva mainly through GCF, and not from the salivary glands (Nibali *et al.*, 2012).

The existing literature has pointed out their importance and critical action in periodontitis (Graves, 2008; Cekici *et al.*, 2014; Nibali *et al.*, 2012). As salivary biomarkers for the early detection and management of periodontitis, they have been already extensively studied, with the hope that the disease would be caught before the tissue destruction starts and CAL is clinically detectable (Jaedicke, Preshaw and Taylor, 2016). The research made in the field, have provided significant advances in our understanding on how realistic the routine use of salivary biomarkers is, the validity of saliva as a source of biomarkers, the precision of the methods to use for their detection, and to what direction the research needs to be conducted and further shed light in the clinical use of biomarkers.

IL-6 is a cytokine which is detected in increased levels in gingivitis and periodontitis. In inflammation it has both anti-inflammatory (through downregulation of neutrophil recruitment and proinflammatory cytokine expression) and pro-inflammatory (ex: induction of acute-phase reactants by the liver) actions. It can be detected in GCF as well as saliva, with GCF being its main source as it is the oral fluid in which it is found in higher concentrations (Nibali *et al.*, 2012). In periodontitis, the levels of IL-6 are affected by periodontal treatment, where there is an association between the short-term inflammatory response to therapy (increase of IL-6 levels) and long-term reduction of its levels relating to clinical improvement in periodontal patients (D'Aiuto *et al.*, 2004; D'Aiuto *et al.*, 2005; Nibali *et al.*, 2012). Both these cytokines are considered in the literature as biomarkers exhibiting good sensitivity and specificity values, in the detection of periodontitis (Arias-Bujanda *et al.*, 2020).

## GCF

Gingival Crevicular Fluid (GCF) is secreted from the gingival sulcus of teeth representing a small fragment in the whole saliva's volume, and its volume is measured in μl (Griffiths *et al.*, 1992). The proteins, exosomes and miRNAs exhibited in the GCF, and their altered profile might be indicative of periodontitis (Saito *et al.*, 2017). In inflammatory conditions, GCF is an exudate originating from the gingival blood capillaries and secreted in the dental sulcus (Barros *et al.*, 2016; Griffiths, 2003). Its composition is mainly blood electrolytes and organic molecules, i.e. albumins, globulins, lipoproteins, peptides, enzymes, fibrinogen and cellular components as well as bacteria. These can vary depending on the state of the gingivae. As the condition of the gingival tissues changes from health to disease, it is reflected in GCF's composition. Being the local product of gingiva, however small in volume, GCF contributes to the whole saliva' proteinic load, exosome and miRNA's amounts. The presence of exosomes and miRNAs in GCF, have attracted interest in the field of periodontology. The research has investigated the relation between exosomes and miRNA in the various aspects of the periodontal disease and how these are expressed (Luan *et al.*, 2018). The main target of research is evidence suggesting the involvement of miRNAs in periodontal tissue homeostasis and pathology (Kebschull and Papapanou, 2015; Olsen, Singhrao and Osmundsen, 2017) around teeth and implants (Asa'ad *et al.*, 2020). The results of studies revealed the important role of miRNAs in the pathogenesis of periodontitis, however, the knowledge obtained so far is limited (Irwandi and Vacharaksa, 2016) and further investigation is still required to determine in a more precise way the function of miRNAs in the complex processes of periodontal tissue homeostasis, pathogenesis and healing. In the literature, the main efforts to date, are concentrated in the establishment of miRNAs involved in the above-mentioned aspects of the periodontal and peri-implant tissues.

This extensive research started to give the grounds for clinical applications. Exosomes have already been used in treatment. A recent review studied the *in vivo* use of exosomes in regenerative periodontal treatment (Gegout *et al.*, 2021) with positive results. However, the methods still need to be optimised and standardised, as these are in their early stages of implementation.

To our knowledge, studies entertaining the miRNA profile of specific periodontal treatment modalities and their healing pathway, have not yet emerged in the literature. The main weight of the present research will be within this context, following the expression of miRNAs after treatment of patients with the aforementioned treatment non-surgical and surgical modalities.

## Hypothesis, Aims and Objectives

The hypothesis of the pilot study is to evaluate whether signature(s) of exosomal miRNAs, IL-1β and IL-6, isolated from GCF and saliva of periodontitis patients prior to any treatment exist and can be used as feasible biomarkers of periodontal treatment.

The objective of the present study is to describe the miRNA (in GCF and saliva), and IL-1β and IL-6 (in saliva) profile expression, and define specific signatures and their association with the clinical parameters informing the periodontal status of patients.

## Materials and Methods

This pilot study is part of an on-going randomised, single-blind, parallel-surgical modalities groups (3 groups of which SD is the control non-surgical group, and SPPF and RPFO are the surgical test groups, conservative and resective flap respectively). The clinical trial includes a total of 87 patient with periodontitis.

This pilot study is based on the analysis of saliva and GCF samples from 12 patients, collected at their baseline appointment following completion of step 1 and step 2 periodontal treatment, prior to any further treatment within the clinical trial mentioned above. From each patient there was a 2ml sample of saliva and 4 GCF samples obtained from the 4 deepest PPD sites per patient, as seen in **Table 3**. The samples used, were from samples collected prior to the commencement of the 2019 pandemic, to avoid contamination from the COVID-19 virus and further isolation precautions while processing them. From the GCF samples, exosomes were isolated, and their RNA was extracted for further analysis comprising of a precipitation protocol, use of the ExoQuick precipitation solution, purification and finally Nanosight analysis. Saliva samples will be processed for investigation of their IL-1β and IL-6 cytokines with the ELISA method.

| | Treatment time point | Sites of treatment/ collection samples | Treatment modality to follow and patient code |
|---|---|---|---|
| GCF | Baseline | Samples from 4 deepest PPD, ideally 1 per quadrant with methylcellulose strips | GROUP A: 51, 54, 59, 60<br>GROUP B: 50, 53, 64, 65<br>GROUP C: 56, 58, 61, 66 |
| Saliva | Baseline | Whole saliva (2ml) | GROUP A: 51, 54, 59, 60<br>GROUP B: 50, 53, 64, 65<br>GROUP C: 56, 58, 61, 66 |

**Table 3. GCF and Saliva collection samples, timeline of collection and treatment modality**

The participants had to fulfil the following criteria to be accepted in the study:

1. Males and females over or 30 years of age who are systemically healthy

2. After reading the "Patient Information Leaflet" and being fully informed about the study, volunteers must agree to read and sign a copy of the "Informed Consent" form.

3. Present with periodontitis and one interdental site with PPD 6mm, BOP, and attachment loss 6mm in any posterior sextant of their mouth (excluding third molars and distal of second molars), or several sites (≥9) with PPD 5mm, BOP, and attachment loss 5mm.

4. Have received maintenance subgingival debridement within 6 months of being assessed for eligibility and have finished a course of non-surgical periodontal therapy within 2 years of starting the trial.

Exclusion criteria were the following:

1. Diabetes, hepatic or renal illness, or other major medical disorders or infectious diseases, such as cardiovascular disease or AIDS, should be mentioned in the medical history.

2. History of rheumatic fever, heart murmur, mitral valve prolapse, artificial heart valve or other conditions requiring prophylactic antibiotic coverage prior to invasive dental procedures.

3. During the month prior to the baseline assessment, prescription of antibiotics, anti-inflammatory, or anticoagulant therapy.

4. History of alcohol or drug abuse.

5. Pregnancy or lactation that was self-reported (due to oral tissue changes related to pregnancy and nursing which can affect interpretation of study results).

6. Other severe acute or chronic medical or psychiatric condition or laboratory abnormality that may increase the risk of trial participation or investigational product administration, or that may interfere with the interpretation of trial results, and that, in the investigator's opinion, would make the subject ineligible for participation in this trial.

Pre-Study Procedures were preliminary to the acceptance of the participants and review of their medical history was also carried out. Clinical and radiographic assessment of periodontal status for diagnostic purposes and to determine eligibility for the study. After an initial discussion with the patients took place to explain the study details, provision of an information sheet was necessary for their consideration.

Following recruitment, all participants were to undergo one session of supra-gingival scaling to promote optimal gingival health and the lowest plaque levels possible of the mouth in preparation for the study treatment, which was followed after the randomization for all posterior sextants. This treatment included:

- Oral hygiene instructions were given to all patients with the goal to reach satisfactory self-control plaque of<25%, prior to treatment visit.
- This treatment was performed by the study dental hygienist or clinician.

## Standard Operating Procedure of Sample Collection, Storage & Analysis.

### 1. Saliva collection

Following the baseline visit, an enrolment and a preparation visit, patients were randomised to their assigned group and the pending treatment (SPPF, RPFO and SD). Non-stimulated saliva sample was collected at baseline, before any other treatment according to the treatment protocol took place or clinical sampling or measurement took place. The study participants were asked to refrain from eating, smoking, drinking, or performing oral hygiene for at least 60 minutes prior to saliva collection. For the baseline, 3, 6 and 12 months post treatment visits, participants were asked if they had eaten, smoked, drank, or performed oral hygiene. If not, they were asked to wait for 60 minutes in the waiting area. The patient will be seated with their head tilted forward in a calm space. Whole saliva samples were taken and pipetted into 2ml cryovials, labelled and immediately stored at -80°C for future analysis.

### 2. GCF collection

Samples of GCF were collected from the 4 deepest sites of individuals with PD (preference for one per quadrant) at baseline, 2 weeks and 3 months post treatment. GCF was collected at baseline, 2 weeks post-surgery and at 3 months post treatment. GCF was collected prior to

periodontal probing to avoid contamination by blood. The selected sites were isolated with cotton rolls and saliva was removed from the supra-gingival area using saliva ejector and cotton ball, avoiding touching the gingival margin. Plaque, if present, was removed from the supra-gingival with the use of a curette to prevent contamination. GCF was collected using methylcellulose strips (pro Flow, Inc., Amityville, NY) carefully and gently placed at the entrance of the sulcus until resistance is felt and left for 30sec. The samples were placed immediately into Eppendorf tubes (on ice), labelled and then placed in the laboratory freezer at -20° to -80°C for storage until time of analysis at a later date.

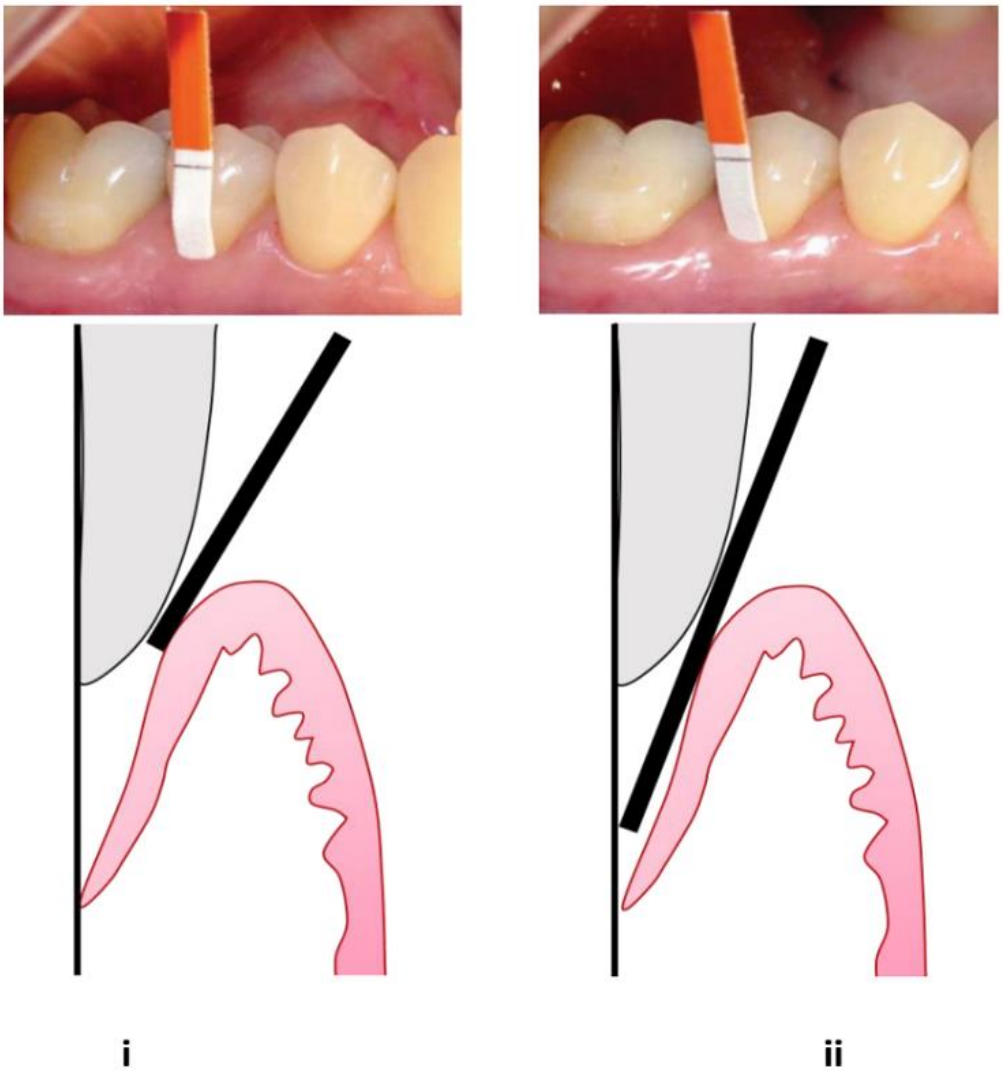



**Figure 3 Collection of GCF with absorbing paper strips of standardised size.** These can absorb volume of GCF ranging between 1.2-2µl. There are two methods of collection: i) Löe and Holm-Pederson technique, positioning of paper strip at gingival margin without penetrating in the gingival sulcus; with low risk of irritation; and ii) Brill's technique, positioning of paper strip into sulcus; this technique may cause irritation to the sulcus (Lagos *et al*., 2011).

## Exosome isolation method (Saliva and GCF)

The procedure of exosome isolation from the saliva and GCF samples followed in this study, is a precipitation protocol. The materials used for this pilot study were12 saliva samples (collected at the baseline examination appointment) and the ExoQuick-TC exosome precipitation solution. The completion of the procedure was as follows:

The frozen samples of GCF and saliva were allowed to thaw on ice for about 15min. GCF was eluted from paper strips by dilution in 100-200ul of proteases-inhibitors-containing PBS buffer. After resuspension for 10min, tubes were centrifuges at 1500rpm, and supernatant collected and transferred to a new tube. 1ml of saliva suspended, collected and placed in new Eppendorf tubes. Consecutive centrifugations of GCF and saliva samples at 3,000xg for 10min, 5,000xg for 20min and 12,000xg at 4°C were performed, with collection of the supernatant (SN) after each cycle in new Eppendorf tubes. The final SN volume of each sample was divided in 1 (GCF) or 2 (saliva) separate Eppendorf tubes. Final volume in each tube was adjusted to 400μL of SN. 200μL of ExoQuick was added to each tube to achieve a final ration of 2:1 (SN:ExoQuick). The tubes were inverted several times to achieve a good mixture. All samples were placed in a rack in an upright position and left to incubate overnight (about 16 hours) at 4°C. The next day, two successive centrifugations at 4°C took place at 2,000xg for 30 and 10min. The SN after both centrifugations were removed and stored at -80°C (for future proteomic analysis with the ELISA method).

Nanosight analysis of extracellular vesicles took place at this point, (Nanoparticle Tracking Analysis - NTA) which is commonly used to determine EV concentration and diameter.

## RNA extraction process

Following overnight incubation, the RNA extraction process from exosomes followed with the use of PureLink®RNA Mini Kit and followed its protocol. Additional PBS and 70% ethanol solution was necessary as materials.

To decontaminate RNases from surfaces and equipment used in this procedure, RNaseZap® Solution was used prior to all processes.

Pellets were resuspended in 100μL and the samples that were separated at the exosome isolation method were merged at this point, in the same Eppendorf tube. The final volume of the tubes as this point is 100μL. 400μL of Lysis buffer was added in the tubes. Sonication with the use of an ultrasonic probe followed to homogenise the samples. 200μL of Lysis buffer was

added to each tube and mixed in a spin mix for about 20sec. Each tube at this point had a final volume of 700µL.

### RNA Purification steps

The 700µL of each sample was transferred into spin cartridges and centrifuged at 12,000xg for 1 min at room temperature (RT). The flow through was discarded. 700µL of Wash Buffer I was added to the spin cartridge, centrifuged at 12,000xg for 1 min at RT and the flow through was discarded. The spin cartridge was placed into a new collection tube. 500µL of Wash Buffer II and Ethanol were added to the spin cartridge and centrifuged at 12,000xg for 1min at RT. The flow through was discarded. This step was repeated once more. Centrifuge at 12,000xg for 2min at RT to dry the membrane with the bound RNA, discard the collection tube and allow to incubate at RT for 1 min. Add 25µL of filtered water at the centre of the membrane with the bound RNA, incubate for 1 min at RT and centrifuge at 12,000g for 2min at RT to elute RNA from the membrane to the recovery tube.

### Protein Analysis (Saliva)

The saliva supernatants were collected and stored at -80°C, until required for ELISA analysis. Human IL-1β and IL-6 (R&D Systems DuoSet® ELISA Development Systems) were used, and protocol was conducted as per the following steps:

### Plate Preparation

The Capture Antibody was diluted in PBS without carrier protein to the working concentration, and the coating of the 96-well microplate was completed with 100µL of the diluted Capture Antibody followed by overnight incubation at RT. The aspiration and wash of each well was completed with Wash Buffer twice, for a total of three washes. 400µL of Wash Buffer was used to fill each well. The remaining Wash Buffer was removed by aspirating or inverting the plate and blotting it against clean paper towels after the last wash. 300µL of Reagent Diluent was used to each well to form a block, followed by incubation at RT for at least 1 hour. The aspiration/ washing process was repeated.

### Assay Procedure

100 µL of sample/ standards in Reagent Diluent was added to each well, covered with adhesive strip, incubated for 2 hours at RT, followed by aspiration/ wash. 100 µL of the

Detection Antibody were added to each well, covered with a new adhesive strip, incubated for 2 hours at RT, followed by aspiration/ wash. 100 µL of the working dilution of Streptavidin-HRP were added to each well, covered with adhesive strip, incubated for 2 hours at RT (away from direct light), followed by aspiration/ wash. 100 µL of Substrate Solution were added to each well and left to incubate at RT away from direct light for 20min. 50 µL of Stop Solution to were added to each well followed immediately by determination of the optical density of each well immediately, using a microplate reader set to 450 nm.

## Results

The clinical data of the patients in each group are shown in **Table 4.**

| | Clinical characteristics at baseline (mean values) | | | | | | |
|---|---|---|---|---|---|---|---|
| **Patients and treatment modalities** | | Patient's code | Plaque (%) | BoP (%) | PPD (mm) | REC (mm) | CAL (mm) |
| | Group A | 51 | 8 | 54 | 9 | 7 | 2 |
| | | 54 | 31 | 64 | 7 | 2 | 5 |
| | | 59 | 19 | 55 | 9 | 5 | 4 |
| | | 60 | 14 | 29 | 7 | 3 | 4 |
| | Group B | 50 | 19 | 69 | 9 | 4 | 4 |
| | | 53 | 25 | 63 | 6 | 4 | 2 |
| | | 64 | 14 | 43 | 7 | 4 | 3 |
| | | 65 | 14 | 43 | 7 | 4 | 3 |
| | Group C | 56 | 22 | 54 | 9 | 2 | 7 |
| | | 58 | 32 | 59 | 12 | 4 | 8 |
| | | 61 | 21 | 57 | 8 | 5 | 3 |
| | | 66 | 21 | 41 | 9 | 5 | 4 |

**Table 4. Table with patients' periodontal clinical data, as collected at their baseline examination. Values are the highest/ deepest per patient for PPD, REC and CAL.**

## Exosome analysis in GCF and Saliva samples.

One of the aims in this pilot study was to determine whether exosomes derived from GCF or saliva from patients with periodontitis, are a source of biomarkers that can inform on the disease status. Our initial approach was to isolate total exosomal RNA from GCF and analyse miRNA expression in order to understand mechanisms regulating gene expression in periodontitis, and whether profiles or signatures of miRNAs were associated with disease status. First, and following extraction of exosomes from GCF, samples were analysed by Nanoparticle tracking analysis (NTA) to obtain information on the size and concentration of

vesicles. The techniques are suitable for size analysis of vesicles with external dimensions in the nano- and sub-micrometre scale range.

As depicted in **Figure 4**, for exosomes or extracellular vesicles derived from GCF, the size distribution analysis showed a non-homogenous size of extracellular vesicles (**Figure 4A**). A representative sample showed a histogram with 5 peaks at 75, 111, 145, 224 and 361nm, and a mode and mean size of particles of 69.99+/-19.30nm, and 143.9+/- 67.88nm, respectively (**Figure 4B and C**). In GCF samples the average concentration of exosomes was $2.41x10^9$+/- 0.91particles/ml (**Figure 4D**). A representative snapshot of GCF extracellular vesicles is shown in **Figure 4E**. Total RNA was extracted from purified GCF extracellular vesicles and the content in 5 preparations was estimated by Nanodrop. **Figure 4F** shows the estimated concentration of RNA (ng/µl) and the corresponding 260/280 ratio. From these, the RNA concentration obtained was low, and the 260/280 ratio which express the purity of RNA, had values <2, indicating possible contamination with DNA.

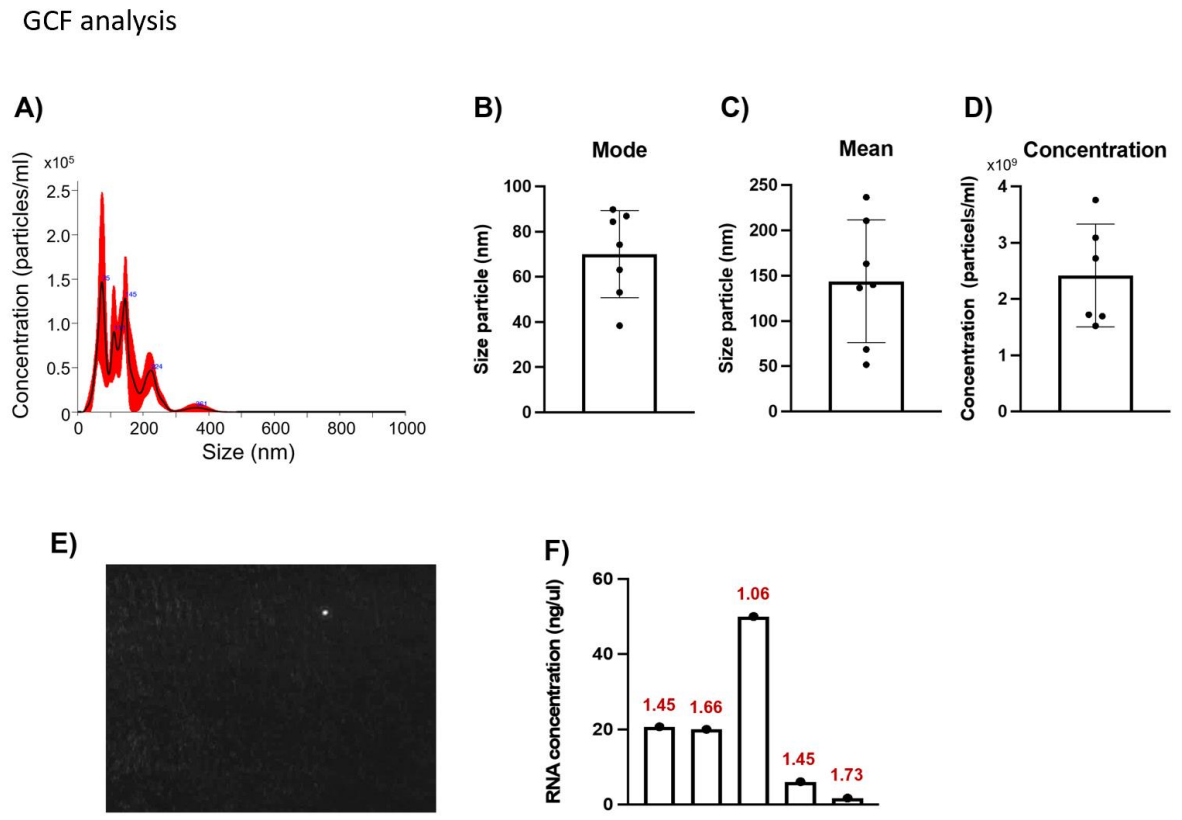



**Figure 4. Nanoparticle Tracking Analysis of exosomes derived from GCF samples.**

**A-C)** size distribution and **D)** Concentration of exosomes in GCF. Nanoparticle Tracking Analysis (NTA) reveals five peaks in this distribution with the size of particles varying between 75 to 361nm. **E)** snapshot from a videoclip of vesicle particles in the sample. **F)** Nanodrop RNA concentrations. The values on top of each bar indicate 260/280 ratios of five different samples analysed.

A similar approach to analyse extracellular vesicles from saliva showed that the presence of bigger vesicles (**Figure 5A**) with sizes in the range of 84 to 307 nm and a mode and mean of 166nm +/- 75.5, and 200nm +/- 98, respectively (**Figure 5B and C**). Extracellular vesicles in saliva samples were present at a higher concentration, compared to GCF, with an average of $1.75x10^{10}$ +/- 1.76 particles/ml (**Figure 5D**). A representative snapshot of GCF extracellular vesicles and the evident higher concentration of particles compared to GCF samples is shown in **Figure 5E** (compare to **Figure 4E**). The reliability of the extracellular vesicles isolation protocol was tested with samples of human serum, which are known to contain a high concentration of particles. The results shown in **Figure 5F** show the homogeneous size of serum particles (mode 104.9 and mean 87.9 nm) and higher concentration compared to GCF and saliva samples ($5.05x10^{10}$ particles/ml**).**

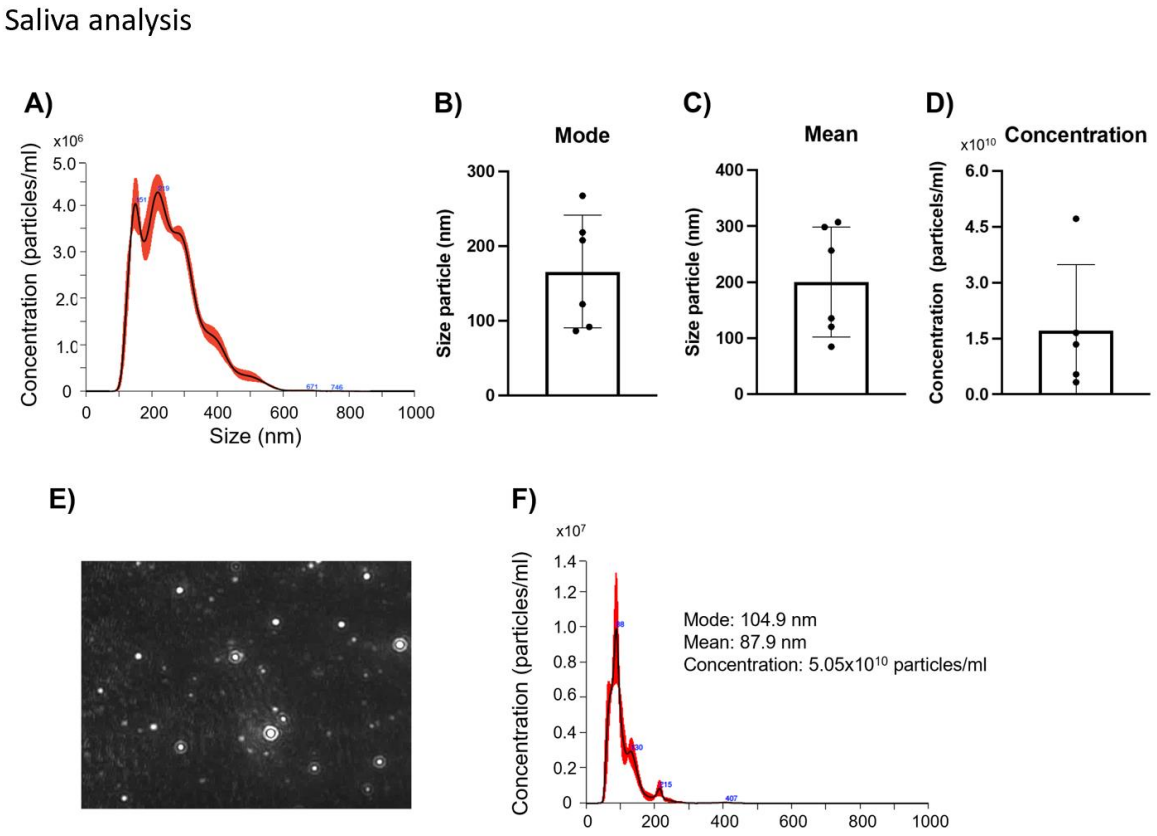
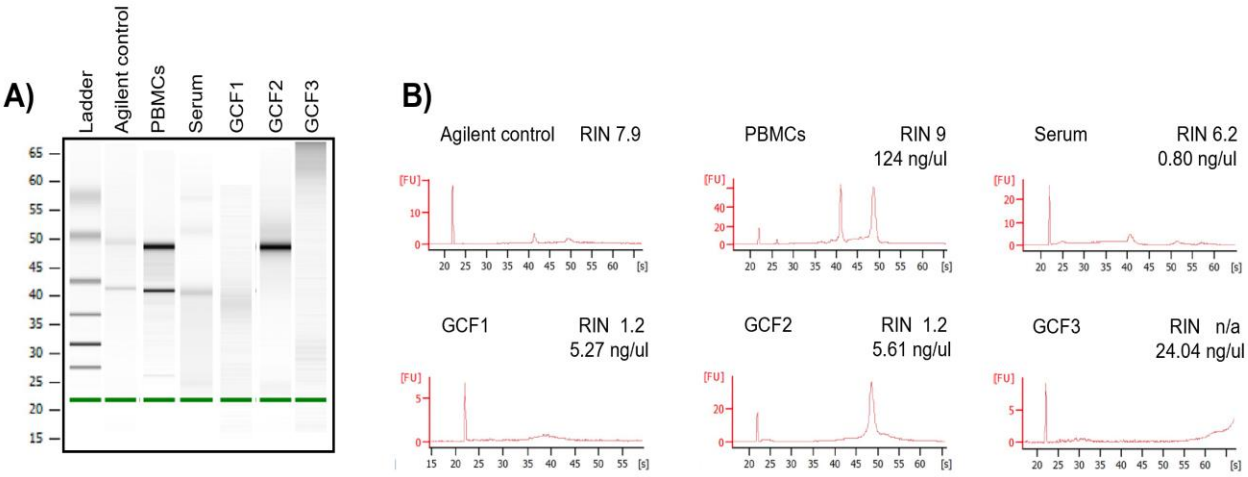



**Figure 5. Nanoparticle tracking analysis of exosomes derived from saliva samples.**

**A-C)** size distribution, and **D)** concentration of exosomes in saliva. Nanoparticle Tracking Analysis gives information on the size of particles varying between 151 and 219nm. **E)** Snapshot of saliva EVs with Nanosight analysis. **F)** Representative histogram for Nanosight analysis of human serum sample used as control for testing reliability of exosomes isolation protocol. The values indicate the mode, mean and concentration of particles in serum samples.

The difference in size of particles in GCF and saliva samples could be the result of higher precipitation of these spherical vesicles due to the higher dilution the samples had. Analysis of both saliva and GCF samples, showed that there were no apoptotic bodies present (large size EVs of ~1000μm in diameter).

**Figure 6** describes the electrophoretic mobility pattern and histograms of three representative GCF samples. In this experiment, in addition to the internal Bioanalyzer Agilent control, a sample of RNA derived from PBMC's was used as control to check the RNA isolation procedure. As indicated by theRIN values of the saliva and GCF samples, these were suboptimal and close to 0 (RIN<2.5), with the Agilent control being at 7.9. The relative high value for PBMC's-derived RNA (9.0) indicates the optimal conditions of the RNA extraction technique (**Figure 6A**). 260/280 and 260/230 optical absorbance ratios were obtained with the use of a Nanodrop Spectrophotometer. The rationale is based on the Beer-Lambert law, which draws a direct correlation between absorbance and concentration of the nucleic acid sample/ aliquot under examination. While nucleic acids absorb at many wavelengths (measured in nm), they have a peak absorbance of UV light at 260nm. The A260/280 ratio is generally used to determine protein contamination of a nucleic acid sample since proteins have a strong UV absorbance at 280 nm. For pure RNA, A260/280 ratios should be close to 2.1. In terms of RNA concentration, the values obtained were low, and the 260/280 ratio that inform on potential DNA contamination and the presence of other contaminants was also low (**Figure 6C**).




C)

| Sample | 260/280 | 260/230 |
|---|---|---|
| GCF1 | 2.64 | 0.11 |
| GCF2 | 1.89 | 0.64 |
| GCF3 | 1.57 | 1.15 |
| PBMCs | 2.14 | 1.39 |

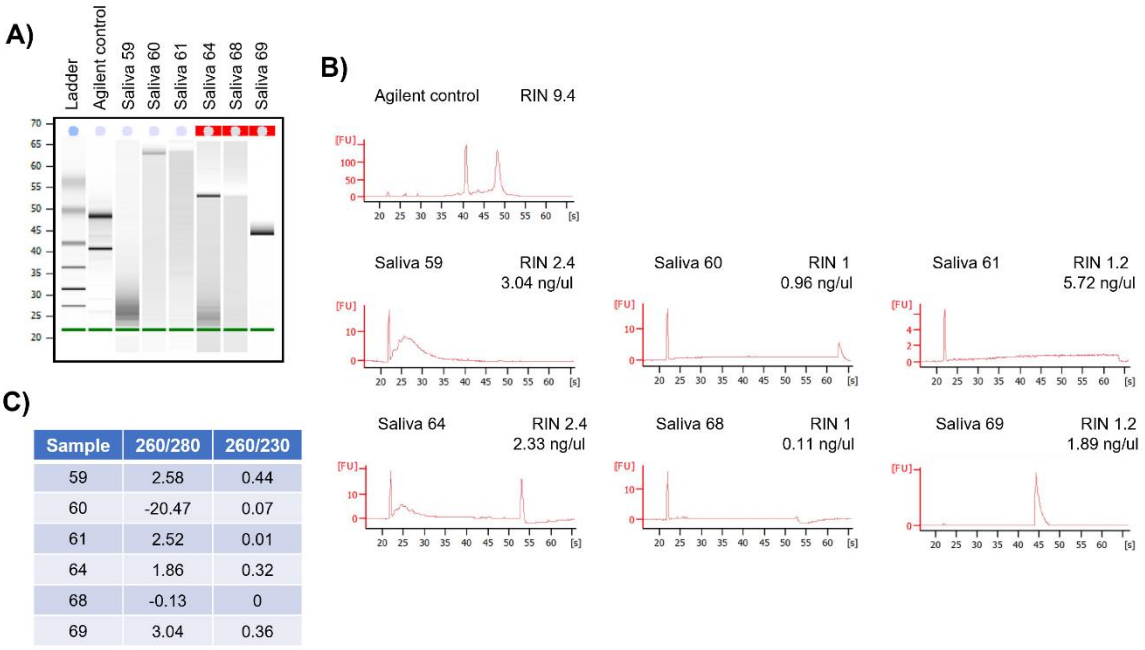
**Figure 6. Bioanalyzer quality control assessment of RNA isolated from GCF-derived exosomes.** Total RNA extracted from GCF-derived exosomes was analysed by Bioanalyser technology to determine RIN values and concentration. In addition to instrument (Agilent) calibration control, GCF samples were compared to RNA isolated from PBMCs and a human serum sample. **A)** Electrophoresis blot showing representative bands and corresponding histograms **(B)** for total RNA in controls and GCF samples (GCF1-GCF3). The RIN value and concentration for each sample are indicated in the histograms. **C)** Ratio of absorbance and 260/280 and 260/ 230nm to assess purity of samples compared to PBMC control.

Electrophoresis by a Bionalayzer instrument is a method of assessment of the overall quality of an RNA preparation (Houde, 2015). RNA analysis is possible with a sample/ aliquot volume from 5ng of total RNA, which is completed and analysed with the help of a software and enables control of Agilent liquid (generated on the Agilent 2100 Bioanalyzer System), providing simultaneously the RNA Integrity Number (RIN). RIN values reflect the quality score of the RNA of a sample (Schroeder *et al.*, 2006), and is able to provide reliable and reproducible characterisation of the total RNA from multiple sample types. An electropherogram is rated on a scale by RIN ranging from 1 to 10, with 10 being the least deteriorated.

The 260/230 ratio is considered as secondary measure of nucleic acid purity, and for pure RNA, these are expected to be within the range of 2.0-2.2 value. In cases where the ratio of a lower value, there is a possible presence of contaminants that absorb at 230nm. In all our

samples, salivary and from GCF, the values are very low (<1.15 for GCF samples and <0.44 for salivary samples), which suggest possible contamination with DNA, making them not suitable for further analysis.

Similar to GCF, Bioanalyzer results for saliva-derived RNA showed samples with low concentration, low RIN values (**Figure 7A and B),** and low 260/280 and 260/230 ratios (**Figure 7C**).




C)

| Sample | 260/280 | 260/230 |
|---|---|---|
| 59 | 2.58 | 0.44 |
| 60 | -20.47 | 0.07 |
| 61 | 2.52 | 0.01 |
| 64 | 1.86 | 0.32 |
| 68 | -0.13 | 0 |
| 69 | 3.04 | 0.36 |

**Figure 7. Bioanalyzer quality control assessment of RNA isolated from saliva-derived exosomes** Total RNA extracted from saliva-derived exosomes was analysed by Bioanalyzer technology (Agilent) to determine RIN values and concentration. Samples were compared to instrument calibration control. **A)** Electrophoresis blot showing representative bands and corresponding histograms **(B)** for total RNA in control and a set of saliva samples (59-61, 64, 68, 69). The RIN value and concentration for each sample are indicated in the histograms. **C)** Ratio of absorbance at 260/280 and 260/230 nm to assess purity of RNA from saliva-derived exosomes.

Altogether, the Bioanalyzer quality control results for GCF and saliva indicate that the amount and quality of isolated RNA samples are not suitable for further downstream analysis of miRNA expression and profiling analysis.

## Analysis of IL-1β and IL-6 in saliva samples and correlation with clinical parameters defining periodontal disease.

This project was based on the analysis of baseline samples derived from patients in three different treatment modalities for periodontal disease. As further analyses will involve evaluation of molecular markers at later stages after treatment onset, we needed to evaluate whether our samples were homogenous in terms of the expression of molecular markers associated with inflammation and clinical parameters and measurements to define periodontitis stage and severity.

Initially, we evaluated by ELISA the levels of pro-inflammatory cytokines IL-1β and IL-6 (**Figure 8.**) where levels have been shown to vary according to periodontitis stage and severity. IL-1β was detected in all three different sets of samples. (**Figure 8A**). From the optical density readings, a standard curve was constructed and the concentration of IL-1β in each sample was determined (**Figure 8B**). The results showed no significant differences in IL-1β in the three different treatment groups (**Figure 8C**). On the other hand, IL-6 was not detected in the saliva samples derived from patients in the three different treatment groups. IL-6 with the ELISA system was detected below the lower limit of 15pg/ml as indicated by the standard curve.

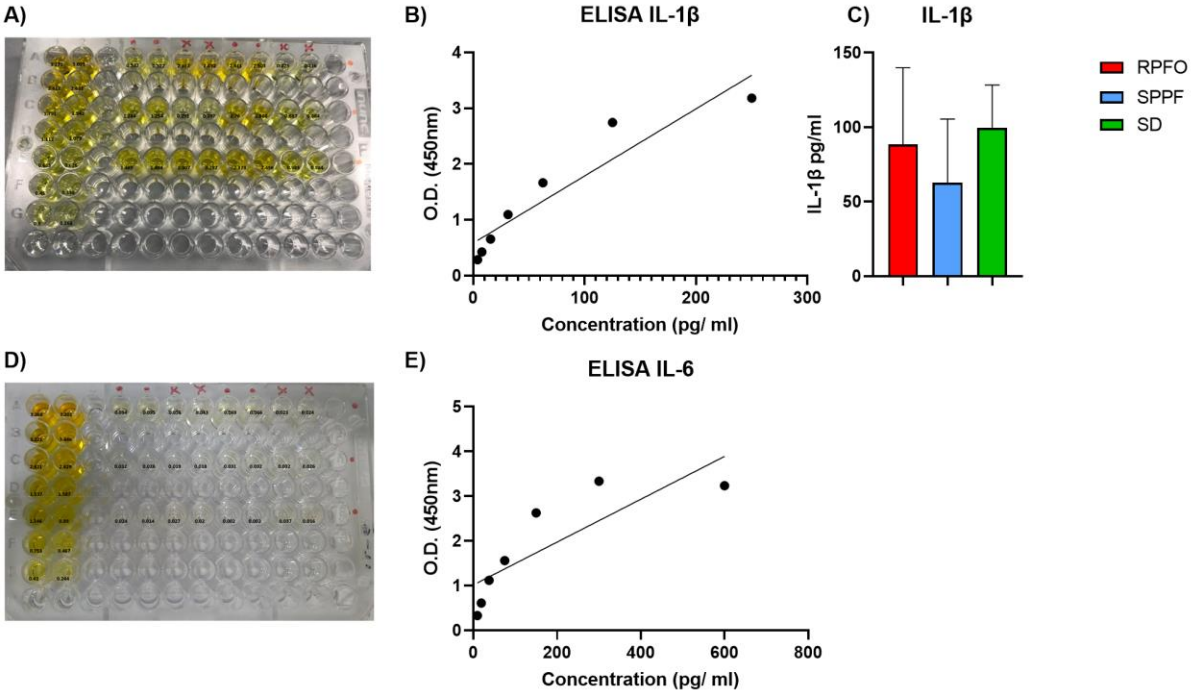



**Figure 8. Levels of IL-1b and IL-6 in saliva samples from patients undergoing three different procedures for periodontitis. A)** and **D)** microplates following completion of ELISA protocol for human IL-1β and IL-6, respectively, and their concentrations (pg/ml) as per the optical density reading through spectrophotometry at 450nm. The labels in each well indicates the identity of the sample. The first 2 columns represent serial dilution samples for IL-1b and IL-6 standard curves standards of each interleukin, and the three Rows A, B and C, include the samples of the 3 patients' groups. Both standards and samples were assayed in duplicated.

**B)** and **E)** represent the standard curves for the IL-1β and IL-6, respectively

**C)** bar representation of the results of the three groups of treatment for human IL-1β concentration in baseline saliva samples from patients that will undergo

(Group A: Resective periodontal flap surgery with osseous recontouring (RPFO), Group B: Simplified Papilla preservation flap technique – (SPPF) or Subgingival debridement – (SD). Data is shown as one-way ANOVA with means and standard error of the mean (SEM).

The low concentration of IL-6 is an expected result for the baseline samples in this study (undetectable levels). IL-6 is a proinflammatory cytokine which is detected in higher levels when periodontitis patients have not received treatment and the inflammation is in a chronic phase. IL-6 levels are increased in saliva and GCF samples of chronic periodontitis patients (Nibali *et al.*, 2012). Studies have shown the decrease in its quantity levels found in saliva, before and after non-surgical treatment for chronic periodontitis (Al-Hamoudi *et al.*, 2018). The salivary IL-6 levels detected in this study (from patients diagnosed with chronic periodontitis and non-obese), were at their baseline examination at 15.1±0.6pg/mL, and 6 months following completion of non-surgical periodontal treatment at 5.8±2.3pg/mL, which is a statistically

significant change. Al-Hamoudi through his findings suggests a drop of about 60% in the quantity detected for IL-6. The baseline samples of our study were collected from patients that previously received Step 1 and 2 periodontal treatment and were ready to receive the Step 3 periodontal treatment or a repeated Step 2, and this particular cytokine is likely to be in relatively low concentration.

At least for IL-1β, the results showed that the three patients' groups were homogeneous, and this would allow detection of changes associated with a particular treatment modality in samples from the same patients collected at later time points.

## Correlation of IL-1b with clinical parameters defining periodontitis.

In order to confirm the homogenous nature of our sample's groups in terms of clinical manifestations and levels of molecular markers (pro-inflammatory proteins), clinical parameters defining periodontitis were plotted against IL-1β concentration and correlation analysis was performed.

The clinical data was obtained for each patient at baseline examination, and these were the bleeding on probing (BoP in %), Periodontal Pocket Depth (PPD in mm), dental plaque (in %), Periodontal Recession (REC in mm) and the Clinical Attachment Loss (CAL in mm). These are values that characterise periodontitis in terms of clinical symptoms, and periodontal destruction (BoP, PPD, REC and CAL) which characterise the disease, and the causative factor of it (plaque).

The analysis of the results was completed using the one-way ANOVA statistical test. The choice of this particular statistical test is because it evaluates the variance in group means within a sample, while taking into account just one independent variable or factor. In the **Figure 9**. below, all clinical parameters were tested with the one-way ANOVA, and the results include the *P*-value.

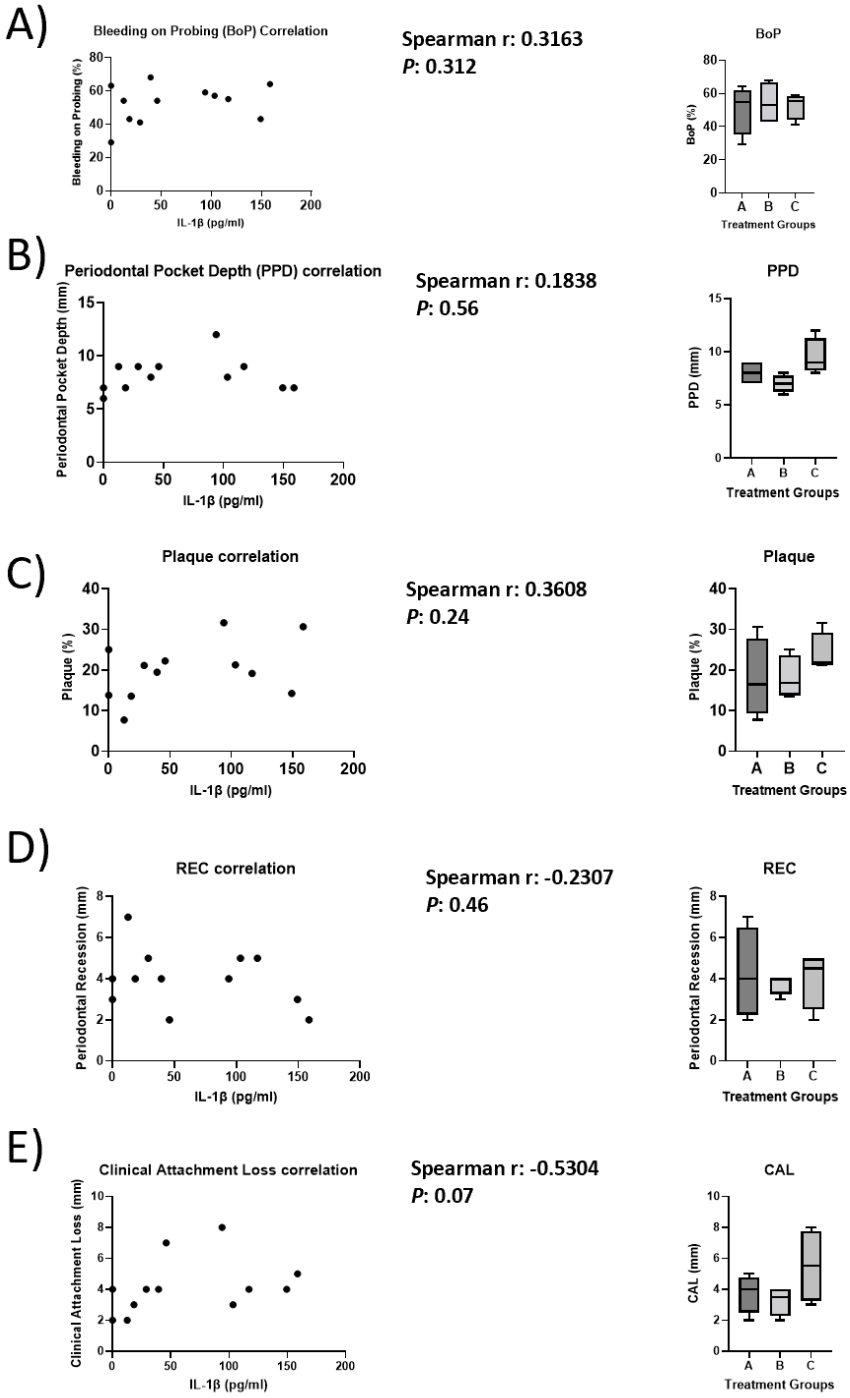



**Figure 9. ANOVA analysis of clinical parameters at baseline and bar charts in relation to the IL-1β cytokine following ELISA protein analysis. A)** Bleeding on Probing – BoP, **B)** Periodontal Pocket Depth – PPD**, C)** Dental Plaque**, D)** Periodontal Recession, – REC **E)** Clinical Attachment Loss – CAL. The Spearman r and P-value are displayed for each clinical parameter.

The *P*-value or probability value is a measure of the likelihood or probability for the correlation of variables examined are due to chance and it is expressed in a decimal number between 0 and 1. A *P*-value close to 1 suggests no correlation other than due to chance, and close to 0 is unlikely to be due to chance. The threshold for this is considered to be the value of 0.01 or less (<0.05 or 5%).

The *P*-values after statistical analysis as depicted with in the correlation charts and bar chartings in **Figure 9.**, indicate no correlation between the clinical parameters and the IL-1β cytokine correlation between the clinical parameters and the IL-1β cytokine concentrations across the samples from patients in the three different treatment groups. The same is reflected with the Spearman's r value, which is for all clinical parameters <0.39 indicating a weak correlation, with the exception of the CAL which is -0.5304, indicating a moderate correlation. Within the same context, CAL's *P*-value is 0.07, making this clinical parameter the closest to the 0.01 threshold, even though still considered as with no strong correlation. Also, to note, that there are 2 r values that are negative (REC and CAL).

From the above statistical results and taking in consideration the small sample size (n=12) of the patients examined, there is no correlation found between the cytokine IL-1β and the clinical parameters examined.

The clinical data was obtained for each patient at baseline examination, and these were the bleeding on probing (BoP in %), Periodontal Pocket Depth (PPD in mm), dental plaque (in %), Periodontal Recession (REC in mm) and the Clinical Attachment Loss (CAL in mm). These are values that characterise periodontitis in terms of clinical symptoms, and periodontal destruction (BoP, PPD, REC and CAL) which characterise the disease, and the causative factor of it (plaque). All these parameters are used in the statistical analysis of the results.

| Clinical Parameter/ IL-1β concentration | Bleeding on Probing (BoP) | Periodontal Pocket Depth (PPD) | Plaque | Periodontal recession | Clinical Attachment Loss (CAL) |
|---|---|---|---|---|---|
| *P*-Value | 0.312 | 0.56 | 0.24 | 0.46 | 0.07 |
| Spearman r value | 0.3162 | 0.1838 | 0.3608 | -0.2307 | -0.5304 |

**Table 5. Spearman r and P-values of clinical parameters.**

## Discussion

Throughout the experiments of GCF and saliva, and the results obtained from them, there was a consistent difficulty on obtaining results which could be used for the main aim of the study: to investigate the presence of salivary and GCF inflammatory biomarkers and protein expression, at baseline examination. This baseline examination appointment was the re-

evaluation session following non-surgical periodontal treatment completed (Step 1 and Step 2), prior to engagement to the clinical trial as mentioned at the Materials and Methods chapter.

In this pilot study, having as an aim to justify further investigation of presence in biomarkers in the available samples, the selection of the number of patients was mainly based in the available literature (Julious, 2005). The specific size of 12 patients is a number supporting the treatment arm of the pilot study in an adequate way through representing results. Such study is proposed to specifically calculate the sample size to be used, in order to preliminarily give clear information on the direction to be followed for the main clinical trial (Bell, Whitehead and Julious, 2018; Whitehead *et al.*, 2016). And even though the sample size is relatively small to allow conclusive results (Ioannidis, 2005), its power is adequate for the purpose it serves in this preliminary pilot study.

The results obtained were negative in the context of presence of molecular cues (miRNA) and IL-6 protein as periodontitis biomarkers. GCF samples were investigated for miRNAs alone, and saliva samples were investigated for miRNAs and proteins together. The correlation between clinical parameters and protein concentration in saliva and GCF was weak for all of them. In the literature, there are multiple studies where the concentrations of the above proteins and miRNA have been investigated, analysed and found to be valuable biomarkers of periodontitis (Chaparro Padilla *et al.*, 2020; Saito *et al.*, 2017).

One of the marked observations in the clinical indices is the high BoP, despite the NSPT the patients had already undergone. This is an expected condition within the periodontal treatment observed at this stage, where the high levels of bleeding are attributed to the residual pocketing the patients are experiencing (Badersten, Nilveus and Egelberg, 1984). This is a well-documented phenomenon following the non-surgical phase of periodontal treatment, where the level of bleeding on probing is a consistent and reliable mark of disease progression (Lang *et al.*, 1990). Within this context, the subjects of this study still exhibit periodontal inflammation, which is reflected as well in their PPD and CAL as presented in **Table 4**.

In the protein results of this study, IL-1β is the protein/ cytokine within detectable levels in the saliva samples. IL-1β is normally found to be in higher levels when it is compared to healthy subjects (Boronat-Catalá, Catalá-Pizarro and Bagán Sebastián, 2014), which is the case in this pilot study. These studies so far have come into the conclusion that this particular cytokine have the characteristic to be present in measurable levels in periodontal inflammatory conditions. Furthermore, a clinical trial demonstrated that it could represent the differentiations in the expression of periodontitis in its phenotypes and forms such as the chronic and incisor-molar pattern, as well as their rate of progression (Kawamoto *et al.*, 2020). Within this context, the detection of IL-1β in the saliva of periodontal patients is a strong representative of

periodontal inflammation and one of the important molecular cues in its early detection. In the present study, the results do not have the expected correlation with the clinical parameters studied (outlined in **Figures 8 and 9.**) as it is found in similar studies advocating for a strong correlation between levels of IL-1β and same clinical parameters (Gümüş *et al.*, 2014). The present results, however detectable, cannot be used for further interpretation of the periodontal status of the 12 patients at the time of their sample collection, which becomes more difficult taking in consideration the lack of a control group.

On the other hand, the IL-6 levels were extremely low, of a non-detectable level as it is depicted in **Figure 8.** This does not present an unusual observation, as a characteristic of this particular cytokine, is the active participation of it in the etiopathology and chronic phase of periodontitis. The levels of IL-6 are found quite reduced or even absent, once periodontal treatment is provided (Nibali *et al.*, 2012; Al-Hamoudi *et al.*, 2018), which reflects the condition of the patients participating in this pilot study. However, in this particular matter in the detection of cytokines in periodontal patients, there is also the aspect of results which can be contradictory (Teles *et al.*, 2009), where the results are not statistically significant. The range of results when studying proinflammatory periodontal cytokines, and specifically IL-6, vary in a way that reveals a noticeable degree of inconsistency. Researchers have interpreted these variations to the presence of inhibitors in the saliva and the complexity periodontitis presents in its pathogenesis (Teles *et al.*, 2009; Al-Hamoudi *et al.*, 2018).

Furthermore, when looking into the most suitable method in detecting proteins, the method of choice is the ELISA, specifically the ‘sandwich method’, presenting the sensitivity, specificity and reproducibility levels for small amounts of proteins in a sample (Aydin, 2015; Leng *et al.*, 2008). It is also preferred over other existing protein detection methods, as it is more reliable (Sakamoto *et al.*, 2018), and is considered as the gold standard when it comes to the enzymatic immunoassay methods. This is a protein detection method based on the reaction between antigens and their equivalent antibodies and ensuring adequate results for clinical and research purposes. In the present pilot study, the IL-6 was of very small amounts, which were lower than the detectable limits (15pg/ml).

Up to now, in the literature there is still no consensus for a more standardised reference when it comes to extracellular levels of exosomes and miRNA in oral fluids, and for these to be used as reference points. The way this is addressed till now, is usually via a control group of healthy subjects (Gürsoy and Kantarci, 2022). The same issue is present for a consensus with regards to a standardised protocol in exosome isolation and miRNA extraction from oral fluids, for optimised use in use in academic research (Wassall and Preshaw, 2016; Wright *et al.*, 2020; Gürsoy and Kantarci, 2022). Of course, we believe that this is due to: i) the relatively new field

of investigation in exosomes and miRNAs as biomarkers in oral fluids, and ii) the particular difficulties and sensitivity when managing nucleic acids (RNA in this study). There are proven results in the literature with regards the potentials and benefits that arise from such studies, that can be regarded as valuable both in the academic field and in clinical applications.

As the present pilot study is part of a larger investigation examining biomarkers in several periodontal treatment modalities, we recommend and encourage studies in the same context, to use biomarkers as a mean of validation and evaluation of the periodontal healing patterns in their early (1$^{st}$, 2$^{nd}$, 3$^{rd}$, 4$^{th}$, 7$^{th}$, 14$^{th}$ day etc), mid and late periods (20$^{th}$, 21$^{st}$, 30$^{th}$ day etc) of periodontal healing. This can be expanded to furthermore periodontal treatment modalities that include surgical and non-surgical treatment, use of regenerative materials implants, and expand in axioms dominating our understanding in periodontology or unclear areas (ex: cell migration and occlusal trauma), all can be verified through biomarkers, and in particular miRNA analysis.

From the analysis of exosomes and miRNA, the samples used in GCF and saliva did not give any values reflecting statistical relevance with the subjects in this study. Of course, there are limitations in every research and failure to obtain positive results that could give rise to valuable conclusions is very likely to happen. However, it is good practice to trace back the possible reasons and pitfalls that may have contributed to this, and so make the most of these in terms of learning and contribution in any other future research.

In the literature, it is emphasized that handling genome is a delicate and difficult procedure. The challenges can be found in multiple levels within a study. The points that arise in the literature can be grouped in the following main topics: i) Sample collection and storage, ii) sample quality and quantity, iii) timing of sample processing, iv) isolation and accurate quantification of miRNA, v) complexity level of the biological medium chosen, vi) contamination of the sample (e.g: with proteins, inhibitors, cells), vii) the study design, viii) the small size of miRNAs ix) the strong association of miRNAs with protein complexes and RNA-binding proteins, x) choice of the commercial extraction kit and extraction protocol vs guanidium thiocyanate/phenol-chloroform RNA isolation methodologies, xi) lack in consensus for an optimised and standardised extraction protocol in the literature so far, and xii) the dexterity and experience of the person conducting the protocol (Wright *et al.*, 2020; Lekchnov *et al.*, 2016; Papagerakis *et al.*, 2019; Tuck *et al.*, 2009; Leshkowitz *et al.*, 2013; Wassall and Preshaw, 2016).

Each of the above topics in itself, presents the possibility of in-depth analysis and has its share of weight and importance in each ongoing research, related to exosome and miRNAs. The genetic material, being particularly sensitive, carries a natural protection (ex. within exosome

membrane, attached to RNA-binding proteins) which in itself presents a shield that must be accessed in order to be analysed in a laboratory. At the same time, the processing must be appropriate so as not to cause its damage or destruction. The complexity of the whole processing is due to the nature of the molecules in question and so far, there is no proposed protocol that is simpler or less time consuming.

The first thing to ensure prior to any investigation is the necessity of proposing a sound experimental study design and choose a valid data analysis. Even though this rationale is sensible, not all research studies reflect this. As part of a good research design, it is proposed to run a validation research on a small panel of identified differentially expressed potential biomarkers, prior to invest time and resources in a larger range study (de Ronde *et al.*, 2018). This will also ensure that the experiments will have representative samples and protect from false positive or false negative results. Within the same context, sample size that is sufficient to detect significant differences between the case and control groups is critical and essential in the whole study design.

Nevertheless, the most important part of the study is the collection, storage and handling of samples.

**Saliva samples**

In the saliva's environment with a variety of cells and all other components, exosomes are already exposed in this complex environment. A study investigating the optimisation of salivary samples collection and storage (Mastazliha, Shima and Berahim, 2018) concluded that exosomes in saliva are 'resilient' in different storage conditions. Our study is advocating this position as the collection and storage conditions of the samples fall within the investigated protocol, with the only difference being the length of storage. Mastazliha's study yields of good results with samples being stored for up to a month. The length of storage of our samples varied between 3 years, and up to about 7 years, till their thawing and processing. However, good quality salivary miRNA could not be isolated. The main possible explanation for this, is the long storage time of the salivary samples. Recent studies though, have considered a protocol that conserves the sample with the use of RNA stabilizers for saliva (Sullivan *et al.*, 2020) when it comes to further analysing them for miRNA.

**GCF samples**

The selection of GCF as biological fluids, as already mentioned, is a very easy method of collection, convenient for the clinicians and patients. However, its management will also determine the quality of the results. All steps of the way in collection and storage of samples, are critical for the preservation of the characteristics of the targeted genome. From contamination, elution, storage temperature and length of storage, all define the quality of the sample (Preianò *et al.*, 2016) and the results to obtain. The same research group came to the conclusion that the length of storage time and temperature have also an impact to the quality and the reproducibility of profiles of the fluid. In short, diagnostic miRNA fingerprints may present variations as a function of GCF collection, storage and handling.

Taken in consideration all of the above and looking retrospectively in the design, and collection steps and storage of the biological fluids followed in this study, there are points and observations which help the understanding of the poor results obtained in the GCF analysis. Samples were well obtained and followed careful collection ways to avoid contamination. However, the immediate storage and freezing without elution of the GCF from the PerioPapers or use of RNA stabilizers and in general to have RNAase-free conditions for collection (Pandit, Cooper-White and Punyadeera, 2013), is now considered an obsolete and not effective method for GCF (Wassall and Preshaw, 2016). The pitfall of the previous understanding of the miRNA ability to escape degradation, hence no needing of further precautions when biological fluids were collected for their extraction and analysis, was attributed to their encapsulation in exosomes (Becker and Lockwood, 2013).

These parameters could have been foreseen at the stage of planning and designing the larger study that the samples derived as the study has been initiated in 2008 and before the optimization collection protocol mentioned above were introduced. At this point, it is important to mention that the knowledge of optimal collection, storage and handling of biological fluids for genomic analysis, have arisen in recent years. The knowledge on the effect of numerous variables in protocol selection came gradually, with various studies proposing ideal protocols to follow (Becker and Lockwood, 2013). One needs to keep in mind, that to the present time, there is no consensus in what can be considered an optimal method to follow in GCF collection (Witwer, 2015; Han, Bartold and Ivanovski, 2022). Prior to this, most studies had as mentioned, study design and protocols that today are no longer in use.

As per the particular difficulties shown in the present study, we strongly believe that further investigations should be continuous in the field of periodontal biomarkers, from which the scientific community will benefit a better understanding in multiple levels. A valuable and rational recommendation is for a consensus in basic level/ rates of exosomes, miRNAs and

cytokines should be set for the scientific community to use as a platform of reference. This will be valuable in terms of communication and research in the inflammatory oral biomarker investigations worldwide.

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