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Targeted Peri-implant Crevicular Fluid Biomarkers in Osteoporotic Patients Receiving a Dental Implant

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A thesis submitted in partial fulfilment for: DClinDent in Periodontology

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Contents

Abstract

Aims and Objectives: The aim of this pilot study was to describe the protein expression during the early and late stage of osseointegration in the peri-implant crevicular fluid (PICF) of a cohort of post-menopausal osteoporotic women.

The primary objective was to explore and compare targeted protein expression in PICF during early days of healing (7 days) and once osseointegration and loading had already been achieved (6 months post loading) in a cohort of post-menopausal osteoporotic women.

The secondary objectives were to determine if inflammatory and bone related markers in PICF correlate with bone mineral density (T-score), use of osteoporosis medications in osteoporotic patients, smoking status or post-operative adverse events (e.g. swelling and pain).

Material and methods: PICF samples of 9 patients were collected at visit 4 (7 days following implant placement) and visit 7 (6 months following loading of the implant) and quantitatively analysed for changes in the expression of pre-determined protein markers using LUMINEX multiplex bead array assays. The difference in the expression of PICF between the two time points was assessed with the Wilcoxon signed rank test ($p<0.05$), while the correlation between marker expression, bone mineral density, use of osteoporosis medications and post operative adverse events was explored through the Spearman rank correlation coefficient.

Results: Inflammatory markers were overall downregulated between visit 4 and visit 7, with CCL18 demonstrating statistical significance. IL1-A was the only inflammatory marker showing a non-statistically significant increase. A non-statistically significant reduction in VEGF and

BMP-2 levels was also observed. Other markers such as osteopontin, osteoprotegrin and BMP9 were not detected in the PICF.

A statistically significant negative correlation (-0.8, p=0.006) between 'no history of periodontal therapy' and CCL2 levels was observed at visit 4. A statistically significant negative correlation (-0.7, P value=0.014) between no previous periodontal therapy and BMP2 levels was also at visit 4. At visit 4, T score was also negatively correlated (-0.8, p value=0.03) with BMP2 levels.

Conclusion: This is a pilot study reporting for the first time on the expression of targeted inflammatory and bone metabolism PICF proteins at early (visit 4) and late stages (visit 7) of healing during osseointegration in osteoporotic post-menopausal women. The overall reduced expression of inflammatory markers (although not significant) between the early and late stages of healing confirm the current knowledge on the biology of osseointegration. Similarly, the non-significant reduction in the values of markers related with bone matrix deposition, angiogenesis, maturation, and remodelling markers at 6 months post loading were anticipated. There was an unexpected non-statistically significant increase for IL1-A at 6 months post loading, which may be related to the limited population sample or high inter-individual variability. Future studies in a larger population sample and with more time-points are warranted to provide a wider range of data and increased accuracy.

1. Background

1.1. Osteoporosis

1.1.1. Definition and clinical manifestations

Osteoporosis is defined as a systemic skeletal disease with features of reduced bone mass and microarchitectural deterioration of bone creating fragility which can lead to fractures (Consensus Development Conference, 1993). The high prevalence (Hernlund et al., 2013) and mortality rate class this condition as high risk, particularly as many cases are undiagnosed until a fracture occurs (Major et al., 2020).

1.1.2. Diagnosis and pathogenesis

According to the World Health Organization, the diagnosis of osteoporosis is ascertained with a bone mineral density (defined through a T score) of at least 2.5 standard deviations below the average mean for young healthy adults (T score ≤-2.5) (Kanis et al., 2008) .

The pathophysiology of osteoporosis is linked to a dysregulation of osteoclasts, osteoblasts and osteocyte activity leading to an increased bone degradation and reduced bone formation (Poole and Compston, 2006). The prevalence of osteoporosis increases in women after menopause, since reduced oestrogen induces an upregulation of RANKL and a reduction of OPG, which leads to bone loss. The increase in parathyroid hormone resulting from reduced calcium intake and increased excretion can also lead to the same effect (Sipos et al., 2009, Cosman et al., 1993). Furthermore, oestrogen deficiency can influence the action of oestrogen responsive target genes, which leads to an increased production and release of IL-1, IL-6, and tumour necrosis factor (Eastell et al., 2016), and therefore to a pro-inflammatory status. The main effects of oestrogen deficiency are summarised in **Error! Reference source not found.**.

The onset and severity of the disease is determined and influenced by multiple factors such as genetics, smoking, alcohol intake, vitamin D deficiency and low body mass index (Pouresmaeili et al., 2018).

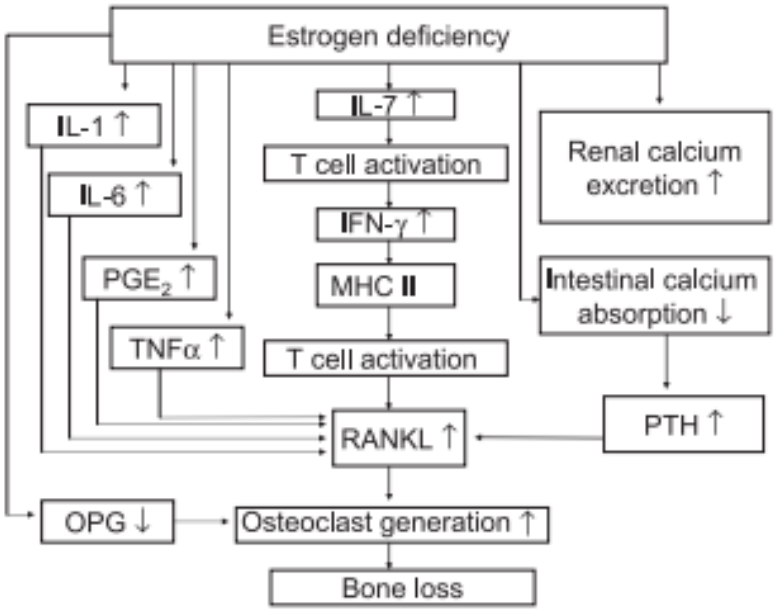

Figure 1. A diagram illustrating the effects of oestrogen deficiency on bone loss (Sipos et al., 2009)

1.1.3. Pharmacological therapy and its effects of on biomarkers

Osteoporotic medications aim to reduce the incidence of fractures and can be categorised into anti-catabolic (antiresorptive) and anabolic, each differing in its mode of action. Anticatabolic medications include Bisphosphonates, Denosumab, hormone replacement therapy, Selective Oestrogen Receptor Modulators and calcitonin.

The most prescribed anti-resorptive medications for osteoporosis are bisphosphonates. Bisphosphonates have a high affinity to hydroxyapatite thereby binding to bone and being

acquired by osteoclasts at sites of bone resorption. Bisphosphonates impede osteoclastic function by inhibiting farnesyl pyrophosphate synthase (FPPS) which prevents prenylation of GTPase proteins resulting in loss of the ruffled border. In addition, osteoclastic apoptosis is accelerated in osteoclasts exposed to bisphosphonates (Brown, 2021). In contrast to other anti-resorptive medications (e.g., Denosumab) Bisphosphonates are bound to bone for years and their pharmacological effects therefore continue following medication discontinuation (Brown, 2021).

In contrast, Denosumab is a human monoclonal antibody that has high affinity and specificity to RANKL, a protein involved with osteoclastic function and maturation. It prevents the interaction of RANKL with the RANK receptor on osteoclasts which reduces osteoclast maturation and activity. Denosumab is not incorporated into bone and therefore the therapeutic benefits do not continue following medication discontinuation (Brown, 2021). Common examples of antiresorptive medications and their efficacy are described in Table 1.

Hormone replacement therapy aims at replacing the declining oestrogen levels either alone or in combination with progesterone. The combination of conjugated oestrogens with bazedoxifene is FDA approved for use in postmenopausal women with an intact uterus for prevention of osteoporosis and treatment of vasomotor symptoms (Tu et al., 2018).

Selective oestrogen receptor modulators (SERMs) selectively bind oestrogen receptors to help reduce the incidence of complications such as breast cancer, for example Raloxifene selectively activates oestrogen receptors in bone tissue whilst acting as an antagonist in breast tissue. It functions by downregulating osteoclast activity, reducing bone resorption (An, 2016).

Calcitonin functions by inhibiting osteoclastic function and reducing reabsorption of calcium and phosphate by the kidneys. The mechanism of action is through disruption of osteoclastic cytoskeletal organisation and impairment of the cellular polarity (Yamamoto et al., 2006)

The anabolic medications, such as Teriparatide is a synthetic form of parathyroid hormone, promoting bone formation by increasing the activity of osteoblasts(Rodan and Fleisch, 1996, Sato et al., 1991, Hanley et al., 2012).

As expected, anti-resorptive medications have been shown to reduce bone turnover markers (BTM) (Silva-Fernandez et al., 2013, Hu et al., 2023, Sawamura et al., 2017, Lotz et al., 2020, Eastell et al., 2011, McClung et al., 2013).Lotz et al. (2020) reported that MG63 human cells cultured on different titanium surfaces treated with bisphosphonates and a control demonstrated reduced marks of osteocalcin, osteoprotegerin, osteopontin, BMP2, PGE2, TGFβ1, IL10, and VEGF in MG63 cells. Denosumab has shown similar results, Bone et al. (2008) reported reduced bone turnover markers of serum C-telopeptide, tartrate-resistant acid phosphatase-5b, and N-terminal propeptide of type 1 procollagen in a randomised control trial of 332 postmenopausal women receiving either denosumab or a placebo over 2 years.

Reports demonstrate that BTMs revert to increased levels following discontinuation of the antiresorptive medications, albeit the levels were still consistently higher than baseline levels (Naylor et al., 2018). Reversion to baseline levels of bone markers tends to be quicker for patients discontinuing denosumab as compared to bisphosphonates which reflects the pharmacology of the medications (Bone et al., 2011). Bone et al. (2011) reported serum bone turnover markers reverting to baseline levels within 48 weeks of denosumab

discontinuation, whereas Naylor et al. (2018) demonstrated bone turnover markers in urine did not reduce back to baseline levels even after 2 years of bisphosphonate discontinuation. To the best of our knowledge these have not been measured in PICF.

		Reduction of Fracture risk	
Drug	**Vertebral**	**Nonvertebral**	**Hip**
Abaloparatide (Tymlos)	Yes	Yes	No effect demonstrated
Alendronate (Fosamax)	Yes	Yes	Yes
Calcitonin (Miacalcin, Fortical)	Yes	No effect demonstrated	No effect demonstrated
Denosumab (Prolia)	Yes	Yes	No effect demonstrated
Ibandronate (Boniva)	Yes	No effect demonstrated	No effect demonstrated
Raloxifene (Evista)	Yes	No effect demonstrated	No effect demonstrated
Risedronate (Actonel, Atelvia)	Yes	Yes	Yes

Table 1. A table of main medications used to treat osteoporosis and their efficacy in fracture reduction (Camacho et al., 2020).

1.2. Osteoporosis of the jawbone

The effect of osteoporosis on the jaw bones is conceivable considering that, as a systemic disease, it can potentially have an impact on different bones of the body. Pre-clinical studies have reported loss of bone mass in the mandible (Elsubeihi and Heersche, 2009), maxilla and the mandibular condyle (Constancio et al., 2017) in osteoporotic conditions.

This evidence is reciprocated with some clinical trials which have demonstrated a reduction in alveolar crest height with increasing osteoporotic severity (Wactawski-Wende et al., 2005). However, other clinical studies did not confirm the influence of systemic bone mineral density on the resorption of edentulous jaws (Ozola et al., 2011, Elders et al., 1992, Springe et al., 2014).

Several clinical studies investigated the relationship between bone density measured in different systemic skeletal sites and in the jawbones in subjects with different T scores. Although many of these studies found a positive correlation (Erdogan et al., 2009, Drozdzowska et al., 2002, Jonasson et al., 2001, Makker et al., 2012, Takaishi et al., 2005, Vishwanath et al., 2011, Horner et al., 1996, Esfahanizadeh et al., 2013, Kribbs, 1990), others reported that jawbone density is not, or only to a limited degree, correlated to the density in other anatomic sites (Jonasson, 2009, Holahan et al., 2011, Kingsmill and Boyde, 1999).

In a systematic review, showed a moderate association between skeletal BMD and jaw BMD in osteoporotic subjects. However, due to the limited number and heterogeneity of the studies (mainly in terms of methodology used to calculate jawbone mineral density and populations examined) the outcome of the article was inconclusive. Despite the contrasting results, during the last twenty years, an increasing number of studies have assessed whether different linear measurements/quantitative indices or qualitative evaluations performed on panoramic X-rays could predict for skeletal bone health (Calciolari et al., 2015).

1.3. Osteoporosis and bony healing

Bone healing is defined by an anabolic stage characterised by tissue formation and overlapped by a catabolic phase involving remodelling of woven bone into mature trabecular and cortical bone. It is a complex biological process that requires the orchestration of different cells, cytokines and growth factors (Tarantino et al., 2011).

Evidence related to the effect of osteoporosis on bone healing is mainly limited to fracture models. Pre-clinical studies have overall reported a delay in fracture healing, reduced biomechanical properties and regenerative outcomes in ovariectomised rats (Chen et al.,

2016, Oliver et al., 2013) (Fig. 3). A pre-clinical study involving guided bone regeneration determined a non-statistically significant trend for reduced bone formation, which was of lower quality on ovariectomised rats compared to healthy controls (Calciolari et al., 2017b). Remarkably, the aforementioned study also indicated specific proteins and signalling pathways that seem to be differentially expressed in osteoporotic conditions and that can therefore be considered as potentially useful targets to improve the regenerative outcomes.

A systematic review of pre-clinical studies by Chen et al. (2016) reported multiple impairments in the bone healing cascade involving progenitor cell recruitment, angiogenesis, extracellular matrix production, callus formation and remodelling as shown in Figure 2.

These potentially detrimental effects of osteoporosis are thought to be due to reduced osteoprogenitor cell recruitment and decreased osteogenic capacity (Shi et al., 2010). Furthermore, Rodriguez et al. (1999) showed that mesenchymal cells derived from osteoporotic donors had a diminished ability to differentiate into osteogenic cells.

Very limited clinical studies have assessed the healing potential of osteoporotic bone. A clinical study carried out by Nikolaou et al. (2009) showed a similar pattern of delayed bone healing and consolidation. However, there were limitations with this study in terms of recruitment bias and significant baseline differences between the assessed groups (Cortet, 2011).

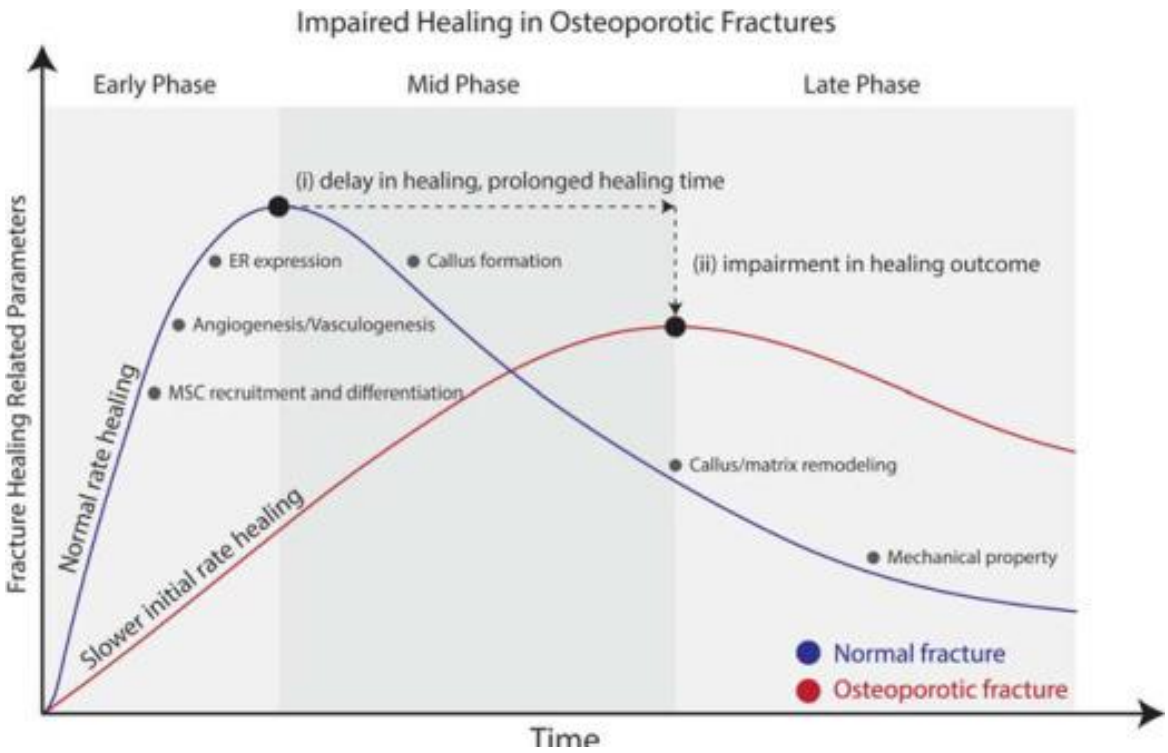

Figure 2. Graphical representation of the available evidence on the effect of osteoporosis on bone healing based mainly on pre-clinical studies on fracture models (Oliver et al., 2013).

1.4. Osteoporosis and dental implants

It is biologically plausible that altered bone metabolism in osteoporotic patients may lead to inferior osseointegration and a systematic review of pre-clinical studies have reported a significantly reduced bone to implant contact in the presence of osteoporosis (Dereka et al., 2018). Nevertheless, other studies have also shown limited or no difference in bone to implant contact (Giro et al., 2011, Nasu et al., 1998, Fujimoto et al., 1998). However, care must be taken with the interpretation of these preclinical studies due to inconsistencies with

the region of implant placement, method of inducing osteoporosis and confirmation methods to determine the level of osteopenia, which create difficulties in translating the findings to clinical studies.

Clinical studies have demonstrated less certainty, with some reports of higher failure rates for implants placed in osteoporotic patients leading to suggestions to consider alternative options (Alsaadi et al., 2008, August et al., 2001). This is particularly the case for implants placed in augmented bone, which may be associated with increased failure rates, as reported by Pinholt (2003). In contrast other studies showed no difference in implant failure between osteoporotic and non-osteoporotic patients (Holahan et al., 2008). A systematic review concluded there is a lack of evidence to define osteoporosis as an absolute contraindication for dental implants (Tsolaki et al., 2009). Due to this lack of clarity, Donos and Calciolari (2014) suggested a pragmatic approach by allowing a longer healing time before prosthesis insertion and expecting a higher rate of complications for bone augmentation procedures.

1.5. Osseointegration of dental implants

1.5.1. Stages of osseointegration

Brånemark and Schroeder were the first that documented bone apposition on titanium surfaces which later led to the term osseointegration (Branemark et al., 1969, Schroeder et al., 1976). Osseointegration is defined as: 'A direct structural and functional connection between ordered, living bone and the surface of a load-bearing implant' (Listgarten et al., 1991). The wound healing around dental implants involves a coordinated sequence of events recapitulating intramembranous ossification, with bone forming directly from mesenchymal tissue without an intermediate cartilage. In particular, the process of osseointegration is

initiated by the haemostasis and inflammatory stages following the implant surgery. A clotting cascade is initiated leading to the cleavage of soluble fibrinogen to fibrin, facilitated through the intrinsic and extrinsic pathways. Together with platelet adhesion to the fibrin matrix, a blood clot is formed. The early inflammatory stage starts within minutes and involves the non-specific innate host defence system including the complement cascade and cellular elements, such as polymorphonuclear leukocyte and macrophage aggregation. The late-stage adaptive immune cells involve lymphocytes and plasma cells, which together with the innate system build the host defence mechanism (Susin et al., 2015, Terheyden et al., 2012).

Abrahamsson et al. (2004), Berglundh et al. (2003) were the first to histologically describe the osseointegration process in a dog model receiving a solid screw type of implant with an SLA surface configuration. Within 2 hours of implant installation the peripheral threads of the implant have shown to be in close contact with surrounding bone. Coagulum occupies the experimental compartments with the presence of erythrocytes, neutrophils and macrophages in a matrix of fibrin. Following 4 days of healing, the coagulum is replaced by granulation tissue, characterized by the presence of mesenchymal cells surrounded by vascular structures. Angiogenesis is a prerequisite to bone formation and is favoured through hypoxic conditions stimulating release of growth factors (Terheyden et al., 2012). At this stage, osteoclasts are present in the cut bone surface with signs of osteoclastic resorption. One week after, the experimental chambers are filled with a collagen-rich provisional matrix, with the presence of newly formed woven bone and vascular structures. Newly formed bone can be seen around the vascular structures and in direct contact with the SLA surface. Osteoblasts and osteocytes are present in the newly formed trabeculae bone. Bone formation intensifies at 2 weeks, with extension from parent bone to all compartments. The pitch regions responsible for primary stability show remodelling with resorption and apposition.

It should be noted that new bone forms around implants in two distinct but overlapping routes. The first is through contact osteogenesis, whereby osteoprogenitor cells adhere to the implant surface differentiating into osteoblasts. Conversely, distance osteogenesis is the apposition of bone from lateral walls of the osteotomy preparation. Both routes of osteogenesis occur around topographically microroughened implant fixtures, but this is not the case for polished or smooth fixtures, where the primary route is distance osteogenesis (Terheyden et al., 2012, Moreo et al., 2009, Sela et al., 2007).

Bone formation continues at 4 weeks with newly formed bone projecting from the cut surface of bone but also extending onto the surface of the implant. The central portion of the chamber is rich with different fibroblast morphotypes and vascular structures. Bone remodelling in the pitch regions increases and signs of secondary osteons and bone remodelling can be observed.

At 6 weeks, healing demonstrates vast bone infill of experimental chambers (Abrahamsson et al., 2004). The bone is characterised as woven or parallel fibred and lamellar bone. Primary and secondary osteons are present with close contact to the SLA surface. At 8- and 12-weeks increased signs of bone remodelling and mineralisation can be appreciated. As a matter of fact, remodelling starts after approximately 3 months and may last several years. It is the process whereby the initial woven bone is replaced by organised lamellar bone with parallel orientation of the collagen fibres. Osteoclasts appear after a few days and resorb initial bone to implant contacts to create space for load-oriented bone, which in turn reduces the bone to implant contact for a period (Schenk and Buser (1998).

Human studies have shown a similar pattern of osseointegration, albeit at a slower rate. A study by Lang et al. (2011) compared the rate and degree of osseointegration between

moderately rough hydrophobic implants and chemically modified hydrophilic moderately roughened hydrophilic implants (Lang et al., 2011). At 7-day healing, biopsy samples demonstrated very limited new bone formation, predominantly osteoid lined with osteoblasts on old bone and bone debris away from the implant surface. A bone to implant contact of 6% was reported at this early time point. The osteoid randomly oriented collagen fibres in the form of woven bone and showed small foci of mineralisation. Most of the tissue formed between the implant and bone bed was primitive matrix with interspersed bone debris. Bone resorption was rare and presented away from the implant surface (Bosshardt et al., 2011).

At 2 weeks the bone to implant contact reached about 12%. Bone formation was noted on old bone as well as forming on the implant surface. These areas of new bone formation on the implant are the first sign of osseointegration. Areas of resorption were evident via Howship's lacunae adjacent to the implant fixture. The resorption assists removal of old bone in contact with the implant surface, which provides in return the initial primary stability. On hydrophilic moderately rough surfaces bone apposition was noted on larger areas of implant surfaces (Lang et al., 2011).

At 28 days, Lang et al. (2011) reported a statistically significant difference in bone to implant contact between hydrophobic and hydrophilic moderately rough implant surfaces, about 32% and 48%, respectively. Remodelling features became apparent, with areas of resorption and woven bone production being visible close to the implant surface.

At 42 days the bone to implant contact was again similar between both hydrophilic and hydrophobic surfaces, with about 60% bone to implant contact. Large amounts of woven bone were present adjacent to the implant, as well as lamellar bone and marrow.

Remarkably, the available data showed that between the periods of 4 to 6 weeks there was pronounced new bone formation and reduction of bone debris and old bone. This suggests that the highest rate of osseointegration occurs during this time. Figure 3 is a histogram summarising these events on hydrophobic and hydrophilic moderately rough implant surfaces and showing the different composition of peri-implant tissue at the different time points.

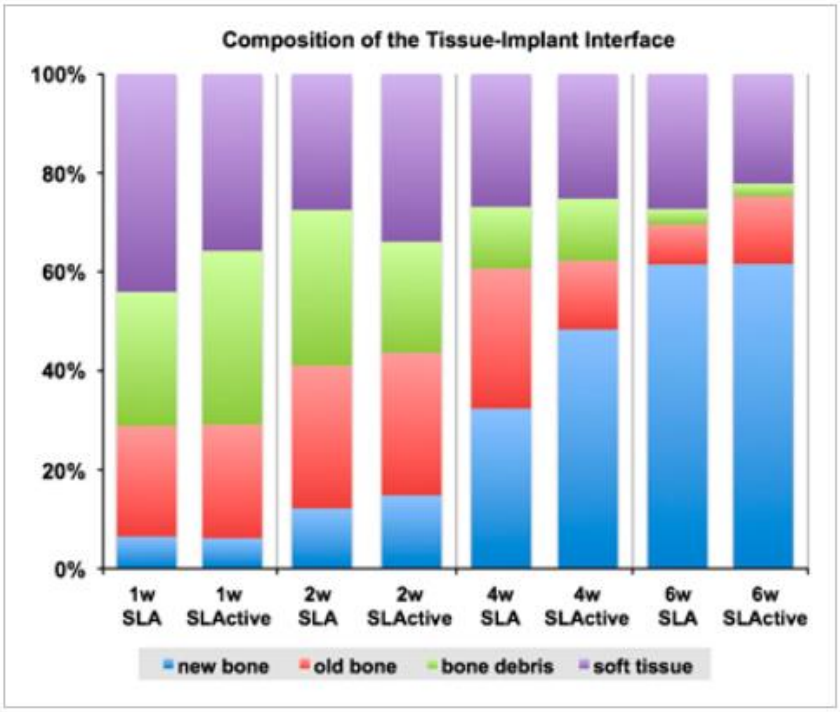

Figure 3. A histogram showing the association of different implant surfaces with percentages of new bone, old bone, bone debris and soft tissue covering the implant at different time intervals (Bosshardt et al., 2011)

1.5.2. Gene expression

A description of the genomic events in osseointegration is provided to support the biological process involved in osseointegration. Ivanovski et al. (2011) described the gene expression at

4, 7 and 14 days following implant expression. The gene profile at day 4 was reflective of localised proliferation and immune-inflammatory processes with genes associated with cytokines such as IL-6, TNF-α and IL2 being upregulated. In contrast the gene expressions at day 14 were related to a maturing wound with extracellular matrix-associated genes, morphogenesis and differentiation-related genes being upregulated. This sequence of events can be compared to the histological findings demonstrating provisional osteoid presence by day 7 and a maturing mineralised osteoid by day 14 (Lang et al., 2011, Ivanovski et al., 2011).

Osteoblastic secretion of extracellular matrix (ECM) proteins provides the initial scaffold for subsequent mineralisation and is a reliable indicator for osteogenesis (Hughes, F.J et al, 2006). Upregulated genes associated with ECM proteins include: collagens (Col 1 to 11), the ECM protein essential for mineralisation: osteopontin (OPN), osteonectin (ON), the bone specific ECM: osteocalcin (OCN), bone sialoprotein (IBSP), periostin (POSTN), and ECM protein-1), alkaline phosphatase (ALP), and bone-specific adhesion proteins (integrins (ITGB4, ITGB5), laminins (LAMA2, LAMA3), and cadherins (CDH11) (Shanbhag, S et al, 2015). Transcription factors are intermediary proteins acting between cytokines such as bone morphogenic proteins (BMPs) and cells, regulating the expression of target genes (Suter, 2020). Specific transcription factors such as runx2, OSX and TAZ have a role in the osteoblastic lineage differentiation. TAZ commits the mesenchymal cells to the osteoblast lineage early on, whilst Runx2 and OSX having importance further downstream (Figure 4). The latter two can be upregulated by BMPs and are essential for osteoblasts differentiation (Marie, 2008, Hughes et al., 2006, Donos et al., 2011).

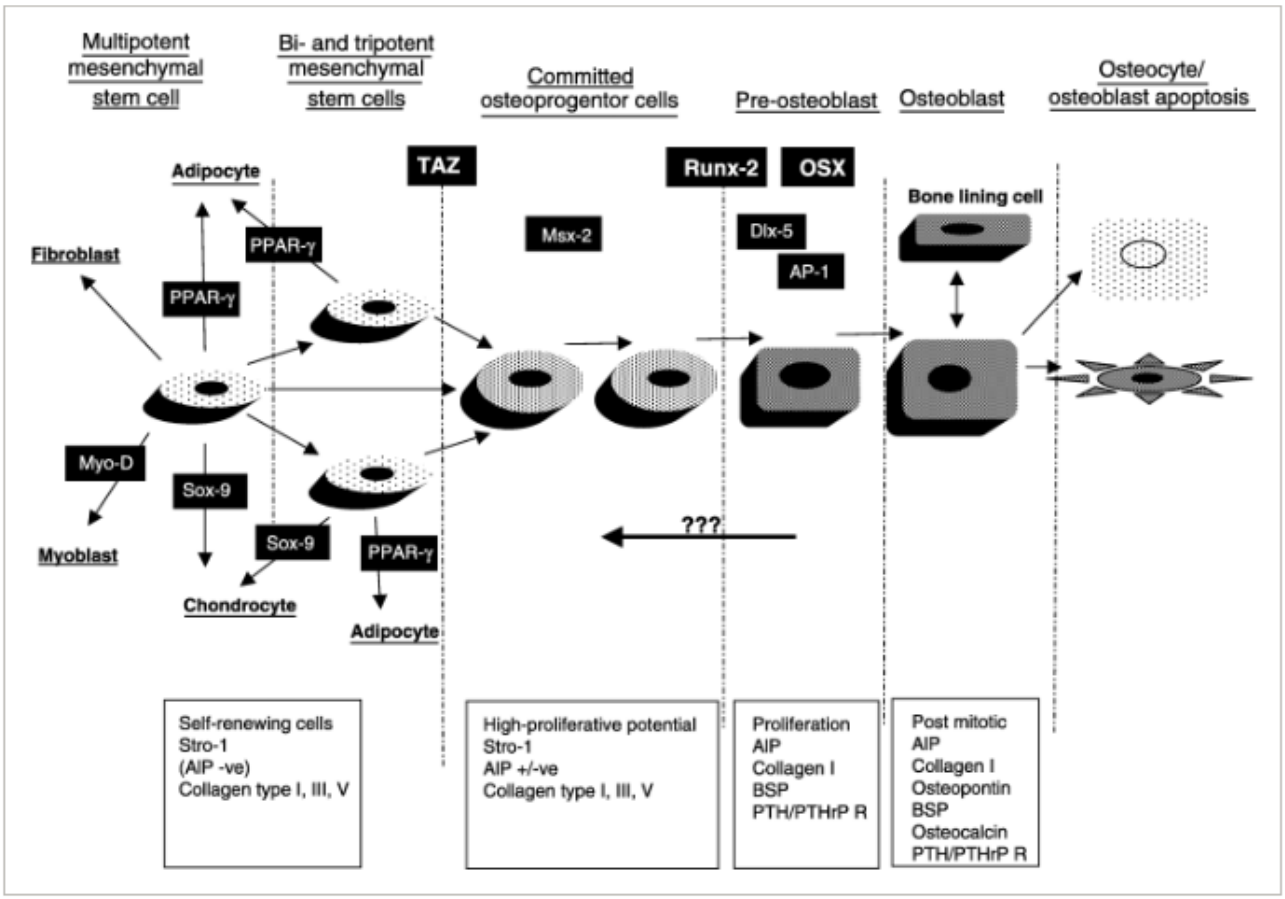

Figure 4. Demonstrates the osteoblastic lineage with some phenotypic features seen at different stages. Cbfa-1, transcription factor expression. AIP, alkaline phosphatase; AP-1, activator-protein-1; BSP, bone sialoprotein; OSX, osterix; PTH, parathyroid hormone; PTHrP R, PTH-related protein receptor (Hughes et al., 2006).

1.5.3. The use of modified titanium surfaces

Implant surface topography can have varying levels of roughness and the extent of this characteristic can be described as macro (from 1 to 100mm), which is related to the overall geometry, micro (from 1µm to 10 µm) and nano (from 1nm to 1µm) (Le Guehennec et al., 2007). The categorisation of surface microstructure was proposed by Albrektsson and Wennerberg (2004) and presented in the consensus report of the European association of osseointegration in 2009 (Lang et al., 2009). The classification was described as follows:

- Smooth surfaces: Sa *[1] value <0.5µm (polished abutment surface).
- Minimally rough surfaces: Sa value 0.5 to <1µm (turned implants).
- Moderately rough surfaces: Sa value 1 to <2µm (Commonly used types).
- Rough surfaces: Sa value ≥2µm (plasma sprayed surfaces).

The topography of an implant surface can be modified to increase roughness through physical techniques, such as cutting and turning or blasting (Bagno and Di Bello, 2004), Wieland et al, 2000). Alternatively, there are chemical methods such as acid etching. In vitro studies have reported that rough implant surfaces encourage an osteogenic environment, as evidenced by increases in the SPP1, RUNX2 and BSP osteogenic markers (Wall et al., 2009). Furthermore, this surface modification increases the responsiveness of osteoblast-like cells to 1α,25-$(OH)_2D_3$ and promotes production of hormones and cytokines involved in bone formation such as TGF-B1 and PGE2 in vitro (Boyan et al., 1998) .

Methods to modify surface chemistry have been developed to provide increased wettability and hydrophilicity of the surface to promote osteogenic potential (Lang et al., 2011), such as the case for modified SLA implants (SLActive). In vitro studies suggest this promotes conditioning by proteins and cell adhesion in the early stages of osseointegration (Rupp et al., 2006, Kieswetter et al., 1996). There is consistent evidence that the increased surface energy promotes osteoblast maturation and increased bone apposition during the early stages of osseointegration (Zhao et al., 2005, Gittens et al., 2013, Buser et al., 2004). A study on rabbits using SLA and SLActive domes further reported on the proteins and signalling pathways during early healing under the respective domes secured to the parietal bones of rabbits. Following mass spectrometry analysis of the tissue formed under the domes, the authors reported a reduced inflammatory

[1] A parameter used to measure surface roughness. It is the three-dimensional average height of all measured points in the dataset.

response and enhanced early osteogenic response in the hydrophilic SLActive domes (Calciolari et al., 2018).

Clinical trials have supported earlier loading times and superior clinical attachment levels of modified SLA implants compared to non-modified SLA implants (Bornstein et al., 2010). Lang et al. (2011) reported superior osseointegration of SLA active implants compared to SLA implants at 2 and 4 weeks after implant placement, while later healing points were comparable. It was suggested that the superior osseointegrative features of SLActive could be explained by an upregulation of osteoblastic recruitment and a downregulation of the early inflammatory response (Donos et al., 2011).

1.5.4. The use of modified titanium surfaces in systemically compromised patients

Different pre-clinical and clinical studies have tested the use of modified titanium surfaces to enhance osseointegration in challenging clinical scenarios and in patients affected by systemic diseases (Retzepi et al., 2010, Schlegel et al., 2013).

For instance, Mardas et al. (2011) demonstrated a higher level of bone formation and surface contact under SLActive domes compared to SLA domes in osteoporotic rats. A possible biological explanation to this outcome lies in the fact that osseous healing in osteoporotic condition has been associated with a tendency for an enhanced inflammatory and stress response and a delayed organization and maturation of the granulation (Calciolari et al., 2017a, Calciolari et al., 2017b). Since SLActive surfaces are able to modulate inflammatory and osteogeneis-related pathways, they might be particularly beneficial in osteoporotic patients, where the same pathways are negatively affected. Likewise, a preclinical study

reported the use of modified dental implant surfaces in untreated diabetes mellitus conditions with promising results (Schlegel et al., 2013).

A limited number of clinical studies have supported the use of SLA or SLActive implants in challenging situations such as in irradiated patients (Heberer et al., 2011, Nack et al., 2015), in poorly controlled type 2 diabetes patients (Khandelwal et al., 2013) and in immediate and early implant loading in the posterior maxilla/mandible (Bornstein et al., 2009, Morton et al., 2010, Roccuzzo and Wilson, 2009, Salvi et al., 2004).

There is currently no clinical evidence to indicate that SLActive implants are advantageous in osteoporotic patients. However, based on the aforementioned, it is plausible to consider these implants as a good modality of treatment for osteoporotic patients, particularly due to their modulating effect on inflammation and bone formation.

1.6. Peri-implant crevicular fluid (PICF)

1.6.1. Characteristics and composition

Peri-implant crevicular fluid (PICF) is the osmotically mediated fluid originating from the gingival vascular plexus around implants (Akman et al., 2018) and can be considered as the analogue of gingival crevicular fluid (GCF) in natural dentition.

PICF can be defined as a serum transudate in health or an exudate in disease and it contains host-derived cytokines, enzymes, immune cells, epithelial degradation products from the junctional and sulcular epithelium (Bostanci and Belibasakis, 2018). The production mechanism and composition of PICF have been shown to be similar to GCF and significant correlations have been reported between GCF and PICF cytokine concentrations and profiles

(Nogueira-Filho et al., 2014, Apse et al., 1989, Alassy et al., 2019). Apse et al. (1989) reported also very similar crevicular flow rate and microflora between GCF and PICF. The same study found a similar volumetric change between GCF and PICF through increasing grades of inflammation.

Table 2 shows the typical composition of GCF, which has been more characterized and investigated so far, as compared to PICF (Rahnama et al., 2014).

The mediators of the immune response	
Total and subgroups of immunoglobulin Ig G	
The mediators of the inflammatory response	
Arachidonic acid derivatives such as PGE_2	
Cytokines such as IL1-1, IL-2, IL-4, IL-6, TNFα	
Enzymes and enzyme inhibitors	
Proteolytic enzymes	**Hydrolytic enzymes**
Collagenase	Arylsulfatase
Elastase	B-glucuronidase
Cathepsin-B	Alkaline phosphate
Cathepsin-G	Acid phosphatase
Cathepsin-D	Myeloperioxidase
Tryptase	Lactoferrin
	Lysozyme
Bone-specific proteins	
Osteonectin	
Phosphoprotein (N-propeptide)	
Osteocalcin	
Telopeptide of type I collagen	
Tissue breakdown products	
Component	**The product breakdown**
Fibronectin	Split components of fibronectin
Collagen	Hydroxyproline
	The final peptides
Proteoglycans	Glycosaminoglycans
	Heparin Sulfate
	Chondroitin-4-sulphate
	Chondroitin sulphate-6

Table 2. A table to show the typical composition of GCF (Rahnama et al., 2014).

1.6.2. Collection methods

Different methods for PICF collection exist and consist of the same techniques for the collection of GCF. The following description therefore accounts for both GCF and PICF collection.

Microcapillary tubule technique

Microcapillary tubules can be used for pre-determined volume collection. The area is firstly cleaned of plaque without touching the gingival margin and dried. The tubule is rested at the crevice and either held or moved for a period whilst collecting the fluid via capillary action. However, this can be disruptive to the delicate structure of the crevice and may introduce blood and contaminants. Although effective at collecting larger samples of GCF/PICF, it can be time consuming and there are inherent difficulties with removing the entire sample from the tubing with it either being forced out with a jet of air, centrifuged or passing a fixed volume of solution through the tubing (Griffiths, 2003).

Absorbent string or filter paper

An alternative method includes placing an absorbent string or filter paper into the crevice for a predetermined collection time (Kaklamanos and Tsalikis, 2002). The paper can be placed either extra-crevicular to prevent trauma to the sulcus or more commonly intra-crevicular. This is a commonly used, straightforward technique but has shortcomings of potential contamination with plaque, serum, or blood and therefore isolation of the site must be adequate (Nazar Majeed et al., 2016, Griffiths, 2003).

Gingival washing methods

The use of two needles, one placed within the other allows ejection of an isotonic solution through the thinner internal needle at the base of the sulcus and collection from the outer needle which is at the gingival margin. The collected sample contains cells and plasma proteins. However, it is difficult to ensure the entire sample is aspirated and therefore it is not possible to determine an accurate quantification of GCF volume due to a precise dilution factor not being obtained (Griffiths, 2003).

1.6.3. PICF as potential sources of biomarkers to describe osseointegration

A biomarker is defined as an objectively measured entity of normal biological processes, pathological processes, or a pharmacologic response (Biomarkers Definitions Working, 2001). Biomarkers can be used for describing a healing process, for early diagnosis or prognostication of disease, as well as disease monitoring and measuring response to treatment.

Current diagnostic parameters, such as clinical and radiographic assessment, provide information on the history of the implant but have limited validity to predict disease activity or to prognosticate treatment outcomes (Bielemann et al., 2018b). This missing information may be provided by PICF markers that describe the molecular events taking place in the peri-implant environment. Few studies in the past years have explored the composition of PICF in different stages of osseointegration and in condition of health or peri-implant diseases.

Based on the osseointegration phases described above (section 1.5.1), pro-inflammatory cytokines play a crucial role in the early inflammatory stage of osseointegration and Bieleman et al. (Bielemann et al., 2018b) described an increase in their expression within PICF within 7 days from implant installation. For instance, TNF-α is an inflammatory cytokine involved in

the normal bone remodelling process, thereby modulating activity of osteoblasts and osteoclasts (Kaklamanos and Tsalikis, 2002) but its role in osseointegration remains unclear. A study demonstrated higher levels of TNF-α up to 4 weeks followed by a decrease (Bielemann et al., 2018b). Likewise, Gurkan et al. (2019) reported a statistical reduction by 8 weeks compared to a 2-week timepoint.

Another well-known cytokine involved in the initial inflammatory stage is IL-1 β, which modulates the degradation of extracellular matrix components and the collagenase activity in inflammation and wound healing (Casado et al., 2013). IL-1 β concentrations were reported to be high during the first week following surgical bone trauma. The levels remained relatively constant up to 8 weeks after implant placement (Gokmenoglu et al., 2014, Bielemann et al., 2018b). Reports of a protective role have been made with an associated increase of IL1-β with plaque scores. Similarly, patients with clinically healthy peri-implant tissues have been reported to have increased levels of PICF IL-1 β with increased biofilm (Petkovic et al., 2010). Gurkan et al. (2019) showed a peak at 2 weeks followed by reductions at week 4, 8 12 and 24 weeks.

Bielemann et al. (2018b) also reported elevated levels of the pro-inflammatory marker IL-6 in PICF during the first week following implant installation, which is most likely associated with the surgical trauma. These levels gradually reduced from weeks 2 to 8. The reported increased levels at week 12 in that study are likely related to mucosal trauma from prosthesis settlement. Another study indicated a peak at 2 weeks followed by statistically significant reductions at week 4, 8 12 and 24 (Gurkan et al., 2019).

Chemokines are a specific type of cytokine which induces migration of cells such as monocytes, lymphocytes and neutrophils during inflammation (Zlotnik and Yoshie, 2012).

CCL2 and CCL18 are examples of chemokines that can be found in gingival crevicular fluid (Stadler et al., 2016, Fageeh et al., 2021). To the best of our knowledge, they have not been explored in PICF. Interestingly, CCL18 has a role in the initial inflammatory recruitment of leukocytes and release of proinflammtory markers whilst in the latter stages of inflammation it can induce M2 polarisation of macrophages which promotes wound healing, angiogenesis and mesenchymal cell differentiation into osteoblasts to repair and regenerate bone (Pajarinen et al., 2019).

Genomic studies have documented a gradual reduction in inflammation after the initial days of healing. A study by Thalji et al. (2014) described the placement of implants in systemically healthy individuals followed by removal at interval periods up to 7 days. The cells adherent to the implants underwent a genomic assessment. There was downregulation of pro-inflammatory genes such as IL-1α and IL-1β together with upregulation of anti-inflammatory genes, CCL18 and CCL22, between days 3 and 7. This varies with different implant surface characteristics, as demonstrated by a study reporting a reduced inflammatory profile on super-imposed nanoscale topography compared to micron-scale topography surfaces at day 3 (Bryington et al., 2014).

While the initial inflammatory stage subsides (4 to 14 days following implant installation), undifferentiated mesenchymal cell recruitment and blood vessels begin to form the foundations of granulation tissue. Angiogenesis is stimulated through the hypoxic conditions attracting low oxygen tolerant cells like macrophages. These cells release vascular endothelial growth factor (VEGF), which stimulates production of and aggregation of endothelial cell precursors (Bosco et al., 2008). Angiogenesis is also enhanced by platelet derived growth factor (PDGF) released by platelets and the release of fibroblast growth factor (FGF) by

macrophages amongst other growth factors. In response to VEGF, pericytes detach from vessels and migrate towards the area of hypoxia. They condense and form tubular structures which anastomose with existing blood vessels. Verrastro Neto et al. (2018) reported an association between the levels of VEGF in PICF and insertion torque of immediately placed implants. Lower torque levels corresponded with higher PICF levels of VEGF at 7 and 30 days, suggesting torque levels may modulate the angiogenic biomarker (Verrastro Neto et al., 2018).

Maturation of the granulation tissue into woven bone is facilitated through molecules, including soluble signalling proteins called growth factors which facilitate cell division, matrix synthesis and tissue differentiation, through receptor–ligand binding. Growth factors found in bone include transforming growth factor-β, basic fibroblast growth factor, insulin-like growth factors, platelet-derived growth factor and bone morphogenic proteins (BMPs) (Hughes et al., 2006).

Bone morphogenic proteins are secreted signalling molecules, belonging to the TGF-B superfamily, with specific roles in osteoblast commitment and differentiation as well as angiogenesis (as described in the preceding sections). The BMPs, particularly BMPs 2,4,6,7,9 have shown an osteogenic potential by committing pluripotent cells to the osteoblastic lineage (Hughes et al., 2006, Mostafa et al., 2019). Dolanmaz et al. (2015) reported the presence of BMP-2 and BMP-7 in PICF with similar levels between SLA and SLActive implants at 1 and 3 months. Verrastro Neto et al. (2018) revealed BMP-9 presence in PICF at day 7 with similar levels in low torque and conventional torqued implants from 7 to 120 days, after which conventional torqued implants demonstrated higher levels.

Growth factors in peri-implant sulcular fluid have been explored (Dursun and Tozum, 2016, Bielemann et al., 2018a). Amongst growth factors, insulin growth factors play an important role in the maturation and proliferation of osteoblast function. Insulin-like growth factor-1 increases the expression of osterix (OSX), the osteoblast associated transcription factor and can act synergistically with BMP-2 (Celil and Campbell, 2005). Platelet derived growth factors are mitogens for connective tissue cells and facilitate chemotaxis of osteoblasts as well as having a role in mesenchymal cell recruitment in bone development and remodelling (Hughes and McCulloch, 1991). Fibroblast growth factors play a role in angiogenesis and mesenchymal cell mitogenesis.

The preceding cellular differentiation provides the foundations for bone formation which involves various proteins and signalling molecules working in harmony. New bone formation is also assisted by matrix metalloproteinases (MMPs) which are a group of enzymes that cleave collagen in response to certain stimuli such as LPS or cytokines (Kaklamanos and Tsalikis, 2002). They participate in converting the initial fibrin matrix into new bone through extracellular matrix degradation. MMP-1, MMP-8 and collagenase in PICF are increased in the initial week following surgery. Upregulation of MMP inhibitors such as TIMP-1-2,-3 has also been reported in the early healing phase (Nomura et al., 2000, Thalji et al., 2014), thus suggesting their potential use as early biomarkers. Alkaline phosphatase (ALP) is a marker of osteoblastic differentiation and activity but can also be produced by polymorphonuclear cells during inflammation. Mandic et al. (2015) reported increased levels of ALP in PICF in week 1 compared to week 4 which are believed to reflect the early inflammatory phase.

This immature woven bone eventually undergoes mineralisation and maturation. As previously described, this may take several months before becoming notable. Osteopontin

(OPN) is required for ECM mineralisation and is a marker for bone maturation and resorption. Prati et al. (2013) reported on immediately loaded vs non-loaded dental implants concluding that OPN is present in PICF at 15 days but immediately loaded levels peaked earlier than conventionally loaded implants. Prati et al. (2013) also reported the presence of osteocalcin in PICF, a protein released by osteoblasts in the mineralisation process, with higher levels found for immediately loaded implants between 7 and 30 days before becoming comparable.

The process of maturation requires a dynamic process which is regulated by cytokines, notably, osteoprotegrin (OPG) and RANKL produced by osteoblasts. Whilst the osteoblast bound molecule RANKL stimulates osteoclasts, OPG acts as a decoy molecule and counteracts this process. Several studies have demonstrated the presence of osteoprotegrin in peri-implant crevicular fluid and have attempted to correlate its presence to clinical parameters. There is conflicting evidence regarding OPG levels and clinical parameters, with studies showing a correlation between increased inflammation and OPG levels (Guncu et al., 2012, Arikan et al., 2008) but another study showing no association between implant health and OPG levels(Hall et al., 2011). Prati et al. (2013) reported negligible levels of OPG in the PICF of immediately placed implants, although there may have been blood contamination of the sample leading to inaccuracies. Implants placed at lower torques have shown higher levels of OPG and VEGF at 7 days post placement which suggests lower torques encourage microcirculation and peri-implant bone formation (Verrastro Neto et al., 2018). A progressive increase of OPG from weeks 2 to 12, followed by a decrease from 12 to 24 weeks has been reported with a suggested association with implant stability (Peker Tekdal et al., 2016, Ghiraldini et al., 2016).

Later stages of osseointegration involve bone remodelling which replaces woven bone with a more organised and load bearing lamellar bone. Osteoblast release of ALP contributes to the initiation of mineralisation and growth of hydroxyapatite crystals of mineralisation. Osteoclasts work in harmony with osteoblasts to resorb bone with enzymes such as tartrate resistant acid phosphatase (TRAP), Metalloproteinase-9 (MMP-9) and cathepsin K (Verrastro Neto et al., 2018). To the best of our knowledge these markers have not been investigated in PICF but are plausible for markers of remodelling in the later stages of osseointegration.

The aforementioned studies highlight the importance of certain markers in promoting/guiding the different stages of osseointegration. It is also important to mention a general marker of bone metabolism, vitamin D, which has a role in the homeostasis of calcium and phosphate through upregulation of intestinal absorption, downregulation of renal excretion, and calcium mobilisation (Atkins et al., 2007). It influences protein production and has a direct effect on osteoblasts, osteoclasts, and osteocytes (Atkins et al., 2007). A systematic review including pre-clinical and clinical studies showed a potential for vitamin D deficiency to have a negative effect on implant osseointegration both in terms of bone to implant contact and new bone formation (Werny et al., 2022). Although this article did not include implant crevicular fluid and concluded that further investigations are required, it does suggest biological plausibility.

1.6.4. PICF as a source of biomarkers relevant for bone mineral density and osteoporosis status

Traditionally, serum and urinary biomarkers have been utilised to determine the rate of disease progression in osteoporotic patients and, together with BMD measurement, they are a valuable tool in early assessment of patients at high risk of osteoporosis (Kuo and Chen,

2017). Biomarkers of bone remodelling do not predict bone mass or fracture risk but can be used to determine changes in bone remodelling during short intervals before alterations in bone mineral density can be detected (Nih Consensus Development Panel on Osteoporosis Prevention and Therapy, 2001).

As a non-invasive alternative to serum or urinary samples, collection of saliva is a practical source for measuring biomarkers, many of which have been identified as diagnostic aids in systemic diseases (Meleti et al., 2020, Mirzaii-Dizgah and Riahi, 2013, Vivacqua et al., 2019).

Salivary samples may potentially be used as a screening tools for metabolic bone conditions, as few studies suggested significant increases in salivary alkaline phosphatase and calcium in cases of osteoporosis and osteopenia (Saha et al., 2017).

Crevicular fluid from teeth and implants is another proposed alternative which has been explored to a lesser extent. Studies on gingival crevicular fluid levels of inflammatory markers have been carried out on post-menopausal osteoporotic patients to assess whether they reflect systemic findings. For instance, Reinhardt et al. (1994) reported an increased expression of pro-inflammatory marker IL1-B in the gingival crevicular fluid of patients with low oestrogen levels as compared to the oestrogen-treated patients. This reflects studies (Stock et al., 1989) which documented blood mononuclear cell and bone cell production of bone-resorbing cytokines in the presence of menopausal -induced oestrogen deficiency.

Gingival crevicular fluid levels of IL-8 and IL-1β have also been reported to correlate with the patient's oestrogen status (Payne et al., 1993). Similarly, (Streckfus et al., 1997) concluded that there was no difference in gingival crevicular fluid levels of IL-6, IL-8, or proteins between healthy pre-menopausal and menopausal women on oestrogen therapy.

Osteocalcin is a reliable marker of bone turnover typically assessed by a patient blood test. While Wilson et al. (2003) reported osteocalcin as undetectable in gingival crevicular fluid, Bullon et al. (2005) demonstrated the correlation between periodontal diseases and osteocalcin but showed no significant association between different densitometric groups and the mean osteocalcin concentrations in serum, saliva, and GCF.

Osteoclastic activity is reflected by the breakdown products of type 1 collagen which forms the major organic component of bone. C-telopeptides and N-terminal cross-linked telopeptide of type I collagen (NTx) are released during bone resorption (Hanson et al., 1992). Therefore, C-telopeptides and NTx are a marker for subtle bone resorption changes and are used as a marker of bone resorption. They have been isolated in GCF and PICF, (Wilson et al., 2003, Sakamoto et al., 2018, Becerik et al., 2011, Talonpoika and Hamalainen, 1994), but to the best of our knowledge their concentration in patients with different BMD has not yet been investigated.

Vitamin D is important in calcium absorption and bone mineralisation with a positive association with bone mineral density (Laird et al., 2010). In its active biologically form, 1,25(OH)2D3, is a modulator of osteoblast and osteoclast activity stimulating osteoblastic bone production and alkaline phosphatase activity (Kraichely and MacDonald, 1998). It preserves bone mass by increasing bone matrix proteins such as osteopontin and osteocalcin (Haussler et al., 2013, Kogawa et al., 2010). Vitamin D deficiency has been associated with a reduced bone mineral density and presents as a risk factor in bone healing (Waskiewicz et al., 2018). Vitamin D has been found in GCF (Balci Yuce et al., 2017, Liu et al., 2010, Acipinar et al., 2019, Zhang et al., 2014), but to the best of our knowledge the relationship between peri-implant crevicular fluid and osteoporosis has not been investigated.

1.6.5. Types of analyses on PICF

The term proteome is a term used to describe all the proteins an organism can express. Proteomics is the study of these proteins and their interactions (Garrels JI. 2011). Varying complexities of laboratory procedures exist for determining protein content in PICF. These range from procedures used to identify single proteins, multiple proteins simultaneously or all the proteins present in the sample.

A quantitative analysis of pre-determined proteins can be carried out using enzyme-linked immunosorbent assay (ELISA) to provide a single plex data of one protein per assay. The ELISA analyses protein samples in microplate wells using specific antibodies. The target antigen is immobilised on a microplate and then engaged with a specific antibody linked to a reporter enzyme. The activity of the reporter enzyme is measured by incubation with a substrate to produce a measurable signal such as light, colour or fluorescent (Lequin, 2005) (Wang et al., 2016, Hornbeck et al., 2001).

The resultant signals provided by the enzyme-antibody complex product include colorimetric techniques involving a chromogenic substrate which reacts with the conjugated enzyme system producing a visual change. Chemiluminescent techniques use a similar principle but has light as the end-product (Alegria-Schaffer, 2014). Fluorescent techniques involve antibody conjugation with a fluorophore instead of an enzyme. A specific wavelength light can elicit this complex to produce its own specific wavelength light which can be detected via digital equipment such as imagers (Kondo et al., 2018). The less used radioactive technique uses radioisotope instead of the enzyme with the emitted radiation captured on Xray films.

Different ELISA techniques are available as illustrated in Figure 5 (Japp et al., 2021). The direct technique uses an antibody-enzyme complex which reacts directly with the immobilised antigen on the assay plate. The indirect technique uses a labelled secondary antibody which binds to the primary antibody-antigen complex. The sandwich ELISA assay indirectly immobilises the antigen with an antibody-antigen complex following which there is indirect detection with another antibody-antigen complex of a different antigen epitope.

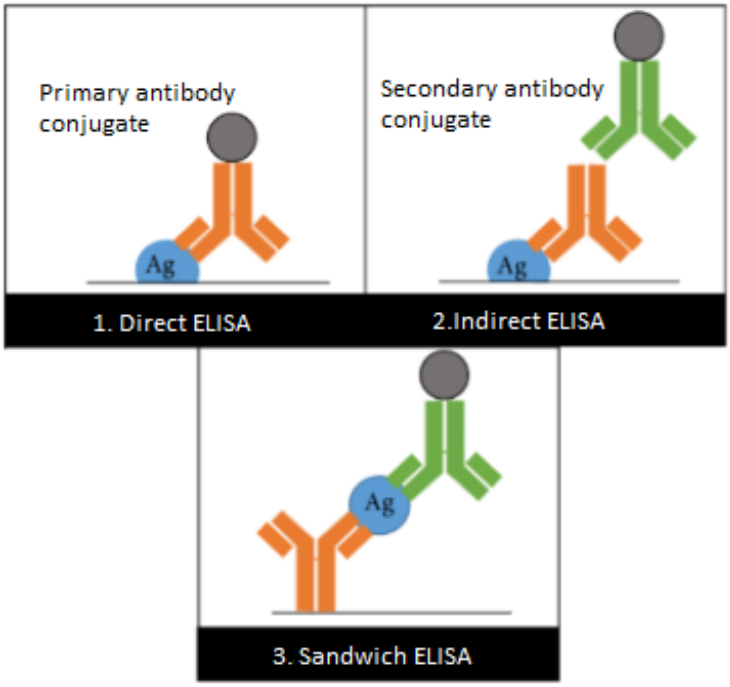

Figure 5. Different ELISA techniques: 1. The direct Elisa 2. The Indirect ELISA 3. The Sandwich ELISA (Japp et al., 2021)

The Western blot technique is a semi quantitative technique for specific protein analysis allowing the identification of a predetermined protein from a mixture of proteins in solution (Mahmood and Yang, 2012). The technique is summarised in Figure 6 and involves separation of proteins based on molecular weight through gel electrophoresis, followed by transfer to a membrane resulting in a band for each protein. The membrane undergoes blocking to prevent

non-specific binding of the specific antibody introduced during the next step. The membrane is exposed to a specific antibody for the protein of interest with the resultant enzyme-antibody complex reaction leading to a light, colour or fluorescence signal similar to the ELISA (Towbin et al., 1979).

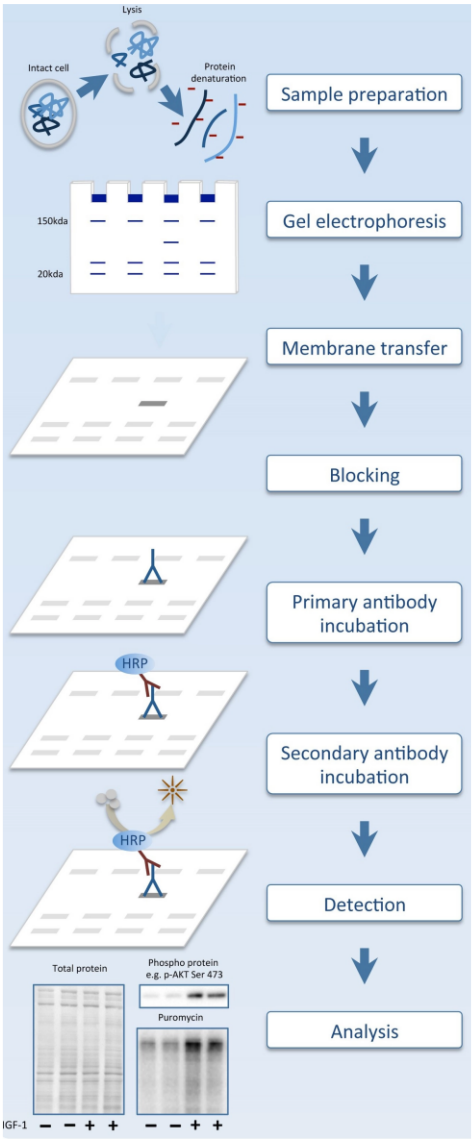

Figure 6 . An illustration of the sequence of steps involved in the western blot (Bass et al., 2017). As detailed in the text this involves the separation of proteins through gel electrophoresis, followed by transfer to a membrane leaving a band for each protein. The membrane undergoes blocking to prevent non-specific binding of the specific antibody introduced during the next step. The membrane is exposed to a specific antibody for the protein of interest with the resultant enzyme-antibody complex reaction leading to a light, colour or fluorescence.

The ELISA is commercially available and optimised for the assay. It does not require complex equipment and is technically less challenging to implement than a western blot. Throughput can also be increased with the use of automated plate handling and detection systems (Van Gool et al., 2020). The western blot technique and ELISA have high specificity, but the ELISA may be prone to higher false positives from cross-reactions of the detecting antibody with undesired proteins in the sample. These techniques are limited by the inability to perform

analyses of multiple proteins which can make these methods time consuming with the requirement for increased sample volumes. Multiplex arrays resolve this by allowing multiple and simultaneous analysis in a single assay which is more conservative of the limited sample volume.

Multiplex assays function with the use of magnetic microscopic beads with designated colours which have specific analyte capture antibody coatings. The analyte of interest is introduced and is bound by the capture antibody. Analyte specific biotinylated detection antibodies are introduced to form an antibody-antigen conjugation. Phycoerythrin conjugated streptavidin is added and binds to the detection antibodies. The results are read by flow cytometry as the beads are distinguished by fluorescent signatures. An example of a multiplex assay is Luminex and the steps are highlighted in Figure 7 (R&D Systems, 2022)(Renvert et al., 2015).

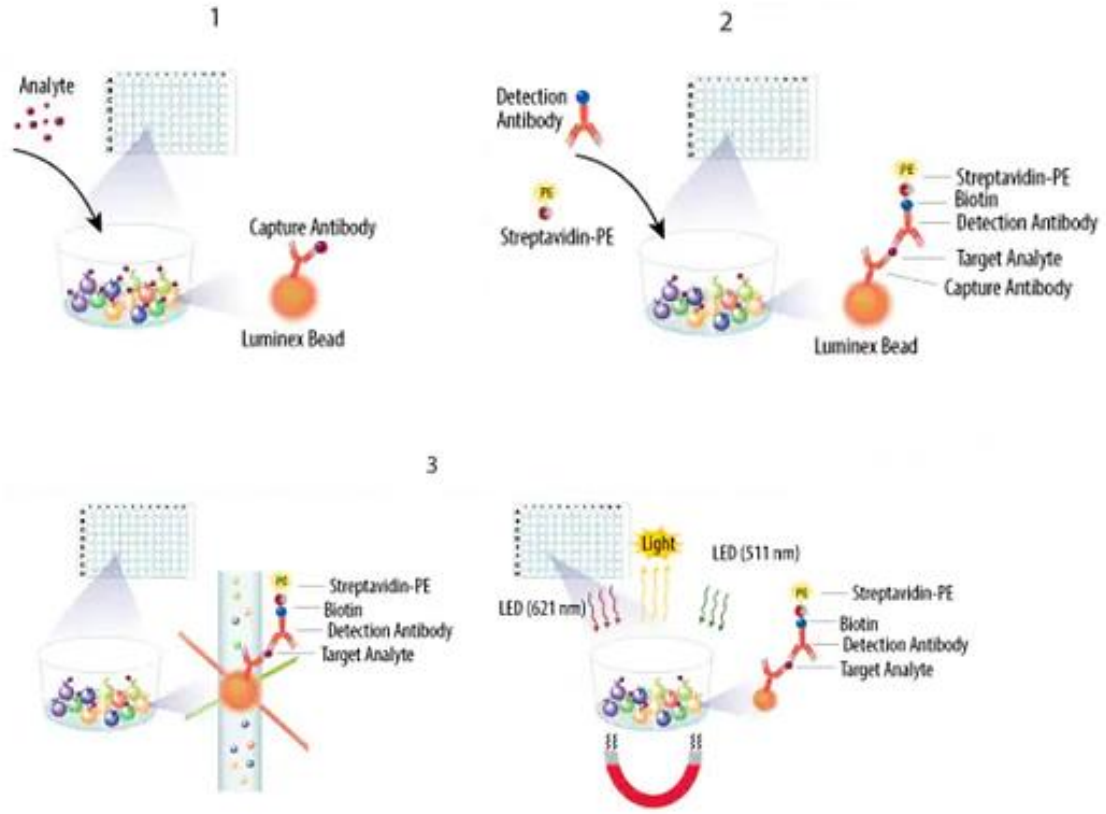

Figure 7. An illustration of the sequence of steps involved in a Luminex multiple assay.

1. Colour coded beads coated with analyte specific capture antibodies are exposed to the substrate. The antibodies bind to the analytes of interest.

2. Biotinylated detection antibodies and Phycoerythrin (PE)-conjugated streptavidin specific to the analytes form an antibody-antigen sandwich.

3. A dual laser flow instrument such as Luminex 200 or Flexmap analyser read the beads. The bead is determined by one laser whilst the second laser determines the magnitude of the Phycoerythrin derived signal which reflects the amount of analyte bound.

Another technique uses a magnet in the MAGPIX analyser to capture and hold the magnetic beads, while two distinct light-emitting diodes (LEDs) illuminate the beads. One LED identifies the analyte, and the second LED determines the magnitude of the PE-derived signal.

An enzyme-linked lectin assay (ELLA) also provides a multiplex Assay but differs in that it provides a simplified microfluidic simplex plex cartridge system. The sample and buffer are added to the cartridge and placed into the Ella which automates the immunoassay, the process is outlined in Figure 8 (R&D systems, 2017) Arnold et al. (2016).

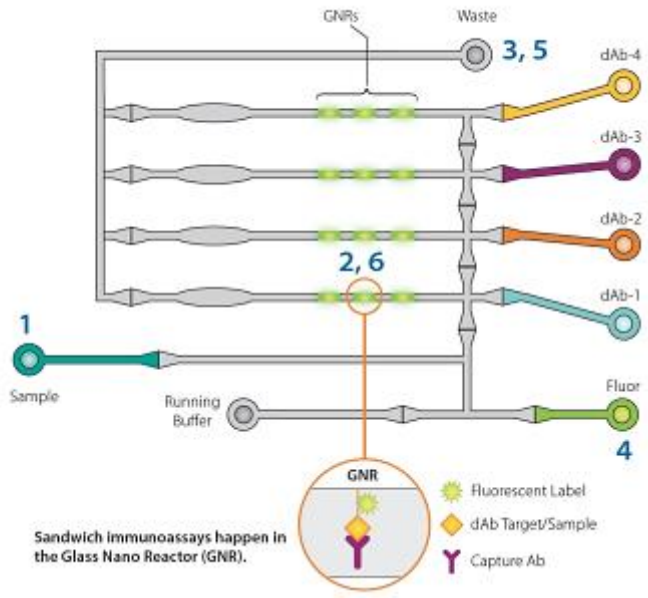

Figure 8. A graphic to Illustrate the sequence of steps involved in an Ella assay (R&Dsystems, 2017).

1. The analyte is introduced through microfluidic channels
2. The capture antibody binds the analyte
3. Stringent analyte removes the unbound analyte
4. Migration of detection antibody through the channels
5. Removal of unbound detection antibody with stringent wash
6. Sandwich immunoassays occur in the Glass Nano Reactor (GNR)

Finally, the more complex assessment of compounds can be determined with full proteomics via mass spectrometry, which provides higher sensitivity analyses and the possibility to search for previously undetected markers. It measures the mass to charge ratio of atoms/molecules in a sample which can be used to determine the exact molecular weight of a sample and determine unknown analytes. The process involves transforming the molecules into a gas phase, followed by separation of ions-based ion the mass/charge ratio in a mass analyser which has an electric/magnetic field. Finally, the ions are measured based on their mass/charge ratios (Yates Iii, J.R, 2011) (Fiorellini JP et al, 2000).

2. Aims and Objectives:

2.1. Aims

This project is part of a larger study which aimed to assess radiographic peri-implant bone level stability in post-menopausal osteoporotic women 12 months after implant loading.

The aim of this thesis project is to describe targeted PICF protein expression at 7 days and 6 months following implant loading in post-menopausal osteoporotic women.

2.2. Objectives

The primary objective was to explore and compare protein expression in PICF during early days of healing (7 days) and once osseointegration and loading have already been achieved (6 months post loading) in a cohort of post-menopausal osteoporotic women.

The secondary objectives include:

- To explore if inflammatory and bone related markers in PICF correlate with bone mineral density (T-score) and use of osteoporosis medications in osteoporotic patients.
- To explore whether inflammatory and bone related markers in PICF correlate with smoking status and post-operative adverse events (e.g. swelling, pain).

3. Materials and Methods

3.1. Study design

This project is part of a prospective case series study which consisted of 8 visits and involved up to 20 post-menopausal osteoporotic women followed up to 12 months post implant loading (Figure 10). The primary outcome was to determine radiographic peri-implant alveolar bone changes after 12 months of implant loading. Secondary outcomes included assessing changes in implant stability by applying resonance frequency analysis; evaluating implant success and survival 12 months after implant loading; describing the proteins and miRNAs expressed in the saliva at different time points; determining 3D geometrical facial changes and this thesis project, which aims to describe the proteins expressed in the peri-implant crevicular fluid at 7 days after implant placement and 6 months post loading.

Post-menopausal osteoporotic women in need for a single implant were recruited from the rheumatology department, DXA clinic and Barts health dental hospital to take part in this prospective case series study. Nine of the patients from the total patient pool had PICF collected at visit 4 and 7 and were included in this thesis project.

3.2. Inclusion and exclusion criteria

The inclusion criteria required for the participant to be enrolled in the study were as follows:

- Participants must be diagnosed with osteoporosis based on DXA measurement of the bone mineral density at the femur neck and/or total hip and/or lumbar spine (T value 2.5 SD or more below the young female adult mean) within the past 24 months.

- No longer than 4 consecutive years of treatment with anti-resorptive agents (like bisphosphonates and denosumab), to reduce the risk of medication-related osteonecrosis of the jaws Lo et al. (2010).

- ≥ 50 years old.

- In self-reported menopause, defined as the permanent cessation of ovulation, for at least one year Soules et al. (2001).

- The edentulous area must involve a maximum of two teeth (wisdom teeth and second molars are excluded) and present at least one neighbouring tooth (e.g. gap in the area of a second premolar and first molar, with first premolar in place).

- Residual alveolar width ≥ 4 mm at bone level Milinkovic and Cordaro (2014), residual alveolar height >8 mm, enough inter-arch space for a crown (at least 5 mm) and a minimum distance of 7 mm from the adjacent teeth (Shah and Lum, 2008). The width and height will be confirmed after x-ray examination in Visit 2.

- Possibility to restore a functional occlusion with a minimum of four occlusal units (i.e. pairs of occluding posterior teeth).

- Willingness to replace the missing tooth/teeth with dental implants

- Registration with a General dental practitioner (GDP).

Exclusion criteria demonstrate unsuitable patients as those:

On chronic treatment (i.e., two weeks or more) with any medication severely affecting oral status (e.g. participants with gingival hypertrophy caused by anti-epileptics, calcium antagonists, cyclosporine and other immunosuppressive) or bone metabolism (e.g. anticoagulant medications, long-standing steroid medications – i.e. equal or more 2.5 mg of prednisolone a day taken for more than 3 months -, anticonvulsants, immunosuppressants).

- Affected by systemic diseases recognized to severely affect bone metabolism (e.g. Cushing's syndrome, Addison's disease, diabetes mellitus type 1, leukaemia, pernicious anaemia, malabsorption syndromes, chronic liver disease, rheumatoid arthritis).

- Knowingly affected by HIV or viral hepatitis.

- History of local radiation therapy in the last five years.

- Affected by limited mental capacity or language skills such that study information cannot be understood, informed consent cannot be obtained, or simple instructions cannot be followed.

- Presenting an acute endodontic/periodontal lesion in the neighbouring areas to the implant site.

- Completely edentulous

3.3. Collection of Peri-implant crevicular fluid

Peri-implant crevicular fluid sample collection was done at visits 4 and 7 before any other clinical measurement. The visits are summarised in Figure 9. Straight after collection, the PICF samples were stored at -80 degrees for future Luminex analysis.

The patients were asked not to smoke, eat, or drink for at least 60 minutes before the sample collection. The site had plaque removed as required, using a curette, and was isolated using cotton rolls. A salivary ejector and cotton balls were used to remove any saliva carefully ensuring no contact with the gingival margin. PICF was collected using methylcellulose strips (Pro Flow, Inc., Amityville, NY) placed gently at the entrance of the peri-implant sulcus until slight resistance is felt. Wherever possible, the mesiobuccal sites were used to facilitate access.

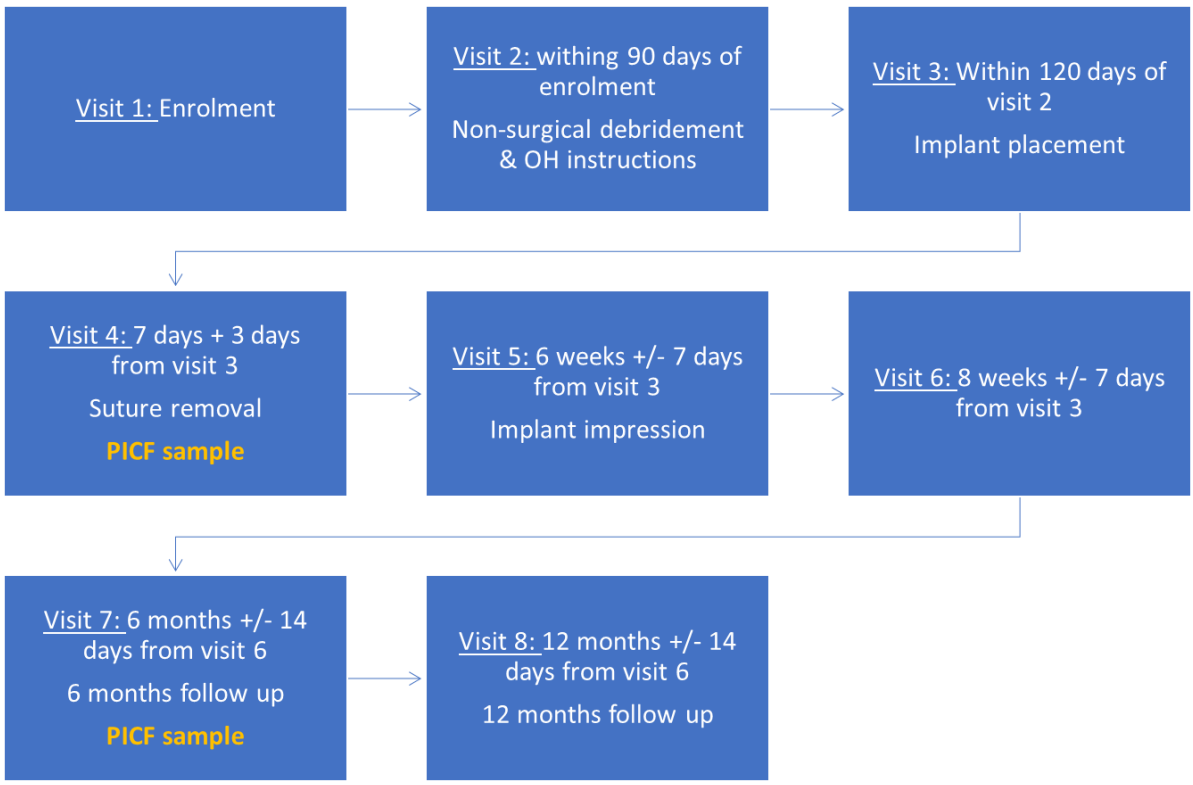

Figure 9. A summary of the visits highlighting visit 4 and visit 7 as the time points for PICF collection.

3.4. Elution of samples

The microcentrifuge was set to 4˚C before preparing the phosphate buffering solution (PBS) and defrosting the samples. The samples were carefully defrosted on ice and the Periopaper transferred, using tweezers, to a specialised centrifuge tube (Costar Spin-X Centrifuge Tube with Filters 0.22um) with the white part of Periopaper placed directly in contact with the filter (Figure 10). The Eluent Buffer (PBS + Protease Inhibitor Cocktail - PBS-PIC, complete ULTRA Tablets, Mini, EDTA-freeEASYpack Cat.No. 05892791001) was prepared by adding one tablet to 10ml PBS (filtered) and vortexed until completely dissolved.

Figure 10 A specialised centrifuge tube with the Periopaper placed against the filter.

The initial tube containing the Periopaper was washed with 50µl of the eluent buffer. The samples were shaken for 2 minutes and then centrifuged at 4˚C at 11,000rpm for 15 minutes (rpm was calculated on COCR Samples Lab's centrifuge with rotor radius=85mm). A further 50µl of eluent buffer was then added, resulting in a total of 100µl, the samples shaken for another 2 minutes and centrifuged for a further 15 minutes at 4˚C at 11,000rpm. The PerioPaper filter was removed from the microcentrifuge tube and a 6µl aliquot was taken for

protein quantification. The eluted PICF solution was stored at -80C until required for further analysis.

3.5. Quantification

The total protein concentration of the eluted PICF solution was determined using the LVis plate on a microplate reader from BMG LABTECH-ClarioStar (Dentistry Lab in Blizard Institute).

3.6. Quantification method

A series of dilutions were prepared for bovine Serum albumin (BSA) and PBS-PIC buffer in the order of: 2000ug/ml, 1000 ug/ml, 500 ug/ml, 250 ug/ml, 125 ug/ml, 62 ug/ml, 0 ug/ml. Following this, 2µl of each diluted protein standard was measured and placed into the micro-drop wells in duplicate. The absorbance of these standards at 280nm was measured and plotted against the concentration to produce a linear regression. Similarly, 2µl of the PICF sample was added in duplicate to the microwells and measured at 280nm. The standard curve was then used to calculate the protein concentrations of the PICF samples.

3.7. Dilution of the samples

The dilution process provides a concentration of analyte within the Luminex range of analysis. Therefore, an excessively high or low concentration will be outside the range of the standard curve. Due to the limited evidence regarding PICF dilutions, the dilutions were initially based on serum and plasma. A preliminary analysis of the PICF samples was carried

out in order to determine the optimal dilution for multiplex analysis. The 11-plex samples were diluted with calibrator diluent RD6-52 to a dilution of 1:2 as advised by the certificate of analysis. CCL18/PARC was initially diluted to 1:50 as advised by the certificate of analysis, but this was outside the range of analysis for the Luminex, necessitating the change of dilution to 1:10. Vitamin D binding protein was initially diluted to 1:500, but this was too concentrated and therefore it was later diluted to 1:2000.

3.8. Selection of biomarkers

The list of proteins analysed in this project was based on a literature review and on a thorough analysis of the biological processes involved in osseointegration and in the etiopathogenesis of osteoporosis. Table 3 shows the list of proteins analysed during the study. The literature review reported in the introduction explains the importance of the proteins expressed at different stages of healing during osseointegration and the proteins of significance in the pathogenesis of osteoporosis.

Table 3. The following table shows the markers of osseointegration, and osteoporosis analysed during the project. The table highlights the relevant healing phases of osseointegration, and the metabolic markers involved in osteoporosis.

	Markers of osseointegration	
Pro-inflammatory	**Bone matrix deposition and Angiogenesis**	**Maturation and Remodelling**
IL1-β	VEGF	Osteopontin
IL- 6	BMP-2	Osteoprotegrin
TNF-α	BMP-9	
CCL2	Osteopontin	
CCL18	Periostin	
IL-1 α		
	Markers of osteoporosis	
Pro-inflammatory	**Bone metabolism markers**	
IL1-β	Osteopontin	
IL- 6	Periostin	
TNF-α	Vitamin D BP	
CCL2	Osteoprotegrin	
IL-1 α		

3.9. Reagent preparation

The wash buffer

This was prepared by adding 20ml of wash buffer concentrate to 480ml of distilled water to prepare 500ml of wash buffer.

Standards

Standards are a set of known analytes and concentrations which are analysed in a spectrophotometer to determine their absorbance. The concentration and absorbance are plotted to produce a standard curve. Similarly, an unknown sample is analysed by the

spectrophotometer and the absorbance identified, this is plotted against the standard curve to determine the concentration of the unknown sample.

The standards were reconstituted with different volumes of the calibrator diluent, RD6- 52, dependent on the standard cocktail, as shown in Table 4.

Table 4. A table showing the biomarkers assessed and their respective standards. Each standard has a unique reconstitution volume as detailed.

Luminex Human Discovery Assay : 11-Plex	**Standards used**	**Reconstitution volume**
Analyte		
BMP-2	Standard C	0.225ml
BMP-9	Standard A	0.275ml
CCL2/JE/MCP-1	Standard B	0.225ml
IL-1 α/IL-1F1	Standard J	0.275ml
IL-1 β/IL-1F2	Standard N	0.250ml
IL-6	Standard G	0.250ml
Osteopontin/OPN		
Osteoprotegerin/TNFRSF11B		
Periostin/OSF-2		
TNF-α		
VEGF		
Luminex Human Discovery Assay : 1-Plex	**Standard used**	**Reconstitution volume**
Analyte		
CCL18/PARC	Standard I	0.200ml
Luminex Human Discovery Assay: 1-Plex	**Standard used**	**Reconstitution volume**
Analyte		
Vitamin D BP	Standard K	0.225ml

The 11-plex assay was provided with six standard cocktails and therefore following reconstitution, 100 µL of each standard was added into a combined tube called the standard 1 tube. 400 µL of Calibrator diluent was added to the standard 1 tube to obtain a 1000 µL final volume.

The 1 plex, CCL18/PARC was provided with 1 standard complex and therefore 100 µL of the standard I was added to a standard tube 1 with an additional 900 µL calibrator diluent to obtain a final volume of 1000 µL.

Similarly, the 1 plex, Vitamin -D BP was provided with 1 standard complex and therefore 100 µL of the standard K was added to a standard tube 1 with an additional 900 µL calibrator diluent to obtain a final volume of 1000 µL. A simple formula to determine the standard tube 1 volume is provided in figure 11.

100 µL X the number of standard cocktails provided= X µL

Following the addition of 100µL of each standard cocktail to a combined standard tube 1, the volume required to obtain 1000µL of final volume is added

X µL + volume of calibrator diluent(µL) =1000 µL

Figure 11. A simple formula to determine the final volume in standard tube 1

Standard dilutions

The standard was left to sit for 15 minutes with gentle agitation prior to the dilution step.

200 µl of calibrator RD6-52 was pipetted into 5 test tubes labelled 2-6. Standard 1 was used to produce a 3-fold dilution series as shown in figure 12.

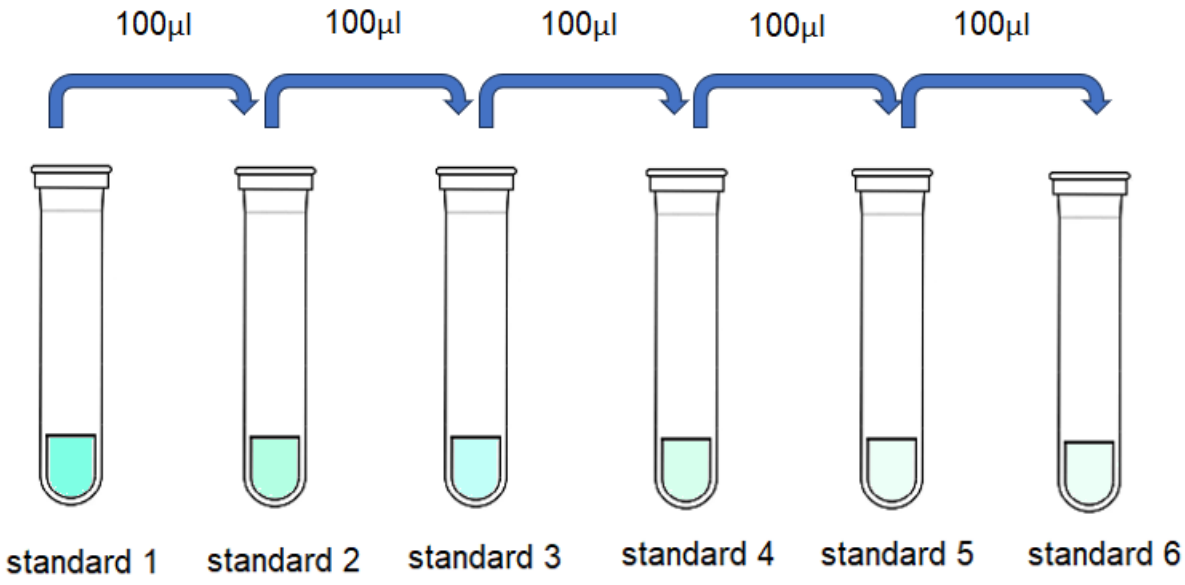

Figure 12. A diagram showing the Serial dilution of standard 1 in calibrator RD6-52

3.10. Diluted microparticle cocktail preparation

The microparticle cocktail vial was centrifuged for 30 seconds at 1000 x g prior to removing the cap. The vial was agitated with a vortexer to resuspend the microparticles. Based on the analysis of 9 patients at 2 visits and 6 standards, with duplicates of each well, 48 wells were required for each of the three assays (11-plex, 1-plex (Vit D BP), 1-plex (CCL18/PARC).

250 µL of microparticle cocktail was diluted using 2.50ml of diluent RD2-1.

3.11. Diluted Biotin-antibody cocktail preparation

The Biotin-Antibody Cocktail vial was centrifuged for 30 seconds at 1000 x g prior to removing the cap. The vial was gently vortexed ensuring not to invert the vial.

250 µL of antibody-biotin cocktail was diluted using 2.50ml of diluent RD2-1. The solution was gently mixed.

3.12. Streptavidin-PE preparation

The Streptavidin-PE was protected from light during handling and storage with the use of a polypropylene amber bottle.

The microparticle cocktail vial was centrifuged for 30 seconds at 1000 x g prior to removing the cap. The vial was agitated with a vortex to resuspend the microparticles. Based on a 48 number well the 110 µL microparticle cocktail was diluted using 2.65ml of diluent RD2-1.

Assay Procedure

Once all reagents, standards and samples were prepared as above, 50 µL of standard or sample were added to each well using a plate layout for recording purposes. The diluted microparticle cocktail was resuspended with the vortex and 50 µL were added to each well. A foil plate sealer securely covered the plate. This was incubated overnight at room temperature on a horizontal orbital microplate shaker (0.12" orbit) set at 800 rpm.

A magnetic device was used on the bottom of the microplate to retain the magnetic beads during the washing procedure. 100 µL of wash buffer was used in each well and allowed to stay for 1 minute before uniform removal of the liquid. The wash procedure was repeated three times.

50 µL of diluted Biotin-antibody cocktail was added to each well. A foil plate sealer securely covered the plate. This was incubated for 1 hour at room temperature on a horizontal orbital microplate shaker (0.12” orbit) set at 800 rpm. 100 µL of wash buffer was used in each well and allowed to stay for 1 minute before uniform removal of the liquid. The wash procedure was repeated three times.

A total of 50 µL of diluted Streptavidin-PE was added to each well. A foil plate sealer was used to securely cover the plate. This was incubated for 30 minutes at room temperature on a horizontal orbital microplate shaker (0.12” orbit) set at 800 rpm. 100 µL of wash buffer was used in each well and allowed to stay for 1 minute before uniform removal of the liquid. The wash procedure was repeated three times.

The microparticles were resuspended by adding 100 µL of wash buffer to each well. This was incubated for 2 minutes on the shaker at 800rpm.The microparticles were resuspended immediately prior to a Luminex reading (Magpix $_{tm}$). A summary of the assay procedure is shown in Figure .

Figure 13. A flowchart summarising the assay procedure.

3.13. Sample size and statistical analysis

There is limited literature on the use of peri-implant crevicular fluid markers in relation to postmenopausal osteoporosis and osseointegration, with no previous study using the same methodology. Therefore, a precise sample size calculation was not possible, and a convenience sample of 9 patients was deemed sufficient for this pilot study.

Descriptive statistics were used to summarize patients' demographic data. The primary outcome was to test for protein marker expression differences between visit 4 and visit 7. Due to a lack of normality distribution in the data set, the difference in the expression of protein markers between the two times points was assessed with a non-parametric test, the Wilcoxon signed rank test.

As Secondary outcomes the correlation between different parameters such as smoking, T-score, adverse events, previous periodontal treatment, and osteoporotic medications with expression of protein markers were investigated. For such outcomes, the spearman rank correlation coefficient was used. A regression model was not implemented due to the limited sample size.

4. Results

4.1. Overall study population

The overall study enrolled 27 post-menopausal osteoporotic women from September 2016 to July 2017. A total of 9 patients withdrew before visit 3 due to inadequate periodontal health (n=1), inadequate alveolar bone to place an implant without adjunctive bone augmentation or a sinus lift procedure (n=7) or personal reasons (n=1). After visit 7, there was 1 patient withdrawal due to personal reasons and her data were analysed up to visit 7, following intention to treat analysis.

4.2. Demographics of the overall study population

The patient population was predominantly white, with 2 patients of Asian ethnicity and 1 from a Black/African ethnic background. The age ranged from 53 to 75 years (mean of 62.33±6.08 years). The T-score ranged from -3.7 to -2.5 (mean of -2.91±0.35). The current smoking status of all patients was non-smoker, but 5 had smoked in the past. Nine patients

were currently receiving bisphosphonate medications for less than 4 years. 6 patients reported a history of periodontal treatment.

Nine implants were placed in the maxilla (50% of the implants), and 9 were placed in the mandible (50%). The majority were placed in the posterior molar premolar regions (n=15), while the canine/incisor region received 3 implants. This is summarised in Table 5.

Table 5. The following table reports the characteristics and demographics of the patients included in the overall study.

Ethnicity Number (%)	White: 15 (83.3%)
	Asian: 2 (11.1%)
	Black or African American: 1 (5.6%)
Age: min-max (mean±SD)	53 – 75 (62.33±6.08)
T score: min-max (mean±SD)	-3.7 – 2.5 (-2.91±0.35)
Smoking status: Number (%)	Smoker: 0 (0%)
	Past smoker: 5 (27.8%)
	Non-smoker: 13 (72.8%)
Osteoporosis medications: Number (%)	Past use: 2 (11.1%)
	Current use: 9 (50%)
	No medication: 7 (38.9%)
Previous periodontal treatment: Number (%)	6 (33.3%)
Implant site: Number (%)	Maxilla: 9 (50%)
	Mandible: 9 (50%)
	Incisors: 2 (11.1%)
	Canines: 1 (5.6%)
	Premolars: 6 (33.3%)

4.3. Demographics of the sample population

The current thesis focused on 9 patients who had PICF samples collected at visit 4 and visit 7 and their demographics are presented in Table 6 and Figures 14. The patient demographic was predominantly white and one patient was from a Black/African ethnic background.

The age ranged from 61 to 81 years (mean of 67.7±6.5 years). The T-score ranged from -3.7 to -2.5 (mean of -3.09±0.37). The current smoking status of all patients was non-smoker but 3 had smoked in the past. Osteoporosis medication (bisphosphonates) was currently taken by

3 patients, 5 patients did not use any osteoporosis medication and 1 had previous use. Three patients reported a history of periodontal treatment.

Three implants were placed in the maxilla (33.3% of the implants), and 6 were placed in the mandible (66.7%). The majority were placed in the posterior molar/premolar regions (n=10) whilst zero were placed in the anterior segments.

Table 6. The following table reports the characteristics and demographics of the sample of patients included in this aspect of the study.

Ethnicity Number (%)	White: 8 (88.9%)
	Asian: 0 (0%)
	Black or African American: 1 (11.1%)
Age: min-max (mean±SD)	61 – 81 (67.7±6.5)
T score: min-max (mean±SD)	-2.5 to -3.7 (-3.09 ± 0.37)
Smoking status: Number (%)	Smoker: 0%
	Past smoker:3 (33.3%)
	Non-smoker: 6 (66.7%)
Osteoporosis medications: Number (%)	Past use: 1 (11.1%)
	Current use: 3 (33.3%)
	No medication: 5 (55.6%)
Previous periodontal treatment: Number (%)	3 (33.3%)
	Maxilla: 3 (33.3%)
	Mandible: 6 (66.7%)
	Incisors: 0 (0%)
Implant site: Number (%)	Canines: 0 (0%)
	Premolars:5 (55.6%)
	Molars: 4 (44.4%)

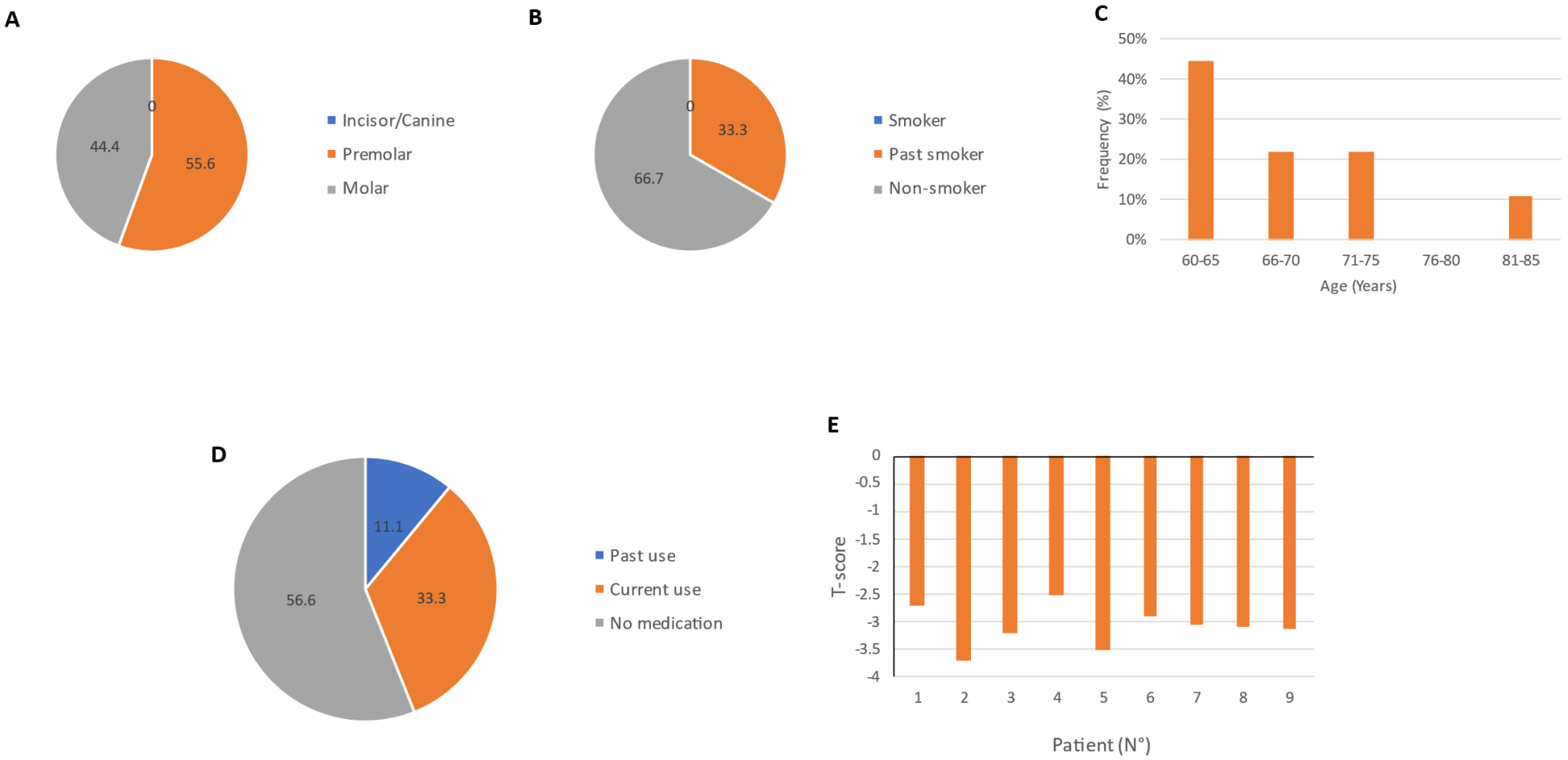

Figure 14. A series of graphs demonstrating the following demographic data: A: Implant placement site B: The smoking status of the patient C. The age distribution of the patients D: The use of bisphosphonate medications E: T-score values for each patient

4.4. Post-surgical adverse events

Post-surgical adverse events were those expected from a minor oral surgery. Specifically, two out of the nine patients experienced an adverse event expected from this type of procedure which involved pain, swelling and a headache. No significant adverse events were otherwise reported by the patients. Table 6 shows the adverse events related to surgery for each patient.

Table 7. The following table reports the adverse events associated with each patient following surgery.

Patient	Surgical Site	Adverse events related to surgery
1	14	Pain, swelling, headache
2	36	nil
3	45	Pain, swelling
4	14	nil
5	14	nil
6	35	nil
7	36	nil
8	46	nil
9	36	nil

4.5. Protein expression analysis

A total of 11 markers were analysed for this study, as previously described. These markers identify key biological phases in early and late osseointegration and/or may relate with bone metabolism (thus being linked to the underlying osteoporosis status). Table 8 shows the concentration of each marker measured at the two visits and highlights the markers not detected. The markers not detected were osteopontin, osteoprotegrin, periostin, BMP9 and TNF-α. IL6 was not detected at visit 7 but was present at visit 4.

Table 8. The following table shows the concentration (pg/ml) of each marker measured at the two visits and highlights the markers not detected. ND: Not detected

Marker	V4 Median (pg/ml)	V4 Mean (pg/ml)	IQR (pg/ml)	Std deviation	V7 Median(pg/ml)	V7 Mean (pg/ml)	IQR (pg/ml)	Std deviation
CCL2	16.91	23.71	20.59	19.11	14.27	14.94	16.61	18.36
CCL18	411.56	414.79	7.9	6.17	410.83	410.85	0.09	0.11
IL-1α	84.37	216.91	242.81	231.78	249.85	201.84	270.32	166.19
IL-1β	177.38	190.06	162.64	158.96	51.32	132.54	270.73	154.06
TNF-α	84.37	216.91	345.77	231.78	249.85	201.84	291.35	166.19
IL6	214.24	99.25	213.75	117.28	ND	ND	ND	ND
Osteopontin	ND	ND	ND	ND	ND	ND	ND	ND
Osteoprotegrin	ND	ND	ND	ND	ND	ND	ND	ND
Periostin	ND	ND	ND	ND	ND	ND	ND	ND
VitD bp	63151077.08	63152280.9	21668.62	10053.81	63161911.39	63157096.1	10834.31	5710.18
BMP2	15.36	15.27	0.2	0.18	15.06	15.1	0.3	0.18
BMP9	ND	ND	ND	ND	ND	ND	ND	ND
TNF Alpha	ND	ND	ND	ND	ND	ND	ND	ND
VEGF	40.51	36.9	16.38	12.05	21.05	37.54	54.91	43.48

4.5.1. Pro-Inflammatory markers

There was a trend for reduction in the inflammatory markers between visit 4 and visit 7. IL1-α conflicted with this trend and resulted in a non-statistically significant increase. They have been described individually below and summarised in a boxplot in figure 20.

CCL2

There was a non-significant decrease in the median value of CCL2 between Visit 4 and visit 7 as shown in figure 15. However, this trend was not statistically significant (p=0.110). The large standard deviation and interquartile range indicated a high inter-individual variability.

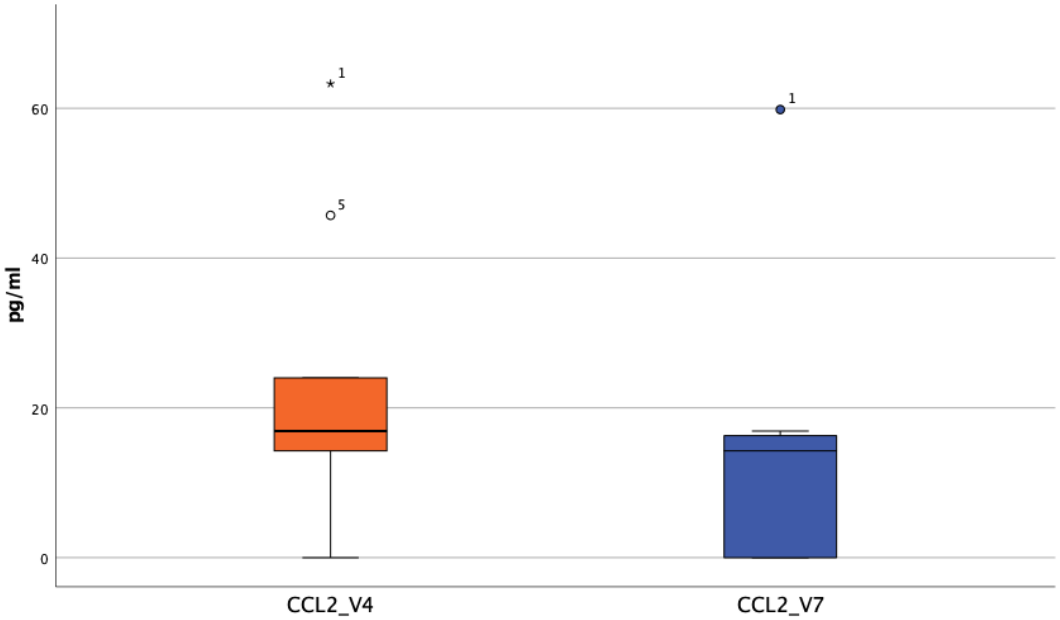

Figure 15. The box plot illustrates the concentrations (pg/ml) of CCL2 at visit 4 and visit 7. The difference between the visits was not statistically significant.

CCL18

There was a decrease in CCL18 levels between visit 4 and visit 7 which was statistically significant (p=0.049) as shown in figure 16. This was supported by a small interquartile range and standard deviation.

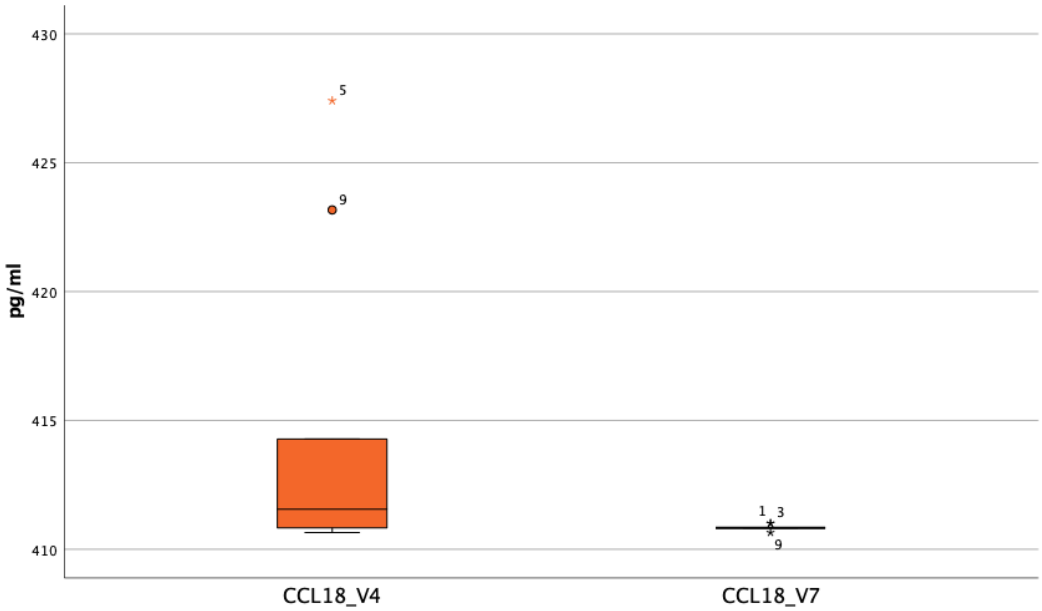

Figure 16. The box plot illustrates the concentrations (pg/ml) of CCL18 at visit 4 and visit 7. The difference between the visits was statistically significant.

Il1-β

As for the other inflammatory markers, IL-1-B concentration tended to reduce from visit 4 to visit 7 as shown in figure 17 . This was not statistically significant (p=0.260) and large standard deviation and interquartile range indicated a high inter-individual variability.

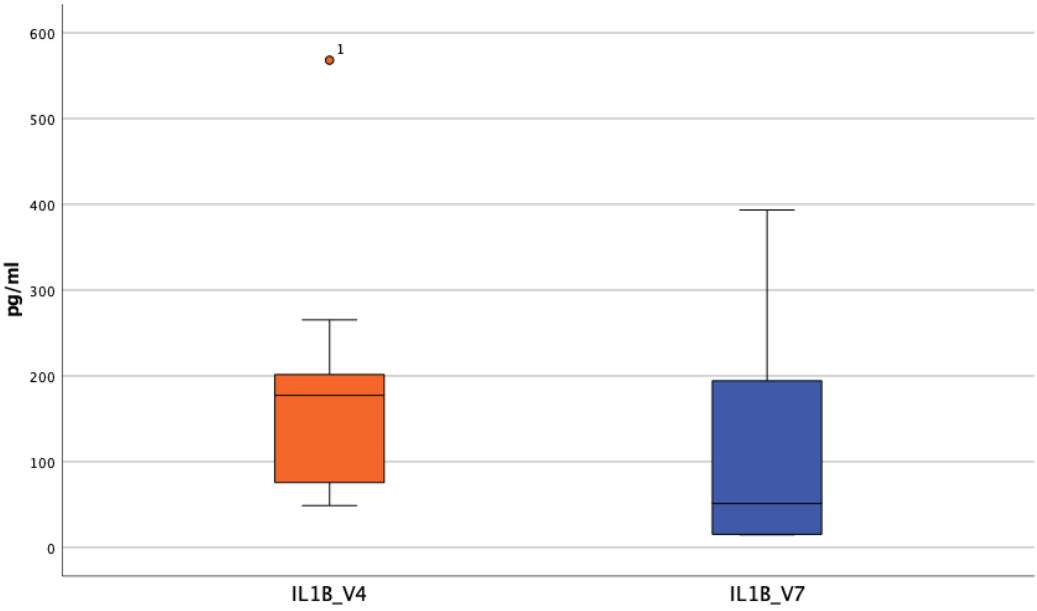

Figure 17. The box plot illustrates the concentrations (pg/ml) of IL-1β at visit 4 and visit 7. The difference between the visits was not statistically significant.

IL-1 α

This is the only inflammation-related markers where there was a non-statistically significant increase in the median value from visit 4 to visit 7 (p=0.767) as shown in figure 18 . As for most of the other inflammatory markers, there was a high inter-individual variability as indicated by the large standard deviation and interquartile range.

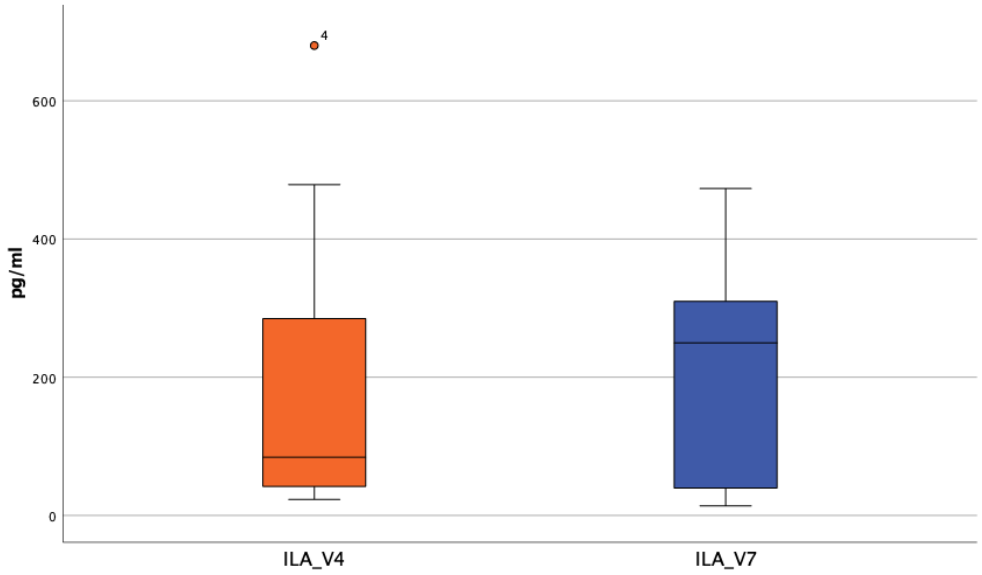

Figure 18. The box plot illustrates the concentrations (pg/ml) of IL-1α at visit 4 and visit 7. The difference between the visits was not statistically significant.

IL-6

IL-6 was detected in all patients at visit 4 but only detected in patient 1 at visit 7 as shown in figure 19. This was at a reduced level compared to visit 4. All other patient samples did not have IL6 detection at visit 7, which suggest that this marker was not expressed at detectable level at this time point.

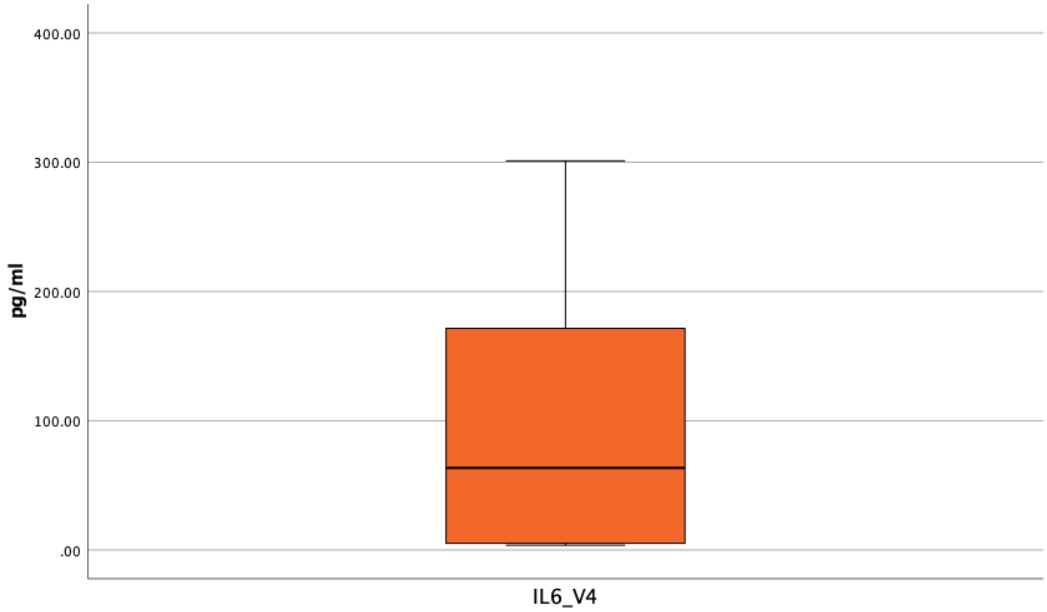

Figure 19. The box plot illustrates the concentrations (pg/ml) of IL-6 at visit 4 and its absence at visit 7. Patient 1 was not plotted for visit 7 due to the inability for comparison.

TNF-α

TNF-α was only detected in patient 1 and 4 at visit 4 and patient 1 in visit 7, therefore no assumptions can be done in relation to this marker.

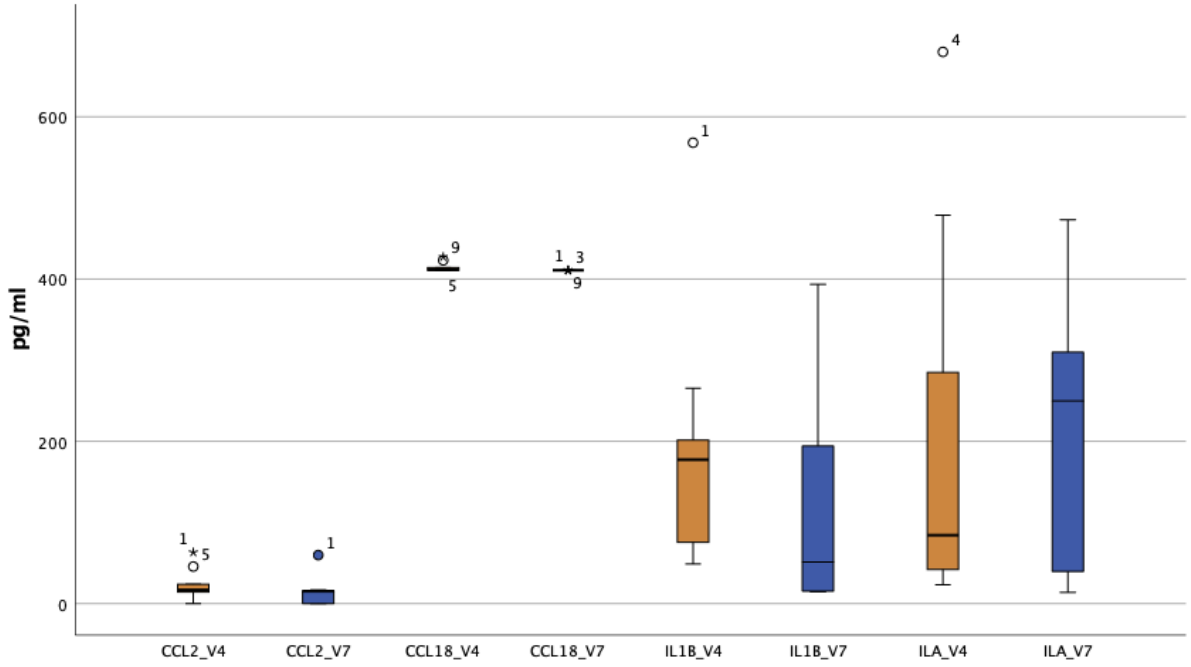

Figure 20. A summary box-plot showing all the inflammatory markers and their differential expression between visit 4 and visit 7

4.5.2. Bone matrix deposition, angiogenesis, maturation, and remodelling

The markers of bone matrix deposition, angiogenesis, maturation, and remodelling showed a trend for reduction between visit 4 and visit 7. However, the markers osteopontin, osteprotegerin, BMP9, periostin were not detected and therefore could not be reported on. They have been described individually below.

VEGF

There was a tendency for a decrease in VEGF levels between visit 4 and visit 7 which was not statistically significant (p=0.515) as shown in figure 21. A large standard deviation and interquartile range, particularly at visit 7, indicated a high inter-individual variability.

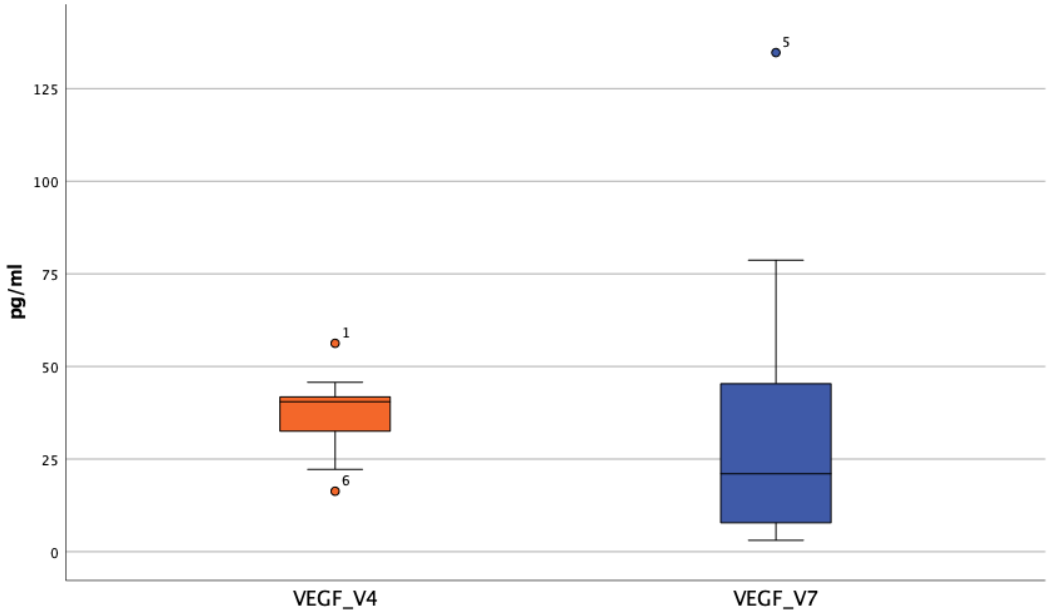

Figure 21. The box plot illustrates the concentrations (pg/ml) of VEGF at visit 4 and visit 7. The difference between the visits was not statistically significant.

BMP-2

There was a decrease in BMP-2 levels between visit 4 and visit 7 which was not statistically significant (p=0.066) as shown in figure 22. A large standard deviation and interquartile range indicated a high inter-individual variability.

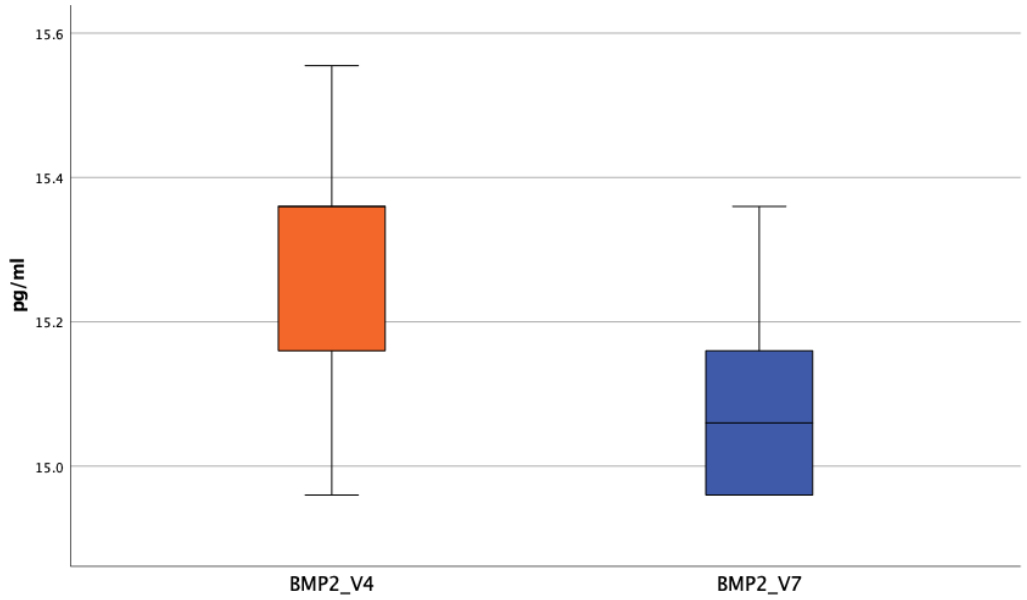

Figure 22. The box plot illustrates the concentrations (pg/ml) of BMP-2 at visit 4 and visit 7. The difference between the visits was not statistically significant.

BMP-9

BMP-9 was not detected in samples at visit 4 or visit 7 and therefore could not be assessed for correlation.

OsteopontIn

Osteopontin was not detected in any sample and therefore could not be assessed for correlation.

Osteoprotegrin

Osteoprotegrin was only detected in patient 5 at visit 7, therefore due to the limited results it was not possible to make any assumptions on the expression trend.

Periostin

Periostin was only detected in patients 1,5 and 9 at visit 4, while it was not detected at visit 7.

Vitamin D BP

There was an increase in Vitamin D BP levels between visit 4 and visit 7 which was not statistically significant (p=0.194) as shown in figure 23. A large standard deviation and interquartile range, particularly at visit 4, indicated a high inter-individual variability.

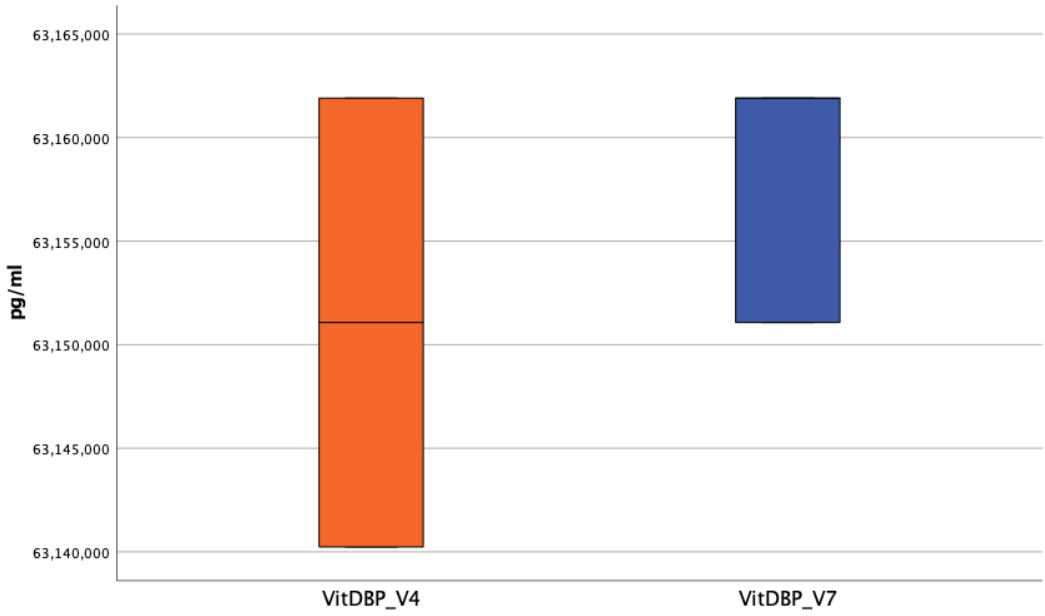

Figure 1. The box plot illustrates the concentrations (pg/ml) of Vit D BP at visit 4 and visit 7. The difference between the visits was not statistically significant.

4.6. Secondary outcomes

The correlation between inflammatory and bone related markers in PICF with bone mineral density (T-score), the use of osteoporosis medications in osteoporotic patients, smoking status and post-operative adverse events (e.g. swelling and pain) was explored as part of the secondary outcomes through the spearman rank correlation coefficient (See appendix 2).

The variable 'no history of periodontal therapy' was negatively correlated with CCL2 levels (-0.8; p value=0.006) at visit 4, thus suggesting higher CCL2 levels when there was no history of periodontal therapy. Likewise, the lack of previous periodontal therapy was negatively correlated with BMP2 levels at visit 4 (-0.7; p value=0.014), thus suggesting higher BMP2 levels at visit 4 with patients with no history of periodontal therapy. Finally, T scores were negatively correlated with BMP2 levels at visit 4 (-0.8; p value=0.03), thus suggesting higher BMP2 levels at visit 4 in more severe cases of osteoporosis. For all the other investigated variables no significant correlations were found.

5. Discussion

This study described for the first time the protein expression in PICF following implant placement and compared protein expression during early (7 days) and late-stage healing (6 months post loading) in post-menopausal osteoporotic women. The markers analysed were key markers related to the different stages of osseointegration as well as to the inflammatory and bone metabolism aspects of osteoporosis. These markers were then correlated against T-scores, osteoporotic medication, smoking status, and post-operative adverse events.

The general trend for the inflammatory markers was a reduction in values from visit 4 to visit 7, with CCL2, CCL18, IL-6 IL1-B showing a non-statistically significant reduction. This is

expected as the inflammatory process is more active during the first weeks following surgical placement of the implant compared to later timeframes of healing (Bielemann et al., 2018b). However, the only marker that showed statistical significance was CCL18, while IL-6 was not detected at visit 7. The main limitation of this result related to the small population size, which may have led to a type 2 error due to a reduced power in the statistical test.

The markers associated with bone matrix deposition, angiogenesis, maturation, and remodelling demonstrated a non-statistically significant reduction for VEGF and BMP-2 levels between visit 4 and visit 7. This trend is expected, as there is an increase in these markers during the initial wound healing stages, with gene expression for VEGF and BMP pathways both being upregulated at days 7 and 14 according to previous studies (Donos et al., 2011). Surgical flap perfusion is also increased during the first few days, reaching baseline levels of perfusion at day 15 (Retzepi et al., 2007). The lack of statistical significance in our data may be related to markers tapering off towards visit 7 (14 days). The other markers in this category were not detected, probably because their expression was at a concentration below the detection threshold of the Luminex kit. The only marker of bone metabolism that was detected at visit 4 and visit 7 was Vitamin D BP, which increased without statistical significance. Vitamin D BP has been shown to be inversely correlated to inflammation, both intraorally and systemically, which may explain this finding (Yin and Agrawal, 2014, Dietrich et al., 2005, Lu, 2023).

Overall, very few parameters were found to be significantly related to marker levels. The spearman rank correlation coefficient demonstrated a statistically significant negative correlation (-0.8) between ‘no history of periodontal therapy’ and CCL2 levels at visit 4 and a statistically significant negative result (p value=0.014) was associated with no previous

periodontal therapy and BMP2 levels at visit 4, suggesting higher BMP2 levels at visit 4 with patients with no history of periodontal therapy. These correlations are difficult to explain and are likely due to chance particularly due to the limited sample size and high inter-individual variability but deserve further investigation in a larger sample size.

A significant correlation (p value=0.03) was found between T-scores and BMP2 levels at visit 4. The lower the T-score (and therefore the more severe the osteoporotic status), the higher was the BMP-2 level at visit 4. This negative correlation might be explained by a higher compensatory level of BMP2 in response to the lower bone mineral density to promote bone formation (Liu et al., 2018). However, this speculation needs to be validated in a larger population, since this correlation might also be due simply to chance.

In conclusion, this project was a proof of principle pilot study, assessing for the first-time marker expression at different stages of osseointegration and osteoporosis in PICF of post-menopausal osteoporotic women. The study confirmed the possibility to study inflammatory-related, angiogenesis-related and bone metabolism-related markers in the PICF with enzyme-linked immunosorbent assays, although few specific markers of interest could not be identified (BMP-9, Osteopontin, Osteoprotegrin, Periostin).

When looking at the marker expression trend, the study demonstrated a differential expression between early and late stages of osseointegration, with statistically significant changes being reported for CCL18. Further to this, the secondary outcomes highlighted a possible correlation between 'no previous periodontal therapy' and CCL2 levels and BMP2 levels at visit 4. The T-score was also shown to be correlated with statistical significance to BMP2 levels at visit 4. However, the biological explanation of such correlations remains still doubtful and would deserve confirmation in a larger sample size.

Due to the pilot nature of this project, the main shortcoming is the small sample size, which does not allow to draw any robust conclusions on the expression trends of the different markers and may have led to a type 2 error. The limited number of time points precluded also from a more detailed characterization of the osseointegration process. However, considering the non-invasive collection of PICF, in the future it would be interesting to collect PICF at multiple early and late time points to better characterize the osseointegration stages. These pilot data can be used to properly power a future larger study that will aim to assess in a larger population, markers of osseointegration, with the aim to identify key specific candidate markers that can be representative of the early and late stages and that can be used to monitor the osseointegration process.

Another important limitation is the lack of a healthy control group. In the future it would be interesting to assess whether markers are expressed differently in healthy versus osteoporotic patients and to correlate the marker expression with clinically relevant parameters (such as peri-implant marginal bone levels and implant success/survival).

Appendix 1:

Protocol Procedures	Visit 1	Visit 2	Visit 3	Visit 4	Visit 5	Visit 6	Visit 7	Visit 8
	Enrolment	Non-surgical debridement and OH instructions	Implant placement (*GBR)	Suture removal	Implant impression	Implant loading	6-months follow-up	12-months follow-up
		Within 90 days from enrolment	Within 120 days from Visit 2, unless the bone quantity/quality is insufficient and a staged technique is necessary, so implant is placed 6 months ±14 days after GBR	7 days + 3 days after Visit 3 (in case of GBR 14 days ± 5 days)	6 weeks ± 7 days after Visit 3 (in case of GBR 11 weeks + 7 days)	8 weeks ± 7 days after Visit 3 (in case of GBR 12 weeks + 7 days)	6 months ± 14 days after Visit 6	12 months ± 14 days after Visit 6
Verification Inclusion/Exclusion	X							
Informed consent	X							
Medical/Dental History and Updates Demographics	X	X	X	X	X	X	X	X
Basic Periodontal Examination (BPE)	X							
Six-point pocket chart (PPD, REC, furcation and mobility)	X (If BPE ≥3)						X	X
PPD, REC, BOP and PI in the teeth adjacent to the implant	X						X	X
Saliva sample	X			X			X	
Peri-implant crevicular fluid collection				X			X	
Peri-apical intra-oral x-ray		(X) **				X		
3D facial scan	X		X	X	X			
CBCT scan		(X) **	X	(X) if not performed in Visit 3				X
Full-mouth plaque (FMPS) and bleeding (FMBS) scores		X					X	X
Periodontal causal therapy, polishing and hygiene instructions		X					X	X
Implant placement (*GBR)			X					
Resonance frequency analysis			X		X	X		X
Suture removal				X				
Implant impression					X			
Implant loading						X		
Recording of any AE		X	X	X	X	X	X	X

Correlations

			Smoking_status	Osteopor_medic	Previous_periotx	AE_surgery	Tscore	CCL2_V4	CCL2_V7	CCL18_V4	CCL18_V7	IL1B_V4	IL1B_V7	ILA_V4	ILA_V7	IL6_V4	VitDBP_V4	VitDBP_V7	BMP2_V4	BMP2_V7	VEGF_V4	VEGF_V7
Spearman's rho	Smoking_status	Correlation Coefficient	1.000	.816**	.000	-.378	-.274	-.138	.513	.367	-.546	-.091	-.321	-.365	.183	.138	.146	-.316	.097	.241	-.365	-.365
		Sig. (2-tailed)	.	.007	1.000	.316	.476	.724	.158	.332	.129	.815	.400	.334	.638	.724	.707	.407	.804	.533	.334	.334
		N	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9
	Osteopor_medic	Correlation Coefficient	.816**	1.000	.000	.000	-.298	-.131	.266	.337	-.156	-.373	-.243	-.410	.224	-.019	.299	-.194	.119	.059	-.596	-.186
		Sig. (2-tailed)	.007	.	1.000	1.000	.436	.737	.488	.375	.689	.323	.528	.273	.563	.962	.435	.618	.761	.880	.090	.631
		N	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9
	Previous_periotx	Correlation Coefficient	.000	.000	1.000	-.378	.639	-.825**	-.187	-.321	-.164	-.091	-.642	.639	-.183	-.596	-.634	-.316	-.777*	.048	-.091	-.548
		Sig. (2-tailed)	1.000	1.000	.	.316	.064	.006	.631	.400	.674	.815	.062	.064	.638	.090	.067	.407	.014	.902	.815	.127
		N	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9
	AE_surgery	Correlation Coefficient	-.378	.000	-.378	1.000	.104	.520	.106	-.156	.866**	.207	.520	-.207	.104	.312	.553	-.060	.330	.109	.207	.518
		Sig. (2-tailed)	.316	1.000	.316	.	.791	.152	.787	.689	.003	.593	.152	.593	.791	.414	.122	.879	.385	.780	.593	.154
		N	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9
	Tscore	Correlation Coefficient	-.274	-.298	.639	.104	1.000	-.343	.196	-.619	.149	.233	-.519	.533	-.500	-.452	-.249	-.433	-.709*	.290	.283	-.483
		Sig. (2-tailed)	.476	.436	.064	.791	.	.366	.614	.075	.701	.546	.152	.139	.170	.222	.517	.244	.032	.449	.460	.187
		N	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9
	CCL2_V4	Correlation Coefficient	-.138	-.131	-.825**	.520	-.343	1.000	.145	.265	.260	.301	.769*	-.243	.234	.567	.371	-.087	.837**	.415	.351	.753*
		Sig. (2-tailed)	.724	.737	.006	.152	.366	.	.709	.491	.499	.431	.015	.529	.544	.111	.325	.824	.005	.267	.354	.019
		N	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9
	CCL2_V7	Correlation Coefficient	.513	.266	-.187	.106	.196	.145	1.000	-.239	.046	.170	-.137	-.426	-.323	.286	.564	-.310	-.005	.278	.017	-.375
		Sig. (2-tailed)	.158	.488	.631	.787	.614	.709	.	.535	.907	.661	.726	.253	.396	.455	.114	.417	.991	.469	.965	.321
		N	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9
	CCL18_V4	Correlation Coefficient	.367	.337	-.321	-.156	-.619	.265	-.239	1.000	-.330	.067	.353	.084	.326	.571	-.255	.261	.646	-.018	.218	.343
		Sig. (2-tailed)	.332	.375	.400	.689	.075	.491	.535	.	.386	.864	.352	.831	.391	.108	.508	.498	.060	.964	.574	.366
		N	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9
	CCL18_V7	Correlation Coefficient	-.546	-.156	-.164	.866**	.149	.260	.046	-.330	1.000	-.050	.470	-.090	-.129	.170	.463	.155	.143	-.173	.090	.398
		Sig. (2-tailed)	.129	.689	.674	.003	.701	.499	.907	.386	.	.899	.202	.819	.740	.662	.209	.690	.713	.656	.819	.288
		N	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9
	IL1B_V4	Correlation Coefficient	-.091	-.373	-.091	.207	.233	.301	.170	.067	-.050	1.000	.293	.183	.367	.544	-.143	-.433	.168	.553	.817**	.233
		Sig. (2-tailed)	.815	.323	.815	.593	.546	.431	.661	.864	.899	.	.444	.637	.332	.130	.714	.244	.665	.122	.007	.546
		N	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9
	IL1B_V7	Correlation Coefficient	-.321	-.243	-.642	.520	-.519	.769*	-.137	.353	.470	.293	1.000	-.109	.435	.718*	.192	.130	.864**	.141	.393	.929**
		Sig. (2-tailed)	.400	.528	.062	.152	.152	.015	.726	.352	.202	.444	.	.781	.242	.029	.620	.738	.003	.717	.295	.000
		N	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9
	ILA_V4	Correlation Coefficient	-.365	-.410	.639	-.207	.533	-.243	-.426	.084	-.090	.183	-.109	1.000	-.200	-.201	-.882**	-.173	-.257	.237	.500	-.017
		Sig. (2-tailed)	.334	.273	.064	.593	.139	.529	.253	.831	.819	.637	.781	.	.606	.604	.002	.656	.504	.539	.170	.966
		N	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9
	ILA_V7	Correlation Coefficient	.183	.224	-.183	.104	-.500	.234	-.323	.326	-.129	.367	.435	-.200	1.000	.293	-.053	-.346	.452	.351	-.017	.583
		Sig. (2-tailed)	.638	.563	.638	.791	.170	.544	.396	.391	.740	.332	.242	.606	.	.444	.891	.361	.222	.354	.966	.099
		N	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9
	IL6_V4	Correlation Coefficient	.138	-.019	-.596	.312	-.452	.567	.286	.571	.170	.544	.718*	-.201	.293	1.000	.233	.130	.748*	.123	.577	.494
		Sig. (2-tailed)	.724	.962	.090	.414	.222	.111	.455	.108	.662	.130	.029	.604	.444	.	.547	.738	.020	.752	.104	.177
		N	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9
	VitDBP_V4	Correlation Coefficient	.146	.299	-.634	.553	-.249	.371	.564	-.255	.463	-.143	.192	-.882**	-.053	.233	1.000	.139	.237	-.178	-.330	.062
		Sig. (2-tailed)	.707	.435	.067	.122	.517	.325	.114	.508	.209	.714	.620	.002	.891	.547	.	.722	.539	.646	.386	.873
		N	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9
	VitDBP_V7	Correlation Coefficient	-.316	-.194	-.316	-.060	-.433	-.087	-.310	.261	.155	-.433	.130	-.173	-.346	.130	.139	1.000	.092	-.913**	-.087	.000
		Sig. (2-tailed)	.407	.618	.407	.879	.244	.824	.417	.498	.690	.244	.738	.656	.361	.738	.722	.	.814	.001	.825	1.000
		N	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9
	BMP2_V4	Correlation Coefficient	.097	.119	-.777*	.330	-.709*	.837**	-.005	.646	.143	.168	.864**	-.257	.452	.748*	.237	.092	1.000	.224	.231	.816**
		Sig. (2-tailed)	.804	.761	.014	.385	.032	.005	.991	.060	.713	.665	.003	.504	.222	.020	.539	.814	.	.562	.551	.007
		N	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9
	BMP2_V7	Correlation Coefficient	.241	.059	.048	.109	.290	.415	.278	-.018	-.173	.553	.141	.237	.351	.123	-.178	-.913**	.224	1.000	.334	.246
		Sig. (2-tailed)	.533	.880	.902	.780	.449	.267	.469	.964	.656	.122	.717	.539	.354	.752	.646	.001	.562	.	.380	.524
		N	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9
	VEGF_V4	Correlation Coefficient	-.365	-.596	-.091	.207	.283	.351	.017	.218	.090	.817**	.393	.500	-.017	.577	-.330	-.087	.231	.334	1.000	.283
		Sig. (2-tailed)	.334	.090	.815	.593	.460	.354	.965	.574	.819	.007	.295	.170	.966	.104	.386	.825	.551	.380	.	.460
		N	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9
	VEGF_V7	Correlation Coefficient	-.365	-.186	-.548	.518	-.483	.753*	-.375	.343	.398	.233	.929**	-.017	.583	.494	.062	.000	.816**	.246	.283	1.000
		Sig. (2-tailed)	.334	.631	.127	.154	.187	.019	.321	.366	.288	.546	.000	.966	.099	.177	.873	1.000	.007	.524	.460	.
		N	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

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