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Bioavailability of Vitamin D and Impact of Supplementation on

Clinical and Inflammatory Outcomes in Cystic Fibrosis

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B.A. Covenant College 1999

M.S. University of Iowa 2001

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An abstract of

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ABSTRACT

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ABSTRACT

Cystic fibrosis (CF) is the most common, heritable disease that significantly shortens life expectancy among Caucasians in the United States. Morbidity and mortality in CF is primarily related to respiratory failure, the result of chronic pulmonary infection and inflammation.

Vitamin D insufficiency may affect up to 90% of individuals with CF and may be related to the high prevalence of fat malabsorption in CF. Vitamin D insufficiency has been associated with an increased risk for low bone mineral density, diabetes, respiratory infection, reduced lung function and inflammation; as well as, mortality. Therefore, repletion of vitamin D may impact the primary causes of morbidity and mortality in CF and should be evaluated.

The study of the clinical impact of vitamin D supplementation in CF has been complicated by the identification of a reliable vitamin D repletion strategy. We conducted a systematic review to determine whether the vehicle of the supplement impacted bioavailability both in CF and non-CF populations. We concluded that vehicle substance does not appear to have an impact on supplement bioavailability in non-CF subjects, and

there was inadequate research to determine whether vehicle substance impacts supplement bioavailability in CF subjects.

In a pilot study of vitamin D supplementation in CF, we randomized adults with CF, hospitalized for a pulmonary exacerbation, to either a high-dose, oral bolus of vitamin D in a non-lipid vehicle or placebo. Subjects were followed for up to 1-year and were evaluated for changes in vitamin D status, vitamin D toxicity, and clinical outcomes. We found a significant increase in vitamin D status; as well as, improved clinical outcomes in the vitamin D group compared to placebo without signs vitamin D toxicity. We also evaluated changes in markers of inflammation and the antimicrobial peptide, LL-37 for 12-weeks after randomization. We found a significant decrease in systemic concentrations of TNF-α and a trend for decreased IL-6 concentrations in the vitamin D group compared to placebo.

We have also shown that vitamin D supplementation in a non-lipid vehicle may improve clinical outcomes and markers of inflammation in CF.

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Above all, to my Lord Jesus Christ, thank you for creating such a wonderful world to study. "For by him all things were created, in heaven and on earth, visible and invisible, whether thrones or dominions or rulers or authorities–all things were created through him and for him. And he is before all things, and in him all things hold together" (Colossians 1).

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CHAPTER 1: INTRODUCTION

Approximately thirty years ago, vitamin D deficiency was a disorder considered to only be associated with diseases of bone mineralization. Since then, many additional diseases have been linked with vitamin D deficiency. At the same time, the prevalence of vitamin D deficiency has come to the attention of the scientific community and the public. In spite of this, the prevalence of vitamin D deficiency and the diseases associated with vitamin D deficiency are increasing in prevalence in the US population (1-3).

Vitamin D has been well-known for its importance in the development of bone-related disorders including rickets and osteomalacia. Recently, additional roles for vitamin D have been hypothesized from genetic and epidemiologic evidence. Genetic analysis has revealed that vitamin D may contribute to the regulation of 900 to more than 1100 genes in the human genome (4, 5). The vitamin D receptor and the enzymes that activate vitamin D have been isolated from immune cells, skeletal and cardiac muscle, brain tissue, respiratory epithelium, pancreatic β-cells, among many others (6, 7). There has also been epidemiologic data that correlates markers of vitamin D status with the risk for many diseases, including; autoimmune disease, cancer, infectious disease, and cardiovascular disease (8-10). At this point, the exact role of vitamin D in these disorders and the intake necessary to reduce risk are still unknown.

The causes of vitamin D deficiency include dietary and lifestyle factors, as well as, physiologic factors (11-13). The US diet includes few vitamin D containing foods and US lifestyle habits have reduced sun exposure which together contribute to inadequate vitamin D status among Americans (3). In addition, physiologic factors common in the

US population such as obesity, age, and chronic conditions, increase the risk for vitamin D deficiency (14-16).

It is important to evaluate the bioavailability of supplemental vitamin D formulations, since a large proportion of vitamin D intake in the US is from fortified foods and supplements (17). There have been a large number of manuscripts addressing the impact of the form of vitamin D on supplement bioavailability, but few have addressed the issue of the vehicle of the supplement (18-21). Vehicle may impact bioavailability to a greater extent in individuals with disorders that impact nutrient absorption (22, 23).

Cystic fibrosis (CF) is a life-shortening disease that occurs in approximately 1:3000 live births in the United States. It is caused an inherited mutation of the CF transmembrane conductance regulator gene that disrupts the function of many systems throughout the body (24, 25). The majority of the morbidity and mortality in CF is due to progressive lung disease that results in respiratory failure (26). This lung disease is characterized by chronic pulmonary infection and inflammation that leads to lung damage and respiratory failure. Vitamin D has been shown to reduce systemic inflammatory markers and their release from respiratory epithelium, as well as increase the production of antimicrobial peptides (27-29)

The CFTR mutation also causes macronutrient malabsorption. Reduced absorption of lipids may result in deficiencies of the lipid soluble nutrient, vitamin D. The prevalence of vitamin D insufficiency has been estimated to be from 70%-100% and the best method for vitamin D repletion continues to be debated (19, 30-35). Over the past ten years, the CF Foundation has updated their recommendations for vitamin D repletion

two times. The most recent recommendations will be published in March 2012 and have not yet been evaluated. A reliable method of vitamin D repletion is necessary to allow studies to evaluate the long term impact of vitamin D supplementation in CF.

Therefore, the primary focus of this dissertation is to evaluate the current literature regarding the impact of supplement vehicle on vitamin D bioavailability and to evaluate the impact of a single, bolus dose of cholecalciferol on vitamin D status, clinical outcomes and inflammatory markers in adults with CF.

The *central hypothesis* for this body of work is that the vehicle substance may contribute to the bioavailability of vitamin D supplements and that vitamin D supplementation of CF adults during pulmonary exacerbation will improve clinical outcomes and produce anti-inflammatory changes in markers of inflammation. There are three sub-hypotheses that were evaluated in this thesis project.

The *first hypothesis* of this thesis is that vitamin D supplements have equal bioavailability independent of the supplement vehicle in healthy subjects and that vitamin D supplements will have greater bioavailability in subjects with fat malabsorption when the primary substance of the supplement vehicle is non-lipid. The rationale for this hypothesis is that the absorption and bioavailability of vitamin D from a lipid vehicle supplement is reduced in subjects with fat malabsorption compared to healthy subjects. This hypothesis may explain the difficulty that has been encountered in reliably increasing the vitamin D status of individuals with nutrient malabsorption, such as those with CF.

The *second hypothesis* is that vitamin D supplementation during a CF pulmonary exacerbation will increase vitamin D status and improve clinical outcomes. The rationale

for this hypothesis is that adequate vitamin D status is associated with higher lung function in healthy persons and in persons with CF. Thus, improving vitamin D status may help improve recovery of lung function during a pulmonary exacerbation and improve clinical outcomes.

The *third hypothesis* is that vitamin D supplementation will reduce serum concentrations of inflammatory markers and increase serum concentrations of the anti-microbial peptide, LL-37. The rationale of this hypothesis is that vitamin D supplementation has been demonstrated to reduce serum inflammatory cytokine concentrations in clinical trials, as well as in *in vitro* analyses. Vitamin D status has been positively correlated with serum concentrations of LL-37 and vitamin D supplementation has been found to increase the production of LL-37 in isolated immune and respiratory cells *in vitro*. Therefore, improving vitamin D status may reduce concentrations of inflammatory markers and increase LL-37 in individuals with CF during a pulmonary exacerbation.

Specific Aim 1: Determine the impact of the vehicle substance on the bioavailability of vitamin D contained in supplements in healthy subjects and in subjects with cystic fibrosis-related fat malabsorption.

The approach for this aim was a comprehensive literature search that identified manuscripts that directly compared the bioavailability of vitamin D from supplements with different vehicles. The hypothesis of this aim is that in healthy subjects, supplement vehicle has no impact on the bioavailability of vitamin D. However, in subjects with CF-related fat malabsorption, vitamin D will have greater bioavailability from a non-lipid vehicle compared to a lipid vehicle.

Specific Aim 2: Evaluate the impact of a single, oral bolus of cholecalciferol on vitamin D status and clinical outcomes in adults with CF.

The approach for this aim was a double-blinded, controlled clinical trial that randomized adults with CF to either a single bolus dose of cholecalciferol or placebo. Serum 25(OH)D, PTH and calcium concentrations were assessed at baseline, 1-week and 12-weeks. Clinical outcomes were evaluated up to a year post-randomization.

Specific Aim 3: Evaluate the impact of cholecalciferol supplementation on circulating concentrations of inflammatory markers and the anti-microbial peptide, LL-37 in adults with CF.

The approach of this aim was a randomized, controlled clinical trial that randomized adults with CF to either a single bolus dose of cholecalciferol or placebo. Serum 25(OH)D, PTH and calcium concentrations were assessed at baseline, 1-week and 12-weeks. Clinical outcomes were evaluated up to a year post-randomization.

CHAPTER 2: LITERATURE REVIEW

VITAMIN D

Vitamin D: History, structure and metabolism

History of discovery

Physical descriptions of the disease that is now known as rickets date back to early Roman, Chinese and Greek writings (36). The disease of rickets was first described in Great Britain in 1645 by the physician, Daniel Whistler, but it was not until the late 1800's that the occurrence of rickets was associated with nutrition (37). Jules Guérin (1838) produced rickets in puppies by removing them from their mother's milk and keeping them indoors. He concluded that a nutritional deficiency caused rickets (38).

Influenced by Guérin's work, Armand Trousseau also determined that rickets was a nutritional deficiency. He recommended treatment with cod liver oil and sunlight. Trousseau also identified osteomalacia as an adult form of rickets (38). In 1890, Theobald Palm described the geographical distribution of rickets and concluded that the prevalence of rickets was inversely associated with the amount of sunlight the region received (39).

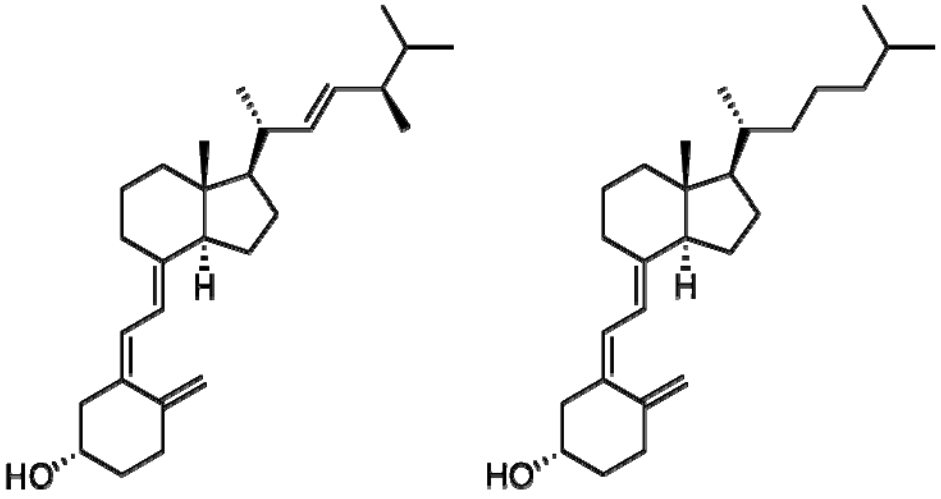
Kurt Huldshinsky determined that the UV spectrum of light was able to prevent rickets and used mercury vapor quartz lamps as therapy and prevention of rickets (39). In 1919, Sir Edward Mellanby rediscovered the anti-rachitic activity of cod liver oil and proposed that this was due to the vitamin A content of the oil (40). However, Elmer McCollum, heated cod liver oil to destroy the vitamin A and found that the anti-rachitic

activity was not destroyed. He isolated the nutrient and named it vitamin D (41). Since then, it has become accepted the vitamin D deficiency is the most common cause of rickets and osteomalacia.

Structure, synthesis and forms

Vitamin D is a secosteroid and is related to the other steroid hormones, such as estrogen, progesterone and testosterone (42). In the epidermis, endogenous 7-dehydrocholesterol is converted to pre-vitamin D when UVB breaks (290-310 nm) the β-ring of the cholesterol structure. Vitamin D is formed through thermal rearrangement of the pre-vitamin. The maximal concentration of pre-vitamin D in the skin is reached with a few hours of sun exposure, after which pre-vitamin D is reversibly converted into two inactive vitamin D compounds, lumisterol or tachysterol. This provides a natural limit on the amount of vitamin D produced (43).

There are several forms of vitamin D, but the two that are most biologically relevant are cholecalciferol (vitamin D_3) and ergocalciferol (vitamin D_2) (Figure 1). These two forms differ in their side chains; ergocalciferol contains a double bond (C22-23) and an additional methyl group attached to C24. Physiologically, they are recognized by the same enzymes and receptors; and overall their metabolism is very similar (42). Cholecalciferol is synthesized in the epidermis and can be obtained from a limited number of animal food sources in the diet such as salmon, cod liver oil, and egg yolks. Ergocalciferol is primarily obtained from mushrooms that are irradiated to convert naturally occurring ergosterol to ergocalciferol (44). Both forms can be found in supplements and fortified foods, although the introduction of cholecalciferol fortification is more recent than ergocalciferol.



Ergocaciferol Cholecaciferol

Figure 1. Structures of the two major forms of vitamin D: Ergocalciferol and cholecalciferol

Bioavailability of vitamin D

After synthesis in the skin, vitamin D enters the circulation and binds to the vitamin D binding protein (DBP, also referred to as $G_c$globulin) or albumin with high affinity leaving only a small portion of the vitamin D unbound (45). DBP binds 85-88% and albumin binds 12-15% of the vitamin D metabolites (46). Only a small proportion, approximately 2%, of DBP is associated with vitamin D and its metabolites; therefore DBP has other functions such as scavenging actin released from cells and activation of macrophages and osteoclasts (47). Blood concentrations of vitamin D increase slowly with cutaneous synthesis, peaking at 24 hours after UVB exposure and returning to pre-exposure concentrations at approximately seven days. (45).

Absorption of vitamin D from the gastrointestinal tract is dependent on its incorporation into mixed micelles. Increased micelle formation increases the absorption of vitamin D; and vitamin D supplements have often been formulated using a lipid vehicle to increase absorption (48). A large percentage of vitamin D that is absorbed from the digestive tract is initially associated with chylomicrons rather than DBP; and inhibition of chylomicron formation reduces vitamin D absorption by more than 40% (49-51). Over time, vitamin D in the circulation becomes more associated with DBP or enters hepatocytes to be metabolized.

Oral vitamin D produces an increase in blood concentrations of vitamin D more quickly than cutaneous synthesis, reaching peak concentrations at 10 hours and returning to baseline by approximately 2 days (45). Uptake of vitamin D by hepatocytes is impacted by the method by which vitamin D is transported in the blood and reaches the liver. Vitamin D transported in chylomicrons is more rapidly taken up by hepatocytes than vitamin D transported by DBP. Therefore, the source of vitamin D, cutaneous versus dietary, will impact the kinetics of vitamin D metabolism and blood concentrations of vitamin D (45).

Metabolism of vitamin D

Vitamin D is transported first to the liver where it is hydroxylated at the C25 to form 25-hydroxyvitamin D (25(OH)D); this structure was first isolated by Hector DeLuca in 1968 (52). This reaction may be catalyzed by either Cyp2R1 or Cyp27A1. These enzymes are considered high capacity, therefore the conversion of vitamin D into 25(OH)D is non-rate-limiting (53). Cyp27A1 is able to hydroxylate vitamin D at the 24, 25 and 27 carbons. Hydroxylation at the 24 position increases the probability that the

molecule will be catabolized for excretion. Hydroxylation at C25 is a step in the activation pathway of vitamin D to the hormonally active form, $1,25(OH)_2D$. There is some evidence that vitamin D_2 may be preferentially 24-hydroxylated rather than 25-hydroxylated, causing increased excretion and reducing the bioavailability of vitamin D_2 supplements. In contrast, a greater proportion of vitamin D_3 may be 25-hydroxylated to 25(OH)D, which serves as the substrate for the synthesis of $1,25(OH)_2D$, the active form of vitamin D. These differences in hydroxylation may contribute to differences in the biologic activity of the two forms (54).

25(OH)D is hydroxylated at the 1α-carbon to form 1α,25-dihydroxyvitamin D ($1,25(OH)_2D$) by Cyp27B1, a mitochondrial mixed-function oxidase. This metabolite is the most biologically active metabolite of vitamin D (55). Cyp27B1 is primarily found in the kidneys, specifically the renal tubules. However, it is distributed throughout the body in many cell types, such as macrophages, respiratory epithelium, bone, placenta, brain, heart, testes and intestine (56-58). Regulation of Cyp27B1 in the kidney is by parathyroid hormone (PTH), FGF23, $1,25(OH)_2D$, calcium and phosphate (59). Extra-renal regulation of Cyp27B1 and the synthesis of $1,25(OH)_2D$ takes place by a different mechanism than in the kidney. For, example in macrophages and monocytes, activation of the toll-like receptor (TLR) pathway increases the transcription of Cyp27B1, thereby increasing the cellular production of $1,25(OH)_2D$ (42, 60, 61).

Another important reaction which metabolites of vitamin D may undergo is 24-hydroxylation. The enzyme, Cyp24A1 catalyzes the conversion of 25(OH)D to $24,25(OH)_2D$, or $1α,25(OH)_2D$ to $1α,24,25(OH)_3D$. Cyp24A1 is primarily found in the kidney, but similar to Cyp27B1, has a wide distribution in the body (42). 24-

hydroxylation initiates a series of catabolic events that inactivate vitamin D metabolites. Cyp24A1 preferentially hydroxylates 1α-hydroxylated vitamin D metabolites, specifically 1α,25$(OH)_2$D. This acts as an important regulator of the activity of 1α,25$(OH)_2$D. PTH and 1α,25$(OH)_2$D are the primary regulators of Cyp24A1 in the kidney. PTH decreases the expression of Cyp24A1 in order to maintain 1α,25$(OH)_2$D concentrations in the circulation and increase calcium absorption. 1α,25$(OH)_2$D acts to inhibit the expression of Cyp24A1 by binding to vitamin D receptor elements (VDRE) in its promoter region (62).

Genomic and non-genomic actions

Genomic actions of vitamin D

1α,25$(OH)_2$D acts a transcriptional regulator in the genomic action of vitamin D. 1α,25$(OH)_2$D binds to vitamin D receptors that translocate to the nucleus where they bind DNA motifs to modify gene transcription. The intracellular vitamin D receptor (VDR) functions as a heterodimer with the retinoic acid receptor. This complex enters the nucleus and binds to specific DNA sequences, known as VDR elements (VDRE).

VDREs have been found in hundreds of genes throughout the human body (4, 63). 1α,25$(OH)_2$D may function to activate or repress the transcription of its target genes. In order to exert these actions on DNA transcription, 1α,25$(OH)_2$D requires a co-regulator, either a co-activator or co-repressor. These co-regulators are tissue specific and provide a level of tissue specificity on the activity of vitamin D as a transcriptional regulator.

Non-genomic actions of vitamin D

$1,25(OH)_2D$ changes cell function through regulation of gene transcription as described above; and $1,25(OH)_2D$ also transduces vitamin D signaling through membrane-bound VDRs. These VDRs have been found in osteoblasts, liver, muscle and the intestine (64, 65). Associated with caveolae-enriched areas of the plasma membrane, these membrane-bound VDRs activate tyrosine phosphorylation cascades (66, 67). Some of the actions of vitamin D on brush border cells of the small intestine to increase calcium absorption are transduced in this way (68). The non-genomic activities of vitamin D are produced through activation of a G-protein coupled signal cascade that includes phospholipase C activation, diacyl glyceride, and inositol triphosphate; leading to release of endoplasmic recticulum calcium stores and increased intracellular calcium concentrations (67, 69).

Functions of Vitamin D

Calcium metabolism

Disorders of bone metabolism prompted the discovery of vitamin D; therefore, the regulation of calcium from the small intestine is known as the classical function of vitamin D. Adequate vitamin D status increases the amount of dietary calcium absorbed by the small intestine from 10% to 30% (44). Active vitamin D, $1,25(OH)_2D$ impacts three aspects of calcium absorption in intestinal epithelial cells. First, $1,25(OH)_2D$ increases the expression of calcium channels in the apical membrane of the intestinal epithelium which allows calcium to move down its electrochemical gradient into the cell. Second, it increases the expression of calbindins, calcium binding proteins that facilitate the transport of calcium through the cell. Third, $1,25(OH)_2D$ increases the function of the

CaATPase that transports calcium across the basolateral membrane and into the circulation. $1,25(OH)_2D$ also increases serum calcium concentrations through stimulating osteoclast activity to increase bone resorption and by inhibiting calcium excretion in the distal tubules of the kidney (70).

Hormone secretion

$1,25(OH)_2D$ participates in regulating the secretion of several hormones. These include hormones that help regulate bone mineralization and blood glucose concentrations. The role of vitamin D in bone metabolism includes the regulation of PTH and fibroblast growth factor (FGF) 23. Although serum ionized calcium concentrations are the main regulator of PTH secretion through the parathyroid calcium receptor, $1,25(OH)_2D$ also regulates PTH gene transcription (71)(Silver, Mayer 1986). However, $1,25(OH)_2D$ regulation of PTH transcription can be superseded by hypocalcemia which may down-regulate the expression of the VDR in the parathyroid, thereby allowing serum calcium concentrations to be maintained in the presence of normal concentrations of $1,25(OH)_2D$ (72).

$1,25(OH)_2D$ regulates the production of FGF23 from osteocytes. $1,25(OH)_2D$ increases the synthesis and secretion of FGF23, which increases the excretion of phosphate through the kidney (73). In turn, FGF23 suppresses the production of 1,25(OH)D from the kidney. Imbalances in this system have been linked to rickets and/or osteomalacia (74, 75).

$1,25(OH)_2D$ also has a role in regulating the release of insulin. Epidemiologic studies have linked insufficient vitamin D status with an increased risk for type I and type II diabetes (76, 77). The VDR is expressed in the beta cells of the pancreas that produce

insulin and *in vitro* treatment with $1,25(OH)_2D$ increases the synthesis of insulin (78, 79). In adults at risk for type II diabetes, vitamin D supplementation has been associated with improved β cell function (80).

Proliferation and differentiation

$1,25(OH)_2D$ has a role in regulating the cell proliferation and differentiation. Vitamin D status has been inversely correlated with risk for some types of cancer and this protective effect has been associated with the regulation of cell proliferation, differentiation and apoptosis by $1,25(OH)_2D$ (81). Vitamin D supplementation has been shown to increase cell differentiation in adenoma patients (82). The VDRE has been found directly or indirectly associated with genes that regulate cell cycling in many tissues (83).

Immune function

The VDR and the 1α-hydroxlyase that activates 25(OH)D is expressed in many cell types throughout the immune system (79). Vitamin D insufficiency has been linked to an increased risk of auto-immune and infectious diseases (84). The role of vitamin D in the innate and adaptive immune systems is discussed below.

Innate immunity

In the innate immune system, adequate $1,25(OH)_2D$ increases the production of antimicrobial peptides, such as cathelicidin and the β-defensins. In the case of LL-37, $1,25(OH)_2D$ regulates transcription of the hCAP18 gene; and the peptide produced is then cleaved to form LL-37 (85, 86). TLR activation in monocytes and macrophages increases the synthesis of the VDR and 1α-hydroxylase, leading to an increase in the intracellular concentration of the active form of vitamin D, $1,25(OH)_2D$. The increased concentrations

of the VDR and $1,25(OH)_2D$ upregulate the transcription of hCAP-18, (87-90). $1,25(OH)_2D$ also upregulates the transcription of a second antimicrobial peptide, defensin-β2 (91). Therefore, $1,25(OH)_2D$ is important for the production antimicrobial peptides and supports innate immunity through this function.

Vitamin D also supports the innate response of macrophages and epithelial cells to antigen. There is evidence that $1,25(OH)_2D$ regulates the expression of pattern recognition receptors, such as NOD2. By increasing the expression of these receptors on monocytes and epithelial cells, $1,25(OH)_2D$ increases the sensitivity of these cells to bacterial derived antigens (92). $1,25(OH)_2D$ also stimulates the chemotactic, phagocytic and oxidative burst responses of macrophages, thereby increasing the innate response of macrophages to antigens (88, 93). Therefore, $1,25(OH)_2D$ increases the innate immune response to antigen by increasing the function of innate immune cells and the production of antimicrobial peptides.

In the clinical setting, vitamin D has been associated with increased concentrations of LL-37. Systemic concentrations of LL-37 in septic patients were positively associated with 25(OH)D in an ICU setting (94). Peripheral blood mononuclear cells produced greater concentrations of hCAP-18 when treated with $1,25(OH)_2D$ or when isolated from subjects treated with high dose vitamin D therapy compared to those left untreated (87). Vitamin D supplementation increases neutrophil concentrations of LL-37 in neonates (95). UVB therapy increased 25(OH)D concentrations in subjects with psoriasis and increased the concentrations of LL-37 in skin lesions (96). These results indicate the vitamin D supplementation may improve *in vivo* production of LL-37.

Adaptive Immunity

Vitamin D is also an important modulator of the adaptive immune response. Activation of a dendritic cell (DC) increases the production of T-cell stimulating molecules and cytokines, resulting in a Th1 phenotype. Excess activation of the Th1 phenotype increases systemic inflammation that can cause damage in Th1 dominant chronic diseases (97). $1,25(OH)_2D$, inhibits the maturation of DC and maintains a more tolerogenic, Th2 phenotype (97, 98). $1,25(OH)_2D$ reduces the expression of markers of DC activation, including MHC-II, co-stimulatory molecules such as CD40, and surface markers of DC maturation (84). $1,25(OH)_2D$ also inhibits the DC production of IL-12 and IL-23, cytokines that favor the Th1 phenotype, while increasing the release of IL-10, an anti-inflammatory cytokine (99). In the presence of adequate $1,25(OH)_2D$, DC are less likely to stimulate an exaggerated immune response.

In T-cells, $1,25(OH)_2D$ reduces the expression of markers related to activation in a way that is similar to the changes found in the DC (100). In T-cells, $1,25(OH)_2D$ also inhibits the expression of pro-inflammatory cytokines, such as IL-1, IL-6, TNF-α, IL-8, and IL-12 (101-103). There are several mechanisms whereby $1,25(OH)_2D$ may suppress the production of inflammatory cytokines, including the NFκB pathway, the Rho/ROCK and the p38-MAPK pathways (104-106). It is well established that $1,25(OH)_2D$ inhibits NFκB from increasing the transcription of pro-inflammatory proteins by increasing the phosphorylation of IκBα. This pathway also limits inflammation *in* vivo. In middle-aged adults, 25(OH)D concentrations were inversely associated with NFκB activation and concentrations of IL-6 (107). $1,25(OH)_2D$ also blocked the NFκB-linked increase of inflammatory markers produced in airway epithelium in response to respiratory syncytial

virus (28). Recent studies have found that $1,25(OH)_2D$ is able to activate the RhoA/ROCK (rho associated coiled-kinase) pathway, inhibiting inflammation and cell proliferation(105). Also, $1,25(OH)_2D$ may inhibit the production of IL-6 and TNF-α from macrophages through inhibition of LPS induced phosphorylation (activation) of p38. $1,25(OH)_2D$ upregulates mitogen activating protein kinase (MAPK) phosphatase-1 which inhibits p38 phosphorylation. Therefore, vitamin D modifies several pathways to limit the production of inflammatory messengers.

Several clinical trials have found that vitamin D supplementation may reduce concentrations of inflammatory markers (108, 109). In subjects with chronic kidney disease, 50,000 IU of vitamin D per week reduced serum concentrations of IL-8, IL-6 and TNF-α (29). Vitamin D supplementation of 2000 IU/day reduced TNF-α concentrations in patients with cardiovascular disease, in a double-blind, randomized, placebo-controlled trial (110). $1,25(OH)_2D$ reduced the concentration of inflammatory cytokines, TNF-α, IL-6, IL-1, and IL-8, produced by monocytes isolated from type 2 diabetic subjects (103). Not all studies have shown reductions in circulating concentrations of inflammatory markers, although these studies used lower doses of vitamin D or found inconsistent results on the impact of vitamin D supplementation on pro and anti-inflammatory markers (111, 112). Evaluation of the impact of vitamin D supplementation on inflammation and clinical outcomes must continue.

Evaluating and correction vitamin D status

Definition of vitamin D status and vitamin D intake

Serum 25(OH)D concentrations from venous blood greater than 20 ng/ml are considered adequate by the IOM and the American Academy for Pediatrics to prevent

vitamin D related bone disorders (113). The Endocrine Society defines deficiency as 25(OH)D < 20 ng/ml and insufficiency as 20-29 ng/ml (114). The maximum concentration of 25(OH)D considered safe is 100 ng/ml (11).

The 2010 IOM recommendations for vitamin D intakes set the RDA for healthy adults at 600 IU. For older adults over the age of 70 years, the RDA was established at 800 IU. The tolerable upper limit for children <1 year is daily intake of 400 IU, for all individuals >1 year is 4000 IU. These recommendations were based on the available evidence for health outcomes. Due to the lack of research into the impact of vitamin D on outcomes other than bone health, such as density and risk of fracture, the IOM committee recommendations were based on the vitamin D intake that has been shown to reduce the risk for reduced bone density and bone fracture(113). The IOM recommended lower intakes of vitamin D than suggested by some researchers in the vitamin D field, including those from the Endocrine Society (114-116).

The Endocrine Society evaluated requirements for vitamin D based on the intake required to reach 30 ng/ml. The Society chose this outcome based on the serum 25(OH)D concentrations necessary to suppress the production of parathyroid hormone and optimize calcium absorption (114). Evidence suggests that PTH concentrations are negatively correlated with 25(OH)D concentrations until 25(OH)D reaches a concentration of approximately 30 ng/ml (117). Although the recommendations for intake differ, it is agreed that the 25(OH)D cutoff for vitamin D deficiency is <20 ng/ml (Table 1).

Table 1 Recommended Dietary Allowances for Vitamin D

Recommended Dietary Allowances (RDAs) for Vitamin D**

	IOM		Endocrine Society	
Age	Daily Requirement	Tolerable Upper Limit	Daily Requirement	Tolerable Upper Limit
0–12 months*	400	400	400-1000	2000
1–18 years	600	600	600-1000	4000
19–70 years	600	600	1500-2000	10,000
>70 years	800	800	1500-2000	10,000

*Adequate Intake (AI)

**Adapted from the Office of Dietary Supplements Dietary Fact Sheet (February 27, 2012, http://ods.od.nih.gov/factsheets/VitaminD-HealthProfessional) and the Endocrine Society Clinical Practice Guidelines(118)

Evaluation of vitamin D status

Several methods for evaluating vitamin D status have been proposed. The three most often discussed are vitamin D, 25(OH)D, and 1,25 $(OH)_2$D. Vitamin D itself, has a relatively short half-life in the circulation, 2-3 days, and therefore blood concentrations only indicate vitamin D intake or cutaneous synthesis in the previous few days (57). 1,25$(OH)_2$D has a short half-life as well, approximately 4 hours, and concentrations may fluctuate diurnally. Also, the conversion of 25(OH)D to 1,25$(OH)_2$D is much more regulated than the conversion of vitamin D to 25(OH)D. There are several factors that regulate the synthesis of 1,25$(OH)_2$D including PTH, calcium, and FGF23. Therefore, measuring 1,25$(OH)_2$D may only be a surrogate for the concentration or activity of the compounds that regulate the synthesis of 1,25$(OH)_2$D. Further 1,25$(OH)_2$D concentrations are maintained even when 25(OH)D concentrations are decreased, thereby masking deficiency (119). Because of the many factors that regulate 1,25(OH)D and its changing concentrations in the serum, it is a poor choice as a marker of vitamin D status.

Serum 25(OH)D is the generally accepted method of evaluating vitamin D status. Cyp27A1, the enzyme that converts of vitamin D to 25(OH)D, is a high efficiency enzyme that does not appear to be highly regulated(54). Further, 25(OH)D has a long half-life, 2-3 weeks. Because of this half-life, the serum levels of 25(OH)D do not fluctuate rapidly and concentrations are reproducible within individuals over short periods of time. Finally, 25(OH)D concentrations have been shown to be associated with health outcomes and is therefore the preferred marker when evaluating vitamin D status (9, 115).

Prevalence of vitamin D deficiency and insufficiency

Estimates of the prevalence of vitamin D deficiency and insufficiency in the US population and around the world range from 20—100% (11). In the US, an estimated 42% of Black American women of reproductive age are vitamin D deficient. Others have estimated that 57% of general medicine patients are deficient and 32% of healthy, free-living adults (12, 120, 121). Vitamin D deficiency impacts a considerable proportion of our population.

The prevalence of vitamin D deficiency and insufficiency is increasing in the US population. The overall prevalence of vitamin D deficiency (25(OH)D < 10 ng/ml) in the NHANES 2001-2004 was 14% and of vitamin D insufficiency (25(OH)D 10 - 30 ng/ml) was 71%; therefore, only 23% of the US population had adequate 25(OH)D concentrations (≥ 30 ng/ml) (1). The greatest increases in severe vitamin D deficiency from NHANES III (1988-1994) to the 2001-2004 NHANES have been in the non-Hispanic black population. The prevalence of adequate vitamin D status in non-Hispanic

blacks is only 3%, and the prevalence of vitamin D deficiency and insufficiency are 29% and 68% respectively (1).

The vitamin D status of the US population had decreased over the past 25 years; and the prevalence of vitamin D related morbidities has increased. Although more study must be done to determine the 25(OH)D concentrations and the necessary intake to prevent chronic disease morbidity and mortality, it is apparent that vitamin D nutriture is an important aspect of health and disease in the US today.

Causes of vitamin D deficiency and insufficiency

Vitamin D status usually follows the seasons of the year and climate. During the winter months and in regions with fewer sunny days, 25(OH)D concentrations are lower than in the summer months and in sunnier regions (122, 123). Also, vitamin D insufficiency is primarily due to reduced UVB exposure either because of lifestyle or season. People who have regular UVB exposure, such as those who regularly use tanning beds, have higher serum 25(OH)D concentrations than those who do not (124, 125). Changes in the habits of US citizens have contributed to the reduction in UVB exposure. Sun-avoidance behavior, as recommended to reduce the risk of skin cancers, has reduced UVB exposure. This includes, avoiding the sun at the times of the day when cutaneous vitamin D is most efficient (10AM-2PM), use of sunscreens, clothing and other shade devices to avoid direct exposure to the sun (120). Other behaviors that contribute to vitamin D insufficiency are increased time spent indoors and decreased exercise and play habits that take place outdoors. The unintended consequence of this is a reduction in cutaneous vitamin D synthesis and an increase in the prevalence of vitamin D insufficiency and deficiency. In the non-Hispanic black population, the high prevalence

of vitamin D deficiency and insufficiency may be related to increased skin pigmentation, which has been associated with reduced UVB conversion of 7-DHC to pro-vitamin D (126, 127).

The prevalence of obesity and overweight has increased in the US population as vitamin D status has decreased. Individuals with a greater percentage of body fat compared to those with a normal percentage, exhibit an up to 50% lower 25(OH)D response to an equal dose of vitamin D or UVB (14, 128). Also, excess adipose tissue may sequester vitamin D, thereby reducing the impact of a given dose on vitamin D status (129). Therefore, the high prevalence of overweight and obesity may be contributing to the high prevalence of vitamin D insufficiency (14, 128).

The vitamin D intake of the US population does not meet the estimated average requirement (EAR) recommendations for vitamin D intake. In the US, 68% of adults and 73% children (NHANES 2003-2006) do not meet recommendations (17). More than half of the oral vitamin D intake for the US population (≥ 2 years of age) is met by supplements and more than 1/3 is from fortified foods (17). The limited intake of vitamin D containing foods and supplements has contributed to the prevalence of vitamin D insufficiency in the US (130).

As individuals age, there is decreased cutaneous synthesis of vitamin D that may increase the risk of vitamin D insufficiency. Older adults in long-term care facilities are also at increased risk for vitamin D insufficiency. This may be related to reduced exposure to UVB, decreased cutaneous synthesis and reduced dietary intake and absorption (16, 131-133).

Dietary sources

Naturally occurring sources of vitamin D are limited. The primary source of 25(OH)D is cutaneous synthesis (43). Selected food sources of vitamin D in the US diet and the average number of IUs of vitamin D per serving are noted in Table 2.

Table 2 Selected Food Sources of Vitamin D

Selected Food Sources of Vitamin D*

	Food	IUs per serving
Fatty Fish	Swordfish, cooked, 3 ounces	566
	Salmon (sockeye), cooked, 3 ounces	447
	Tuna fish, canned in water, drained, 3 ounces	154
	Sardines, canned in oil, drained, 2 sardines	46
Animal foods	Liver, beef, cooked, 3 ounces	42
	Egg, 1 large (vitamin D is found in yolk)	41
Fortified foods	Orange juice fortified with vitamin D, 1 cup (check product labels, as amount of added vitamin D varies)	137
	Milk, nonfat, reduced fat, and whole, vitamin D-fortified, 1 cup	115-124
	Yogurt, fortified with 20% of the DV for vitamin D, 6 ounces	80
	Margarine, fortified, 1 tablespoon	60
	Ready-to-eat cereal, fortified with 10% of the DV for vitamin D, 0.75-1 cup	40
	Mushrooms, some varieties when exposed to UV light	Variable

*Adapted from the Office of Dietary Supplements Dietary Fact Sheet (February 27, 2012, http://ods.od.nih.gov/factsheets/VitaminD-HealthProfessional)

Vitamin D repletion

Correction of vitamin D status is may be achieved through exposure to sunlight or through oral supplementation; however, intramuscular injection of vitamin D has not been found to be an effective method for repletion (132, 134). The dose of vitamin D

necessary to correct vitamin D status is dependent on a number of factors, including the initial serum 25(OH)D concentrations, malabsorptive disorders and adiposity (32, 129, 135). Individuals with lower 25(OH)D concentrations will more efficiently convert oral vitamin D to 25(OH)D, but still require larger doses to regain an adequate 25(OH)D concentration (3, 13). A vitamin D dose of 100 IU will produce an mean increase in serum 25(OH)D concentrations of 1 ng/ml in healthy individuals (135, 136). In individuals with difficult to correct deficiency, 50,000 IU of vitamin D once a week for eight weeks may be administered to correct vitamin D status (137). Alternative methods, such as UVB light, may be useful in individuals with malabsorptive disorders (124, 138).

Vitamin D and health outcomes

Health outcomes related to vitamin D nutriture

Hypertension, myocardial infarction, and stroke, as well as other cardiovascular-related diseases, such as diabetes, congestive heart failure, peripheral vascular disease, atherosclerosis, and endothelial dysfunction have been associated with vitamin D deficiency and insufficiency (2, 139, 140). One mechanism by which vitamin D sufficiency may impact the risk for these outcomes is the reduction of pro-inflammatory markers. Increased inflammation has been linked to poor outcomes in CVD, diabetes, peripheral vascular disease and endothelial dysfunction (141). $1,25(OH)_2D$ has been shown to reduce activation of the renin-angiotensin pathway as well as improve the flow augmentation rate, which may reduce risk of hypertension (142, 143).

Risk of type 1 and type 2 diabetes mellitus have been linked to vitamin D deficiency (80). Vitamin D supplementation in early childhood or during pregnancy has been associated with a reduced risk for type 1 diabetes (144, 145). Observational studies

have indicated that vitamin D insufficiency may increase the risk of impaired glucose tolerance and for developing type 2 diabetes (77, 146, 147).

Vitamin D deficiency rickets and osteomalacia have been linked with low 25(OH)D concentrations that reduce the absorption of calcium from the GI tract (135, 148). Osteoporosis is also linked to vitamin D deficiency and reduced calcium absorption (70, 149). $1,25(OH)_2D$ may directly stimulate bone mineralization by suppressing osteoblast apoptosis while stimulating bone turnover (150).

The link between vitamin D and autoimmune disease risk was first described as an association between sun exposure and autoimmune disease prevalence. Vitamin D deficiency has been associated with increased risk of rheumatoid arthritis, multiple sclerosis, thyroiditis and inflammatory bowel disease (10). A well-known example is multiple sclerosis (MS). The relationship between northern latitudes, reduced sun exposure and an increased risk for MS was first described in US WWII veterans (151, 152). It is believed that vitamin D alters the risk for autoimmune disorders through its tolerogenic influence on the adaptive immune system (153).

Vitamin D sufficiency has been found to reduce the risk for some types of cancer. The link between serum 25(OH)D or vitamin D supplementation and reduced risk of colon cancer has been well established (82, 154). The regulation of proliferation and cell differentiation by $1,25(OH)_2D$ may reduce the risk for cancer development (105).

Health disparities associated with vitamin D

Non-Hispanic, black Americans are at greater risk of vitamin D insufficiency and deficiency than non-Hispanic white Americans(1). Several health disparities have been associated with increased risk for vitamin D insufficiency; these include diabetes,

cardiovascular disease, hypertension, pregnancy complications, among others. Vitamin D insufficiency is among the risk factors for these health outcomes, and it is postulated that restoring vitamin D status may ameliorate some of the disparities (155-157).

There is an increased risk of pregnancy complications, such as premature birth, bacterial vaginosis and pre-eclampsia among black women, which may be related to vitamin D status (157). It is believed that vitamin D modulates the immune system to preserve tolerance of the fetus during pregnancy. Further, vitamin D may boost the innate immune system to decrease the risk of bacterial vaginosis that can lead to pre-mature labor and birth (158).

Black Americans have a greater risk for cardiovascular disease, than white, non-Hispanic Americans. When analyses of cardiovascular mortality from the NHANES data were controlled for 25(OH)D concentrations, the increased risk for cardiovascular disease was attenuated (156). When the disparity in systolic blood pressure was analyzed in the NHANES data, it was determined that approximately one quarter of the disparity may be attributed to differences in 25(OH)D. (159) Observational studies have linked increased risk of diabetes with vitamin D deficiency, which may explain the high prevalence of type 2 diabetes in black Americans (77, 155, 160).

CYSTIC FIBROSIS

Etiology of cystic fibrosis

Cystic fibrosis (CF) is the most common life-shortening, inherited disease among Caucasians in the United States affecting approximately 1:3000 live births (161). The prevalence of CF varies across races and regions of the globe. Within the Caucasian population, the prevalence of CF varies; for example, among northern European

Caucasians, the rate of CF is 1:1800 in Northern Ireland compared to 1:7300 in Sweden. Furthermore, in some Asian countries, the prevalence of CF is as low as 1:100,000 (162, 163)

CF is caused by a mutation in the CF transmembrane conductance regulator (CFTR) gene (161, 164). The CFTR gene encodes for a transmembrane chloride channel which is expressed in secretory epithelial cells from tissues throughout the body, including the epithelium that lines the respiratory airways and the pancreatic ducts (25). The CFTR channel regulates the movement of chloride out of the cell and thereby also impacts the movement of water and other charged molecules, particularly sodium. The primary site of CFTR expression in the lung is in the sub-mucosal glands and dysfunction causes the production of abnormally thickened sputum (165).

The CFTR gene was isolated in 1989, it contains more than 230 kb and there are more than 1,000 known mutations of the gene. (166-168). Each mutation may affect the functioning of the CFTR protein differently and therefore, there is a spectrum of signs and severity of CF disease severity (162, 163, 169). The most common mutation is the ΔF508 and accounts for more than 70% of the mutations identified (163). The ΔF508 mutation is a three base pair deletion that removes the phenylalanine residue from the amino acid position 508 (164). This mutation causes the production of thickened mucus in the airways of the lungs, ducts of the pancreas and other organs. This thickened mucus is an important factor in CF complications.

Pulmonary pathophysiology in CF

CF patients develop chronic lung infections due to an inability to effectively remove pathogens from the lungs. The thickened mucus produced due to the

dysfunctional CFTR protein traps pathogens and is ineffectively cleared from the lungs. As a result, pathogens develop biofilms that maintain their presence in the lung and increase the inflammatory response (170). Inflammation leads to structural damage to the lung and respiratory failure (26, 171). Most morbidity and mortality in individuals with CF is due to progressive lung disease and recurrent pulmonary infections (172).

The pathophysiology of respiratory failure in CF involves both chronic pulmonary infection and inflammation. The CF lung responds to pulmonary infection with an exaggerated inflammatory response (173, 174). This response may or may not be preceded by the presence of pathogens in the lung. An exaggerated inflammatory response in the CF lung has been demonstrated to be present even in the first months of life when it is possible that the lungs have not yet been infected by any pathogens (175). It is hypothesized that the presence of dysfunctional CFTR within the cell may increase the production of inflammatory mediators by disrupting signaling pathways independent of the presence of infection (175, 176). Therefore, it is thought that the presence of chronic pulmonary infection is not the sole cause of the inflammation that is an important component of lung tissue damage.

The mechanism by which ΔF508 causes inflammation is related to the impact of the mutated polypeptide on the protein synthesis pathways of the cell. The ΔF508 mutation produces a misfolded protein, which remains trapped in the endoplasmic reticulum. This interrupts normal cellular functions and signaling, resulting in the increased production intracellular messengers, such as NFκB, which in turn increase the transcription of inflammatory cytokines such as IL-8, TNF-α and IL-6 (177). The misfolded protein may also increase oxidative stress by altering the intracellular redox

potential, increasing the production of reactive oxygen species and thereby increasing the production of inflammatory mediators (177, 178). Increased concentrations of systemic inflammatory mediators have been linked to reduced lung function (179).

Activated neutrophils are thought to be the main cause of lung parenchymal damage through their release of inflammatory mediators and proteases (180). Neutrophils are found in the lungs of CF patients at greatly increased numbers compared to infected non-CF lungs (173, 180). It is hypothesized that increased recruitment of neutrophils to the lungs, and their release of inflammatory mediators and proteases, are central to the exaggerated inflammatory response in CF (181). Neutrophils release inflammatory cytokines and chemokines, such as IL-1, IL-12, IL-8, that increase inflammation and neutrophil activation. They are also activated by inflammatory mediators produced by other immune cells and respiratory cells. Neutrophils are largely recruited by IL-8, which acts as a chemokine to attract neutrophils to the lungs. Neutrophils are activated by TNF-α and IL-8 to increase the production of proteases (182). Two prominent proteases that neutrophils produce, neutrophil elastase and matrix metalloproteinase-9, have been positively correlated with cytokine concentrations and inversely correlated with lung function (183, 184). These proteases may directly damage pulmonary tissue leading to reduced lung function. The misfolded CFTR proteins cause the increased production of these inflammatory cytokines which increase neutrophil recruitment and lung parenchyma damage.

Anti-inflammatory therapies have been shown to improve lung function and reduce the frequency of pulmonary exacerbations (185). These therapies have included glucocorticoids, ibuprofen and antibiotics. Oral therapy with glucocorticoids and

ibuprofen are limited due to adverse side effects, such as growth inhibition and renal dysfunction (186). The ideal anti-inflammatory therapy would not only suppress the exaggerated inflammatory response in CF, but also enhance the innate response to pathogens (187).

Gastrointestinal pathophysiology in CF

The CFTR channel is present in the gastrointestinal (GI) tract from the stomach through the colon. In CF, there appears to be chronic underlying inflammation throughout the small intestine (188). The mucosa does not function normally in CF and this contributes to macronutrient malabsorption. Malabsorption is due in part to reduced fluid secretion that results in the thickened mucus lining the intestine and increased paracellular permeability in the small intestine (189, 190). However, dysfunction of the CFTR channel in the pancreas and its ducts, also increases the thickness of the pancreatic secretions and contributes to macronutrient malabsorption by reducing the release of digestive enzymes, bicarbonate and fluids into the small intestine. As a result, there is a high prevalence of macronutrient malabsorption in CF and up to 90% of CF patients receive pancreatic enzyme replacement therapy (191).

Fat malabsorption is a prominent component of nutrient malabsorption in CF and has multiple causes. Pancreatic insufficiency results in little or no production of pancreatic lipase and co-lipase, although the production of lingual lipase is not effected. Pancreatic enzyme replacement therapy is frequently used in CF to replace the pancreatic lipase and co-lipase, however it is unable to normalize fat absorption (192). Therefore, lingual lipase is the main endogenous source of fat digestion in the CF GI tract which results in reduced digestion of dietary fats. The inadequately digested, large fat molecules

are less able to form small micelles that facilitate lipid absorption causing reduced fat digestion and absorption (193). Additionally, the pH of the small intestine is lower in CF than non-CF, due to the lack of duodenal bicarbonate, which normally would neutralize gastric acidity. Micelle formation by bile salt interactions are reduced at low pH, therefore, fewer micelles are formed, more bile salts and fats are lost in the stool which may be lead to fat malabsorption (194). Finally, fat malabsorption may also be due to the thickened mucus that lines the walls of the GI tract limiting the interaction of the contents with the brush border transporters (195). Fat malabsorption in the CF digestive tract leads to nutritional deficiencies, including contributing to vitamin D insufficiency.

Vitamin D in Cystic Fibrosis

Prevalence and causes of vitamin D Insufficiency

Adults and children with CF have a high prevalence of vitamin D deficiency, despite increased awareness of the health benefits of adequate vitamin D status and guidelines for treatment of vitamin D deficiency (19, 31, 35, 196). The risk of vitamin D deficiency is greater in the CF than in the non-CF population and has been estimated to affect from 76% to 100% of CF patients (31, 35, 197, 198). In infants diagnosed with CF through newborn screening, 37% were found to have 25(OH)D concentrations <20 ng/ml (199). The causes of vitamin D deficiency in CF include decreased pancreatic exocrine function, resulting in malabsorption of fat soluble vitamins including vitamin D; however, other risk factors for vitamin D deficiency are present in patients with CF. These include decreased sunlight expose due to chronic illness, decreased retention of vitamin D metabolites associated with decreased serum vitamin D binding protein, and

malnutrition-associated wasting of adipose tissue, which may limit endogenous storage of vitamin D (22, 34, 122, 200, 201).

Low concentrations of DBP may contribute to risk of vitamin D deficiency in the CF population by decreasing the retention of vitamin D and its metabolites in the blood. Low concentrations of DBP are associated with decreased serum concentrations of 25(OH)D. Vitamin D status is determined by measuring the total amount of 25(OH)D in the blood, which includes 25(OH)D bound to the DBP or serum albumin and unbound 25(OH)D (119, 202). In non-CF subjects and in subjects with other disease states, concentrations of DBP have been correlated with vitamin D status as measured by 25(OH)D (202, 203). Serum concentrations of DBP have been found to be decreased in CF subjects compared to non-CF subjects and may be an additional factor in the vitamin D deficiency in CF (200, 204).

The increased risk of vitamin D deficiency in CF, and the associated comorbidities, make evaluation and repletion of vitamin D status an important part of CF therapy. Over the past twenty years several methods have been suggested for vitamin D repletion, with limited success (205).

Vitamin D status and clinical outcomes

Vitamin D sufficiency (25(OH)D > 30 ng/mL) may be particularly important in the CF population. CF subjects are at greater risk of comorbidities that have been associated with vitamin D insufficiency. The following CF comorbidities have been associated with reduced vitamin D status: low bone mineral density (BMD), diabetes, decreased lung function; respiratory infections, and dysregulation of the adaptive and

innate immune response (8, 97, 196, 206, 207). Therefore adequate vitamin D replenishment may produce better clinical outcomes for CF patients.

There is a high prevalence of low bone mineral density in CF patients. In CF adults, it may be greater than 85% (Aris 1998). Inadequate vitamin D status is a contributor, as are poor overall nutrition, hypogonadism, reduced physical activity, and excess inflammation (34, 208). Vitamin D repletion may increase bone density both by improving calcium absorption and reducing inflammation in individuals with CF.

Vitamin D insufficiency may also contribute to the increased risk of CF-related diabetes (CFRD). Vitamin D deficiency has been linked to both type 1 and type 2 diabetes (77, 145). CFRD causes increased inflammation and has been linked to reduced lung function (209). Vitamin D has been linked to reduced production of inflammatory mediators from immune cells in diabetic patients (103).

Reduced lung function, as measured by FEV_1 % of predicted, is used to assess respiratory status in CF. Vitamin D status has been correlated with improved lung function in CF and in the general population (196, 210). Respiratory failure is the most common cause of mortality in CF. Vitamin D sufficiency may improve lung function through the increased production of antimicrobial peptides or improved muscular strength (207).

Vtamin D sufficiency has been linked to increased production of the antimicrobial peptides LL-37 and defensin-β2 (211). Defensin-β2 concentrations are correlated with vitamin D status in children with CF (211). LL-37 concentrations can be elevated in CF, but *in vitro* studies have shown that there is a 10-fold increase in the release of LL-37 from CF-derived respiratory epithelial cells in $1,25(OH)_2D$ supplemented media

compared to vitamin D insufficient media (86). Increased concentrations of antimicrobial peptides may reduce bacterial load in the CF lung and lead to reduced frequency/severity of pulmonary exacerbations.

In some clinical analyses, vitamin D supplementation has been associated with a reduced risk for respiratory infection (212). However, there have been other studies that have shown little benefit (213). Vitamin D may benefit reduce respiratory infection through its impact on antimicrobial peptides, muscle function and inflammation (207).

Chronic respiratory infection and a dysregulation of the immune response that leads to a damaging inflammatory state in CF (171). Vitamin D may also be important in regulating the inflammatory response that is considered to be important in the progression to respiratory failure in CF patients (97, 172). Systemic inflammation has been associated with decreased lung function over time in CF as measured by FEV_1 % of predicted (27, 196, 214). Vitamin D insufficiency has been associated with increased systemic inflammation (215-217). $1,25(OH)_2D$ reduces DC stimulation of the Th1 phenotype that increases the production of the inflammatory mediators, which have been linked to increased inflammation and decreased lung function in CF (97). In addition, $1,25(OH)_2D$ has also been found act directly on CF respiratory epithelial cells to decrease the production of inflammatory markers (218). Vitamin D repletion may reduce the production of inflammatory mediators in CF and reduce the deleterious effects of inflammation on lung function.

Vitamin D sufficiency may also have an impact on quality of life through improved mobility. Vitamin D status is associated with greater muscle strength and mobility, particularly in individuals with low body mass and chronic disease (219, 220).

In elderly women who were vitamin D insufficient at baseline, vitamin D supplementation increased muscle strength (221). In CF subjects, greater mobility as assessed by the Life-space questionnaire, has been associated with fewer hospitalizations (222). By improving muscular strength, vitamin D may improve mobility and quality of life in CF.

Vitamin D repletion

The CF Foundation has recently changed its recommendations for evaluation of vitamin D status and vitamin D repletion in CF patients (118). As before, it is recommended healthcare providers evaluate vitamin D status at the end of winter at the expected 25(OH)D nadir. The American Association for Pediatrics has accepted 25(OH)D > 20 ng/ml as adequate to prevent rickets in non-CF children. The additional comorbidities related to vitamin D insufficiency and evidence that 25(OH)D > 35 ng/ml may be necessary to suppress excess PTH production guided the CF Foundation study group to recommend 25(OH)D concentrations between 30 and 50 ng/ml (223, 224).

The new guidelines also recommend using a cholecalciferol supplement with a daily or weekly dosing schedule, evaluation of vitamin D status, confirmation of adherence to recommended therapy and then a step-wise increase in supplement dose. Individuals with refractory insufficiency should be referred to a specialist in vitamin D (118).

The previous high dose protocol for treating vitamin D deficiency in CF recommended by the CF Foundation did not reliably produce vitamin D sufficiency (25(OH)D ≥ 30 ng/ml) in the majority of CF subjects (30, 31). In adults, weekly oral dosing of ergocalciferol, 50,000 IU or 100,000 IU, per the CF Foundation's previous

recommendations, did not produce sufficiency in 92% of adult CF patients (31). In pediatric CF patients, doses up to 150,000 IU of ergocalciferol weekly produced sufficiency in a maximum of 42% of patients (32, 33). A single study found that a very large dose of ergocalciferol was able to produce sufficiency in 17 of 18 subjects. The dose used for this was 700,000 IU of ergocalciferol over two weeks (30). Therefore, these studies found the previous CF Foundation recommendations inadequate.

The difficulties with vitamin D repletion in the CF population may also be related to the common use of high dose vitamin D_2 (ergocalciferol) in a lipid vehicle as the treatment for vitamin D deficiency. Vitamin D is a lipid soluble vitamin and may be given with a lipid vehicle, or it may instead be compounded with a non-lipid vehicle, such as lactose or cellulose, in a pressed-powder tablet. In normal subjects, the lipid and non-lipid vehicles appear to exhibit equal bio-availabilities (18, 225). However, most CF subjects have pancreatic insufficiency and have been shown to have decreased absorption (~50%) of vitamin D from a lipid vehicle compared to healthy subjects (22, 226).

Studies of the impact of vitamin D on CF comorbidities depend on the ability to adequately replete and maintain vitamin D status in CF individuals. If the new guidelines are able to do this, then research may evaluate the importance of vitamin D supplementation and the mechanisms through which vitamin D impacts clinical outcomes in CF.

CHAPTER 3:

EVALUATION OF VEHICLE SUBSTANCES ON VITAMIN D BIOAVAILABILITY: A SYSTEMATIC REVIEW

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Grossmann RE, Tangpricha V 2010 Evaluation of vehicle substances on vitamin D bioavailability: a systematic review. Mol Nutr Food Res 54:1055-1061

Introduction to chapter 4

From our systematic review of the literature, we determined that there is insufficient evidence to conclude that the vehicle substance of a vitamin D supplement impacts the bioavailability of the supplement in healthy, non-CF subjects. Additionally, there were no manuscripts that directly evaluated the impact of vehicle substance on vitamin D supplement bioavailability in CF subjects. We did find two manuscripts that suggested that a vitamin D supplement in a non-lipid vehicle may have greater bioavailability than a lipid vehicle in CF subjects.

In Chapter 4, we will evaluate the impact of a cholecalciferol supplement in a non-lipid vehicle compared to placebo on clinical outcomes in CF adults hospitalized for a pulmonary exacerbation. This is not an evaluation of the efficacy of this supplementation strategy since vitamin D insufficiency, 25(OH)D concentrations < 30 ng/ml, was not included in the eligibility criteria for this pilot study. Rather, this pilot study evaluates the feasibility and clinical impact of vitamin D supplementation in CF adults hospitalized for a pulmonary exacerbation.

In order to optimize bioavailability, a cholecalciferol supplement in a non-lipid vehicle was chosen. The supplement dose, 250,000 IU, was selected as the dose required to raise blood 25(OH)D concentrations at least 10 ng/ml. Previous analyses found that 600,000 IU of cholecalciferol increased 25(OH)D concentrations more than 20 ng/ml when administered over 12 weeks. Our protocol administered the dose in one large, oral bolus within 48 hours of hospital admission for a pulmonary exacerbation. The purpose of this strategy was to evaluate the feasibility of increasing vitamin D status during CF hospitalization.

In chapter 4 we will report the impact of this bolus dose of cholecalciferol on vitamin D status and clinical outcomes; as well as, measures of vitamin D toxicity.

CHAPTER 4:

PILOT STUDY OF VITAMIN D SUPPLEMENTATION IN ADULTS WITH CYSTIC FIBROSIS PULMONARY EXACERBATION: A RANDOMIZED, CONTROLLED TRIAL

In press: Dermato-Endocrinology

Grossmann RE, Zughaier SM, Kumari M, Seydafkan S, Lyles RH, Liu S, Sueblinvong V, Schechter MS, Stecenko AA, Ziegler TR, Tangpricha V. Pilot study of vitamin D supplementation in adults with cystic fibrosis pulmonary exacerbation: A randomized, controlled trial. Dermatoendocrinol 2012; In press.

Introduction to chapter 5

In the fourth chapter, we demonstrated that a single, oral bolus of cholecalciferol produces a rapid increase in serum 25(OH)D concentrations in adult CF subjects hospitalized for treatment of a pulmonary exacerbation. We also found improvements in hospitalization and survival in subjects randomized to vitamin D compared to placebo. There was also a trend for improvements in IV antibiotic therapy and recover of lung function in the vitamin D group.

In chapter 5, we will report changes in markers of inflammation and the antimicrobial peptide, LL-37, from baseline and between the two groups. *In vitro* studies have found that $1,25(OH)_2D$ can regulate pathways that lead to the generation of inflammatory messengers and antimicrobial peptides. Clinical studies have indicated that vitamin D supplementation may reduce systemic concentrations of inflammatory markers. In CF, inflammation, both pulmonary and systemic, has been associated with poor clinical outcomes and reduced survival. The goal of this manuscript is to describe changes in inflammatory markers in this small pilot study. These findings may be useful in the planning of future analyses of the mechanisms through which vitamin D may modulate the immune response in CF.

During treatment for a pulmonary exacerbation, a reduction in inflammation is expected. This may be due to the anti-inflammatory impact of the reduced bacterial burden after pulmonary exacerbation or the direct impact of some antibiotics on markers of inflammation. In this study, we evaluated the use of macrolide antibiotics, which are known to have anti-inflammatory properties, but none of the subjects were prescribed these during their hospitalization. We also evaluated other antibiotic therapies, however

there were no significant differences between the groups. Therefore, differences of antibiotic therapy during hospitalization were unlikely to cause differences between the groups.

In chapter 5, we will describe the change in inflammatory markers and the antimicrobial peptide, LL-37, in response to a single oral bolus dose of cholecalciferol.

CHAPTER 5:

IMPACT OF VITAMIN D SUPPLEMENTATION ON MARKERS OF INFLAMMATION IN ADULTS WITH CYSTIC FIBROSIS HOSPITALIZED FOR A PULMONARY EXACERBATION

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ABSTRACT

Patients with cystic fibrosis (CF) suffer from chronic lung infection and inflammation leading to respiratory failure. Vitamin D deficiency is common in patients with CF and correction of vitamin D deficiency may improve innate immunity and reduce inflammation in patients with CF. We conducted a double-blinded, placebo-controlled, randomized, clinical trial of high dose vitamin D to assess the impact of vitamin D therapy on anti-microbial peptide concentrations and markers of inflammation. We randomized 30 adults with CF hospitalized with a pulmonary exacerbation to 250,000 IU of cholecalciferol or placebo and evaluated changes in plasma concentrations of inflammatory markers and the anti-microbial peptide LL-37 over 12-weeks post-intervention. In the vitamin D group, there was a 50.4% reduction in TNF-α at 12-weeks ($p<0.01$) and there was a trend for a 64.5% reduction in IL-6 ($p=0.09$). There were no significant changes in IL-1β, IL-8, IL-10, IL-18BP, and NGAL. We conclude that a large bolus dose of vitamin D is associated with reductions in two inflammatory cytokines, IL-6 and TNF-α. This study supports the concept that vitamin D may help to regulate inflammation in CF and that further research is needed to elucidate the potential mechanisms involved and impact on clinical outcomes.

INTRODUCTION

Cystic fibrosis (CF) is the most common life-shortening, inherited disease among Caucasians in the United States. Chronic pulmonary infection and inflammation lead to respiratory failure, the most common cause of morbidity and mortality in CF (26). Pulmonary inflammation is associated with poor outcomes and anti-inflammatory therapies have had limited extended use due to adverse side effects (181).

Vitamin D insufficiency has been estimated to affect up to 90% of the adults with CF (34). Vitamin D has been shown to suppress *in vitro* and *in vivo* the production of pro-inflammatory cytokines, such as IL-6, IL-8, and TNF-α; as well as, increase production of the anti-microbial peptide LL-37 from CF respiratory epithelial cells (29, 86, 227).

Increased production of LL-37 and decreased production of inflammatory messengers may reduce pulmonary complications in CF and improve clinical outcomes. The purpose of this communication is to describe the impact of a vitamin D repletion strategy on markers of inflammation and LL-37 in CF adults during pulmonary exacerbation.

METHODS

Study design

As described previously, adult CF patients of the Emory University CF Center hospitalized for treatment of a pulmonary exacerbation were eligible. After consent was obtained, subjects were randomized to either 250,000 IU cholecalciferol or placebo. Blood samples were obtained at baseline, 1-week and 12-weeks (228)

Analytical Methods

Serum IL-1β, IL-10, IL-18 binding protein (IL-18BP) and plasma IL-6, IL-8 and TNF-α were assessed by DuoSet ELISA (R&D Systems, Minneapolis, MN) and plasma LL-37 by ELISA (Hycult Biotech, The Netherlands). All cytokine measurements were performed in duplicate. Antibiotic therapy during hospitalization was determined from hospital patient records. Pathogens present at admission were determined by sputum analysis. Pancreatic insufficiency was assessed by the requirement for physician prescribed pancreatic enzymes.

Statistical analysis

All statistical analyses were completed using SAS 9.3 (SAS Institute, Cary, NC). This was a secondary analysis, the original study was designed to provide 90% power to detect a 10 ng/ml difference in serum 25(OH)D between the groups at a significance level of α=0.05. Standard chi-square tests and Fisher's exact test were used to assess differences between the groups in antibiotic therapy and microbiology. Variables were assessed for normality and the following variables were transformed due to their right-skewed distributions before inclusion in statistical analyses: IL-1β, IL-6, IL-10, and LL-37. Paired t-tests were applied to assess within-group changes in mean serum/plasma concentrations from baseline. Mixed effects linear regression models with a random intercept were used to evaluate the difference in mean serum/plasma concentrations of the vitamin D and placebo group at each time point, based on repeated measurements of IL-1β, IL-6, IL-8, IL-10, IL-18BP, TNF-α, NGAL and LL-37. The following confounders were assessed: age, BMI, sex, race, CFRD, pancreatic insufficiency, lung function measured as baseline FEV_1 % of predicted, and vitamin D intake.

The confounders found to significantly impact the random intercept model were FEV_1% of predicted, CF-related diabetes, pancreatic insufficiency and age. The variables whose means were significantly different at $\alpha<0.10$, were adjusted for these confounders.

RESULTS

Baseline demographics have been reported elsewhere. There were no statistically significant differences in the demographics of the two groups., As previously reported, in the vitamin D group, serum concentrations of 25(OH)D increased at 1-week and 12-weeks, 27.5 ±13 ng/ml and 6.2 ±11 ng/ml, respectively ($p<0.001$ and 0.06), there was no significant change in the placebo group (228).

There were no significant differences between the groups in microbiology at the time of hospitalization or the types of antibiotic therapy during hospitalization. Comparison of the mean concentrations of markers of inflammation and LL-37 are summarized in Table 3. In IL-1β, IL-10, IL18BP, NGAL and LL-37 there were no significant differences in the means between groups at any time point.

At 12-weeks, mean serum TNF-α concentrations in the vitamin D group were significantly less than those in the placebo (Table 3). Compared to baseline, TNF-α decreased 3.54 ±8.3 pg/ml and 27.62 ±8.4 pg/ml at 1-week and 12-weeks, respectively ($p= 0.60, 0.0002$); when adjusted for confounders, the change in TNF-α remained statistically significant. In the placebo group there was no significant change in TNF-α from baseline at 1-week or 12-weeks (Figure 2).

At 1-week, there was a trend for decreased plasma IL-6 concentrations in the vitamin D group compared to placebo (Table 3).Compared to baseline, mean plasma IL-6 concentrations decreased significantly in the vitamin D group 24.91 ±8.4 pg/ml ($p=0.004$)

at 1-week and 18.84 ±11.8 (p=0.4) at 12-weeks; there was no significant change from baseline in the placebo at 1-week or 12-weeks (Figure 2).

DISCUSSION

Inflammation is an important contributor to CF disease progression and anti-inflammatory therapies may improve clinical outcomes (181). Given the high prevalence of insufficiency, vitamin D should be evaluated for its potential to reduce inflammation (34). This is the first evaluation of multiple markers of inflammation and the anti-microbial peptide LL-37 in response to a marked increase in 25(OH)D produced by high dose vitamin D supplementation during a CF exacerbation. The data we present indicates that vitamin D supplementation may reduce systemic concentrations of IL-6 and TNF-α.

Vitamin D may reduce transcription of inflammatory cytokines through modulation of the NFκB and MAPK pathways (181, 229). Serum 25(OH)D concentrations have been negatively correlated with the pro-inflammatory marker, IgG, and positively correlated with lung function in cross-sectional studies of CF subjects (19, 27). Pre-treatment of isolated CF epithelial cells with the hormonal form of vitamin D, $1,25(OH)_2D$, decreases the secretion of IL-8 and IL-6 in response to antigen stimulation (227). $1,25(OH)_2D$ has also been shown to increase the production LL-37, the anti-microbial peptide and hCAP-18 mRNA from which it is synthesized(86, 227). In this randomized, controlled trial, we have found that increasing vitamin D status in CF subjects may also reduce concentrations of pro-inflammatory markers.

Vitamin D has the properties of an ideal anti-inflammatory therapy since it may suppress concentrations of pro-inflammatory cytokines while supporting innate immune functions, such as antimicrobial peptide synthesis. This pilot study provides baseline data

for the design of future studies to assess the impact of vitamin D on pulmonary and systemic inflammation in CF.

Tables

Table 3 Comparison of mean serum and plasma concentrations of inflammatory markers

Mean concentrations at baseline, 1-week and 12-weeks in CF adults hospitalized with a pulmonary exacerbation randomized to either a single oral 250,000 IU dose of cholecalciferol or placebo.

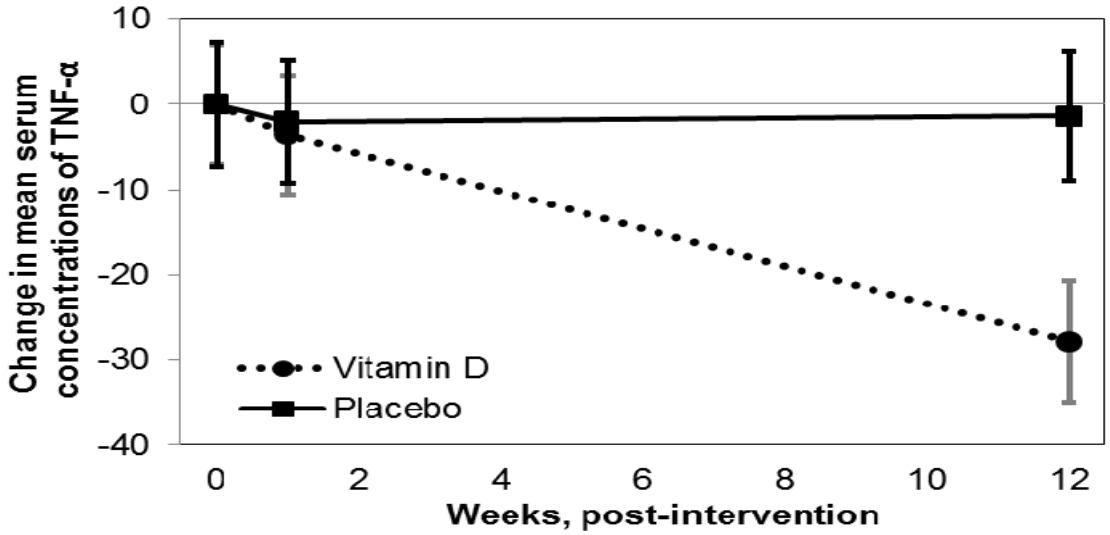
	Time	Vitamin D_3[1]	Placebo[1]	p-value[2]	p-value[4]
	Baseline	1.95 (1.32)	2.44 (1.17)	0.48	
IL-1β[3], pg/ml	1 week	1.27 (0.74)	2.76 (1.08)	0.18	
	12 weeks	2.47 (1.73)	4.74 (3.69)	0.15	
	Baseline	38.63 (9.60)	34.38 (6.76)	0.85	0.50
IL-6[3], pg/ml	1 week	13.72** (2.35)	29.41 (7.20)	0.09	0.09
	12 weeks	22.65 (4.34)	36.62 (7.46)	0.21	0.11
	Baseline	34.64 (1.07)	41.40 (2.79)	0.03	0.05
IL-8, pg/ml	1 week	34.51 (0.84)	40.24 (1.96)	0.07	0.13
	12 weeks	39.59* (1.88)	48.54** (4.28)	0.003	0.01
	Baseline	42.03 (21.17)	28.54 (5.56)	0.99	
IL-10[3], pg/ml	1 week	30.01 (11.22)	29.62 (5.80)	0.73	
	12 weeks	49.92 (28.89)	37.24* (11.22)	0.40	
	Baseline	2.64 (0.16)	2.77 (0.18)	0.57	
IL-18-1BP, pg/ml	1 week	2.65 (0.14)	2.80 (0.18)	0.57	
	12 weeks	2.50 (0.12)	2.92 (0.20)	0.11	
	Baseline	55.67 (8.18)	69.52 (7.50)	0.18	0.41
TNF-α	1 week	52.13 (5.62)	67.48 (7.92)	0.13	0.34
	12 weeks	27.62** (5.82)	68.54 (8.61)	0.0003	0.0049
	Baseline	79.65 (1.71)	78.56 (2.18)	0.77	
NGAL	1 week	76.11 (2.84)	76.85 (1.81)	0.88	
	12 weeks	71.64* (3.13)	74.95 (4.01)	0.39	
	Baseline	73.10 (7.40)	192.60 (46.50)	0.003	
LL-37[3]	1 week	128.10 (33.80)	174.60 (28.40)	0.17	
	12 weeks	142.60 (45.90)	135.30 (28.80)	0.67	

[1]unadjusted mean from mixed model (SEM)
[2]unadjusted p-value comparing means from mixed model
[3]variable was log-transformed before inclusion in model
[4]adjusted for FEV_1% of predicted, CFRD, pancreatic insufficiency and age
*within group comparison, p-value <0.05, compared to baseline
**within group comparison, p-value <0.01, compared to baseline

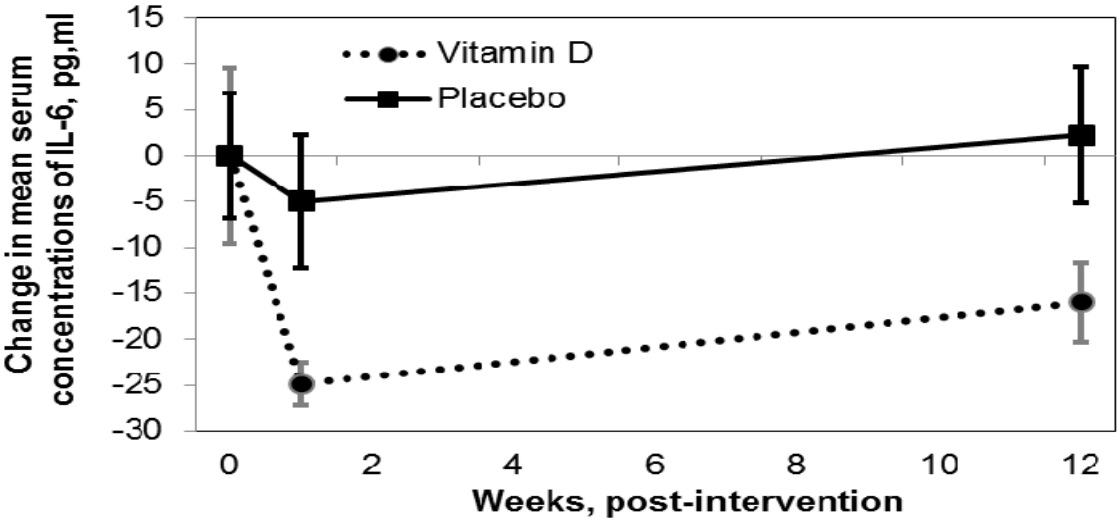
Figures

Figure 2. Mean change in plasma concentrations of TNF-α and IL-6

Mean change in plasma concentrations of TNF-α and IL-6 at baseline, 1-week and 12-weeks in CF adults randomized to 250,000 IU cholecalciferol or placebo.
a. Mean change in TNF-α plasma concentrations. In the vitamin D group, TNF-α decreased 3.56 and 27.83 pg/ml at 1-week and 12-weeks (p=0.6, 0.0002); TNF-α remained unchanged in the placebo. (SEM bars).
b. Mean change in plasma concentrations of IL-6. In the vitamin D group, IL-6 decreased 12.39 and 5.16 pg/ml at 1-week and 12-weeks (p=0.004, 0.35); IL-6 remained unchanged in the placebo. (SEM bars).



Mean change in TNF-α serum concentrations in CF adults randomized to 250,000 IU cholecalciferol or placebo. In the vitamin D group, TNF-α decreased 3.56 and 27.83 pg/ml at 1-week and 12-weeks (p=0.6, 0.0002); TNF-α remained unchanged in the placebo. (SEM bars).



Mean change in serum concentrations of IL-6 in CF adults randomized to either 250,000 IU cholecalciferol or placebo. In the vitamin D group, IL-6 decreased 12.39 and 5.16 pg/ml at 1-week and 12-weeks (p=0.004, 0.35); IL-6 remained unchanged in the placebo. (SEM bars).

CHAPTER 6: CONCLUSIONS

Key Findings

Vitamin D insufficiency is common in the US population and may be linked to the high prevalence of cardiovascular disease, cancer and autoimmune disorders (115). However, much is still unknown about the best strategy for vitamin D repletion, the appropriate vitamin D dose to improve clinical outcomes and how vitamin D repletion may impact clinical outcomes in populations at high risk for vitamin D deficiency.

Chapter 2 of this dissertation provides a systematic review of the current scientific literature regarding the impact of supplement vehicle on the bioavailability of vitamin D. We concluded that in a healthy population there appears to be little difference in bioavailability of vitamin D from lipid, non-lipid or ethanol vehicles. However, the number of manuscripts that compared the bioavailability of vitamin D from more than one vehicle is very limited. No manuscripts directly evaluated the impact of supplement vehicle on the absorption or bioavailability of vitamin D in subjects with CF-related macronutrient malabsorption. Individuals with malabsorptive disorders, such as CF, are at increased risk for vitamin D deficiency and may also have increased risk of vitamin D deficiency related comorbidities. Therefore, additional research into the formulation of vitamin D supplements, including vehicle, may aid in correcting vitamin D status in these populations.

The fourth chapter of this dissertation evaluated the impact of a single, large bolus dose of cholecalciferol in adults with CF hospitalized for a pulmonary exacerbation. We demonstrated a significant increase in vitamin D status over a short period of time without any evidence of vitamin D toxicity. Unadjusted survival and number of days

hospitalized were also significantly improved in the supplemented group and there was a trend for improvements in IV antibiotic therapy and recovery of lung function. Given the small sample size of this pilot study, these outcomes and others should be explored in a larger sample.

In the fifth chapter of this dissertation, we report on the impact of high dose vitamin D supplementation on markers of inflammation and the anti-microbial peptide, LL-37 in adults with CF. Although this was a small, pilot study, there were reductions in serum concentrations of TNF-α, as well as a trend for reduced IL-6 concentrations. The results from this study may be used to inform future study of the impact vitamin D on inflammation in CF.

Implications for vitamin D repletion in the CF population

In the CF population, several comorbidities may be impacted by vitamin D insufficiency. Vitamin D repletion in CF is complicated by pancreatic insufficiency and macronutrient malabsorption. Vitamin D is a lipid-soluble vitamin, often administered in a lipid vehicle. Although the predominant form of nutrient malabsorption in CF is lipid malabsorption, there has not been a direct evaluation of the impact of lipid and non-lipid vehicles on supplement bioavailability in CF. There are two manuscripts that provide some evidence that a non-lipid vehicle may have greater bioavailability in the setting of CF malabsorption.

In subjects with CF, Khazai, *et al*. found that a vitamin D supplement from a non-lipid vehicle had more than a 3 times greater impact on vitamin D status than a lipid vehicle supplement; however, this study was confounded by the form of vitamin D in the supplements. The lipid vehicle supplement contained ergocalciferol and the non-lipid

vehicle supplement contained cholecalciferol (19). Cholecalciferol has been shown to produce a greater increase in serum 25(OH)D concentrations than ergocalciferol (20, 21). Therefore, it cannot be assumed that the difference in bioavailability in the Khazai study was due only to the vehicle of the supplement. A direct comparison of cholecalciferol bioavailability from a lipid and a non-lipid vehicle is necessary to confirm a greater bioavailability from a non-lipid vehicle.

It does appear that lipid malabsorption contributes to the reduced vitamin D status of individuals with CF. Lark, *et al*. found an approximately 50% reduction in the absorption of ergocalciferol from a lipid vehicle supplement in CF subjects compared to non-CF subjects. Also, the bioavailability of the supplement was significantly reduced in the CF group compared to the non-CF group, demonstrated by the reduced 25(OH)D response to the ergocalciferol dose in the CF group. This study did not evaluate the absorption or bioavailability of vitamin D from a non-lipid vehicle supplement, so no direct comparison can be made between lipid and non-lipid supplements. Therefore, it is still important to compare the absorption and bioavailability of vitamin D from a non-lipid vehicle and a lipid vehicle in CF subjects. Although vitamin D may have greater bioavailability from a non-lipid vehicle supplement, individuals with CF may still need larger doses to optimize vitamin D status. An understanding of the absorption kinetics and metabolic conversion to 25(OH)D, may help determine the correct dosing of vitamin D in CF.

There may also be a significant variability in the 25(OH)D response to a given dose of vitamin D among individuals with CF. Lark, *et al*. reported that two subjects in the CF group did not show any change in serum 25(OH)D concentrations after a 100,000

IU dose of ergocalciferol (22). In our sample, we also found one subject in the vitamin D group whose 25(OH)D concentrations were essentially unchanged after a 250,000 IU dose of cholecalciferol in a non-lipid vehicle (unpublished data). This variability implies that it is unlikely that a single repletion strategy will optimize 25(OH)D concentrations in all individuals with CF and the evaluation of vitamin D status after vitamin D therapy remains important.

Individuals with other malabsorptive disorders, including individuals with short bowel syndrome, celiac disease, among others are also at greater risk of vitamin D insufficiency; as well as, at greater risk for vitamin D related co-morbidities such as low bone mineral density, diabetes, and other inflammatory diseases (23, 230-232). Therefore the correction of vitamin D insufficiency in these populations may have greater benefits than in the healthy population. They may require increased dosing of vitamin D or may benefit from different supplement formulations. Therefore, continued evaluation of supplements with greater potential bioavailability in these individuals may lessen the health burden experienced. Additionally, since the population of the US has a high prevalence of individuals at increased risk for vitamin D deficiency related to age, obesity and chronic disease, the repletion methods that benefit individuals with nutrient malabsorption may also provide insight in how to maintain the vitamin D status of the US population.

Implications for the evaluation of vitamin D supplementation in CF

Clinical outcomes are an significant factor in evaluating the importance and the success of vitamin D repletion in CF. Vitamin D supplementation adds another therapy to the already heavy therapy burden experienced by individuals with CF and their healthcare

providers. Therefore, a thoughtful evaluation of the impact of vitamin D supplementation on health outcomes, overall patient compliance with therapy and healthcare costs should be performed.

First, it is essential to address the impact of vitamin D on clinically relevant outcomes. We chose the outcomes of hospitalizations, antibiotic therapy, return to baseline lung function and survival with the advice of CF physicians as outcomes of clinical importance. In the vitamin D group there was an improvement or a trend for improvement in all of these outcomes compared to placebo. Although this pilot study had a small sample size, the clustering of improved clinical outcomes and surrogate markers of disease severity in the vitamin D group provides evidence that vitamin D repletion during CF pulmonary exacerbation may have a beneficial impact on clinical outcomes.

Lung function is important to evaluate in relationship to vitamin D supplementation since vitamin D status has been positively correlated with lung function and lung function is one indicator of disease progression. The majority of lung function deterioration is accumulated during pulmonary exacerbations; therefore, recovery of baseline lung function is an indicator of disease progression (172). Our study found a trend for increased recovery of baseline lung function in the vitamin D group compared to the placebo. The impact of vitamin D on longer-term outcomes such as survival and hospitalizations may be explained by the increase in the return to baseline lung function in the vitamin D group. In future studies, the relationship of vitamin D status to the recovery of lung function and long-term clinical outcomes should be evaluated.

Evaluation of vitamin D toxicity is important in trials of vitamin D supplementation since toxicity has been reported due to the inadvertent intake of large

doses. There were no signs of vitamin D toxicity with a single large, oral bolus of cholecalciferol; no reports of clinical symptoms of hypercalcemia or elevated serum ionized calcium. There were also no statistically significant differences in PTH concentrations between the two groups; therefore, we were unable determine if this strategy impacts PTH concentrations. Larger studies are necessary to evaluate the safety of this dose and any impact on PTH concentrations.

The lack of impact on PTH concentrations may be due to a small sample size or the timing of sample collection. However, it may also indicate that the rapid increase in vitamin D status and its subsequent return to close to pre-intervention concentrations may have not been sufficient to impact PTH concentrations. This is a concern, since adults with CF have a high risk of reduced bone density and increased PTH may cause loss of bone mineralization leading to further reductions in bone density. Longer term dosing regimens should be evaluated to determine if PTH concentrations may be suppressed over longer periods of vitamin D supplementation.

Finally, assessment of the impact of vitamin D supplementation should include an evaluation of patient compliance and economic effects. We did not directly evaluate the impact of this supplementation method on patient compliance or healthcare costs. However, the use of a single, bolus dose of vitamin D to improve vitamin D status may impact both of these outcomes. Previous vitamin D repletion strategies have required multiple, large doses over a period of weeks. These methods have also recommended evaluation of vitamin D status over a period of months after completion of therapy (30-33, 35). A single dose may be administered during patient visits, increasing compliance and reducing the burden of therapy. Additionally, the 25(OH)D response to a single bolus

dose may be evaluated in shorter time intervals due to the large increase in 25(OH)D concentrations at 1-week post-intervention. This may reduce the timespan necessary to replete vitamin D or determine that an individual should be referred to a specialist for additional evaluation. The economic impact of vitamin D repletion is difficult to evaluate; however, fewer days of hospitalization, fewer IV antibiotics prescribed would be expected reduce healthcare costs. This study provides essential preliminary data that will be useful in the design of further analyses of the impact of vitamin D supplementation in CF.

Implications for the evaluation of systemic inflammation in CF

Systemic levels of inflammation are important in CF both for their impact on pulmonary inflammation and their impact on other CF comorbidities. Individuals with CF are at increased risk for both low bone mineral density and diabetes which are both related to increased inflammation (233). Chronic inflammation has a negative impact on bone metabolism and has been linked to impaired linear growth and bone mineral accrual (21). Pre-pubertal high levels of IL-6 have been shown to increase bone mineralization defects (234). Chronic systemic inflammation may increase the risk for low bone mineral density in CF. Vitamin D may reduce systemic levels of IL-6 and lessen the impact of inflammation on the risk for reduced bone mineral density and growth in CF. Vitamin D repletion should be evaluated for its impact on markers of bone turnover.

The reduction in concentrations of the inflammatory cytokines IL-6 and TNF-α in the vitamin D group compared to the placebo was not accompanied by changes in the concentrations of IL-1β. The production of IL-1β is regulated by two pathways, the NFκB and caspase-1 pathways (235). The inflammasome recognizes both exogenous and

endogenous signals leading to the activation of caspase-1 (236). Therefore, the activation of IL-1β may be impacted by multiple signals with which vitamin D supplementation may or may not interact. *In vitro* analysis may be able to assess the impact of vitamin D on both of these pathways.

Concentrations of IL-10 were also unchanged in the vitamin D group compared to the placebo group. This may be related to the many immune cell types that synthesize IL-10 and the complexity of the regulation of its expression (237, 238). Therefore, vitamin D supplementation may not sufficiently interact with all these components to regulate systemic levels of IL-10. However, further research into the impact of vitamin D sufficiency on immune cell function in CF may elucidate a role for vitamin D in the regulation of IL-10 in some immune cell types.

Diabetes and inflammation act as a reciprocal feed-forward loop, increased inflammation causes β-cell dysfunction and reduced insulin sensitivity, that in turn increases inflammation (239). Vitamin D status has been inversely correlated with the risk for type 1 and type 2 diabetes (144, 145). CF-related diabetes (CFRD), has been linked to reduced lung function, bone mineral density and survival (209). Inflammation and reduced lung function are associated with impaired glucose tolerance and diabetes in CF (209, 240). Insulin therapy of insulinopenia in CF has been shown to reduce the deterioration of lung function (241). Vitamin D repletion may reduce both the risk for glucose intolerance, development of full diabetes, and may reduce the inflammatory response that is linked to the deterioration of lung function in CFRD.

Limiting inflammation through the use of anti-inflammatory therapies has shown promise in clinical trials. Trials of glucocorticoids, such as prednisone, have been shown

to reduce deterioration of lung function. However, these trials had to be discontinued due to the rate of side effects such as glucose intolerance, growth impairment and cataracts (242, 243). Ibuprofen has also been shown to slow the progression of lung disease, particularly in those with mild lung disease; and it has been found to have an acceptable safety profile in a Cochrane review (244). However, there is limited use of ibuprofen due to possible side effects and the need to titrate dosage (245). If ibuprofen is not titrated to high enough levels, it may increase inflammation by increasing neutrophil influx into the CF lung (246). These anti-inflammatory interventions have had limited use as a component of CF therapy. However, clinicians and researchers continue to evaluate anti-inflammatory therapies (247, 248). Vitamin D does modulate the immune response and has been shown to have anti-inflammatory effects. Therefore, as a component of anti-inflammatory therapy, vitamin D may benefit CF clinical outcomes.

Vitamin D bolus dosing during hospitalization for a pulmonary exacerbation may be particularly beneficial since hospitalization includes more intensive respiratory therapies that rehydrate airways and breakdown mucus. The BALF of CF patients contains many proteins, polysaccharides, glycosaminoglycans and neutrophil extracellular nets that inhibit the activity of anti-microbial peptides (249). Hydration of the airways and the use of hypertonic saline may increase the activity of endogenous antimicrobial peptides by releasing the antimicrobial peptides from these complexes (250). Therefore, optimal antimicrobial peptide response to vitamin D repletion may depend on other therapies that facilitate the activity of these peptides.

Implications for public health

Addressing vitamin D insufficiency in the US population is a public health issue because of the high prevalence of insufficiency and the numerous health outcomes related to insufficiency (9, 157, 207). Cutaneous synthesis of vitamin D from UVB exposure, the natural source of vitamin D, has been discouraged due to the increased risk for skin cancers associated with excess sun exposure. The first recommendation on the American Cancer Society website for the prevention of skin cancer is to avoid sun exposure between 10AM-4PM (251, 252). This recommendation all but eliminates adequate UVB exposure to produce vitamin D in the epidermis (126). There are also few unfortified sources of vitamin D in the US diet and it is estimated that the US population obtains approximately 75% of their dietary intake of vitamin D from fortified foods or supplements (17). Therefore the formulation of dietary supplements and fortified foods will impact the prevalence of vitamin D insufficiency.

Vitamin D supplement sales have increased dramatically in the past decade, a greater than 6-fold increase since 2001. From 2008 to 2009, sales of vitamin D supplements more than doubled (253).(115) With the recent increase in the RDA for vitamin D from the IOM, these increases in vitamin D supplement sales may remain strong (254). The number of news articles about vitamin D published on the internet has also increased. A Google news search found a more than 15-fold increase in the number of articles published between 2000-2001 and 2010-2011. Many of these reports describe the benefits of supplemental vitamin D. This illustrates the increased interest in the benefits of vitamin D supplementation and increased utilization of the vitamin D supplements by the US population. Therefore, the scientific and medical communities

should communicate the importance of moderate vitamin D intake and the role for moderate sun exposure as components of a healthy lifestyle (255).

In spite of increased sales and information, segments of the population at increased risk for vitamin D deficiency, non-Hispanic black Americans and children less than 1 year of age appear to be experiencing increased prevalence of vitamin D insufficiency (1, 12, 224, 256). Therefore, studies that evaluate repletion strategies in these populations should be emphasized. Healthcare providers should also be educated about the risk factors of vitamin D deficiency and the appropriate methods for evaluation and repletion of vitamin D status.

Our review of the literature regarding supplement vehicle and bioavailability indicates that additional research is necessary to assist in the formulation of supplements with the greatest bioavailability, particularly in those with malabsorption who are at increased risk of vitamin D insufficiency. This research will also be valuable to inform fortification programs and to educate the public about the best supplements to purchase over-the-counter.

Implications for future research

Within the CF population, our study indicates that vitamin D supplementation may reduce the number of days hospitalized and reduce the necessity for IV antibiotic therapy. These improvements in clinical outcomes may lead to financial savings to our health care system. Therefore, further analysis of the impact of vitamin D supplementation within the CF population may provide additional evidence for a positive clinical and economic impact of vitamin D repletion.

There is limited information about the time course of the absorption and bioavailability of a single, large dose vitamin D in CF. An intermittent dosing schedule may be convenient for individuals with CF and increase adherence to a vitamin D supplementation protocol. However, there is little information about the time course of vitamin D absorption and changes in vitamin D status. Given the long circulating half-life of 25(OH)D and the potential role of vitamin D in acute infection, especially during pulmonary exacerbation, a bolus dosing strategy may also improve clinical outcomes. Future studies should evaluate an intermittent, high dose vitamin D supplementation strategy in maintaining 25(OH)D concentrations.

Additionally, long-term maintenance of vitamin D sufficiency should be evaluated to determine whether it may reduce systemic and pulmonary inflammation. Short-term modulation of inflammation by increasing serum 25(OH)D concentrations may act directly on previously activated T-cells and B-cells, reducing the production of inflammatory mediators (100). Long-term dosing of vitamin D may alter the maturation and activity of DC cells that stimulate the production of mature Th1 phenotype T-cells from naïve T-cells (257). The Th1 T-cells produce inflammatory cytokines such as IL-1, IL-6, IL-8, and TNF-α (84). Th1 T-cells have been linked with the exaggerated inflammatory response in CF. Maintenance of vitamin D status may reduce the production of inflammatory mediators through both direct impact on T-cells and the DC stimulation of naïve T-cells.

Evaluation of vitamin D in conjunction with therapies that reduce mucus viscosity or disrupt the structure of neutrophil extracellular traps may increase the effectiveness of vitamin D repletion. A factor that limits the activity of LL-37 on bacterial pathogens in

the CF lung is the increased density of neutrophil extracellular traps produced by neutrophils and the presence of other extra-cellular polysaccharides and glycoproteins (249, 258, 259). If vitamin D repletion increases the production of antimicrobial peptides in the CF lung, therapies that limit the binding and inactivation of antimicrobial peptides should enhance the clinical impact of vitamin D sufficiency.

The reduction of circulating inflammatory cytokines, may be due to their reduced production from circulating monocytes. In order to determine this, the production of vitamin D related enzymes, 1α-hydroxylase, VDR, and 24α-hydroxylase in peripheral blood mononuclear cells may be assessed from both vitamin D supplemented and un-supplemented CF subjects. Stubbs, *et al*. demonstrated that there was a change in these enzymes that occurred with vitamin D supplementation, as well as, a reduction in blood concentrations of IL-8, IL-6 and TNF-α (29). Changes in the vitamin D-related enzymes may help to clarify how vitamin D supplementation impacts levels of circulating inflammatory cytokines.

There is some research that indicates that higher concentrations of 25(OH)D, >30-40 ng/ml, may have additional health benefits in the general population (115). This hypothesis has not been addressed in the CF population. Some of the improved health outcomes that have been associated with higher 25(OH)D concentrations, including cancer, have not had a great impact in the CF population (185). However, it may be beneficial to look at CF specific outcomes that may be improved by increased 25(OH)D concentrations, such as CFRD, frequency of pulmonary exacerbations and deterioration of lung function in relationship with the long-term maintenance of vitamin D status.

Research continues to indicate that increasing the vitamin D status of the US population may relieve some morbidity and mortality. Then, an analysis of the economic costs and benefits of a broader vitamin D fortification program should be performed. Cardiovascular disease, cancer and chronic lower respiratory diseases, are the top three causes of mortality with the US population and have been linked to vitamin D insufficiency (44). Therefore, increasing the vitamin D status of the population may have an impact on public health; as well as, a financial impact.

SUMMARY

There is increased interest in the benefits of vitamin D research both in the general population and in CF. Vitamin D may be a valuable component of CF therapy as it may boost the innate response yet limit excess inflammation.

Addressing the challenges of vitamin D repletion in CF related to the high prevalence of fat malabsorption is an essential component of evaluating the long-term impact of vitamin D on health outcomes. We have found that there is inadequate information to determine the most bioavailable vitamin D supplement in CF. Evidence does suggest that supplement vehicle may have a major impact on supplement bioavailability. However, further research is necessary to determine the importance of supplement vehicle on vitamin D bioavailability.

Understanding the mechanisms by which vitamin D may benefit individuals with CF is still in its initial phases. It appears that vitamin D is likely to be a player in a large, complex system that modulates immune function. Additionally, vitamin D may both maintain the activity of the innate immune response, while assisting in the normal

maintenance of tolerance in the adaptive immune response. Hence, these characteristics may make vitamin D an important component of CF therapy in the future.

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