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Association of vitamin D (1,25OHD,
25OHD and vitamin D binding
protein) and alkaline phosphatase
with orthodontic tooth movement
and osteoblast function

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Boston University

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DISSERTATION

**ASSOCIATION OF VITAMIN D (1,25OHD, 25OHD AND
VITAMIN D BINDING PROTEIN) AND ALKALINE PHOSPHATASE WITH
ORTHODONTIC TOOTH MOVEMENT AND OSTEOBLAST FUNCTION**

by

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Abstract

Introduction

In this study, we identified the association of Vitamin D with orthodontic tooth movement and the impact of Vitamin D 1,25OHD and 25OHD forms on osteoblast function.

Materials and Methods

This study is comprised of two parts; a clinical and a laboratory part. In part I, saliva samples were collected from orthodontic patients each month for the first six months of orthodontic treatment along with casts at the beginning and the end of the study period. The samples were measured for Vitamin D binding protein (VitDBP) and alkaline phosphatase (ALP) and correlated with clinical tooth movement using absolute change in irregularity index (II). In part II, osteoblasts were collected from the calvaria of 3-5 day old healthy wild-type mice and cultured with differing concentrations of 1,25OHD (1, 10 and 100nmol) and 25OHD (100, 200, 400 nmol). ALP, OPG and RANKL were measured as outcomes of Vitamin D treatment of osteoblasts. Intracellular signaling in response to Vitamin D was assessed by identifying the phosphorylation of ERK 1/2, p38 and NLK in primary osteoblasts.

Results

Measurement of salivary Vitamin D binding protein (VitDBP) showed that both low (<2.75 ng/ml) and high (>6.48 ng/ml) logVitDBP were associated with reduced tooth movement. There was no significant correlation between ALP levels and orthodontic treatment. Significant seasonal

changes in VitDBP using a two-season year model were found with lower levels noticed in the summer (Mar-Sept) than in the winter (Oct-Feb) at $p < 0.05$.

A decrease in OPG production with higher concentrations of 1,25OHD and 25OHD with a corresponding increase in RANKL levels in primary osteoblast cultures was found. Similar to the clinical findings, ALP levels were not significantly affected by increasing concentrations of both 1,25OHD and 25OHD. The ERK 1/2 showed upregulation in response to treatment with 1,25OHD and a downregulation in response to treatment with 25OHD concentrations. Meanwhile, p38 and NLK were affected by 1,25OHD and not by 25OHD.

Conclusions

Clinical outcomes of orthodontic treatment are associated with a range of optimal Vitamin D binding protein (VitDBP) as detected in saliva. Different forms of Vitamin D affect osteoblast response and signaling differently.

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Part I: A Longitudinal Assessment of the Link between Vitamin D Binding Protein and Alkaline Phosphatase with Orthodontic Tooth Movement

Introduction

The goal of orthodontic treatment is to move the teeth and dentoalveolar structures into more a harmonious position, restore alignment, and reestablish health and function of the dentition. The success of orthodontic tooth movement is due to multiple mechanical, skeletal and biological factors that regulate the bone turnover and other periodontal tissues. Vitamin D plays a major role in bone biology. Vitamin D deficiency is an etiological factor in several bone turnover diseases such as rickets and osteomalacia. Many other pathologies including, but not limited to, heart disease, hypertension, diabetes, several malignant diseases as well as autoimmune disorders are also being linked to reduced levels or absence of Vitamin D (El-Khoury, et al., 2011). While many studies have focused on the association between orthodontic tooth movement and vitamin D levels, there are still numerous gaps in knowledge (Bartzela, et al., 2009) (Collins, et al., 1988) (Huang, et al., 2014) (Kawakami, et al., 2004) (Shetty, et al., 2015). These include an optimal range of vitamin D levels in individuals undergoing orthodontic treatment and how different forms of vitamin D control the bone turnover during this process. Our study is designed to address these two issues using a combination of *in vivo* and *in vitro* approaches.

Orthodontic Tooth Movement

Teeth are moved through the bone by mechanical forces applied to the teeth. Orthodontic tooth movement differs from physiological migration of teeth in the alveolar socket as it is a forced displacement of teeth in a new position. This process requires the actions of two main types of

cells in bone; osteoblasts and osteoclasts. Osteoblasts are derived from a mesenchymal stem cell line whereas; osteoclasts are derived from a monocyte/macrophage line. Osteoblasts, once encased in mineralize tissue, become osteocytes which are considered terminally differentiated osteoblasts. Their main function is to maintain bone. (Huang, et al., 2014). The balance between the actions of these two cell types allows for bone to be resorbed in the direction where the tooth is to move and for it to be built in the area where it has vacated.

While it is clear that tooth migration in response to orthodontic forces requiring a coupling between the osteoblastic bone apposition and osteoclastic bone resorption, the exact mechanism of orthodontic treatment is still not fully understood after more than 100 years of studies. There are several theories regarding how orthodontic tooth movement (OTM) happens and how the bone responds. The most commonly accepted theory of tooth movement is the pressure-tension theory presented by Sandstedt in 1904, and further described by Oppenheim in 1911 and Schwarz in 1932. (Meikle, 2006). This theory describes the creation of a pressure (compression) side and a tension side during tooth movement. The force is applied at the tension side and forces the tooth into the pressure side. The rate-limiting step is known to be the bone resorption at the compression side while bone is formed at the pressure side. (Huang, et al., 2014). The other common theories are the bone-bending and piezoelectric theories. The bone bending theory was introduced in the 1880s by Kingsly and Farrar. According to this theory, when a force is applied, the bone “bends” into a concave (negative) and convex (positive) surface and the tooth moves due to piezoelectricity (Asiry, 2018) (Proffit, et al., 2000). None of these theories sufficiently explain the biology of bone response underlying orthodontic tooth movement especially in multi-rooted teeth where pressure-tension distinction and bending concepts become complicated. Another limitation of these theories is their biological relevance where the concepts are not fully supported by tissue responses.

Mechanically the three phases of tooth movement during orthodontic treatment have been described as; initial, lag and post-lag (progressive) phases (Burstone, 1962). The initial phase is where the force displaces the teeth during the first 1-3 days. The second phase (the lag phase), is where no tooth movement occurs which usually takes 2-10 weeks. The third phase (the post-lag phase) is where the progressive tooth movement occurs (Burstone, 1962) (Proffit, et al., 2000) (Asiry, 2018). These stages of orthodontic tooth movement have been shown in humans and animal models. It is however, unclear how the biological factors regulate the response to orthodontic tooth movement. In addition, the wide time span of especially the lag phase suggests that orthodontic treatment heavily depends on individual response by the patient especially during the initial stages of tooth movement. It is also not known what biological mechanisms determine how long the subsequent lag phases and progressive phases after the initial stages. Several gaps in knowledge exist in understanding the biology and factors regulating the complex orthodontic migration of the teeth in alveolar bone and stability of the outcomes

Vitamin D and Orthodontic Tooth Movement

Vitamin D is a fat-soluble molecule with a direct effect on mineral ion homeostasis. It regulates calcium absorption and the balance between osteoblasts and osteoclasts. It has also been shown to regulate osteoblastic differentiation (Kawakami, et al., 2004). Vitamin D correlates with parathyroid hormone, calcium and bone health parameters such as bone mineral density, C-terminal polypeptide, bone specific alkaline phosphatase, osteocalcin, and several other key factors that play a role in bone turnover (Tsuprykova, et al., 2017).

Vitamin D metabolism involves two main types of pre-vitamin D; D₂ (ergocalciferol,) and D₃ (cholecalciferol). Ergocalciferol (VitD₂ as it will be referred to) is mainly ingested by the diet,

in the form of fortified foods and supplements. Most fortified foods have the D₂ form with the exception of fatty fish which have the D₃ compound (Tsuprykova, et al., 2017). Cholecalciferol (referred to as VitD₃) is the only vitamin D isoform produced by the mammalian body in response to sun exposure. (van den Ouweland, et al., 2013) (Tsuprykova, et al., 2017). After ingestion of production both VitD₂ and VitD₃ are transported to the liver to become hydroxylated into 25OHD₂ and 25OHD₃, respectively. These two hydroxylated forms of Vitamin D are collectively referred as 25OHD. After the hydroxylation process in the liver, 25-hydroxyvitamin D (25OHD) becomes the major circulating form of vitamin D with a half-life of 2-3 weeks. (Holick, et al., 2008) (van der Meijen, et al., 2014).

The active hormonal form of vitamin D in the human body is 1,25 dihydroxycholecalciferols with two hydroxylation sites. 1,25OHD regulates bone deposition and PTH release through calcium and phosphate serum absorption and reabsorption in the intestines and kidneys, respectively (Bartzela, et al., 2009). It may also have an effect on the immune system by promoting immunosuppression (Bartzela, et al., 2009). 1,25OHD regulates its own metabolism by mediating the 24-hydroxylase activity which leads to its degradation (van Driel, et al., 2004). 1,25OHD is not a good indicator of circulating vitamin D because its half-life is 4-6 hours and the circulating amount is a thousand-fold less than 25(OH)D. Also, as the level of circulating 1,25OHD decreases an imbalance in the calcium levels is observed, increasing the production of PTH, which in turn, increases the renal levels of 1,25(OH)D (Holick, et al., 2008) (van der Meijen, et al., 2014). 1,25OHD also has an important and direct role in the genomic signaling of chondrocytes and osteoblasts on CYP27B1 which is a gene that provides instructions for making an enzyme called 1 α -hydroxylase that carries out one of the reactions to convert vitamin D to 1,25OHD (St- Arnaud, 2008) (van der Meijen, et al., 2014). 1,25OHD induced chondroblasts modulates fibroblast growth

factor 23 (FGF23) secretion in osteoblasts, however, it is still unknown what the secreted factor is. (St- Arnaud, 2008). It is clear however, that these two forms of vitamin D have distinct roles in bone metabolism. How their levels are associated with alveolar bone turnover during orthodontic tooth movement and their impact on bone cells is key to understanding the biological basis of orthodontic treatment responses in alveolar bone.

The main enzymes responsible for Vitamin D metabolism in the liver is CYP2R1, CYP27A1 and CYP3A4. CYP27A1 has been shown to only affect the D₃ form and not the D₂ form and CYP3A4 does not hydroxylate and only works on drug metabolized Vitamin D. (Dusso, et al., 2004) (Anderson, 2017). CYP24A1 works in various tissues to change 25OHD into 24OHD and 1,24,25OHD (Rochel, et al., 2017). (van der Meijen, et al., 2014) (Dusso, et al., 2004). Half of all CYP24A1-null mice die of hyper-vitaminosis D and hypercalcemia before reaching 3 weeks of age (Anderson, 2017). Therefore, it is suggested that CYP24A1 activity is of great importance for healing (Anderson, 2017). From the liver, the 25OHD travels to the kidney where it is transformed into 1,25OHD. In the kidneys CYP27B1 is the only enzyme that acts on the transformation of 25OHD to 1,25OHD (Dusso, et al., 2004).

Vitamin D works on vitamin D receptors in the osteoblasts and other cells to perform its functions (Anderson, 2017). Total vitamin D level is regulated by liver functions, kidney diseases, pregnancy and genetic backgrounds (Tsuprykova, et al., 2017). Studies have shown that 25OHD may assist in bone building by osteoblasts whereas 1,25OHD may stimulate bone resorption. (Eleftheriadis, et al., 2012) Studies done on hemodialysis patients have found that 25OHD suppress osteoclast action in cases of inflammation by increasing expression of RANKL (Receptor

Activator of Nuclear factor Kappa B Ligand) and inducing the production of OPG (osteoprotegerin) (Eleftheriadis, et al., 2012). 1,25OHD induces RANKL activity in immature osteoblasts but not in mature osteoblasts (Anderson, 2017).

Although there is no consensus of the “normal” serum vitamin D levels in humans, the agreement is in the range of >30ng/ml (or >75nmol/L) or 20-80ng/ml (Holick, et al., 2008) (Tsuprykova, et al., 2017). Insufficiency of 25(OH)D is considered in the range of 21-29ng/ml and deficiency as <20ng/ml. The WHO had previously considered levels below 20ng/ml as insufficient and under 10ng/ml as deficient but due to changes in laboratory reference ranges those levels have changed (Rosen, 2011). According to an NHANES study, the mean 25OHD levels were 24ng/ml (60nmol/L) among several age groups (Rosen, 2011). Some experts consider no upper limit to vitamin D levels that would lead to toxicity while others would consider it above 150-200ng/ml based on the study of lifeguards (Holick, et al., 2008).

Many factors affect the variation in pre-vitamin D conversion including skin type, type, amount and duration of sun exposure, surface area exposed to sun, geographical location, and age are some of them (Abboud, et al., 2017). Although sunlight exposure is the main way vitamin D₃ is synthesized in the body, not all sunlight is equal. A Ultraviolet B (UVB) irradiation (wavelength:280-315) threshold of 20mJ/cm² is required to induce the transformation of 7-DHC into pre-vitamin D₃ (Hollis, 2005). For example, in winter (November through February) in Boston where the latitude is above 40⁰ (42⁰ N) the daily accumulated UVB solar irradiation from 11:30-14:20 EST remains below 20mJ/cm² (Hollis, 2005). Sunscreen and skin color also have a major effect on blocking UVB absorption (Hollis, 2005). In areas where cultural norms require more skin coverage by clothing the UVB absorption becomes even less. A very high prevalence of vitamin

D deficiency has been reported in Saudi Arabia despite having one of the highest numbers of sunny days per year (Tuffaha, et al., 2013). In another study testing 60,979 patients from 136 different countries found that hypovitaminosis D is significantly higher in Middle Eastern countries (Haq, et al., 2016).

In order to raise vitamin D levels in the absence of adequate sun exposure, 800-1000 IU vitamin D₃ is prescribed to patients (Holick, et al., 2008). However, the nutritional labeling value in the United States is 400 IU. This level is believed by authors such as Hollis to be inadequate. It seems to be an effective dose to raise the 25OHD concentration to 80-200nmol/L in infants only but seems to have little to no effect on adults (Hollis, 2005). For vitamin D deficiency, Holick recommends 50,000 IU vitamin D₂ once a week or every two weeks for 8 weeks to attain a 25OHD concentration of 75nmol/L (Holick, et al., 2008). For every 40IU of vitamin D intake, we see an increase of 0.70nmol/L of circulating 25OHD over a 5-month regimen (Hollis, 2005) a greater the rise with supplementation is obtained the lower the baseline levels (Rosen, 2011).

Circulating vitamin D is found in three forms; 85-90% is bound to VitDBP, 10-15% is loosely bound to serum albumin and less than 0.03% is found in an unbound and free form. (Bikle, 2014). Vitamin D is brought to the liver by the use of Vitamin D Binding Protein (VitDBP). There, Vitamin D₂ and D₃ are metabolized into the 25-OHD₂ and 25-OHD₃ respectively. (Su, et al., 2014). VitDBP has a higher affinity with the 25OHD form and less with the 1,25OHD (10-100-fold lower) due to the additional hydroxyl group. If 25OHD concentrations are around 20-80ng/ml and 1,25OHD levels are 1000-fold lower, then VitDBP levels are estimated around 20-fold higher than all vitamin D forms together (Tsuprykova, et al., 2017). VitDBP is less than 1% saturated normally considering that its serum concentration ($6.10^{-6}M$) is much larger than the concentration of 25OHD

(4.10^{-8}M) and that the free concentration of 25OHD is not regulated at a constant level (Bouillon, et al., 1977). The most common theory is the free hormone hypothesis, which states that only unbound hormones are biologically free to perform their physiologic activities but protein-bound hormones are biologically inactive (Yousefzadeh, et al., 2014)

Vitamin D Binding Protein (VitDBP) is a polymorphic single-chain serum glycoprotein found in many body fluids and organs, including but not limited to, serum, saliva, breast milk, cerebrospinal fluid and lungs, kidneys, spleen and placenta (Delanghe, et al., 2015). VitDBP is produced by the liver and is responsible for transporting 25OHD and 1,25OHD to the cells. It also is filtered through the kidneys, particularly the molecular sieve of the glomerular filtration barrier and re-uptaken by the megalin/cubulin system in the proximal tubules (Rochel, et al., 2017) (Tsuprykova, et al., 2017). Therefore, diseases that affect the liver and kidneys such as, liver cirrhosis and chronic kidney diseases affect the levels of VitDBP in the blood (Tsuprykova, et al., 2017). The main functions of VitDBP are binding, solubilizing and transport of Vitamin D and its metabolites. There are over 120 unique racial variants of VitDBP but the most common are DBP1F, DBP1S and DBP2 (Delanghe, et al., 2015). Studies have proven that serum VitDBP concentrations and 1,25OHD₃ levels are positively correlated (Delanghe, et al., 2015).

VitDBP is found to vary according to race, age and gender. Differences have been found in measuring free 25OHD between races because African Americans were found to have less circulating free 25OHD but stronger bones. They have lower levels of VitDBP resulting in equivalent levels of calculated free 25OHD₃. (Hong, et al., 2018). In African Americans, higher levels of vitamin D deficiency are noted than in Caucasians. They are also more likely, along with Asians, to carry mutations of the GC1F single nucleotide polymorphism (SNP) which has a high affinity

for 25OHD and lower VitDBP. Caucasians are more commonly found to have the GC1S SNP, which has the opposite affinity. In another study by Bouillon et al. found no racial differences in normal serum concentrations of VitDBP between whites from Belgium and blacks from Zaire (Bouillon, et al., 1977).

Age and gender are also factors that affect VitDBP levels. Vitamin D insufficiency is more common in older than younger individuals. VitDBP is negatively correlated with age in females rather than males (Yousefzadeh, et al., 2014). Other studies have shown no differences in VitDBP levels in aging (Delanghe, et al., 2015). Women were found to have higher mean VitDBP levels than men but no other associations were noticed between VitDBP levels and age, bodyweight, BMI, fat mass or fat percentage. (Pop, et al., 2015). They also found that postmenopausal women had lower Vit DBP levels than premenopausal. (Pop, et al., 2015) (Nimeri, et al., 2013). In another study by Bouillon in 1977, they noticed higher concentrations in women than men in parallel with what Pop et al. concluded. (Bouillon, et al., 1977) (Pop, et al., 2015).

Certain diseases also affect vitamin D and VitDBP levels. Vitamin D deficiency or insufficiency is slightly increased in patients with Diabetes with albuminuria which in turn, coincides with an increase in urinary VitDBP. (Delanghe, et al., 2015). Vitamin D₃ synthesized endogenously was also noted to be different than ingested Vitamin D₂. (Haddad, et al., 1993). Plasma 25OHD levels were found by Zhang et al to be higher in patients with generalized aggressive periodontitis than healthy controls. This was also found to be positively correlated with the gingival bleeding index. Initial periodontal therapy was shown to decrease local and systemic levels of 25OHD. (Zhang, et al., 2014) As for VitDBP levels, they were shown to be increased in salivary levels of patients with periodontitis (Wu, et al., 2009). Another interesting finding to note was that

serum levels of VitDBP were not affected by several diseases of calcium metabolism (primary osteoporosis, primary and secondary hyperparathyroidism, rickets, osteomalacia or vitamin D intoxication) but liver and kidney diseases did. (Bouillon, et al., 1977) (Tsuprykova, et al., 2017).

There is substantial controversy surrounding how to correctly measure vitamin D. Between 25OHD and 1,25OHD, 25OHD is usually considered as the measure of vitamin D status due to its longer half-life (10-15 days) compared to 1,25OHD (4-6 hrs.). Another important factor in choosing 25OHD is that circulating levels are 1000 time more than 1,25OHD which is measured in picograms as opposed to 25OHD's nanograms. 1,25OHD has also been noted to be directly influenced by serum calcium, phosphorus and parathyroid hormone more than 25OHD (Su, et al., 2014). A Vitamin D Standardization Program was established to develop protocols for standardizing the existing serum 25(OH)D data from national surveys around the world. This program includes participants from several different countries including the US, Belgium, UK, Australia, Canada, Germany, Ireland, Mexico and South Korea. They aim toward standardizing the laboratory measurement of vitamin D and improving the detection, evaluation and treatment of vitamin D deficiency. (<https://ods.od.nih.gov/Research/vdsp.aspx#overview>) (Cashman, et al., 2015). However, the problem is not only in how to measure 25OHD but some researchers believe we should be measure VitDBP and serum albumin as well due to the fact that less than 0.03% of 25OHD is unbound (Tsuprykova, et al., 2017). The first assays for measuring total 25OHD were based on a VitDBP competitive protein binding method; however, this method includes other vitamin D metabolites other than only 25OHD which are usually inactive. (Jassil, et al., 2017)

Several methods of measuring vitamin D are now available. The methods are divided into two main groups; immunochemical and chromatographic/physical. Radioactive, enzymatic or

chemiluminescence detection fall under the first group, whereas HPLC and LC-MS/MS fall under the second. (van den Ouweland, et al., 2013) (Enko, et al., 2014). Traditionally, radio-immune assays, high-performance liquid chromatography (HPLC) with UV detection and radioimmunoassay have been used routinely, however, due to the high demand for superior sensitivity and specificity, new methods are being used. Liquid Chromatography coupled with electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI)-mass spectrometry (MS) are now the more popular options (Higashi, et al., 2008). LC-MS is used for the direct measurement of total 25OHD (Holick, et al., 2008). It can distinguish between 25(OH)D₃ and 25(OH)D₂, and therefore can report individual and total levels of 25(OH)D (El-Khoury, et al., 2011). However, Su et al found that though most manufacturers claim that their LC/MS/MS assays equally measure D₂ and D₃, Vitamin D Binding assays overestimate 25OHD₃ by 10.8% and underestimate 25OHD₂ by 38.4% which is problematic in that US prescription supplements of Vitamin D are usually the D₂ form (Su, et al., 2014) One of the issues with LC-MS methods is that they can show up to 15% higher results than protein binding assays and some immunoassays (El-Khoury, et al., 2011). Another problem of the LC-MS method is that the vitamin D molecule is lipophilic and therefore difficult to ionize due to its lack of easily chargeable groups (El-Khoury, et al., 2011).

A Cookson-type reagent has been used with some success in a study by Higashi (Higashi, et al., 2008). Knox and Højskov also reported some progress with a protein precipitation extraction (El-Khoury, et al., 2011). Use of human breast milk and dried blood spots have also been reported to be used for LC-TMS analysis of vitamin D levels (El-Khoury, et al., 2011). The National Institute of Standards and Technology and the Centers for Disease Control and Prevention have chosen LC/MS/MS as the reference method for measuring 25OHD however, there is no reference method

when measuring VitDBP although most experts use polyclonal antibody based immunoassays. (Su, et al., 2014) (Malmstoem, et al., 2017)

A study by Higashi et al. in 2008 developed a method to accurately measure the 25(OH)D levels in 1.0ml sample of saliva after derivatization with 4-phenyl-1-1,2,4-triazolin-3,5-dione (PTAD). (Higashi, et al., 2008) This definitely carries the appeal of being noninvasive compared to serum measurement which is considered the gold standard. There is a proven correlation between the levels of vitamin D in saliva and serum (Higashi, et al., 2008). However, the main disadvantage of using saliva is the low analyte concentration (Higashi, et al., 2008). They also noted that the saliva samples were stable to up to 3 freeze/thaw cycles and that it was possible to store the saliva at -20 degrees Celsius for up to four months without loss of 25(OH)D₃ (Higashi, et al., 2008). In a later study by the same author in 2010, they found that there was no significant effect of storage conditions such as light exposure, room temperature storage for two weeks and 10 freeze/thaw cycles on the measured values (Higashi, et al., 2010). The 25(OH)D₃ levels in saliva were found to be significantly lower (one thousandth) than that of serum. However, a good correlation was also observed between them, proving that there is a reliable ratio between the 25(OH)D₃ levels in saliva and serum (Higashi, et al., 2008) (Higashi, et al., 2010). Therefore, saliva assays can be used for the assessment of vitamin D status of patients. This method proved to be specific, accurate and reproducible and can be used successfully in application to clinical studies (Higashi, et al., 2008).

When measuring VitDBP, immunonephelometry has been used with both monoclonal and polyclonal antibodies. This manages to assay all three major electrophoretic variants (Gc2, Gc1s and Gc1f). (Jassil, et al., 2017). Most studies use enzyme linked immunosorbent assay (ELISA) to

measure VitDBP, which uses 2 monoclonal antibodies in a sandwich format. This may be different when compared to methods using polyclonal antibodies. (Jassil, et al., 2017). Polyclonal techniques have shown no significant racial differences when measuring VitDBP. (Denburg, et al., 2016). According to a study by Hong et al in 2018, Vitamin DBP is a valid measure to determine free 25OH vitamin D₃ status in conjunction with total 25OHD₃. (Hong, et al., 2018). Luminex, which is a bead based immunoassay that uses dye defined beads coated with anti-analyte antibodies and is usually used for multiplexing to measure several analytes for each sample, has also been used successfully in measuring VitDBP levels (Dudal, et al., 2014).

Alkaline Phosphatase

Alkaline phosphatase (ALP) is a glycoprotein produced by fibroblasts, osteoblasts and osteoclasts. It plays a role in mineral formation of hard tissue structures like bone and cementum as first studied by Robinson in 1923 (Robison, et al., 1924) (Farahani, et al., 2015). It is known to be a biological marker of osteoblastic activity during bone deposition (Farahani, et al., 2015) (Rodan, 1992). Normal levels in human osteoblasts were found by Corrado et al to be 29.85 ± 4.93 . (Corrado, et al., 2013). Several studies have measured ALP levels in GCF (gingival crevicular fluid) during tooth movement to better understand the biology behind tooth movement. In a study by Perinetti in 2011, it was found that ALP levels in GCF can be used as a non-invasive clinical biomarker for measuring the skeletal maturation of orthodontic patients. It was compared to CVM (cervical vertebral maturation) and found to be significantly related (Perinetti, et al., 2011). Farahani et al, noticed that the peak ALP levels during orthodontic tooth movement were noticed at day 14 and 28 (Farahani, et al., 2015). ALP activity values were also noted as being higher in the tension rather than compression sides of tooth movement, which may be explained by the greater

bone deposition at that side but were more pronounced after tooth movement had ceased. (Farahani, et al., 2015) (Flórez-Moreno, et al., 2013).

ALP has been studied in saliva as a biomarker of bone resorption occurring with periodontal disease. Patel et al found that in periodontal diseases, the levels of ALP increased as the disease progressed from gingivitis to periodontitis at a statistically significant level $p < 0.001$ (Patel, et al., 2016). They also noted the salivary ALP levels to be 20.70 ± 7.18 U/L in healthy periodontium, 33.06 ± 5.49 U/L in gingivitis and 49.62 ± 16.46 U/L in periodontitis (Patel, et al., 2016). However, in another study by Sophia et al, they had higher levels comparing healthy and periodontally diseased post-menopausal women with healthy levels at 37.26 ± 9.92 and periodontitis 70.43 ± 4.36 (Sophia, et al., 2017). In a study by Jazaeri et al, mean salivary ALP activity levels were found at 5.04 ± 1.866 U/dl measured by the Kinetic method to convert 4-nitrophenyl phosphate to 4-nitrophenol (Jazaeri, et al., 2015). Limited studies focused on salivary ALP levels have also been studied in regards to orthodontic tooth movement by Flórez-Moreno et al where the levels appeared to consistent and show no differences between time points (Flórez-Moreno, et al., 2013). Yet, a clear understanding of ALP levels in saliva is required to understand its biological value as a factor and marker of orthodontic tooth movement.

Hypothesis

Null Hypothesis: (H_0): There is no correlation between Vitamin D Binding Protein and Alkaline phosphatase levels and the rate of orthodontic tooth movement.

Hypothesis: (H_1): Vitamin D binding protein and Alkaline phosphatase levels are correlated with the rate of orthodontic tooth movement

Aims

Overall Aim

The aim of this study was to measure the correlation between salivary vitamin D binding protein and alkaline phosphatase levels and the rate of tooth movement with orthodontic treatment in the orthodontic patient population.

Specific Aims

1. To determine if baseline salivary level of VitDBP and ALP levels changes the rate of orthodontic tooth movement and identify the impact of confounding factors such as the seasonal effects on VitDBP levels
2. To measure the change in VitDBP and ALP levels in response to orthodontic treatment
3. To determine the correlation between salivary VitDBP and ALP levels during orthodontic tooth movement

Materials and Methods

A total of 127 patients were recruited from the Department of Orthodontics and Orthofacial Orthopedics at the Henry M Goldman School of Dental Medicine at Boston University after IRB approval. All subjects were provided with written and oral consent to be included into the study. Participants with any medical conditions that might affect bone metabolism or who were taking any vitamin D supplements were excluded from the study. As part of the orthodontic treatment standards, patients with active periodontal disease do not start orthodontic treatment until their disease is under control; therefore, we excluded patients with signs of periodontal disease from in this study.

The saliva samples were collected at each visit for 6 consecutive, monthly visits during the leveling and aligning stage of orthodontic treatment. Impressions were taken before orthodontic treatment was started as part of the orthodontic treatment records. A second set of alginate impressions were taken at the end of the study period. Whole saliva was collected in 15ml polypropylene Falcon tubes at each appointment and immediately placed in a -80⁰c freezer at Boston University. Once collection was completed, the samples were transported in dry ice to the Forsyth Institute for analysis.

Subjects were enrolled in the study between April 2017 and March 2018 from the patients seeking orthodontic treatment at the Henry M Goldman School of Dental Medicine at Boston University. The patients were asked if they had any medical conditions or were taking any medications including vitamin supplements and were not included if they did. The treatment plans varied between extraction and non-extraction cases and between traditional braces, clear aligner therapy

and others such as expanders or headgears. The treatment was completed by the orthodontic residents at the university under the supervision of the faculty. The initial samples were taken at the treatment planning appointment and then consecutively after treatment had started. Alginate impressions were taken at the 7th and final appointment after 6 months of treatment. The casts were then scanned along with the initial models to be measured for the tooth movement.

Measurement of Irregularity and Crowding

Little's Irregularity Index (II) is an orthodontic treatment index which was developed by Robert Little in 1975. It measures the distance between contact points of the mandibular canine to canine region regardless of the direction of the distance. (Little, 1975) It is a useful tool in determining the amount of incisor irregularity but does not measure crowding only in terms of lack of space. It is considered faster and easier than other indices.

Initial and final casts were scanned using the Ortho Insight 3D from Motion View LLC., (Chattanooga, Tennessee) scanner and software. Teeth were identified using the software and the mesial and distal contact points corrected manually. The irregularity index was then measured using the software for each before and after cast as well as the absolute difference between them for statistical analysis.

Processing and Analysis of Saliva

Saliva samples were thawed and centrifuged at 12,000 rpm for 10 minutes and the supernatants were aliquoted into 2ml Eppendorf tubes. The samples were then measured for ALP using Abcam Alkaline Phosphatase Assay kit (Colorimetric) (ab83369) according to manufacturer's in-

structions (protocol in appendix below). Optical density (OD) of the products at 405 nm was measured by spectrophotometry. Enzyme activity was calculated as the OD of the reaction product multiplied by the reaction volume and normalized to the reaction time and to the total protein. For the VitDBP, the kit used was Human Circulating Cancer Biomarker Magnetic Bead Panel 2 (Cat. #HCCBP2MAG-58K) from EMD Millipore. The samples were run on the Luminex® 200™. All ALP samples were run using the same kit and the VitDBP using kits from the same batch at the same time to reduce possible variation from external factors.

Statistical Analysis

Statistical analysis was performed using SAS 9.4 software with mixed and linear regression models. Descriptive statistics were summarized using mean and standard deviation for continuous variables and frequency for categorical variables, respectively. Data were thoroughly checked for potential data entry errors and outliers. Mixed linear regression models and traditional linear regression models were used to assess the relationship between VitDBP and season and between baseline VitDBP and absolute difference in tooth irregularity.

Results

A total of 127 patients were recruited for the study. 84 patients completed the study but 73 were included in the final analysis due to multiple missing samples and casts. Of the 6 timepoints, only 4 were measured including the first and last sample in order to have consistency in the number of acceptable samples for each participant. Of the final 73 subjects, 43 were female and 30 males, yielding a female/male ratio of 1.38. The age ranged from 8 to 63 years old with a mean age of 21.5 ± 11.07 . In the child group (<18 years old) the mean age was 13.2 and in the adults (>18 years old) the mean age was 30.2. The adult (n=36) to child (n=37) ratio in the study was 0.97. Angle classification of subjects was Class I (n=48), Class II including both division 1 and 2 (n=18) and Class III (n=7) which was consistent with the average normal ratios seen in an orthodontic clinic. The treatment plans were extraction (n=16), non-extraction (n=39), clear aligners (n=10) and others (n=8). The “other” category included were considered any treatment plan that did not involve the above mentioned, such as expansion or splint therapy. Racial ethnicity was self-reported and included; African Americans (n=27), Asians (n=6), Caucasians (n=17) and Hispanics (n=23).

In our analysis, we examined the patterns of change in VitDBP and ALP levels across four time points. Subjects were grouped into one of four groups according to whether their VitDBP or ALP levels changed from quartile to quartile over different time points into changed (fluctuating), monotone increasing, monotone decreasing and no change. Although no statistically significant group differences in the mean absolute differences in II were observed, subsequent analysis revealed that if comparing subjects with any changing patterns in VitDBP (i.e. monotone increasing, monotone decreasing or fluctuating) to those in the no change group, we observed that the no

change VitDBP group showed overall smaller mean absolute differences in II than those in the changed groups.

The tables below show the demographic data for the enrolled subjects according to the change in quartiles of VitDBP over time points as well as the average logVitDBP, ALP and ALP activity measures for all four time points.

Variables	Overall	Change	No Change	P-value
Age	21.63±11.07	21.95±11.71	20.67±11.71	0.6735
Gender				
Male	30(41.1)	23(31.51)	7(9.59)	1
Female	43(58.9)	32(43.84)	11(15.07)	
Classification				
Class I	48(65.75)	35(47.95)	13(17.81)	0.3438
Class II div-1/2	18(24.66)	13(17.81)	5(6.85)	
Class III	7(9.59)	7(9.59)	0(0)	
Treatment Plan				
Extraction	16(21.92)	11(15.07)	5(6.85)	0.9295
Non Extraction	39(53.42)	30(41.1)	9(12.33)	
Clear Aligners	10(13.7)	8(10.96)	2(2.74)	
Other	8(10.96)	6(8.22)	2(2.74)	
Race				
African American	27(36.99)	22(30.14)	5(6.85)	0.5957
Asian	6(8.22)	5(6.85)	1(1.37)	
Caucasian	17(23.29)	13(17.81)	4(5.48)	
Hispanic	23(31.51)	15(20.55)	8(10.96)	

Table 1: Demographic data for enrolled subjects

Variables	Time	Overall	Change	No Change
logVitDBP	1	4.24±2.12	3.91±1.71	5.23±2.86
	2	4.4±2.13	4.13±1.85	5.23±2.73
	3	4.09±1.89	3.75±1.28	5.12±2.9
	4	4.27±2.1	3.97±1.76	5.18±2.77
ALP	1	8.96±9.41	7.38±7.27	13.8±13.21
	2	10.13±9.5	10.08±8.91	10.27±11.39
	3	8.52±7.27	7.29±5.05	12.21±11.05
	4	10.08±7.58	9.75±7.05	11.11±9.18
ALP Activity	1	1.87±1.96	1.54±1.51	2.87±2.75
	2	2.11±1.98	2.1±1.86	2.14±2.37
	3	1.77±1.51	1.52±1.05	2.54±2.3
	4	2.1±1.58	2.03±1.47	2.31±1.91

Table 2: log VitDBP and ALP averages over time points for all subjects

Looking at only baseline logVitDBP, it was not significantly related with the absolute change in tooth II at $p=0.0748$. We hypothesized that subjects with a normal range of VitDBP show higher tooth movement levels, measured by the absolute difference in II. In this study, we ranked the subjects by their VitDBP levels. Subjects with VitDBP in the middle 50% were called “normal”, while the others were classified as “extreme”. Table 3 shows that VitDBP in log scale was between 2.75 and 6.48ng/ml for the normal range group (middle two quartiles), ≤ 2.69 ng/ml for the extremely low subgroup (first quartile) and between 6.59 and 8.42ng/ml for the extremely high subgroup (fourth quartile). Our analysis shows that the normal range group (mean= 2.36 ± 0.28) had significantly higher mean absolute differences in II ($p=0.002$) than the extreme group (mean= 1.22 ± 0.31). The group level ranges for absolute change in II is reported in Table 3 below. Demographic information by normal vs extreme VitDBP groups are shown in Table 4.

	Normal	Extreme	
		Low	High
LogVitDBP	2.75 - 6.48 ng/ml	0 – 2.69 ng/ml	6.59 - 8.42 ng/ml
Absolute change in II	0.14 – 7.48 mm	0.11 – 5.63 mm	0.07 – 3.32 mm

Table 3: LogVitDBP and II between normal and extreme ranges

		Normal	Extreme	
			Low	High
Age		20.05 ± 11.74	22.78 ± 7.89	23.72 ± 12.39
Gender	Male	15 (20.55)	9 (12.33)	6 (8.22)
	Female	22 (30.14)	9 (12.33)	12 (16.44)
Race	African American	12 (16.44)	8 (10.96)	7 (9.59)
	Asian	4 (5.48)	0 (0)	2 (2.74)
	Caucasian	11 (15.07)	2 (2.74)	4 (5.48)
	Hispanic	10 (13.7)	8 (10.96)	5 (6.85)

Table 4: Demographic data of subjects in Normal and Extreme groups

In an exploratory analysis, we found that neither ALP concentration nor ALP activity were not significantly related to both total ALP concentration area under the curve ($p=0.039$) and the absolute change in ALP between the first and last time points ($p=0.0018$)

A mixed regression model with random intercept was used to assess the seasonal effects on VitDBP. A seasonal variable was created. The samples collected between March and September were considered “summer” and those between October and February “winter”. A significant seasonal difference in VitDBP was observed ($p<0.05$). Samples collected in the winter season (4.47 ± 0.30) had significantly higher VitDBP than those collected in the summer season (4.24 ± 0.30).

Discussion

In this study, we compared the salivary VitDBP and ALP levels of orthodontic patients over the first 6 months of orthodontic treatment (leveling and aligning phase) with the absolute change in irregularity index measured before and after the 6 month period. The distribution of subjects in this study correlates with the average population of patients in the Henry M Goldman School of Dental Medicine Orthodontic clinic in terms of age, gender, race, classification and treatment plan. We found this sample distribution to be representative of the population studied.

There is no international standardized level for VitDBP in human saliva or serum. Serum levels are usually estimated between 300 and 600 mg/L in healthy subjects (Delanghe, et al., 2015). However, there are no studies to our knowledge of VitDBP levels in saliva. Our study showed logVitDBP levels between 0 and 8.42 ng/ml. Although previous studies have shown differences in vitamin D and VitDBP levels with age, gender and race, our study showed no statistically significant effects at $p < 0.05$ (Hong, et al., 2018) (Bouillon, et al., 1977) (Nimeri, et al., 2013) (Pop, et al., 2015). Considering the type of vitamin D measured, between 25OHD, 1,25OHD and VitDBP, VitDBP was deemed the most appropriate due to its higher concentration in the body and its expression in saliva. 25OHD was another viable option, however, considering that less than 0.03% of 25OHD is unbound and free, the commercially available kits and the controversy in measurement, we decided that VitDBP would be a more appropriate measurement. According to a study by Hong et al in 2018, Vitamin DBP is a valid measure to determine free 25OH vitamin D₃ status in conjunction with total 25OHD₃ (Hong, et al., 2018).

When considering the technique to measure vitamin D in this study, we looked into several methods. The National Institute of Standards and Technology and the Centers for Disease Control

and Prevention have chosen LC/MS/MS as the reference method for measuring 25OHD however, there is no reference method when measuring VitDBP although most experts use polyclonal antibody based immunoassays. (Su, et al., 2014) (Malmstoem, et al., 2017). The most feasible one was the Luminex kit was used due to ease, accuracy of measurements and availability of the needed equipment at the Forsyth institute. This kit allowed us to reproducibly measure the VitDBP levels in collected saliva.

ALP is a biomarker of osteoblastic activity that reflects tissue responses during orthodontic tooth movement (Farahani et al). There is no consensus on the normal salivary ALP levels, however, several studies have shown means of 20.70 ± 7.18 U/L in healthy patients in a study by Patel et al and 37.26 ± 9.92 by Sophia et al (Patel, et al., 2016) (Sophia, et al., 2017). ALP activity was measured using the kinetic method by Jazaeri et al to be 5.04 ± 1.866 U/dl (Jazaeri, et al., 2015). In our study, we found the mean ALP concentration levels to be 9.33 ± 8.47 pg/ml and ALP activity 1.94 ± 1.76 uM/min/mg protein. ALP concentration and activity were not significantly correlated with absolute change in Irregularity Index over the total 6-month period. This correlates with what was found by Flórez-Moreno et al where ALP levels appeared to consistent and show no differences between time points in orthodontic patients (Flórez-Moreno, et al., 2013).

When dividing the subjects into whether their VitDBP or ALP levels fluctuated between quartiles over time points or remained in the same quartile, there were significant differences. We considered those that changed quartiles, whether increasing, decreasing or fluctuating to be a “changed” group and those that did not as a “no change group”. The changed group had higher mean absolute differences in irregularity index. This shows that fluctuation in VitDBP levels may have an impact of the rate of tooth movement. Further study is needed in this area to determine the

exact pathway of this change. However, with ALP levels, there was no significant differences between the changed and no change groups.

We hypothesized that subjects with a normal range of VitDBP show higher tooth movement levels, measured by the absolute difference in II. In this study, we ranked the subjects by their VitDBP levels. Subjects with VitDBP in the middle 50% were called “normal”, while the others were classified as “extreme”. VitDBP in log scale was between 2.75 and 6.48ng/ml for the normal range group, between 0 and 2.69ng/ml for the extremely low subgroup and between 6.59 and 8.42ng/ml for the extremely high subgroup. Our analysis shows that the normal range group (mean= 2.36±0.28 ng/ml) had significantly higher mean absolute differences in II (p=0.002) than the extreme group (mean= 1.22±0.31 ng/ml). Vitamin D has been studied in regards to accelerating tooth movement in several animal studies (Nimeri, et al., 2013) (Bartzela, et al., 2009) (Diravidamani K., 2012). Local injections of 1,25OHD in cats showed a 60% increase in tooth movement (Collins, et al., 1988). Bone regeneration was also enhanced after local and systemic vitamin D supplementation in dogs according to Hong et al. (Hong, et al., 2018). Accelerated tooth movement in rats was found with Vitamin D injections in the studies of Kale (Kale, et al., 2004), Kawakami (Kawakami, et al., 2004), as well as increased number of osteoclasts and osteoblasts (Kawakami, et al., 2004). In a clinical, split mouth study by Shetty et al, there was a decreased amount of tooth movement in humans after injection with vitamin D₃ (Shetty, et al., 2015). There have also been several cases of systemic reviews that have included vitamin D as a stimulant for tooth movement (Nimeri, et al., 2013) (Bartzela, et al., 2009). Vitamin D has been shown to be a stabilizing agent in orthodontic tooth movement. (Diravidamani K., 2012). As well as a seemingly

opposing view in a study by Tyrovola and Suda (Tyrovola, et al., 2008) (Suda, et al., 2003). Although it seems to show that it may not speed up tooth movement, they have shown that at physiologic levels of Vitamin D, the counter effects do not present themselves (Suda, et al., 2003) .

Previous studies have shown that there is significant seasonal fluctuation in 25OHD (Oleröd , et al., 2017) and 1,25OHD levels (Darling et al). During winter, both free and calculated serum 25OHD levels were found to be higher than in the summer ($0.020 \pm 0.005\%$ vs $0.019 \pm 0.004\%$; $P = 0.007$ according to Oleröd et al (Oleröd , et al., 2017). However, they noticed no significant differences in VitDBP or albumin levels. In our study we noticed a $p < 0.05$ significant difference in salivary VitDBP levels when considering a two-season year model. We found that during the “winter” season defined as October to February, salivary logVitDBP levels were found to be higher than in the “summer” season (March to September). This correlates with changes noted in 25OHD and 1,25OHD but not previous studies of VitDBP. There may be a relation between the binding capacity of VitDBP and the circulating 25OHD levels and its metabolites. Further studies are needed in order to determine the seasonal effects on VitDBP levels and its activity on 25OHD and 1,25OHD levels.

Part II: Impact of Vitamin D (1,25OHD and 25OHD) on Osteoblast Function

Introduction

Bone is made up of three main cell types; osteoblasts that form bone, osteocytes that maintain bone and osteoclasts that resorb bone. Osteoblasts come from mesenchymal stem cells, which are the precursors of four cell lines; adipogenic, myogenic, osteochondral progenitor and pre-osteoblast lines (Rutkovskiy, et al., 2016). In order for the pre-osteoblastic line to occur the Runx/Osterix system must be activated. This activation is regulated by the presence of MMP13. (Rutkovskiy, et al., 2016)

The pre-osteoblastic cells become fully mature osteoblasts by transitioning into several stages. These stages have been divided by Rutkovskiy et al in a 2016 article into three stages (Rutkovskiy, et al., 2016). Stage 1 is when the osteoblasts continue to proliferate and secrete fibronectin, collagen type 1, TGF beta receptors and osteopontin. Stage 2 occurs when the cells exit the cell cycle and start to differentiate and the extracellular matrix begins to mature. Alkaline phosphatase and collagen type I are secreted during this stage. The 3rd stage, which occurs before osteoblasts are fully matured, is characterized by mineralization of the matrix with an organic scaffold of mineral deposition of osteocalcin, osteopontin, bone sialoprotein and calcium phosphate. Mature osteoblasts are then either surrounded by mineralized tissue and become osteocytes or if nearer to the surface become bone lining cells. The late stages of maturation are controlled by the Wnt/Notch pathway and bone morphogenic proteins which have an effect on the Runx2 and Osterix expression to encourage the differentiation of new osteoblasts (Rutkovskiy, et al., 2016).

The osteoblasts are counteracted by osteoclasts. Osteoclasts come from monocyte/macrophage cell lineage and fuse together to form multinucleated cells. These osteoclasts are in charge of resorbing the formed bone using tartrate-resistant acid phosphatase (TRAP), matrix metalloproteinases (MMPs) and cathepsin K, providing a balance to the osteoblasts building bone (Shaw, et al., 2016).

The main pathway osteoblasts and osteoclasts communicate is the OPG/RANKL (osteoprotegerin/ receptor activator of nuclear factor kappa B ligand) pathway. This ensures a balance between both cells giving way to the modeling/remodeling of bone structures. RANKL, when bound to its receptor on the osteoclast determines the differentiation, function and survival (Huang, et al., 2014). OPG is a decoy receptor of RANKL and therefore decreases its effectiveness. OPG inhibits osteoclast formation and encourages osteoblast action. RANKL is a protein that is essential for the development of osteoclasts as well as T cells and dendritic cells (Rutkovskiy, et al., 2016). OPG is decreased and RANKL increased in response to compression, which facilitates tooth movement in orthodontic treatment (Huang, et al., 2014). The active form of vitamin D (1,25OHD) is a major player in this pathway (Lips, 2006) (St- Arnaud, 2008).

In the case of inflammation, several processes become disturbed. The system shifts to the favor of the osteoclast/bone resorption side rather than the osteoblast/bone building side. Different factors and cytokines have been found to either promote or inhibit bone resorption and therefore the activity of osteoblasts and osteoclasts (Huang, et al., 2014) (Shaw, et al., 2016). Cytokines that inhibit bone resorption and aid the osteoblasts include IL-4, IL-10, IFN-g and IL-12/18, whereas those that promote bone resorption and aid osteoclasts include IL-1b, TNF-a, IL-6 and IL-17 (Wan, et al., 2016). Other influencers to bone builders are growth factors such as TGF-b and BMP 2-4,

and hormones, such as calcitonin and estrogens (Wan, et al., 2016). However, lipid mediators such as PGE2 have been found to assist in bone breakdown. Vitamin D is a unique factor in that contradictory studies have found it to either assist or hinder bone formation according to the maturation stage in which it was studied as well as the skeletal origin of the osteoblasts (Wan, et al., 2016). A possible link was also identified by Wu et al. between vitamin D binding protein and generalized aggressive periodontitis (Wu, et al., 2009).

Vitamin D is a fat-soluble molecule that has been shown by many studies to have an effect on mineral ion homeostasis (Kawakami, et al., 2004). Vitamin D is also essential for the differentiation and maturation of osteoblasts (Anderson, 2017) (Tsuprykova, et al., 2017). Pre-vitamin D is obtained either from the skin by sun exposure or ingested from the diet. Fatty fish contain the D₃ compound of the vitamin whereas fortified foods have the D₂ form (van den Ouweland, et al., 2013) (Tsuprykova, et al., 2017). In order for the actions of vitamin D to occur it must be converted into the active form 1,25 OH₂D by processes in the liver and kidneys (van der Meijen, et al., 2014). The active form of vitamin D works on vitamin D receptors in the osteoblasts and other cells to perform its functions (Bikle, 2014). This may be another factor into play with the osteoblast activity in that studies have shown that 25OHD may assist in bone building by osteoblasts whereas 1,25OHD stimulates bone resorption (Anderson, 2017) (Yang, et al., 2016) (Turner, et al., 2014). Studies done on hemodialysis patients have found that 25OHD suppress osteoclast action in cases of inflammation by increasing expression of RANKL and inducing the production of OPG (Eleftheriadis, et al., 2012).

Vitamin D has also been shown to be a potent differentiator of osteoblast-like MC3T3-E1 cells into osteoblasts. Kim et al. showed that 1,25OHD increase cell proliferation and ALP activity.

Gene expressions of ALP, Col-I, OCN and VDR were also increased in osteoblasts with 1,25OHD induction (Kim, et al., 2018). However, Vit D receptors for 1,25OHD have been observed to have different effects depending on the age of the osteoblasts. In early stages, it seems to inhibit the differentiation into osteoblasts. However, in later stages, it seems to increase osteoblastic activity (St- Arnaud, 2008). Van Driel et al found that 1,25OHD decreases osteoblasts proliferation but increases differentiation by increasing collagen type I synthesis, osteocalcin and osteopontin (van Driel, et al., 2004). 2

5OHD was thought to only have effects on osteoblasts after conversion to 1,25OHD, because osteoblasts are capable of synthesizing 1,25OHD from 25OHD by expressing 1α -hydroxylase. While studying the effects of 25OHD on osteoblasts, they found that it increases the ALP activity and osteocalcin expression (van der Meijen, et al., 2014) (van Driel, et al., 2004). It is currently unknown if these effects occur only after conversion or if 25OHD has a cellular effect of its own, but silencing CYP27B1 human osteoblasts cell studies have shown that 25OHD decreases osteonectin and CYP24 mRNA expression compared to controls (van der Meijen, et al., 2014).

Alkaline phosphatase (ALP) is a glycoprotein produced by fibroblasts, osteoblasts and osteoclasts. It plays a role in mineral formation of hard tissue structures like bone and cementum as first studied by Robinson in 1923 (Robison, et al., 1924) (Farahani, et al., 2015). It is known to be a biological marker of osteoblastic activity during bone deposition (Farahani, et al., 2015) (Rodan, 1992).

The MAPK (mitogen-activated protein kinase) pathway is one of the major pathways involved in bone remodeling and has also been shown to be activated during orthodontic tooth move-

ment (Jiang, et al., 2018). It is composed of a multiple Ser/Thr kinases including ERK (extracellular signal-regulated kinase) 1/2, p38 and JNK (c-Jun-N-terminal kinase) (Jiang, et al., 2018). These signals are closely associated with bone formation of osteoblasts and bone resorption of osteoclasts mainly in the areas of proliferation, apoptosis and differentiation (Jiang, et al., 2018). There is a contradictory evidence of the full mechanism of ERK1/2 and p38 pathways in osteogenic differentiation (Li, et al., 2012) (Xiao, et al., 2002) (Jiang, et al., 2018). Li et al showed an increase in osteogenic differentiation when ERK 1/2 and p38 signaling pathways we inhibited in dental follicle cells (Li, et al., 2012). However, Xiao et al reported restrained osteoblastic activity and bone formation due to inhibition of the ERK1/2 pathway (Xiao, et al., 2002).

Hypothesis

Null Hypothesis: (H_0): There is no correlation between different concentrations of 1,25OHD and 25OHD with osteoblast functions.

Hypothesis: (H_1): Different concentrations of 1,25OHD and 25OHD will impact osteoblast functions

Aims

Overall aim

The aim of this study was to determine the impact of different concentrations of 1,25OH Vitamin D and 25OH Vitamin D on osteoblast function and signaling pathway

Specific aims

- 1) To determine the impact of vitamin D's effect on OPG, RANKL and ALP production by the osteoblasts
- 2) To identify the signaling pathways that 1,25OHD and 25OHD stimulate in osteoblasts

Materials and Methods

Cell Culture

Osteoblasts were collected from the calvaria of 3-5 day old healthy FVB wild-type mice purchased from Jackson Laboratories (Bar Harbor, ME) in conformity with the standards of the Public Health Service Policy on Human Care and the Use of Laboratory Animals and were approved by the Institution of Animal Care and Use Committee of the Forsyth Institute.

The frontal and parietal bones were aseptically isolated by dissection of the scalp skin, periosteum and loosely adherent tissues. They were immediately rinsed with ethanol and phosphate-buffered saline (PBS) + antibiotics (penicillin/streptavidin) to remove all extraneous tissue. The calvaria were then washed with a solution of 137 mM NaCl, 2.7 mM KCl and 3 mM NaH₂PO₄ x H₂O and incubated for 10 minutes on a shaker at 37°C in 4 mM After that, a Na₂-EDTA solution, three times to demineralize the extracellular matrix discarding the supernatants. They were then, incubated a total of 7 times at 37°C in a freshly prepared collagenase type 2 solution (Worthington) in 137 mM NaCl, 2.7 mM KCl, 3mM NaH₂PO₄ x H₂O on a shaker to separate the cells from the extracellular matrix. The first 2 supernatants were discarded, and the supernatants from the following five incubations were collected, pooled, centrifuged, and re-suspended in α -MEM media supplemented with 10% FBS, 10,000 IU penicillin (1%), and 10,000 μ g/ml streptomycin (1%). Cells were cultured in 75 ml flasks with MEM Alpha (1X) media with L-Glutamine, Ribonucleotides and Deoxy-ribonucleotides from Gibco™ including 10% FBS (fetal bovine serum) and 1% penicillin and streptomycin until they reached 90% confluency. Once the cells had grown enough, they were seeded depending on the experiment and treated with 25OHD₃ (10⁻⁸ M) and 1 α 25OHD₃ (10⁻⁸ M) in varying dosages, 25OHD (0, 100, 200 or 400 nmol/L) and 1 α 25OHD (0, 1, 10 or 100

nmol/L). The media was changed every 3 days. All experiments were repeated 3-5 times and averages used for statistical calculations.

Measurement of Alkaline Phosphatase (ALP) in Osteoblast Culture Supernatants

Osteoblasts were seeded in 24-well plates at 2×10^4 cells/well in triplicate with MEM Alpha (1X) from Gibco™ including 10% FBS and 1% penicillin/ streptomycin with the appropriate concentrations of 1,25OHD (1, 10, 100nmol) and 25OHD (100, 200, 400 nmol) as well as control sample. Supernatant of cells was collected at days 3, 7, and 10 with each media change. Then the collected samples were measured using Abcam Alkaline Phosphatase Assay kit (Colorimetric) (ab83369) according to manufacturer's instructions. Optical density (OD) of the products at 405 nm was measured by spectrophotometry. Enzyme activity was calculated as the OD of the reaction product multiplied by the reaction volume and normalized to the reaction time and to the total protein.

Measurement of Osteoprotegerin (OPG) and RANKL in Osteoblast Culture Supernatants

Osteoblasts were obtained as explained above and seeded in 24-well plates at 1×10^4 cells/well in triplicate with MEM Alpha (1X) from Gibco™ including 10% FBS and 1% penicillin/ streptomycin with the appropriate concentrations of 1,25OHD (1, 10, 100nmol) and 25OHD (100, 200, 400 nmol) as well as a control sample. Supernatant of cells was collected at days 3, 7, and 10 with each media change. OPG and RANKL levels were measured using the Quantikine DuoSet ELISA (enzyme-linked immunosorbent assay) kit by R&D systems according to manufacturer's instructions. ELISA plates were coated with the OPG, RANKL (R&D Systems) capture antibodies

overnight, then washed with 0.05% Tween[®] 20 in PBS, blocked using 1% BSA in PBS, and incubated with standards and samples. The detection antibody diluted in 1% BSA in PBS was applied to the plates, followed by Avidin-HRP in 1% BSA in PBS. The colorimetric signal developed after incubation with 1:1 mixture of H₂O₂ and Tetramethylbenzidine, and the reaction was stopped using 2N H₂SO₄. Absorbance was read at 450 nm (Spectramax 340PC 384 with a SoftMax Pro software 4.3LS, Molecular Devices), and the background absorbance values were subtracted. Sample concentration was calculated based on a standard curve.

Measurement of Osteoprotegerin (OPG) and RANKL Expression in Osteoblasts

Osteoblasts were seeded into 6-well plates at 1×10^5 cells/well with MEM Alpha (1X) from Gibco™ including 10% FBS and 1% penicillin/ streptomycin with the appropriate concentrations of 1,25OHD (1, 10, 100nmol) and 25OHD (100, 200, 400 nmol) as well as a control sample. Cells were lysed at 3, 7, and 10 days using the RNeasy Plus kit from Qiagen. Media was removed from each well and the appropriate amount of lysate was added to disrupt and homogenize the cells. An equal amount of ethanol was then added to each condition to promote selective binding of RNA to RNeasy membrane. Then the mixture was transferred to a RNeasy spin column and centrifuged at $> 8000 \times g$ for 15 sec. The process was repeated with the appropriate buffers (RW1 once and RPE twice). The spin column was then placed in a new 2ml collection tube and centrifuged at full speed for 1 min to eliminate any residual buffers. 40 µg of RNase free water to elute the RNA. Purity of RNA was confirmed by a 260/280 optical density value of 1.8-2.0 by Nanodrop.

cDNA was generated using reverse transcription using Invitrogen SuperScript IV VILO kit per manufacturer's protocol for qRT-PCR (protocol in appendix below). Reverse transcription reaction was performed using 1 µg RNA using random hexamers, dNTP, buffer, nuclease-free water, ribonuclease (RNase) inhibitor and reverse transcriptase (High-Capacity cDNA Reverse Transcription Kit, ABI). The samples were incubated in the GeneAmp PCR 9700 system (ABI): 25°C for 10 min, 37°C for 2 h, 85°C for 5 min with a total of 20 µl reaction volume. The cDNA products were stored at -20°C and diluted before the real-time reaction.

The expression of the OPG and RANKL genes in osteoblasts was measured with quantitative real-time PCR and analyzed using the ΔC_T and $\Delta\Delta C_T$ method. The expression of each target gene was calculated relative to GAPDH endogenous control, relative to the 3 day controls. The following equations were used to calculate the ΔC_T and $\Delta\Delta C_T$ for OPG and RANKL results normalized to GAPDH.

$$\Delta C_T = xCT(\text{sample}) - xCT(\text{GAPDH})$$

$$c = x\Delta C_T(\text{sample}) - x\Delta C_T(\text{3day control})$$

Measurement of total protein concentration

Protein concentration was measured using the BCA (Bicinchoninic acid assay) kit by ThermoFisher Scientific. A standard curve was prepared using serial dilutions of bovine serum albumin (BSA) at 0, 5, 25, 50, 125, and 250 µg/ml. 200 µl BCA reagent (Pierce™ BCA Protein Assay Kit) were added to 10 µl sample and standards and assayed in duplicates. Absorbance was measured at 562 nm (Spectramax 340PC384 with SoftMax 4.3LS, Molecular Devices). Background absorb-

ance of de-ionized water was subtracted from all measurements. Sample concentrations were extrapolated from the standard curve. Western Blot was then performed according to the methodology below.

Measurement of ERK1/2, p38 and NLK in Osteoblasts

Osteoblasts were seeded into 6-well plates at 1×10^5 cells/well with MEM Alpha (1X) from Gibco™ including 10% FBS and 1% penicillin/ streptomycin with the appropriate concentrations of 1,25OHD (1, 10, 100 nmol) and 25OHD (100, 200, 400 nmol) as well as a control sample. Cells were lysed and scraped at 1-hour and 24-hours using the appropriate amount of Cell Lysate provided by ThermoFisher Scientific supplemented with protease and phosphatase inhibitors. Antibodies chosen according to the MAPK pathway as ERK1/2 (p44/42 MAPK), Total ERK 1/2, p-p38, Total p-38 and NLK using B-actin as a control. Antibodies were purchased from Cell Signaling Technology.

5 µg of lysate of each sample was calculated according to the BCA assay and mixed with 4x sample loading buffer with 2.5% volume β-ME then denatured by heating to 100°C for 8 min. Electrophoresis was done to the lysate samples in 12% polyacrylamide gels in running buffer (25 mM Trizma base, 192 mM glycine, 0.1% sodium dodecyl sulfate (SDS)) at 100 V for 1.5-2 hrs. Molecular weight standard (ladder) was added in a separate well in the gel. A polyvinylidene fluoride (PVDF) membrane was prepared by 1 min incubation in methanol followed by 10 min incubation in blotting buffer (25 mM Trizma base, 192 mM glycine, 20% 79 methanol). Transfer of the proteins to the PVDF membrane was performed at 4°C overnight at 66 mA. The next morning, the membrane was incubated in blocking solution (5% (w/v) BSA in 20 mM Tris-HCl, 150 mM

NaCl, 0.1% Tween-20 + 5% BSA (TBS-T)) for 1 hour and then incubated with the targeted primary antibodies overnight at 4°C in 5% BSA in TBS-T on a shaker. The next day, the membrane was washed 3 times with TBS-T for 10 min and incubated with goat anti-rabbit IgG-horseradish peroxidase (HRP) secondary antibody in blocking solution (5% (w/v) milk in 20 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20 + 5% milk (TBS-T)) for 1 hr. at room temperature. The membrane was then washed 3 times for 10 min in TBS-T, and the HRP conjugate was detected using a chemiluminescence reaction. An X-ray film was exposed to the membrane and protein bands were visualized. Results were normalized to the expression of (β -actin). To assay for additional targets, the membrane was incubated for 20 min in a stripping buffer and washed 3 times in TBS-T before incubation with the next primary antibody.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 8 Software 2019 (San Diego, California) for regression, one way ANOVA, and Tukey Post-hoc analysis as needed. The level of significance was noted at $p < 0.05$ for all analyzed data. All tests were performed 3-5 times in duplicate and the means entered for statistical analysis.

Results

Measurement of Alkaline Phosphatase Concentration and Activity in Osteoblast Supernatant

ALP concentration and activity were measured using the Abcam Alkaline Phosphatase Assay kit (Colorimetric) kit (ab83369) explained above. Supernatants were collected at 3, 7, and 10 days in triplicate of 20,000 cells/well. Results were measured using a statistical analysis system STATA and Graphpad Prism 8. No significant differences were noted between groups or between different time-points of the same group.

	ALP concentration	stdev	ALP Activity	stdev
3 days				
Control	8.572	2.169	1.818	0.515
1,25OHD 1nmol	8.438	1.390	1.805	0.311
1,25OHD 10nmol	8.613	1.580	1.833	0.367
1,25OHD 100nmol	8.777	2.154	1.888	0.495
25OHD 100nmol	8.704	1.828	1.868	0.417
25OHD 200nmol	9.621	3.489	2.101	0.801
25OHD 400nmol	8.748	2.179	1.905	0.480
7 days				
Control	9.230	2.047	1.975	0.474
1,25OHD 1nmol	8.888	2.283	1.909	0.529
1,25OHD 10nmol	9.058	1.730	1.944	0.389
1,25OHD 100nmol	9.687	2.999	2.081	0.703
25OHD 100nmol	8.898	1.977	1.926	0.438
25OHD 200nmol	8.766	2.020	1.864	0.476
25OHD 400nmol	8.824	1.984	1.892	0.457
10 days				
Control	9.242	2.381	1.950	0.569
1,25OHD 1nmol	8/644	2.036	1.812	0.489
1,25OHD 10nmol	9.261	2.192	1.970	0.517
1,25OHD 100nmol	9.503	2.585	1.995	0.621
25OHD 100nmol	8.721	2.270	1.828	0.545

25OHD 200nmol	8.592	2.239	1.812	0.535
25OHD 400nmol	9.421	1.978	1.975	0.475

Table 5: ALP concentration and activity means and standard deviations

Figure 1: ALP Concentration in pg/ml between groups over time. No statistical significance (at $p < 0.05$) between groups over time.

A. at 3 days.

B. at 7 days.

C. at 10 days.

Conditions: control, 1,25OHD 1nmol, 1,25OHD 10nmol, 1,25OHD 100nmol, 25OHD 100nmol, 25OHD 200nmol, 25OHD 400nmol.

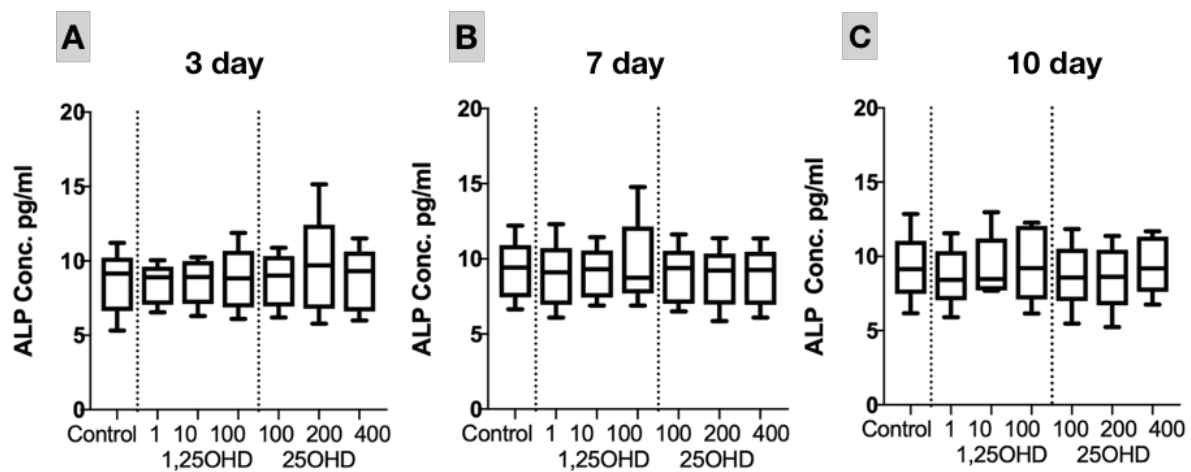


Figure 2: ALP Concentration in pg/ml between time points within groups. No statistical significance (at $p < 0.05$) between time points within groups.

- A. Control condition with days 3, 7, and 10
- B. 1,25OHD 1nmol condition with days 3, 7, 10
- C. 1,25OHD 10nmol condition with days 3, 7, 10
- D. 1,25OHD 100nmol condition with days 3, 7, 10
- E. 25OHD 100nmol condition with days 3, 7, 10
- F. 25OHD 200nmol condition with days 3, 7, 10
- G. 25OHD 400nmol condition with days 3, 7, 10

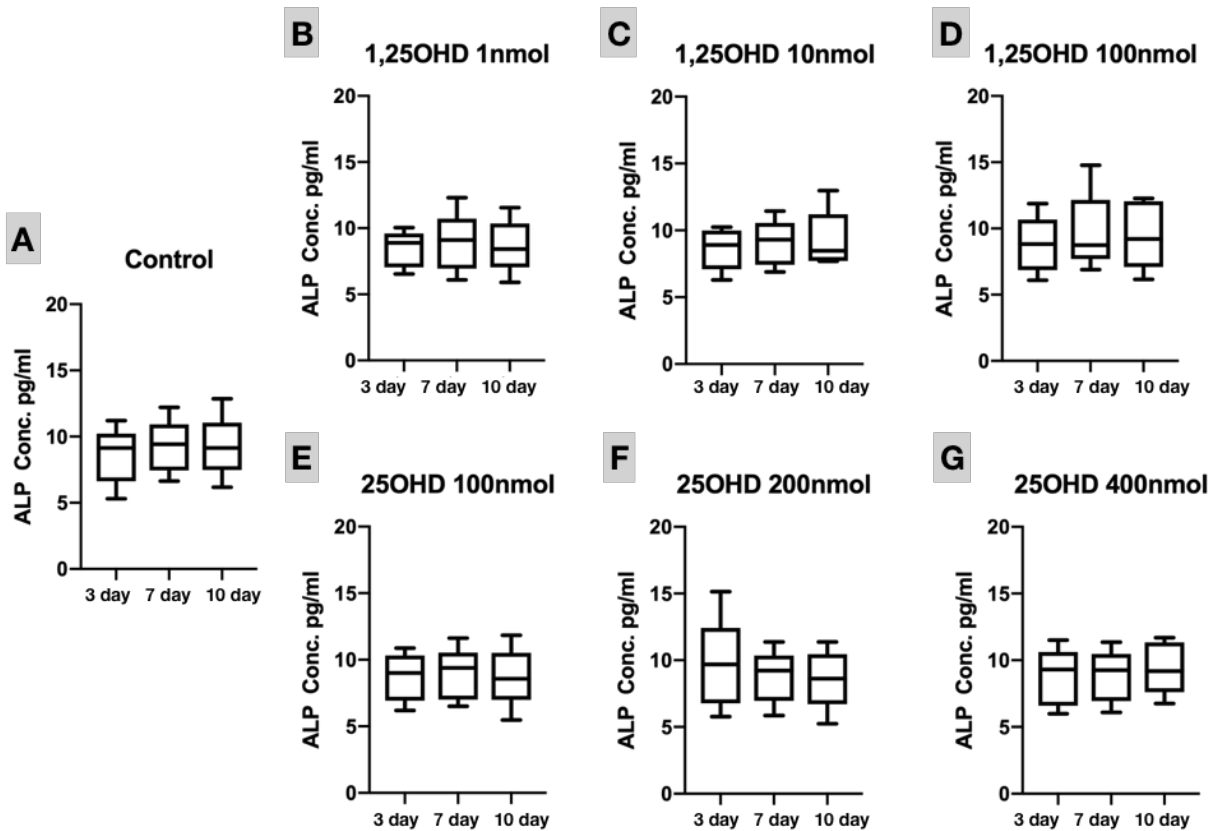


Figure 3: ALP Activity ($\mu\text{M}/\text{min}/\text{mg}$ protein) between groups over time. No statistical significance (at $p < 0.05$) between groups over time.

- A. at 3 days.
- B. at 7 days.
- C. at 10 days.

Conditions: control, 1,25OHD 1nmol, 1,25OHD 10nmol, 1,25OHD 100nmol, 25OHD 100nmol, 25OHD 200nmol, 25OHD 400nmol.

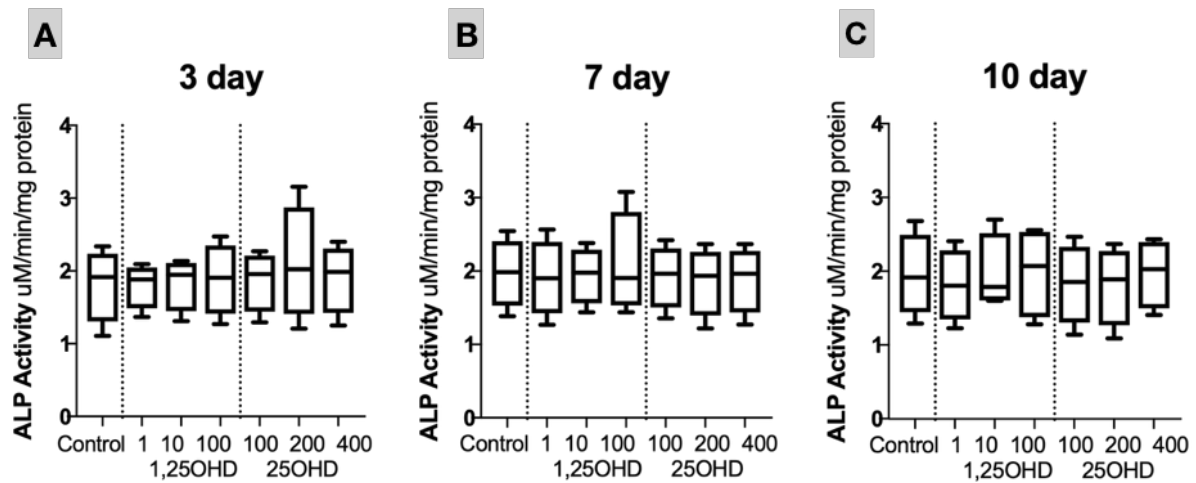
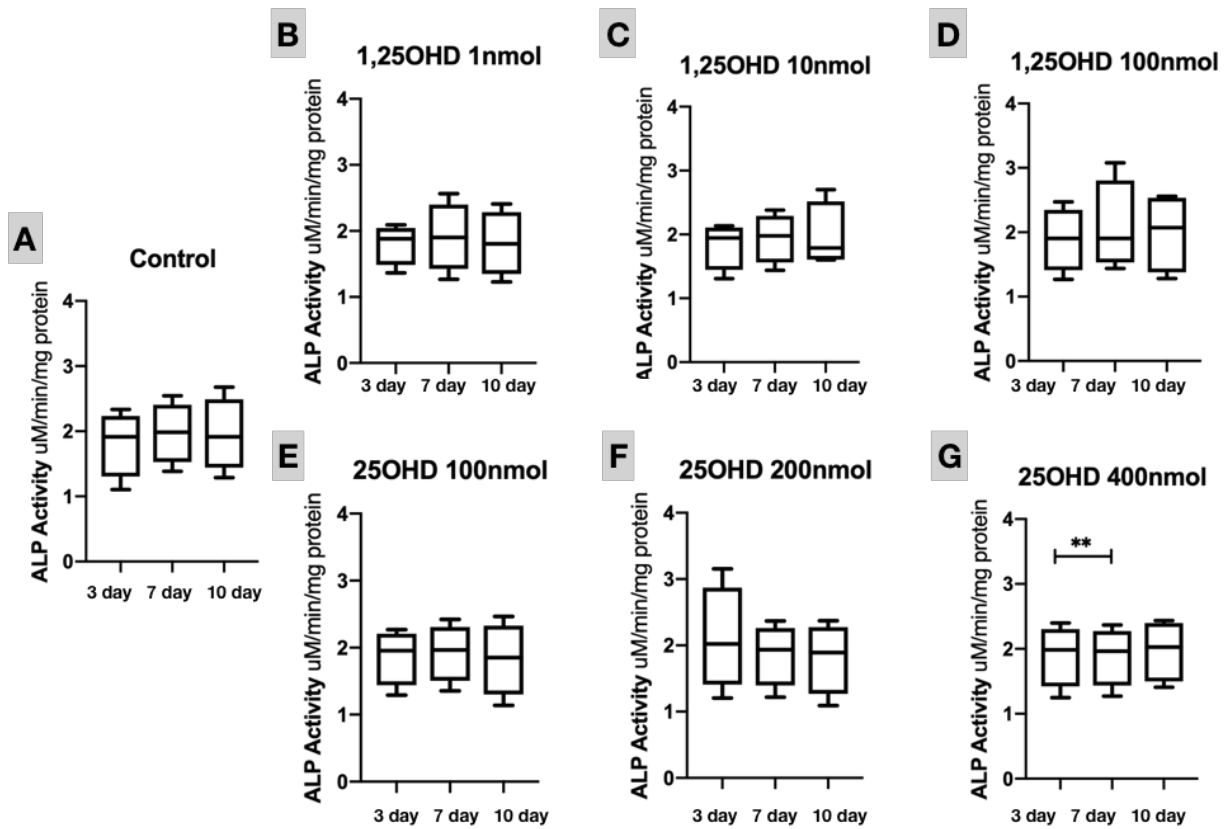


Figure 4: ALP Activity ($\mu\text{M}/\text{min}/\text{mg}$ protein) between time points within groups. No statistical significance (at $p < 0.05$) between time points within groups.

- A. Control condition with days 3, 7, and 10
- B. 1,25OHD 1nmol condition with days 3, 7, 10
- C. 1,25OHD 10nmol condition with days 3, 7, 10
- D. 1,25OHD 100nmol condition with days 3, 7, 10
- E. 25OHD 100nmol condition with days 3, 7, 10
- F. 25OHD 200nmol condition with days 3, 7, 10
- G. 25OHD 400nmol condition with days 3, 7, 10



Measurement of OPG and RANKL Levels in Osteoblasts Supernatant

OPG and RANKL concentration were measured using the Quantikine DuoSet kit by R&D systems according to manufacturer's instructions explained above. Supernatant was collected at 3, 7, and 10 days in triplicate of 10,000 cells/well. Results were measured using statistical analysis system STATA. Using one-way ANOVA and Tukey Post-hoc, the following statistically significant results were observed.

In regards to OPG levels at 3 days, a significant difference was noted between control and 25OHD 100nmol (0.0171), 1,25OHD 1nmol and the 25OHD 100nmol ($p=0.0005$), 1,25OHD 10nmol and 25OHD 100nmol ($p=0.0001$), 1,25OHD 100nmol and 25OHD 100nmol ($p=0.0011$) and 25OHD 100nmol and 25OHD 400nmol ($p=0.0193$). At 7 days, the significant differences were noted between the control and 25OHD 100nmol ($p=0.0043$), 1,25OHD 1nmol and 25OHD 100nmol ($p=0.0001$) and 25OHD 200nmol ($p=0.0224$). 1,25OHD 10nmol was noted to be significantly associated with both 25OHD 100nmol and 200nmol ($p=0.000$ and 0.0116 , respectively). However, 1,25OHD at 100nmol was significant with 25OHD 100 nmol and 400nmol ($p=0.000$ and 0.0016 , respectively). Lastly with 7 days, 400nmol of 25OHD was statistically significant with 25OHD 100nmol and 200nmol ($p=0.000$ and 0.0018 , respectively). With 10 days, the significant differences were noted with control and 25OHD 100nmol ($p=0.0175$), 25OHD 100nmol with 1,25OHD 1nmol ($p=0.0118$), 1,25OHD 10nmol ($p=0.0094$) and 1,25 100nmol ($p=0.0020$). Finally, 10 day 25OHD 100nmol and 25OHD 400nmol ($p=0.0018$).

When considering OPG levels by group over time points, the only differences were found in 25OHD 200nmol and 25OHD 400nmol. For the 200nmol concentration the difference was noted between day 7 and day 10 ($p=0.0000$). For the 400nmol concentration the difference was noted between days 3 and 10 ($p=0.0377$).

	OPG	stdev	RANKL	stdev
3 days				
Control	20,353.07	25,513.30	19.14	17.62
1,25OHD 1nmol	11,316.26	8,562.44	29.77	31.71
1,25OHD 10nmol	6,510.57	2,832.54	54.25	32.11
1,25OHD 100nmol	3,678.50	1,044.30	97.63	118.04
25OHD 100nmol	14,706.47	13,323.73	26.40	20.44
25OHD 200nmol	11,993.36	10,688.58	22.67	19.56
25OHD 400nmol	7,799.88	6,645.57	41.22	36.94
7 days				
Control	13,495.80	12,363.04	26.96	22.11
1,25OHD 1nmol	10,695.70	7,366.23	25.95	18.41
1,25OHD 10nmol	5,6775.88	1,228.76	56.01	40.62
1,25OHD 100nmol	1,364.49	479.27	44.86	26.66
25OHD 100nmol	17,773.36	18,71.96	27.36	20.89
25OHD 200nmol	15,627.60	21,733.22	22.67	23.27
25OHD 400nmol	4,615.41	5,586.43	64.03	72.28
10 days				
Control	6,222.13	930.27	23.94	18.66
1,25OHD 1nmol	16,123.50	19,227.94	21.92	12.66
1,25OHD 10nmol	9,911.01	14,806.31	34.04	19.62
1,25OHD 100nmol	2,358.66	3,173.24	130.76	187.96
25OHD 100nmol	20,514.57	24,658.17	21.22	17.16

25OHD 200nmol	17,909.90	25,030.23	16.16	11.03
25OHD 400nmol	3,497.77	4,696.84	59.40	74.97

Table 6: OPG and RANKL concentrations and standard deviations

Figure 5: OPG concentration in pg/ml by time points over groups.

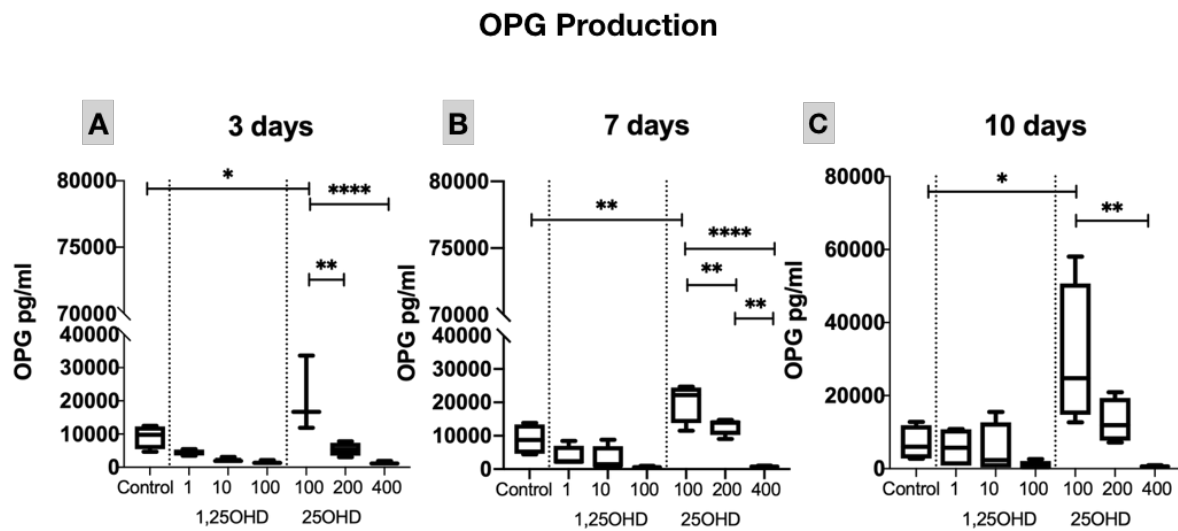
A. at 3 days.

B. at 7 days.

C. at 10 days.

Conditions: control, 1,25OHD 1nmol, 1,25OHD 10nmol, 1,25OHD 100nmol, 25OHD 100nmol, 25OHD 200nmol, 25OHD 400nmol.

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$



3 day	Con- trol	1,25OHD 1nmol	1,25OHD 10nmol	1,25OHD 100nmol	25OHD 100nmol	25OHD 200nmol	25OHD 400nmol
Control	-	0.6289	0.2140	0.1457	0.0171	0.8262	0.1212
1,25OHD 1nmol	0.6289	-	0.9821	0.9427	0.0005	0.9998	0.9137
1,25OHD 10nmol	0.2140	0.9821	-	>0.9999	0.0001	0.9029	0.9999
1,25OHD 100nmol	0.1457	0.9427	>0.9999	-	<0.0001	0.8081	>0.9999
25OHD 100nmol	0.0171	0.0005	0.0001	<0.0001	-	0.0011	<0.0001
25OHD 200nmol	0.8262	0.9998	0.9029	0.8081	0.0011	-	0.7552
25OHD 400nmol	0.1212	0.9137	0.9999	>0.9999	<0.0001	0.7552	-

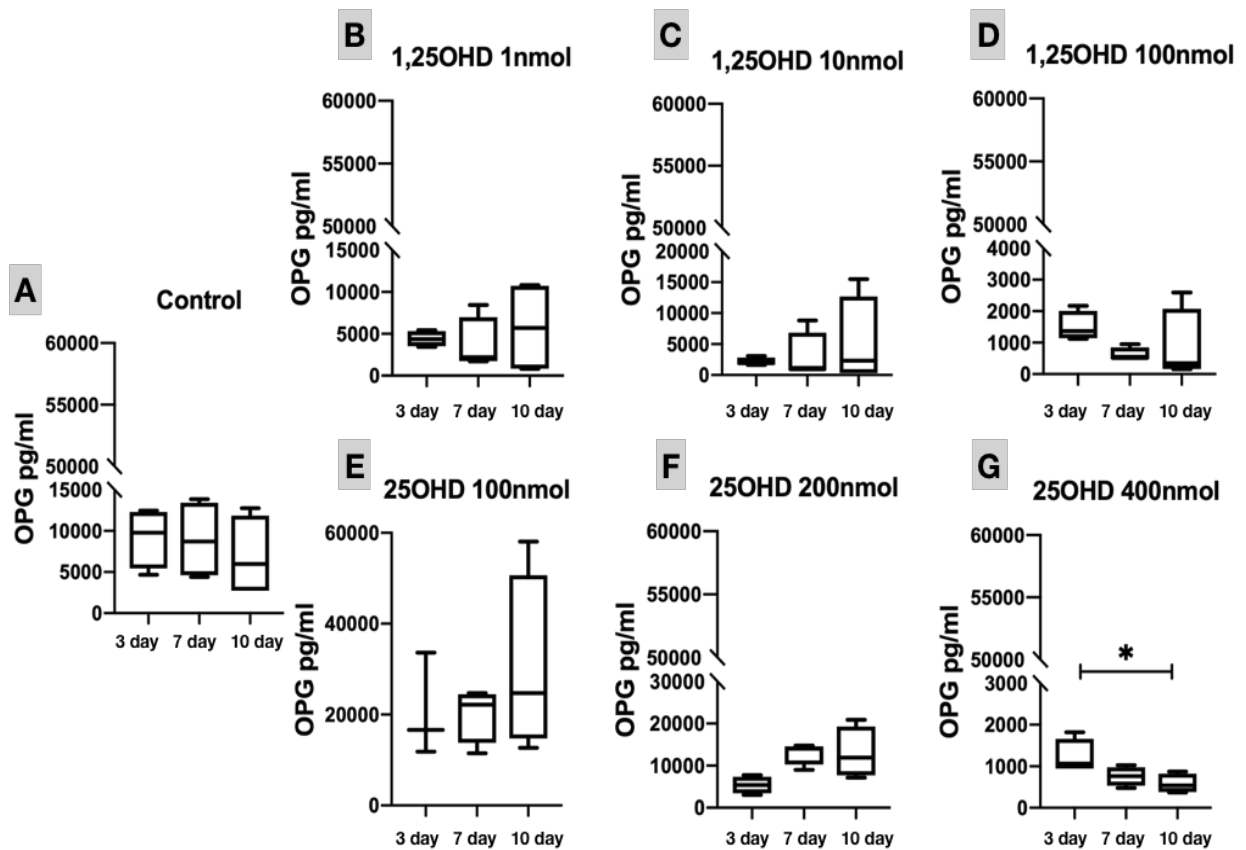
7 day	Con- trol	1,25OHD 1nmol	1,25OHD 10nmol	1,25OHD 100nmol	25OHD 100nmol	25OHD 200nmol	25OHD 400nmol
Control	-	0.4099	0.2645	0.0517	0.0043	0.7024	0.0579
1,25OHD 1nmol	0.4099	-	0.9999	0.8958	0.0001	0.0224	0.9148
1,25OHD 10nmol	0.2645	0.9999	-	0.9722	0.0000	0.0116	0.9798
1,25OHD 100nmol	0.0517	0.8958	0.9722	-	0.0000	0.0016	1.0000
25OHD 100nmol	0.0043	0.0001	0.0000	0.0000	-	0.1222	0.0000
25OHD 200nmol	0.7024	0.0224	0.0116	0.0016	0.1222	-	0.0018
25OHD 400nmol	0.0579	0.9148	0.9798	1.000	0.0000	0.0018	-

10 day	Con- trol	1,25OHD 1nmol	1,25OHD 10nmol	1,25OHD 100nmol	25OHD 100nmol	25OHD 200nmol	25OHD 400nmol
Control	-	1.0000	0.9999	0.9559	0.0175	0.9520	0.9455
1,25OHD 1nmol	1.0000	-	1.0000	0.9836	0.0118	0.9005	0.9783
1,25OHD 10nmol	0.9999	1.0000	-	0.9922	0.0094	0.8589	0.9890
1,25OHD 100nmol	0.9559	0.9836	0.9922	-	0.0020	0.4716	1.0000
25OHD 100nmol	0.0175	0.0118	0.0094	0.0020	-	0.1329	0.0018
25OHD 200nmol	0.9520	0.9005	0.8589	0.4716	0.1329	-	0.4454
25OHD 400nmol	0.9455	0.9783	0.9890	1.0000	0.0018	0.4454	-

Table 7: OPG statistical significance by one way ANOVA over time points

Figure 6: OPG concentration in pg/ml by groups over time points

- A. Control condition with days 3, 7, and 10
- B. 1,25OHD 1nmol condition with days 3, 7, 10
- C. 1,25OHD 10nmol condition with days 3, 7, 10
- D. 1,25OHD 100nmol condition with days 3, 7, 10
- E. 25OHD 100nmol condition with days 3, 7, 10
- F. 25OHD 200nmol condition with days 3, 7, 10
- G. 25OHD 400nmol condition with days 3, 7, 10



	3-7 day	3-10 day	7-10 day
Control	0.9966	0.7539	0.7972
1,25OHD 1nmol	0.9573	0.8712	0.7204
1,25OHD 10nmol	0.9736	0.6625	0.7890
1,25OHD 100nmol	0.2563	0.4631	0.8884
25OHD 100nmol	0.9985	0.6666	0.5929
25OHD 200nmol	0.0625	0.0623	>0.9999
25OHD 400nmol	0.1346	0.0377	0.6981

Table 8: OPG statistical significance over groups by time points

When looking into RANKL levels over time, the following combinations were considered statistically significant. At 3 days, control with 25OHD 400nmol at $p=0.0125$ and 25OHD between the 100 and 400nmol concentrations ($p=0.0128$). In the 7-day time-point, the 25OHD 400nmol was statistically significant with all of the following, control ($p=0.0010$), 1,25OHD 1nmol ($p=0.0054$), 1,25OHD 10nmol ($p=0.0040$), 1,25OHD 100nmol ($p=0.0309$), 25OHD 100nmol ($p=0.0008$) and 25OHD 200nmol ($p=0.0008$). However, the 10-day time point showed no statistically significant difference between any combinations. When considering by group, the only significance was in the 1,25OHD 10nmol group between days 3 and 7 ($p=0.0009$) and days 3-10 ($p=0.0001$).

Figure 7: RANKL concentration in pg/ml by time points over groups

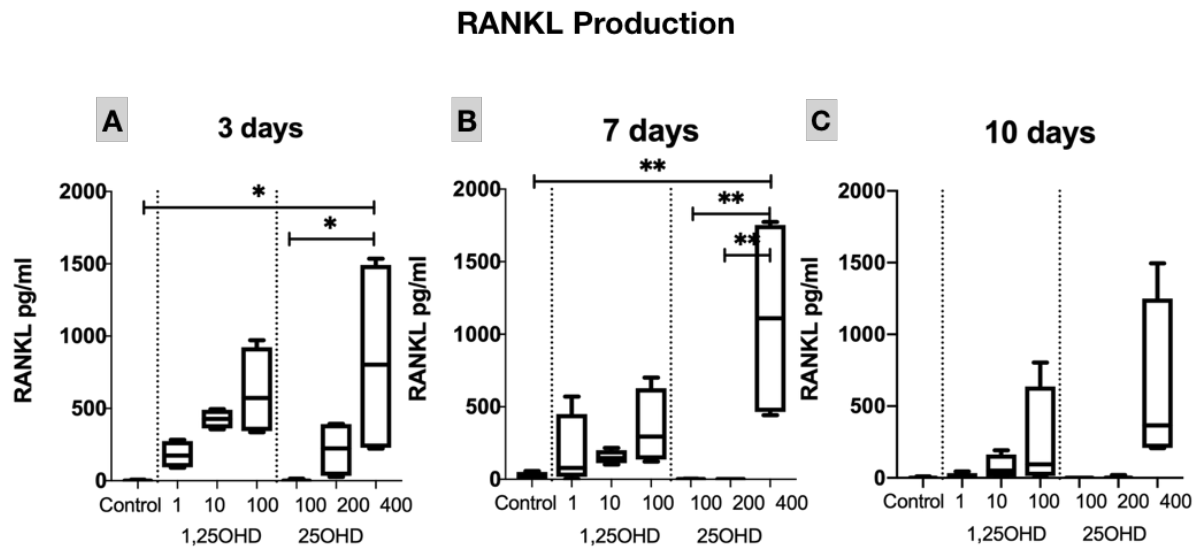
A. at 3 days.

B. at 7 days.

C. at 10 days.

Conditions: control, 1,25OHD 1nmol, 1,25OHD 10nmol, 1,25OHD 100nmol, 25OHD 100nmol, 25OHD 200nmol, 25OHD 400nmol.

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$



3 day	Con- trol	1,25OHD 1nmol	1,25OHD 10nmol	1,25OHD 100nmol	25OHD 100nm ol	25OHD 200nmol	25OHD 400nmol
Control	-	0.9789	0.4593	0.1160	0.8031	0.9494	0.0125
1,25OHD 1nmol	0.9789	-	0.9073	0.4443	0.9799	>0.9999	0.0735
1,25OHD 10nmol	0.4593	0.9073	-	0.9754	0.4639	0.9538	0.4901
1,25OHD 100nmol	0.1160	0.4443	0.9754	-	0.1177	0.5428	0.9328
25OHD 100nmol	0.8031	0.9799	0.4639	0.1177	-	0.9512	0.0128
25OHD 200nmol	0.9494	1.0000	0.9538	0.5428	0.9512	-	0.1019
25OHD 400nmol	0.0125	0.0735	0.4901	0.9328	0.0128	0.1019	-

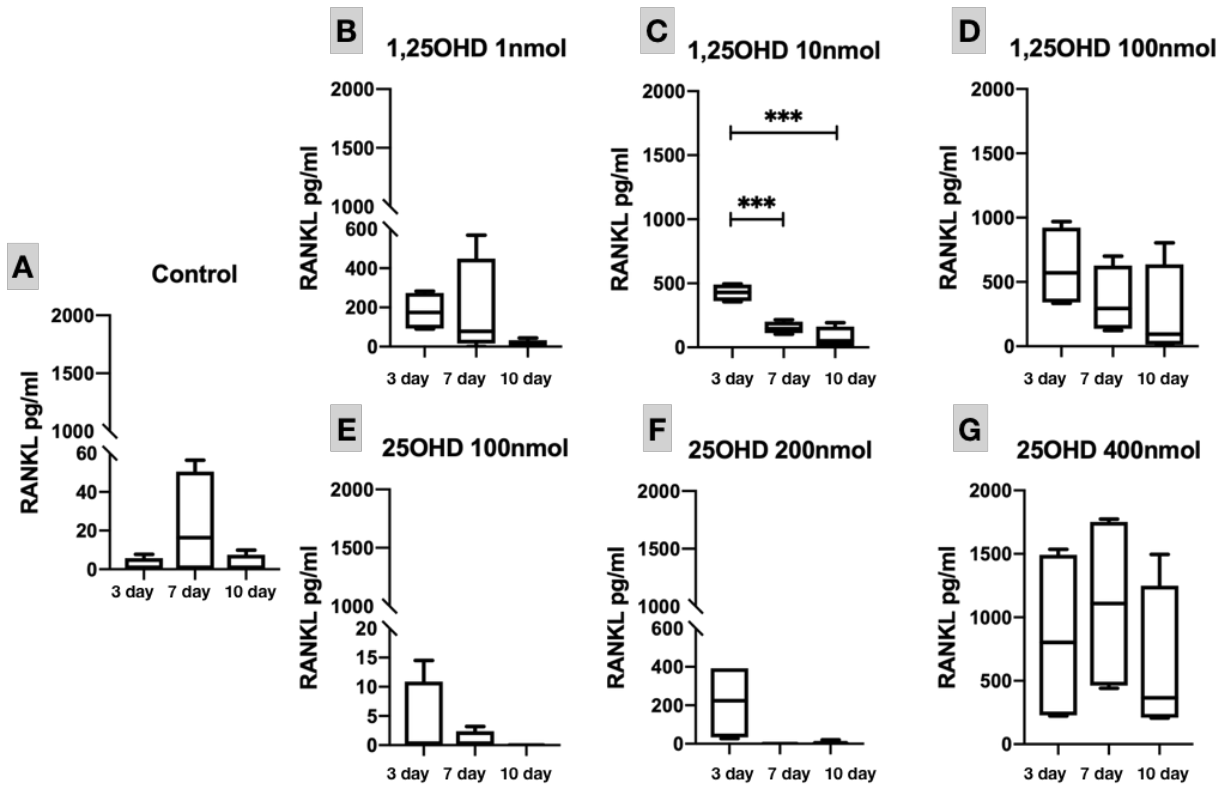
7 day	Con- trol	1,25OHD 1nmol	1,25OHD 10nmol	1,25OHD 100nmol	25OHD 100nmol	25OHD 200nmol	25OHD 400nmol
Control	-	0.9888	0.9962	0.7307	1.0000	1.0000	0.0010
1,25OHD 1nmol	0.9888	-	1.0000	0.9837	0.9787	0.9783	0.0054
1,25OHD 10nmol	0.9962	1.0000	-	0.9647	0.9913	0.9912	0.0040
1,25OHD 100nmol	0.7307	0.9837	0.9647	-	0.6726	0.6709	0.0309
25OHD 100nmol	1.0000	0.9787	0.9913	0.6726	-	>0.9999	0.0008
25OHD 200nmol	1.0000	0.9783	0.9912	0.6709	>0.9999	-	0.0008
25OHD 400nmol	0.0010	0.0054	0.0040	0.0309	0.0008	0.0008	-

10 day	Control	1,25OH D 1nmol	1,25OHD 10nmol	1,25OH D 100nmol	25OHD 100nmol	25OHD 200nmo 1	25OHD 400nmo 1
Control	-	1.0000	0.9997	0.8544	>0.9999	>0.9999	0.0617
1,25OHD 1nmol	1.0000	-	0.9999	0.8732	>0.9999	>0.9999	0.0675
1,25OHD 10nmol	0.9997	0.9999	-	0.9671	0.9997	0.9998	0.1270
1,25OHD 100nmol	0.8544	0.8732	0.9671	-	0.8487	0.8603	0.5193
25OHD 100nmol	>0.999 9	>0.9999	0.9997	0.8487	-	0.9942	0.0601
25OHD 200nmol	>0.999 9	>0.9999	0.9998	0.8603	0.9942	-	0.0634
25OHD 400nmol	0.0617	0.0675	0.1270	0.5193	0.0601	0.0634	-

Table 9: RANKL statistical significance by one way ANOVA over time points

Figure 8: RANKL concentration in pg/ml by groups over time points

- A. Control condition with days 3, 7, and 10
- B. 1,25OHD 1nmol condition with days 3, 7, 10
- C. 1,25OHD 10nmol condition with days 3, 7, 10
- D. 1,25OHD 100nmol condition with days 3, 7, 10
- E. 25OHD 100nmol condition with days 3, 7, 10
- F. 25OHD 200nmol condition with days 3, 7, 10
- G. 25OHD 400nmol condition with days 3, 7, 10



	3-7 day	3-10 day	7-10 day
Control	0.2336	0.9987	0.2500
1,25OHD 1nmol	>0.9999	0.3434	0.3409
1,25OHD 10nmol	0.0009	0.0001	0.2931
1,25OHD 100nmol	0.5133	0.2931	0.8912
25OHD 100nmol	0.6336	0.4843	0.9630
25OHD 200nmol	0.0665	0.0730	0.9980
25OHD 400nmol	0.8458	0.8806	0.5717

Table 10: RANKL statistical significance over groups by time points

Figure 9: RANKL/OPG by time points showing each condition (control, 1,25OHD 1nmol, 1,25OHD 10nmol, 1,25OHD 100nmol, 25OHD 100nmol, 25OHD 200nmol, 25OHD 400nmol) over days 3, 7, and 10.

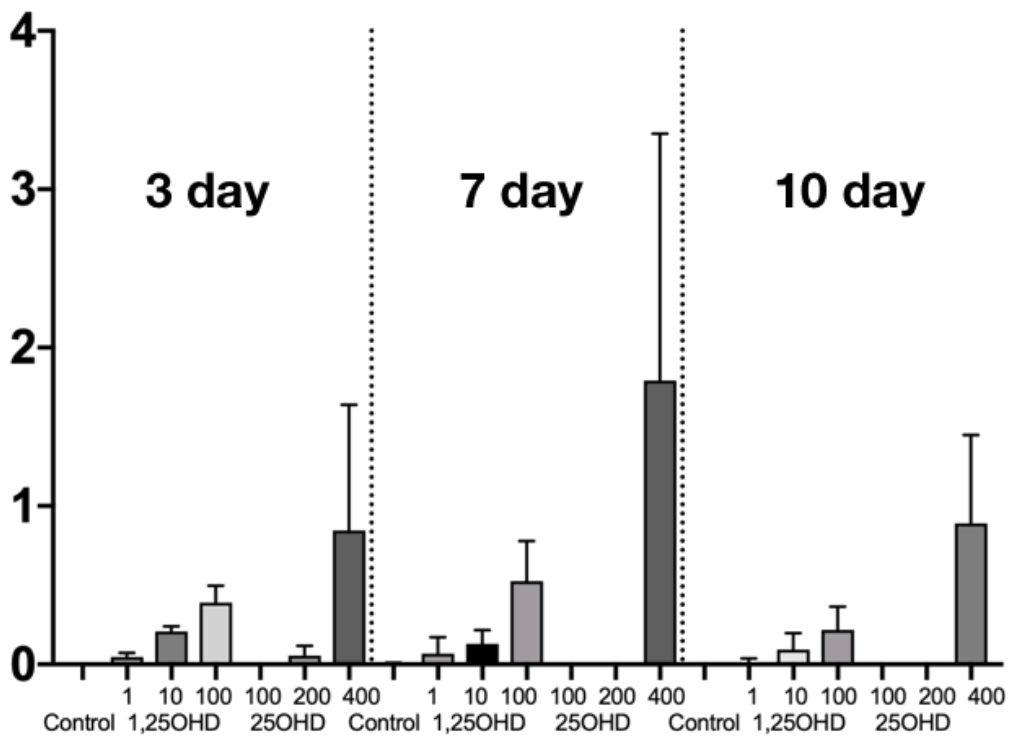
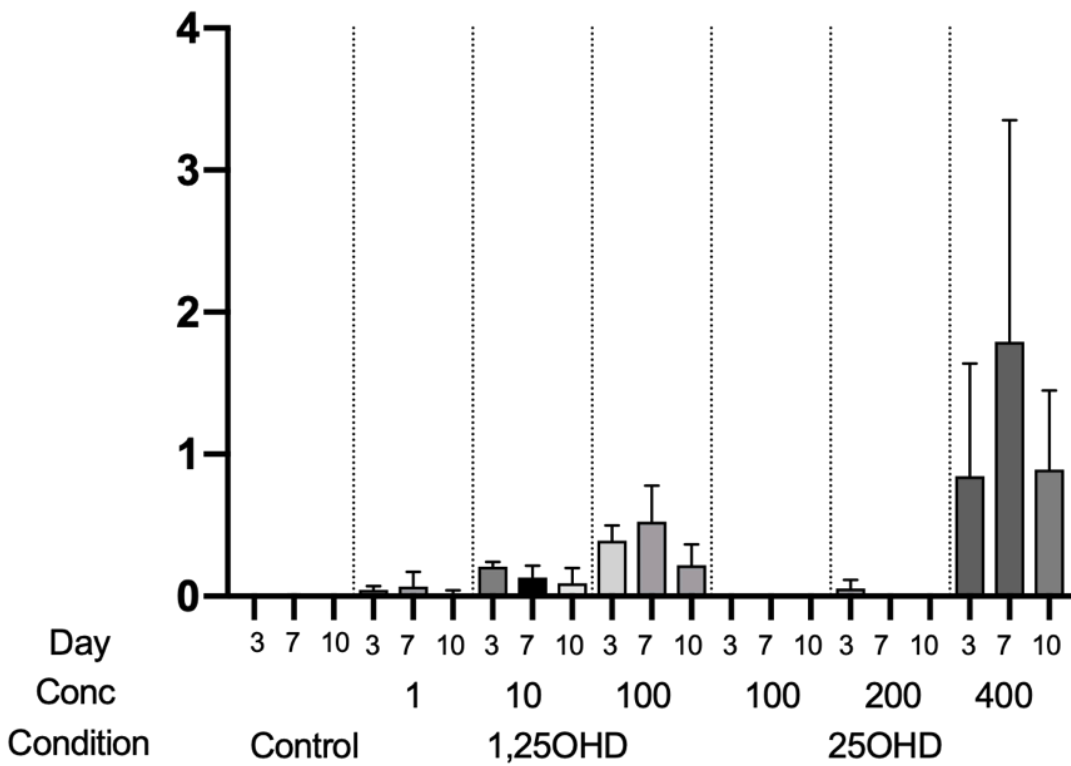


Figure 10: RANKL/OPG by groups showing days 3, 7, and 10 for each condition; control, 1,25OHD 1mol, 1,25OHD 10nmol, 1,25OHD 100nmol, 25OHD 100nmol, 25OHD 200nmol, 25OHD 400nmol.



Measurement of OPG and RANKL levels from cDNA by qrtPCR

qRTPCR results for OPG and RANKL from the cDNA were measured at 3, 7, and 10 days using GADPH as the endogenous control as explained above. Results were measured using statistical analysis system STATA.

With regression analyses, a significant difference ($p=0.007$) was noted between the 3-day control and the 10-day 25OHD at 400nmol concentration for OPG. The $\Delta\Delta CT$ was measured to the 3-day control for both OPG and RANKL. Significant differences were noted by ANOVA at day 10 with 25OHD between the control and the 400nmol concentration ($p=0.04$) and between the 200nmol and 400nmol concentrations ($p=0.035$). For RANKL, the regression analysis to the 3-day control showed significant differences with 3-day 25OHD 400nmol concentration ($p=0.006$), 7-day 1,25OHD 100nmol ($p=0.026$) and 10-day 25OHD 400nmol ($p=0.031$). Using ANOVA, we noticed significant differences in 3-day 25OHD 400nmol with control ($p=0.037$) and 25OHD 100nmol ($p=0.019$). Another significant difference was noted in the 7-day data between the 1,25OHD 1nmol and 1,25OHD 100nmol ($p=0.025$).

Paired T-tests were performed between the same groups over different days for both OPG and RANKL. With OPG, statistically significant differences were found in the control groups between days 3 and 7 ($p=0.019$) and days 7-10 ($p=0.0009$). In the 1,25OHD 1nmol group, significant differences were found between all combinations of days; 3-7 ($p=0.0036$), 7-10 ($p=0.0152$) and 3-10 ($p=0.0029$). A p value of 0.01 was noted between days 3-10 within the 1,25OHD 100nmol and the 25OHD 100nmol groups. In the 25OHD 200nmol group, significant differences were found between all group combinations; 3-7 days ($p=0.04$), 7-10 day ($p=0.01$) and 3-10 days ($p=0.042$).

Regarding RANKL, the significant differences were found in the 1,25OHD 1nmol category between day 7-10 ($p=0.04$) and in the 25OHD 400nmol category between day 3-7 ($p=0.015$) and days 7-10 ($p=0.011$).

In regards to the $\Delta\Delta$ CT measurements with 3day control as the control, significant differences in OPG in group 1,25OHD 1nmol between day 3 and 7 ($p=0.0071$) and between days 3 and 10 ($p=0.0016$). With the RANKL $\Delta\Delta$ CT, 1,25OHD 1nmol showed differences in days 3-7 ($p=0.054$) and 25OHD 400nmol between days 3-7 ($p=0.0063$) and days 7-10 ($p=0.014$).

OPG

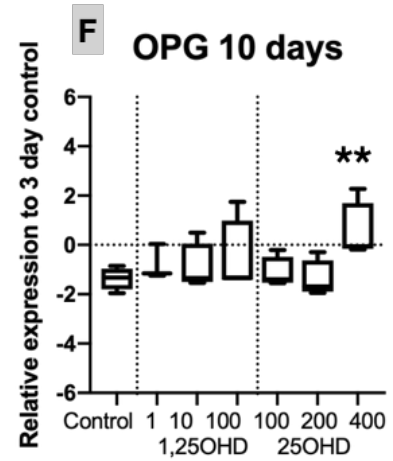
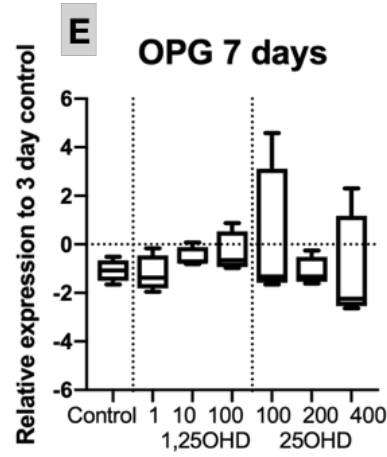
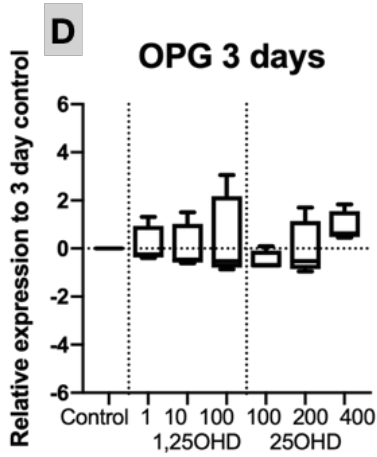
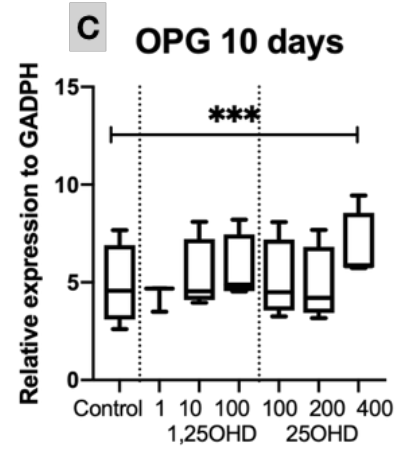
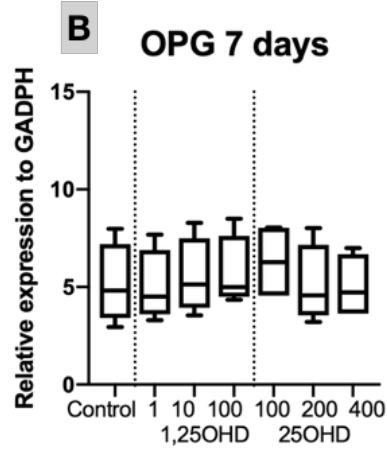
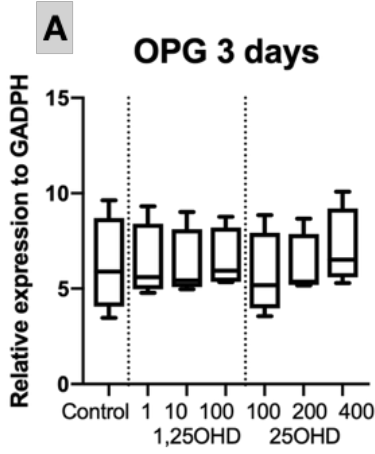
	OPG Δ CT	STDev	OPG $\Delta\Delta$ CT	STDev
3 days				
Control	6.218	2.546	0.000	0.000
1,25OHD 1nmol	6.327	2.032	0.109	0.812
1,25OHD 10nmol	6.210	1.886	-0.008	1.010
1,25OHD 100nmol	6.500	1.606	0.282	1.853
25OHD 100nmol	5.694	2.240	-0.525	0.419
25OHD 200nmol	6.143	1.691	-0.075	1.202
25OHD 400nmol	7.102	2.069	0.884	0.643
7 days				
Control	5.139	2.087	-1.079	0.464
1,25OHD 1nmol	4.999	1.874	-1.219	0.754
1,25OHD 10nmol	5.520	1.987	-0.544	0.416
1,25OHD 100nmol	5.703	1.887	-0.352	0.844
25OHD 100nmol	6.285	1.992	0.067	3.016
25OHD 200nmol	5.086	2.054	-1.132	0.599
25OHD 400nmol	5.013	1.654	-1.205	2.347
10 days				
Control	4.854	2.093	-1.364	0.454
1,25OHD 1nmol	4.292	0.689	-0.789	0.713
1,25OHD 10nmol	5.285	1.895	-0.933	0.956

1,25OHD 100nmol	5.627	1.752	-0.591	1.560
25OHD 100nmol	5.080	2.086	-1.138	0.628
25OHD 200nmol	4.815	1.976	-1.403	0.750
25OHD 400nmol	6.711	1.825	0.493	1.189

Table 11: OPG Δ CT and $\Delta\Delta$ CT values

Figure 11: OPG ΔC_T by time points over groups using GADPH as control and $\Delta\Delta C_T$ by time points over groups using 3-day control as control

- A. 3day ΔC_T showing sample normalization to GADPH for each of control, 1,25OHD 1mol, 1,25OHD 10nmol, 1,25OHD 100nmol, 25OHD 100nmol, 25OHD 200nmol, 25OHD 400nmol
- B. 7day ΔC_T showing sample normalization to GADPH for each of control, 1,25OHD 1mol, 1,25OHD 10nmol, 1,25OHD 100nmol, 25OHD 100nmol, 25OHD 200nmol, 25OHD 400nmol
- C. 10day ΔC_T showing sample normalization to GADPH for each of control, 1,25OHD 1mol, 1,25OHD 10nmol, 1,25OHD 100nmol, 25OHD 100nmol, 25OHD 200nmol, 25OHD 400nmol
- D. 3day $\Delta\Delta C_T$ showing sample normalization to GADPH and 3day control for each of control, 1,25OHD 1mol, 1,25OHD 10nmol, 1,25OHD 100nmol, 25OHD 100nmol, 25OHD 200nmol, 25OHD 400nmol
- E. 7day $\Delta\Delta C_T$ showing sample normalization to GADPH and 3day control for each of control, 1,25OHD 1mol, 1,25OHD 10nmol, 1,25OHD 100nmol, 25OHD 100nmol, 25OHD 200nmol, 25OHD 400nmol
- F. 10day $\Delta\Delta C_T$ showing sample normalization to GADPH and 3day control for each of control, 1,25OHD 1mol, 1,25OHD 10nmol, 1,25OHD 100nmol, 25OHD 100nmol, 25OHD 200nmol, 25OHD 400nmol



Regression	ΔC_T			$\Delta\Delta C_T$		
	3 days	7 days	10 days	3 days	7 days	10 days
1,25OHD 1nmol	0.941	0.921	0.684	0.894	0.764	0.480
1,25OHD 10nmol	0.996	0.788	0.736	0.992	0.265	0.566
1,25OHD 100nmol	0.849	0.692	0.547	0.731	0.137	0.312
25OHD 100nmol	0.737	0.423	0.876	0.319	0.422	0.697
25OHD 200nmol	0.962	0.970	0.978	0.884	0.970	0.947
25OHD 400nmol	0.573	0.929	0.213	0.105	0.928	0.007

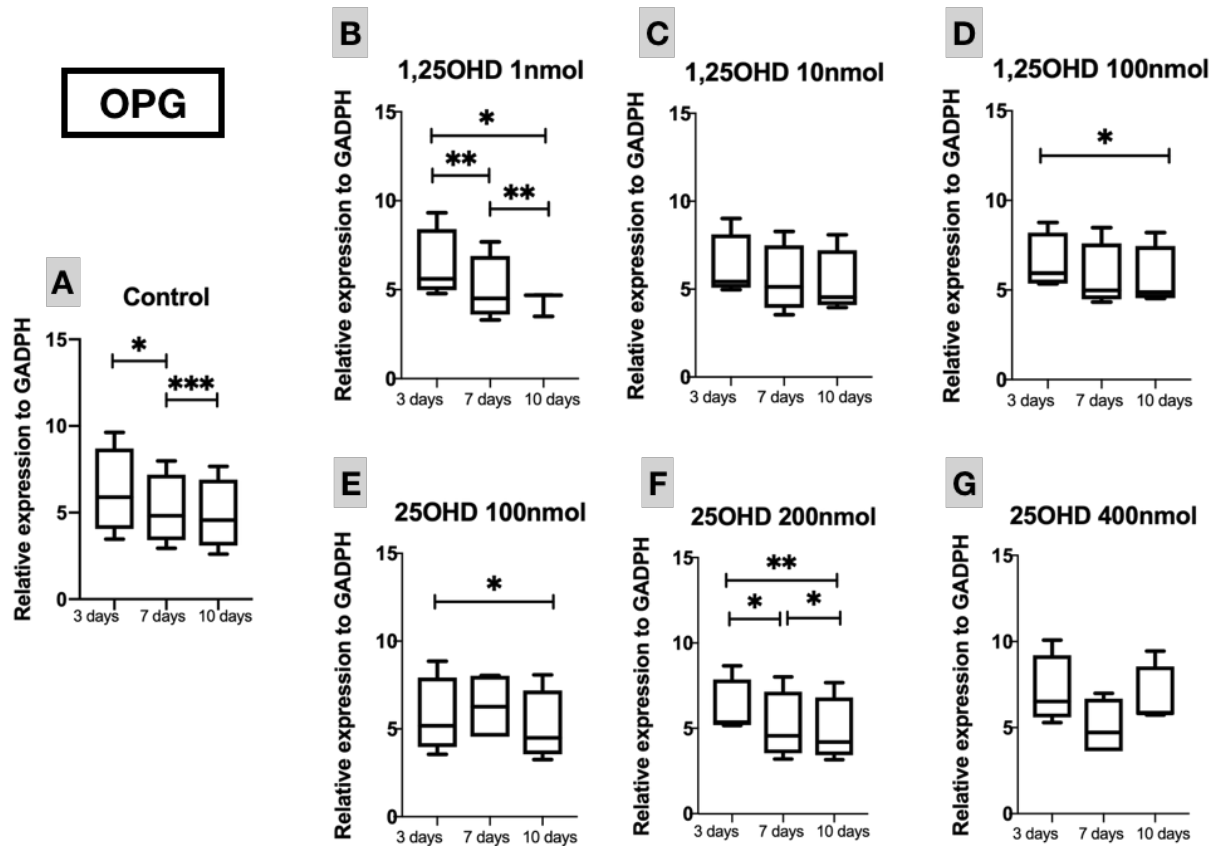
Table 12: OPG ΔC_T and $\Delta\Delta C_T$ regression analysis between groups

Delta CT OPG			
	3 days	7 days	10 days
1,25OHD	0.9967	0.9519	0.7771
25OHD	0.8261	0.7669	0.5074
Delta Delta CT OPG			
	3 days	7 days	10 days
1,25OHD	0.981	0.228	0.7562
25OHD	0.090	0.765	0.017
	Between day 10 and 25OHD		
	Control	25OHD 100nmol	25OHD 200nmol
25OHD 100nmol	1		
25OHD 200nmol	1	1	
25OHD 400nmol	0.040	0.085	0.035

Table 13: OPG ΔC_T and $\Delta\Delta C_T$ ANOVA over time points

Figure 12: OPG ΔC_t by time points using GADPH as control

- A. Control condition with days 3, 7, and 10
- B. 1,25OHD 1nmol condition with days 3, 7, 10
- C. 1,25OHD 10nmol condition with days 3, 7, 10
- D. 1,25OHD 100nmol condition with days 3, 7, 10
- E. 25OHD 100nmol condition with days 3, 7, 10
- F. 25OHD 200nmol condition with days 3, 7, 10
- G. 25OHD 400nmol condition with days 3, 7, 10

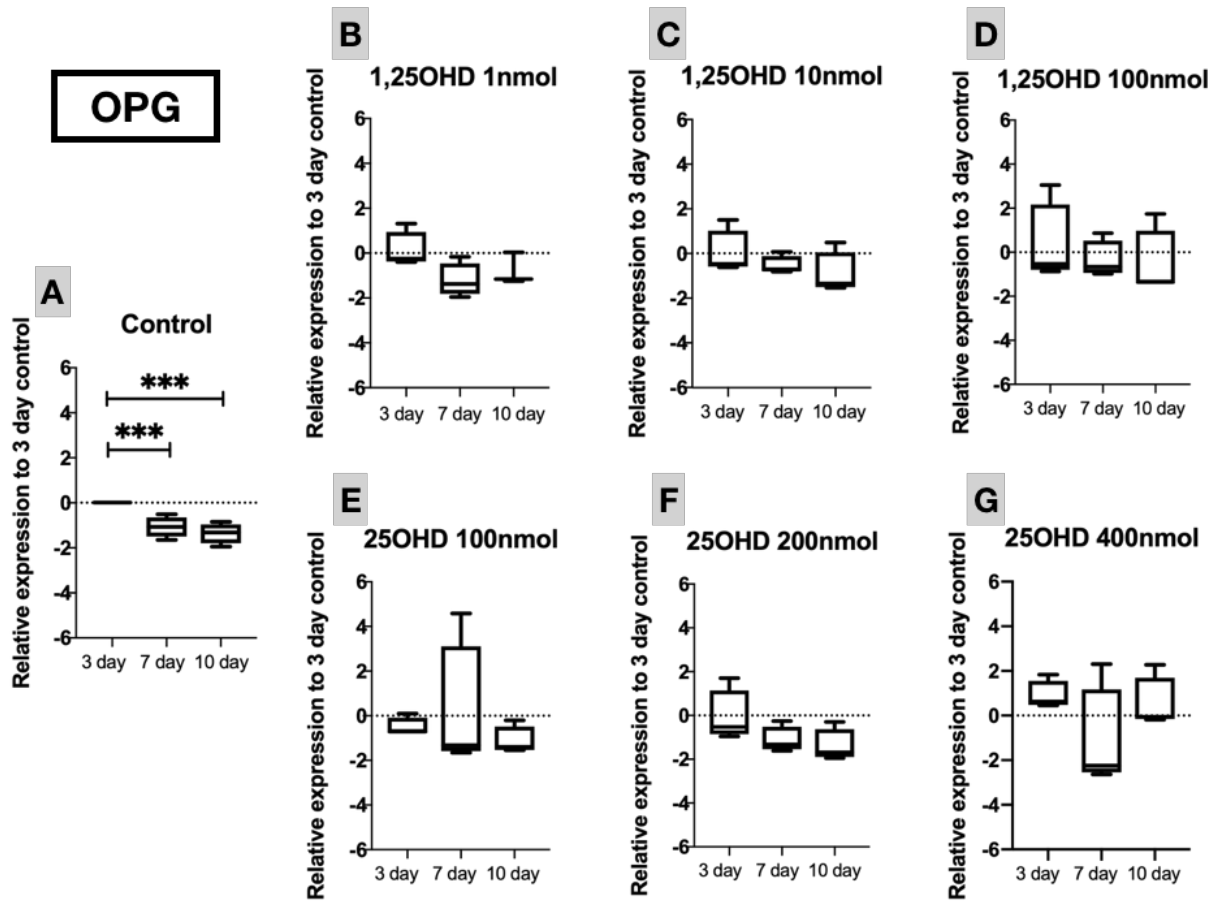


	Day 7 - Day 3	Day 10 – Day 3	Day 10 – Day 7
Control	0.0188	0.0092	0.0009
1,25OHD 1nmol	0.0026	0.0152	0.0029
1,25OHD 10nmol	0.0813	0.0564	0.1470
1,25OHD 100nmol	0.3950	0.0113	0.0501
25OHD 100nmol	0.6798	0.0100	0.3880
25OHD 200nmol	0.0399	0.0101	0.0421
25OHD 400nmol	0.0923	0.2559	0.0610

Table 14: OPG ΔC_T paired T Test between groups

Figure 13: OPG $\Delta\Delta C_T$ values to 3 day control by time points

- A. Control condition with days 3, 7, and 10
- B. 1,25OHD 1nmol condition with days 3, 7, 10
- C. 1,25OHD 10nmol condition with days 3, 7, 10
- D. 1,25OHD 100nmol condition with days 3, 7, 10
- E. 25OHD 100nmol condition with days 3, 7, 10
- F. 25OHD 200nmol condition with days 3, 7, 10
- G. 25OHD 400nmol condition with days 3, 7, 10



	Day 7 - Day 3	Day 10 – Day 3	Day 10 – Day 7
Control	0.0071	0.0016	0.5511
1,25OHD 1nmol	0.0826	0.2683	0.7127
1,25OHD 10nmol	0.6508	0.3098	0.7932
1,25OHD 100nmol	0.8211	0.6926	0.9716
25OHD 100nmol	0.8884	0.8808	0.6245
25OHD 200nmol	0.2632	0.1415	0.9034
25OHD 400nmol	0.1969	0.9337	0.3206

Table 15: OPG $\Delta\Delta C_t$ paired T Test between groups

RANKL

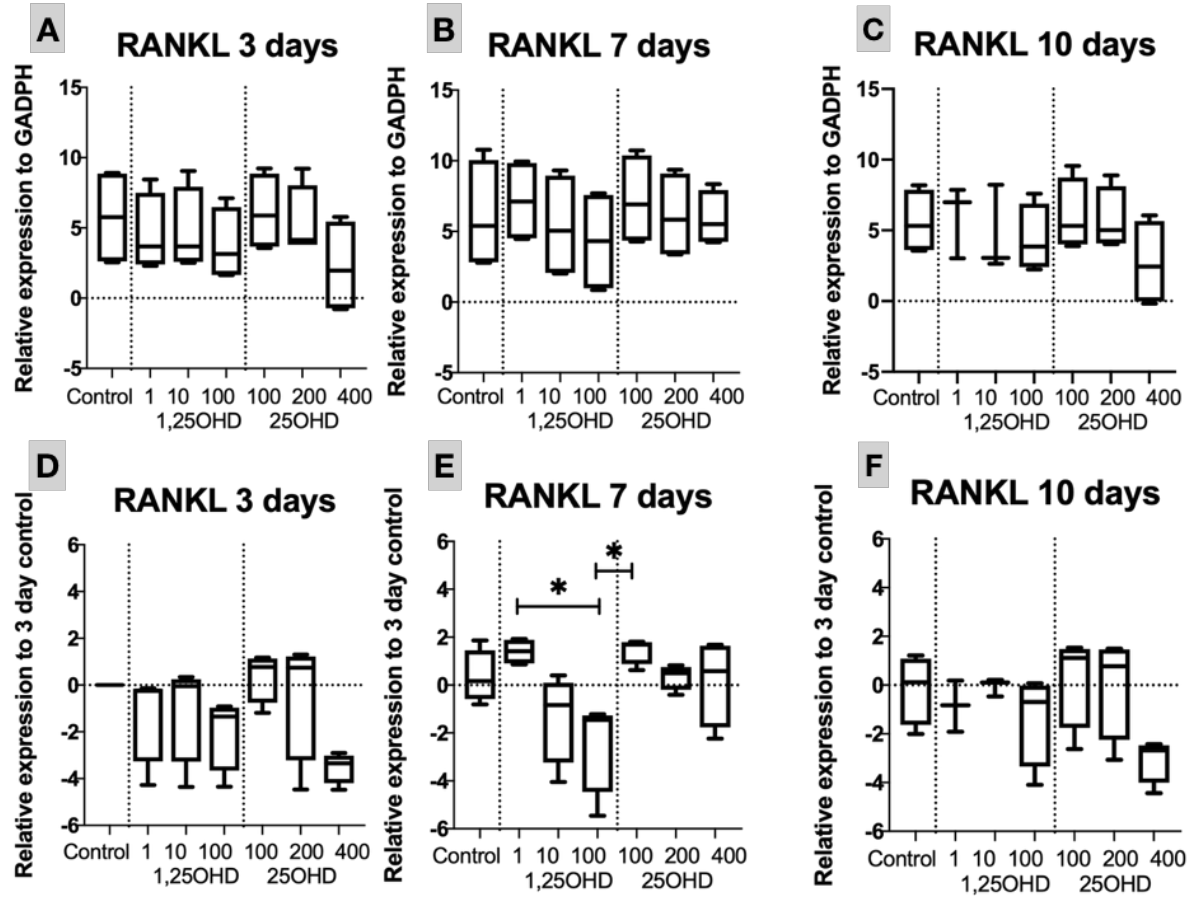
	RANKL Δ CT	STDev	RANKL $\Delta\Delta$ CT	STDev
3 days				
Control	5.743	3.533	0.000	0.000
1,25OHD 1nmol	4.522	2.810	-1.221	2.035
1,25OHD 10nmol	4.710	3.019	-1.023	2.228
1,25OHD 100nmol	3.755	2.622	-1.988	1.596
25OHD 100nmol	6.130	2.788	0.387	1.080
25OHD 200nmol	5.318	2.614	-0.425	2.717
25OHD 400nmol	2.224	3.377	-3.520	0.670
7 days				
Control	6.087	3.923	0.343	1.112
1,25OHD 1nmol	7.150	3.004	1.407	0.537
1,25OHD 10nmol	5.354	3.777	-1.327	1.901
1,25OHD 100nmol	4.287	3.660	-2.394	2.01
25OHD 100nmol	7.207	3.307	1.464	0.559
25OHD 200nmol	6.095	3.173	0.352	0.532
25OHD 400nmol	5.898	1.985	0.155	1.840
10 days				
Control	5.600	2.308	-0.144	1.437
1,25OHD 1nmol	5.962	2.579	-0.851	1.052
1,25OHD 10nmol	4.637	3.109	-0.050	0.368

1,25OHD 100nmol	4.391	2.397	-1.352	1.892
25OHD 100nmol	6.026	2.568	0.283	1.959
25OHD 200nmol	5.740	2.254	-0.003	2.121
25OHD 400nmol	2.693	3.049	-3.050	0.930

Table 16: RANKL Δ CT and $\Delta\Delta$ CT values

Figure 14: RANKL ΔC_T over time points by groups using GADPH as control and $\Delta\Delta C_T$ by time points over groups using 3-day control as control

- A. 3day ΔC_T showing sample normalization to GADPH for each of control, 1,25OHD 1mol, 1,25OHD 10nmol, 1,25OHD 100nmol, 25OHD 100nmol, 25OHD 200nmol, 25OHD 400nmol
- B. 7day ΔC_T showing sample normalization to GADPH for each of control, 1,25OHD 1mol, 1,25OHD 10nmol, 1,25OHD 100nmol, 25OHD 100nmol, 25OHD 200nmol, 25OHD 400nmol
- C. 10day ΔC_T showing sample normalization to GADPH for each of control, 1,25OHD 1mol, 1,25OHD 10nmol, 1,25OHD 100nmol, 25OHD 100nmol, 25OHD 200nmol, 25OHD 400nmol
- D. 3day $\Delta\Delta C_T$ showing sample normalization to GADPH and 3day control for each of control, 1,25OHD 1mol, 1,25OHD 10nmol, 1,25OHD 100nmol, 25OHD 100nmol, 25OHD 200nmol, 25OHD 400nmol
- E. 7day $\Delta\Delta C_T$ showing sample normalization to GADPH and 3day control for each of control, 1,25OHD 1mol, 1,25OHD 10nmol, 1,25OHD 100nmol, 25OHD 100nmol, 25OHD 200nmol, 25OHD 400nmol
- F. 10day $\Delta\Delta C_T$ showing sample normalization to GADPH and 3day control for each of control, 1,25OHD 1mol, 1,25OHD 10nmol, 1,25OHD 100nmol, 25OHD 100nmol, 25OHD 200nmol, 25OHD 400nmol



Regression	ΔC_T			$\Delta\Delta C_T$		
	3 days	7 days	10 days	3 days	7 days	10 days
1,25OHD 1nmol	0.577	0.684	0.857	0.332	0.344	0.521
1,25OHD 10nmol	0.640	0.779	0.634	0.413	0.147	0.932
1,25OHD 100nmol	0.369	0.494	0.521	0.125	0.026	0.248
25OHD 100nmol	0.863	0.627	0.818	0.721	0.190	0.726
25OHD 200nmol	0.850	0.997	0.939	0.696	0.991	0.908
25OHD 400nmol	0.135	0.934	0.135	0.006	0.819	0.031

Table 17: RANKL ΔC_T and $\Delta\Delta C_T$ regression analysis between groups

Delta CT RANKL			
	3 days	7 days	10 days
1,25OHD	0.8282	0.7237	0.8268
25OHD	0.3123	0.9331	0.2704
Delta Delta CT RANKL			
	3 days	7 days	10 days
1,25OHD	0.4601	0.0185	0.5635
25OHD	0.0118	0.3891	0.0517
Between day 7 and 1,25OHD			
	Control	1,25OHD 1nmol	1,25OHD 10nmol
1,25OHD 1nmol	1		
1,25OHD 10nmol	0.885	0.157	
1,25OHD 100nmol	0.156	0.025	1
Between day 3 and 25OHD			
	Control	25OHD 100nmol	25OHD 200nmol
25OHD 100nmol	1		
25OHD 200nmol	1	1	
25OHD 400nmol	0.037	0.019	0.077

Table 18: RANKL ΔC_T and $\Delta\Delta C_T$ ANOVA over time points

	Day 7 - Day 3	Day 10 – Day 3	Day 10 – Day 7
Control	0.5806	0.8544	0.6971
1,25OHD 1nmol	0.0647	0.5066	0.3990
1,25OHD 10nmol	0.06789	0.7277	0.1129
1,25OHD 100nmol	0.5899	0.0502	0.9252
25OHD 100nmol	0.1991	0.8301	0.3600
25OHD 200nmol	0.6137	0.3289	0.7576
25OHD 400nmol	0.0155	0.097	0.0107

Table 19: RANKL ΔC_T paired T Test between groups

	Day 7 - Day 3	Day 10 – Day 3	Day 10 – Day 7
Control	0.8921	0.9806	0.7988
1,25OHD 1nmol	0.0540	0.9219	0.0981
1,25OHD 10nmol	0.9643	0.7096	0.5594
1,25OHD 100nmol	0.9502	0.8784	0.7158
25OHD 100nmol	0.5174	0.9925	0.4542
25OHD 200nmol	0.8501	0.9513	0.9674
25OHD 400nmol	0.0063	0.8583	0.0140

Table 20: RANKL $\Delta\Delta C_T$ paired T Test between groups

Figure 17: RANKL/OPG ratio ΔC_T by time points over groups

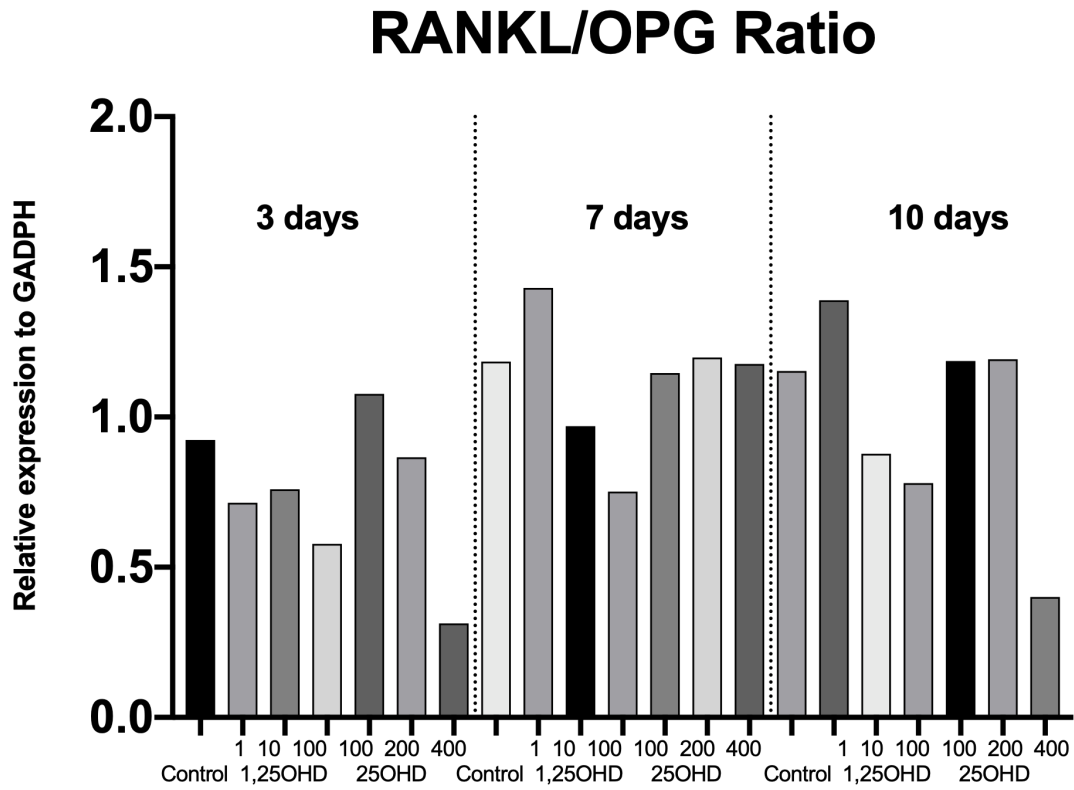
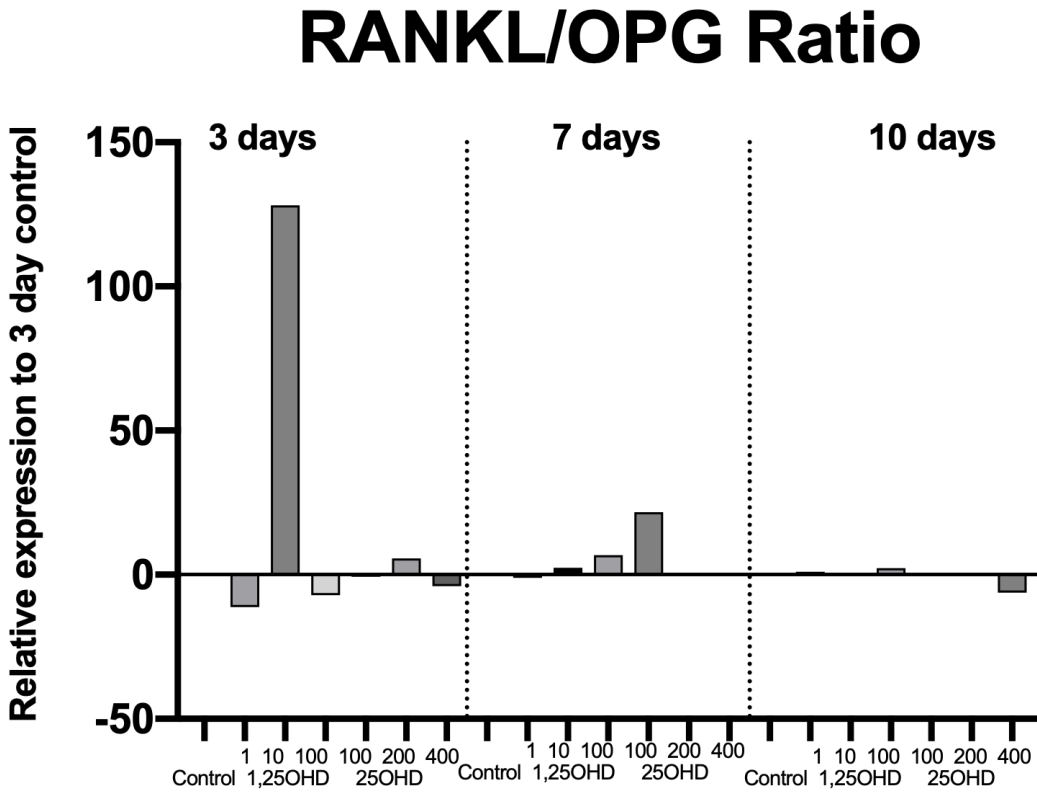


Figure 18: RANKL/OPG Ratio $\Delta\Delta C_T$ by time points over groups using 3 day control as control



Western Blot

Western blot was performed using the above-mentioned technique on the cell lysates collected 24hrs treatment with the appropriate concentration of 1,25OHD or 25OHD. The signaling pathway was assessed according to phosphospecific antibodies in the MAPK pathway; pERK1/2 (p44/42 MAPK), ERK 1/2(p44/42 MAPK), p-p38, p-38 and NLK using B-actin as a control. These antibodies were chosen according to the MAPK pathway to indicate proliferation, apoptosis and differentiation, respectively. Quantification was done using ImageJ software. After quantification, percentage change was calculated from the plots shown in figure 20 below by the following formula for each of ERK 1/2 and p38.

$$\frac{\frac{pERK}{Bactin}}{ERK} \quad \text{and} \quad \frac{\frac{pp38}{Bactin}}{p38}$$

1,25OHD seems to upregulate all three pathways with varying degrees with increasing concentrations. 25OHD seems to have an effect only on the ERK 1/2 portion of the pathway which downregulates further with increasing the concentration, as shown in figure 21 below.

Figure 19: Western blot showing control, 1,25OHD and 25OHD with all concentrations with phospho- and total ERK 1/2, p38 and NLK with B actin as an endogenous control.

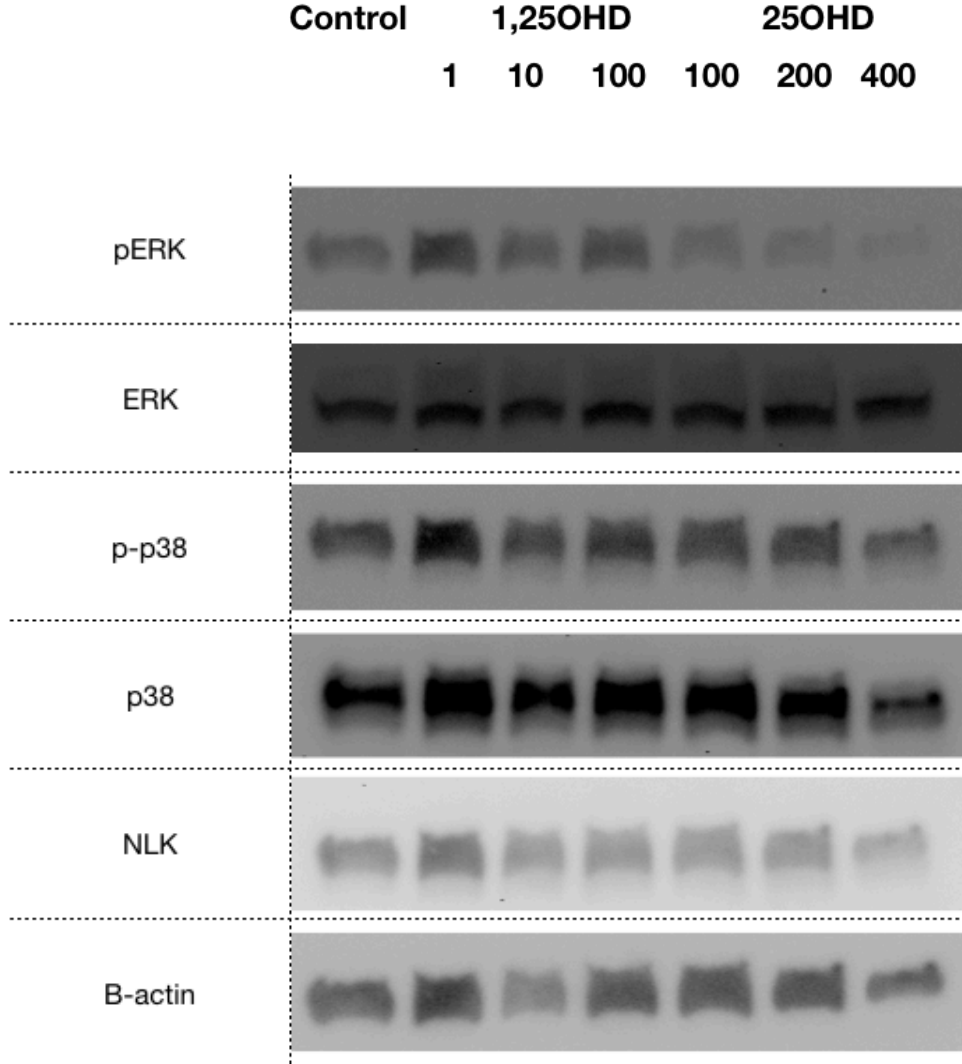
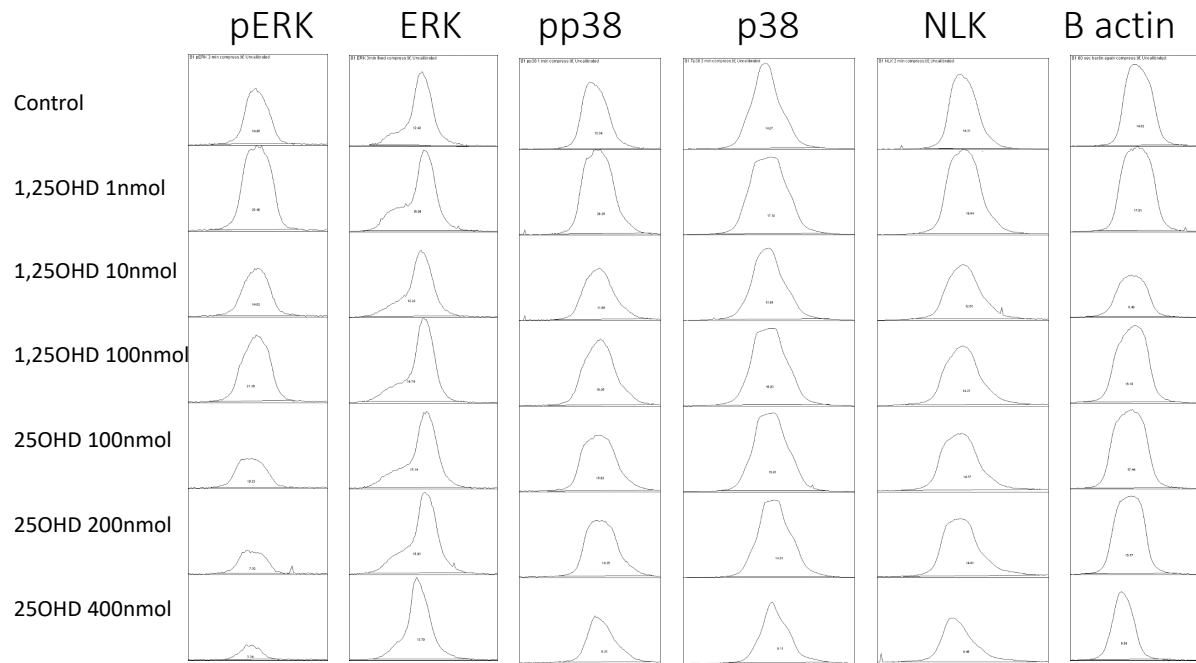


Figure 20: Quantification plots for pERK 1/2, ERK 1/2, p-p38, p38, NLK and B actin for control, 1,25OHD (1nmol, 10nmol and 100nmol), 25OHD (100nmol, 200nmol, 400nmol)

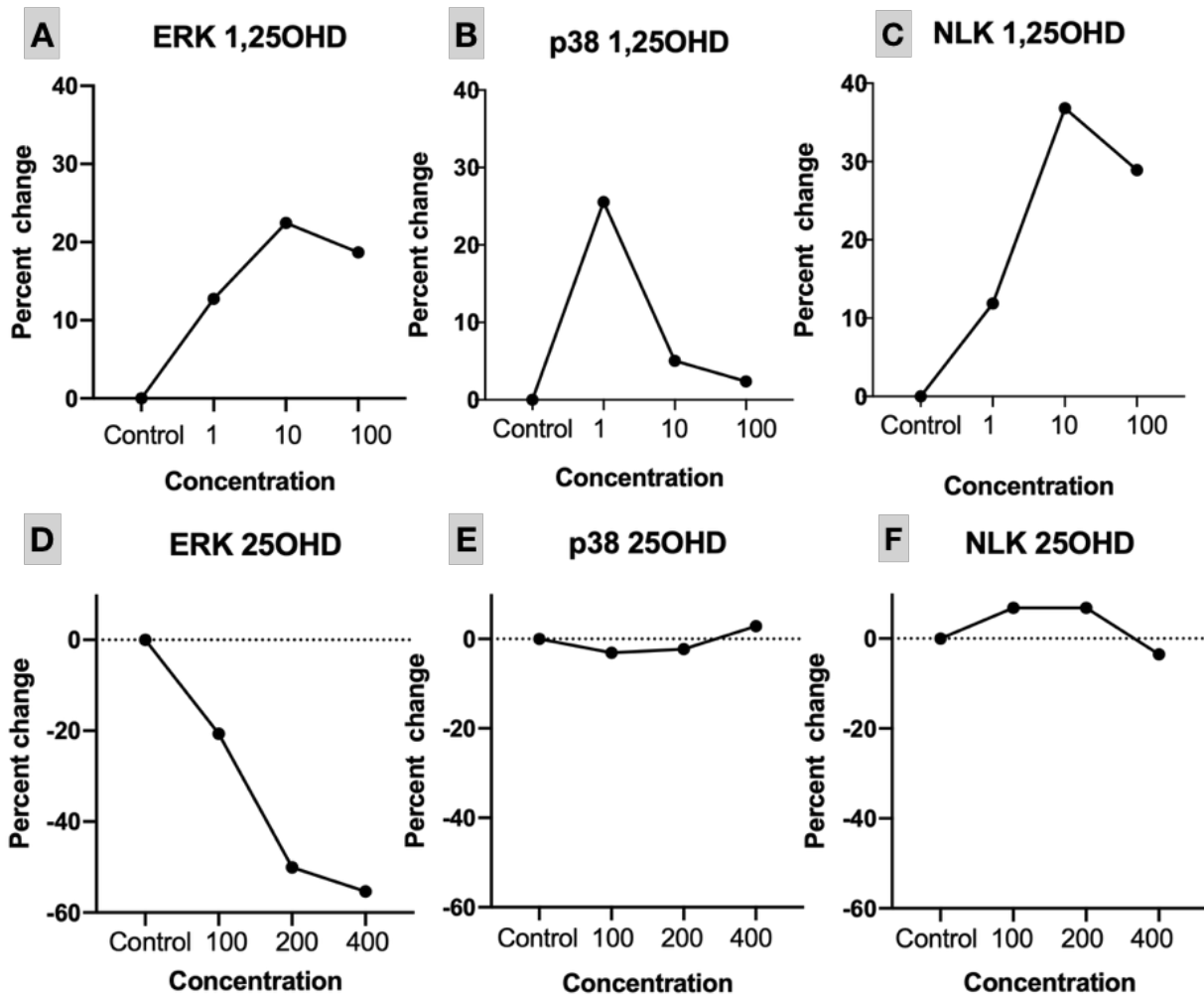


	ERK 1/2	p38	NLK
1,25OHD 1nmol	12.74%	25.54%	11.89%
1,25OHD 10nmol	22.44%	5.05%	36.81%
1,25OHD 100nmol	18.68%	2.39%	28.93%
25OHD 100nmol	-20.67%	-3.07%	6.85%
25OHD 200nmol	-50.05%	-2.28%	6.81%
25OHD 400nmol	-55.39%	2.85%	-3.50%

Table 21: Western blot percentage changes in 1,25OHD and 25OHD to control

Figure 21: Percentage change in 1,25OHD and 25OHD with increase in concentration with pERK 1/2, p-p38 and NLK calculated as phospho/B actin/total for each.

- A. pERK/B actin/ERK percentage change for 1,25OHD with 1nmol, 10nmol and 100nmol
- B. p-p38/B actin/p38 percentage change for 1,25OHD with 1nmol, 10nmol and 100nmol
- C. NLK/B actin percentage change for 1,25OHD with 1nmol, 10nmol and 100nmol
- D. pERK/B actin/ERK percentage change for 25OHD with 100nmol, 200nmol and 400nmol
- E. p-p38/B actin/p38 percentage change for 25OHD with 100nmol, 200nmol and 400nmol
- F. NLK/B actin percentage change for 25OHD with 100nmol, 200nmol and 400nmol



Discussion

In this study we compared the impact of vitamin D on the activity of murine calvarial osteoblasts. We used both the active form (1,25OHD) and the circulating form (25OHD) in varying concentration (1,25OHD: 1nmol, 10nmol and 100nmol and 25OHD: 100nmol, 200nmol, 400nmol) along with a control.

ALP production and activity were used to measure the osteoblastic bone activity of the cells. 1,25OHD and 25OHD have been shown to increase ALP activity (Kim, et al., 2018) (van der Meijen, et al., 2014) (van Driel, et al., 2004). However, in our study there were no significant differences in ALP concentration or activity with different concentrations of 1,25OHD and 25OHD over any of the measured time points (3 days, 7 days and 10 days). We also measured each condition over the 3 timepoints and also found no significant differences. We conclude that at 3, 7 and 10 days, different concentrations of 1,25OHD and 25OHD may not impact osteoblastic ALP concentration or activity.

The OPG/RANKL balance is extremely important in bone metabolism and remodeling. In previous studies, 1,25OHD and 25OHD reduced primary human osteoblasts proliferation and increased differentiation (Anderson, et al., 2007) (van der Meijen, et al., 2014). We measured levels in supernatant using ELISA and in cDNA using qRT-PCR. There seems to be a trend of decreased OPG production with the increase of concentration with both 1,25OHD and 25OHD. 25OHD seems to have a greater effect on OPG production than 1,25OHD with most of the statistically significant correlations around the 100nmol concentration. Changes also seemed to increase as time progressed with higher levels at the 10 day time point than the 3 or 7 day. The 25OHD 400nmol condition showed the greatest change over time especially between the 3 and 10 day time

points. Saturation of product may also be a factor in play in this dynamic. At the 10 day concentration, the most changes occur with the 100nmol concentration, this may be due to saturation of the system which may explain the decrease as the concentration increased. We also seem to see an opposing reaction with the RANKL levels, which appear to increase as the concentration increases. The most significant correlations here appear at the 7 day time point, specifically around the 25OHD 400nmol condition. When looking at the conditions by groups over timepoints, the levels also seemed to increase with increased concentration. The only statistically significant correlations were in the 1,25OHD 10nmol condition, specifically between days 3 and 7 and days 3 and 10. When considering the RANKL/OPG ratio, the differences seem more pronounced in the 7 day time point than in the 3 and 10 day time points. The trend continues toward the RANKL side as the concentration increases for both 1,25OHD and 25OHD. These mentioned results lead to the conclusion that 25 OHD may affect OPG levels more than 1,25OHD and the opposite for 1,25OHD and RANKL levels. 25OHD may be closer related with the bone building aspect of bone remodeling and 1,25OHD with the bone resorptive aspect. These effects seem to increase with both concentration and time.

When looking at the OPG and RANKL expression by PCR, we compared the results both to an endogenous control GADPH and to the 3 day control. Although the statistics mostly showed p values greater than 0.05, there is a clear trend that correlates with the above mentioned ELISA results for both OPG and RANKL. OPG cDNA levels seemed to decrease over time for all the conditions studied with the most significant differences between days being at the control, 1,25OHD 1nmol and 25OHD 200nmol concentrations. With RANKL cDNA levels the significance was mainly with the 25OHD 400nmol concentration with a slightly similar trend. The dif-

ferences in the RANKL/OPG ratio with the endogenous control were inconsistent but when comparing to the 3 day control we noticed the greatest changes occurring around 3 days. This is divergent to the results seen with the RANKL/OPG ratio with supernatant levels measured by ELISA, which may be due to a difference in what is happening on a genetic level (peak day 3) to a protein level (peak day 7). However, both levels seem to plateau off at the 10 day time point. This corroborates with what was seen in previous studies by van der Meijen et al (van der Meijen, et al., 2014).

The Western blot technique showing the protein concentrations of the measured sample lysates was done according to the method mentioned above. Using the MAPK pathway, we chose ERK 1/2, p38 and NLK as antibodies to indicate proliferation, apoptosis and differentiation of the cascade reaction of the pathway. There is a contradictory evidence of the full mechanism of ERK1/2 and p38 pathways in osteogenic differentiation (Li, et al., 2012) (Xiao, et al., 2002) (Jiang, et al., 2018). In our study, 1,25OHD shows upregulation of each of ERK 1/2, p38 and NLK, whereas 25OHD only downregulates the ERK 1/2 portion of the pathway.

Conclusions

In part I, we show that clinical outcomes of orthodontic treatment are associated with a range of optimal Vitamin D binding protein (VitDBP) as detected in saliva. LogVitDBP levels between 2.75 and 6.48 ng/ml are associated with higher changes in II than above or below suggesting that too low or too high VitDBP is linked to a disruption in clinical response to orthodontic treatment. We also show no significant correlation between ALP levels and absolute changes in II but significant correlation with VitDBP levels, suggesting that salivary ALP is not a clinically relevant measure of orthodontic treatment outcomes. Furthermore, we note significant seasonal variation with higher VitDBP levels measured during winter months.

In part II, we show a decrease in OPG production with higher concentrations of 1,25OHD and 25OHD with a corresponding increase in RANKL levels. ALP levels were not significantly affected by increasing concentrations of both 1,25OHD and 25OHD which correlates with the clinical findings. The ERK 1/2 shows upregulation with 1,25OHD concentrations but downregulation with 25OHD concentrations. P38 and NLK were mainly affected by 1,25OHD and not by 25OHD suggesting a differential action between both forms of Vitamin D on osteoblast signaling.

We conclude that vitamin D and its metabolites have an impact on orthodontic tooth movement and osteoblast cellular functions.

Appendix

RESEARCH SUBJECT INFORMATION AND CONSENT FORM- ADULT

TITLE: The Effects of Vitamin D on Orthodontic Tooth Movement

INVESTIGATOR: Nada Tashkandi

This consent form may contain words that you do not understand. Please ask the study doctor or the study staff to explain any words or information that you do not clearly understand. You may take home an unsigned copy of this consent form to think about or discuss with family or friends before making your decision.

PURPOSE OF THE STUDY:

The purpose of this research study is to measure the effects of Vitamin D levels on the rate of Orthodontic tooth movement. We think that hypothesized levels of vitamin D in your body will be correlated with how your teeth move when you have your braces on and that that will affect the length of treatment.

DESCRIPTION OF THE STUDY

You will be one of approximately 128 subjects asked to participate in this study. Participating in this study will mean that you will be required to spit into a glass tube for each of your first 6 routine adjustment appointments after your braces are on. We will measure the level of vitamin D in your saliva to compare with the speed of your orthodontic treatment. You will also have an impression of your upper and lower teeth made after 6 months (at the end of the study period). This will be done for research purposes only. No other changes will be made from your normal orthodontic treatment than if you had not participated in the study.

RISKS AND DISCOMFORTS

The only discomfort will be in collecting the saliva into the tube. You will be given ample time to allow the saliva to pool into your mouth and dribble it into the tube. The impressions taken will be the same as the ones taken in the initial records appointment.

BENEFITS

Your participation into this study will not affect your orthodontic treatment in any way (neither benefit nor worsen). However, it will help in assessing the effects of vitamin D levels on tooth movement which will give us a better understanding into how teeth move.

COSTS

There are no charges for the study visits and they will be taking up 5-10 minutes of your normal orthodontic appointment. You will not be reimbursed for participation in this study.

AUTHORIZATION TO USE AND DISCLOSE INFORMATION FOR RESEARCH PURPOSES

Federal regulations give you certain rights related to your health information. These include the right to know who will be able to get the information and why they may be able to get it. The study doctor must get your authorization (permission) to use or give out any health information that might identify you.

Boston Medical Center wants to use and/or share your health information as part of this research study. The law requires Boston Medical Center to get your authorization (permission) to do so.

Health information that might be used or given out during this research includes:

- Information from your hospital or office health records at Boston Medical Center or elsewhere. This applies to information that is reasonably related to the aims, conduct, and oversight of the research study. If health information is needed from your doctors or hospitals outside of Boston Medical Center, you will be asked to give permission for these records to be sent to the researcher.
- New health information from tests, procedures, visits, interviews, or forms filled out as part of this research study.

The reasons that your health information might be used or given out to others are:

- To do the research described here
- To make sure we do the research according to certain standards set by ethics, law, and quality groups or otherwise as required by law

The people and groups that may use or give out your health information are:

- Researchers involved in this research study from Boston Medical Center
- Researchers from other institutions or organizations that are involved in this research study

Other people at Boston Medical Center who may need to access your health information to do their jobs such as for treatment, research administration, payment, billing, or health care operations

- People or groups that the researchers use to help conduct the study or to provide oversight for the study
- The Institutional Review Board that oversees the research and other people or groups that are part of the Human Research Protection Program that oversees the research
- Research monitors, reviewers, or accreditation agencies and other people or groups that oversee research information and the safety of the study

Some people or groups who get your health information might not be obligated to follow the same privacy laws that we follow. We ask anyone who gets your health information from us to protect the privacy of your information. However, after your information has been shared with others, we cannot promise that it will be kept private.

The time period for using or giving out your health information:

- Because research is an ongoing process, we cannot give you an exact date when we will either destroy or stop using or sharing your health information.

Your privacy rights are:

- You have the right not to sign this form that allows us to use and give out your health information for research. If you do not sign this form, you cannot be in the research. This is because we need to use the health information to do the research. Your decision not to sign the form will not affect any treatment, health care, enrollment in health plans, or eligibility for benefits.
- You have the right to withdraw your permission to use or share your health information in this research study. If you want to withdraw your permission, you must write a letter to the Principal Investigator at the address listed on the first page of this form. If you withdraw your permission, you will not be able to take back information

that has already been used or shared with others. This includes information used or shared to do the research study or to be sure the research is safe and of high quality. If you withdraw your permission, you cannot continue to be in the study.

- You have the right to see and get a copy of your health information from the Principal Investigator that is used or shared for research.

The results of this research study may be published in a medical book or journal, or used to teach others. However, your name or other identifying information will not be used for these purposes without your specific permission.

CONFIDENTIALITY

Each participant will be given a code and all samples and data will be labeled with this code. Nobody other than the PI will be able to identify you with this code. Only the PI will have the master code and will keep it in a locked cabinet. Absolute confidentiality cannot be guaranteed because of the need to give information to other parties. The results of this research study may be presented at meetings or in publications. Your identity will not be disclosed in those presentations.

VOLUNTARY PARTICIPATION AND WITHDRAWAL

Taking part in this study is voluntary. You have the right to refuse to take part in this study. If you decide to be in the study and then change your mind, you can withdraw from the research. Your participation is completely up to you. Your decision will not affect your being able to get health care at this institution or payment for your health care. It will not affect your enrollment in any health plan or benefits you can get.

If you choose to take part, you have the right to stop at any time. If there are any new findings during the study that may affect whether you want to continue to take part, you will be told about them as soon as possible.

The investigator may decide to discontinue your participation without your permission because he/she may decide that staying in the study will be bad for you, or the sponsor may stop the study.

Do not sign this consent form unless you have had a chance to ask questions and have received satisfactory answers to all of your questions. You will be given a copy of this form to keep.

CONSENT

I have read the information in this consent form (or it has been read to me). All my questions about the study and my participation in it have been answered. I freely consent to participate in this research study.

By signing this consent form I have not waived any of the legal rights which I otherwise would have as a subject in a research study.

You may contact the PI with any questions or concerns at nadatash@bu.edu

CONSENT SIGNATURE:

Signature of Subject

Date

Printed Name of Subject

Signature of PI

RESEARCH SUBJECT INFORMATION AND CONSENT FORM-CHILD

TITLE: The Effects of Vitamin D on Orthodontic Tooth Movement

INVESTIGATOR: Nada Tashkandi

This consent form may contain words that you do not understand. Please ask the study doctor or the study staff to explain any words or information that you do not clearly understand. You may take home an unsigned copy of this consent form to think about or discuss with family or friends before making your decision.

PURPOSE OF THE STUDY:

The purpose of this research study is to measure the effects of Vitamin D levels on the rate of Orthodontic tooth movement. We think that hypothesized levels of vitamin D in your child's body will be correlated with how his/her teeth move when they have braces on and that that will affect the length of treatment.

DESCRIPTION OF THE STUDY

You will be one of approximately 128 subjects asked to participate in this study. Participating in this study will mean that your child will be required to spit into a glass tube for each of your first 6 routine adjustment appointments after the braces are on. We will measure the level of vitamin D in his/her saliva to compare with the speed of your orthodontic treatment. They will also have an impression of your upper and lower teeth made after 6 months (at the end of the study period). This impression will be done for research purposes only. No other changes will be made from your normal orthodontic treatment than if you had not participated in the study.

RISKS AND DISCOMFORTS

The only discomfort will be in collecting the saliva into the tube. S/he will be given ample time to allow the saliva to pool into your mouth and dribble it into the tube. The impressions taken will be the same as the ones taken in the initial records appointment.

BENEFITS

Your participation into this study will not affect your orthodontic treatment in any way (neither benefit nor worsen). However, it will help in assessing the effects of vitamin D levels on tooth movement which will give us a better understanding into how teeth move.

COSTS

There are no charges for the study visits and they will be taking up 5-10 minutes of your normal orthodontic appointment. You will not be reimbursed for participation in this study.

AUTHORIZATION TO USE AND DISCLOSE INFORMATION FOR RESEARCH PURPOSES

Federal regulations give you certain rights related to your health information. These include the right to know who will be able to get the information and why they may be able to get it. The study doctor must get your authorization (permission) to use or give out any health information that might identify you.

CONFIDENTIALITY

Each participant will be given a code and all samples and data will be labeled with this code. Nobody other than the PI will be able to identify you with this code. Only the PI will have the master code and will keep it in a locked cabinet. Absolute confidentiality cannot be guaranteed because of the need to give information to other parties. The results of this research study may be presented at meetings or in publications. Your identity will not be disclosed in those presentations.

VOLUNTARY PARTICIPATION AND WITHDRAWAL

The language below refers to the information we will use from your child's medical record.

Use and Disclosure of Your Health Information

Boston Medical Center wants to use and/or share your health information as part of this research study. The law requires Boston Medical Center to get your authorization (permission) to do so.

Health information that might be used or given out during this research includes:

- Information from your hospital or office health records at Boston Medical Center or elsewhere. This applies to information that is reasonably related to the aims, conduct, and oversight of the research study. If health information is needed from your doctors or hospitals outside of Boston Medical Center, you will be asked to give permission for these records to be sent to the researcher.
- New health information from tests, procedures, visits, interviews, or forms filled out as part of this research study.

The reasons that your health information might be used or given out to others are:

- To do the research described here
- To make sure we do the research according to certain standards set by ethics, law, and quality groups or otherwise as required by law

The people and groups that may use or give out your health information are:

- Researchers involved in this research study from Boston Medical Center
- Researchers from other institutions or organizations that are involved in this research study
- Other people at Boston Medical Center who may need to access your health information to do their jobs such as for treatment, research administration, payment, billing, or health care operations
- People or groups that the researchers use to help conduct the study or to provide oversight for the study
- The Institutional Review Board that oversees the research and other people or groups that are part of the Human Research Protection Program that oversees the research
- Research monitors, reviewers, or accreditation agencies and other people or groups that oversee research information and the safety of the study

Some people or groups who get your health information might not be obligated to follow the same privacy laws that we follow. We ask anyone who gets your health information from us to protect the privacy of your information. However, after your information has been shared with others, we cannot promise that it will be kept private.

The time period for using or giving out your health information:

- Because research is an ongoing process, we cannot give you an exact date when we will either destroy or stop using or sharing your health information.

Your privacy rights are:

- You have the right not to sign this form that allows us to use and give out your health information for research. If you do not sign this form, you cannot be in the research. This is because we need to use the health information to do the research. Your decision not to sign the form will not affect any treatment, health care, enrollment in health plans, or eligibility for benefits.
- You have the right to withdraw your permission to use or share your health information in this research study. If you want to withdraw your permission, you must write a letter to the Principal Investigator at the address listed on the first page of this form. If you withdraw your permission, you will not be able to take back information that has already been used or shared with others. This includes information used or shared to do the research study or to be sure the research is safe and of high quality. If you withdraw your permission, you cannot continue to be in the study.
- You have the right to see and get a copy of your health information from the Principal Investigator that is used or shared for research.

The results of this research study may be published in a medical book or journal, or used to teach others. However, your name or other identifying information will not be used for these purposes without your specific permission.

Taking part in this study is voluntary. You have the right to refuse to take part in this study. If you decide to be in the study and then change your mind, you can withdraw from the research. Your participation is completely up to you. Your decision will not affect your being able to get health care at this institution or payment for your health care. It will not affect your enrollment in any health plan or benefits you can get.

If you choose to take part, you have the right to stop at any time. If there are any new findings during the study that may affect whether you want to continue to take part, you will be told about them as soon as possible.

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Do not sign this consent form unless you have had a chance to ask questions and have received satisfactory answers to all of your questions. You will be given a copy of this consent form to keep.

CONSENT

I have read the information in this consent form (or it has been read to me). All my questions about the study and my participation in it have been answered. I freely consent to participate in this research study.

The study has been fully described to my child in a manner that is understandable to him/her. My child has provided assent to participate in this study by allowing us to view his/her medical records

By signing this consent form I have not waived any of the legal rights which I otherwise would have as a subject in a research study.

You may contact the PI with any questions or concerns at nadatash@bu.edu

CONSENT SIGNATURE:

Signature and Printed Name of Parent

Date

Printed Name of Subject

Signature of PI

Protocols

ALP (ab83369) from Abcam

- 1- Reaction wells set up:
 - a. Standard wells = 120 μL standard dilutions.
 - b. Sample wells = 2-80 μL samples (adjust volume to 80 μL /well with Assay Buffer).
 - c. Sample Background Control wells (for colored samples only) = 2-80 μL samples (adjust volume to 80 μL /well with Assay Buffer).
- 2- ALP Reaction:
 - a. Add 20 μL Stop Solution to Sample Background Control wells to terminate ALP activity in these samples. Mix well by pipetting up and down.
 - b. Add 50 μL of 5 mM pNPP Solution to each well containing Sample and Background Sample Controls. Do not add solution to Standard wells.
 - c. Add 10 μL of ALP enzyme solution to each pNPP Standard well. Mix by pipetting up and down.
 - d. Incubate plate at 25°C for 60 minutes protected from light. The enzyme will convert pNPP substrate to an equal amount of colored p-Nitrophenol (pNP).
 - e. Stop reaction in Sample wells and Standard wells by adding 20 μL Stop Solution.
- 3- Measurement:
 - a. Gently shake the plate.
 - b. Measure output at OD 405 nm on a microplate reader.

VitDBP (CAT. # HCCBP2MAG-58K) from EMD Millipore

- 1- Add 200 μL of Assay Buffer for Blocking (L-AB) into each well of the plate. Seal and mix on a plate shaker for 30 minutes at room temperature (20-25°C).
- 2- Decant Assay Buffer for Blocking (L-AB) and remove the residual amount from all wells by inverting the plate and tapping it smartly onto absorbent towels several times.
- 3- Add 25 μL of Assay Buffer (L-AB4) to the Background and sample wells.
- 4- Add 25 μL of each Standard or Control into the appropriate wells.
- 5- Add 25 μL of appropriate matrix to Background, Standard and Control wells. When assaying serum or plasma, use the Assay Buffer (L-AB4) provided in the kit. When assaying tissue culture or other supernatant, use proper control culture medium as the matrix solution.
- 6- Add 25 μL of diluted sample into the appropriate wells. When assaying serum or plasma, use the Assay Buffer (L-AB4) provided in the kit to dilute the sample. When assaying tissue culture or other supernatant, use proper control culture medium as the diluent.
- 7- Vortex Mixing Bottle and add 25 μL of the Mixed Beads to each well. (Note: During addition of Beads, shake bead bottle intermittently to avoid settling.)
- 8- Seal the plate with a plate sealer. Wrap the plate with foil and incubate with agitation on a plate shaker overnight (16-18 hr.) at 4°C
- 9- Gently remove well contents and wash plate 3 times following instructions listed in the **plate washing** section.
- 10- Add 25 μL of Detection Antibodies into each well.
- 11- Seal, cover with foil, and incubate with agitation on a plate shaker for 1 hour at room temperature (20-25°C).
- 12- Add 25 μL Streptavidin-Phycoerythrin to each well containing the 25 μL of Detection Antibodies.
- 13- Seal, cover with foil and incubate with agitation on a plate shaker for 30 minutes at room temperature (20-25°C).
- 14- Gently remove well contents and wash plate 3 times following instructions listed in the **plate washing** section.
- 15- Add 100 μL of Sheath Fluid (or Drive Fluid if using MAGPIX) to all wells. Re-suspend the beads on a plate shaker for 5 minutes.
- 16- Run plate on Luminex® 200™, HTS, FLEXMAP 3D® or MAGPIX® with xPONENT® software.
- 17- Save and analyze the Median Fluorescent Intensity (MFI) data using a 5-parameter logistic or spline curve-fitting method for calculating analyte concentrations in samples. (Note: Multiply the calculated concentration of the samples by the dilution factor, which is 10000.)

PLATE WASHING

1.) Solid Plate

If using a solid plate, use either a handheld magnet or magnetic plate washer.

A.) Handheld magnet (**EMD Millipore Catalog #40-285**) - Rest plate on magnet for 60 seconds to allow complete settling of magnetic beads. Remove well contents by gently decanting the plate in an appropriate waste receptacle and gently tapping on absorbent pads to remove residual liquid. Wash plate with 200 μ L of Wash Buffer by removing plate from magnet, adding Wash Buffer, shaking for 30 seconds, reattaching to magnet, letting beads settle for 60 seconds and removing well contents as previously described after each wash. Repeat wash steps as recommended in Assay Procedure.

B.) Magnetic plate washer (**EMD Millipore Catalog #40-094, #40-095, #40-096 and #40-097**) - Please refer to specific automatic plate washer manual for appropriate equipment settings. Please note that after the final aspiration, there will be approximately 25 μ L of residual wash buffer in each well. This is expected when using the BioTek® plate washer and this volume does not need to be aspirated from the plate.

If using an automatic plate washer other than BioTek® 405 LS or 405 TS, please refer to the manufacturer's recommendations for programming instructions.

2.) Filter Plate (EMD Millipore Catalog #MX-PLATE)

If using a filter plate, use a vacuum filtration manifold to remove well contents. Wash plate with 200 μ L/well of Wash Buffer, removing Wash Buffer by vacuum filtration after each wash. Repeat wash steps as recommended in the Assay Procedure.

Osteoblast Isolation

Before procedure

- 1- Prepare solution A
 - a. 137 mM NaCl (8g/L)
 - b. 2.7 mM KCl (201 mg/L)
 - c. 3mM NaH₂PO₄ x H₂O (414 mg/L)
 - d. 1L dH₂O
 - e. Adjust pH=7.2
- 2- Prepare solution B
 - a. 4 mM NaEDTA (0.74g/500mL Solution A)
 - b. adjust pH=7.2
- 3- Filter solution A and B
- 4- Prepare solution C
 - a. It should be prepared fresh (on the same day, just before use)
 - b. Worthington Collagenase Type 2 (180 µ/mL) in Solution A
 - c. 40 mL/L
 - d. Keep on ice and heat to 37°C for 1 min just before use

The procedure

- 1- 2-4 day old mice, sterilized surgical procedure, 6 well plate on ice with one well 70% Ethanol 2mL and three wells PBS + 1% FBS
- 2- Euthanize pups by CO₂ then decapitation
- 3- Spray the pup with ethanol in hood
- 4- Remove skin from skull
- 5- Cut around the sutures to remove the frontal and parietal bones
- 6- Remove all remnants of soft tissue and sutures around the calvarial bones
- 7- Wash in ethanol then PBS/FBS 3 times. Keep in last well on ice.

In the lab

- 1- Move calvaria from PBS/FBS to 25mL flask
- 2- Wash calvaria with 5mL Solution A then suction
- 3- Incubate calvaria and shake for 10 min at 37°C at 100rpm as follows
 - a. 5 mL solution B x 3 times and discard
 - b. 5 mL solution C x 2 times and discard
 - c. 5 mL solution C x 5 times and collect supernatant
- 4- Centrifuge supernatants at 300g x 5 min at 24°C
- 5- Aspirate supernatant and Re-suspend in alpha-MEM and move to 75 cm² flask with 10% FBS and 1% antibiotic (p/s)
- 6- Incubate in CO₂ at 37°C
- 7- Change media every 3 days
- 8- View under microscope until reaching 90% confluence
- 9- Passage into plates for treatment

Detachment of osteoblasts

- 1- Aspirate media from flasks
- 2- Add 3mL Trypsin/EDTA with gentle tapping
- 3- Incubate for 3 min at 37⁰C in CO₂
- 4- View under microscope to evaluate detachment
- 5- Add 9 mL of alpha-MEM/FBS/PBS
- 6- Centrifuge then aspirate media
- 7- Count cells under microscope
- 8- Calculate number of cells per well needed and plate

ELISA (Quantikine ELISA Mouse Osteoprotegerin/TNFRSF11B Immunoassay (Cat. # MOP00) and Mouse TRANCE/RANK L/ TNFSF11 (Cat. # MTR00)) from R&D systems

Plate Preparation

- 1- Dilute the Capture Antibody to the working concentration in PBS without carrier protein. Immediately coat a 96-well microplate with 100 µl per well of the diluted Capture antibody. Seal the plate and incubate overnight at room temperature
- 2- Aspirate each well and wash with Wash Buffer, repeating the process two times for a total of three washes. Wash by filling each well with Wash Buffer (400 µl) using a squirt bottle, manifold dispenser or an autowash. Complete removal of liquid at each step is essential for good performance. After the last wash, remove any remaining Wash Buffer by aspirating or by inverting the plate and blotting it against clean paper towels.
- 3- Block plates by adding 300 µl of Reagent Diluent to each well. Incubate at room temperature for a minimum of 1 hour.
- 4- Repeat the aspiration/wash step as in step 2. The plates are now ready for sample addition

Assay procedure

- 5- Add 100 µl of sample or standards in Reagent Diluent or an appropriate diluent, per well. Cover with an adhesive strip and incubate 2 hours at room temperature.
- 6- Repeat the aspiration/wash step as in step 2 of plate preparation
- 7- Add 100 µl of the Detection Antibody, diluted in Reagent Diluent to each well. Cover with a new adhesive strip and incubate 2 hours at room temperature.
- 8- Repeat the aspiration/wash step as in step 2 of plate preparation
- 9- Add 100 µl of the working dilution of Streptavidin-HRP to each well. Cover the plate and incubate for 20 min at room temperature. Avoid placing the plate in direct light.
- 10- Repeat the aspiration/wash step as in step 2 of plate preparation
- 11- Add 100 µl of Substrate Solution to each well. Incubate for 20 min at room temperature. Avoid placing the plate in direct light.
- 12- Add 50 µl of Stop Solution to each well. Gently tap the plate to ensure thorough mixing.
- 13- Determine the optical density of each well immediately, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 or 570 nm from the readings at 450 nm. The subtraction will correct for optical imperfections in the plate. Readings made at 450 nm without correction may be higher and less accurate.

RNA Extraction (RNeasy kit) from Qiagen

Procedure

- 1- Harvest cells: Cells grown in a monolayer (do not use more than 1×10^7 cells): Cells can be either lysed directly in the cell-culture vessel (up to 10 cm diameter) or trypsinized and collected as a cell pellet prior to lysis. Cells grown in cell-culture flasks should always be trypsinized.

To lyse cells directly:

Determine the number of cells. Completely aspirate the cell-culture medium, and proceed immediately to step 2.

Note: Incomplete removal of cell-culture medium will inhibit lysis and dilute the lysate, affecting the conditions for binding of RNA to the RNeasy membrane. Both effects may reduce RNA yield.

- 2- Disrupt the cells by adding Buffer RLT.

For direct lysis of cells grown in a monolayer, add the appropriate volume of Buffer RLT to the cell-culture dish. Collect the lysate with a rubber policeman.

Pipet the lysate into a microcentrifuge tube (not supplied). Vortex or pipet to mix, and ensure that no cell clumps are visible before proceeding to step 3.

- 3- Homogenize the lysate.

If processing $\leq 1 \times 10^5$ cells, homogenize by vortexing for 1 min. After homogenization, proceed to step 4.

Note: Incomplete homogenization leads to significantly reduced RNA yields and can cause clogging of the RNeasy spin column. Homogenization with a rotor-stator or QIAshredder homogenizer generally results in higher RNA yields than with a syringe and needle.

Pipet the lysate directly into a QIAshredder spin column placed in a 2 ml collection tube, and centrifuge for 2 min at full speed. Proceed to step 4.

- 4- Add 1 volume of 70% ethanol to the homogenized lysate, and mix well by pipetting. Do not centrifuge.

Note: The volume of lysate may be less than 350 μ l or 600 μ l due to loss during homogenization.

Note: When purifying RNA from certain cell lines, precipitates may be visible after addition of ethanol. This does not affect the procedure.

- 5- Transfer up to 700 μ l of the sample, including any precipitate that may have formed, to an RNeasy spin column placed in a 2 ml collection tube (supplied). Close the lid gently, and centrifuge for 15 s at $\geq 8000 \times g$ ($\geq 10,000$ rpm). Discard the flow-through.

Reuse the collection tube in step 6.

- 6- Add 700 μ l Buffer RW1 to the RNeasy spin column. Close the lid gently, and centrifuge for 15 s at $\geq 8000 \times g$ ($\geq 10,000$ rpm) to wash the spin column membrane. Discard the flow-through. Reuse the collection tube in step 7.

Note: After centrifugation, carefully remove the RNeasy spin column from the collection tube so that the column does not contact the flow-through. Be sure to empty the collection tube completely.

- 7- Add 500 μ l Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge for 15 s at $\geq 8000 \times g$ ($\geq 10,000$ rpm) to wash the spin column membrane. Discard the flow-through. Reuse the collection tube in step 8.

Note: Buffer RPE is supplied as a concentrate. Ensure that ethanol is added to Buffer RPE before use.

- 8- Add 500 μ l Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge for 2min at $\geq 8000 \times g$ ($\geq 10,000$ rpm) to wash the spin column membrane. The long centrifugation dries the spin column membrane, ensuring that no ethanol is carried over during RNA elution. Residual ethanol may interfere with downstream reactions.

Note: After centrifugation, carefully remove the RNeasy spin column from the collection tube so that the column does not contact the flow-through. Otherwise, carryover of ethanol will occur.

- 9- Optional: Place the RNeasy spin column in a new 2 ml collection tube (supplied), and discard the old collection tube with the flow-through. Close the lid gently, and centrifuge at full speed for 1 min. Perform this step to eliminate any possible carryover of Buffer RPE, or if residual flow-through remains on the outside of the RNeasy spin column after step 8.
- 10- Place the RNeasy spin column in a new 1.5 ml collection tube (supplied). Add 30–50 μ l RNase-free water directly to the spin column membrane. Close the lid gently, and centrifuge for 1 min at $\geq 8000 \times g$ ($\geq 10,000$ rpm) to elute the RNA.
- 11- If the expected RNA yield is $>30 \mu\text{g}$, repeat step 10 using another 30–50 μ l RNase free water, or using the eluate from step 10 (if high RNA concentration is required). Reuse the collection tube from step 10

cDNA Extraction

1- Prepare RT and No RT control reaction mixes (on ice)

Component	RT reaction	No RT Control reaction
SuperScript IV VILO Master Mix	4uL	-
SuperScript IV VILO No RT Control	-	4uL
Template RNA (1pg to 2.4ug total RNA)	varies	varies
Nuclease-free water	to 20uL	to 20uL

2- Anneal primers: gently mix and incubate at 25⁰C for 10 min

3- Reverse transcribe RNA: incubate at 50⁰C for 10 min

4- Inactivate enzyme: incubate at 85⁰C for 5 min

5- qPCR amplification: use diluted or undiluted cDNA for qPCR or store at -20⁰C for up to one week or -70⁰C for long term storage

Cell lysis by CellLytic M (Cat. #C2978) from Sigma

- 1- Wash cells and treat with CellLytic M.
 - a. For adherent cells: Remove the growth medium from the cells to be assayed. Rinse the cells once with DPBS being careful not to dislodge any of the cells and discard DPBS. Add appropriate volume of CellLytic M reagent.
- 2- Incubate the cells for 15 min on a shaker
- 3- Collect lysed cells
 - a. For adherent cells: Remove cells from plates (cell scraping might increase total protein yield)
- 4- Centrifuge the lysed cells for 15 min at 12,000 -20,000 x g to pellet the cellular debris
- 5- Remove the protein containing supernatant to a chilled test tube

Note: Lysate preservation requires low temperatures. Therefore, for long term storage it is recommended to store lysate at -70°C .

BCA protein concentration

- 1- Pipette 25 μ l each standard or protein sample replicate into individual wells of a 96-well plate
- 2- Add 200 μ l BCA working reagent to each well. Mix on plate shaker for 30 seconds. Cover plate.
- 3- **Standard assay:** incubate reaction at 37^oC for 30 min or at room temperature for 2-16 hrs.

Enhanced assay: incubate reactions at 60^oC for 15 min

- 4- Cool plate to room temperature
- 5- Measure absorbance at 562 nm on a plate reader

Note: if the plate reader does not have a 562 nm filter, wavelengths from 540-590 nm have been used successfully

- 6- To obtain corrected absorbance, subtract the absorbance of the blank standard from the absorbance measurement or all other standard and protein samples
- 7- Plot the corrected absorbance versus the known mass of the BSA standards to generate the standard curve.

Note: if a curve fitting algorithm associated with the microplate reader is used for preparing the standard curve, a four parametric (quadratic) or best-fit curve will provide the most accurate results

- 8- Using the standard curve, interpolate the recorded corrected absorbance reading for the samples assayed which fall within the linear range of the standard curve
- 9- Calculate the amount of protein present in the original sample by correcting for dilution and sample volume

Western Blot

- 1- Wash cells 1x with DPBS
- 2- Collect cells and follow Lysis buffer specific protocol, centrifuge and use supernatant
- 3- Perform BCA or Bradford assay to determine protein concentration. Freeze samples at -20 or -80°C for later use or continue.
- 4- Thaw samples completely and mix well. Centrifuge at 10,000 x g for 8 min to pellet debris.
- 5- Calculate amount/volume of sample needed. Always load equal amounts of sample, volumes may vary or bring to equal volumes with PBS.
- 6- LDS buffer to calculation.
- 7- Boil for 5-8min at 100°C in boiling water bath.
- 8- Let cool and centrifuge at 10,000 x g for 1 min
- 9- Load all sample to gel.
- 10- Remove green casting frame, place gels in inner electrophoresis chamber, wells facing inward. Squeeze gel molds together and lock into place. Put inner chamber into running tank.
- 11- Fill inner chamber with running buffer to just overflow it.
- 12- Pour out some buffer from inner chamber until level is about halfway filling the gel wells.
- 13- Use 1ml pipette to clean each well and remove excess Acrylamide, using 800ul buffer/bubbles.
- 14- Refill inner chamber with running buffer (if leaky dump out all running buffer, remove inner chamber, re-lock the gels into place, and try again)
- 15- Load samples into wells (always load 10ul of ladder at one or both ends of the gel)
- 16- Add more running buffer in outer chamber to be around 2 inches from the bottom.
- 17- Put lid in place, red-red and black-black. Run gel at 80-85V for 2 hrs or 100V for 1 hr.

Transfer

- 18- Soak two filter papers and two foam pads in Blotting buffer (BB) for 10 min
- 19- Fill one tray with methanol to activate membrane (1min)
- 20- Fill second tray with BB to equilibrate gel, gently open glass gel frame and remove top glass pane. Cut off gel comb and any excess gel. Gently rock the gel off the glass plate into BB to equilibrate (5-10 min).
- 21- Dump methanol from membrane and pour in BB (it will float so keep it wet)
- 22- Dump gel's BB into the transfer apparatus and refill with new BB.
- 23- Slide filter paper under the gel in the tray in BB, center the gel on top of filter paper.
- 24- On the BLACK side of the clamp case, layer as follows:
 - a. Bottom: 1 foam pad
 - b. Filter paper with gel on top
 - c. PVDF membrane (cut with razor to just cover the gel, ensure no bubbles and keep wet with BB)
 - d. Filter paper (do NOT move once laid down)
 - e. Top: 1 foam pad
- 25- Close case, squeeze and keep pressure in the center of the case, slide lock over.
- 26- Insert into inner chamber with black side to black side.
- 27- Fill outer tank with BB (do NOT pour over clamp/case) until level with top edge of outer tank.

- 28- Run at 4⁰C with ice pack inside chamber at constant mAmps at 66mA overnight
- 29- Remove membrane from transfer apparatus. Ensure all of the visible ladder has been transferred from the gel to the membrane. Handle the membrane with forceps gently.
- 30- Discard BB in hazardous waste container

Blocking

- 31- Use milk or BSA depending on the primary antibody incubation buffer recommendation
- 32- Prepare 10ml block buffer per membrane (5% milk or BSA): 20ml TBS-T + 1g dried milk or BSA
- 33- Remove membrane, place in tray with TBS-T for 5 min then replace with blocking buffer for 1-2 hours at room temperature.

Primary Antibody

- 34- Prepare 10 ml of 5% milk or BSA per membrane depending on antibody manufacturer's datasheet with appropriate concentration of antibody
- 35- Make pouches for membrane, seal three sides with heat sealer
- 36- Add antibody/buffer (10ml per pouch), seal and shake overnight at 4⁰C or 2 hr at RT

Secondary Antibody

- 37- Remove from pouches and wash 3 x 10 min in TBS-T
- 38- Make 10ml 5% milk per membrane with appropriate concentration of secondary antibody.
- 39- Replace TBS-T with antibody/buffer and shake for 1-2 hrs at room temperature.

Developing

Stripping/Reprobing

- 40- Rinse membranes in TBS-T for 3 x 5min
- 41- Place in pouch and add 10ml Strip Buffer and incubate at 37⁰C in shaker for 20 min.
- 42- Discard strip buffer in Hazardous waste container
- 43- Rinse 3 x 10 min in TBS-T
- 44- Reblock for 1 hour at room temperature in 5% milk or BSA (antibody dependent)
- 45- Continue with new target antibody as above.

To save membrane

- 46- After transferring, wash membranes in ultrapure water 3 x 10min
- 47- Dry membrane completely on filter paper and place in pouch
- 48- When ready to use
 - a. Activate membrane in methanol for 2 min
 - b. Wash in ultrapure water 3 x 10min
 - c. Block in 5% milk or BSA for 1-2 hrs at room temperature
 - d. Continue with WB procedures

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Curriculum Vita

