EXPRESSION PROFILES OF TGF-β AND TLR PATHWAYS IN *PORPHYROMONAS GINGIVALIS* AND *PREVOTELLA INTERMEDIA* CHALLENGED OSTEOBLASTS

by

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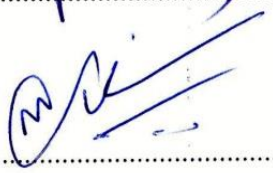
Asst. Prof. Mayur Tamhane ..... 

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To my beloved family…

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ABSTRACT

EXPRESSION PROFILES OF TGF-β AND TLR PATHWAYS IN *P. GINGIVALIS* AND *P. INTERMEDIA* CHALLENGED OSTEOBLASTS

Although many microorganisms have been related to loss of bone in dental implant failures, it is still unknown as to which of these pathogens play a role in bone loss following dental implant surgery. As the long term survival of dental implants depends in part, on the control of bacterial infection, the presence of these oral pathogens in and around the failing implant site activates an immune response in the human body. Interaction between bacteria and host cells invariably results in the release of one or more cytokines. This study is focused on determination of the effects of oral pathogens known to be isolated from around the failing implant site on viability, matrix mineralization and the expression pattern of proinflammatory mediators and other cytokine receptors of bone cells For this purpose osteoblasts were challenged by *Porphyromonas gingivalis* and *Prevotella* intermedia, one of the most predominantly isolated bacteria, from dental implant failures. Results showed that these challenges reducing viability and matrix mineralization of osteoblasts. These pathogens affect osteoblast viability in a dose dependent manner. In addition QRT-PCR results of TGF-β and TLR showed that these pathogens affect the expressions of TGF-β pathway cytokine and receptors in a different direction however TLR pathway genes were affected in the same direction. BMPER and BMPR1B are the receptors of TGF-β pathway that were found to be upregulated by *P.g* and *P.i* challenge. In the TLR pathway highly expressed genes were found to be IL8 and NFRKB. These findings indicate that the presence of both *P. gingivalis* and *P.* intermedia may inhibit osseointegration and induce bone resorption. In the light of this knowledge we can conclude that *P.gingivalis* and *P.intermedia* are both risk factors for dental implant failure.

ÖZET

TGF-β VE TLR YOLAKLARININ *PORPHYROMONAS GINGIVALIS* VE *PREVOTELLA INTERMEDIA* İLE ETKİLEŞTİRİLMİŞ OSTEOBLASTLARDAKİ EXPRESYON PROFİLİ

Diş implant başarısızlıklarında görülen kemik kaybının farklı mikroorganizmalar tarafından kaynaklandığı bilinmesine rağmen, bu patojenlerden hangisinin diş implant cerrahisini takiben kemik kaybına yol açtığı halen bilinmemektedir. Diş implantının uzun süreli kullanılabilmesi; kısmi olarak bakteriyel enfeksiyonun kontrolüne bağlı olup, başarısız diş implantı çevresinde bulunan oral patojenlerin varlığı, vücutta bağışıklık/immün sistem cevabını aktive eder. Bakteri ve konakçı hücreler arasındaki ilişki sonucunda daima bir veya birden fazla sitokin konakçı hücrelerden salınır. Çalışmamız kemik hücrelerinde, başarısız diş implant bölgelerinden izole edilen ağız patojenlerinin, osteoblast canlılğı, mineralizasyonu ve enflamatuar cevabı tetikleyici işaretleyicilerinin ve diğer sitokin reseptörlerinin ekspresyon mekanizmalarının tanımlanmasına odaklanmıştır. Bu amaçla başarısız implant bölgelerinde sıkça rastlanan bakterilerden *Porphyromonas gingivalis* ve *Prevotella intermedia* ile insan osteoblast hücreleriyle etkileştirilmiştir. Buna göre *P.gingivalis* ve *P.intermedia* patojenlerinin osteoblast hücre canlılığına ve mineralizasyonuna zarar verdiği görülmüştür. Enfeksiyon derecesi arttıkç bu ptojenlerin hücre calılığını zararının da arttığı görülmüştür. QRt-PZR deneylerinde TGF- β yolağındaki sitokin ve reseptörlerin bu iki bakteri varlığında farklı yönde ekspresyon gösterdiği ancak TLR yolağında benzer ekspresyonlar gözlendiği saptanmıştır. TGF- β yolağında yüksek oranda ekspresyonu artan genler BMER ve BMPR1B olmuştur. TLR yolağında ise en cok IL8 ve NFRKB'nin anlatımı arttmıştır. Bu sonuçlar *P.gingivalis* ve *P.intermedia* patojenlerininin varlığında implant bölgesinde osseointegrasyonun engellenebileceği ve kemik emilimine teşvik edilebileceğini göstermektedir. Buna bağlı olarak *P.gingivalis* ve *P.intermedia* patojenlerinin başarısız diş implantlarında bir risk faktörü olarak kabul edilebileceği bu çalışmayla gösterilmiştir.

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LIST OF SYMBOLS / ABBREVIATIONS

ACTB	Actin, beta
ACVR2A	Activin A receptor, type I
ARA	Arabinose
ATCC	American Type Culture Collection
BHIA	Brain heart infusion agar
BHIB	Brain heart infusion broth
BGLAP	Bone gamma-carboxyglutamate (gla) protein
BMPER	BMP binding endothelial regulator
BMPR1A	Bone morphogenetic protein receptor, type IA
BMPR1B	Bone morphogenetic protein receptor, type IB
BMPR2	Bone morphogenetic protein receptor, type II
BMP	Bone morphogenetic proteins
CAT	Catalase
CBF	Core binding factor
CEL	Cellobiose
COL1A1	Collagen, type I, alpha 1
COL1A2	Collagen, type I, alpha 2
COL3A1	Collagen, type III, alpha 1
DMEM F-12	Dulbecco's modified Eagle's nutrient mixture F-12
ECM	Extracellular matrix proteins
ERK	Extracellular-regulated protein kinase
ESC	Esculin
FAA	Fastidious anaerobe agar
FAB	Fastidious anaerobe broth
FBS	Fetal bovine serum
GCF	Gingival crevicular fluid
GDF	Growth and differentiation factors
GEL	Gelatin
GEN	Gentamycin

GLU	Glucose
GLY	Glycerol
h.FOB	Human fetal osteoblasts
HKG	House keeping gene
HPRT1	Hypoxanthine phosphoribosyltransferase 1
IND	Indole
IL12A	Interleukin 12A
IL1A	Interleukin 1A
IL1B	Interleukin 1B
IL6	Interleukin 6
IL8	Interleukin 8
IRAK1	Interleukin-1 receptor-associated kinase 1
IRAK2	Interleukin-1 receptor-associated kinase 2
IRF3	Interferon regulatory factor-3
JNK	Jun N-terminal kinase
LAC	Lactose
LTBP1	Latent transforming growth factor beta binding protein 1
LTBP2	Latent transforming growth factor beta binding protein 2
LTBP4	Latent transforming growth factor beta binding protein 4
LPS	Lipopolysaccharide
MAN	Mannitol
MAL	Maltose
MAP2K3	Mitogen-activated protein kinase kinase 3
MAP2K4	Mitogen-activated protein kinase kinase 4
MAP3K1	Mitogen-activated protein kinase kinase kinase 1
MAP4K4	Mitogen-activated protein kinase kinase kinase kinase 4
MET	Metronidazole
MLZ	Melezilose
MNE	Mannose
MOI	Multiplicity of infection
NFKB1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1
NFKBIA	NFKB inhibitor, alpha
NFRKB	Nuclear factor related to kappaB binding protein

OD	Optical density
PBS	Phosphate buffer saline
RAF	Raffinose
RHA	Rhamnose
RPL13A	Ribosomal protein L13a
RUNX1	Runt-related transcription factor 1
SAC	Saccharose
SAL	Salicin
SEM	Scanning electron microscopy images
SERPINE1	Serpin peptidase inhibitor, clade E, member 1
SMAD1	SMAD family member 1
SMAD2	SMAD family member 2
SMAD3	SMAD family member 3
SMAD4	SMAD family member 4
SMAD5	SMAD family member 5
SMURF1	SMAD specific E3 ubiquitin protein ligase 1
STAT1	Signal transducer and activator of transcription 1, 91kDa
SOR	Sorbitol
TGF-β	Transforming growth factors beta
TLR	Toll-like receptors
TRE	Trehalose
TGFB1	Transforming growth factor, beta-induced, 68kDa
TGFBR1	Transforming growth factor, beta receptor 1
TGFBR2	Transforming growth factor, beta receptor 2
TGFBR3	Transforming growth factor, beta receptor 3
TGIF1	TGFB-induced factor homeobox 1
URE	Urease
XYL	Xylose

1. INTRODUCTION

1.1. ORAL CAVITY

The teeth are highly mineralized appendages of the mouth and they are necessary for tearing, scraping and chewing the ingested food [1]. Teeth consist of mainly two types of tissues; hard tissue and soft tissue. Hard tissue contains an extremely hard material called "enamel", a layer of bony material, "dentine" and a bone-like structure called "cementum" (Figure 1.1). Soft tissue is formed by dental pulp and is divided into crown and root. The teeth are surrounded and supported by the periodontium [2].

The periodontium supporting tissues of the teeth is comprised of the gingiva, periodontal ligament, cementum and alveolar bone [3]. All these tissues function as a unit.

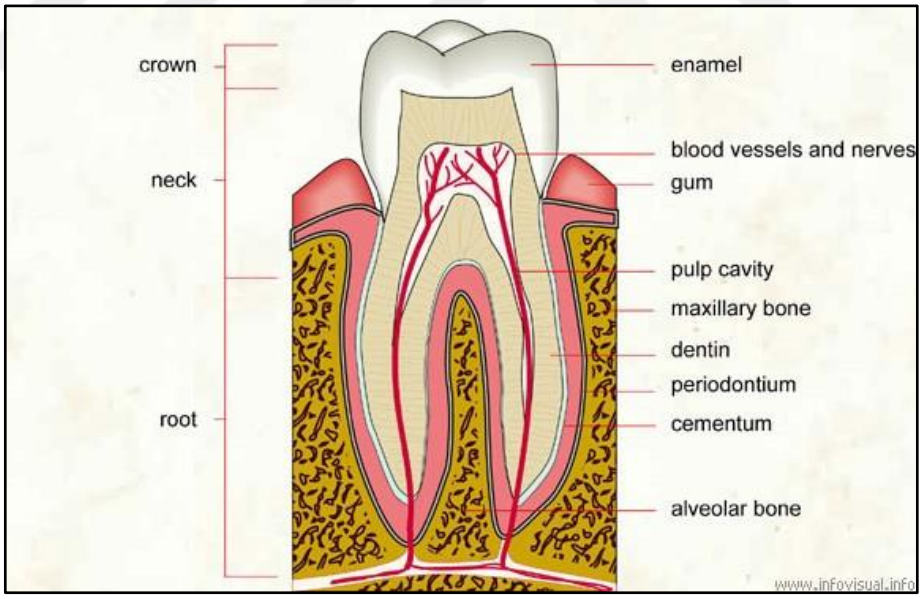

Figure 1. 1. Section of a molar tooth [3]

The gingiva is one portion of the oral mucosa and is the most peripheral component of the periodontium, it is highly vascular and receives blood supply. It helps in the support of the teeth, and protects the alveolar bone and periodontal ligaments from bacterial invasion [4]. In a healthy periodontium it can be seen stippled with pigmentation, in pale pink or coral

pink in the Caucasian. Change in size and colour of the gingiva is evidence of gingival disease [5].

The cementum is a hard bone like avascular tissue that surrounds the root of the tooth. Because of this function of cementum, it is also classified as a part of the periodontium although it is an extension of the tooth. Cementum is a highly mineralized tissue that comprises of hydroxypatite almost 50% of the inorganic and orgnanic matrix. Hydroxyapatite constitutes up almost 50% of the inorganic matrix and types I and types III collagens constitutes up to 90% of the organic matrix [6].

The peridontal ligament is a soft, specialized connective tissue placed between the cementum covering the bone forming socket wall (alveo-dental ligament) and the root of the tooth. It is a highly fibrous tissue which is largely constitutes of collagen types I and III [7].

Alveolar bone is a specialized type of bone which is designed to hold teeth. In humans, alveolar bone is found in the mandible, (lower part of the jaw), and the maxilla (the upper part of the jaw). Alveolar bone is especially thick and dense when compared to other types of bone so providing support for the teeth, along with attachment points for muscles [8].

This bone is also known as the alveolar process. The gums attach to the alveolar process, and the bone allows blood vessels to enter for the purpose of supplying blood to the teeth. Damage to the alveolar bone can have serious consequences, including the risk of loss of teeth and septicemia if the damage is caused by an infection [9].

Bone is composed of an extracellular matrix and cells. The extracellular matrix of bone is comprised of two thirds inorganic material and one third organic material. The inorganic material consists mainly of calcium and phosphate mineral and also hydroxyl, carbonates, citrates and some trace elements such as fluoride [10]. These minerals are organized into hydroxyapatite crystal-like structures. The organic material in bone is made up of mostly type 1 collagen (>90%), with much smaller amounts of non-coIlagenous proteins (including osteopontin, osteocalcin, osteonectin, phosphoproteins and bone morphogenetic

proteins) and proteoglycans [11]. Some of these noncollagenous proteins are considered to be growth factors, involved in the regulation of bone cell metabolism. The major cell types of bone are osteoblasts and osteoclast (Figure 1.2).

Osteoblasts, (bone forming cells), are derived from a heterogeneous population of local precursor cells from the bone marrow [12]. While several aspects of the cellular origins of osteoblasts are not known, they are widely believed to be derived from stroma1 cells differentiating from early mesenchymal progenitors [12]. Fully differentiated osteoblasts are responsible for the production of bone matrix, and the regulation of its mineralization. They are cuboidal cells found lining bone osteoid and have the morphology of a typical protein-producing cell, ie. prominent golgi and well developed endoplasmic reticulum [13].

Osteoclasts, (bone resorbing cells), are also derived from precursor cells in bone marrow but these cells are haematopoietic in origin, are widely believed to be derived from the monocyte macrophage lineage as it is a common differentiation pathway with macrophages until the final differentiation steps [14].

A third major cell type found in bone tissue, is the osteocyte. These cells are mature osteoblast cells that have become surrounded by matrix during bone formation. They are the most abundant cell type within bone, involved in adaptation and remodelling of bone in response to mechanical stress. Osteocytes occupy spaces (lacunae) within bone in isolation, but maintain contact with other cells by means of cell processes connected via gap junctions. These not only permit communication between cells, but also exchange of nutrients and waste products [15].

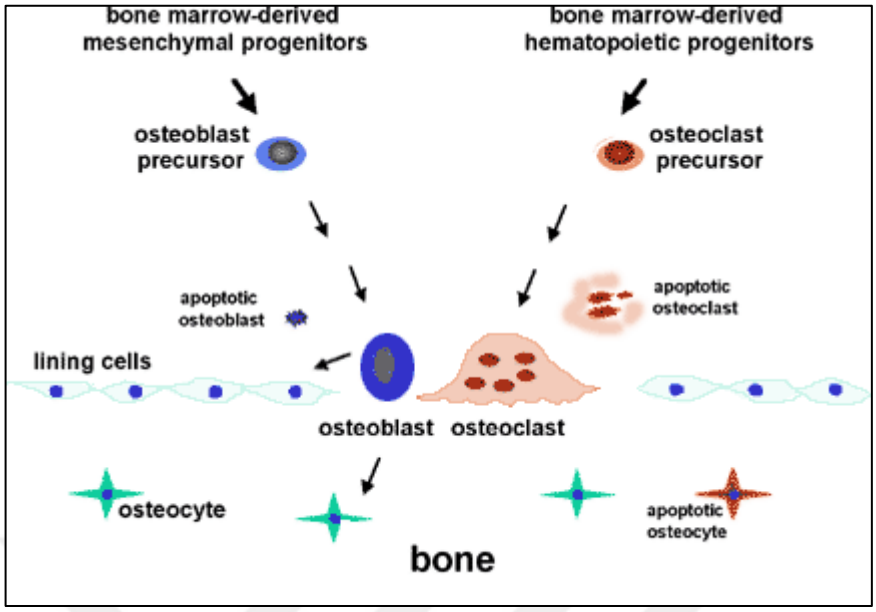

Figure 1. 2. The origin and types of bone cell [16]

1.2. ORAL DISEASE IN THE PERIODONTUM

Periodontal diseases are infections of the structures around the teeth and range from simple gum inflammation to serious disease that result in major damage to the soft tissue and bone that support the teeth [17]. In the worst cases, teeth are lost.

In poor oral hygiene gingivitis which is the mildest form of periodontal diseases occurs. It is defined as the early stage of gingival inflammation. Gingival inflammation increases the gingival crevicular fluid (GCF). The increase in GCF flow causes the microbiota to shift from a largely gram positive dominating community to Gram negative putative periodontal pathogens (e.g. *Fusobacteria, Porphyromonas, Prevotella species)* along with spirochaetes such as Treponema species [18]. At this stage bacteria in plaque build up, causing the gums to become inflamed and to easily bleed during tooth brushing (Fig 2.2.B). There are four stages of gingivitis which leads to periodontitis. Although the gums may be irritated, the teeth are still firmly planted in their sockets. Gingivitis is the reversible stage of periodontal disease with daily tooth brushing and flossing. However if gingivitis is left untreated, it can advance to periodontitis.

Periodontitis (Figure 1.3) is chronic inflammation of the periodontal membrane which results in irreversible loss of connective tissue attachment and alveolar bone [19]. In a person with periodontitis, the inner layer of the gum and bone pull away from the teeth and form pockets. It has been reported that 30 per cent of adult population have periodontitis with a periodontal pocket depth ≥ 4 mm and 5-15 per cent of these patients have advanced periodontitis (pocket depth ≥ 6mm)[20].

These small spaces between teeth and gums collect debris and can become infected. The body's immune system fights the bacteria as the plaque spreads and grows below the gum line [21]. Toxins or poisons produced by the bacteria in plaque can induce the host immune response which can lead to the production of pro-inflammatory cytokines. This process can lead to the breakdown of the bone and connective tissue that holds teeth in place. As the disease progresses, the pockets deepen and more gum tissue and bone are destroyed. When this happens, teeth are no longer anchored in place, they become loose, and tooth loss can occur. At this stage, the most preferred treatment is dental implants [22].

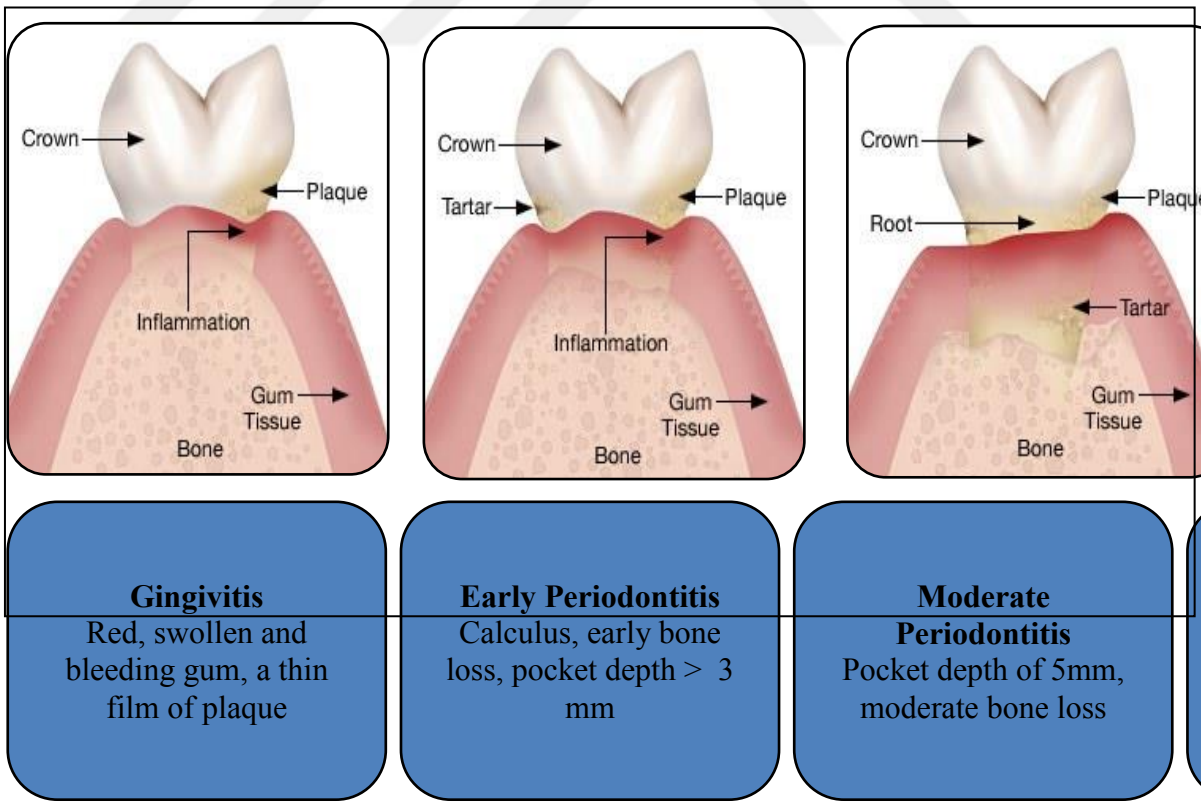

Figure 1. 3. Initiation and progress of Periodontitis [23]

1.3. DENTAL IMPLANTS AND OSSEOINTEGRATION

Dental implants are inert, alloplastic materials fixed in the maxilla and/or mandible for the management of tooth loss and to aid replacement of lost orofacial structures as a result of trauma, neoplasia and congenital defects (Figure 1.4). Dental implants were first introduced by Branemark in 1971. Since then, 80 different manufacturers have produced 220 implant brands and approximately one million endosseous dental implants are placed annually [24].

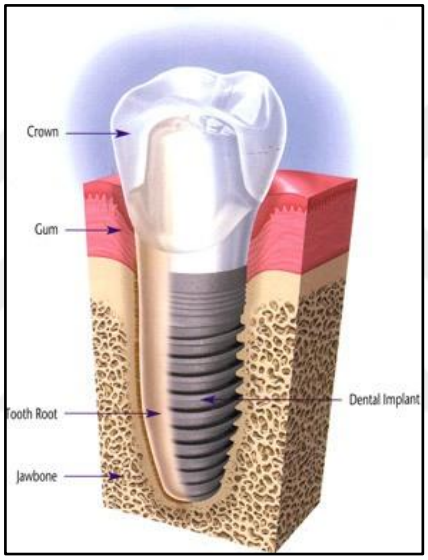

Figure 1. 4. Anatomy of a dental implant embedded into maxilla [25]

The term of osseointegration was firstly introduced by Branemark in 1971 with the discovery that a piece of titanium embedded in rabbit bone became firmly anchored. His observations released that soft tissue formed an attachment between titatium and bone as soon as the titanium implant was embedded in the bone. He called this process osseous integration now known as osseointegration [26].

The process of osseointegration starts as soon as the endosseous implant fixtures are surgically inserted into the bone. At first a water molecule layer is formed around the implant area within nanoseconds. In the second stage, extracellular matrix covers the implant surface within 30s to hours following implantation [27]. Blood and tissue fluids

bring proteins to the wound site and these proteins and other molecules easily adsorb on the implant surface with the help of the water layer. In the third stage, interaction of cells with implant surface via the adsorbed protein layer takes place, initiating cellular adhesion, migration and differentiation, which occur within a few hours to several days. This stage is vastly regulated by extracellular matrix proteins (ECM), cell surface-bound and cytoskeletal proteins, chemical ions released by the surface [28]. ECM has the information that is interpreted by cells via adhesion structures and influences cell shape, cytoskeletal organization, cell motility and polarity, gene expression, proliferation and survival. ECM is composed of collogenous (90%) and non collagenous proteins such as type I collagen, fibronectin, thrombospondin, osteonectin, osteopontin, osteoadherin, and bone sialoprotein proteoglycans including biglycans [29].

By the end of one week the adherence of osseous matrix to implant surface is easily observed, simultaneously ECM gets anchored in the cavities on the surface. Bone to implant contact ratio reaches 35.8 ± 7.2% [30]. By day 16 a mixture of mineralized tissue, osteoid and dense matrix integrates extensively and covers the surface of implant surface [31]. By day 28, there is close contact between bone and the entire length of the implant surface and also at the neck. Furthermore collagen fibers and osteoblast form an enormous tissue layer adjacent to implant, collagen fibers move themselves parallel to the implant surface. Cells, ECM proteins and mineralized bone tissue is observed in direct contact with implant and the propotion of bone to implant contact reaches 46.3 ± 17.7% [32].

At the end of 12 weeks, a close contact of mature lamellar bone with titanium surface occurs with newly developed bone. All these process are the normal process of implantation. At the end of 3 months if the implant meets the criterias below, it is called a successful implant [31].

Despite high success rates, implant fixture failure may occur and is defined as ‘the inadequacy of the host tissue to establish or maintain osseointegration’ [32].

Succes criterias of implants include;

- absence of persistent signs/symptoms such as pain, infection, neuropathies, parathesias, and violation of vital structures;
- implant immobility;
- no continuous peri-implant radiolucency;
- negligible progressive bone loss (less than 0.2 mm annually) after physiologic remodeling during the first year of function
- patient/dentist satisfaction with the implant supported restoration [25]

If the implant does not meet the criteria above, implants can be described as failing or failed [33]. A failing implant demonstrates a progressive loss of supporting bone but is clinically immobile, whereas a failed implant is clinically mobile. Signs of a failing dental implant are detected both clinically and radiographically with the diagnosis made in a similar way to periodontitis (Figure 1.5). This involves measuring clinical parameters including peri-implant loss of gingival attachment, bleeding on probing, plaque/gingivitis indices, suppuration and mobility [32].

Contamination of the implanted are reported in 5-8% of cases within selected implant systems and is considered ‘an inflammatory process affecting the tissues around an osseointegrated implant in function, resulting in loss of supporting bone [34]. It has also been described as ‘‘a site-specific infection yielding many features in common with chronic adult periodontitis’’ or ‘‘an inflammatory, bacterial driven destruction of the implant-supporting apparatus’ [35].

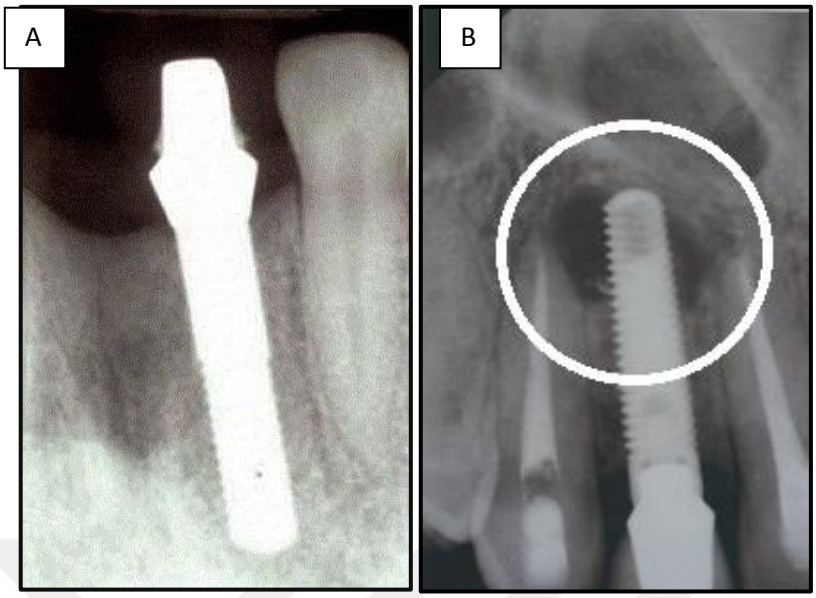

Figure 1. 5. A. Succesful implant B. Lack of osseointegration [36]

1.3.1. Microorganisms Involved in Failed Implants

Contamination of an implant is considered as one of main factor contributing to the failure of dental implants. Since now, no single micro-organism has been closely associated with colonisation or infection of any implant system [37]. Failing dental implants are associated with a microbial flora traditionally associated with periodontitis. Thus, a shift from a predominately gram-positive non-motile, aerobic and facultative anaerobic composition towards a flora with a greater proportion of gram-negative, motile, anaerobic bacteria is observed. *P. gingivalis* and *P. intermedia*, gram negative anaerbose, are mostly isolated from failing implant site (Table 1.1) [37]. These are member of black pigmented anaerobes that are known to produce endotoxins such as collagenase, hyaluronidase and chondroitin sulphates. These toxins are shown to initiate an acute inflammatory response in addition to producing bone destruction [38]. Therefore identifying the interactions between host tissue and model anaerobes is an essential way for both developments of prevention and treatment methods.

Table 1. 1. Most prevalent microbes associated with failing/failed implants[38]

Prevotella intermedia
P. nigrescens
Actinobacillus actinomycetemcomitans
Staphylococci, coliforms, Candida spp.
Bacteroides forsythus
Spirochetes
Fusobacterium spp.
Peptostreptococcus micros
Porphyromonas gingivalis
Bacteroides spp.
Staphylococcus spp.
P. nicrescens, P. micros.
Fusobacterium nucleatum
Actinobacillus actinomycetemcomitans
Capnocytophaga spp.
Eikenella corrodens
Porphyromonas gingivalis
Campylobacter rectus
Treponema denticola
Tannerella forsythia
Steptococcus anginosus (milleri) group
Enterococcus spp.
Yeast spp.

1.4. THE BIOLOGICAL ROLE OF CYTOKINES AND RECEPTORS

Cytokines are small cell-signaling protein molecules that are secreted by numerous cells of the immune system, especially by monocytes and T lymphocytes and also many cells in such as endothelial cells and fibroblasts. They are extremely potent and act at very low concentrations. They are very specific, and act through specific receptors of the target cell membrane [39]. Receptors are protein molecules, embedded in either the plasma membrane (cell surface receptors) or the cytoplasm or nucleus (nuclear receptors) of a cell, to which one or more specific kinds of signaling molecules may attach. Numerous receptor types are found within a typical cell and each type is linked to a specific biochemical pathway [40]. Furthermore, each type of receptor recognizes and binds to certain ligand shapes; hence, the selective binding of specific a ligand to its receptor activates or inhibits a specific biochemical pathway. In a bacterial infection many types of pathways are induced by bacterial components [39]. This study focused on two major pathways which are Transforming Growth Factors (TGF-Beta) super family and Toll-like receptors (TLRs) because of their relations to inflammation and bone regeneration.

1.4.1. TGF-β Pathway

TGF- β super family comprises a large and diverse group of polypeptide morphogens including the prototype of the family–the TGF- β themselves as well as the Bone Morphogenetic Proteins (BMPs), and the Growth and Differentiation Factors (GDFs) (Figure 1.6). The members of the TGF- β family are expressed in distinct temporal and tissue-specific patterns and therefore play an important role in the development, homeostasis and repair of most tissues in organisms [41]. All immune cell lineages, including B-Cell, T-Cell and dendritic cells as well as macrophages secrete TGF- β, which negatively regulates their proliferation, differentiation and activation by other cytokines. Thus, TGF- β is a potent immunosuppressor and perturbation of TGF- β signaling is linked to autoimmunity, inflammation and cancer [42]. TGF- β are possibly the most pleiotropic secreted proteins functioning as morphogens mediating several physiological processes including hematopoiesis, regulation of hormone secretion, in immune response, angiogenesis, tissue morphogenesis and regeneration, and bone induction and modulation [43].

TGF-β receptor signaling also helps control the process by which cells mature to carry out special functions (differentiation), and plays a role in the formation of the extracellular matrix [41].

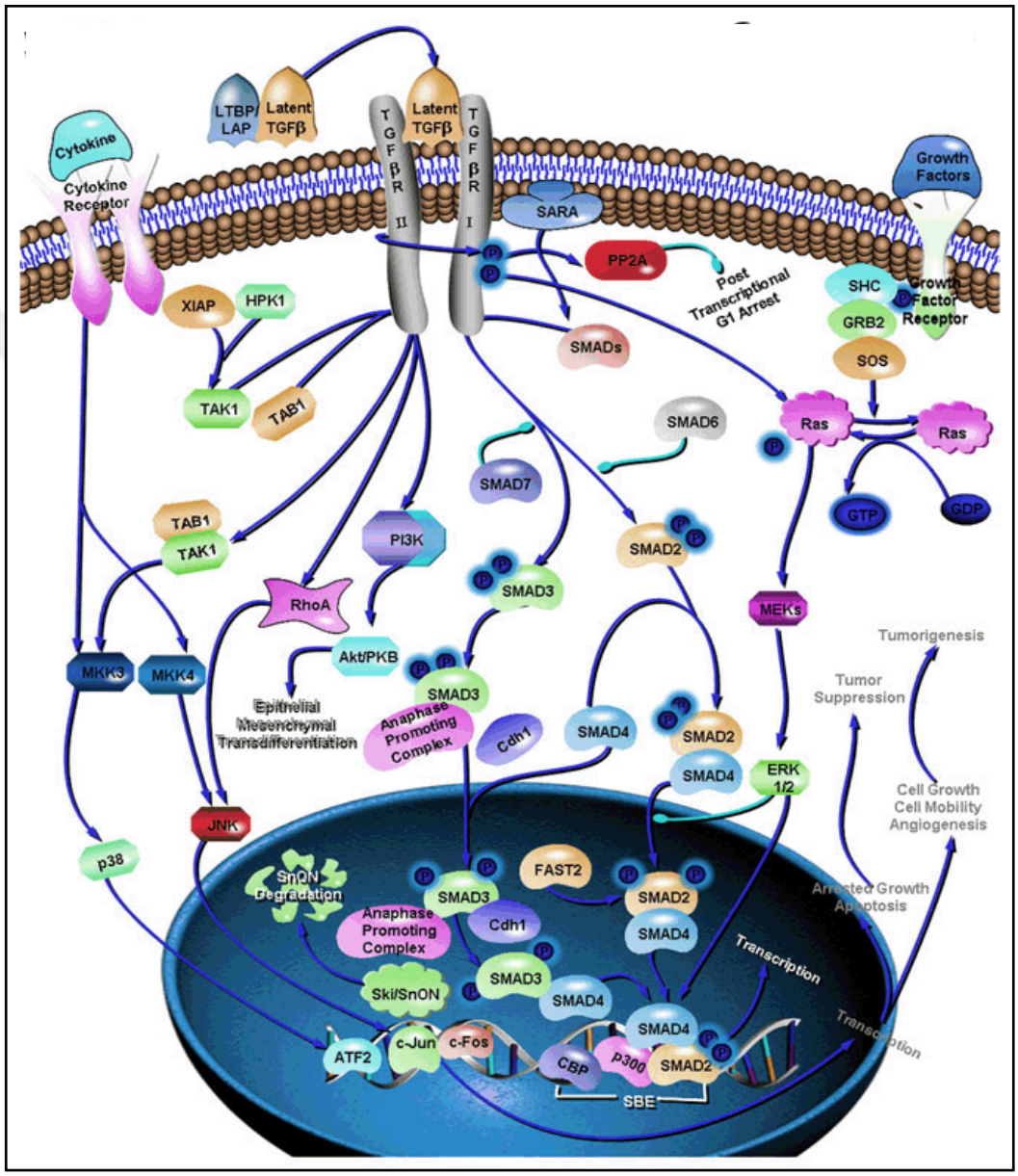

Figure 1. 6. Genes and signaling pathway of TGF-β [44]

In this study Activin receptor type-2A (ACVR2A), BMP binding endothelial regulator (BMPER), BMP receptors type IA (BMPR1A), BMP receptors type IB (BMRP1B), BMP receptors type 2 (BMPR2), extracelular matrix proteins including; Bone gamma-

carboxyglutamic acid-containing protein (BGLAP), COL1A1, COL1A2, COL3A1, Runt-related transcription factor 1 (RUNX1), SERPINE1, SMAD1, SMAD2 SMAD3, SMAD4, SMAD5, SMURF1, STAT1, Transforming growth factor beta 1 (TGF-β1), transforming growth factor, beta receptor I (TGFBR1), transforming growth factor, beta receptor 2 (TGFBR2), transforming growth factor, beta receptor 3 (TGFBR3), TGF-β -induced factor homeobox 1 (TGIF1), LTBP1, LTBP2, and LTBP4 are studied. Their known functions are listed in Table 1.2.

Activin receptor type-2A, dimeric growth and differentiation factor transmembrane proteins, composed of a ligand-binding extracellular domain with cysteine-rich region, a transmembrane domain, and a cytoplasmic domain with predicted serine/threonine specificity [G1]. BMP ligands are known to signal via ACVR2A to activate SMADs 1, 5, and 8, as well as members of the mitogen-activated protein kinase family [G1]. A previous study showed soluble activin receptor type IIA fusion protein (ActRIIA.muFc) stimulates osteoblastogenesis, promotes bone formation and increases bone mass in vivo [45].

BMP receptors are a family of transmembrane serine/threonine kinases, are closely related to the activin receptors, ACVR1 and ACVR2. The BMPR1A receptor protein and its ligands are involved in transmitting chemical signals from the cell membrane to the nucleus.BMP Type II receptors phosphorylate and activate type I receptors which autophosphorylate, then bind and activate SMAD transcriptional regulators [46]. The BMPR2 gene belongs to a family of genes originally identified for its role in regulating the growth and maturation (differentiation) of bone and cartilage [47]. Recently, researchers have found that this gene family plays a broader role in regulating the growth and differentiation of numerous types of cells. BMPER gene encodes a secreted protein that interacts with, and inhibits BMP function. It has been shown to inhibit BMP2- and BMP4-dependent osteoblast differentiation and BMP-dependent differentiation of the chondrogenic cells. Mutations in this gene are associated with a lethal skeletal disorder, diaphanospondylodysostosis [48]. Collagens are a family of proteins that strengthen and support many tissues in the body, including cartilage, bone, tendon, skin, and the white part of the eye (the sclera). Type I collagen is the most abundant form of collagen in the human body. The COL1A1 gene produces a component of type I collagen called the pro-α1(I) chain [49]. The COL1A2 gene produces a component of type I collagen called the

pro-α2(I) chain. The COL3A1 gene produces the components of type III collagen, called pro-alpha1(III) chains [49]. *P. gingivalis* LPS can activate osteoclasts directly and causes the release of prostaglandin E2 and of the cytokines IL-1b and TNF-a from macrophages, monocytes, and fibroblasts. These compounds are potent local mediators of bone resorption and, moreover, can inhibit collagen synthesis by osteoblasts and induce the production of host metalloproteases that destroy connective tissue and bone [50]. BGLAP also known as osteocalcin is a noncollagenous protein found in bone and dentin. The expreesion of osteocalcin was shown to suppressed by *P.g* LPS in rat calvarial cells [51].

RUNX1 also called as Core binding factor (CBF) is a heterodimeric transcription factor that binds to the core element of many enhancers and promoters [52]. It is a critical osteoblast differentiation factor which may be inhibited by pro-inflammatory cytokines, such as IL-1β and TNF-α. The bacterial *P. gingivalis* LPS may result in activation of IL-1β and TNF-α as mention and in turn result in in inhibition of, RUNX2 so bone coupling [53].

Smad proteins play central roles in intracellular signaling by members of the TGF-β superfamily. BMP type I receptor phosphorylates a set of intracellular substrate signaling proteins, Smads. Smad proteins are classified into three subgroups, i.e., receptor-regulated Smads, a common-partner Smad and inhibitory Smads. The phosphorylated Smad proteins form complexes with the Co-Smad, Smad4, translocate into the nucleus, bind to the regulatory elements of target genes, and regulate their transcription [54]. Smads are critically involved in osteoblast differentiation. Smad1 and Smad5, but not Smad8, are activated by BMP-6 and BMP-7, whereas Smad1, Smad5 and Smad8 are all activated by BMP-2. Non-Smad signaling mediated by several kinases, including mitogen-activated protein (MAP) kinases p38, ERK and Jun N-terminal kinase (JNK), and phosphoinositol-3 (PI3) kinase, is also activated by BMP receptors and regulates cell differentiation [55].

Latency-associated proteins and latent TGF-β binding proteins regulates the secretion and activation of TGF-β. The product of this gene targets latent complexes of transforming growth factor beta to the extracellular matrix, where the latent cytokine is subsequently activated by several different mechanisms [56].

TGFβ-1 is particularly abundant in tissues that make up the skeleton, where it helps regulate bone growth, and in the intricate lattice that forms in the spaces between cells (the extracellular matrix). Within cells, this protein is turned off (inactive) until it receives a chemical signal to become active. TGF-β receptor signaling helps control the process by which cells mature to carry out special functions (differentiation), and plays a role in the formation of the extracellular matrix [57]. TGF-β binds to its receptor, TGF-β receptor 2 (TGFBR2), which forms a heteomeric complex with TGF-β receptor 1 (TGFBR1). This leads to the transduction of the TGF-β signal from the cell surface to the cytoplasm [56].

There has been no study to show changes in the expressions of ACVR2A, BGLAP, BMPER, BMPR1A, BMRP1B, BMPR2, SMAD1, SMAD2 SMAD3, SMAD4, SMAD5, SMURF1, STAT1, LTBP1, LTBP2 and LTBP4 genes following *P.g* or *P.i* challenge to human fetal osteoblast.

Table 1. 2. Genes studied with their known functions in TGF-β pathway

Gene Symbol	Known function
ACVR2A	Activins are dimeric growth and differentiation factors which belong to the transforming growth factor-beta (TGF-beta) superfamily of structurally related signaling proteins [58].
BGLAP	BGLAP also called as Osteocalcin is a small, noncollagenous and highly conserved and secreted protein that is associated with the mineralized matrix of bone [48].
BMPER	This gene encodes a secreted protein that interacts with, and inhibits bone morphogenetic protein (BMP) function. It has been shown to inhibit BMP2- and BMP4-dependent osteoblast differentiation and BMP-dependent differentiation of the chondrogenic cells. Mutations in this gene are associated with a lethal skeletal disorder, diaphanospondylodysostosis[48].
BMPR1A	The BMPR1A receptor protein and its ligands are involved in transmitting chemical signals from the cell membrane to the nucleus [48].
BMPR1B	Type II receptors phosphorylate and activate type I receptors which autophosphorylate, then bind and activate SMAD transcriptional regulators. Receptor for BMP7/OP-1 and GDF5[54].
BMPR2	The BMPR2 gene belongs to a family of genes originally identified for its role in regulating the growth and maturation (differentiation) of bone and cartilage. Recently, researchers have found that this gene family plays a broader role in regulating the growth and differentiation of numerous types of cells [55].
COL1A1	Collagens are a family of proteins that strengthen and support many tissues in the body, including cartilage, bone, tendon, skin, and the white part of the Type I collagen is the most abundant form of collagen in the human body. The COL1A1 gene produces a component of type I collagen called the pro-α1(I) chain [49].

Table 1. 3. Genes studied with their known functions in TGF-β pathway (Continued)

COL1A2	The COL1A2 gene produces a component of type I collagen called the pro-α2(I) chain [49].
COL3A1	Type III collagen is found in tissues such as the skin, lungs, intestinal walls, and the walls of blood vessels. The *COL3A1* gene produces the components of type III collagen, called pro-alpha1(III) chains [49].
RUNX1	RUNX1 also called as Core binding factor (CBF) is a heterodimeric transcription factor that binds to the core element of many enhancers and promoters. The protein encoded by this gene represents the alpha subunit of CBF and is thought to be involved in the development of normal hematopoiesis
SERPINE1	This gene is an important component of the coagulation system that down-regulates fibrinolysis in the circulation [59].
SMAD1	SMAD proteins are signal transducers and transcriptional modulators that mediate multiple signaling pathways. This protein mediates the signals of the bone morphogenetic proteins (BMPs) [54].
SMAD2	This protein mediates the signal of the transforming growth factor (TGF)-beta, and thus regulates multiple cellular processes, such as cell proliferation, apoptosis, and differentiation. This protein is recruited to the TGF-beta receptors through its interaction with the SMAD anchor for receptor activation (SARA) protein[54].
SMAD3	SMAD3 gene have been found to cause Loeys-Dietz syndrome type III. This a disorder that affects the connective tissue, which gives structure and support to blood vessels, the skeleton, and many other parts of the body [60].
SMAD4	Component of the heterotrimeric SMAD2/SMAD3-SMAD4 complex that forms in the nucleus and is required for the TGF-mediated signaling [61].
SMURF1 & SMAD5	3 ubiquitin-protein ligase SMURF that acts as a negative regulator of BMP signaling pathway. Mediates ubiquitination and degradation of SMAD1 and SMAD5, 2 receptor-regulated SMADs specific for the BMP [62].

Table 1. 4. Genes studied with their known functions in TGF-β pathway (Continued)

STAT1	STAT (signal transducer and activator of transcription) proteins are latent cytoplasmic transcription factors that become activated by tyrosine phosphorylation in response to cytokine stimulation. Stat1 is essential for gene activation in response to interferon stimulation [63].
TGFB1	TGFβ-1 is particularly abundant in tissues that make up the skeleton, where it helps regulate bone growth, and in the intricate lattice that forms in the spaces between cells (the extracellular matrix). Within cells, this protein is turned off (inactive) until it receives a chemical signal to become active [45].
TGFBR1	TGF-β receptor type 1 is sometimes called a tumor suppressor. Tumor suppressors keep cells from growing and dividing too fast or in an uncontrolled way. TGF-β receptor signaling also helps control the process by which cells mature to carry out special functions (differentiation), and plays a role in the formation of the extracellular matrix [45].
TGIF1	The TGIF1 gene provides instructions for making a protein called TG-interacting factor. This protein is important for normal development of the front part of the brain (forebrain). TG-interacting factor is a transcription factor, which means that it regulates the activity of certain genes [64].
LTBP1	The secretion and activation of TGF-betas is regulated by their association with latency-associated proteins and with latent TGF-beta binding proteins. The product of this gene targets latent complexes of transforming growth factor beta to the extracellular matrix, where the latent cytokine is subsequently activated by several different mechanisms[65] .

1.4.2. TLR Pathway

TLRs (Toll-like receptors) are transmembrane proteins expressed by cells of the innate immune system, which recognize invading microbes and activate signaling pathways that launch immune and inflammatory responses to destroy the invaders. Toll receptors were first identified in Drosophila [66].

Activation of TLRs occurs after binding of the cognate ligand. Upon activation, TLRs activates two major signaling pathways. The core pathway activated by most TLRs leads to activation of the transcription factor NF-KappaB (Nuclear Factor-KappaB) and the MAPKs (Mitogen-Activated Protein Kinases) p38 and JNK (c-Jun Kinase) (Figure 1.7). The second pathway is activated by TLR3 (Toll-Like Receptor-3) and TLR4 (Toll-Like Receptor-4) and leads to activation of both NF-KappaB and another transcription factor IRF3 (Interferon Regulatory Factor-3), allowing for an additional set of genes to be induced, including anti-viral genes such as Interferon-Beta. In this way, TLRs can tailor the innate response to pathogens. TLRs that recognize nucleic acids signal from endosomes, whereas cell-surface TLRs sense lipids and proteins. Plasma membrane localized TLRs include TLR1, TLR2, TLR4, TLR5, TLR6 and TLR10. Newly discovered TLR11, TLR12 and TLR13 are also believed to be Plasma membrane localized, whereas endosomal TLRs include TLR3, TLR7, TLR8 and TLR9 [67].

In addition to the innate immune response, evidence implicates the involvement of the TLR family in a spectrum of systemic disorders following bacterial infections including Sepsis, Cardiac Ischemia, Peridontitis, and Cerebral palsy. The TLRs that control the onset of an acute inflammatory response are critical antecedents for the development of adaptive acquired immunity. Genetic and developmental variation in the expression of microbial pattern recognition receptors may affect the individual's predisposition to infections in childhood and may contribute to susceptibility to severe neonatal inflammatory diseases, allergies, and autoimmune diseases [67].

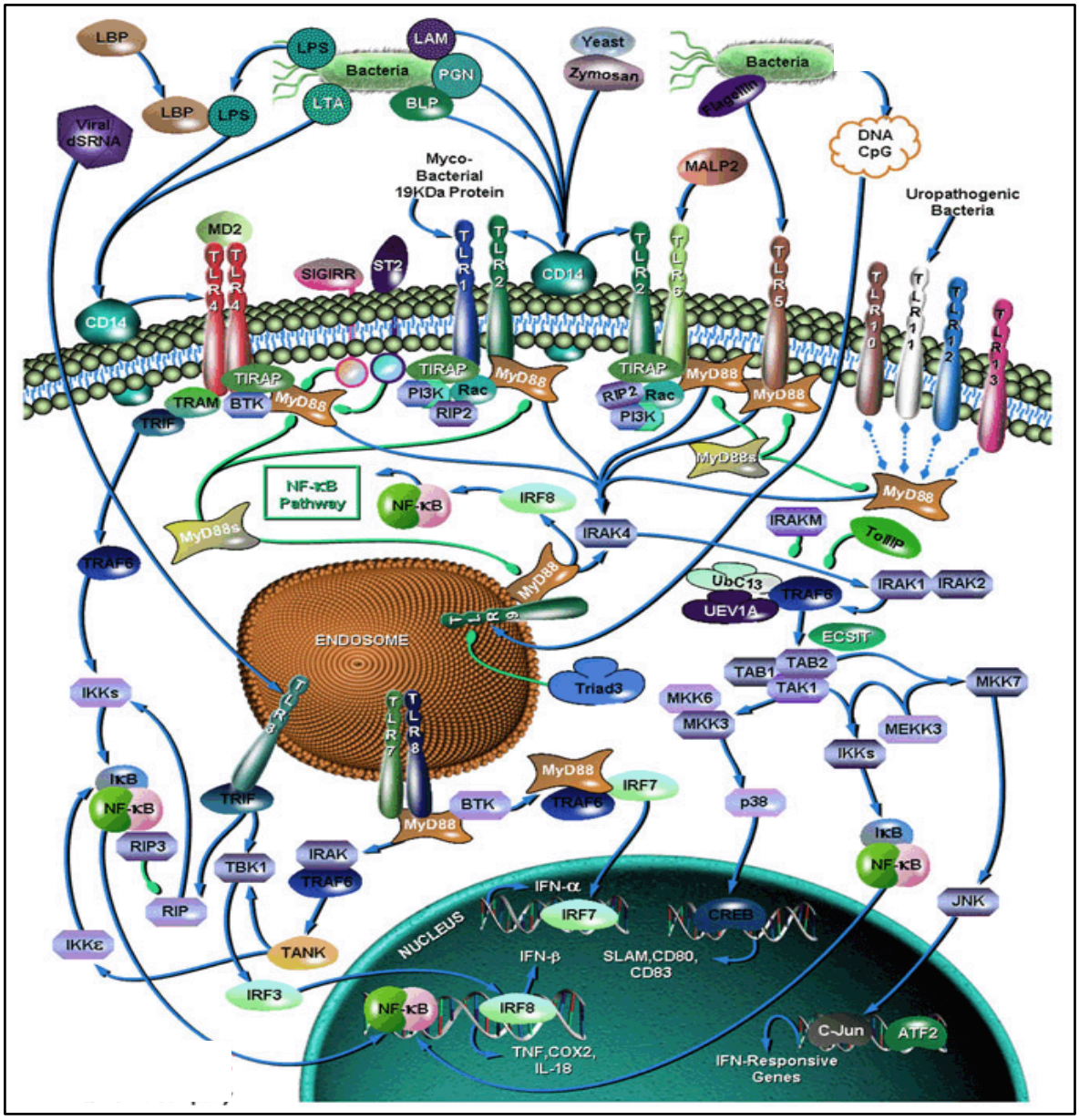

Figure 1. 7. Genes and signaling pathway of TLR [68]

In this study IL1A, IL1B, IL6, IL8, IRAK1, IRAK2, MAP2K3, MAP2K4, MAP3K1, MAP4K4,, NFKB1, NFKBIA, NFRKB, TLR3, TLR4, TLR5, TLR6, MYD88, TOLLIP, TICAM1,TICAM2 are studied. Their known functions are listed in Table 1.3.

IL-1 is a key inflammatory cytokine that mediates profound effects in virtually every organ system of the body [69]. It activates the rapidly acting innate immune response, is a potent stimulator of both hematopoesis and the adaptive immune system, and places the organism

in a state of readiness to deal with injury or infection. Because of this important role, IL-1 family is extensively studied by both *P.g* and *P.i* studies. *P. g* have been reported to induce expression of pro-inflammatory mediators such as IL-1b, IL-8, IL-6 from gingival epithelial or fibroblast cells [70]. IRAK-1 and IRAK-2 are protein kinases that mediate signaling by Toll/IL1/Plant R (TIR) domain-containing receptors including the IL-1, IL-18, and Toll-like receptors (TLRs). IRAK 1 is also partially responsible for IL1-induced upregulation of the transcription factor NF-kappa B. The interleukin-1 receptor-associated kinase 2 (IRAK-2) has been implicated in multiple TLR signaling pathways, because the overexpression of IRAK2 activates NF-kappa B [71]. In a recent study, peritoneal macrophages from IRAK2-deficient mice showed impaired production of cytokines in response to multiple TLR ligands. A study showed the upregulation of IRAK in *P.g* LPS treatment of gingival fibroblast [72].

MAPKs are involved in directing cellular responses to a diverse array of stimuli, such as mitogens,osmotic stress, heat shock and proinflammatory cytokines. They regulate proliferation,gene expression, differentiation, mitosis, cell survival, and apoptosis[73]. In a study with H9c2 myocardial cell , it was shown that *P. g* -secreted factors may initiate MAPK) and extracellular-regulated protein kinase (ERK) signal pathways and lead to myocardial cell hypertrophy and apoptosis [74].

Recent studies have implicated Toll-like receptors (TLR) in bacterial signalling, which trigger an intracellular cascade that leads to activation of the transcription factor nuclear factor-kB (NFkB). NFkB1 and NFkB2 are members of the Rel/NFkB family of transcription factors that regulate the expression of genes that participate in immune, apoptotic and oncogenic processes [58]. A study showed that *P. g* challenged bone marrow stromal cells activates Components of the NF-κB complex, NF-κB1, NF-κB2 [75].

Table 1. 5. Genes studied with their known functions in TLR pathway

Gene Symbol	Known function
IL1A	Interleukin-1 alpha is described as "pro-inflammatory" because it stimulates the activity of genes involved in inflammation and immunity. This protein plays a critical role in protecting the body from foreign invaders such as bacteria and viruses. It is also involved in bone resorption, the breakdown and removal of bone tissue that is no longer needed [69].
IL1B	This cytokine is produced by activated macrophages as a proprotein, which is proteolytically processed to its active form by caspase 1. This cytokine is an important mediator of the inflammatory response, and is involved in a variety of cellular activities, including cell proliferation, differentiation, and apoptosis [76].
IL6	The protein is primarily produced at sites of acute and chronic inflammation, where it is secreted into the serum and induces a transcriptional inflammatory response through interleukin 6 receptor alpha. The functioning of this gene is implicated in a wide variety of inflammation-associated disease states, including suspectibility to diabetes mellitus and systemic juvenile rheumatoid arthritis [77].
IL8	The protein encoded by this gene is a member of the CXC chemokine family. This chemokine is one of the major mediators of the inflammatory response. This chemokine is secreted by several cell types. It functions as a chemoattractant, and is also a potent angiogenic factor [78].
IRAK1& IRAK2	IRAK1 and IRAK2 are two putative serine/threonine kinases that become associated with the interleukin-1 receptor upon stimulation. This gene is partially responsible for IL1-induced upregulation of the transcription factor NF-kappa B [79].
MAP2K3& MAP2K4	This kinase is activated by mitogenic and environmental stress, and participates in the MAP kinase-mediated signaling cascade [80].

Table 1. 6. Genes studied with their known functions in TLR pathway (Continued)

MAP3K1	Activates the ERK and JNK kinase pathways by phosphorylation of MAP2K1 and MAP2K4. Activates CHUK and IKBKB, the central protein kinases of the NF-kappa-B pathway [81].
MAP4K4	Serine/threonine kinase that may play a role in the response to environmental stress and cytokines such as TNF-alpha. Appears to act upstream of the JUN N-terminal pathway. Phosphorylates SMAD1 on Thr-322 [81].
NFKB1	NF-kappaB (nuclear factor-kappa B) is a rapidly acting primary transcription factor found in all cell types. It is involved in cellular responses to stimuli such as cytokines and stress and plays a key role in regulating the immune response to infection. In unstimulated cells NF-kappaB dimers are sequestered inactively in the cytoplasm by a protein complex called inhibitor of kappa B (IkappaB) [82].
NFKBIA	Inhibits the activity of dimeric NF-kappa-B/REL complexes by trapping REL dimers in the cytoplasm through masking of their nuclear localization signals. On cellular stimulation by immune and proinflammatory responses, becomes phosphorylated promoting ubiquitination and degradation, enabling the dimeric RELA to tranlocate to the nucleus and activate transcription [83].
NFRKB	Putative regulatory component of the chromatin remodeling INO80 complex which is involved in transcriptional regulation, DNA replication and probably DNA repair [84].
TLR3	TLR3 is a nucleotide-sensing TLR which is activated by double-stranded RNA, a sign of viral infection. Acts via MYD88 and TRAF6, leading to NF-kappa-B activation, cytokine secretion and the inflammatory response [85].
TLR4	Cooperates with LY96 and CD14 to mediate the innate immune response to bacterial lipopolysaccharide (LPS). Acts via MYD88, TIRAP and TRAF6, leading to NF-kappa-B activation, cytokine secretion and the inflammatory response [34].

Table 1. 7. Genes studied with their known functions in TLR pathway (Continued)

TLR5	Participates in the innate immune response to microbial agents. Mediates detection of bacterial flagellins. Acts via MYD88 and TRAF6, leading to NF-kappa-B activation, cytokine secretion and the inflammatory response [85]
TLR6	The polymorphism of TLR6 tends to be associated with asthma emergency visits [86].
MYD88	Adapter protein involved in the Toll-like receptor and IL-1 receptor signaling pathway in the innate immune response. Acts via IRAK1, IRAK2, IRF7 and TRAF6, leading to NF-kappa-B activation, cytokine secretion and the inflammatory response. Increases IL-8 transcription. Involved in IL-18-mediated signaling pathway [87].
TOLLIP	Component of the signaling pathway of IL-1 and Toll-like receptors. Inhibits cell activation by microbial products. Recruits IRAK1 to the IL-1 receptor complex. Inhibits IRAK1 phosphorylation and kinase activity [66].
TICAM1& TICAM2	Involved in innate immunity against invading pathogens. Adapter used by TLR3 and TLR4 (through TICAM2) to mediate NF-kappa-B and interferon-regulatory factor (IRF) activation, and to induce apoptosis [85]. Ligand binding to these receptors results in TRIF recruitment through its TIR domain TICAM2 Functions in LPS-TLR4 signaling to regulate the MYD88-independent pathway during the innate immune response to LPS. Also involved in IL1-triggered NF-kappa-B activation, functioning upstream of IRAK1, IRAK2, TRAF6, and IKBKB. Physically bridges TLR4 and TICAM1 and functionally transmits LPS-TRL4 signal to TICAM1 [88].

2. MATERIALS

2.1. BACTERIAL STRAINS

Porphyromonas gingivalis ATCC 33277 (ATCC, USA)
Prevotella intermedia ATCC25611 (ATCC, USA)

2.2. CELL LINE

Human fetal osteoblast (hFOB 1.19) (ATCC, USA)

2.3. CHEMICALS and BUFFERS

- Fastidious Anaerobe Agar (FAA) (LabM, UK)
- Brain Heart Infusion Agar (BHIA) (Salubris, USA)
- Fastidious Anaerobe Broth (FAB) (LabM, UK)
- Brain Heart Infusion Broth (BHIB) (Salubris, USA)
- Anaerobic Gas Mixture (80 % N2, 10 % H2, 10 % CO2) (Habas, Turkey)
- Barium Chloride Solution (Sigma, Germany)
- Hydrosulfuric Acid Solution (Sigma, Germany)
- Fetal Bovine Serum (FBS), Heat Inactivated (Gibco, Germany)
- Trypsin-EDTA (Gibco,USA)
- Phosphate Buffer Saline (PBS) (Gibco, Germany)
- Gentamycin (Gen) (Gibco,Germany)
- Metronidazole (Met) (Gibco,Germany)
- Sodium cacodylate buffer (Sigma, Germany)
- Hydrochloric acid (Sigma, Germany)
- Gluteraldehyde (Sigma, Germany)
- Paraformaldehyde (Sigma, USA)
- Silver nitrate (Sigma, USA)
- Sodium hydroxide (Merck, Germany)

- WST1 reagent (Roche, France)
- β mercaptoethnanol (Merck, Germany)
- Taq polymerase (5 units/µl), (Fermentas, Canada)
- Taq polymerase buffer, (Fermentas, Canada)
- Magnesium chloride (Fermentas, Canada)
- dNTP mix (Promega, USA)
- Gene Specific Primers (alpha DNA, Canada)
- Tris Base (Sigma, USA)
- Ethidium bromide (Merck, Germany)
- Loading dye (Sigma, Germany)
- DNA ladder (Invitrogen, USA)
- SYBR Green qPCR master mix (SABiosceinces, Germany)

2.4. KITS

- API 20A anaerobic identification kit (BioMerieux, USA)
- WST-1 Cell proliferation Kit (Roche, USA)
- RNeasy RNA isolation kit (Qiagen, USA)
- RT² First Strand Kit (SABiosciences, USA)
- 10X Taq Buffer (Fermentas, USA)
- RT Profiler PCR Array (SABiosciences, USA)

2.5. LABORATORY EQUIPMENTS

- Anaerobic workstation (Don Whitley, UK)
- Autoclave (HV-85 (HICLAVE, Hirayama, Japan)
- Centrifuge (Hettich mikro 22R and SIGMA 2-5 centrifuge, Germany)
- Biological Safety Cabinet (NuAire, USA)
- CO2 incubator (Nuaire NU5510/E/G, USA)
- ELISA plate reader (Bio-Tek EL x 800, USA)
- Inverted Light Microscopy (Nikon Eclipse TS100, USA)

- Laminar flow cabinet (ESCO Labculture Class II Biohazard Safety Cabinet 2A, Singapore)
- Microwave (Arçelik, Turkey)
- NanoDrop (Implen, Germany)
- Light Microscopy (Olympus, USA)
- pH meter (Hanna instruments PH211, Germany)
- Real Time Thermal Cycler (Biorad, Germany)
- Scaning Electron Microscopy (Zeiss Evo40, Germany)
- Turbidemeter ((BIOLOG,Hayward CA)
- Thermalcycler (BioRad, Germany)
- UV Illuminator (BioRad, Germany)
- Vortex (Stuart SA8, UK)
- -80 °C freezer (Thermo Forma -86 C ULT Freezer, USA).

3. METHODS

3.1. BACTERIAL CULTURE

Porphyromonas gingivalis ATCC 3744 and *Prevotella intermedia* ATCC 2563 were obtained from American Type Cell Culture, USA. Both bacteria were subcultured by the streaking method every 72h on brain heart infusion agar (BHIA) supplemented by 5% defibrinated sheep blood (Salubris, USA) and incubated at 37 °C in an anaerobic workstation (Don Whitley Scientific, UK) in an atmosphere of 10% CO_2, 10% H_2 and 80% N_2 gas mixture (Habaş, Turkey)

3.1.2. Bacterial identification

API 20 A system (Biomerieux, France) was applied according to the manufacturer's instructions for the identification of anaerobes. Briefly *P.g* and *P.i* were subcultured onto sheep blood agar and incubated at 37 °C in an anaerobic cabinet for 16-24 h. Following incubation, bacteria were harvested with a sterile cotton swab and suspended in API 20A medium inside the anaerobic cabinet. The turbidity of the bacterial suspension was Mc. Farland standard No.3. Bacterial suspensions were inoculated into API 20A strip and incubated at 37 °C for 24 h in the anaerobic cabinet. Following incubation API 20A strip was read according to the reading table (Table 3.1) and an 8-digit number obtained which corresponded to the bacteria's ID.

Table 3. 1. Reading table for API 20A commercial kit

API 20A READING TABLE			
TESTS	REACTIONS / ENZYMES	RESULTS	
		NEGATIVE	POSITIVE
IND	Indole formation	XYL – mix /2-3 min + EHR / 5 min.	
		Yellow	red
URE	Urease	Yellow-orange	red
		BCP	
GLU	Acidification of Glucose	Purple	Yellow / yellow-green
MAN	Acidification of Mannitol		
LAC	Acidification of Lactose		
SAC	Acidification of Saccharose		
MAL	Acidification of Maltose		
SAL	Acidification of Salicin		
XYL	Acidification of Xylose		
ARA	Acidification of Arabinose		
GEL	Hydrolysis of gelatin	No diffusion of pigment	Diffusion of black pigment
ESC	Hydrolysis of esculin	Yellow (2)	Brown-black (2)
		in UV (365 nm)	
		Fluorescence	no fluorescence
		BCP	
GLY	Acidification of Glycerol	Purple	Yellow / Yellow-green
CEL	Acidification of Cellobiose		
MNE	Acidification of Mannose		
MLZ	Acidification of Melezilose		
RAF	Acidification of Raffinose		
SOR	Acidification of Sorbitol		
RHA	Acidification of Rhamnose		
TRE	Acidification of Trehalose		
CAT	Catalase	After 30 min. in air, add H2O2 in a positive tube	
		No bubbles	Bubbles
SPOR	Spores	Absent	Present
GRAM	Gram reaction	Pink	Violet
COCC	Morphology	Rod	Coccus

3.2. TISSUE CULTURE

Human fetal osteoblasts (h.FOB 1.19, ATCC, USA) were used in this study as a model for bone regeneration and bone resorption. The human fetal osteoblast cell line were cultured in Dulbecco's modified Eagle's Nutrient Mixture F-12 (DMEM F-12) (Gibco, Germany) supplemented with 10% heat inactivated fetal bovine serum (FBS) (v/v) and 100 U/ml penicillin and 1000 μg/ml streptomycin (Gibco, Germany) in a humidified atmosphere of 5% CO_2 (Habaş, Turkey) at 37°C.

3.3. ANTIBIOTIC CONCENTRATION FOR TOTAL INHIBITION OF BACTERIA

The antibiotic concentration to kill *P.g* and *P.i* at a density of 10^9cfu/ml with gentamicin-metronidazole (Gibco, Germany) combination was determined) to kill the remaining extracellular bacteria following incubation with h.FOB. For this purpose osteoblasts were grown in 96 well plates at a density of 10^4 cells per well and incubated overnight in a humidified atmosphere of 5% CO_2 at 37°C before challenging with bacteria. *P.g* and *P.i* were subcultured on BHIA and incubated anaerobically for 18-24h. Following incubation a loop of bacteria was dissolved in 5 ml of PBS and centrifuged at 1000 x g for 5 min and was resuspended in antibiotic free DMEM F-12 medium supplemented with 10% FBS. After resuspension, the optical density of the bacterial suspension was measured at 600 nm, and diluted to an optical density of 0.5 corresponding to 5×10^8 CFU/ml. Bacteria suspensions were then further diluted in DMEM F-12 medium to generate 1000 multiplicity of infection (MOI). The MOI was confirmed retroactively in parallel experiments by plating bacteria onto BHIA and counting bacterial colonies after incubation for 72h. Osteoblasts were than challenged by either *P.g* or *P.i* at a MOI of 1000 for 2h in a humidified atmosphere of 5% CO_2 at 37°C. After challenging for 2 h, the monolayers were washed three times with 100μl of PBS to remove nonattached bacteria and the cultures were further incubated for 2h in fresh culture medium containing different combinations of gentamicin (gen) and metronidazole (met) mixture (0.2 mg/ml gen + 0.2 mg/ml met/, 0.3 mg/ml gen+ 0.2 mg/ml met, 0.4 mg/ml gen + 0.2 mg/ml met, 0.4 mg/ml gen+ 0.25 mg/ml met, 0.45 mg/ml gen + 0.25 mg/ml met, 0.5 mg/ml gen+ 0.3 mg/ml met) to kill the remaining extracellular bacteria. Following incubation 10μl of medium was inoculated onto BHIA and incubated under anaerobic conditions for 72h to observe bacterial growth.

3.4. WST ASSAY

To measure the effect of antibiotic combinations on osteoblast viability, osteoblasts were prepared as described in Section 4.3. Cell viability of osteoblasts following bacterial challenges with *P.g* or *P.i* was measured by using a commercially available WST assay (Roche, France). The WST-1 assay is a nonradioactive, spectrophotometric method. The stable tetrazolium salt WST-1 is cleaved to a soluble formazan by a complex cellular mechanism that occurs primarily at the cell surface. This bioreduction is largely dependent on the glycolytic production of NAD(P)H in viable cells. Therefore, the amount of formazan dye formed directly correlates to the number of metabolically active cells in the culture.

Osteoblasts were then treated with different concentrations of antibiotic combinations (0.2 mg/ml gen + 0.2 mg/ml met/, 0.3 mg/ml gen+ 0.2 mg/ml met, 0.4 mg/ml gen + 0.2 mg/ml met, 0.4 mg/ml gen+ 0.25 mg/ml met, 0.45 mg/ml gen + 0.25 mg/ml met, 0.5 mg/ml gen+ 0.3 mg/ml met) and incubated for 2h in a humidified atmosphere of 5% CO_2 at 37°C. Following incubation WST protocol was conducted according to the manufacturer's instructions. Osteoblasts were washed three times with 100µl of PBS. Tenµl of WST reagent and 90 µl of culture medium (without antibiotics were mixed together and 100 µl of final volume was added to each well of a 96 well tissue culture plate. Following the addition of WST reagent, the plates were wrapped in aluminum foil and incubated in the dark at 37°C for 2h in a humidified atmosphere of 5% CO_2 at 37°C. The absorbance of each well was measured at 450 nm with an ELISA plate reader. Unchallenged cells were used as a negative control.

To measure the effect *P.g* or *P.i* on osteoblast viability, osteoblasts and bacterial suspensions of *P.g* and *P.i* were prepared as described in Section 4.3. Bacterial suspensions were then diluted in antibiotic free DMEM F-12 medium to generate 50, 100, 250, 500 and 1000 MOI. Osteoblasts were then challenged by either *P.g* or *P.i* at different MOIs as indicated for 2h. Following challenging monolayers were washed three times with 100µl of PBS and further incubated for 2h in culture medium containing 0.3 mg/ml gen+ 0.2 mg/ml met. To determine the cell viability WST protocol was applied as previously described..

3.5. SCANNING ELECTRON MICROSCOPY

The osteoblast monolayers were inoculated onto 13-mm-diameter sterile thermanox plastic cell-culture coverslips (Nalge Nunc, Germany). Osteoblasts were then challenged by either *P.g* or *P.i* at a 1000 MOI as described in section 4.2. Following challenging, cells were washed with 1 ml of PBS three times. Unchallenged cells were used as negative control. Cells were fixed with freshly prepared 2% glutaraldehyde in 0.1 M sodium cacodylate buffer for 2h. Following fixation, cells were washed three times with 0.1M cacodylate buffer (pH 7.2) for 5 min. Cells were dehydrated through a graded alcohol series and critical point dried. Samples were coated with gold for 5 min with sputter coater (Baltec SCD 005, USA) and imaged on a scanning electron microscope (247 Zeiss evo40, Germany).

3.6. VON KOSSA STAINING

The von kossa staining method was carried out to represent mineralized matrix of osteoblasts [60]. Briefly, osteoblast were inoculated into 6 well plates at a density of $2.5x10^4$cells/well and incubated overnight in a humidified atmosphere of 5% CO_2 at 37°C. Osteoblasts were then challenged by either *P.g* or *P.i* at a 1000 MOI for 2h as described in section 4.2. Unchallenged cells were used as negative control. Following incubation cells were rinsed three times by 1 ml of PBS then fixed in 2% paraformaldehyde (pH 7.2) for 30 min. Following fixation, cells were rinsed again with PBS and were incubated with a 3% silver nitrate solution (Sigma, Germany) under UV light for 1h using UV lamb (Name, Country). Images were captured under inverted microscope (Nikon Eclipse TS100, USA) at 40X magnification. Mineralized areas were identified with bright colour.

3.7. RNA ISOLATION

Total RNA was isolated from challenged and unchallenged cells by RNeasy mini kit (Qiagen, USA) according to manufacturer's instructions. Briefly, osteoblast were inoculated into 6 well plates at a density of $2.5x10^4$ cells/well and incubated overnight in a humidified atmosphere of 5% CO_2 at 37°C. Osteoblasts then were challenged by either *P.g* or *P.i* at a 1000 MOI for 2h as described in section 4.2. Following incubation, cells were

rinsed three times with 1 ml of PBS and trysinized and centrifuged at 300 x g for 5 min. Supernatant was discarded and the pellet was lysed and homogenized by 350 µl of RLT buffer. 1 volume of 70% ethanol was added to the lysate to create conditions that promote selective binding of RNA to the RNeasy membrane. Lysates were transferred to the RNeasy spin column placed in a collection tube and centrifuged for 15s at 8000 x g.The flowthrough was discarded. 700 µl of Buffer RW1 was then added to the RNeasy spin column and centrifuged again for 15s at 8000 x g. Following centrifugation 500 µl of buffer RPE was added to RNeasy spin column and centrifuged for 15s at 8000 x g. This step was repeated with the same conditions. To collect total RNA 50 µl of RNase free water was added to RNeasy spin column and centrifuged for 1 min at 8000 x g. The concentration of total RNA was determined by Nanodrop (Implen, Germany) In addition total RNA was run on 2% agarose gel at 90V for 30 min. The gel was visualized under UV illuminator (Biorad, Germany).

3.7.1. Gel electrophoresis

2 mg of agarose (Sigma, Germany) was dissolved in 100ml of 0.5X TBE buffer (Sigma, Germany) to obtain a 2% agarose gel. Agarose was heated in a microwave (Arçelik, Turkey) for 2 mins. The solution was then left to cool to 50°C- 55°C. 1 µl of EtBr (Sigma, Germany) was added to the solution and the solution thenpoured into the casting tray. RNA sample was prepared by mixing 5 µl of RNA and 1 µl of 6X Loading dye (Sigma, Germany). RNA samples were then loaded onto the agarose gel and run for 30 min at 90V. Gels were visualized under a UV trans-illuminator (Bio-Rad, Germany).

3.8. REVERSE TRANSCRIPTION OF TOTAL RNA

Total RNA was reversedly transcribed to cDNA by using the commercial RT First Strand Kit (SABiosciences, USA). All the reagents were thawed on ice and genomic DNA elimination mixture was prepared by mixing 2 µl of gDNA elimination buffer (Buffer GE), 5 µl of RNA and 3 µl of RNase free water. Genomic elimination mixture was incubated for 5 min at 42 °C then immediately chilled on ice for 5 min. Following incubation reverse transcription coctail was prepared with 4 µl of 5x Reverse Transcriptase Buffer 3 (Buffer BC3), 1 µl of primer and external control mix (Control P2), 2 µl of Reverse Transcriptase

Enzyme mix (RE3) and 3 µl of RNAse free water. The genomic elimination mix was mixed with reverse transcription mix. The mixture was placed into thermal cycler (BioRad, Germany). The thermal cycler was programmed at 42°C for 15 min then 95°C for 5 min. Polymerased chain reaction was carried out from the synthesized cDNA to confirm functionality. PCR mix was prepared with 2 µl of 10X PCR Buffer 2 µl of 10 mM MgCl2, 0.2 µl of Taq Polymerase (Fermentas, USA), 4 µl template, 0,4 µl 10mM GAPDH primer and 11 µl of nuclease free water (Gibco, USA) and programmed as follows; denaturation (1 cycle) at 94°C for 5 min, anneling (35 cycle) 94°C for 30 s, 55°C for 30s, 72°C for 30 sn and elongation (1 cycle) at 72°C for 10 min. The pcr products were run on 2% agarose gel at 90V for 30 min and visualized under UV illuminatior (BioRad, Germany).

3.9. GENE EXPRESSION ANALYSIS

3.9.1. RT Profiler PCR Array

Gene expressions of the TGF β and BMP family were identified by RT Profiler PCR Array (PAHS-035, PAHS018) (SABiosciences, USA). Further the expressions of selected gene (COL1A) and 2 more genes (ALP and BGN) were analysed manually by quantitative real time pcr experiments.

PCR mastermix was prepared with 1350 µl of 2X RT SYBR Green Mastermix, 102 µl cDNA synthesis reaction and 1248 µl of nuclease free water (Gibco, USA). The mastermix was transferred into the reservoir and 25 µl of PCR mastermix was added to each well of RT Profiler PCR plate with a multichannel pipettor. The flow of the preparation is shown in Figure 3.1. The sealed plate was placed into a real time thermal cycler (Biorad ICycler, Germany) and programmed as shown in Table 3.2.

Table 3. 2. Programme of thermal cycler for qPCR

Cycle	Repeat	Step	Time	Temperature °C	Melt Curve
1	1	1	10 min	95	
2	40	1	15 s	95	
		2	60 s	60	
3	1	1	60 s	95	
4	1	1	60 s	55	
5	80	1	10 s	55	+ 0.5 (each cycle)

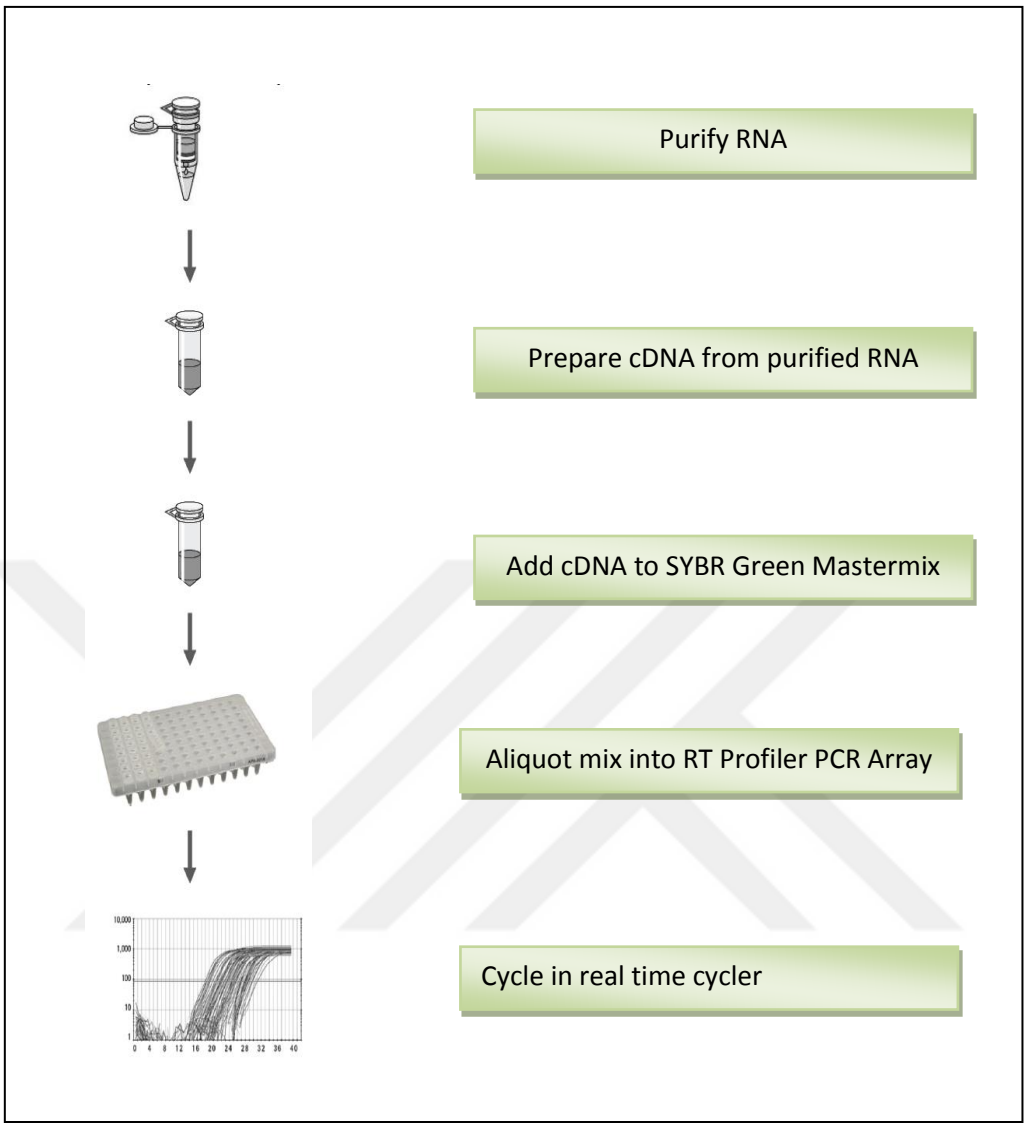

Figure 3. 1. RT Profiler PCR Array Procedure [89]

3.9.2. Manual Quantitative Real Time PCR

Selected genes from RT Profiler Array; Col1A and genes ALP and BGN that are related to matrix mineralization and osseointegration were also studied. qPCR was conducted to see the altered gene expression in h.FOB after challenging with *P.g* and *P.i*. RT master mix was prepared with 12,5 µl of RT SyBr Green qPCR mastermix, 10.5 µl of nuclease free water, 1 µl of DNA template and 1 µl of gene specific primer. Primer sequences are listed in Table 3.3. Real time pcr was performed in BioRad ICycler. The real time machine programmed as given in Table 3.2.

Table 3. 3. Sequence list of gene specific primers

Target RNA	Primer sequence	Size (bp)
ALP	F. 5'- GCATAACATCAGGGACATTGACGTG -3' R. 5- GTGGGAGTGCTTGTATCTCGG -3'	124
COL1A	F. 5'- ATGCCTGGTGAACGTGGT -3' - R.5'-AGGAGAGCCATCAGCACCT-3'	87
BGN	F. 5'-CTCAACTACCTGCGCATCTCAG-3' R. 5'-GATGGCCTGGATTTTGTTGTG-3'	105

4. RESULTS AND DISCUSSION

In this study the effects of *Porphyromonas gingivalis* and *Prevotella intermedia* on human fetal osteoblast viability and cytokine and receptor network were evaluated.

4.1. ANTIBIOTIC APPLICATION TO BACTERIA AND OSTEOBLASTS

The antibiotic concentration required for total inhibition of non adherent bacteria following challenging was determined. No bacterial growth was observed in the plates treated with antibiotic combinations of 0.3 mg/ml gen+ 0.2 mg/ml met, 0.4 mg/ml gen + 0.2 mg/ml met, 0.4 mg/ml gen+ 0.25 mg/ml met, 0.45 mg/ml gen + 0.25 mg/ml met, 0.5 mg/ml gen+ 0.3 mg/ml met), meaning that these concentrations were enough to kill all bacteria in the culture. Bacterial growth was only observed in plates treated with the antibiotic combination of 0.2 mg/ml gen + 0.2 mg/ml met. This antibiotic concentration was excluded from all further experiments.

The effect of different antibiotic combinations on the viability of osteoblast cells were also determined, in order to determine any cytotoxic effects of the antibiotics that could interfere with later results. Figure 4.1 shows the effects of the antibiotic combinations on osteoblast viability. The antibiotic combination of 0.3 mg/ml gen+ 0.2 mg/ml met did not affect the cell viability (% cell viability: 100). However higher concentrations of antibiotics showed a reduction in osteoblast viability. The antibiotic combinations of 0.4 mg/ml gen + 0.2 mg/ml met and 0.4 mg/ml gen+ 0.25 mg/ml displayed a slight decrease in cell viability of 97.3 % and 95.2% respectively. Furthermore 0.45 mg/ml gen + 0.25 mg/ml met reduced the viability of osteoblasts to 92.7%. The highest concentration of antibiotic combination, 0.5 mg/ml gen+ 0.3 mg/ml met was found to reduce osteoblast viability more than other antibiotic combinations with a viability of 87.5%.

These results indicated that the antibiotic combination of 0.3 mg/ml gen+ 0.2 mg/ml should be used for further experiments as this concentration did not affect cell viability at all. This concentration has also be used by Lamont et al. and Dashper et al. were they also

found 0.3 mg/ml gen+ 0.2 mg/ml met antibiotic combination to be enough to kill 10^9 bacteria in an hour while exhibiting no toxicity to gingival epithelial cells [90][91].

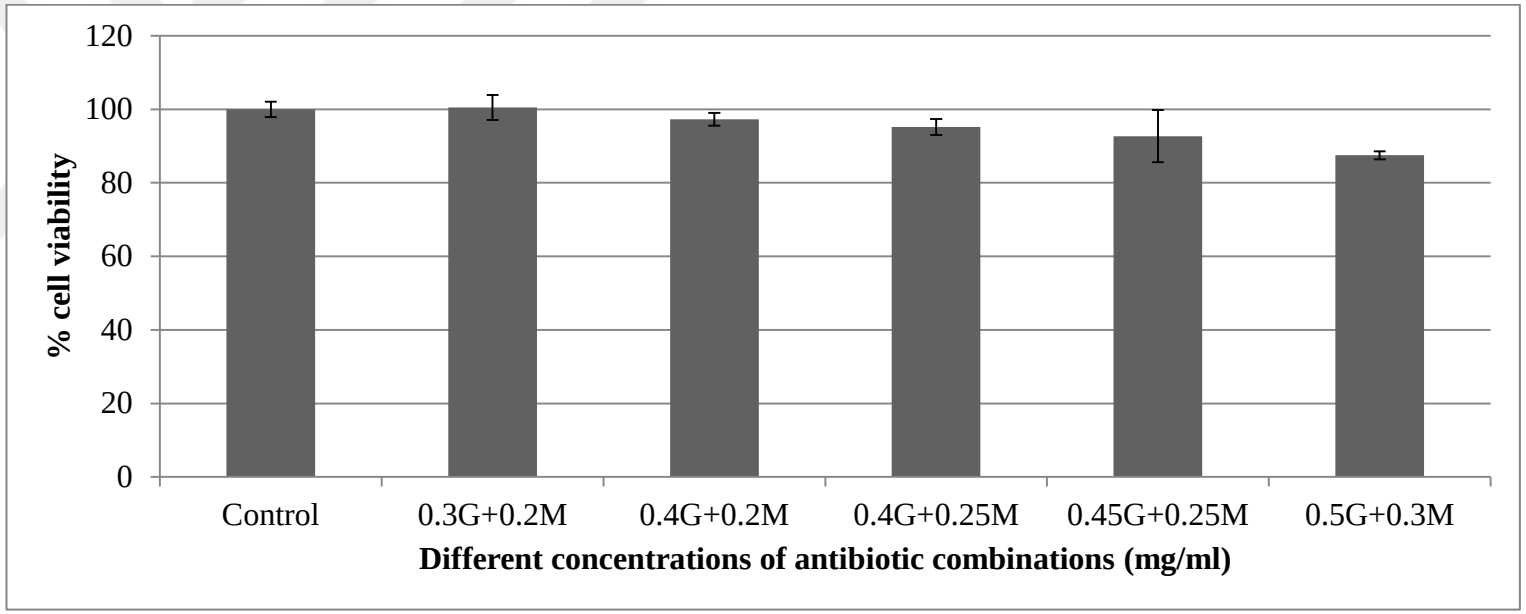

Figure 4. 1 Cell viability of human fetal osteoblast cells following incubation with different concentrations of antibiotic combinations for 2h. G: Concentration of Gentamicin, M: Concentration of Metronidazole. Control used was unchallenged cells. Values given as means of standard deviations of triplicate independent determinations from a typical experiment

4.2. THE EFFECT OF *P.g* AND *P.i* CHALLENGE ON HUMAN FETAL OSTEOBLAST VIABILITY

h. FOBs were challenged with *P.g* and *P.i* at concentrations of 50, 100, 250, 500 and 1000 MOI for 2h. The potential cytotoxic effects of these challenges were investigated by WST assay. As expected, Figure 4.2 and 4.3 shows the reduced viability of osteoblasts following challenges by *P.g* and *P.i* at all MOIs. The figures show that the increasing multiplicity of infection showed reduction incell viability in a dose-dependent manner. At a challenge of 50 MOI of *P.g* the viability of osteoblasts was 94.05%. At the same MOI of *P.i* challenge showed more reduction in the viability of osteoblasts with a rate of 88.9%. However at challenges of 100, 250 and 500 MOI of both *P.g* and *P.i,* the reduction rate of osteoblasts viability was similar. At 100 MOI a 80.14% and 80.5% reduction for *P.g* and *P.i* challenged cells was observed respectively, while at 250 MOI a 74.78% (*P.g*) and 74.49% (*P.i*) decrease was seen.A 500 MOI displayed a further reduction of 66.6% (*P.g*) and 66.95% (*P.i*). At the highest MOI of1000, the viability was shown to reduce to 56.8% by *P.g* challenge whereas viability reduced to 52.75% by *P.i* challenge.

These results indicate that *P.i* challenges at MOI of 100 and 1000 were slightly more destructive than *P.g* challenges to osteoblasts. These findings show that both *P.g* and *P.i* negatively affected osteoblast viability in a dose dependent manner. These findings are similar to those of Kadono et al, who found that *P.g*. cell invasion affected osteoblast differentation and activity adversely [92]. Although a previous study by Wenjian Zhang et al. found that an increase in bacterial concentration gave way to more bacterial invasion of the cell, this did not affect cell viability or proliferation [93]. The difference between these results could be due to different methods used for measuring cell viability. The latter study used manual cell counting as opposed to WST1 which relies on mitochondrial dehydrogenase enzyme activity which is said to be more accurate.

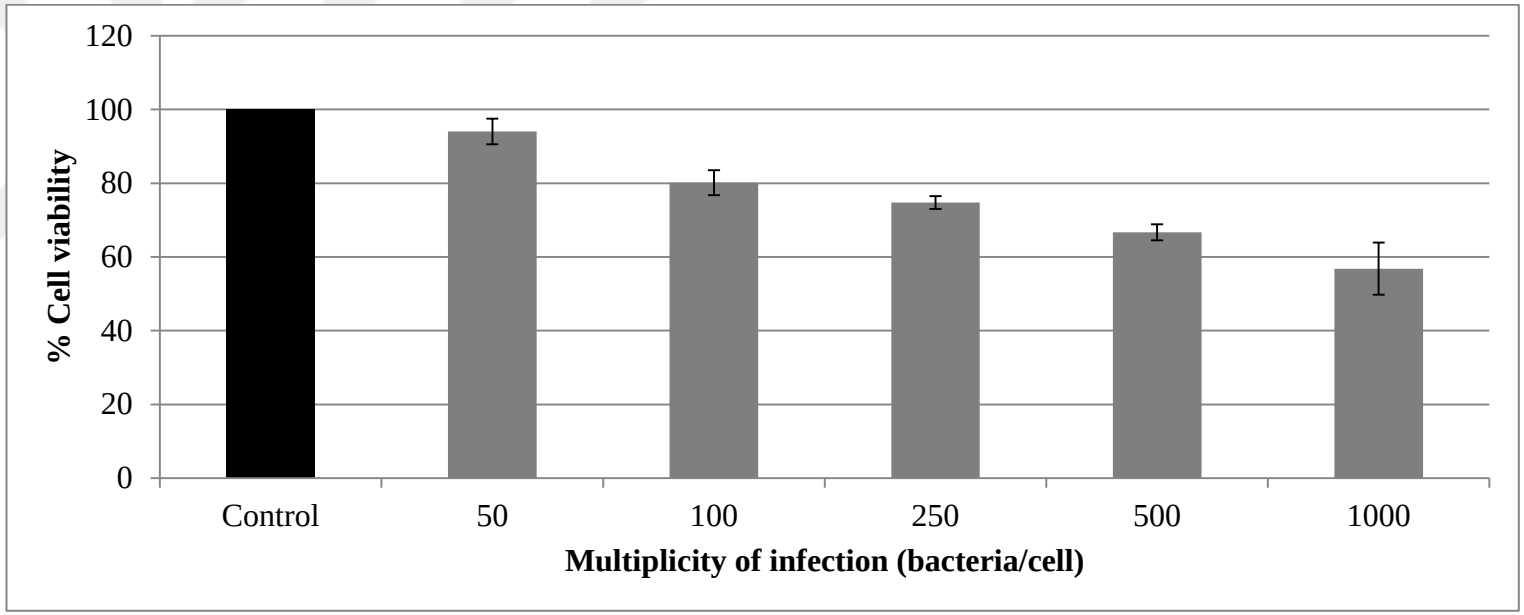

Figure 4. 2. The reduced viability of osteoblasts as a result of *P. gingivalis* challenge

Control used was unchallenged cells. Values given as means of standard deviations of triplicate independent determinations from a typical experiment

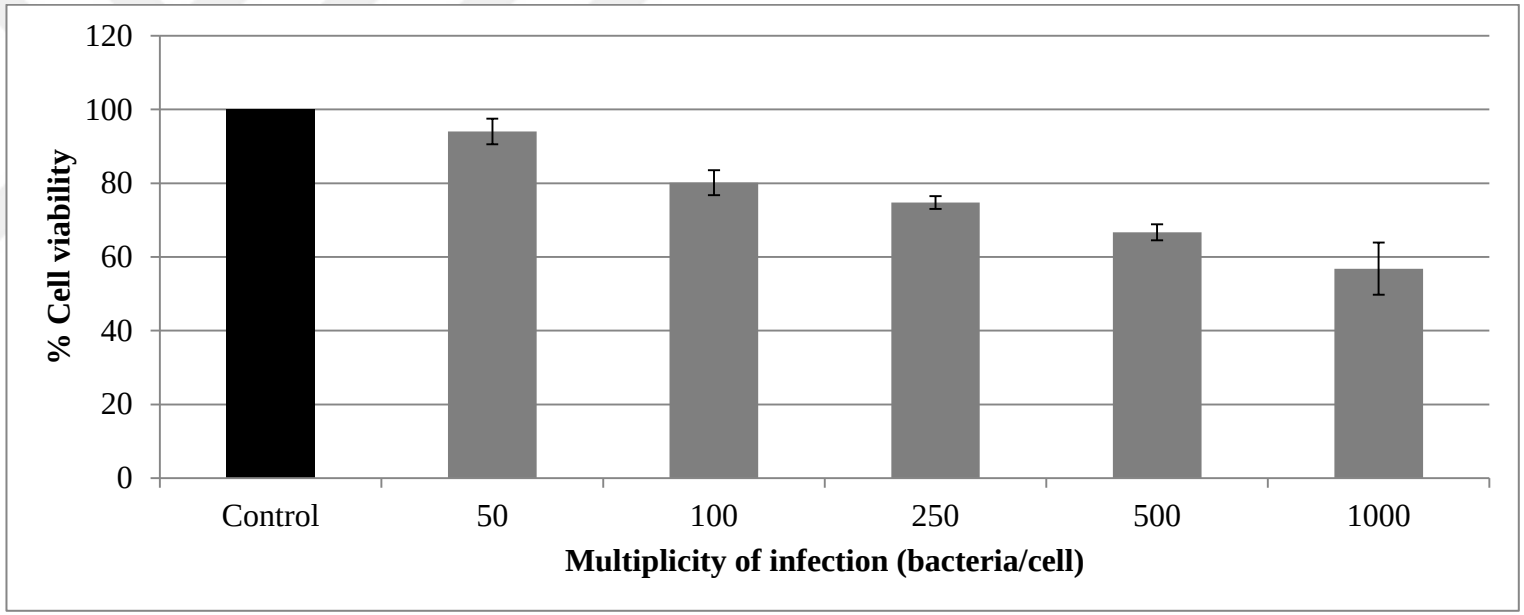

Figure 4. 3. The reduced viability of osteoblasts as a result of *P. intermedia* challenge

Control used was unchallenged cells. Values given as means of standard deviations of triplicate independent determinations from a typical experiment

4.3. SEM

Osteoblasts were challenged by *P.g* and *P.i* at a MOI of 1000 for 2h. The effects of bacterial challenges on osteoblast morphology were observed by SEM microscopy. Osteoblasts normally have a flattened fibroblast-like morphology (Figure 4.A) however, following challenging the cell morphology changed *P.g* challenged cells at a 1000 MOI showed detachment of osteoblasts from the slide along with a more spherical morphology, suggesting cell death (Figure 4.4B). Similar changes in morphology and detachment were also observed for, *P.i* challenged osteoblasts at the same MOI of 1000 (Figure 4.4C). These results are compatible with WST assay results which showed the highest reduction in osteoblast viability at 1000 MOI in for both *P. g* and *P. i*. These gram negative invasive pathogens are known to use their fimbria for facilitating attachment to host cells and thereby invading them. Many studies have confirmed this method of attachment for *P.g* and *P.i* as a major factor for infection of host cells. Such attachments have previously been shown to negatively induce major immune pathways in host system [50][94].

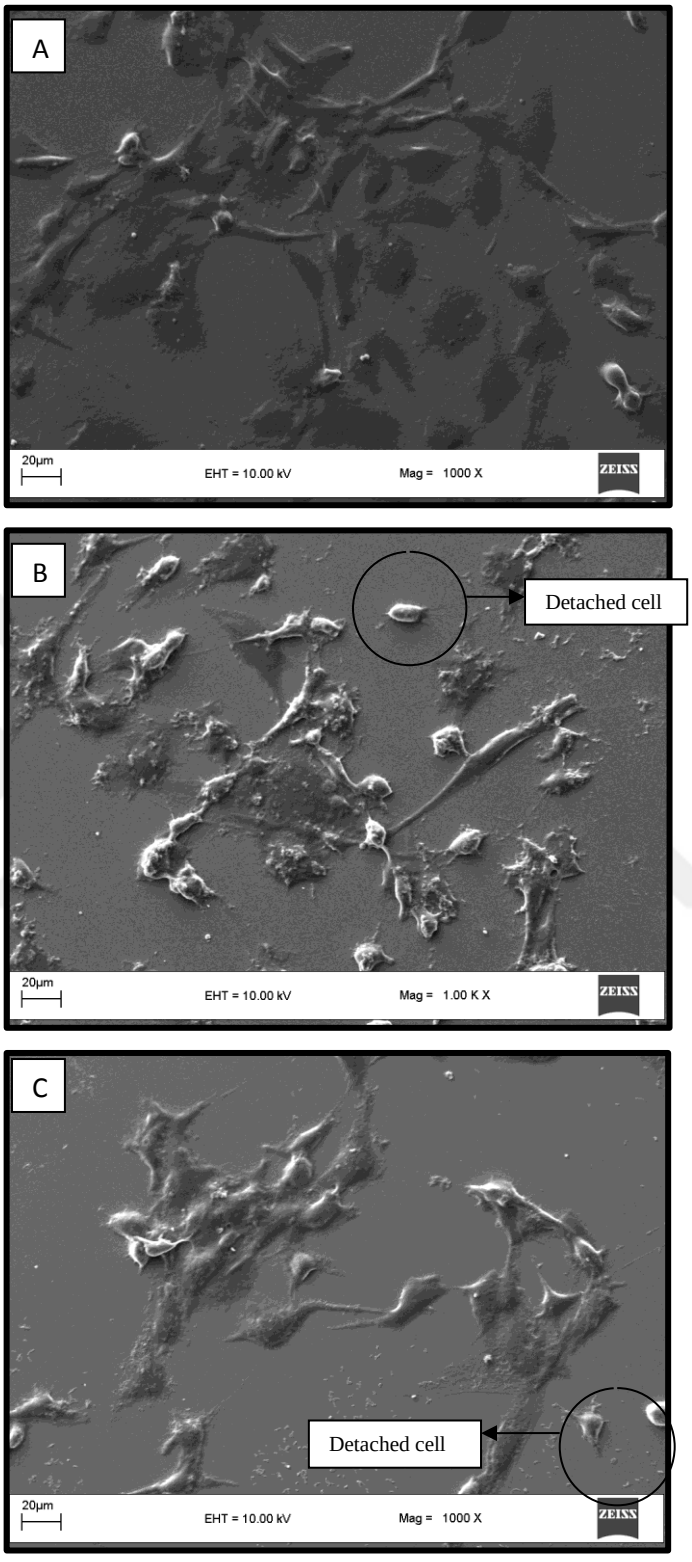

Figure 4. 4. Scanning electron microscopy images A. Unchallenged h.FOBs B. h.FOBs challenged by *P.g.* at a MOI of 1000 for 2h C. h.FOBs challenged by *P.i.* at a MOI of 1000 for 2h**.**

4.4. MATRIX MINERALIZATION OF OSTEOBLAST FOLLOWING BACTERIAL CHALLENGES

Following *P.g* or *P.i* challenge matrix mineralization of osteoblasts were identified by the Von Kossa staining method. This method is based on binding of silver ions to the anions (phosphates, sulfates, or carbonates) of calcium salts to form dark brown to metallic silver staining. Following staining, the plates were captured under inverted microscopy and the images of challenged and unchallenged osteoblasts were compared. Mineralized areas were identified by a bright colour (Figure 4.5A). The challenge of osteoblasts by both *P.g* and *P.i* showed a large reduction in mineralization (Figure 4.5B and C). These results indicate that *P.g* and *P.i* challenge might lead to bone resorption via reducing matrix mineralization. These findings are supported by a previous study by Zhang et al., where a reduction in mineralization of mouse calvarial osteoblast following *P.g* invasion were observed [93]. Additionally Wang et al. showed that the lipids of *P.g* inhibited osteoblast differentiation [95]. Similar results have also been found in *P.i* challenged cells. A previous study showed that *P.i* challenge of calvarial osteoblasts inhibited bone formation by reducing alkaline phosphatase activity and calcium incorporation [96].

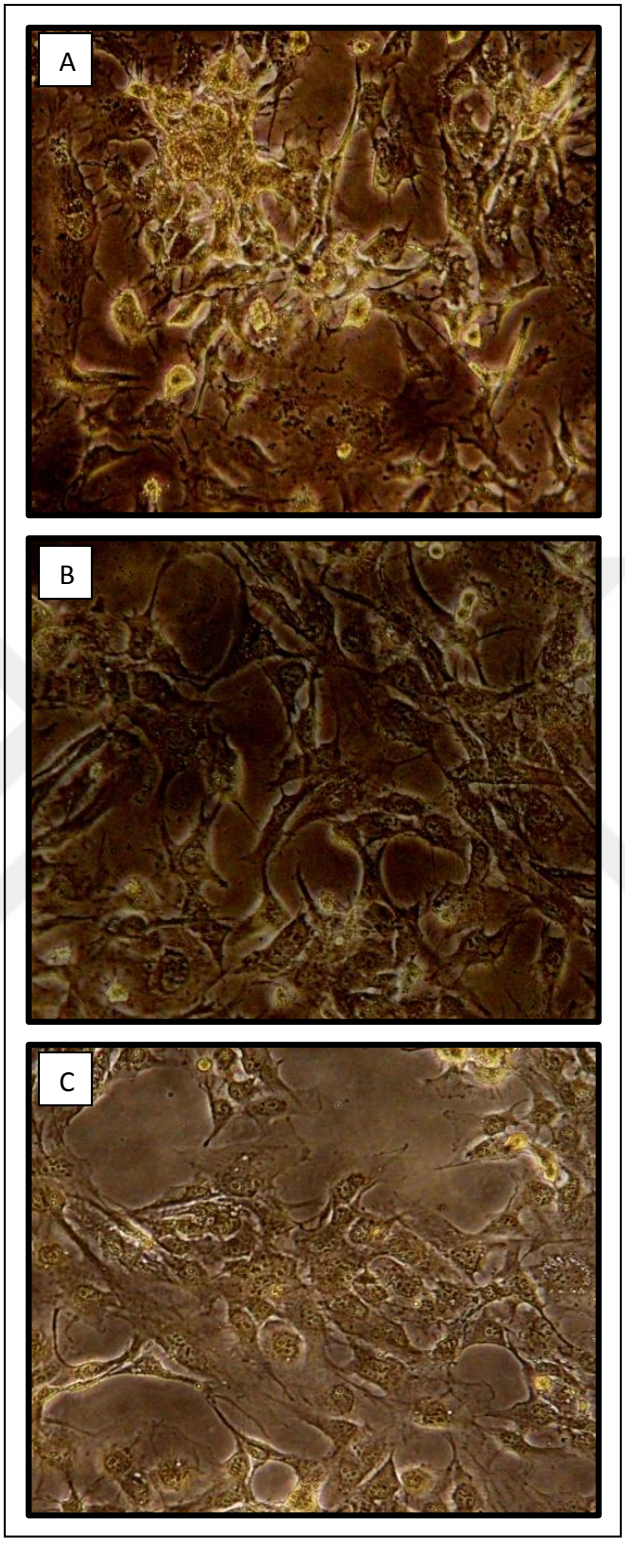

Figure 4. 5. Mineralized nodule formation examined by Von Kossa staining in the unchallenged osteoblasts. B. Mineralized nodule formation following *P. g* challenge at MOI of 1000. C. Mineralized nodule formation examined by Von Kossa staining in osteoblast cultures following *P.i* challenge at a MOI of 1000.

4.5. RNA ISOLATION

The absorbance ratio of 260/280 nm and total RNA concentration was determined by Nanodrop (Implen, Germany). The absorbance ratio was approximately 1.8-2.0 for all samples which met with the criteria for RT-PCR experiments (Table 4.1).

Table 4. 1. Total RNA concentration isolated from h.FOB following *P.g* and *P.i* challenge

	P.g	*P.i*	N.C	N.C.	N.C	N.C
RNA concentration (ng/µl)	98,2	100,2	96,4	128,3	123,7	135,2

P.g: RNA isolated from *P.g* challenged osteoblast *P.i*: RNA isolated from *P.i* challenged osteoblast, NC: RNA isolated from unchallenged osteoblasts.

In addition total RNA was run on 2% agarose gel at 90V for 30 min to visualize the stability. Gel electrophoresis results revealed that the RNAs were stable (Figure 4.6). Two clear and bright bands were seen for each RNA sample.

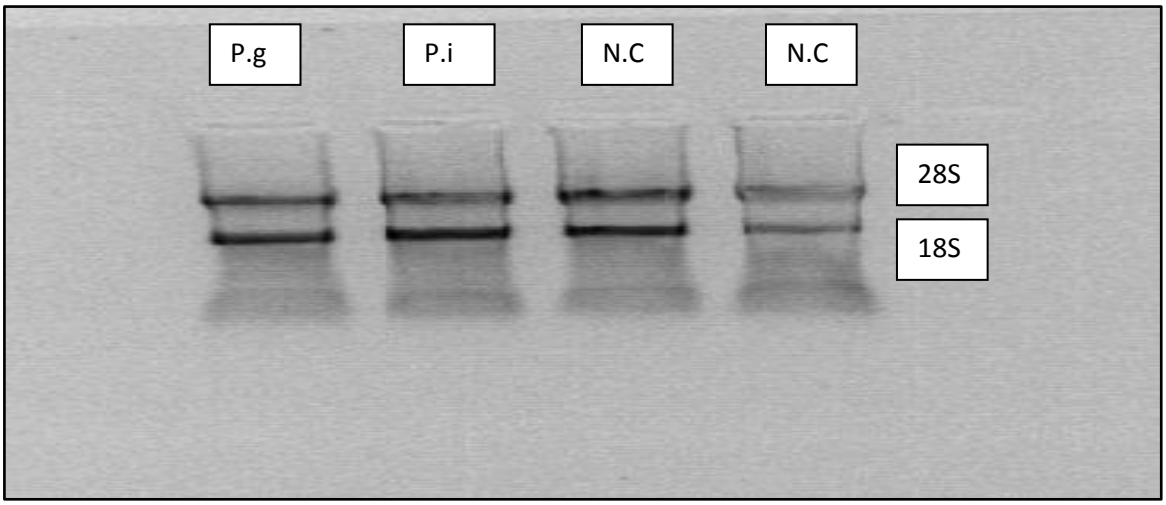

Figure 4. 6. Gel electrophoresis of isolated RNA samples
P.g: RNA isolated from *P.g* challenged osteoblast P.i: RNA isolated from *P.i* challenged osteoblast, NC: RNA isolated from unchallenged osteoblast

4.6. REVERSE TRANSCRIPTION OF TOTAL RNA

PCR was carried out from synthesized cDNAs to confirm functionality. PCR products were run in 2% agarose gel and visualized under UV illuminator (Figure 4.7). Results showed that each cDNA was synthesized successfully.

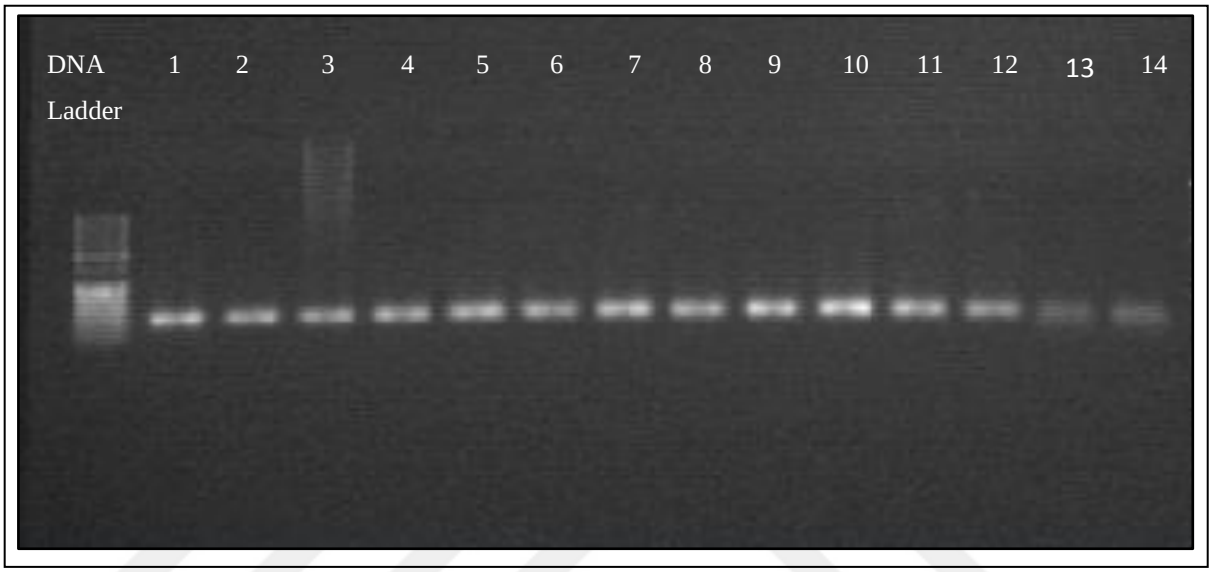

Figure 4. 7. Gel electrophoresis of GAPDH amplified cDNA samples1-4.cDN synthesized from *P.g* challenge RNA, 5-8 cDNA synthesized form *P.i* challenged RNA, 8-14 cDNA synthesized from unchallenged osteoblasts.

4.7. GENE EXPRESSION ANALYSIS

4.7.1. RT Profiler PCR Array

The panel of cytokine genes and receptor pathways were selected either according to their association with failing implants or bone morphogenesis or because they are genes coding for important inflammation-associated molecules that may be involved in the bacterial challenge process.

Gene expression analysis was performed with both the array and manual quantiative PCR. The relative expression of genes were analysed with SABiosciences RT² Profiler PCR Array Data Analysis 3.1.2 [97].

Bone morphogenetic proteins (BMPs) are secreting signaling molecules belonging to the transforming growth factor-b (TGF-b) superfamily. Individual bone morphogenetic proteins and activins are prominent at many sites during embryogenesis and are likely to be key regulators of early development and organogenesis. In vertebrates, one of the functions of bone morphogenetic proteins is to induce formation of bone, cartilage, and connective tissues associated with the skeleton. BMPs and activins signal via type I and type II receptors for BMPs (BMPRs) and activins (ActRs), respectively. The expression patterns of these receptors were identified in this study (Figure 4.8 and 4.9). Although ACVR2A is known to promote osteoblast proliferation suprisingly the results showed increased expression of ACVR2A following both *P.g* and *P.i* challenges (Fold Change: 3.3404 and 6.6807 respectively). Funaba et al showed that activin receptors were localized in proliferating chondrocytes and osteoblasts in developing bone in neonatal rats [98]. But as expected, expression of BMPER was increased by both *P.g* and *P.i* challenges (Fold Change: 5.8159 and 23.2636 respectively) since this gene encodes a secreted protein that interacts with and inhibits bone morphogenetic protein (BMP) function. This gene has been shown to inhibit BMP2- and BMP4-dependent osteoblast differentiation and BMP-dependent differentiation of the chondrogenic cells [99].

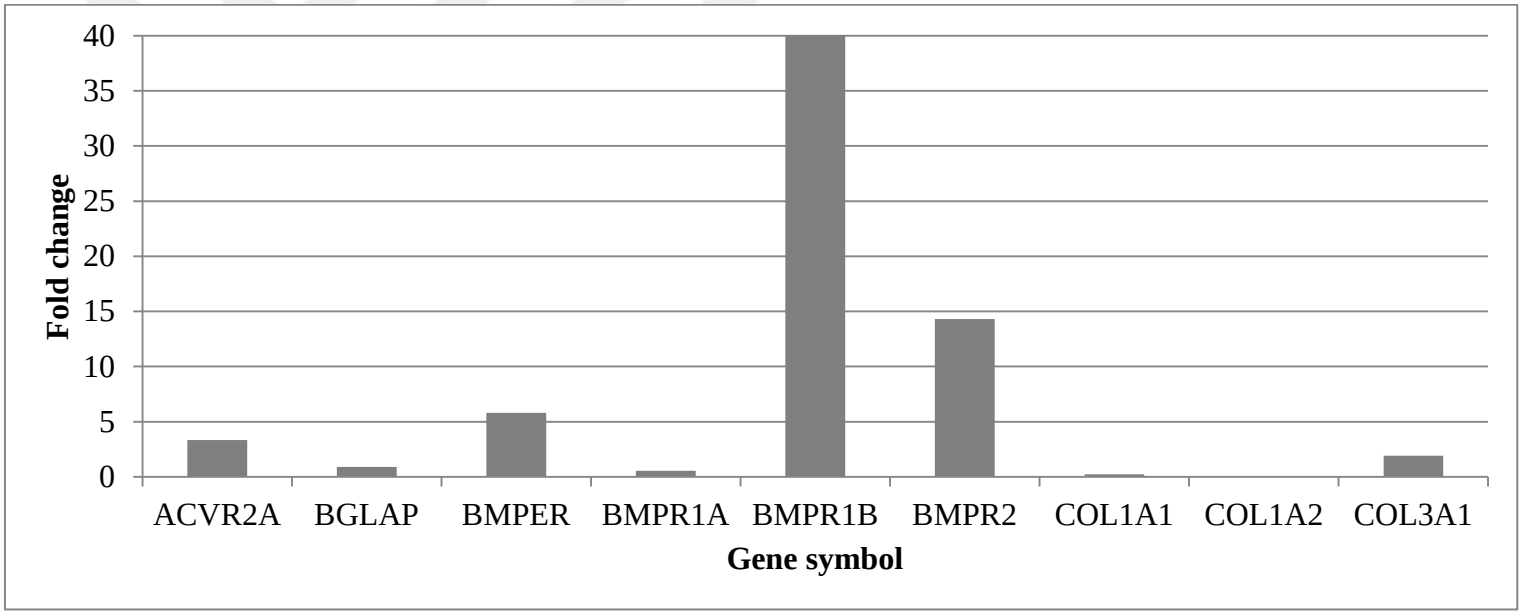

Figure 4. 8. The altered gene expressions of ECM proteins and BMP receptor genes in osteoblasts due to the *P.gingivalis* challenge

Fold change of mRNA level was calculated relative to the expression value in non-challanged cells

Fold-change values less than one indicate a negative or down-regulation

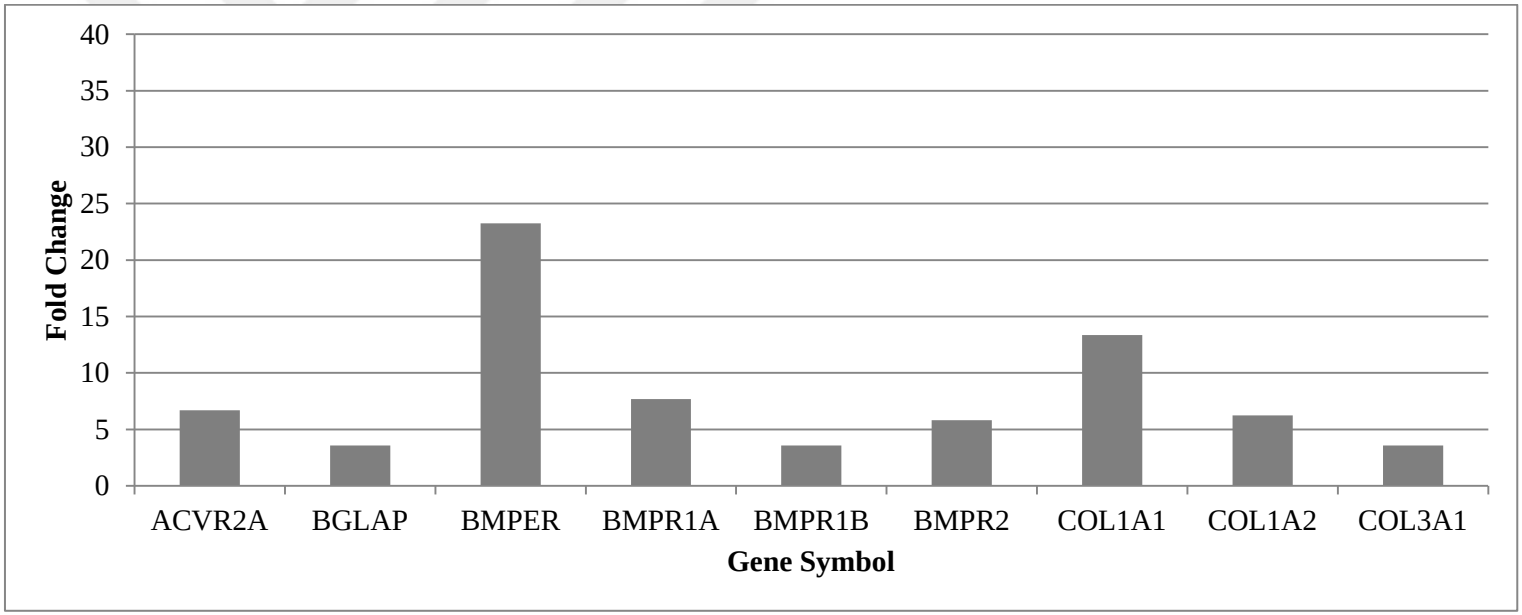

Figure 4. 9. The altered gene expressions of ECM proteins and BMP receptor genes in osteoblasts due to the *P. intermedia* challenge

Fold change of mRNA level was calculated relative to the expression value in non-challanged cells

Fold-change values less than one indicate a negative or down-regulation

Expressions of BMPR1A were differentially regulated by *P.g* and *P.i* challenges on the contrary to BMPER in this study. The expression of BMPR1A was found to be downregulated by *P.g* challenge (Fold Change: 0.551). However the gene was upregulated by *P.i* challenge (Fold Change: 7.6741). A previous study showed that deletion of BMPR1A in differentiated osteoclasts increased osteoblastic bone formation, thus suggesting that BMPR1A signaling in osteoclasts regulates coupling to osteoblasts by reducing bone-formation activity during bone remodeling [100]. Thus the reduced viability of osteoblasts following *P.g* challenge could be due to decreased levels of BMPR1A. The expression of type IB (BMPR1B) and bone morphogenetic protein receptor was highly upregulated with a fold change of 40.5042 in *P.g* challenge and an upregulation of 3.5801 fold change in *P.i* challenged cells. Although in a previous study with 3 different bone cells in vivo, Singhatanadgit et al. indicated that BMPR-B could be a therapeutic target for enhancing bone regeneration in vivo [101]. These findings could suggest that osteoblasts were still trying to induce repairing mechanism to inhibit the effect of bacterial challenges. The higher fold regulation of BMPR1B following *P.g* challenge to compare *P.i* challenge confirms the WST assay results which showed the more reduced viability of osteoblasts by *P.i* challenge at a MOI of 100 and 1000.

Another receptor studied was BMPR2, which showed upregulation in both *P.g* and *P.i* challenges (Fold Change: 14.3204, 5.8159 respectively). Although there is limited information about this gene it is found to be related to a heterozygous loss of function underlying the majority of familial cases (BMPR-II) pulmonary arterial hypertension [102].

The expression of bone gamma-carboxyglutamate protein BGLAP was found to be downregulated (Fold Change: 0.895) by *P.g* challenge whereas the gene were upregulated by *P.i* challenge (Fold Change 3.5801). BGLAP is also known as osteocalcin. BGLAP is a terminal marker of osteoblastic differentiation and has been suggested to play an inhibitory role during bone formation [103]. Ducy et al showed that osteocalcin-deficient mice showed an increase in bone formation suggesting that presence of osteocalcin is a marker of bone resorption [104]. The upregulation of BGLAP by *P.i* challenge therefore could inhibit bone formation, suuportingly the reduced viability of osteoblast was shown by

WST assay. On the other hand the downregulation of BGLAP by *P.g* challenge shows that *P.g* and *P.i* might have different mechanisms in affecting viability of osteoblasts.

Collagen is the most abundant organic component of bone. It constitutes a superfamily of extracellular matrix proteins whose primary function is structural. The major collagenous component of bone is type I collagen, but types III, V, and X collagens are also found in bone tissue. It was reported that collagen is essential for the growth and differentiation of various kinds of cells including osteoblasts [49]. Maehata et al. indicated that Type I and III collagens function as regulatory molecules for the differentiation and proliferation of human osteoblastic cells, and discusses the possibility that collagen metabolism is a possible new target for the treatment of osteoporosis [105]. COL1A1, COL1A2 and COL3A1 were studied in this study. Expressions of all collagens studied were found to be upregulated by *P.i* challenge (Fold change: 13.3614, 6.233 and 3.5801). COL3A1 was also found to be upregulated by *P.g* challenge (Fold change: 1.9185) however expressions of COL1A1 and COL1A2 showed downregulation (Fold change: 0.2398 and 0.008 respectively). The reduction in matrix mineralization in *P.g* challenged cells following Von Kossa staining is in accordance with the downregulation of COL1A1 and COL1A2.

Genes from the SMAD family were also examined (Figure 4.10 and 4.11). The SMAD gene family provides instructions for producing proteins that help regulate the activity of particular genes as well as cell growth and division (proliferation). The proteins carry out these functions as part of the transforming growth factor beta (TGF-β) pathway, which transmits signals from the outside of the cell to the nucleus. This type of signaling pathway allows the environment outside the cell to affect how the cell produces other proteins. SMAD1 mediates the signals of the BMPs, which are involved in a range of biological activities including cell growth, apoptosis, morphogenesis, development and immune responses [54]. Findings showed SMAD1 expression to be increased following both *P.g* and *P.i* challenge (Fold change: 4.1125 and 15.3482 respectively). All other SMAD genes studied, SMAD2, SMAD3, and SMAD4 were downregulated (Fold change, 0.985, 0.043, 0.0026 respectively) by *P.g* challenge. SMAD3 and SMAD5 were downregulated *P.i* challenge as well however expression pattern of SMAD4 was observed to be different since it was upregulated by *P.i* challenge (Fold change 4.724).

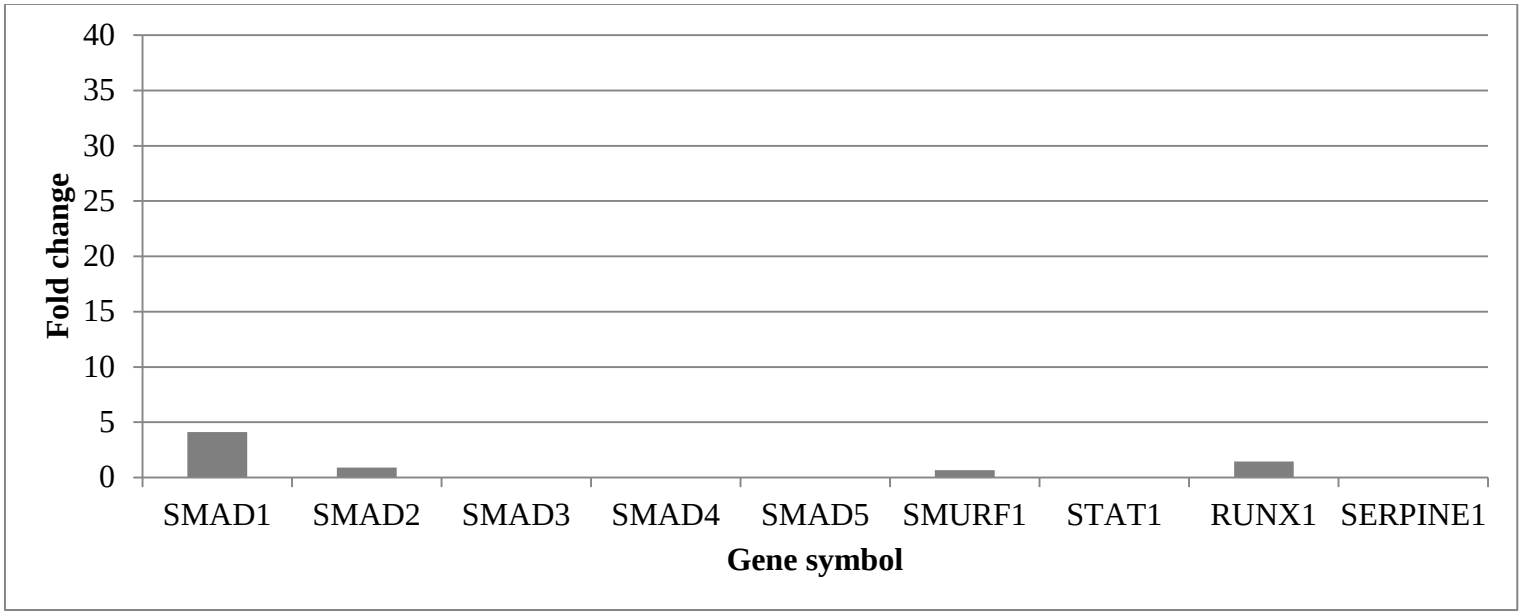

Figure 4. 10. The altered gene expressions of SMAD family genes in osteoblats due to the *P. gingivalis* challenge
Fold change of mRNA level was calculated relative to the expression value in non-challanged cells
Fold-change values less than one indicate a negative or down-regulation

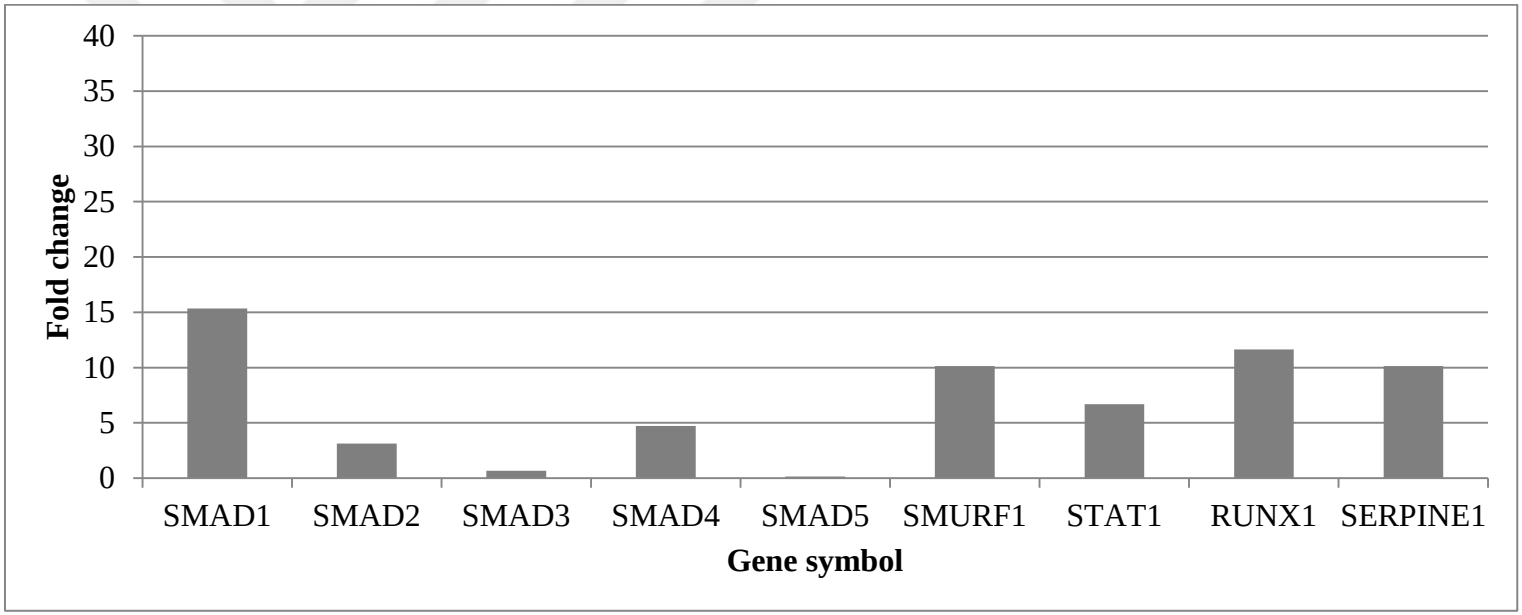

Figure 4. 11. The altered gene expressions of SMAD family genes in osteoblats due to the *P. intermedia* challenge

Fold change of mRNA level was calculated relative to the expression value in non-challanged cells

Fold-change values less than one indicate a negative or down-regulation

Wang et al. observed that osteoblast-specific SMAD1 conditional knockout (cKO) mice showed impaired osteoblast proliferation and differentiation suggesting SMAD1 to have play a role in osteoblast proliferation [106] . However in our results we observed the reduction of osteoblast viability possibly meaning that the bacterial must have another mechanism of inducing reduction of osteoblasts. However our results are in consistent with Murakami, et al. who indicated that BMPR-IB activates SMAD1-dependent pathways [107]. In this study the upregulation of both BMPR-IB and SMAD1 in both bacterial challenges was observed, thereby demonstrating the the activation of SMAD1 dependent pathways. A previous study by Hellingman et al showed that blocking Smad2/3P after the onset of chondrogenesis resulted in a halt in collagen II production [108]. Similarly by *P.g* challenge SMAD2 and SMAD3 and COL1A1 and COL1A2 showed downregulation. Another receptor SMURF1 was also seen to be downregulated by *P.g* challenge further supporting findings by Zhao et al. who found that SMURF1 inhibits osteoblast differentiation and bone formation in vitro and in vivo [109]. This reduction in gene expression is probably the reason for reduced matrix mineralization. Furthermore, Borton et al demonstrated that loss of SMAD3 resulted in a lower rate of bone formation [110]. As indicated in our results SMAD3 was downregulated by in both bacterial challenges. This downregulation further supports our findings of reduced osteoblast viability. In anoher study Moser et al. showed that BMPER is a secreted protein that directly interacts with BMP2, BMP4, and BMP6 and antagonizes BMP4-dependent Smad5 activation [111]. An upregulation of BMPER and at the same time downregulation of SMAD5 show that this mechanism was induced by *P.g* and *P.i* challenges. STAT was another that was downregulated by *P.g* challenge, and this gene was also studied by Tajima et al. and supportingly they found that inhibition of STAT1 accelerated bone fracture healing [112]. However the upregulation of both SMURF1 and STAT by *P.i* challenge (Fold Change 10.1261 and 6.6807) shows that *P.i* affects osteoblasts in another way to reduce both viability and mineralization.

The expression of RUNX1 slightly changed upon *P.g* challenge (Fold change: 1.454) but was highly upregulated by *P.i* challenge (Fold change: 11.6318). We expected reduced regulation since it is a critical regulator of osteoblast differentiation in vertebrates, also a regulator of bone formation by differentiated osteoblasts beyond development. Smith et al.

showed that Runx1 plays a role in mediating early events of endochondral and intramembranous bone formation in mutant mouse models [113].

The expression of SERPINE1 was also studied and showed differential regulation by *P.g* and *P.i* challenge. By a challenge of *P.g* this gene was found to be downregulated (Fold change: 0.0212) however by *P.i* challenge fold regulation was as high as 10.1261. Bizzarro et al. indicated that Periodontitis is characterized by elevated SERPINE11 activity [114]. Therefore such a high regulation of this gene is not suprising since *P.i* is one of the most abundant pathogens isolated from periodontitis patients. However the downregulation of SERPINE1 by *P.g* challenge confirms again that these microorganisms have a different inhibitory or destructive mechanism on osteoblasts.

It has been shown that endogenous TGF-β acts directly on osteoblasts to regulate bone remodeling, structure and biomechanical properties [115]. Thereby these results are confirming TGF-β receptors as key element for the activation of this pathway on osseointegration. In this study, TGF-β receptors were also studied for their relation to bone formation (Figure 4.12 and 4.13). The expressions of, TGFBR1 and TGFBR3 were increased in *P.g* challenged cells (3.5801 and 3.1167 respectively). Furthermore, the expression of TGFB1, TGFBR2 and TGIF1 were decreased by *P.g* challenge (0.0424, 0.0974, and 0.0974 respectively). In the *P.i* challenged cells the expressions of TGFB1, TGFBR1 were found to be upregulated (6.2333 and 1.4540 respectively), while the expression of TGFBR2, TGFBR3 and TGIF1 were decreased (0.1817, 0.5141, 0.6329 respectively). Filvarof et al. also showed that Inhibition of TGF-beta receptor signaling in osteoblasts leads to decreased bone remodeling and increased trabecular bone mass[116]. So we assume that the downregulation of TGF-β receptors observed in our study could be another factor leading to decreased bone remodeling that was seenin the WST assay. The increase in the other type of TGF-β receptors that was observed shows that not all receptors have function to reduce bone remodeling in the case of *P.g* and *P.i* challenged cells.

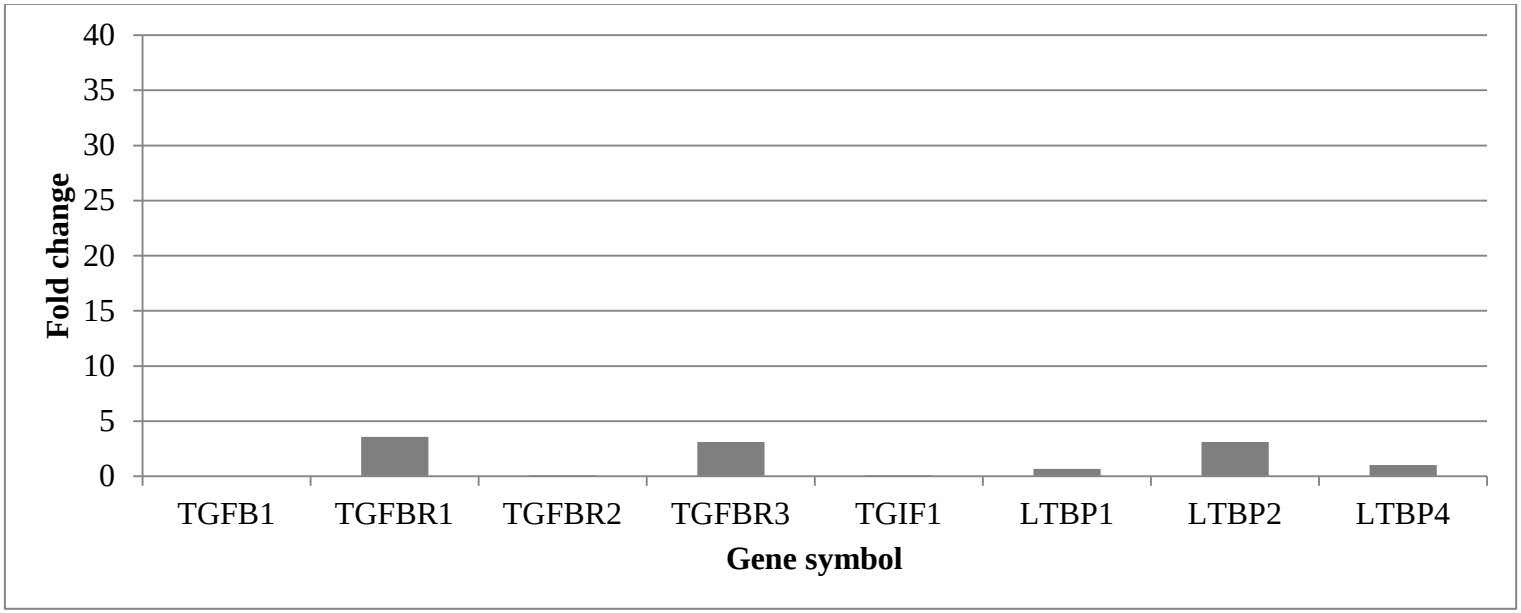

Figure 4. 12. The altered gene expressions of TGF-β receptors and LTP genes osteoblast due to the *P. gingivalis* challenge

Fold change of mRNA level was calculated relative to the expression value in non-challanged cells

Fold-change values less than one indicate a negative or down-regulation

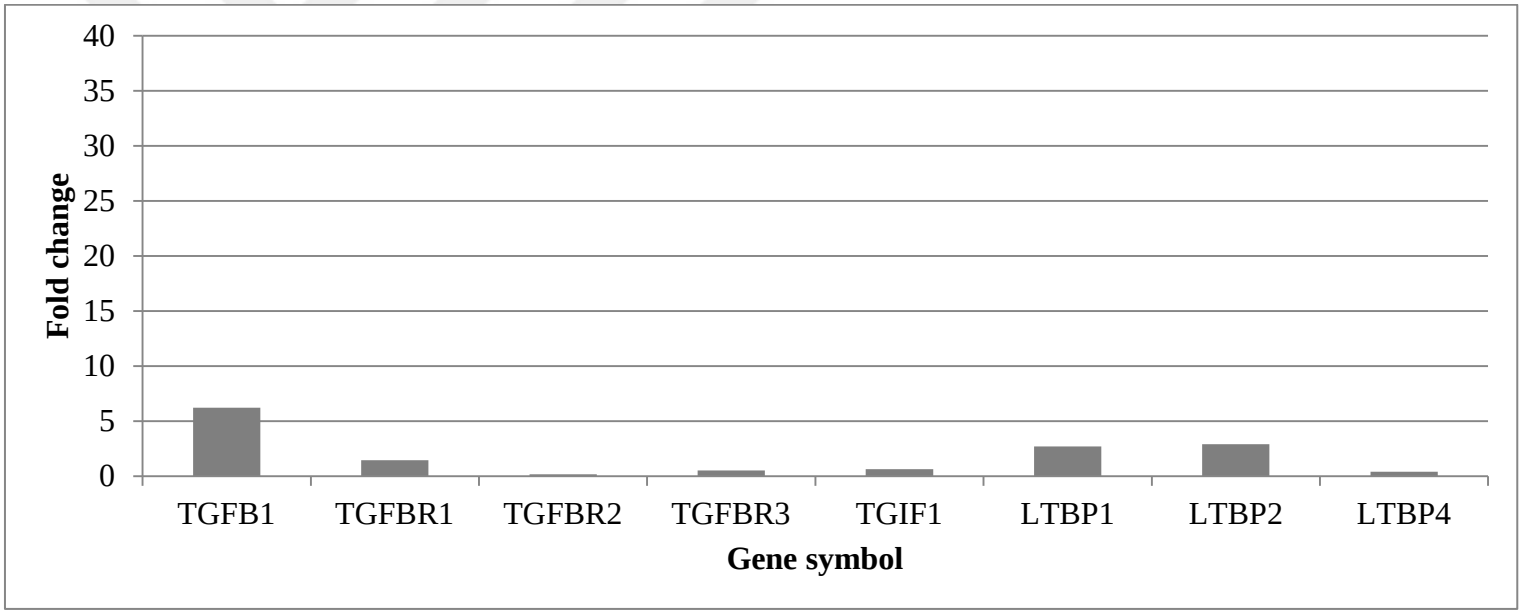

Figure 4. 13. The altered gene expressions of TGF-β receptors and LTP genes in osteoblasts due to the *P. intermedia* challenge

Fold change of mRNA level was calculated relative to the expression value in non-challanged cells

Fold-change values less than one indicate a negative or down-regulation

The latent TGF-beta binding proteins (LTBP), which is a family of carrier proteins, that were originally identified by their association with the latent form of transforming growth factors [117]. They interact with a variety of extracellular matrix proteins and may play a role in the regulation of TGF beta bioavailability. Our findings showed LTBP1 and LTBP4 to be differentially regulated by *P.g* and *P.i* challenges. In the *P.g* challenged cells LTBP1 were downregulated (Fold Change: 0.6783) and LTBP4 expression was slightly upregulated with a fold change of 1.0281. On the other hand, *P.i* challenged cells expressed an increase in LTBP1 (Fold Change: 2.7132) and a decrease in LTBP4 (Fold Change: 0.4175). However, LTBP2, expression was found to be very similar for both bacteria, with a fold change of 2.9079 in *P.i* challenged cells and 3.1167 in *P.g* challenged cells. The latent TGF-β binding protein is a structural extracellular matrix protein involved in the formation of large fibrillar structures in the extracellular matrix of bone cells [118]. So decreased matrix mineralization in both challenges is might be due to different latent factors since their differential regulations. Additionally LTBP appears to play a role in bone formation in vitro and its localization in vivo is also suggestive of a role in bone development and formation [119]. So LTBP could also be another factor that plays a role in reduced viability of osteoblasts.

In the TLR pathway, genes related to toll like receptor, Interleukin 1 (IL), mitogen-activated protein kinase (MAP), and nuclear factor-kappa-B (NF-κB) family were examined. All these pathways are known to have a role in bone regeneration and the cell cycle.

TLRS are members of IL1 receptor superfamily. These are pattern recognition receptors that recognize foreign substances in the body and activate immune system. TLR4 was seen to be upregulated by both *P.g* and *P.i* (Fold change: 1.2311 and 2.1435 respectively) (Figure 4.14 and 4.15). Since this gene is related to recognition of LPS on bacterial cells such a finding was not surprising. This receptor has also shown to be upregulated by *P.gingivalis* fimbrae in human aortic endothelial cells and other bacterial infections [120]. In addition Sun et al. showed an increase in gene expression of TLR4 in human periodontal ligament cells by *P.i* challenged cells [121]. Sartori et al. indicated that TLR-4 signaling activates MYD88-dependent pathways to subsequent activation of IRAK, TRAF6, and, ultimately NF-κB, which is required for cytokine induction [122]. Upregulation of MYD88, IRAK2 and NF-κB observed in this study is consistent with these findings.

Conversely all other TLR studied TLR3, TLR5 and TLR6 were downregulated by *P.g* and *P.i* challenge (0.0206, 0.003, 0.0048 and 0.0254, 0.0015, 0.0021 respectively). Similar results have been seen in a study with filarial infection, where a downregulation of TLR3 and TLR5 were observed in human dendritic cells following helminth parasite infection [123]. Our findings suggest that *P.g* and *P.i* are likely to act via TLR4.

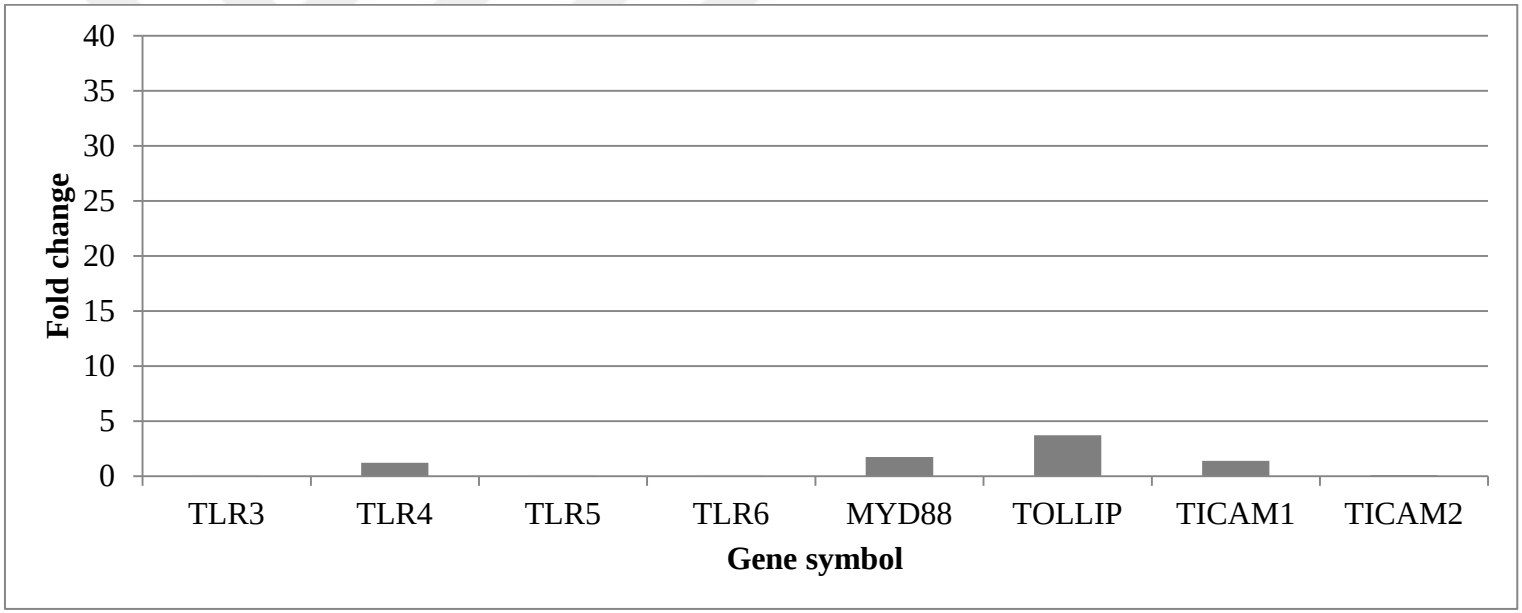

Figure 4. 14. The altered gene expressions of TLR family genes in osteoblasts due to the *P.gingivalis* challenge

Fold change of mRNA level was calculated relative to the expression value in non-challanged cells

Fold-change values less than one indicate a negative or down-regulation

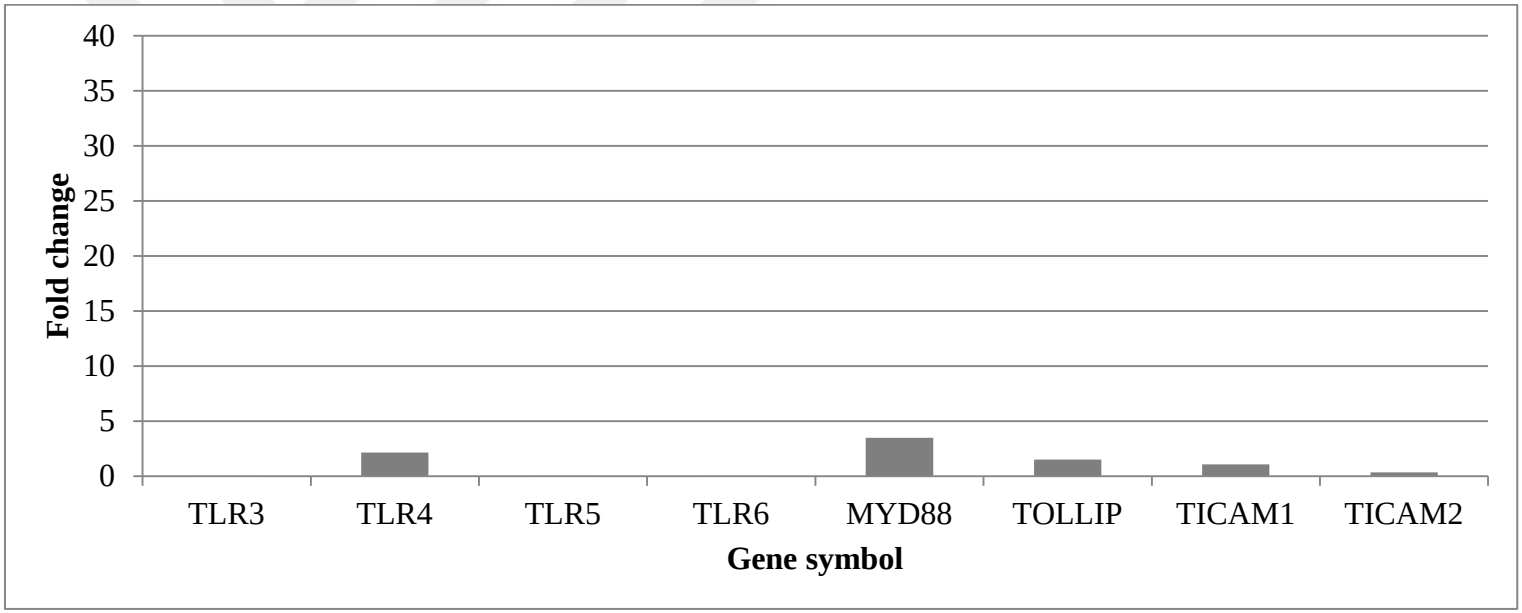

Figure 4. 15. The altered gene expressions of TLR family genes in osteoblasts due to the *P. gingivalis* challenge

Fold change of mRNA level was calculated relative to the expression value in non-challanged cells

Fold-change values less than one indicate a negative or down-regulation

MYD88 is a universal adapter protein as it is used by all TLRs (except TLR 3) to activate the transcription factor NF-κB. The expression of MYD88 was found to be upregulated in both *P.g* and *P.i* challenged cells (1.7411 and 3.4822 respectively). Similar findings have been reported in a study were an increase in the expression of MYD88 was found following *Salmonella* infection [124]. In addition a higher regulation of TLR4 and MYD88 by *P.i* challenge might be the reason for more destructive result of osteoblast viability by *P.i* challenge compared to *P.g* challenge.

TOLLIP is an inhibitory adaptor protein which was upregulated in this study by both *P.g* and *P.i* challenge (3.7321 and 1.5157 respectively). TOLLIP negatively regulates LPS/TLR4 signaling [125], therefore the suppressed expressions of TLRs could be due to upregulation of this gene.

TICAM1 and TICAM2 are domain-containing adaptor proteins involved in TLR4 signaling. In this study the expression of TICAM1 was found to be upregulated in both both *P.g* and *P.i* challenge (1.4142 and 1.0718 respectively). However the expression of TICAM2 was downregulated in both *P.g* and *P.i* challenge (0.0947 and 0.3536 respectively). So TICAM1 and TICAM2 are affected in the same way by both bacterial challenges. Oshiumi et al indicated that TICAM-2 physically bridges TLR4 and TICAM-1 and functionally transmits LPS-TLR4 signaling to TICAM-1 [126]. However, according to our results although TICAM-1 showed an upregulation, TICAM-2 did not. This could mean that there is another adaptor protein that acts on TLR and TICAM1 following *P.g* and *P.i* challenges. It has also been shown that TICAM1 is dominant in host protection and innate immune responses against poliovirus infection [127].

Cytokines from the IL-1 family were also studied (Figure 4.16 and 4.17). The IL1 family plays a central role in the regulation of immune and inflammatory responses. Interestingly the expressions of all interleukins apart from IL8 were downregulated by both bacterial challenges. IL8 showed an upregulation with a large fold change of 17.1484 in *P.g* challenge, and 12.1257 in *P.i* challenge. Similar finding have shown IL8 to be upregulated by oxidative stress [128]. IL8 is a member of the proinflammatory cytokines that change the microenvironment and even regulate deleteriously host cellular mechanisms at the site of infection. Such anupregulation might could be indicative of osteoblast contamination by bacterial invasion or adhesion. Expressions of IL1A, IL1B, IL6 and IL12A were decreased by both *P.g* and *P.i* challenges (Fold Change: 04665, 05359, 0.3536, 0.009 and 0.3789, 0.5359, 0.0825, 0.0055). Although IL1B and IL6 are proinflammatory cytokines, IL1B and IL6 have also been seen to be dowregulated by LPS induction in sepsis [98]. Since TGF-β is an inhibitor of IL12 production [129] perhaps the the upregulation of TGF- β (in *P.i* challenge) suppressed the expression of IL12 observed in our study. IL1A was found to be upregulated by *P.g* challenge in epithelial cells however also a high expression of IL8 might have suppressed the IL1A.

Challenge of osteoblasts by *P.g*. and *P.i* lead to an upregulation in IRAK 2 expression (Fold Change: 3.0314 and 5.6569). Since IRAK2 expression plays a role in the induction of ILs which in turn activates the immune system, we would expect to see such a response [130]. A previous study also showed such a relationship between IRAK2 and IL in relation to bone resorption [131]. Previous studies have shown IRAK2 polymorphisms to play a role in post-menopausal bone loss [132]. The higher upregulation of IL8 observed in our results could be related to the increased expression of IRAK2. Conversely the expression of IRAK1 was found to be downregulated following bacterial challenge in our study. These findings are supported by a previous study that suggests a significantly independent regulation of IRAK-1 and IRAK-2 in human astroglial cells (HAG) [133]. The same study also suggested inducible NF-κB-sensitive, miRNA-146a-mediated down-regulation of IRAK-1 results in NF-κB induced up-regulation of IRAK-2 expression which drives an extensively sustained inflammatory response.

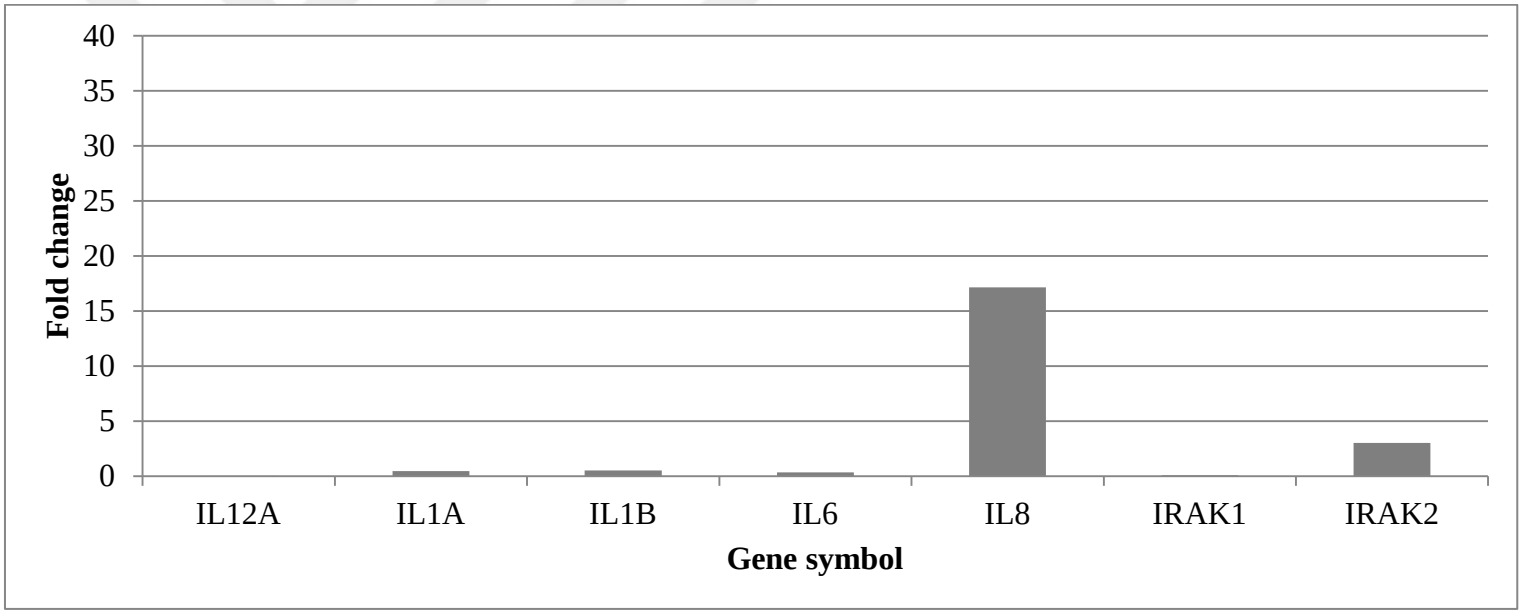

Figure 4. 16. The altered gene expressions of IL1 family and IL1 receoptor genes in osteoblats due to the *P. gingivalis* challenge

Fold change of mRNA level was calculated relative to the expression value in non-challanged cells

Fold-change values less than one indicate a negative or down-regulation

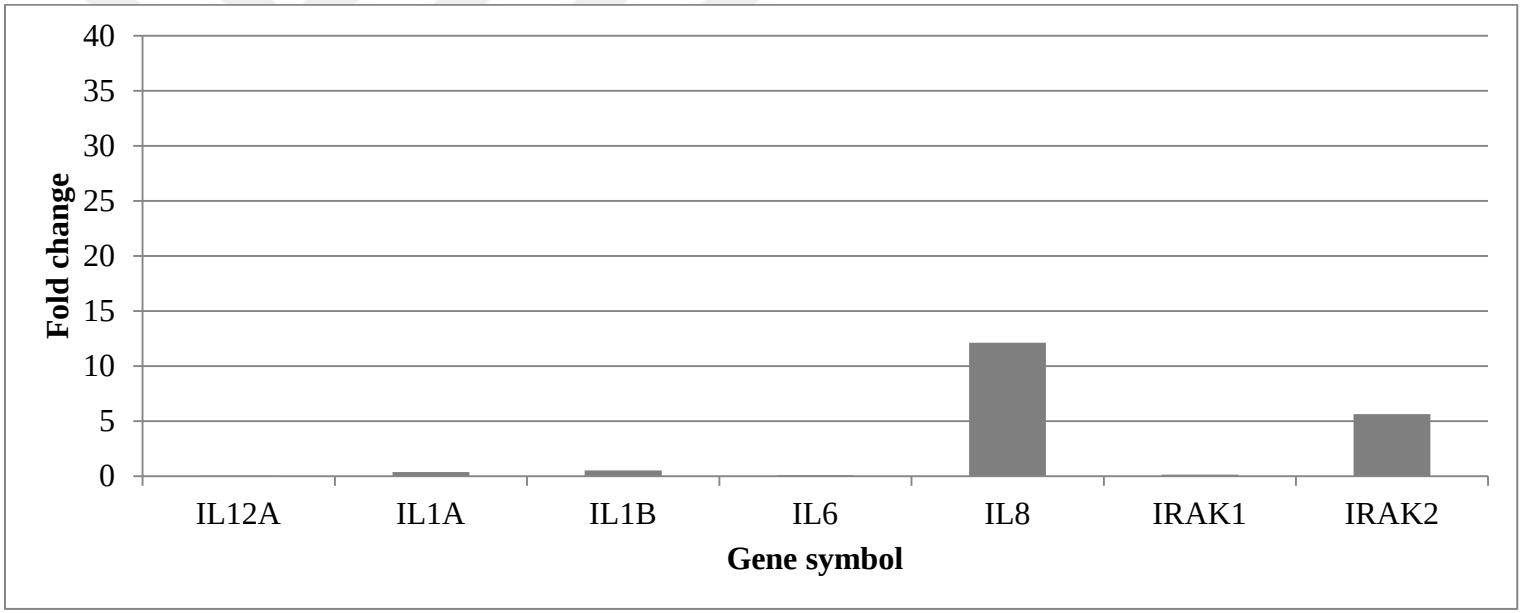

Figure 4. 17. The altered gene expressions of IL1 family and IL1 receoptor genes in osteoblats due to the *P. intermedia* challenge

Fold change of mRNA level was calculated relative to the expression value in non-challanged cells

Fold-change values less than one indicate a negative or down-regulation

These findings are consistent with our results since an upregulation of NF-κB1 was found by both *P.g* and *P.i* challenge NF-κB is activated by many divergent stimuli, including bacteria and lipopolysaccharides (LPS), viruses, viral proteins, double-stranded RNA, and physical and chemical stresses [134].

The expression of NF-κB1 was seen to be upregulated by both *P.g* and *P.i* challenge (Fold Change: 2.0 and 1.8661 respectively) (Figure 4.18 and 4.19). This upregulation of the gene is probably is a result of stimulation by the LPS of the bacteria. Similar findings have been seen in a study by Chou et al., where *P. gingivalis* fimbria-mediated invasion upregulated NF-κB1 expression in human aortic endothelial cells. LPS of *P.i* was also shown to enhance NF-κB1 binding activity in human gingival fibroblasts [135].

The Upregulation of NF-κB1 gives rise to the upregulation of NFRKB. NFRKB was also upregulated when challenged by both bacteria. *P.g* challenged cells were seen to increase NFRKB expression 6 fold more than unchallenged osteoblasts. Furthermore, this expression was much higher in *P.i* challenged osteoblasts (21.1121 fold) compared to unchallenged cells. Although there are limited studies on NFRKB, it has been suggested that NFRKB may be involved in the disorders of transcriptional regulation commonly observed in minimal change nephrotic syndrome relapse [136]. Our findings saw that cells challenged with *P.g* and *P.i* decreased the level of NFKBIA expression. This is not suprising since NFKBIA is an inhibitor of NFKB. This data reveals the NF-κB pathway was induced by bacterial challenge. In addition the increase in NF-κB could be one of the reasons for IL8 upregulation since many studies have shown an induction of IL8 by NF-κB expression [137].

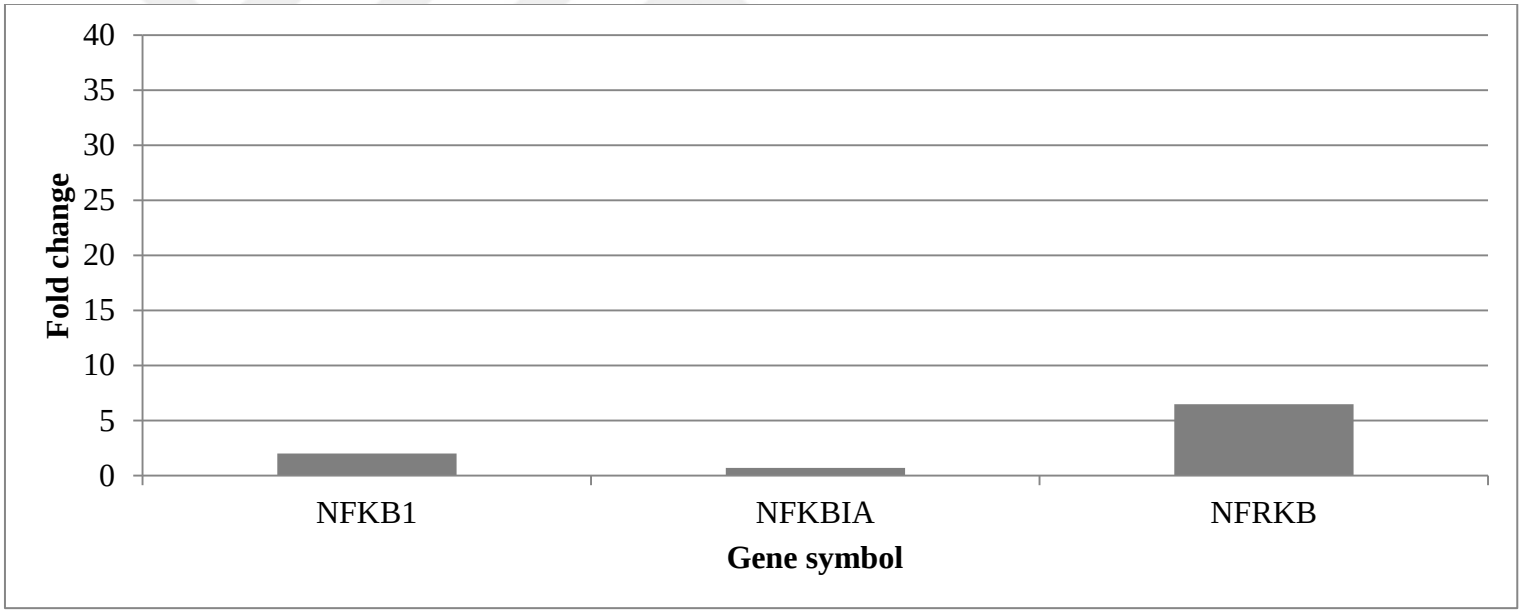

Figure 4. 18. The altered gene expressions of NF-κB family genes in osteoblats due to the *P. gingivalis* challenge
Fold change of mRNA level was calculated relative to the expression value in non-challanged cells
Fold-change values less than one indicate a negative or down-regulation

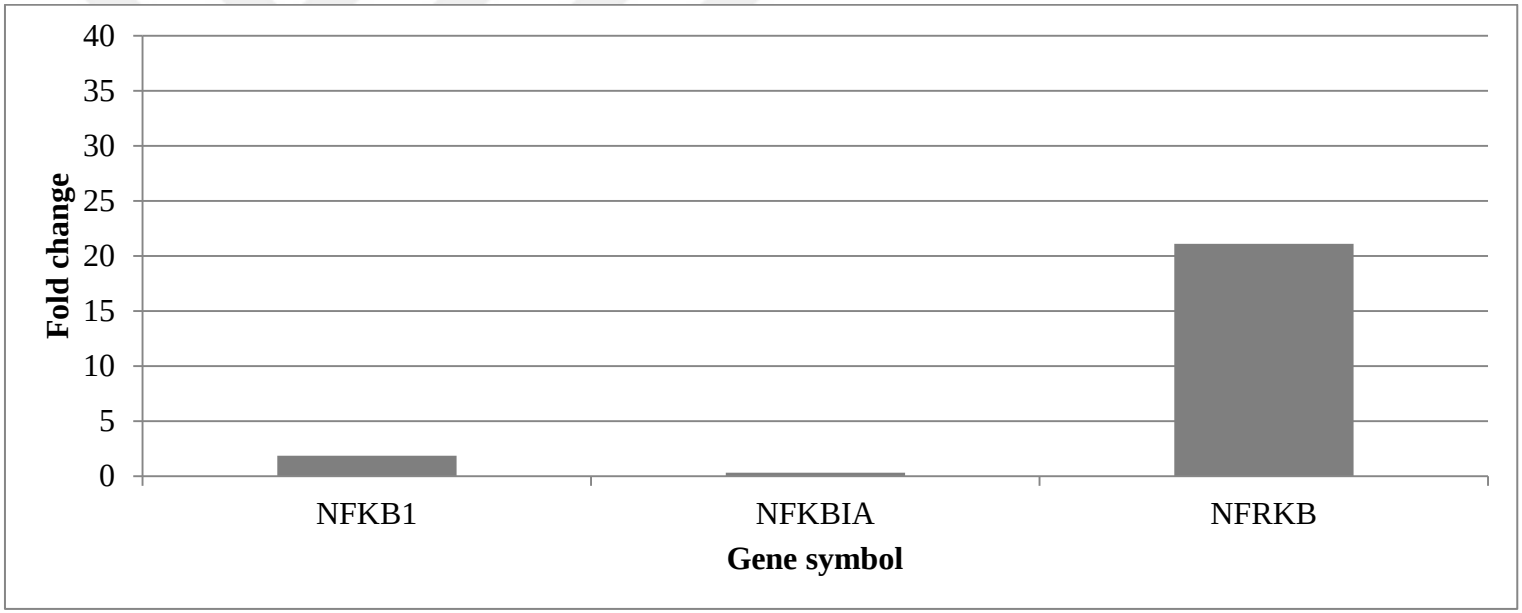

Figure 4. 19. The altered gene expressions of NF-κB family genes in osteoblats due to the *P. intermedia* challenge

Fold change of mRNA level was calculated relative to the expression value in non-challanged cells

Fold-change values less than one indicate a negative or down-regulation

The mitogen-activated protein (MAP) kinase phosphatase from the (MKP) family, plays an important function in regulating the pro-inflammatory cytokines by deactivating MAP kinases.

The levels of MAP2K3, MAP2K4 and MAP3K1 expression were decreased by *P.g* challenge (Fold Change: 0.2031, 0.1166, 0.0136 respectively) (Figure 4.20 and 4.21) Similar results were shown in the *P.i* challenged cells. Expressions of MAP2K3, MAP2K4 and MAP3K1 were also found to be reduced (Fold Change 0.0947, 0.1539, 0.0156). Conversely expression of MAP4K4 showed an upregulation by *P.g* challenge (Fold Change: 1.3195). However, interestingly expression of MAP4K4 did not change in *P.i* challenged cells.

Riewe et al. showed that *P. gingivalis* infection induced phosphorylation and activation of MAPK2K3 in human extravillous trophoblasts [138]. Different results could be due to the different cell line and strains used. Although there are not many studies on MAP3K1, an overexpression of MAP3K1 in extramammary Paget disease has been previously found [139]. Similarly a increased levels of MAPK4K and NFKB1 after phagocytosis of bacterial pathogens was also observed [140].

Interestingly *P.g* and *P.i* challenges showed same direction in the expressions of all genes studied apart from MAP4K4 on the contrary to TGF-β pathway genes in which most of the genes showed differential regulation.

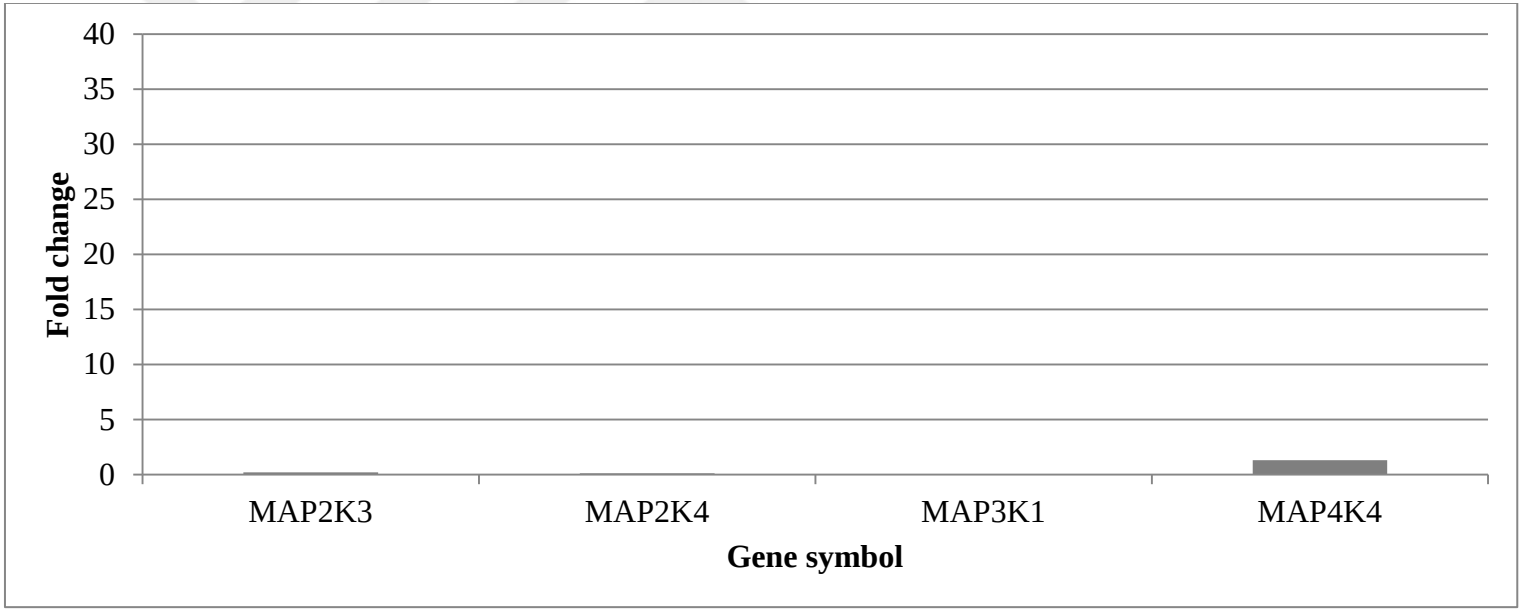

Figure 4. 20. The altered gene expressions of MAP family genes in osteoblasts due to the *P. gingivalis* challenge
Fold change of mRNA level was calculated relative to the expression value in non-challanged cells
Fold-change values less than one indicate a negative or down-regulation

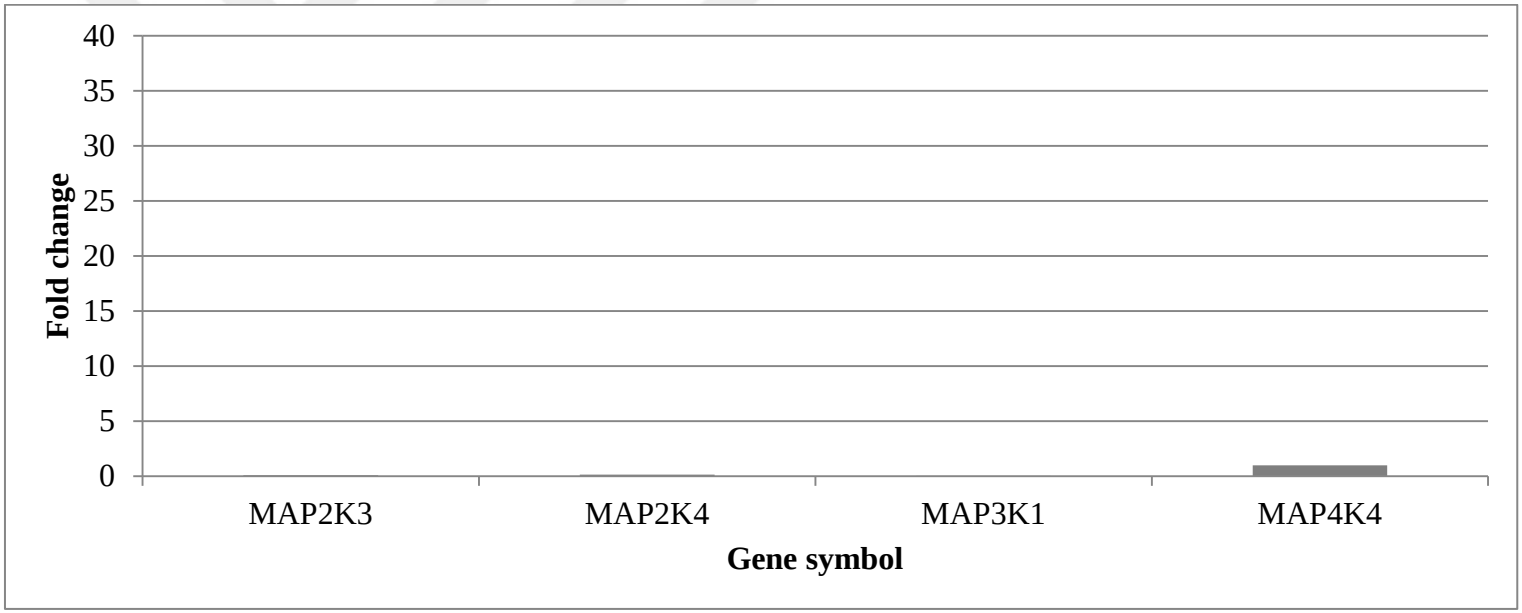

Figure 4. 21. The altered gene expressions of MAP family genes in osteoblasts due to the *P. intermedia* challenge

Fold change of mRNA level was calculated relative to the expression value in non-challanged cells

Fold-change values less than one indicate a negative or down-regulation

Table 4. 2. Fold Change of TGF-β pathway genes in hFOB cells following challenge by *P.g*

Gene description	Gene symbol	Accession no*	Fold change [a]
Activin A receptor, type I	ACVR2A	NM_001616	3,340
Bone gamma-carboxyglutamate (gla) protein	BGLAP	NM_199173	0,895
BMP binding endothelial regulator	BMPER	NM_133468	5,816
Bone morphogenetic protein receptor, type IA	BMPR1A	NM_004329	0,551
Bone morphogenetic protein receptor, type IB	BMPR1B	NM_001203	40,504
Bone morphogenetic protein receptor, type II	BMPR2	NM_001204	14,320
Collagen, type I, alpha 1	COL1A1	NM_000088	0,240
Collagen, type I, alpha 2	COL1A2	NM_000089	0,008
Collagen, type III, alpha 1	COL3A1	NM_000090	1,919
SMAD family member 1	SMAD1	NM_005900	4,113
SMAD family member 2	SMAD2	NM_005901	0,895
SMAD family member 3	SMAD3	NM_005902	0,004
SMAD family member 4	SMAD4	NM_005359	0,003
SMAD family member 5	SMAD5	NM_005903	0,016
SMAD specific E3 ubiquitin protein ligase 1	SMURF1	NM_020429	0,678
Signal transducer and activator of transcription 1, 91kDa	STAT1	NM_007315	0,002
Runt-related transcription factor 1	RUNX1	NM_001754	1,454
Serpin peptidase inhibitor, clade E, member 1	SERPINE 1	NM_000602	0,021
Transforming growth factor, beta-induced,	TGFB1	NM_000660	0,042
Transforming growth factor, beta receptor 1	TGFBR1	NM_004612	3,580
Transforming growth factor, beta receptor 2	TGFBR2	NM_003242	0,097
Transforming growth factor, beta receptor 3	TGFBR3	NM_003243	3,117
TGFB-induced factor homeobox 1	TGIF1	NM_003244	0,097

Table 4. 3. Fold Change of TGF-β pathway genes in hFOB cells following challenge by *P.g* (Continued)

Latent transforming growth factor beta binding protein 1	LTBP1	NM_000627	0,678
Latent transforming growth factor beta binding protein 2	LTBP2	NM_000428	3,117
Latent transforming growth factor beta binding protein 4	LTBP4	NM_003573	1,028

*Accession numbers indicate the sequence used as SuperArray analysis

[a] Fold change of mRNA level was calculated relative to the expression value in non-challanged cells.

Fold-change values less than one indicate a negative or down-regulation.

Table 4. 4. Fold Change of TGF-β pathway genes in hFOB cells following challenge by *P.i*

Gene description	Gene symbol	Accession no*	Fold change [a]
Activin A receptor, type I	ACVR2A	NM_001616	6,681
Bone gamma-carboxyglutamate (gla) protein	BGLAP	NM_199173	3,580
BMP binding endothelial regulator	BMPER	NM_133468	23,264
Bone morphogenetic protein receptor, type IA	BMPR1A	NM_004329	7,674
Bone morphogenetic protein receptor, type IB	BMPR1B	NM_001203	3,580
Bone morphogenetic protein receptor, type II	BMPR2	NM_001204	5,816
Collagen, type I, alpha 1	COL1A1	NM_000088	13,361
Collagen, type I, alpha 2	COL1A2	NM_000089	6,233
Collagen, type III, alpha 1	COL3A1	NM_000090	3,580
SMAD family member 1	SMAD1	NM_005900	15,348
SMAD family member 2	SMAD2	NM_005901	3,117
SMAD family member 3	SMAD3	NM_005902	0,678
SMAD family member 4	SMAD4	NM_005359	4,724
SMAD family member 5	SMAD5	NM_005903	0,148
SMAD specific E3 ubiquitin protein ligase 1	SMURF1	NM_020429	10,126
Signal transducer and activator of transcription 1	STAT1	NM_007315	6,681
Runt-related transcription factor 1	RUNX1	NM_001754	11,632
Serpin peptidase inhibitor, clade E, member 1	SERPINE1	NM_000602	10,126
Transforming growth factor, beta-induced, 68kDa	TGFB1	NM_000660	6,233
Transforming growth factor, beta receptor 1	TGFBR1	NM_004612	1,454
Transforming growth factor, beta receptor 2	TGFBR2	NM_003242	0,182
Transforming growth factor, beta receptor 3	TGFBR3	NM_003243	0,514
TGFB-induced factor homeobox 1	TGIF1	NM_003244	0,633
Latent transforming growth factor beta binding protein 1	LTBP1	NM_000627	2,713
Latent transforming growth factor beta binding protein 2	LTBP2	NM_000428	2,908

Table 4. 5. Fold Change of TGF-β pathway genes in hFOB cells following challenge by *P.i* (Continued)

Latent transforming growth factor beta binding protein 4	LTBP4	NM_003573	0,418

*Accession numbers indicate the sequence used as SuperArray analysis

[a] Fold change of mRNA level was calculated relative to the expression value in non-challanged cells.

Fold-change values less than one indicate a negative or down-regulation.

Table 4. 6. Fold change of TLR pathway in hFOB cells following challenge by *P. g*

Gene description	Gene symbol	Accession no*	Fold change[a]
Toll-like receptor 3	TLR3	NM_003265	0,025
Toll-like receptor 4	TLR4	NM_138554	1,231
Toll-like receptor 5	TLR5	NM_003268	0,002
Toll-like receptor 6	TLR6	NM_006068	0,002
Myeloid differentiation primary response gene (88)	MYD88	NM_002468	1,741
Toll interacting protein	TOLLIP	NM_019009	3,732
Toll-like receptor adaptor molecule 1	TICAM1	NM_182919	1,414
Toll-like receptor adaptor molecule 2	TICAM2	NM_021649	0,095
Interleukin 12A	IL12A	NM_000882	0,009
Interleukin 1A	IL1A	NM_000575	0,467
Interleukin 1B	IL1B	NM_000576	0,536
Interleukin 6	IL6	NM_000600	0,354
Interleukin 8	IL8	NM_000584	17,148
Interleukin-1 receptor-associated kinase 1	IRAK1	NM_001569	0,095
Interleukin-1 receptor-associated kinase 2	IRAK2	NM_001570	3,031
Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	NFKB1	NM_003998	2
NFKB inhibitor, alpha	NFKBIA	NM_003998	0,707
Nuclear factor related to kappaB binding protein	NFRKB	NM_006165	6,498
Mitogen-activated protein kinase kinase 3	MAP2K3	NM_002756	0,203
Mitogen-activated protein kinase kinase 4	MAP2K4	NM_003010	0,117
Mitogen-activated protein kinase kinase kinase 1	MAP3K1	NM_005921	0,014
Mitogen-activated protein kinase kinase kinase kinase 4	MAP4K4	NM_004834	1,32

*Accession numbers indicate the sequence used as SuperArray analysis

[a] Fold change of mRNA level was calculated relative to the expression value in non-challanged cells.

Fold-change values less than one indicate a negative or down-regulation.

Table 4. 7. Fold change of TLR pathway in hFOB cells following challenge by *P.i*

Gene description	Gene symbol	Accession no*	Fold change[a]
Toll-like receptor 3	TLR3	NM_003265	0,021
Toll-like receptor 4	TLR4	NM_138554	2,144
Toll-like receptor 5	TLR5	NM_003268	0
Toll-like receptor 6	TLR6	NM_006068	0,005
Myeloid differentiation primary response gene (88)	MYD88	NM_002468	3,482
Toll interacting protein	TOLLIP	NM_019009	1,516
Toll-like receptor adaptor molecule 1	TICAM1	NM_182919	1,072
Toll-like receptor adaptor molecule 2	TICAM2	NM_021649	0,354
Interleukin 12A	IL12A	NM_000882	0,006
Interleukin 1A	IL1A	NM_000575	0,379
Interleukin 1B	IL1B	NM_000576	0,536
Interleukin 6	IL6	NM_000600	0,083
Interleukin 8	IL8	NM_000584	12,126
Interleukin-1 receptor-associated kinase 1	IRAK1	NM_001569	0,144
Interleukin-1 receptor-associated kinase 2	IRAK2	NM_001570	5,657
Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	NFKB1	NM_003998	1,866
NFKB inhibitor, alpha	NFKBIA	NM_003998	0,33
Nuclear factor related to kappaB binding protein	NFRKB	NM_006165	21,112
Mitogen-activated protein kinase kinase 3	MAP2K3	NM_002756	0,095
Mitogen-activated protein kinase kinase 4	MAP2K4	NM_003010	0,154
Mitogen-activated protein kinase kinase kinase 1	MAP3K1	NM_005921	0,016
Mitogen-activated protein kinase kinase kinase kinase 4	MAP4K4	NM_004834	1

*Accession numbers indicate the sequence used as SuperArray analysis

[a] Fold change of mRNA level was calculated relative to the expression value in non-challanged cells.

Fold-change values less than one indicate a negative or down-regulation.

4.2. MANUAL QUANTITATIVE REAL TIME PCR (QRT-PCR)

Selected genes from RT Profiler Array; Col1A and genes ALP and BGN that are related to matrix mineralization and osseointegration were also studied by manual qPCR. Fold regulations of these genes are shown in Figure 4.22 and 4.23. Collagen type 1A expression were slightly upregulated in both by *P.g* and *P.i* challenges (Fold Change: 1.3535 and 1.528 respectively) Expression of alkaline phosphatase was also shown to increase in *P.g* challenge (Fold Change: 1.5547), however the expression was found to decrease in *P.i* challenge (Fold Change 0.7657). In case of biglycan expression the situation was vice versa. Expression of BGN was downregulated by *P.g* challenge (Fold Change: 0.5116) and upregulated by *P.i* challenge (Fold Change: 2.0946). This finding was also observed in a study by Murata et al, where extracts of *P.i* and *Actinobacillus actinomycetemcomitans* inhibited alkaline phosphatase activity in osteoblastic cells in vitro[141]. This observation (reduced expression of ALP), could be related to the reduction in mineralization in *P.i* challenged cells is due to. Furthermore, the reduction in mineralization of osteoblasts by *P.g* challenge could be due to decreased expression of BGN It has previously been shown that *P.g* LPS's to significantly delay normally high expression levels of BGN in rat alveolar bone osteoblasts [142].

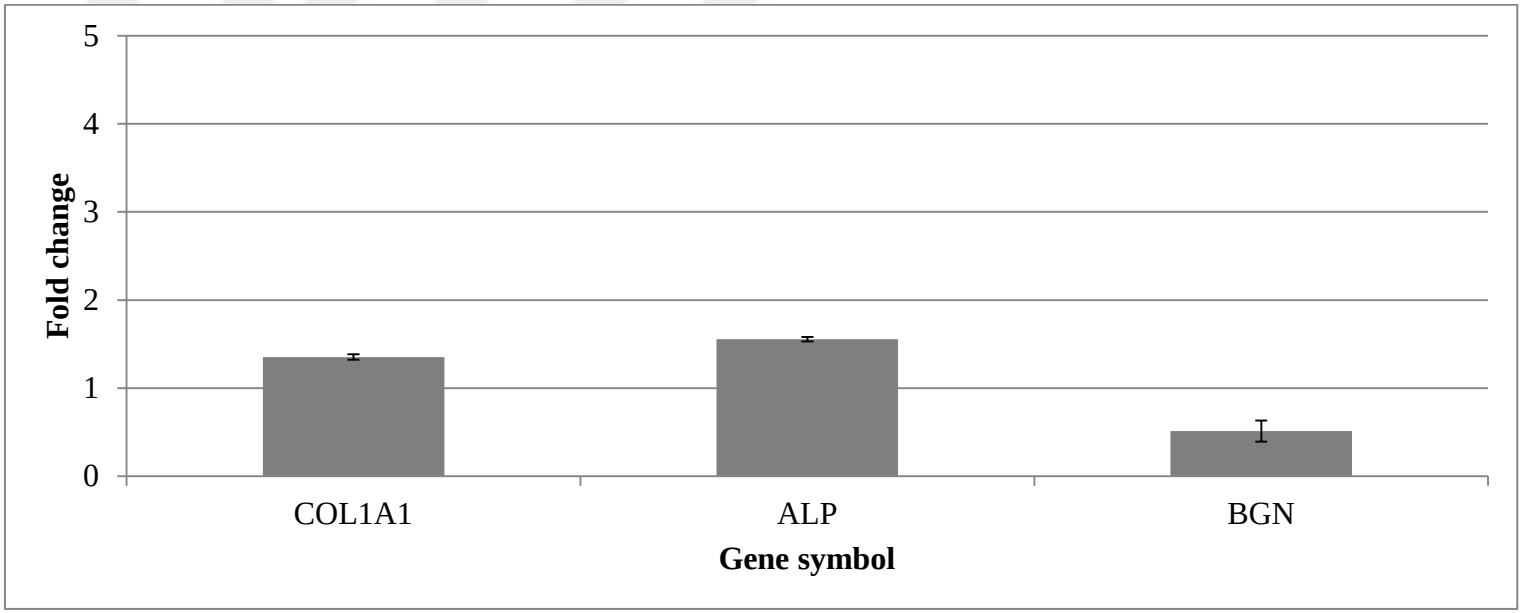

Figure 4. 22. The altered gene expressions of COL1A, ALP and BGN genes in osteoblasts due to the *P.gingivalis* challenge.

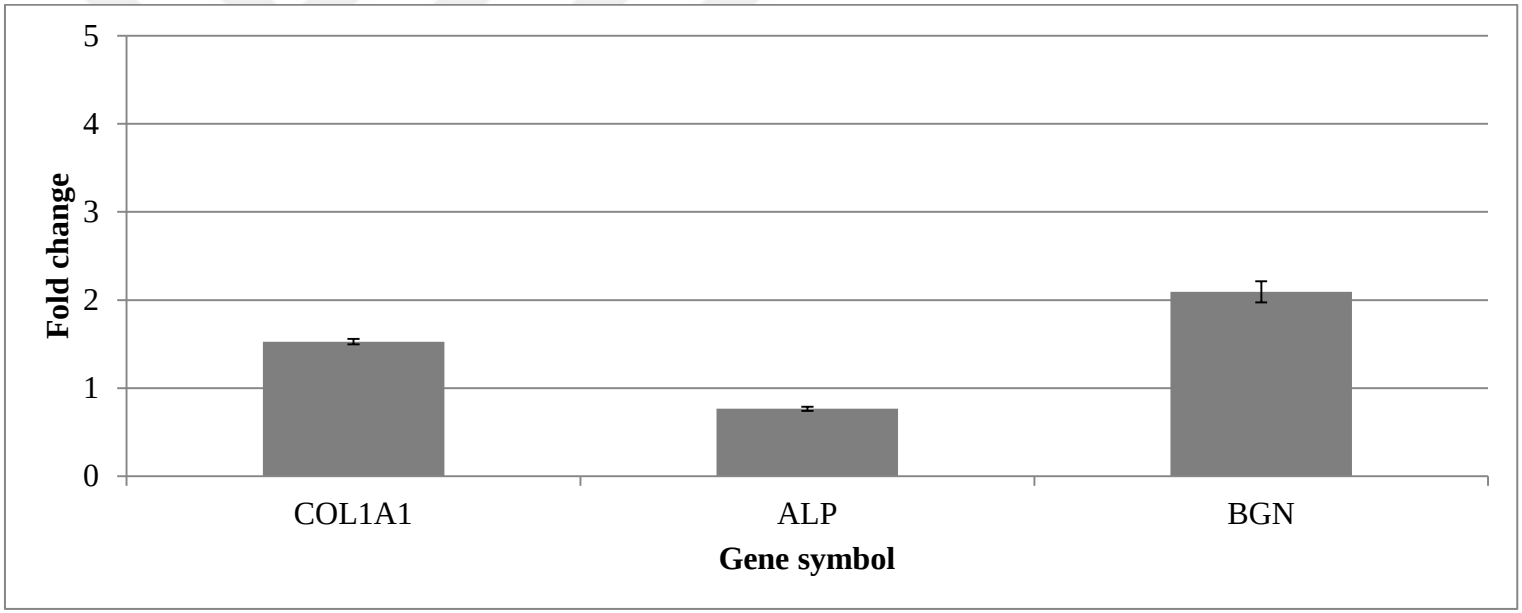

Figure 4. 23. The altered gene expressions of COL1A, ALP and BGN genes in osteoblasts due to the *P. intermedia* challenge.

4.7.3. Validation of superarray gene expression

P.g and *P.i* induced transcript expression results from superarray studies were analyzed by qRT-PCR for COL1A. GAPDH was used as a House Keeping Gene (HKG) and the manual qRT-PCR analyses were performed three times. The expression level of COL1A was positively regulated in both *P.g* and *P.i* challenges (Fold Change: 1.3535 and 1.528 respectively). However RT profiler array showed that COL1A expression was downregulated following *P.g* challenge (Fold Change: 0.2398) and upregulated in *P.i* challenge (13.3614). The direction and magnitude of the expression level of COL1A with qRT-PCR did not show equivalent expression changes with the superarray fold change outcomes. These differences could bedue to the difference in primer designs. The primer used in qPCR experiments might have a lower binding affinity compared to the primer used in the RT Profiler array. This may affect the PCR efficiency. Another reason could be that in qPCR, gene expressions were normalized by only the HKG GAPDH. However in RT Profiler Array a mix of HKG (Hypoxanthine phosphoribosyltransferase 1 (HPRT1), Ribosomal protein L13a (RPL13A), Actin, beta (ACTB) and GAPDH) expressions were used for normalization purposes.

5. CONCLUSION

Early studies have emphasized the potential risk related to pathogenic bacterial contamination in or around the dental implant area. In light of this knowledge, two well documented oral pathogens, *P.g* and *P.i* were studied for their effects on HFOB cells. Studies were undertaken in terms of, changes to cell morphology, mineralization, cell viability and detailed expression profiles of genes related to inhibition and formation of bone.

The results of this study showed that both *P.g* and *P.i* were capable of inhibiting osteoblast proliferation and therefore bone formation. According to cell viability results, *P.i* showed a more lethal effect on the viability of osteoblasts compared to *P.g*. Both of these anaerobes were also seen to reduce matrix mineralization in osteoblast cells. Moreover gene expression studies showed that *P.g* and *P.i* induced TLR and TGF beta related genes, by upregulation or downregulation of their cytokines and receptors. Furthermore, differences were observed in the expression of several genes following *P.g* and *P.i* challenge. These differences suggest that *P.g* and *P.i* could have different mechanisms of inhibiting or reducing bone formation. The changes observed in the expression of key genes involved in bone resorption and formation indicates an important role for these bacteria in lack of osseointegration. In conclusion the findings of this study confirm that both *P. gingivalis* and/or *P. intermedia* are risk factor for bone loss.

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