

VITAMIN D-MEDIATED INDUCTION OF INNATE IMMUNITY IN GINGIVAL  
EPITHELIAL CELLS

By

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To my wife and family

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By

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Vitamin D, while classically thought of as important for the health and development of bones, is now known to play an important part immune regulation. My goal was to show if vitamin D contributed to immune regulation of gingival epithelial cells.

Using OKF6/Tert-1 cell cultures as a model of gingival epithelial cells (GEC), I provide evidence that GEC have the cytochrome P450 enzyme machinery required to convert vitamin D<sub>3</sub> to active 1,25-dihydroxyvitamin D<sub>3</sub> (1,25-D<sub>3</sub>) using RT-qPCR, and PCR. OKF6/Tert-1 cells convert 25-dihydroxyvitamin D<sub>3</sub> (25-D<sub>3</sub>) to 1,25-D<sub>3</sub> as demonstrated with measurement of 1,25-D<sub>3</sub> in culture media and increased CYP24A1 expression. The 1,25-D<sub>3</sub> regulated key antimicrobial and inflammatory responses of GECs. Pro-inflammatory cytokines IL-6 and IL-8 were decreased in OKF6/Tert-1 cells following treatment with 10nm 1,25-D<sub>3</sub>. Similarly, IL-1A expression was decreased in C57BL/6 mice with treatment of 10um 1,25-D<sub>3</sub>. The antimicrobial peptide (AMP) LL-37 was exported from OKF6/Tert-1 cells at an increased rate following 10nm 1,25-D<sub>3</sub> treatment. *Porphyromonas gingivalis* (*Pg*) CFUs and 16s rRNA expression were

decreased upon invasion of TIGK and OKF6/Tert-1 cells respectively following treatment with 1,25-D<sub>3</sub>. These results show that treatment with topical vitamin D<sub>3</sub> may be effective in regulating innate immunity within human gingival epithelial cells and the oral environment.

## CHAPTER 1 VITAMIN D: A REVIEW

### **Introduction**

Vitamin D, classically thought of as important for the growth and development of bones and associated with calcium absorption, has now gained acceptance as having a far wider reaching scope of importance to the human body. Vitamin D is now recognized as playing a role in overall systemic health and functions within immunity, inflammation, and infection. Vitamin D deficiency affects health on a global level, and assessing how to best treat the problem has grown increasingly complex and involved with recent literature showing distinct differences in vitamin D functions between men and women, those of differing ethnic and racial backgrounds, the young and the elderly, as well as in different locations around the world. The treatment outlook and continued supplementation for vitamin D deficiency or associated disease is determinant on many factors including: type of vitamin D or analog compound used, delivery system including storage and half-life issues, drug interactions and effects on a systemic level, and even what it means to be “vitamin D deficient”.

### **Brief History**

The history of vitamin D begins with the discovery of vitamin A by McCollum and Davis in mid-1913 which led to further studies focusing on vitamin discovery.<sup>1</sup> Vitamins B and C quickly followed, and the term “vitamin D” was coined by McCollum in a paper published in June of 1922.<sup>2</sup> Within the paper, McCollum builds off the previous work of Mellanby and describes vitamin D in terms of the effects of increased calcium deposition within sufferers of rickets.<sup>2,3</sup> The vitamin D compound was first isolated by Askew through distillation in 1930 and then further by crystallization in 1932.<sup>4,5</sup> The

compound discovered was what we know now as vitamin D<sub>2</sub>. It was not until 1936 when the compound we now describe as vitamin D<sub>3</sub> was identified by Windhaus.<sup>6</sup> While both vitamin D<sub>2</sub> and vitamin D<sub>3</sub> had a similar effect in treating rickets, the differences in form as well as function began to emerge with further research.

### **Vitamin D<sub>2</sub> and D<sub>3</sub>**

Vitamin D<sub>2</sub>, or ergocalciferol, was used to treat rickets by Hess in 1930 in the form of “viosterol” or irradiated ergosterol, while vitamin D<sub>3</sub>, or cholecalciferol, was used in the original Mellanby experiments in the form of cod liver oil.<sup>3,7</sup> In general, D<sub>2</sub> is found mainly in plants or yeasts (occasionally in mushrooms) and vitamin D<sub>3</sub> is the natural form of vitamin D found in most primates and other animals.<sup>8-10</sup> The vitamin D<sub>3</sub> precursor, 7-dehydrocholesterol (DHCR7), absorbs UVB radiation through the skin and isomerizes into vitamin D<sub>3</sub>.<sup>9-12</sup> While vitamin D<sub>2</sub> can be ingested from other sources and utilized within the human body, the natural presence of vitamin D<sub>3</sub> within humans has led to further research on the two showing that D<sub>2</sub> is less effective as supplement in form and function when compared to vitamin D<sub>3</sub>.<sup>8,13-15</sup> In order to develop a further understanding of the complex cellular machinery required, and the process undergone by the human body to convert this natural vitamin D<sub>3</sub> into an active and useable form, it is necessary to discuss what are known as cytochrome P450 enzymes.

### **Cytochrome P450 Enzymes and Vitamin D Conversion**

The process to activate vitamin D<sub>3</sub> to a biologically active metabolite is a sequence of hydroxylation reactions involving many different cytochrome P450 (cyp) enzymes.<sup>16-18</sup> Following conversion of DHCR7 within the skin, Vitamin D<sub>3</sub> is transported to the liver where it is stored and further converted to 25-hydroxyvitamin D<sub>3</sub> (25-D<sub>3</sub>); this conversion is a result of 25-hydroxylation by Cyp2R1 and Cyp27A1.<sup>16,19-22</sup> The 25-D<sub>3</sub> is

the primary circulating form of vitamin D<sub>3</sub>, and it is further converted by 1 $\alpha$ -hydroxylation to the active form 1,25-dihydroxyvitamin D<sub>3</sub> (1,25-D<sub>3</sub>) by Cyp27B1 upon transport to the kidney.<sup>23,24</sup> The concentration of active 1,25-D<sub>3</sub> present within a system are regulated by the 24-hydroxylase Cyp24A1 which converts 1,25-D<sub>3</sub> to 1,24,25-D<sub>3</sub> to recirculate for future degradation.<sup>16,22,25-27</sup>

Within all of these steps, the transport of vitamin D is facilitated by the vitamin D-binding protein (DBP). Due to being responsible for the entirety of vitamin D transport, the concentration of DBP present within a subject can affect concentration of certain vitamin D forms as well as having an effect on immune response.<sup>28-31</sup> Finnish researchers concluded that variation in DBP concentration may be affecting levels of bone mass within adolescents.<sup>31</sup> Changes in concentration of DBP can also affect serum 25-D<sub>3</sub> concentration.<sup>29</sup> The concentration of DBP should be considered as well as the levels of vitamin D itself when trying to determine if an individual is vitamin D deficient.<sup>30</sup>

Although classic 1 $\alpha$ -hydroxylation of 25-D<sub>3</sub> takes place within the kidney, it has been shown that other tissues possess the 1 $\alpha$ -hydroxylase enzyme Cyp27B1 and may convert circulating 25-D<sub>3</sub> to 1,25-D<sub>3</sub> at other such sites.<sup>32-34</sup> This extra-renal conversion has been shown to also exist within respiratory epithelium, gingival fibroblasts, and periodontal ligament cells.<sup>35-37</sup>

Following conversion, the DBP and active 1,25-D<sub>3</sub> complex enters into cells via endocytosis following binding interactions with the megalin receptor.<sup>38</sup> 1,25-D<sub>3</sub> undergoes active function and gene regulation within cells by binding to a specialized transcription factor known as the vitamin D receptor.

## **The Vitamin D Receptor**

The vitamin D receptor (VDR) is a ligand-specific transcription factor that is present in most tissues and immune cells in the human body.<sup>39-42</sup> The 1,25-D<sub>3</sub> is the most potent ligand for the VDR, and upon ligation to the VDR it binds to the retinoid X receptor (RXR) which accompanies VDR translocation to the nucleus and localization at specific vitamin-D response elements (VDREs) located in promoter and enhancer regions of many genes.<sup>40,42,43</sup> The VDR and RXR have zinc-finger protein regions that act as DNA binding domains on a potential VDRE.<sup>42</sup> VDREs generally consist of a PGTTCa region that will bind VDR followed by a three-to-six base spacer region and a PGGTCA region to bind with RXR.<sup>42</sup> These binding events allow for regulation both up and down for many gene products including the VDR itself as well as many products with immune function.<sup>40,42-44</sup>

## **Genome-Wide Studies**

Through the usage of techniques such as chromatin immunoprecipitation and sequencing (ChIP-seq) and microarray analysis, it has been possible to find VDREs throughout the human genome and determine exactly which genes and gene products may be effected by vitamin D regulation.<sup>45-48</sup> One study has shown greater than 1,000 chromatin domains that contain one or more VDR binding sites as well as the potential for up to 13,000 VDREs total within the human genome, while another has shown differing numbers between 1,800 to 2,700 total binding domains in various immune cells.<sup>45,46</sup> It has also been shown that 1,25-D<sub>3</sub> does bind to these potential VDREs by showing location shifts of VDR to be within proximity of potential VDREs.<sup>49</sup> Overall, genomic studies and indentifying VDREs has shown that vitamin D binding events may have a large effect on both innate and adaptive immunity, antigen presentation,

inflammation, and expression of antimicrobials responsible for fighting potential local and systemic infection.<sup>47</sup>

## **Vitamin D Status, Treatment, and Supplementation**

### **Sufficiency, Deficiency, Toxicity**

Numerous efforts have been made in many different ways to try and better establish a standard for proper concentration of vitamin D in humans, but with multiple influences ranging from sun exposure and skin pigmentation to age and weight, it is difficult to determine if what is good for one individual will be good for another.<sup>50</sup> Knowing this, the recommended limits of vitamin D<sub>3</sub> supplementation recommended by some studies remain around 1,000 IU/day or 10,000IU/week, while others show that 500-700IU/day may be sufficient.<sup>51,52</sup> One international unit (IU) of vitamin D is equivalent to 0.025µg or 25ng of vitamin D<sub>3</sub>.<sup>53</sup> This would make the 1,000IU/day amount equivalent to 25µg/day of vitamin D.

Another statistical approach has been attempted to determine the risk-benefit assessment and discover the method of treatment that will allow for the maximum net health gains resultant from vitamin D supplementation.<sup>55</sup> Utilizing Quality of Life for Benefit Risk Assessment (QALIBRA) software, an advanced analysis takes place using an equation created to weigh loss versus gain as in an actuarial table to determine exactly how much vitamin D should be supplemented.<sup>55</sup> The completed equation given as an example in the study goes on to show that a serum concentration of 25-D<sub>3</sub> of 87.5nmol/L provides the lowest risk of mortality in a human population.<sup>55</sup> This estimate can be directly compared to other studies which show estimates of >50nmol/L, 75nmol/L, or even as high as 90-100nmol/L.<sup>55</sup> Judging the study based purely on the numerical proximity to other studies, this statistical method of analysis to develop a

“best fit” number for all seems more useful than the prior vitamin D index and biomarker individual method.

Another potential pitfall of trying to reach sufficient levels of vitamin D in a population with so much variability is the toxicity that arises from overconsumption or supplementation. Vitamin D created in the skin is not an issue, because the body will regulate excess levels of the hormone, but chronic consumption of ~40,000IU/day in supplements will result in vitamin D toxicity and hypercalcemia.<sup>56</sup> Symptoms arising from vitamin D poisoning include: severe gastrointestinal pain (and possible nausea, vomiting, constipation, and diarrhea), bone pain, heart beat irregularity, joint and muscle pain, loss of appetite, frequent urination and thirst, nervousness and itching, and potential kidney stones.<sup>56</sup> However, in the case of an accidental overdose of 2,000,000 IU within two elderly nursing home residents, no short term results aside from slightly elevated plasma calcium levels were observed.<sup>57</sup> This does not, however, exclude the potential for a dosage of that size to result in long term vitamin D toxicity.<sup>57</sup>

### **Food and Nutrition**

While there are foods like milk, which provide adequate amounts of vitamin D for the prevention of rickets, and foods historically proven to be rich in vitamin D, like the cod liver oil in Mellanby’s experiments on rickets, many alternative foods today are being experimentally fortified with vitamin D.<sup>3,58,59</sup> The fortification of various foods with vitamin D is an important potential option for the treatment of deficiency. Fortification in most cases requires a stable carrier for the compound to be introduced, and study has shown that the milk protein alpha-lactalbumin is a good candidate for encapsulation with vitamin D<sub>3</sub> and subsequent use a carrier in fortified foods.<sup>60</sup>

Hens that have been fortified with vitamin D lay vitamin D enriched eggs, and the effectiveness of these eggs on the vitamin D status of humans who consume them is an important topic of research.<sup>61-63</sup> Consumption of seven total vitamin D-enhanced eggs a week over the course of an 8 week winter has shown complete stabilization of serum 25-D<sub>3</sub> concentration when compared to the significantly lower concentration of 25-D<sub>3</sub> in individuals in the control group.<sup>61</sup> Another positive aspect is that the fortification of the eggs does not have any negative implications or detrimental results on the production process.<sup>62</sup> This process also improves the antioxidant quality of the eggs to be consumed.<sup>63</sup>

Eggs may be successful as a vitamin D fortified food, but fortification is still required in many countries to assist in increasing serum 25-D<sub>3</sub> to healthy levels. In Ireland, a 10 year study was performed to assess the intake of citizens, and while it did not involve measured amounts of serum 25-D<sub>3</sub> taken from patients, the survey showed that residents of Ireland had a slight increase in vitamin D consumed over the 10 year period.<sup>64</sup> Their intake, however, was still nowhere near the recommended amount of intake for healthy adult vitamin D status.<sup>64</sup>

A somewhat overlooked and non-fortified option for dietary vitamin D intake is the mushroom. Most mushrooms develop large amounts of vitamin D<sub>2</sub> when exposed to sunlight or UV radiation, and while vitamin D<sub>2</sub> may not be as useful or potent a treatment as vitamin D<sub>3</sub>, it is an often underestimated source to control potential serum vitamin D concentration<sup>65</sup>, and even so there are even some mushrooms that can also produce vitamin D<sub>3</sub>.<sup>66</sup> Shitake mushrooms in particular can form small amounts of vitamin D<sub>3</sub> as well as vitamin D<sub>2</sub> when exposed to UV light, and the important part of

mushrooms as a potential source of vitamin D is that they bioavailability and amount gained from intake is equal to an oral vitamin D supplement of the same IU dosage.<sup>66</sup>

### **Vitamin D<sub>2</sub> and D<sub>3</sub>, Drug Interactions, Bioavailability**

Supplementation with vitamin D has been attempted with D<sub>2</sub> as well as vitamin D<sub>3</sub>, and while vitamin D<sub>3</sub> is clearly the better option due to increasing serum 25-D<sub>3</sub> concentration in greater amount, vitamin D<sub>2</sub> as a supplement may have its own interesting implications.<sup>67</sup> Even at lower values of increase, vitamin D<sub>2</sub> supplementation has shown increases in DBP and the 24,25-D degradation product, and those increases may allow for similar increases in available 25-D and 1,25-D.<sup>68</sup>

It is important to understand potential drug interactions that vitamin D may have within the body if taken as a supplement. Atorvastatin intake lowers serum 25-D<sub>3</sub> concentration, while subsequent supplementation with 25-D<sub>3</sub> will lower Atorvastatin concentrations in kind.<sup>69</sup> Thiazide diuretics in combination with vitamin D or calcium supplementation will result in hypercalcemia within suffers of hyperparathyroidism, decreased kidney function, or in the elderly.<sup>69</sup> Aside from the two interactions mentioned, evidence of other drugs interfering with vitamin D supplementation status was not of sufficient quality of level to prove a connection.<sup>69</sup>

The bioavailability of vitamin D is the final important issue to deal with when it comes to supplementation and amounts necessary. Bioavailability is the amount of a supplement that will be absorbed within the gastrointestinal tract following digestion, and it is almost always greatly lower than the amount of the compound that is originally taken due to factors like how much interaction and degradation there is when met in direct contact with other recently ingested compounds.<sup>70</sup> There is little known data about the absorption of vitamin D<sub>3</sub> compounds, but it is fat-soluble and 25-D<sub>3</sub> is known to be

absorbed in greater quantities than non-hydroxylated D<sub>3</sub> or D<sub>2</sub>.<sup>70</sup> The amount of fat ingested with supplementation does not seem to have an effect, but tetrahydrolipstatin (orlistat) and sucrose polyester compounds such as Olestra most likely lower the amount of vitamin D absorbed.<sup>70</sup>

## **Immunity, Inflammation, and Infection**

### **Immunity**

Vitamin D signaling events occur within antigen-presenting-cells (APCs) such as dendritic cells (DCs), adaptive immune cells like T cells and B cells, and even innate cells such as monocytes, macrophages and neutrophils.<sup>71-74</sup> In regards to antigen presentation and adaptive immune activity, 1,25-D<sub>3</sub> functions in a generally immunosuppressive manner and primes dendritic cells which present antigen and encourage the development of T regulatory cells (Tregs) which function to regulate immunity through tolerance and suppression of active effector T cells.<sup>75-77</sup> To further show potential as a potential anti-inflammatory substance, vitamin D as an oral supplement in humans has also been shown to increase and decrease levels of CD38+ cells and circulating IL-17+ T cells respectively.<sup>78</sup> The innate immune protection provided by neutrophils, monocytes, and macrophages can be thought of as result of the chemoattractant properties of antimicrobial peptides (AMPs) such as  $\beta$ -defensins and the human cathelicidin LL-37.<sup>71-74</sup> The aforementioned regulatory effects on the immune system showcase vitamin D as a rather potent anti-inflammatory substance.

### **Inflammation**

As previously mentioned, the levels of anti-inflammatory IL-10-producing Tregs and pro-inflammatory IL-17+ T cells can be regulated by vitamin D.<sup>77,78</sup> These ILs, or interleukins, are small protein products known as cytokines that can control and affect

the cells and tissue environments around them.<sup>79</sup> ILs, as well as chemokines, interferons, and tumor necrosis factor may be produced and released from the various cells affected by vitamin D such as T cells, B cells, macrophages, and neutrophils.<sup>79</sup> Much like the binding of vitamin D to VDR, these cytokines function through distinct specific bind events on the surface or internally within cells and release further cytokine and chemokine products which result in a complicated interplay of control and self-regulation.<sup>79</sup>

Mast cells have been shown to be capable of metabolizing 25-D<sub>3</sub> which in turn reduces mast cell activation and allergic inflammation.<sup>80</sup> Following the discovery of VDR binding, many synthetic VDR agonists have been produced, and several of these vitamin D analogs have shown to reduce release of CXCL10, a potent inflammatory chemokine.<sup>81,82</sup> 1,25-D<sub>3</sub> and 25-D<sub>3</sub> both reduce levels of pro-inflammatory IL-6 released from human B and T cells.<sup>83</sup> In a study of an opposite approach, many pro-inflammatory cytokines (IL-2, IL-1 $\beta$ , TNF, and IFN- $\gamma$ ) were to shown to be elevated and in high circulation within adults with significant vitamin D deficiency.<sup>84</sup> Within mice, it was shown that vitamin D deficiency was responsible for disruption of normal fecal microbiota and resulted in increased intestinal inflammation.<sup>85</sup> 1,25-D<sub>3</sub> and 25-D<sub>3</sub> were both responsible for inhibition of pro-inflammatory cytokines IL-6 and TNF within human monocytes treated with LPS to stimulate inflammation.<sup>86</sup> 1,25-D<sub>3</sub> treatment lowers levels of IL-1 $\beta$ , TNF, IFN- $\gamma$ , and IL-8 in monocytes treated with heat-killed bacteria.<sup>87</sup> In many cases, such bacteria and other such microbes may infect and cause harmful inflammation which can be readily prevented utilizing vitamin D.

## **Infection and AMPs**

Vitamin D treatment may increase antimicrobial activity in certain circumstances, and vitamin D deficiency is known to have an association with increased infection incidence in the respiratory system, the GI tract, and many other places.<sup>88–92</sup> As previously discussed, AMPs like LL-37 or  $\beta$ -defensin can be a powerful weapon to fight against infection both directly and indirectly.<sup>71–74</sup> LL-37 and human  $\beta$ -defensin 2 both contain a VDRE binding region and are positively regulated by 1,25-D<sub>3</sub> to fight against infection.<sup>93</sup> Supplementation with 1,25-D<sub>3</sub> and 25-D<sub>3</sub> leading to increased levels of LL-37 can be used to treat many different types of infection which may result from vitamin D deficiency.<sup>94–100</sup>

Vitamin D<sub>3</sub> treatment in patients with sepsis or septic shock shows increased levels of LL-37.<sup>94</sup> Low concentrations of 25-D<sub>3</sub> and 1,25-D<sub>3</sub> are commonplace within victims of community-acquired pneumonia as well as those suffering from sepsis.<sup>95,96</sup> There is also a trend of severe vitamin D deficiency within those suffering from infection in areas in proximity to prosthetic attachments and implants.<sup>97</sup> Increased levels of LL-37 and other antimicrobials from 1,25-D<sub>3</sub> treatment has been shown to inhibit and control infection of *Mycobacterium species marinum* and *tuberculosis* respectively.<sup>98,99</sup> However, vitamin D cannot be involved in all infection processes. It was shown that there is no association between genetic polymorphisms in the vitamin D pathway and incidence of *Chlamydia trachomatis* infection.

## **Racial, Territorial, Sexual, Age-Dependent and Organismal Differences**

The evolution of pigment within the skin of human beings is a story that involves the importance of vitamin D to the human condition, survival, and development of strong immune function and bone and calcium homeostasis.<sup>101–103</sup> The development of lighter

skin pigments as well as beneficial mutations of 7-dehydrocholesterol (DHCR7) allowed for human beings to move away from the equator to more northern latitudes where UVB radiation was not plentiful enough to properly regulate vitamin D in non-mutated individuals of dark skin.<sup>104</sup> These variations in latitude-dependent UVB radiation as well as the tendency for individuals to move around the globe to locations where their skin color, diet, or life style may not be suitable for proper vitamin D development are a growing concern to the deficiency status of our global community.

### **Racial and Ethnic Variability**

Vitamin D deficiency is more prevalent in those of African descent and darker pigment when compared to those of European descent with lighter pigment.<sup>105,106</sup> A darker pigment of skin is more concentrated in melanin and thus is more resistant to incoming UVB radiation and thus it is more difficult for darker skinned individuals to make vitamin D<sub>3</sub> within their skin.<sup>107</sup> In America, where there are so many individuals of differing descent and skin color living in similar areas, it is thought that the darker skinned individuals will naturally have lower vitamin D intake from the sun and thus more foods fortified in vitamin D should be made available to the general public.<sup>106</sup> Studies have shown that even with vitamin D fortified milk available, non-Hispanic whites still have the highest dietary intake levels of total vitamin D.<sup>106</sup> That would mean that the population that latitude affects the least is also getting the highest levels of dietary assistance towards fighting vitamin D deficiency. One study shows that serum concentrations of DBP are similar within black versus white individuals of the same area, so it can be said that the problem lies within total amount of vitamin D<sub>3</sub> available and not the levels of associated, required compounds.<sup>108</sup>

This deficiency in vitamin D among individuals of darker skin can be compounded synergistically when other problems that are greater among darker pigmented individuals are taken into account. African American men are diagnosed with prostate cancer more often than European American men, and it has been shown that deficiency in vitamin D<sub>3</sub> also increases mortality in prostate cancer.<sup>109</sup> This comes coupled with the fact that far more African American men are deficient in vitamin D than European American men, and it presents a terrible problem for the health of individuals based purely upon the color of their skin.<sup>109</sup> Efforts have been made to supplement and normalize the vitamin D levels of African Americans, and the research has led to evaluating dosage requirements for helping those suffering from deficiency.<sup>110</sup>

It should also be noted, however, that even among individuals of the same pigment and living in the same area there are great discrepancies in vitamin D concentration.<sup>111</sup> This was shown recently in a study of over 1,800 middle-aged Caucasian French adults who still showed varying levels of vitamin D deficiency that seemed to be independent of dietary intake.<sup>111</sup> Adding to that fact, Caucasian individuals in more northern latitudes have their own problems when it comes to vitamin D production due to the longer winters and lesser levels of sunlight overall in addition to lower UVB radiation.<sup>112</sup> These issues have led to individuals in places like Norway needing to supplement vitamin D by scheduling therapy trips to tanning salons, solar simulators, sunbeds, and other such locations with UVB lamps available.<sup>112</sup> These problems arising from location are not distinct to Scandinavia, and vitamin D deficiency and status vary on a global level.

## Worldwide Populations

The health of populations and individuals on worldwide scale is affected by potential vitamin D deficiency and the varying degree by which they are able to supplement and/or prevent the problems associated with said deficiency.<sup>113-123</sup> The highest concentrations of serum 25-D<sub>3</sub> are present in North America, and this is most likely due to the fortification of foods present in North American with vitamin D when compared to many other countries.<sup>113</sup>

In Iran, studies have shown that vitamin D deficiency is commonplace in young men and women during the winter months, but status improved greatly during the summer months and also had a minor improvement with weekly supplementation.<sup>114</sup> Office workers both male and female were observed in Ankara, Turkey, as it could be assumed those who work indoors receive less essential sunlight when compared to others around them.<sup>115</sup> Results showed that the majority of workers were greatly deficient in vitamin D even within the summer months and it should be considered a significant problem to public health.<sup>115</sup> In a place like Saudi Arabia, the summer months can be the greatest time of deficiency and cause for alarm due to the tendency to avoid the sun and the intense heat.<sup>116</sup> Research has gone as far as to equate the most efficient times for Saudi citizens to expose themselves to the sun and the summer elements to more optimally create their essential vitamin D<sub>3</sub>.<sup>116</sup>

Even in the tropical country of Malaysia, where again the sunny climate may lead one to thinking there could be cause for worry or alarm, vitamin D deficiency among women is an issue.<sup>117</sup> Women residing both in urban and rural areas were observed for deficiency, and a clear difference was observed as the urban women, who were most likely exposed to less sun, were a great deal more vitamin D deficient than those

residing in rural areas.<sup>117</sup> In New Zealand, citizens of non-Māori heritage are far more deficient than those of a Māori background, and this is even true when both parties are taking weekly vitamin D supplements.<sup>118</sup>

On the Mediterranean island of Crete, efforts have been made to observe the mean 25-D<sub>3</sub> concentration present within an isolated population and to redefine what deficiency meant to them based on health issues without making comparison of their deficiency to outside populations.<sup>119</sup> The study showed that a serum concentration of around 20ng/mL was the cut off where deficiency would be determined.<sup>119</sup>

In Norway, adolescent populations of natives as well as immigrants were sampled for vitamin D deficiency and to determine potential childhood health risks.<sup>120,121</sup> Deficiency was shown to be much more common during the school year, especially in young boys, due to long hours spent indoors when compared to their summer months away from school.<sup>120</sup> Among the immigrant children observed, very few were found to be deficient by the standards of Norway, but almost half of them were under a level which would be considered “sufficient”.<sup>121</sup>

A large part of the problem of deficiency comes from the inability to come to global standard of what is “deficient” and to recommend a level of intake from food or sunlight to combat this deficiency.<sup>122</sup> This is a great problem in Europe where many small countries lie in close proximity to one-another and yet have very different standards of care and treatment when it comes to vitamin D deficiency and supplementation.<sup>122</sup>

The problem that lies within Olmsted County, Minnesota is not one of deficiency, but rather of concentrations of vitamin D high enough to result in toxicity and illness due

to hypercalcemia.<sup>123</sup> Over a 10 year period of study, 9.2% of individuals sampled had concentrations of serum 25-D<sub>3</sub> high enough to be considered toxic.<sup>123</sup> It is thought that this trend toward a toxic concentration of vitamin D present within the population may be a result of over-treatment with high-dose prescription supplements.<sup>123</sup> It is no surprise that this may happen in world that is finally growing informed of the potential health issues caused by vitamin D deficiency.

### **Sexuality and Pregnancy**

While the majority of studies on vitamin D deficiency in relation to the sex of an individual may be focused on pregnant females, there are still several issues that pertain explicitly to male sufferers. In one study, a significant portion of patients suffering from erectile dysfunction (ED) were shown to be deficient in vitamin D.<sup>124</sup> It is believed this trend towards erectile dysfunction may have been due to vitamin D being necessary for the health of endothelial tissue within the penis and surrounding areas.<sup>124</sup> It is unlikely that it may be caused by low testosterone, because vitamin D supplementation has not had significant results on increasing testosterone concentrations in clinical trials.<sup>125</sup> Vitamin D is important in the reproductive system, and this has been shown in males where data has suggested a relationship between serum 25-D<sub>3</sub> concentration and the ability of semen to cause pregnancy during vaginal intercourse.<sup>126</sup> Vitamin D is still more important to the female, however, in regards to pregnancy and the health of a fetus.

Levels of vitamin D, and supplementation if required, are important to a healthy outcome during pregnancy and post-pregnancy for both the expectant mother and the unborn child. While already thought of as necessary for the development of healthy bones within a growing fetus, the status of vitamin D is also important balancing

antimicrobial protection for both mother and child while being sure to regulate immune self-tolerance to prevent fetal rejection and miscarriage.<sup>127,128</sup> Vitamin D deficiency leads to an increase chance of miscarriage during the first trimester of pregnancy.<sup>129</sup> This may be due to a lack of self-tolerance as a result of impaired Treg function within vitamin D deficient pregnant women.<sup>130</sup> Misregulation of vitamin D levels and subsequent deficiency may lead to problems such as infection, gestational diabetes, small fetal size, preterm birth, and preeclampsia.<sup>131</sup> Preeclampsia, or pregnancy-induced hypertension, has shown to be increased in mothers of ethnic minorities that suffer from low concentration of serum 25-D<sub>3</sub>.<sup>132</sup>

Requirements for supplementation to combat these issues have been tested, and while doses in the 1,000IU/day range have shown little to no effect, dosage of 4,000 IU/day within pregnant females has been shown to normalize serum concentrations of vitamin D and improve chances of a normal, healthy birth.<sup>133-135</sup> In an almost opposition as to what one would normally expect, vitamin D supplementation slightly suppresses levels of the beneficial LL-37 AMP within the macrophages of newborns.<sup>136</sup> However, this suppression does not seem to affect the killing capacity of macrophages.<sup>136</sup>

Post-pregnancy, the psychological health and mood of new mothers may also be directly affected by vitamin D deficiency, but different studies have shown opposing results. In 2015, a study showed little to no links of post-partum depression (PPD) to vitamin D deficiency, while a study in 2017 resulted in 76% of surveyed PPD sufferers being deficiency in vitamin D.<sup>137,138</sup> In addition to vitamin D supplementation having a positive effect on weight at birth, bone mass and fetal calcium levels, the kidney health of a individual may also show benefits from increased vitamin D even into early

childhood.<sup>139</sup> Following birth, infants still require and benefit from vitamin D in a number of ways as do adults and the elderly.

### **From Infancy to Elderly**

Previously, vitamin D supplementation in infant children has been only to combat rickets and thus no standard treatment levels have been agreed upon to eliminate the health problems that may apply to the youth of today.<sup>140</sup> In Finland, researchers are busy evaluating the levels of vitamin D necessary to allow for strong bones, a strong immune system and resistance to infection, less incidence of allergy and asthma, and greater cognitive development of infants.<sup>140</sup>

As previously discussed, children around the world may be deficient in vitamin D.<sup>121</sup> This deficiency may lead to problems with bone health as well as many non-skeletal issues which may affect a child well into the onset of puberty or even into adulthood.<sup>141</sup> The most dangerous of these problems may very well be respiratory tract infections (RTIs) such as pneumonia which can lead to severe illness and death within a great number of children.<sup>142</sup> Efforts are being taken eliminate the onset of these RTIs with vitamin D supplementation, but proper treatment amounts are not well defined or documented for each potential infection.<sup>142</sup> Until proper supplementation amounts are agreed upon, this will continue to be a problem for children as they may suffer well into adulthood and even old age as a result of ill-developed immune systems caused by vitamin D deficiency.

While discussing populations around the world, it was shown that the elderly in New Zealand suffer from high levels of vitamin D deficiency, and this is also true for those of increasing age in all walks of life.<sup>118,143</sup> This trend towards sub-optimal concentrations of circulating serum vitamin D in an aging world population may lead to

increased bone fractures, as well as several other problems that may lead to early mortality unless the deficiency is treated.<sup>143</sup> Chronic pain is a problem for most, if not all, individuals of advanced age, and low concentrations of 1,25-D<sub>3</sub> are associated with severe bouts of chronic pain in men over the age of 70.<sup>144</sup> Increasing levels of chronic inflammation and swelling are also a problem for the elderly, and individuals over the age of 65 undergoing treatment for chronic inflammation show significant links between low levels of vitamin D and high levels of pro-inflammatory cytokines.<sup>145</sup> The decline of cognitive function, onset of dementia, and development of brain abnormalities is a problem that many older individuals must confront. Low concentrations of serum 25-D<sub>3</sub> are present within African American and Hispanic persons of increased age and are associated with a greater rate of cognitive decline.<sup>146</sup> It is yet to be determined if vitamin D supplementation can assist in lowering the rate of this decline.<sup>146</sup>

Individuals of all races, locales, ages, and sexes have shown to be challenged in various ways when it comes to the issue of vitamin D deficiency, but it is not just humans that deal with the associated health problems and further issues related to vitamin D.

### **The Animal Kingdom and Beyond**

While the knowledge base of vitamin D in research and the effects of deficiency within other creatures may not be as great as it is in humans, many important things can be learned from looking into the lives and vitamin D status of other organisms.

Canines and felines are very different from humans in that they lack the ability to create vitamin D<sub>3</sub> within their skin.<sup>147,148</sup> The diet of such creatures is important, as vitamin D<sub>3</sub> will be gained from a mainly animal-based diet while D<sub>2</sub> will be acquired if plant sources are consumed.<sup>147,148</sup> Felines in particular have a hard time utilizing D<sub>2</sub> as

well as vitamin D<sub>3</sub>, though canines may utilize both forms efficiently.<sup>147,148</sup> Canines and felines may well be a potential species for research to be compared to humans, as many of the same conditions and diseases suffered by humans from vitamin D deficiency extend to cats and dogs as well.<sup>147,148</sup>

Farm animals have also been the subjects of vitamin D research for their own health as well as the health of the human population that may feed upon such animals. Vitamin D metabolism has been well researched within dairy cattle, and much like humans their immune health, bone formation, and reproductive health are affected by daily sun exposure and varying concentrations of serum 25-D<sub>3</sub>.<sup>149</sup> In opposition to cattle, which have been studied for benefit to their own health, research has been performed on pigs to improve the health of the human population.<sup>150</sup> In order to explore another avenue for potential vitamin D fortification within the food we eat, experiments have been performed in order to determine the optimal conditions for the formation of vitamin D<sub>3</sub> within the skin of pigs.<sup>150</sup> Pork or other foodstuffs that contain pig skin may be fortified with vitamin D through the use of UV-producing LED lights within living areas and processing plants for pigs.<sup>150</sup>

Some species of fish contain great levels of dietary vitamin D<sub>3</sub>, and research has been performed to determine whether this vitamin D<sub>3</sub> is from a dietary source or converted in the skin through UV radiation as within humans.<sup>151</sup> Rainbow trout have been found to form vitamin D<sub>3</sub> within their skin, but the potential for formation of vitamin D<sub>3</sub> within species of fish that live within extremely deep waters is unknown.<sup>151</sup>

Although it is thought that plants mainly contain vitamin D<sub>2</sub>, certain plants within the *Solanaceae* or nightshade family also contain vitamin D<sub>3</sub>, which may have the

potential for consumption and supplementation of vitamin D within individuals that do not consume animal products.<sup>152</sup> The tomato, potato, eggplant, bell pepper, and other plants commonly eaten as food belong to the nightshade family.<sup>152</sup> While the fruits and other commonly eaten parts of such plants have not shown significant or any sign of vitamin D<sub>3</sub>, it has been shown that small amounts of vitamin D<sub>3</sub>, 25-D<sub>3</sub>, and 1,25-D<sub>3</sub> exist within the leaves of tomato and bell pepper plants exposed to UV radiation.<sup>152</sup> This is a problem for potential plant-based fortification, as the leaves of such plants are extremely poisonous in large quantities, but perhaps future research will show possible vitamin D treatment or supplementation from plants.<sup>152</sup>

### **Disease and Oral Health**

Vitamin D deficiency is associated with systemic health problems as well as common diseases. Vitamin D is an excellent localized topical for the treatment of psoriasis and certain skin diseases.<sup>153</sup> Vitamin D and calcium are necessary in the proper amounts if the human body is to develop, remodel, and grow a healthy bone structure.<sup>154</sup> Within muscle, vitamin D is important to strength, flexibility and proper function, and vitamin D deficiency may result in muscle weakness, and even muscle atrophy.<sup>155,156</sup> Vitamin D deficiency is associated with obesity in that serum concentrations of 25-D<sub>3</sub> are lower in obese individuals than the non-obese.<sup>157,158</sup> Deficient concentrations of serum 25-D<sub>3</sub> are associated with both type 1 diabetes (T1D) and type 2 diabetes (T2D).<sup>159,160</sup> Within cystic fibrosis, treatment with vitamin D may be used to regulate anti-inflammatory and anti-microbial activity in the airway.<sup>161</sup> Patients with liver disease are commonly vitamin D deficient and this applies to alcoholics, non-drinkers, adults, and children suffering from liver disease.<sup>162</sup> As VDR is expressed in mammary glands and in many breast cancers, 1,25-D<sub>3</sub> treatment and the resultant anti-

inflammatory and pro-apoptotic effects from induced immune regulation are well documented as beneficial to breast cancer patients.<sup>163,164</sup> This trend of vitamin D deficiency as a symptom, or even causal agent of disease can be directly related to my current research interests of periodontal disease and oral health.

Characterized by both chronic inflammation and infection as well as bone and tooth loss, periodontal disease has symptoms that mimic the normal effects of vitamin D deficiency.<sup>165</sup> Low serum concentrations of 25-D<sub>3</sub> may have an association with periodontal disease, and a strong association has been shown between increasing 1,25-D<sub>3</sub> concentration after alleviation of periodontal disease symptoms and reduction of inflammation.<sup>166,167</sup> 1,25-D<sub>3</sub> treatment inhibits the expression of pro-inflammatory cytokine IL-8 within a cell culture model of periodontal disease, and 25-D<sub>3</sub> can downregulate TLR4 and the JAK1/STAT3 pathway to have potential implications in further alleviating periodontal inflammation.<sup>168,169</sup> 25-hydroxylase activity that can convert vitamin D<sub>3</sub> to 25-D<sub>3</sub> has been confirmed in human gingival fibroblasts and periodontal ligament cells, and this is a positive discovery because 25-D<sub>3</sub> treatment in human oral keratinocytes can increase expression of the AMP LL-37.<sup>37,170</sup> 25-D<sub>3</sub> has also shown upregulation of VDR and downregulation of NF-kB which resulted potent anti-inflammatory effects in mice.<sup>171</sup> Overall, vitamin D is a candidate for research on immune regulation within oral health.

## CHAPTER 2 VITAMIN D: CONVERSION WITHIN HUMAN GINGIVAL EPITHELIAL CELLS

### **Background and Purpose**

As described in chapter 1, vitamin D is important to the regulation of immunity, alleviating harmful inflammation, and fighting infection within the human body.<sup>73,74,82,88-90,172</sup> Within a great number of diseases, vitamin D is at levels below what could be considered sufficient to proper immune function.<sup>173</sup> Along with its recent role as an immune regulator, vitamin D is classically associated with successful bone formation and continued bone health.<sup>154,174-176</sup> As a disease that involves both harmful inflammation and bone/tooth loss, periodontitis is a prime candidate for potential phenotypic alleviation following treatment with vitamin D.<sup>165</sup>

Serum 25-D<sub>3</sub> concentrations are lower in those suffering from periodontitis, and treatment with 25-D<sub>3</sub> in diabetic mice with periodontitis has been shown to ameliorate symptoms of harmful inflammation and potential alveolar bone loss.<sup>169,171,177</sup> 25-D<sub>3</sub> and 1,25-D<sub>3</sub> induce innate immunity and increase antimicrobial activity within human gingival epithelium.<sup>72,170</sup> While 1,25-D<sub>3</sub> is an effective treatment, the enzyme Cyp24A1 exists to metabolize, negatively regulate, and degrade the hormone which gives it a short half life of ~6-8 hours.<sup>27</sup> Topical treatment of periodontitis with the more stable, and readily available over-the counter, vitamin D<sub>3</sub> may be a better treatment option if it can be shown to convert to active 1,25-D<sub>3</sub> within an oral environment.

While presence of the cytochrome P450 machinery required to convert vitamin D<sub>3</sub> to active 1,25-D has been shown in gingival fibroblasts and ligament cells, it has not been shown within gingival epithelium.<sup>37</sup> The overall goal of my research is to show the presence of cytochrome P450 enzymes within human gingival epithelium as evidence of

vitamin D conversion and increased innate immune regulation following topical vitamin D treatment. I hypothesize that vitamin D<sub>3</sub> is converted to active 1,25-D<sub>3</sub> within human gingival epithelium and that vitamin D<sub>3</sub> treatment will lower the expression of pro-inflammatory cytokines in human gingival epithelium.

## **Methods**

### **Cell Culture**

Cell lines used within the following experiments were the human oral keratinocytes OKF6/Tert-1 and telomerase-immortalized gingival keratinocytes (TIGK).<sup>178,179</sup> Cells were grown to confluency in 6-well or 12-well plates at 37°C and 5% CO<sub>2</sub> within keratinocyte serum-free media (KSFM) supplemented with 10% FBS, L-glutamine, and penicillin/streptomycin. All cell line experiments utilized both biological and assay triplicates.

### **Oligonucleotide Primers**

All primers used within the following experiments were designed and ordered through Integrated DNA Technologies (IDT, Coralville, IA) and are listed in table 2-1 following the results section. Primers were validated with the first derivative of melt curve analysis following RT-qPCR containing a single peak, and products were run on agarose gels to reveal bands of predicted amplicon size by design.

### **Cytochrome P450 Enzyme Presence**

Cytochrome P450 enzyme presence was examined by RT-qPCR. OKF6/Tert-1 cells were treated in triplicate with 10nm vitamin D<sub>3</sub>, 10nm 1,25-D<sub>3</sub>, or EtOH vehicle control for 6 hours at which point RNA was isolated from the cells according to the RNeasy Plus Minikit (Qiagen, Valencia, CA). Genomic DNA contamination was minimized by running the samples through gDNA Eliminator spin columns (Qiagen)

prior to RNA purification. RNA was reverse transcribed using iScript Reverse Transcription Supermix for RT-qPCR (Bio-Rad, Hercules, CA). RT-qPCR was carried out with SYBR Green Supermix (Bio-Rad) and individual qPCR primers for CYP2R1, CYP27A1, CYP27B1, and CYP24A1. Data was analyzed using Bio-Rad CFX Manager Software.  $\beta$ -actin was used as a control.

For cytochrome p450 enzyme presence by PCR, OKF6/Tert-1 cells were treated in triplicate with EtOH, 10nm vitamin D<sub>3</sub>, or 10nm 1,25-D<sub>3</sub>. RNA was purified from cells and reverse transcribed to cDNA with the absence of reverse transcriptase (RT-) as a negative control for potential genomic DNA contamination. The cDNA and oligonucleotide primers for enzymes CYP2R1, CYP27A1, and CYP27B1 were used in PCR amplification using an iProof High-Fidelity Master Mix (Bio-Rad) and then run on a 3% agarose gel with gel-loading buffer in each sample and 100bp Quickload ladder for determining presence or absence by amplicon size.

To assess concentrations of total 1,25-D<sub>3</sub> or 25-D<sub>3</sub> for the purposes of ascertaining if direct conversion was taking place, OKF6/Tert-1 cells were treated in triplicate with vitamin D<sub>3</sub>, 25-D<sub>3</sub>, or EtOH vehicle control for 6 or 24 hours. Media fractions were removed and kept at -80°C until samples were sent for analysis of total 1,25-D<sub>3</sub> or 25-D<sub>3</sub> content by radioimmunoassay (Heartland Assays, Ames, IA). Untreated media was used as a control. Vitamin D<sub>3</sub> treated samples were analyzed for total 25-D<sub>3</sub> content, 25-D<sub>3</sub> treated samples were analyzed for total 1,25-D<sub>3</sub> content, and EtOH samples as well as untreated media control were analyzed for 25-D<sub>3</sub> and 1,25-D<sub>3</sub> content for purposes of control. The assay to measure 25-D<sub>3</sub> had a lower detection limit

of 2.5 ng/mL while the assay to measure 1,25-D<sub>3</sub> content had a lower detection limit of 5.5 pg/mL and an upper detection limit of 210 pg/mL.

### **Vitamin D Anti-Inflammatory and Anti-Microbial Effects**

Pro-inflammatory cytokine expression was observed using RT-qPCR. OKF6/Tert-1 cells were treated in triplicate for 6 hours with either 10nm 1,25-D<sub>3</sub> or EtOH vehicle control in the presence or absence of polyinosinic:polycytidylic acid (poly(I:C)) as an inflammatory stimulus. Poly (I:C) interacts with TLR3 unlike the TLR2 interactions with *Pg* in periodontitis, but it was used as a stimulus because both pathways can function to increase inflammation via NF-κB activation.<sup>180–182</sup> RNA purification, reverse transcription, and RT-qPCR were carried out as described previously but using primers for IL-6 and IL-8 as they are both pro-inflammatory cytokines. β -actin was used as a control.

Regulatory effects on pro-inflammatory cytokines were also observed in mice. C57BL/6 mice (n = 15) were treated with an oral rinse of either 1 μm vitamin D<sub>3</sub>, 1 μm 1,25-D<sub>3</sub>, or EtOH vehicle control, all of which were suspended in mineral oil to allow for lengthened treatment through continual adhesion to the gingival surface. 6 hours following treatment, mice were euthanized by CO<sub>2</sub> exposure and all gingival epithelial tissue was harvested and placed in RNAlater Stabilization Solution (Thermo Fisher) for stability and then utilized for RT-qPCR as previously described. CYP24A1 expression was examined as a control of vitamin D activity, and IL-1A expression was quantified to determine regulation of inflammation as it is commonly expressed in inflammatory activated epithelial cells. Mouse β-actin was used as a control.

For quantifying levels of the antimicrobial peptide LL-37, OKF6/Tert-1 cells were treated in triplicate with 10nm 1,25-D<sub>3</sub> or EtOH vehicle control for 24 hours. Media

fractions were removed and kept for analysis while cells were washed 2x in PBS and lysed with cell scraping following the addition of cell lysis buffer. LL-37 levels were measured and the ratio between levels in both cell and media fractions to determine cellular LL-37 export was determined by Sandwich ELISA with a kit from MyBioSource (San Diego, CA).

To view the effects of vitamin D treatment on *Pg* invasion, untreated TIGK cells in triplicate and TIGK cells pre-treated in triplicate for 24 hours with 10nm 1,25-D<sub>3</sub> or EtOH were washed 4x with PBS and infected with *Pg* (MOI = 100) and incubated for 90 minutes. Within the previously untreated TIGK cells, this infection also came with a concurrent 90 minute treatment of either 10nm 1,25-D<sub>3</sub> or EtOH, while the pre-treated cells received no treatment along with the infection. Cells were then washed 4x again in PBS and treated with metronidazole/gentamicin in media for another 60 minute incubation to kill externally adherent *Pg* that had not yet invaded. Cells were washed 4x more in PBS, scraped on ice with ice-cold PBS, and lysates transferred to cryovials containing DMSO and *Pg* growth media and kept at -80°C until CFU counts were to be determined by spot plating of lysate serial dilutions.

For assessment of total bacterial load by visualization of *Pg* 16s rRNA, OKF6/Tert-1 cells were treated in triplicate with either 10nm vitamin D<sub>3</sub> or 10nm 1,25-D<sub>3</sub> for 24 hours, and washed 4x in PBS followed by infection with *Pg* (MOI = 100). Cells were then supplied with plain media or media supplemented with additional 10nm vitamin D<sub>3</sub> or 1,25-D<sub>3</sub> and allowed to incubate for a further 24hours, washed 4x in PBS, at which point RNA was purified, reverse transcribed, and RT-qPCR was carried out as previously described but using primers for *Pg* 16s rRNA to show total bacterial load.

Primers were developed to identify *Pg* exclusively and not other potential microbes.<sup>183</sup>

Human 18s rRNA was used as a control.

## Results

### Conversion of Inactive Vitamin D by Gingival Epithelial Cells

In RT-qPCR for the presence of cytochrome P450 machinery (Fig 2-1), RNA for CYP24A1, CYP27A1, CYP27B1, and CYP2R1 was present in OKF6/Tert-1 cells whether treated with EtOH, vitamin D<sub>3</sub>, or 1,25-D<sub>3</sub> for 6 hours. Expression of CYP24A1 was significantly increased in vitamin D<sub>3</sub> (Fig 2-1A) and 1,25-D<sub>3</sub> (Fig 2-1B) treated samples when compared to EtOH vehicle control samples. No significant difference was measured in the expression levels of CYP27A1, CYP2R1, or CYP27B1 amongst all treatments. Statistics were performed using Student's t-test with a p-value of < 0.05 showing significance. Error bars represent standard error.

PCR for cytochrome P450 presence (Fig 2-2) resulted in bands of proper amplicon size (CYP2R1: ~110bp, CYP27A1: ~285bp, CYP27B1: ~245bp) being present for each gene observed. CYP2R1 lanes showed single banding and no genomic DNA contamination in RT- control. CYP27A1 lanes showed faint alternate banding at approximately 400bp but nothing in RT- control. CYP27B1 shows strong presence in all treatments, but also 2 more unexpected bands at ~335bp and <100bp and contamination from genomic DNA in RT- control treatments at ~335bp as well.

Radioimmunoassay for the presence of 25-D<sub>3</sub> and 1,25-D<sub>3</sub> varied by treatment type, but not by treatment length. Plain media as a control, as well as samples treated with vitamin D<sub>3</sub> and EtOH vehicle control for 6 or 24 hours, showed no measureable amount of 25-D<sub>3</sub> present within the limits of the assay (<2.5ng/mL) (Table 2-2). Samples treated with 25-D<sub>3</sub> for 6 and 24 hours showed 1,25-D<sub>3</sub> present in levels above the upper

limits of the assay (>210pg/mL), while media and EtOH showed no 1,25-D<sub>3</sub> present within lower limits of the assay (<5.5pg/mL) (Table 2-2).

### **Anti-Inflammatory Effects of Vitamin D in Gingival Epithelial Cells**

RT-qPCR for immune markers of inflammation resulted in no significant difference in the levels of IL-6 or IL-8 between samples treated with EtOH or 1,25-D<sub>3</sub>, but IL-6 and IL-8 levels were significantly decreased in 1,25-D<sub>3</sub> treated samples that had undergone prior inflammatory stimulus with poly(I:C) versus EtOH treated samples (Fig 2-3). Statistics were performed using Student's t-test with a p-value < 0.05 showing significance. Error bars represent standard error.

RT-qPCR within mice showed significant differences in expression of CYP24A1 in 1,25-D<sub>3</sub> treated versus EtOH and vitamin D<sub>3</sub> treated mice, and significant differences in expression of IL-1A in EtOH treated versus vitamin D<sub>3</sub> and 1,25-D<sub>3</sub> treated mice. CYP24A1 was ~300-fold upregulated in 1,25-D<sub>3</sub> treated mice when compared to other treatments (Fig 2-4A). IL-1A was appreciably downregulated within both 1,25-D<sub>3</sub> (Fig 2-4A) and vitamin D<sub>3</sub> (Fig 2-4B) treated mice compared to those treated with EtOH. Statistics were performed using Student's t-test with a p-value < 0.05 showing significance. Error bars represent standard error.

### **Antimicrobial Effects of Vitamin D in Gingival Epithelial Cells**

ELISA showed a significantly higher level of free LL-37 in growth media than in the cell lysate fraction of samples treated with 1,25-D<sub>3</sub> when compared to EtOH treated samples (Fig 2-5). Statistics were performed using Student's t-test with a p-value < 0.05 showing significance. Error bars represent standard error.

To determine the effects on antimicrobial activity within gingival epithelium, cells were treated with vitamin D and infected with *Pg*. CFUs of *Pg* present following invasion

and re-plating were decreased 2-3 times in samples treated with 1,25-D<sub>3</sub> than samples treated with EtOH. Levels of overall *Pg* present were lower in pre-treated samples than samples treated with 1,25-D<sub>3</sub> and EtOH concurrent to the introduction and invasion of *Pg* (Fig 2-6A). CFU counts were lower in re-plated samples of the same dilution treated with 1,25-D<sub>3</sub> (Fig 2-6B) versus EtOH (Fig 2-6C). Statistics were performed using Student's t-test with a p-value < 0.05 showing significance. Error bars represent standard error.

RT-qPCR to estimate total bacterial load showed significantly lower levels of 16s rRNA expression following dual treatment in vitamin D<sub>3</sub> (2-7A) and 1,25-D<sub>3</sub> treated cells when compared to cells that were only pre-treated with either. A greater reduction in 16s rRNA levels was shown in the 1,25-D<sub>3</sub> treated samples when compared to those treated with vitamin D<sub>3</sub>. Statistics were performed using Student's t-test with a p-value < 0.05 showing significance. Error bars represent standard error.

Table 2-1. List of Primers Used

<b>Table 2-1: List of Primers Used</b>		
<b>Gene</b>	<b>Forward Primer (5' - 3')</b>	<b>Reverse Primer (5' - 3')</b>
B-Actin	GGATCAGCAAGCAGGAGTATG	AGAAAGGGTGTAACGCAACTAA
Cyp24A1	TTGGCTCTTTGTTGGATTGTCCGC	TGAAGATGGTGCTGACACAGGTGA
Cyp2R1	GATGCAGTTGTACGTGGTTATTC	GCTCAGGATGGAACACTTCT
Cyp27A1	GGACATGGCTCAACTCTTCTAC	CAGTTGGGCCTCCATATCTTC
Cyp27B1	CTCTTTGAGAGGAAGGGTGAAG	TAATAGTGGCCAGAGAGCAAAG
IL-6	GGAGACTTGCCTGGTGAAA	CTGGCTTGTTCTCACTACTC
IL-8	GCAGCTCTGTGTGAAGGTGCAGTTTTGC	TTTCTGTGTTGGCGCAGTGGTCC
Mouse B-Actin	GAGGTATCCTGACCCTGAAGTA	CACACGCAGCTCATTGTAGA
Mouse Cyp24A1	ACCACCGCCTAGAGAGATATT	GACAGCAGCGTACAGTTCTT
Mouse IL-1A	GCTTGAGTCGGCAAAGAAATC	GAGAGATGGTCAATGGCAGAA
Human 18s rRNA	CTGAGAAACGGCTACCACATC	GCCTCGAAAGAGTCCTGTATTG
Pg 16s rRNA	CTTGACTTCAGTGGCGGCAG	AGGGAAGACGGTTTTACCA

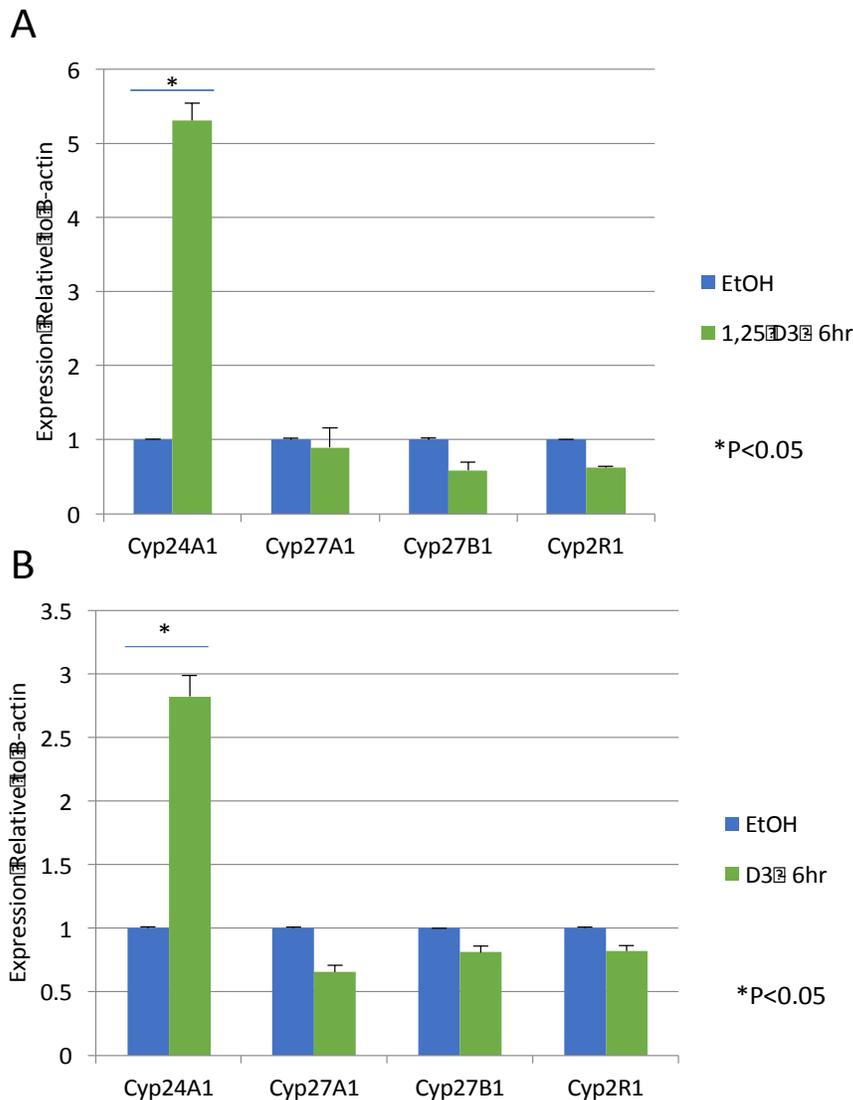


Figure 2-1. Cytochrome P450 Enzymes - qPCR

OKF6/Tert-1 cells were treated in triplicate with EtOH, 10nm vitamin D<sub>3</sub>, or 10nm 1,25-D<sub>3</sub> for 6 hours. RNA was purified and reverse transcribed to cDNA for use in RT-qPCR to observe the presence and expression of CYP24A1, CYP27A1, CYP27B1, and CYP2R1.  $\beta$ -actin was used as a control. All cytochrome P450 enzymes observed were present, while CYP24A1 was significantly upregulated within both vitamin D<sub>3</sub> (A) and 1,25-D<sub>3</sub> (B) treated cells when compared to EtOH control. The presence of Cyp-family enzymes allows for the potential conversion of vitamin D<sub>3</sub> to active 1,25-D<sub>3</sub> within gingival epithelium, and the significant increase in CYP24A1 shows active degradation of 1,25-D<sub>3</sub> which provides evidence for the possibility of conversion within the vitamin D<sub>3</sub> treated cells. Statistics were performed using Student's t-test with a p-value of < 0.05 showing significance. Error bars represent standard error.

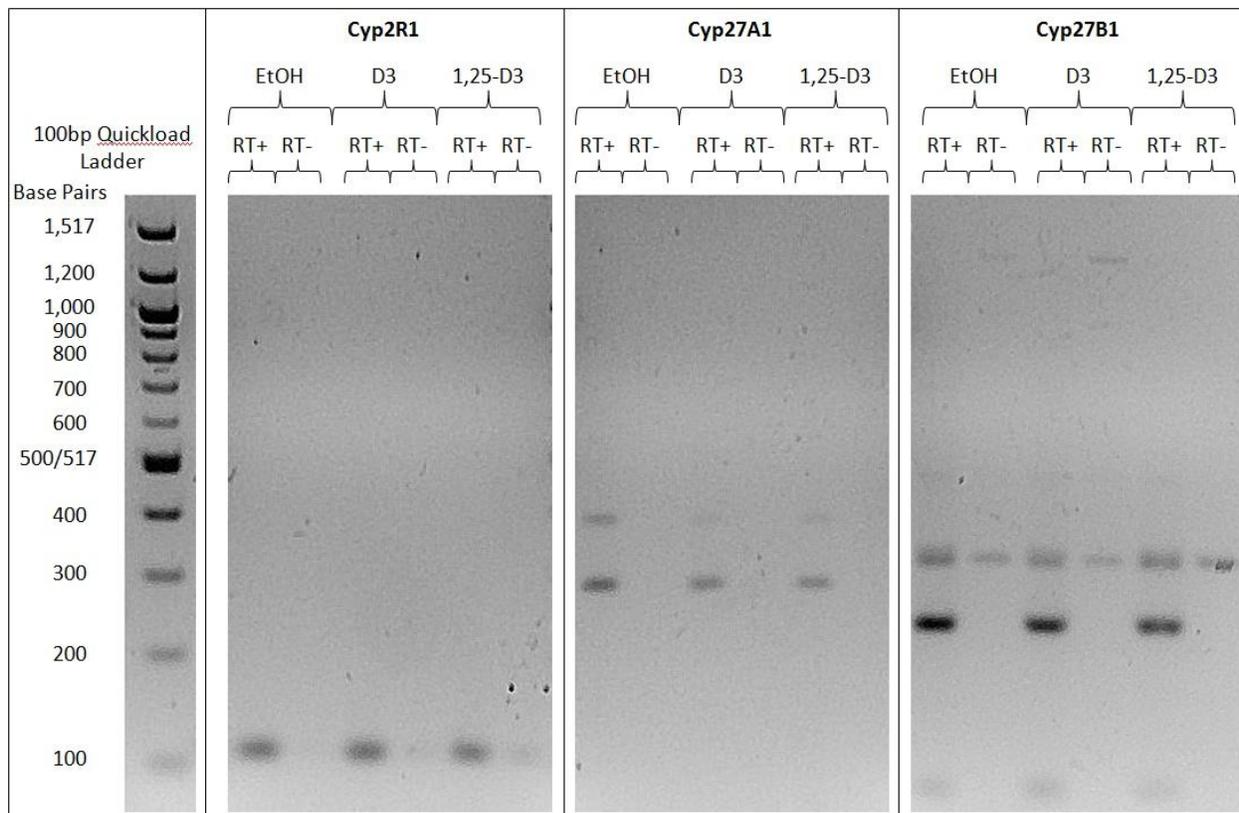


Figure 2-2. Cytochrome P450 Enzymes – PCR and Gel Electrophoresis

Gel electrophoresis was performed on PCR products to show cytochrome p450 enzyme presence within OKF6/Tert-1 cells. OKF6/Tert-1 cells were treated in triplicate with EtOH, 10nm vitamin D<sub>3</sub>, or 10nm 1,25-D<sub>3</sub>. RNA was purified from cells and reverse transcribed to cDNA with the absence of reverse transcriptase (RT-) as a negative control for potential genomic DNA contamination. cDNA and oligonucleotide primers for enzymes CYP2R1, CYP27A1, and CYP27B1 were used in PCR amplification and run on a 3% agarose gel for determining presence or absence by amplicon size. Bands of proper amplicon size (CYP2R1: ~110bp, CYP27A1: ~285bp, CYP27B1: ~245bp) were found for each gene observed. CYP2R1 lanes showed single banding and no genomic DNA contamination in RT- control. CYP27A1 lanes showed faint alternate banding at approximately 400bp but nothing in RT- control. CYP27B1 shows strong presence in all treatments, but also 2 more unexpected bands at ~335bp and <100bp and contamination from genomic DNA in RT- control treatments at ~335bp as well. Amplification of PCR products shows no quantifiable difference between EtOH, vitamin D<sub>3</sub>, and 1,25-D<sub>3</sub> treatments, but overall presence of gene products is observed in all RT+ treatment groups which shows the presence of RNA that would code for the enzymes in OKF6/Tert-1 cells. Future study should excise and purify gel bands to send out for Sanger sequencing to ensure the observed bands were the proper amplicon sequences and thus the observed gene products.

Table 2-2. 25-D<sub>3</sub> and 1,25-D<sub>3</sub> Quantification in OKF6/Tert-1 – Radioimmunoassay

To quantitatively observe vitamin D conversion, OKF6/Tert-1 cells were treated in triplicate for 6 or 24 hours with EtOH, 10nm vitamin D<sub>3</sub>, or 10nm 25-D<sub>3</sub> respectively. Media fractions were collected and kept at -80°C until analyzed by radioimmunoassay for total concentration of either 25-D<sub>3</sub> or 1,25-D<sub>3</sub>. Untreated media was used as an additional control. Results should interpreted as “presence” of 25-D<sub>3</sub> or 1,25-D<sub>3</sub> if measurement is over the detection limit of the assay or “absence” of 25-D<sub>3</sub> or 1,25-D<sub>3</sub> if under the detection limit of the assay. Cells treated with media, EtOH, and vitamin D<sub>3</sub> showed no detectable 25-D<sub>3</sub> within the limits of the assay (<2.5 ng/mL). Cells treated with media and EtOH showed no detectable 1,25-D<sub>3</sub> (<5.5 pg/mL), but 25-D<sub>3</sub> treated cells showed 1,25-D<sub>3</sub> presence above the limits of the assay (>210 pg/mL). This potentially shows conversion of 25-D<sub>3</sub> to 1,25-D<sub>3</sub> within gingival epithelium, but not conversion of vitamin D<sub>3</sub> to 25-D<sub>3</sub>. This is possibly because all of the vitamin D<sub>3</sub> used within treatment fully underwent conversion to 1,25-D<sub>3</sub> within both the 6 and 24 hour timepoints. In that case, a shorter timepoint may have shown detectable 25-D<sub>3</sub> within the vitamin D<sub>3</sub> treated cells. Another potential explanation may be that the 10nm vitamin D<sub>3</sub> treatment used is close to being under the detection limit (2.5ng/mL) of the 25-D<sub>3</sub> assay. Future study should use 100nm concentration as a treatment level to ensure that initial treatment is over the lower limit of the radioimmunoassay. Vitamin D<sub>3</sub> treated cells have not been tested for 1,25-D<sub>3</sub> presence yet due to time constraints and cost.

<b>Table 2-2: Radioimmunoassay Quantification of 25-D<sub>3</sub> and 1,25-D<sub>3</sub> in OKF6/Tert-1</b>							
<b>25-D<sub>3</sub> ng/mL following D<sub>3</sub> treatment</b>				<b>1,25-D<sub>3</sub> pg/mL following 25-D<sub>3</sub> treatment</b>			
	<b>Treatment</b>				<b>Treatment</b>		
<b>Time</b>	Media	EtOH	D <sub>3</sub>	<b>Time</b>	Media	EtOH	25-D <sub>3</sub>
6 Hrs	<2.5 ng/mL	<2.5 ng/mL	<2.5 ng/mL	6 Hrs	<5.5 pg/mL	<5.5 pg/mL	>210 pg/mL
24 Hrs	<2.5 ng/mL	<2.5 ng/mL	<2.5 ng/mL	24 Hrs	<5.5 pg/mL	<5.5 pg/mL	>210 pg/mL

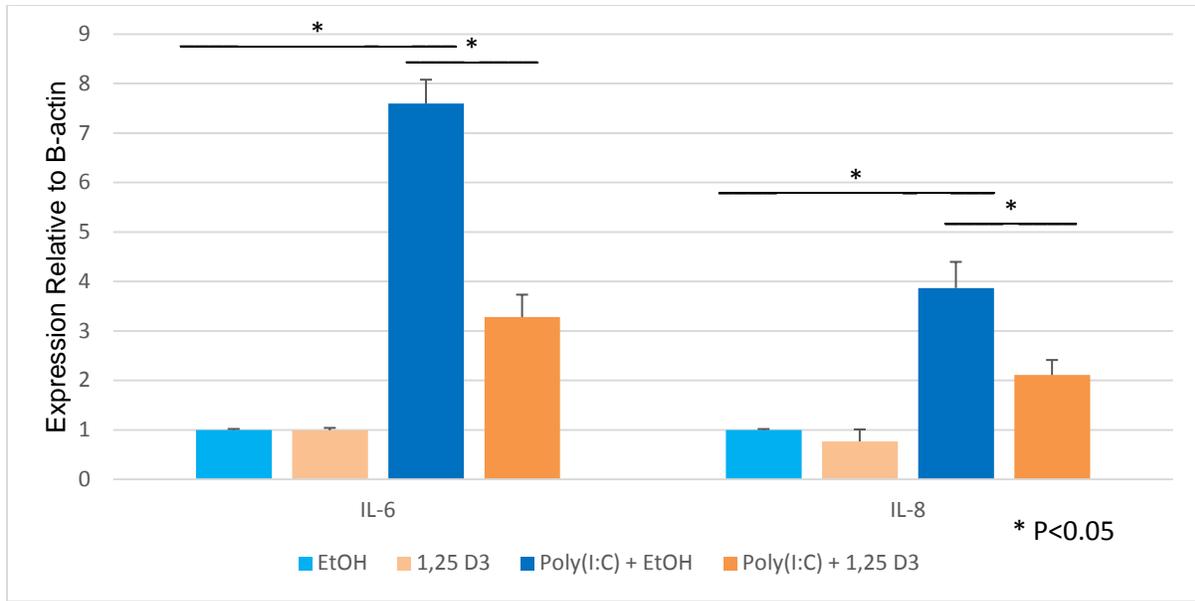


Figure 2-3. Inflammatory Markers and 1,25-D<sub>3</sub> - qPCR

OKF6/Tert-1 cells were treated in triplicate for 6 hours with EtOH or 10nm 1,25-D<sub>3</sub> in the presence or absence of 1ug/mL poly(I:C) as an inflammatory stimulus. While poly(I:C) may be a TLR3 agonist, it can activate the NF-κB pathway much like TLR2 activation by *Pg* within periodontitis, which makes it adequate for our stimulation of inflammation. RT-qPCR was used to view expression of pro-inflammatory cytokines IL-6 and IL-8 relative to β-actin as a control. Both IL-6 and IL-8 were significantly increased in poly(I:C) treated cells when compared to those not exposed to inflammatory stimulus. Levels of IL-6 and IL-8 were significantly reduced in the samples treated with poly(I:C) and 1,25-D<sub>3</sub> when compared to the poly(I:C) stimulated cells treated with EtOH. The reduction of IL-6 and IL-8 pro-inflammatory cytokines in OKF6/Tert-1 cells treated with 1,25-D<sub>3</sub> following the induction of inflammation by poly(I:C) shows the potential for amelioration of symptoms within an inflammatory phenotypic environment. Statistics were performed using Student's t-test with a p-value < 0.05 showing significance. Error bars represent standard error.

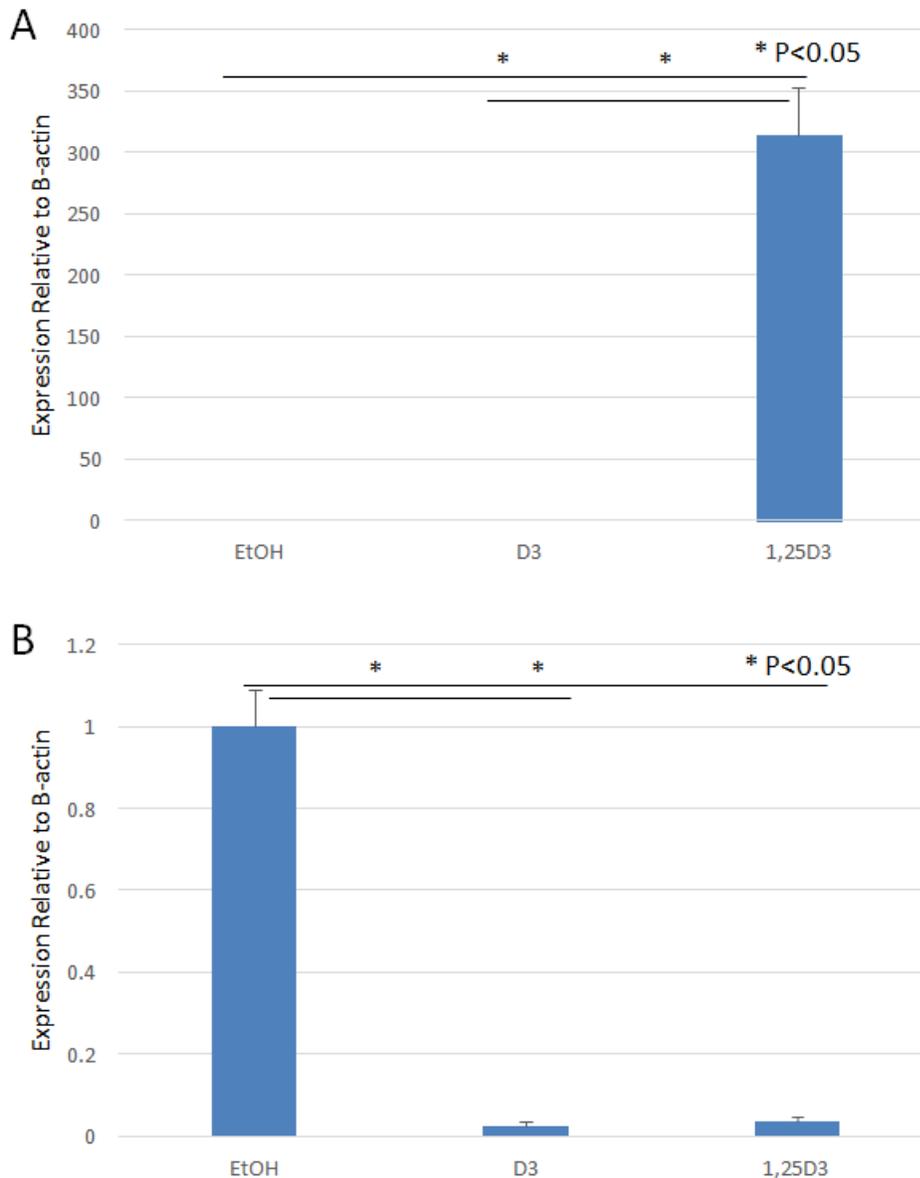


Figure 2-4. Inflammatory Regulation in Mice - qPCR

C57BL/6 Mice (n = 15) were treated with an oral rinse of EtOH, 1 $\mu$ m vitamin D<sub>3</sub>, or 1 $\mu$ m 1,25-D<sub>3</sub> for 6 hours to further observe innate immune regulation by vitamin D treatment. Mouse gingival tissue was harvested and RT-qPCR was performed to determine the levels of CYP24A1 as a measure of vitamin D activity and IL-1A as a pro-inflammatory cytokine that is present in inflammatory epithelial tissue. Mouse  $\beta$ -actin was used a control. Results showed significant increase of CYP24A1 in 1,25-D<sub>3</sub> treated mice (A) and significant reduction of pro-inflammatory cytokine IL-1A in vitamin D<sub>3</sub> (B) and 1,25-D<sub>3</sub> treated (A) mice. The increase in CYP24A1 provides evidence of 1,25-D<sub>3</sub> activity, and the decreased levels of IL-1A can be attributed to the anti-inflammatory effects of vitamin D treatment. Statistics were performed using student's t-test with a p-value of < 0.05 showing significance. Error bars represent standard error.

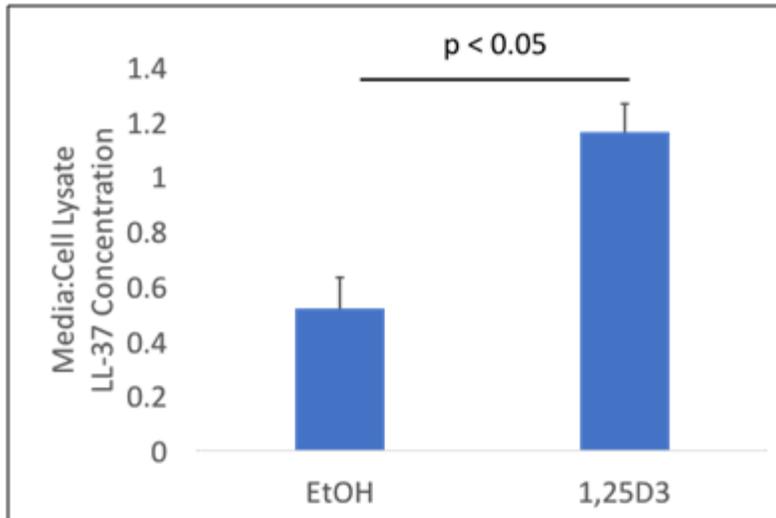
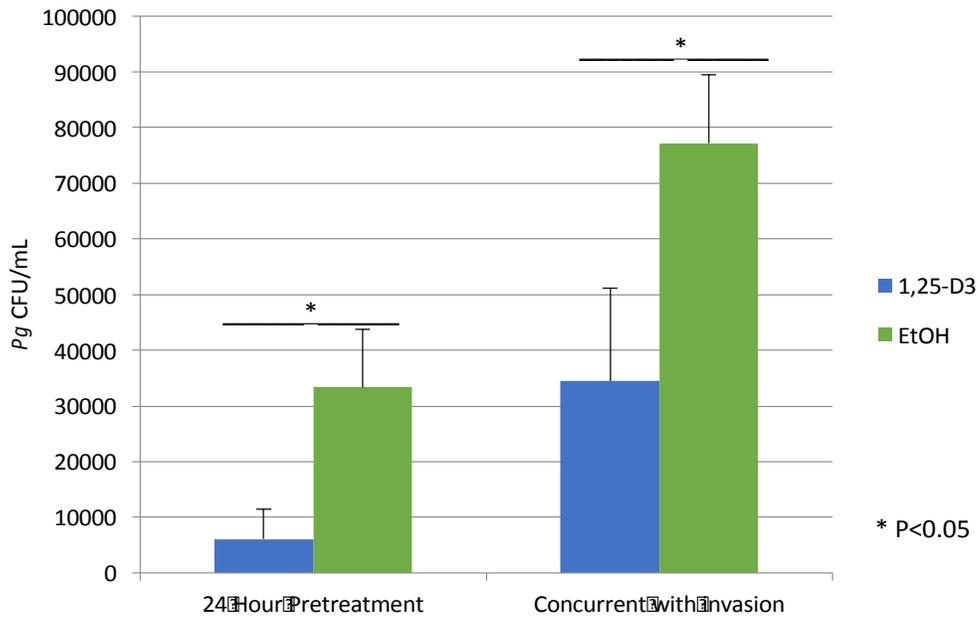


Figure 2-5. Export of LL-37 - ELISA

OKF6/Tert-1 cells were treated in triplicate for 24 hours with either 10nm 1,25-D<sub>3</sub> or EtOH vehicle control to observe the effects of vitamin D treatment on the levels of the antimicrobial peptide LL-37. Media and cell lysate fractions were taken from cell samples and used in sandwich ELISA to show levels of total LL-37. Results showed that a greater ratio of total free LL-37 resided in the media fraction within the 1,25-D<sub>3</sub> treated cells when compared to the EtOH treated cells. This shows greater levels of LL-37 export from vitamin D treated cells when compared to EtOH control treated cells. This increased level of extra-cellular LL-37 could mean potentially increased antimicrobial activity in the oral cavity upon vitamin D treatment of human gingival epithelium. Statistics were performed using Student's t-test with a p-value of < 0.05 showing significance. Error bars represent standard error.

A



B



C



Figure 2-6. Vitamin D and *Porphyromonas gingivalis* - Invasion Assay

TIGK cells initially left untreated and TIGK cells pre-treated for 24 hours with 10nm 1,25-D<sub>3</sub> or EtOH were infected in triplicate with *Pg* (MOI=100) to show the antimicrobial effects of vitamin D treatment. The initially untreated cells then received a 90 minute treatment with either 10nm 1,25-D<sub>3</sub> or EtOH while undergoing *Pg* invasion. Following invasion, cells were washed, lysed to release invaded *Pg*, and plated to count CFUs to determine viable *Pg*. CFUs were 2-3 times lower in all vitamin D treated cells (A) which shows potential antimicrobial activity, with overall levels of *Pg* being higher in cells that were not pre-treated with either 1,25-D<sub>3</sub> or EtOH. Plate images show cells pre-treated with 1,25-D<sub>3</sub> (B) or EtOH (C) at equivalent dilutions. It is yet unclear whether vitamin D is acting directly or indirectly to fight *Pg*. Statistics were performed using Student's t-test with a p-value of < 0.05 showing significance. Error bars represent standard error.

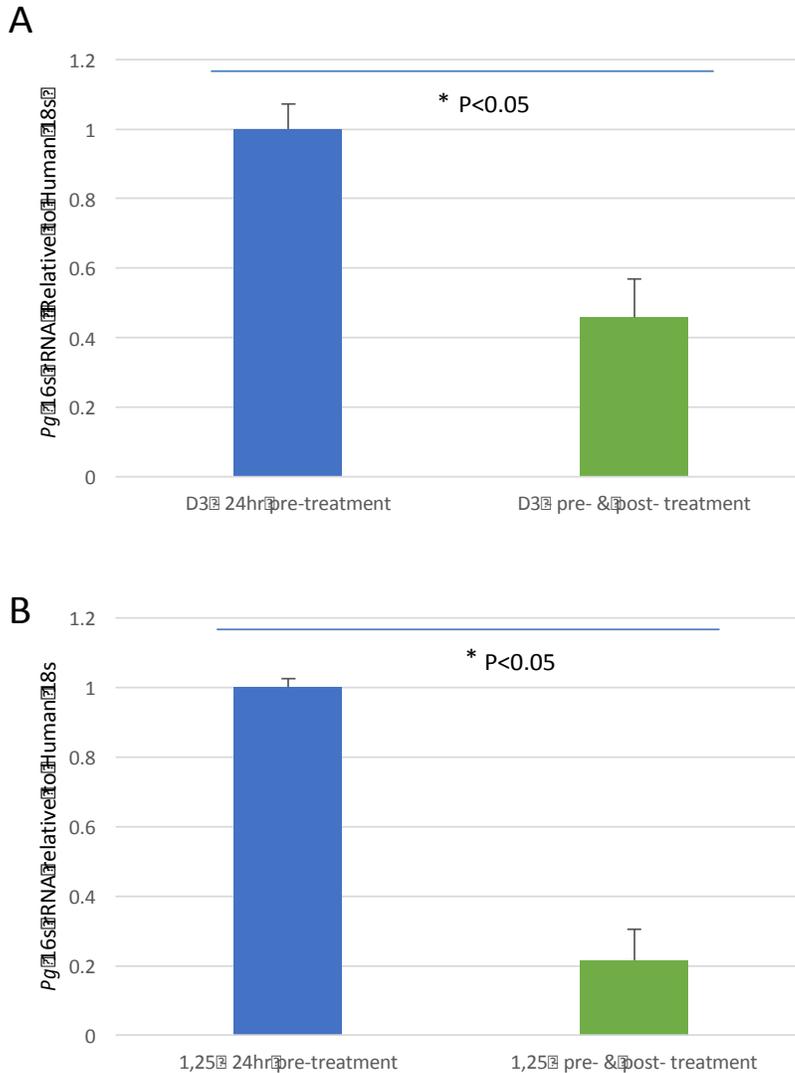


Figure 2-7. *Pg* 16s rRNA – Total Bacterial Load - qPCR

To observe the synergy of dual (pre- and post- invasion vitamin D treatment), OKF6/Tert-1 cells were treated in triplicate with either 10nm vitamin D<sub>3</sub> or 10nm 1,25-D<sub>3</sub> for 24 hours, infected with *Pg* (MOI=100), and incubated for 24 hours or supplemented with additional 10nm vitamin D<sub>3</sub> or 1,25-D<sub>3</sub> and then incubated 24 hours. RNA was purified and RT-qPCR was then carried to observe expression levels of *Pg* 16s rRNA to show total bacterial load. Human 18s rRNA was used as a control. 16s rRNA expression was significantly lower with dual treatment in both vitamin D<sub>3</sub> (A) and 1,25-D<sub>3</sub> (B) treated cells when compared to cells only pre-treated with either. This may mean that vitamin D treatment has both active and passive/bolstering effects on antimicrobial activity and immunity that contribute synergistically. Statistics were performed using Student's t-test with a p-value of < 0.05 showing significance. Error bars represent standard error.

## CHAPTER 3 DISCUSSION OF RESULTS

The presence of cytochrome P450 enzymes within human gingival epithelium was successfully shown by RT-qPCR and PCR. While this does not explicitly mean that conversion is taking place, the presence of local conversion machinery means that there is a good chance for vitamin D<sub>3</sub> applied for treatment to be hydroxylated to 1,25-D<sub>3</sub>. If vitamin D<sub>3</sub> is transformed to 1,25-D<sub>3</sub>, it would mean that vitamin D<sub>3</sub> treatment alone could result in VDR and RXR binding that would facilitate the inflammatory regulation that occurs upon interactions with vitamin D response elements in specific genes. In the case of an inflammatory disease like periodontitis, this would mean vitamin D<sub>3</sub> may be effective local/topical treatment for symptom alleviation.

Within the RT-qPCR in figure 2-1, the insignificant difference in the levels of CYP2R1, CYP27A1, and CYP27B1 is to be expected, as the hydroxylation events that take place with the conversion of vitamin D<sub>3</sub> and 25-D<sub>3</sub> would not require additional machinery except to potentially speed up the reaction. While CYP2R1 and CYP27A1 levels are not affected by treatment, CYP27B1, as way to maintain calcium homeostasis, is known to be downregulated by Ca<sup>2+</sup> ions that result from increased vitamin D activity.<sup>16</sup> The upregulation of CYP24A1 levels in accordance with vitamin D treatment can be explained by the increased need to degrade active 1,25-D<sub>3</sub> to prevent further binding to VDR and unnecessary events of immune regulation in the case of excess 1,25-D<sub>3</sub>.<sup>16</sup> In the case of vitamin D<sub>3</sub> treated cells, the CYP24A1 upregulation would result from conversion of vitamin D<sub>3</sub> to 1,25-D<sub>3</sub> which would require the enzyme to degrade excess vitamin D. Samples in the future should also be treated with 25-D<sub>3</sub> to ensure similar data amongst all vitamin D treatment types.

Amplification of PCR products shows no quantifiable difference between EtOH, vitamin D<sub>3</sub>, and 1,25-D<sub>3</sub> treatments, but overall presence of gene products is observed in all RT+ treatment groups which shows the presence of RNA that would code for the enzymes in OKF6/Tert-1 cells. CYP27B1 bands are dark and thus levels are higher than the other enzymes, and this points to a tendency to focus on 1 $\alpha$ -hydroxylation and the importance of converting 25-D<sub>3</sub> to 1,25-D<sub>3</sub> to take place in VDR binding events for potential genetic regulation.<sup>43</sup> The comparatively low levels of the CYP27A1 and CYP2R1 enzymes, may be due to cells so far away from the normal site of 25-hydroxylation in the liver not requiring much if any at all of the 25-hydroxylase enzymes. This would also explain the higher levels of CYP27B1, as the gingival cells would have developed seeing vitamin D mainly in the serum 25-D<sub>3</sub> form upon cellular localization for conversion to active 1,25-D<sub>3</sub> following hydroxylation in the liver. Future study should excise and purify gel bands to send out for Sanger sequencing to ensure the observed bands were the proper amplicon sequences and thus the observed gene products. It is also of note that more specific primers must be developed to eliminate the 1 or 2 bands that are not specific to our target amplicon size and also to further lower amplification of genomic DNA with our primers.

The attempt to directly show conversion of vitamin D by radioimmunoassay in table 2-2 showed that treatment with 25-D<sub>3</sub> resulted in conversion to 1,25-D<sub>3</sub>, but treatment with vitamin D<sub>3</sub> did not show presence of 25-D<sub>3</sub>. The reasons for this are most likely because vitamin D<sub>3</sub> was fully converted to 1,25-D<sub>3</sub> in both the 6 and 24 hour treatments and there was no residual 25-D<sub>3</sub> left. A shorter timepoint (2 hours, 4 hours) may subvert these issues and allow 25-D<sub>3</sub> to be detected. Another thought and potential

explanation is that our treatment concentration of 10nm vitamin D<sub>3</sub> may be too close to the lower limit of detection (2.5 ng/mL) to be observed by the assay. In that case, we should raise our treatment level to 100nm concentration of vitamin D<sub>3</sub> and retest to see if vitamin D<sub>3</sub> can be detected. As our treatment samples are on both the upper and lower limits of the assay used, it is important to think of the results as more of a “presence” and “absence” of the detected compound to determine if conversion is happening but not truly quantify the extent of conversion. If vitamin D<sub>3</sub> does fully convert to 1,25-D<sub>3</sub>, as it was shown with the 25-D<sub>3</sub> treated cells, then it is good evidence of our hypothesis of vitamin D conversion taking place within human gingival epithelium. This would result in being able to potentially use over-the-counter vitamin D<sub>3</sub> as a topical treatment for periodontitis or other inflammatory diseases within the oral cavity. Future analysis should analyze vitamin D<sub>3</sub> treated cells for 1,25-D<sub>3</sub> concentration.

Assuming conversion is successful, it is also important to show the potential anti-inflammatory and/or antimicrobial effects of active 1,25-D<sub>3</sub> treatment within gingival epithelium. This has been shown by visualizing levels of pro-inflammatory cytokines following vitamin D treatment as well as cellular export levels of the antimicrobial peptide LL-37.<sup>72,173</sup> Antimicrobial activity can also be observed by testing the effects of vitamin D on the periodontal pathogen *Pg* in regards to cellular invasion and survival.

The decreased levels of pro-inflammatory cytokines in figure 2-3 as a result of vitamin D treatment in the cells that have undergone inflammatory stimulation are a direct example of the potential for vitamin D<sub>3</sub> to topically treat harmful inflammation. While the poly(I:C) used is not directly relevant to periodontal disease as it is a TLR3 agonist and not a TLR2 agonist, both pathways can lead to induction of inflammation

through NF- $\kappa$ B.<sup>180-182</sup> IL-6 and IL-8 were shown to be downregulated as in previous studies, but other pro-inflammatory cytokines such as IL-1B, IL-1A and TNF must be observed to see if on-site topical vitamin D treatment is truly responsible for alleviating harmful inflammation.<sup>83,86,87</sup>

Figure 2-4 continues to show the decrease of harmful inflammation, but within mice. The increased levels of CYP24A1 indicate that 1,25-D<sub>3</sub> is being utilized, but the cytokine is not upregulated in the vitamin D<sub>3</sub> treated mice which would have supported our working hypothesis and shown conversion to active vitamin D within mice as well. The significant downregulation of the pro-inflammatory cytokine IL-1A in mice treated with EtOH compared to 1,25-D<sub>3</sub> treated mice shows active vitamin D to be anti-inflammatory. The mice treated with vitamin D<sub>3</sub> also showed significant IL-1A downregulation which may further support vitamin D<sub>3</sub> as an anti-inflammatory treatment. Further mouse experiments should be expanded to include inflammatory stimulus prior to vitamin D treatment as well as viewing other cytokines such as IL-1B, TNF, IL-6, and IL-8.

The increase in the levels of the antimicrobial peptide LL-37 in the external media fraction of cells treated with vitamin D within figure 2-5 shows a potential increase in the release of antimicrobial peptides from cells to combat potentially pathogenic bacteria. This export of LL-37 from cells may function to fight against *Pg* in periodontitis, but it must be recognized that *Pg* is known to utilize peptides similar in form to LL-37 as a protein source.<sup>184</sup> The effects of direct application of LL-37 to *Pg* growth and survival must be observed in a future experiment to ensure that this upregulation of cellular antimicrobial activity is relevant to periodontitis.

Vitamin D treatment affects the survival and viability of *Pg* invasion as shown in figure 2-6. The CFU counts of viable *Pg* are reduced 2-3 times from treatment with 1,25-D<sub>3</sub> as both a pre-treatment prior to invasion as well as a treatment during potential invasion. This shows proper treatment with and/or maintaining sufficient levels of vitamin D may have a bolstering effect to immunity that can prevent *Pg* invasion as a pre-emptive deterrent within healthy individuals as well as when used as a topical treatment in those suffering from periodontitis. The differences in CFU counts between the pre- and concurrent treatment levels are most likely due to unintended differences in levels of viable *Pg* introduced for the invasion assay, but future studies will make an attempt to standardize the invasion assay further and only use living/replicating organisms. The difference between 1,25-D<sub>3</sub> and EtOH treatment and the effects on *Pg* colony viability are clear enough, but it remains to be seen whether vitamin D is acting as a direct or indirect antimicrobial on *Pg* so that will need to be researched in the future.

Another way of exploring the potential for vitamin D treatment of periodontitis with action on *Pg* is to measure *Pg* 16s rRNA levels for total bacterial load. Figure 2-7 attempts to perform this measurement as well as observe the synergy between pre-emptive immune bolstering with vitamin D treatment in a healthy cellular environment and treatment post-invasion of *Pg* when harmful inflammation would be present. *Pg* 16s rRNA levels are reduced following treatment with both vitamin D<sub>3</sub> and 1,25-D<sub>3</sub>, and this shows again that vitamin D<sub>3</sub> treatment may have an active effect that is nearly comparable to that of treatment with 1,25-D<sub>3</sub>. A good direction for 16s rRNA studies and a meaningful future experiment would be to directly correlate the levels of 16s rRNA to viable *Pg* colonies in a sample in order to be able to more easily quantify the effects of

vitamin D treatment on *Pg* without needing to re-plate and determine CFU values by colony counting.

While conversion from inactive to active vitamin D has been previously shown in gingival fibroblasts and ligament cells, it has never before been shown in gingival epithelium.<sup>37</sup> The data presented shows the potential for vitamin D conversion within human gingival epithelium, and thus a direct topical application of vitamin D<sub>3</sub> may be viable as an anti-inflammatory and anti-microbial treatment in periodontitis. The idea of a topical treatment brings many new challenges however, such as determining the necessary concentration with which to treat humans, how to ensure the treatment stays localized to the affected areas following application, or the potential for harmful side effects related to the direct application of a tightly regulated hormone to a single/specific bodily surface. The implications are great, but further study must be performed on the mechanics of potential conversion within the human oral environment as well as on the direct effects that vitamin D may have on invading periodontal pathogens and alleviation of harmful inflammation within human patients suffering from periodontitis.

What was once thought off as a simple regulator of bone deposition is now showing proof as an integral regulatory substance in immune function throughout the human body. With vitamin D proving itself as much more than a simple hormone produced in the skin, the field of vitamin D research is wide-open and continued studies will lead to the improved immune health of all suffering the harmful effects of vitamin D deficiency.

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## BIOGRAPHICAL SKETCH

William Ruddick was born in Hinsdale, Illinois and raised by his grandparents in Oak Forest, Illinois. He currently resides in Clermont, Florida with his wife Amy.

He completed his primary and secondary education while continuing to reside in Oak Forest and graduated from Oak Forest High School. He received his associate's degree in science at Moraine Valley Community College, in Palos Hills, Illinois, and his bachelor's degree in biology at Governor's State University, in University Park, Illinois. William worked for a short time as a laboratory chemist before deciding to further his education. He was admitted to the Interdisciplinary Program in Biomedical Sciences at the University of Florida in the fall of 2014.

His scientific fields of interest include immunology, microbiology, ecology, and ornithology. Outside of science, his interests include film, gaming, hunting, fishing, spelunking, skydiving, reading, theme parks, music, singing, crochet, cooking, board games, baseball, and golf.