THE EFFECTS OF REGULAR TANNING BED USE AND INCREASED VITAMIN D STATUS ON BONE MINERAL DENSITY AND SERUM INFLAMMATORY MARKERS IN HEALTHY WOMEN

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MARY ELIZABETH HEFFERNAN

Dr. Catherine Peterson, Thesis Supervisor

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The undersigned, appointed by the dean of the Graduate School, have examined the thesis entitled

THE EFFECTS OF REGULAR TANNING BED USE AND INCREASED VITAMIN D STATUS ON BONE MINERAL DENSITY AND SERUM INFLAMMATORY MARKERS IN HEALTHY WOMEN

Presented by Mary Elizabeth Heffernan,

a candidate for the degree of Master of Science,

and hereby certify that, in their opinion, it is worthy of acceptance.

Catherine Peterson, PhD, RD

Laura Hillman, MD

Pam Hinton, PhD

Mark Ellersieck, PhD

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ABSTRACT

Vitamin D is synthesized in the skin in response to UVB radiation and has an essential function in optimal bone health. Recent evidence has also implicated a role for vitamin D in a properly functioning immune system. The objective of this study was to determine the relationship between vitamin D status, inflammatory markers, and bone mineral density in healthy pre- and post-menopausal women who regularly use tanning beds. This observational study examined 69 healthy female subjects: 49 Tanners and 20 Non-Tanners. Subjects provided medical and dietary information, a blood specimen, and bone mineral density was measured. Blood specimens were analyzed for serum 25(OH)D, parathyroid hormone (iPTH), estradiol (E_2), cortisol, and inflammatory markers. **Results:** Tanners had serum 25(OH)D concentrations that were significantly higher ($P<0.0001$) and iPTH concentrations that were significantly lower ($P<0.0001$) than Non-Tanners. There were no differences in bone density between groups. Tanners had significantly lower serum TNFα ($P<0.0200$) and a linear regression revealed that 25(OH)D had a significant inverse relationship with TNFα ($P<0.0463$), which remained significant after controlling for potential covariates. **Conclusions:** Serum 25(OH)D status is inversely related to TNFα concentrations in healthy women, which may in part explain its role in the prevention and treatment of numerous diseases.

INTRODUCTION

Vitamin D is an essential nutrient that is provided by both dietary and exogenous sources. Until recently, it had been assumed that vitamin D deficiency had been eliminated as a significant health problem. However, new evidence has indicated a re-emergence of vitamin D-deficient rickets, as well as an alarming prevalence of low circulating levels of vitamin D in the United States population. These findings indicate potential health problems related to both the calcemic functions of vitamin D, particularly associated with bone health; and the more recently discovered non-calcemic functions of vitamin D, specifically related to proper immune functioning.

Vitamin D is classically known for its function in calcium homeostasis. When serum calcium concentrations drop, active vitamin D ($1,25(OH)_2D$) acts as a hormone and signals the intestine to increase both calcium and phosphorus absorption. Additionally, vitamin D functions at the level of the bone and promotes its mineralization. Thus, vitamin D plays an important role in building and maintaining strong bones, preventing diseases such as rickets and osteoporosis.

In addition to its calciotropic functions, vitamin D also functions as an immunomodulatory hormone capable of influencing nearly every cell in the immune system, often mediated through cytokine secretion. Additionally, vitamin D has been implicated in the protection against and treatment of numerous autoimmune diseases, including multiple sclerosis, rheumatoid arthritis, inflammatory bowel diseases, and type I diabetes. A relationship has also been identified between vitamin D and the prevention and treatment of cardiovascular disease; several cancers, specifically breast, prostate, colon, lung, and ovarian; as well as infectious diseases such as tuberculosis and influenza.

The link between vitamin D and this expansive list of diseases and condition indicates a need to examine the relationship between vitamin D and inflammatory marker status in not only those suffering from disease, but also otherwise healthy individuals.

EXTENDED LITERATURE REVIEW

I. Vitamin D

Structure

Structure. Vitamin D is a fat soluble, secosteroid hormone that exists in two forms: vitamin D_2 and vitamin D_3. Vitamin D_2 is a photoproduct of the irradiation of ergosterol, a fungal sterol also known as provitamin D_2. Vitamin D_3, also known as cholecalciferol, is produced following the irradiation of provitamin D_3 (7-dehydrocholestrol). Exposure of provitamin D_3 to ultraviolet radiation produces a photoproduct called previtamin D_3. Previtamin D_3 is thermodynamically unstable and undergoes rearrangement of its double bonds to form vitamin D_3 in a temperature dependant process. The only structural difference between vitamin D_2 and vitamin D_3 is found in their side chains (Figure 1). Vitamin D_2 contains a double bond between C_{22} and C_{23} and a methyl group on C_{24}.(1) The term *vitamin D* without a subscript refers to either vitamin D_2, vitamin D_3 or both.

Synthesis in the Skin

Synthesis. Ultraviolet B (UVB) radiation (209-315 nm), absorbed by provitamin D_3, is responsible for vitamin D formation in the skin. Approximately 80-90% of the previtamin D_3 formation occurs within the plasma membrane of the actively growing layers of the epidermis.(2) Previtamin D_3 isomerizes to vitamin D_3 and it is ejected from the plasma membrane into the extracellular space. The vitamin D binding protein (DBP) in circulation then attracts vitamin D_3 into the dermal capillary bed and vitamin D enters circulation (Figure 2).(3)

Regulation in skin. The skin naturally regulates the synthesis of previtamin D_3 in the skin via several mechanisms. First, melanin (responsible for skin pigment) competes with provitamin D_3 for UVB photons, therefore, limiting production of previtamin D_3. Second, previtamin D_3 can also absorb ultraviolet radiation, converting it into biologically inert isomers lumisterol and tachysterol (Figure 2). Additionally, provitamin D_3 is rapidly converted to previtamin D_3 in the first few minutes of UVB exposure; however, previtamin D_3 photodegradation occurs during prolonged exposure. (4) Finally, vitamin D_3 is also susceptible to degradation upon exposure to sunlight. Vitamin D_3 must exit the epidermis and move into the dermal capillary bed, or it will be rapidly degraded into photoisomers, primarily 5,6-*trans*-vitamin D3, supersterol I, and supersterol II.(5)

Factors Affecting Synthesis. Several other factors, aside from photochemical regulation, affect the skin's ability to produce vitamin D_3. Season, latitude, and time of day all affect vitamin D_3 production by altering UVB photons absorbed by the ozone layer. The solar zenith angle of the sun is more slanted as winter approaches, in higher latitudes, and in the early morning and late afternoon, causing the total number of photons reaching the Earth's surface during these times to diminish. During the day, UV levels are also at their most intense when the sun is at its highest point, which occurs around noon. At this point, the radiation has the least distance to travel through the atmosphere and more UVB photons are present to enable the production of vitamin D_3.(6) Webb *et al*, showed that exposure to sunlight in Boston (42º N) caused synthesis of previtamin D_3 in human skin from March through October; however, by November, the amount of UVB photons present was insufficient to promote vitamin D formation. Moving south, a sufficient amount of UVB photons to produce previtamin D_3 year-round

was discovered in both Los Angeles (34º N) and Puerto Rico (18ºN). Further north, in Edmonton, Canada (52º N), the period of provitamin D_3 conversion lasted from only mid-March to mid-October.(7) In addition to the ozone layer, sunscreens also block UVB photons. A sunscreen with a sun protection factor (SPF) of 8 blocks approximately 95% of previtamin D_3 production.(8) Finally, age also affects cutaneous vitamin D_3 synthesis. The conversion of provitamin D_3 to previtamin D_3 is limited by the concentration of the provitamin in the skin. MacLaughlin *et al*, showed that the concentrations of protvitamin D_3 in the epidermis are inversely related to age.(9) Upon further investigation, Holick *et al*, exposed healthy young and elderly subjects to identical amounts of UVB light and discovered peak circulating concentrations of 25-hydroxyvitamin D (25(OH)D) were 30% lower in the elderly compared with the young subjects.(10)

Vitamin D from Food

Food Sources. Only a few foods, such as fish liver oils, fatty fish, and sun-dried mushrooms, naturally contain vitamin D. Fortified foods include breakfast cereals, milk, milk products, grain products, pastas, margarine, and some brands of orange juice (Table 1). In the United States fortified milk is the primary dietary source of vitamin D. Nevertheless, when testing the actual vitamin D content in milk, Holick *et al*, found 71% of tested samples contained either less than 80% or greater than 120% the amount of vitamin D claimed on the label.(11) A follow-up study conducted over a year later again found discrepancies between the label and the actual amount of vitamin D found in the milk. Eighty percent of the samples contained either 20% less or more than the label claimed, and14% had undetectable amounts of vitamin D.(12) The type of vitamin D

used to supplement food can also vary. Both D_2 and D_3 can be used as a supplement; however, recent research has shown that vitamin D_2 (ergocalciferol) potency is less than 1/3 that of vitamin D_3 (cholecalciferol). Both produce a similar initial increase in serum 25(OH)D, but serum values following D_3 supplementation continue to rise past those with D2 supplementation. Furthermore, serum 25(OH)D values also decrease more rapidly following D_2 supplementation.(13, 14)

Digestion and Absorption. Upon ingesting vitamin D from food or drink, it becomes incorporated into chylomicrons and approximately 80% is absorbed into the lymphatic system. Following ingestion, it takes only a few hours for blood levels of 25(OH)D to increase. A peak occurs approximately 12 hours later and values fall back to baseline near 72 hours.(1)

Metabolism

Circulation. Once vitamin D enters the blood stream, either via intestinal absorption or cutaneous synthesis, it is bound by the vitamin D-binding protein (DBP). DBP is primary made by hepatic parenchymal cells. It has been detected in the cerebrospinal fluid, seminal fluid, saliva, and breast milk.(15) DBP binds both 25(OH)D and $1,25(OH)_2D$, solublizing these two sterols for serum transport. Under normal physiological conditions DBP circulates in the blood at concentrations 20-fold higher than the total amount of vitamin D metabolites, thus most of the circulating vitamin D metabolites are bound to DBP.(16) Both vitamin D_2 and vitamin D_3 are biologically inert compounds, and must be hydroxylated twice in order to become active and capable of binding to its receptor. Vitamin D is first transported to the liver, where its first

hydroxylation occurs on carbon 25 via the enzyme 25-hydroxylase (25-OH-ase), forming 25(OH)D. This is the major circulating form of vitamin D and is the primary determinant of vitamin D status. 25(OH)D can also be stored in fat tissue and then released into circulation as needed.

Conversion to Active Form. The enzyme 25-hydroxyvitamin D 1α-hydroxylase (1α-OH-ase) is responsible for converting 25(OH)D to its active form, 1,25-dihydroxyvitamin D ($1,25(OH)_2D$). This enzyme is classically identified in the proximal tubules of the kidney. Nevertheless, recent studies have identified 1α-OH-ase expression in a wide range of extrarenal tissues, including epidermal keratinocytes, activated macrophages, and epithelial cells in the prostate, breast, and colon.(17-19) Renal activation of 25(OH)D results in elevated levels of circulating $1,25(OH)_2D$, which subsequently bind to the vitamin D receptor (VDR) in target tissues. Extrarenal formation of $1,25(OH)_2D$, however, is not released into the bloodstream, but instead appears to act locally by binding to VDRs present with the same or adjoining cells. This local interaction may be responsible for effects of $1,25(OH)_2D$ on regulation of cell proliferation, differentiation, and apoptosis.(20)

Degradation. In addition to being converted to its active form, 25(OH)D can also be hydroxylated on carbon 24 by the enzyme vitamin D 24-hydroxylase (24-OH-ase), forming $24,25(OH)_2D$. This metabolite does not readily bind to VDR, and its production is the first step in the degradative pathway of 25(OH)D. 24-hydroxylase is also capable of hydroxylating $1,25(OH)_2D$, producing the inactive metabolite $1,24,25\ (OH)_3D$.(21) The final metabolic fate of vitamin D is excretion in bile as calcitroic acid, an inactive and water-soluble molecule.(22)

Regulation of Metabolism

Skin. Vitamin D is regulated at several steps throughout its metabolic pathway. As stated previously, the first regulated step is at the level of synthesis. When exposed to excessive sunlight, previtamin D3 is converted into vitamin D3, as well as biologically inactive metabolites, lumesterol or tachysterol. Furthermore, excessive sunlight can also degrade vitamin D3 into inert suprasterols I and II.(5) These mechanisms prevent vitamin D intoxication via sunlight exposures.

Liver. Once vitamin D enters circulation, regulation can also occur at the liver, the site of the first hydroxylation step. The enzyme 25-OH-ase, which is responsible for converting vitamin D to 25(OH)D, is regulated via feedback inhibition by $1,25(OH)_2D$. Thus, hepatic production of 25(OH)D is inhibited by high levels of circulating $1,25(OH)_2D$. (23)

Kidney. The primary mechanism of regulation occurs at the level of the kidney. When circulating ionized calcium concentrations decrease, the parathyroid gland detects this change and increases production and secretion of parathyroid hormone (PTH). PTH increases cyclic AMP (cAMP) and changes the phosphate concentration within the renal tubule, which causes increased production of $1,25(OH)_2D$ by the enzyme 1α-OH-ase. $1,25(OH)_2D$ can then enter circulation and bind to its receptor in the small intestine and increase the efficiency of intestinal calcium absorption (Figure 3).(24)

Vitamin D Status

Vitamin D status is measured by serum 25(OH)D concentrations. A 25(OH)D concentration of < 20 to 25 nmol/L is the accepted range for clinical deficiency, and thus, high risk for osteomalacia and rickets.(25) However, these conditions are most commonly diagnosed by a serum 25(OH)D below 40 nmol/L. Recently, there has been considerable debate regarding the classification of "normal," "insufficient," and "deficient" 25(OH)D status in humans. Because vitamin D status influences PTH, calcium, and bone mineral density (BMD), these biomarkers have recently been used to estimate normal, insufficient, and deficient values of 25(OH)D. Due to the inverse relationship between vitamin D and PTH, secondary hyperparathyroidism is the primary indicator of poor vitamin D status.(26-28) Recently, several researchers have exploited this relationship as a means to determine optimal 25(OH)D levels.(27-30) The concentration of 25(OH)D at which PTH reaches its nadir is ≥ 80-100 nmol/L; while, the elevated PTH values occurring at serum 25(OH)D concentrations below this level reflect impaired absorption of dietary calcium. Furthermore, Heaney *et al.* shows that humans with mean serum 25(OH)D levels of 50 nmol/L had significantly reduced calcium absorptive performance compared with a mean 25(OH)D level of 86 nmol/L.(30) Due to the effects of calcium on bone, BMD is also affected by low 25(OH)D levels. Using the NHANES III data, bone mineral density and serum 25(OH)D levels were compared and a significant positive association between 25(OH)D levels and bone mineral density was found in whites, Mexican Americans, and blacks.(31) Higher 25(OH)D levels were associated with greater bone density in all groups, indicating that 25(OH)D levels at the upper end of the reference range (90 -100 nmol/L) is important for maximal bone mineral

density. In addition, Trivedi *et al.* reported a reduced fracture risk in men and women over age 65 with vitamin D supplementation that raised 25(OH)D to levels to 74.3 nmol/L compared with the placebo group, whose average 25(OH)D was 53.4 nmol/L.(32) Furthermore, there was a 33% reduction in typical osteoporotic fractures relative to the placebo. Hollis B.W. argues, that to properly define normal status in healthy subjects, we must look to sunbathers, fieldworkers, and individuals who work outside and do not wear sunscreen.(27) Since humans did not evolve in a "sun-shy" environment, we must look to those who do not prevent sun exposure in order to develop a range of normal. Serum 25(OH)D levels range from 135 – 225 nmol/L in individuals living in sun-rich environments. In response to the significant amount of recent research on appropriate vitamin D status, Grant, W.B. and Holick, M.F. developed new recommendations for 25(OH)D levels (Table 2).(33)

The Food and Nutrition Board (FNB) of the Institute of Medicine established the current recommendations for vitamin D intake in 1997.(34) The recommendation is 200 IU for children and adults up to 50 years of age. For adults ages 51 to 70, the recommendation is 400 IU, and for those over 70 years of age, 600 IU is recommended. These guidelines were set based on intake required to prevent bone diseases. However, a significant amount of research has been completed in the past 10 years to indicate that these numbers are insufficient for both health and disease prevention. Bischoff-Ferrari *et al.* summarized evidence from randomized controlled trials, consistent evidence from prospective and cross-sectional epidemiologic studies, strong mechanistic evidence, and dose-response relationships to determine an optimal serum 25(OH)D concentration.(35) They showed that for all endpoints, optimal 25(OH)D status began at 75 nmol/L, and was

at best between the range of 90 – 100 nmol/L. Furthermore, they concluded that these concentrations could not be reached following the current recommendations, and that an intake of $\geq$ 1000 IU per day was needed to achieve a concentration of 75 nmol/L in no less than 50% of the adult population. Heaney, R.P. has examined the vitamin D input needed to achieve an optimal serum 25(OH)D concentration of 80 nmol/L.(36) Using a regression of vitamin D supplementation-induced increases in serum 25(OH)D, Heaney created guidelines for daily oral vitamin D_3 (cholecalciferol) intake (Table 3). As is clearly evident from this table, these recommendations significantly exceed those proposed by the FNB in 1997. In fact, it is suggested that those with 25(OH)D concentrations between 20 and 40 nmol/L ingest an oral dose of 2200 IU of D_3, which is 200 IU over the tolerable upper limit (UL). However, the UL has been repeatedly disputed, with research showing that supplementation up to 4000 IU of vitamin D_3 per day is safe for up to 6 months.(37-39) Furthermore, Hathcock *et al.* applied the risk assessment method used by the FNB to derive an updated UL for vitamin D.(40) Results revealed an absence of hypervitaminosis D in healthy adults ingesting 10,000 IU vitamin D_3 daily, and therefore concluded that this value is an appropriate UL. Furthermore, Vieth maintains that the decision by the FNB to set up the UL at 2000 IU/day was based on limited review of the literature and ignored studies indicating that higher doses were safe.(41)

Significant concern has been raised since the onslaught of research in the past decade has revealed deficient 25(OH)D status spanning all ages, both sexes, and across the world.(25, 42-52) Table 4 shows the percentage of young adults in Boston, MA with vitamin D insufficiency, as characterized by a serum 25(OH)D level less than 50 nmol/L.

An editorial written by 15 of the top researchers in the field had the following to say regarding the current recommendations for vitamin D status and intake:

> "Regrettably, we are now stuck in a revolving cycle of publications that are documenting the same vitamin D inadequacy . . . the phenomenon will continue for as long as the levels of vitamin D fortification and supplementation and the practical advice offered to the public remain essentially the same as they were in the era before we knew that 25(OH)D even existed."(53)

Vitamin D Receptors

Genomic effects. The receptor protein for vitamin D is a member of the steriod hormone receptor family.(54) Consistent with all nuclear receptors, the vitamin D receptor (VDR) contains an activation domain located at the N-terminous, a DNA-binding region containing two zinc fingers, a ligand-binding domain, and a hinge region. Once the ligand binds to its domain, the receptor dimerizes with the retinoic X receptor (RXR), and binds to the DNA-response element in the promoter region of the gene. Binding to the DNA-response element causes gene transcription.(55) Additionally, there are several coactivating or corepressing proteins can influence gene expression.(56, 57)

Non-genomic effects. In addition to altering gene expression, $1,25(OH)_2D$ can also cause rapid, nongenomic effects on signal transduction pathways to induce responses such as opening voltage-gated calcium and chloride channels.(58, 59) Some of these effects were discovered to be steroid specific, thus, there is an ongoing debate as to whether the vitamin D steroid receptor could also be responsible for the non-genomic effects of vitamin D. Erben *et al.* showed that disruption of the VDR caused an inhibition of the rapid nongenomic effects of vitamin D.(60)

Biological Functions of Vitamin D

Calciotropic functions. The primary physiological function of vitamin D is to maintain intracellular and extracellular calcium concentrations. This is accomplished via the effect of $1,25(OH)_2D$ in the intestine and bone. After binding to the VDR, transcription occurs, followed by the translation of numerous proteins, including a calcium-binding protein.(1) This protein is required for calcium transport from the intestine, enabling increased absorption of calcium and phosphorus into circulation. In addition to acting in the intestine through the VDR, $1,25(OH)_2D$ is capable of inducing non-genomic rapid transport of calcium across the lumen of the intestine.(61) This hormonally-stimulated transport is termed transcaltachia and it is thought to be mediated locally by the ionized calcium concentration of the vasculature.

$1,25(OH)_2D$ enhances mobilization of calcium and phosphorus from bone stores during times of calcium deprivation by inducing stem cell monocytes to become mature osteoclasts, which initiates the release of calcium and phosphorus into circulation.(62) In addition, steroid receptors for $1,25(OH)_2D$ are also present on the plasma membrane of osteoblasts and $1,25(OH)_2D$ has been shown to not only inhibit osteoblast differentiation, but also stimulate bone formation by mature osteoblasts.(63, 64) Thus, the role of $1,25(OH)_2D$ in bone is complex and multidimensional.

Non-calciotropic functions. The VDR is also found in significant concentrations in T lymphocytes and macrophages.(65) Furthermore, 1α-OH-ase, the enzyme responsible for the final and rate-limiting hydroxylation step of the synthesis of $1,25(OH)_2D$, is expressed by activated macrophages, making them capable of synthesizing and secreting $1,25(OH)_2D$ in a regulated fashion.(66) Additionally, 24-

hydroxylase, the enzyme responsible for $1,25(OH)_2D$ degradation, is also expressed in monocytes, macrophages, and human tumors.(67-70) In addition, VDR has also been identified in numerous cancer cell lines and epithelial cells.(71-78) Taken together, these findings suggest a paracrine role for vitamin D in the immune system.(79)

The relationship of low serum 25(OH)D levels and autoimmune disease, particularly multiple sclerosis, inflammatory bowel diseases, Type I diabetes, and rheumatoid arthritis, has been appreciated for several years.(80-86) More recently, studies have shown defects in macrophage functions, such as chemotaxis, phagocytosis, and the production of proinflammatory cytokines under vitamin D-insufficient conditions.(87) Finally, the link between 25(OH)D status and cancer risk, particularly of the breast, prostate, and colon, has been repeatedly demonstrated.(87-96) Therefore, these finding also indicate that vitamin D plays a significant role in proper immune functioning.

II. Bone

Bone is a connective tissue that serves three main functions in the body. First, it acts as support and a site of muscle attachment. Second, bone acts as a protectant to the vital organs of the body, as well as bone marrow. Finally, bone serves as a storage site for ions, particularly calcium and phosphate, which are essential for the maintenance of serum homeostasis.(97)

Two types of bone are present in the mature human skeleton: cortical and trabecular. Cortical bone is dense, compact bone that comprises 80% of the skeleton, has a slow turnover rate, and makes up the outer part of all skeletal structures. Its primary

function is to provide mechanical strength, but can also function in metabolic processes when prolonged mineral deficit occurs. Trabecular bone comprises only 20% of the skeleton. This type of bone is less dense, more elastic, and has a higher turnover rate than cortical bone. The primary functions of trabecular bone are mechanical support and mineral supply during states of acute deficiency.(98)

Structure

Bone Mineral. The mineral of bone is comprised of spindle-shaped crystals of hydroxyapatite [$3Ca_3(PO_4)_2(OH)_2$]. Calcium makes up 37 to 40%, phosphate is 50 to 58%, and carbonate is 2 to 8% of the hydroxyapatite crystals. Bone mineral also contains small amounts of sodium, potassium, magnesium, citrate, and other extracellular ions present in the extracellular fluid at the time the mineral was deposited. The bone mineral is located on and within the extracellular matrix.(99)

Extracellular Matrix. The extracellular matrix can be further divided into the type I collagen fibers, which comprises about 90% of the matrix, and noncollagenous proteins, which contribute the other 10% of the organic matrix of bone. The type I collagen fibers are long fibrous proteins that form tight cross-linkages between fibers via covalent bonds. These fibers provide elasticity and flexibility to bone and determine structural organization. Noncollagenous proteins have various functions including, serving as chemoattractants at the bone surface for osteoclast recruitment, as well as stimulating osteoblastic activity. Thus, these proteins contain chemical signals for bone remodeling.(100)

Bone Cells. There are four primary bone cells, including lining cells, osteoblasts, osteoclasts, and osteocytes. Lining cells form a membrane that covers the bone surface and protects the bone from circulating cells and hormones. Osteoblasts synthesize, deposit, and arrange the fibrous matrix proteins to enable mineralization of the matrix. Osteoclasts are multinucleated cells that resorb bone and release the breakdown products into the extracellular fluid, which eventually enables entry into the bloodstream. Finally, osteocytes are former osteoblasts that have stopped synthesizing bone matrix and have become embedded within the layers of bone. Osteocytes monitor bone strain and are responsible for relaying that information to lining cells, which can initiate bone remodeling.(99)

Bone Remodeling

The adult skeleton is in a continual state of breakdown and reformation via the actions of osteoblasts and osteoclasts. Bone remodeling occurs at specific locations and is characterized by a sequence of events beginning with activation, followed by resorption, reversal, and finally formation. In the activation phase, lining cells retract, exposing the bone surface to the circulating blood. The mineralized bone then serves as a chemoattractant for osteoclast precursors by expressing the receptor activator of NF-κβ ligand (RANKL). RANKL can interact with a receptor on the osteoclast precursor known as RANK. The interaction of RANK with its ligand causes activation and differentiation of osteoclasts, which can then initiate resorptive activity via lysosomal enzymes.(101) These enzymes are secreted into the extracellular compartment, which has been sealed off by a ring of actin, allowing the enzyme to reach a sufficiently high

concentration. The osteoclast then acidifies the extracellular compartment by secreting protons across a gradient. The hydroxyapatite crystals are mobilized due to the digestion of their collagen link and then dissolved by the acidic environment.(97) Once a suitable volume of bone has been removed, osteoclasts undergo apoptosis. At this point, the reversal phase begins and osteoblasts are recruited to replace the bone removed from the cavity. Osteoblasts originate from undifferentiated mesenchymal stem cells in response to local growth factors. Once stimulated, they undergo proliferation and differentiation into preosteoblasts and finally mature osteoblasts. Mature osteoblasts line the layers of uncalcified bone matrix, called the osteoid tissue, and lay down the matrix between and beneath themselves, pushing backwards as new bone is added. Osteoblasts then secrete proteins into the newly formed matrix that aid in creating the three-dimensional configuration that attracts calcium and phosphate ions and arranges them in the hydroxyapatite crystals. Once the revision process is complete, the remaining surface osteoblasts become lining cells, sealing in the new bone.(99) The new bone that is formed is called a bone structural unit, or BSU.

Regulation of Bone Remodeling

For bone remodeling to begin, osteoclasts must be activated, thus factors that directly stimulate or inhibit osteoclast activity have significant influence on this process. Resorption can be stimulated by increased proliferation of osteoclast precursors, causing differentiation into mature cells, as well as by activation of mature cells to resorb bone. In addition, osteoclasts can be inhibited by blockage of precursor proliferation, inhibition

of proliferation, or inactivation of mature cells.(102) This process of remodeling can be induced via systemic hormone regulation or local hormone and cytokine regulation.

Systemic Regulation. The three most influential systemic regulators of bone remodeling include PTH, calcitonin, and $1,25(OH)_2D$. PTH is the primary regulator of bone remodeling due to its principal role in the regulation of calcium homeostasis. During periods of continuous increased PTH secretion, as is the case when calcium concentrations are low, bone resorption is stimulated. However, intermittent secretion of PTH stimulates bone formation.(103) Calcitonin is a small peptide hormone produced by the thyroid gland in response to increased calcium concentration, and its primary function is to inhibit bone resorption by acutely suppressing osteoclastic activity.(104) $1,25(OH)_2D$ is capable of inducing both bone resorption and formation via numerous mechanisms. It is a potent stimulator of osteoclastic bone resorption by both stimulating the differentiation of osteoclast precursors and activating mature osteoclasts. $1,25(OH)_2D$ also indirectly promotes bone mineralization by increasing intestinal absorption of calcium and phosphorus. Finally, due to its role in immune function,$1,25(OH)_2D$ may also inhibit resorption via inhibition of cytokine production.(102)

Several other hormones can also have regulatory effects on bone remodeling. Thyroid hormones, specifically thyroxine and triiodothyronine can stimulate bone resorption, whereas, glucocorticoids can inhibit osteoclast formation and decrease bone resorption. Finally, decreased estrogen concentrations are also associated with increased osteoclastic activity, resulting in increased bone resorption following menopause.(98)

Local Regulation. Local hormones and cytokines play a significant, yet extremely complex role in the regulation of bone remodeling. Several important local

stimulators and inhibitors of osteoclastic activity have recently been identified, inducing their effects primarily through the regulation of RANK.

As previously discussed, RANKL binds to its receptor on the surface of osteoclastic precursor cells and is necessary for differentiation, activation, and survival of osteoclasts.(101) Macrophage colony-stimulating factor (M-CSF) also works in conjunction with RANKL to induce osteoclastic bone resorption. M-CSF binds to its receptor on osteoclast precursor cells and has been shown to be necessary for osteoclast development.(105) Osteoprotegrin (OPG) is a secreted glycoprotein that is capable of binding to RANKL and blocking its effects.(106) Several cytokines modulate bone remodeling by stimulating M-CSF production and directly increasing RANKL expression. Furthermore, other cytokines can promote osteoclast maturation by regulating cellular production of OPG and RANKL.

Interleuken-1 (IL-1) is a cytokine that can be found in the form of two different molecules, IL-1α and IL-1β. Both molecules bind to the same receptor and induce the same effects on bone. IL-1 is released by both activated monocytes and osteoblasts and is a potent stimulator of osteoclasts. Thus, IL-1 has been implicated as a mediator of bone resorption and increased bone remodeling in osteoporosis.(100) Tumor Necrosis Factor-α (TNF-α) is another cytokine that is functionally related to IL-1, and it has been suggested that they can act synergistically to increase each other's secretion.(107) Interleuken-6 (IL-6) is a pleiotropic cytokine that is expressed and secreted by osteoblasts, osteoclasts, and stromal cells in response to PTH, 1,25$(OH)_2$D, and IL-1. IL-6 is a stimulator of osteoclast formation, and thus, bone resorption.(98) However, IL-6

has also been shown to promote osteoblast generation during conditions of high bone turnover.(108)

III. Immune Function

The immune system can be divided into two primary responses: innate immunity and adaptive immunity. The function of the innate immunity is to elicit a rapid response to microbes. The principle components of this type of immunity include (1) physical and chemical barriers, such as antimicrobial substances produced at epithelial surfaces; (2) phagocytic cells, including neutrophils, macrophages, and natural killer (NK) cells; (3) blood proteins and mediators of inflammation; and (4) cytokines that regulate and coordinate activities of innate immune cells. Adaptive immunity induces a more vigorous and specific response to infection and has the capability to "remember" microbes following repeated exposures. The primary components of the adaptive immunity include T and B lymphocytes. The adaptive immune response can also be broken down into two types of responses: humoral immunity and cell-mediated immunity. Humoral immunity is mediated by B lymphocytes (B cells), which acts as the primary defense against extracellular microbes due to B cells' ability to secrete antibodies. T lymphocytes (T cells) are involved in cell-mediated immunity, and primarily provide defense mechanisms against intracellular microbes by promoting destruction of microbe-infected cells. Although identified as two distinct responses, the innate and adaptive immune response work in conjunction to form an integrated defense system against microbes.(109)

Cells of the Immune System

Hematopoietic cells, also known as leukocytes and white blood cells, are formed in the bone marrow from a precursor cells known as the hematopoietic stem cell. Hematopoietic cells can be further divided into either myeloid or lymphoid lineage. Myeloid cells include neutrophils, monocytes/macrophages, eosinophils, and basophils. Cells that are included in the lymphoid lineage are T and B lymphocytes and natural killer cells. Dendritic cells can arise from either myeloid or lymphoid cells.(110)

Myeloid Cells. Monocytes are incompletely differentiated cells that originate in the bone marrow and enter circulation. Once they enter tissues, monocytes mature and become macrophages. Macrophages are present in all organs and function by ingesting microbes and activating T cells. T cells can then activate the macrophage to kill the microbe. Monocytes and macrophages are important for both innate and adaptive immunity because they phagocytose microbes and then produce cytokines that recruit and activate other inflammatory cells.(109)

Neutrophils, basophils, and eosinophils are granulocytes, which means that they are myeloid leukocytes that contain large intracellular granules that contain enzymes that can kill microbes. Neutrophils are the most numerous of the leukocytes and respond immediately to tissue injury. They are drawn from the circulation and into infected tissue, at which point they begin to phagocytose foreign cells or macromolecules. These cells or molecules are contained in intracellular vesicles called phagosomes. Cytoplasmic granules fuse with the phagosomes and release the contents of the granule into the vesicles, causing hydrolysis of the foreign molecules. Although neutrophils are critical for the initial stages of immune stimulation, they are also capable of causing significant

damage to host tissue during a prolonged inflammatory response. Eosinophils are predominantly found in connective tissue and contain large cytoplasmic granules that are filled with enzymes. When the granule contents are released from the cell (known as degranulation), they are effective at killing large parasites. Although their primary function is parasite removal, they are also capable of phagocytosing pathogens. In addition, eosinophils play a role in allergies by secreting cytokines and releasing granules that cause tissue damage. Finally, basophils are the least common of granulocytes and are important during the inflammatory response because their granules contain heparin and vasoactive amines. These compounds thin the blood and elicit vasodilation, which enables other immune cells to quickly reach the site of infection.(110)

Mast cells are another type of myeloid cells that can be found in the connective tissues, and in some cases, the gastrointestinal mucosa. Degranulation is rapidly triggered by tissue injury, and the histamine, cytokines, and growth factors released from the granules initiate the inflammatory response and can also play a role in allergy.(110)

Lymphocytes. Lymphocytes arise from stem cells in the bone marrow and undergo a complex maturation process. B cells mature in the bone marrow and T cells mature within the thymus. After maturation, lymphocytes leave the marrow or thymus, circulate, and enter peripheral lymphoid organs. These mature cells are called naïve lymphocytes. Once naïve lymphocytes encounter antigen, they differentiate into effector and memory cells. Some mature lymphocytes can also differentiate into memory cells, which function to induce rapid immune responses to subsequent antigen exposure.

Effector B lymphocytes produce antibodies, which can recognize extracellular and cellular surface antigens, in addition to differentiating into plasma cells that secret

antibodies. The antigen receptor, called the B cell receptor (BCR), is a complex containing a membrane-protein, known as an immunoglobulin (Ig), which interacts specifically with the antigen. The antibody that is secreted by the activated B cell is a soluble form of the Ig molecule.

Effector T lymphocytes can be further divided into helper T (Th) cells and cytolytic (also known as cytotoxic) T cells (CTLs). Most helper T cells express membrane proteins called CD4 and are often referred to as $CD4^+$ T cells. In addition, CTLs express proteins called CD8, and therefore are often called $CD8^+$ T cells.(109) Th cells can also be divided into to Th1 and Th2 cells. The differentiation of Th1 versus Th2 cells depends on the surrounding cytokine environment. The presence of IFNγ and IL-12 favors Th1 development, whereas the presence of IL-4 favors Th2 development.(110) Th1 cells secret IFNγ, IL-2, and TNFα and its activation is essential for proper cell-mediated immune responses, including response to tumors and intracellular pathogens. When autoimmune diseases develop, these Th1 cells are responsible for attacking self proteins, causing the onset of symptoms. Examples of Th1 induced disease include multiple sclerosis, type I diabetes mellitus, and inflammatory bowel disease. Th2 cells are required for a proper response to extracellular pathogen, particularly bacteria and parasites. In addition allergies and asthma are examples of diseases induced by Th2 cells.(111) All T cells only recognize peptide antigens attached to host proteins. These host proteins are encoded by genes in a major histocompatibility complex and are expressed on the surface of antigen-presenting cells (APCs). APCs are a population of cells whose primary function is to capture antigens and display (or present) them to T lymphocytes. Examples of APCs include monocytes, macrophages, and dendritic cells.

Once stimulated, Helper T cells secrete cytokines that stimulate proliferation and differentiation of other T cells, B cells, macrophages, and other leukocytes. Activated CTLs lyse cells that are infected by viruses and intracellular microbes that produce foreign antigens. Natural Killer cells are another subset of lymphocytes that have a primary role in innate immunity. They directly kill microbes by secreting the cytokine interferon-γ (IFN-γ).(109)

Dendritic Cells. As was previously stated, dendritic cells can arise from either myeloid or lymphoid precursors. They are the most potent APC and are located in most organs, including the epithelia. Dendritic cells function by capturing antigens and transporting them to peripheral lymph organs. Once inside the lymph organs, dendritic cells act as APCs for naïve T cells. Dendritic cells are the primary initiators of T cell mediated immune responses.(110) More recently, it has been shown that dendritic cells not only function during an immune response to an antigen, but also play a role in tolerance, an essential feature of a properly functioning immune system.(112-114) Tolerance involves the immune system's ability to recognize and eliminate foreign antigens while not reacting to the individual's own (self) antigens. Abnormalities in this function of the immune system causes improper immune responses against self antigens and results in autoimmune disorders.(109)

Vitamin D in the Immune System

The identification of VDRs in human leukocytes first implicated vitamin D as an immune system modulator.(115, 116) In addition, expression of VDRs has also been found on APCs such as macrophages and dendritic cells, providing greater support for a

role of vitamin D in immune functioning.(117) Furthermore, $1,25(OH)_2D$, as well as its analogs, have been shown to induce antiproliferative, prodifferentiatve, and immunomodulatory effects on numerous cells of the immune system (Table 5). The effects of vitamin D on these cells include decreasing the production of inflammatory cytokines and increasing the production of anti-inflammatory cytokines. These cytokines also serve as surrogate markers of disease incidence and progression in autoimmune diseases, cardiovascular disease, and many cancers, indicating biological plausibility for a role of vitamin D in the prevention and treatment of these diseases.

Monocytes, Macrophages, and Dendritic Cells. Macrophages in diseased conditions, such as sarcoidosis and tuberculosis have demonstrated the capacity to convert 25 (OH) D to $1,25(OH)_2D$ (118-120), and the expression of 1α-OH-ase on macrophages has since been confirmed.(67, 121) However, although the 1α-OH-ase enzyme found on the macrophage is identical to that found in the kidney, macrophage 1α-OH-ase is predominantly controlled by IFNγ, and $1,25(OH)_2D$ is not capable of inducing negative feedback on the enzyme, unlike the 1α-OH-ase of the kidney.(121) The enzyme 1α-OH-ase was also indentified in dendritic cells, and Hewison, ***et al.*** showed in vitro that these cells synthesize $1,25(OH)_2D$ as a result of increased 1α-OH-ase expression.(122)

Numerous studies have shown that $1,25(OH)_2D$ induces differentiation of monocyte cell lines toward macrophages.(123-125) In addition, monocytes exposed to $1,25(OH)_2D$ have a diminished capacity to stimulate T cell activation as APCs.(126) Nevertheless, exposure to $1,25(OH)_2D$ has also been shown to enhance the ability of monocytes and macrophages to achieve both phagocytosis and chemotaxis.(126) Human

peritoneal macrophages removed from patients treated by continuous ambulatory peritoneal dialysis and treated with both 25(OH)D and $1,25(OH)_2D$, followed by stimulation by lipopolysaccharide, revealed that $1,25(OH)_2D$ inhibited both the expression of TNFα mRNA and protein content in a dose dependant manner.(127) 25(OH)D incubation also down-regulated TNFα expression. Thus, $1,25(OH)_2D$ has a direct immunomodulatory effect on monocytes and macrophages.

$1,25(OH)_2D$ and its analogs have also been shown to inhibit dendritic cell differentiation and maturation and induce spontaneous apoptosis (128-132), which is of particular significance since inappropriate dendritic cell maturation can cause unnecessary T cell responses, or autoimmunity. Furthermore, the antigen-presentation function of dendritic cells treated with $1,25(OH)_2D$ during maturation is significantly reduced.(130) Studies have also shown that treatment of dendritic cells with $1,25(OH)_2D$ and its analogs cause decreased IL-12 production, an immunostimulatory cytokine, and increased IL-10 production, an immune-suppressing cytokine, which also leads to decreased T cell activation.(130, 133, 134) IL-12 production is essential for the development of Th1 cells and cellular immunity. Inhibiting the activation of IL-12 has also been shown to prevent the development and block disease progression of experimental autoimmunity(135), and D'Ambrosio, *et al.* demonstrated that $1,25(OH)_2D$ can negatively regulate IL-12 production by both macrophages and dendritic cells.(136) The capacity of dendritic cells to inhibit T cell maturation, decrease IL-12 production, and increase IL-10 production are mechanisms by which dendritic cells demonstrate their tolerogenic properties.

Inhibition of monocyte, macrophage, and dendritic cell differentiation, maturation, function and survival can lead to decreased T cell responsiveness, and may help explain the immunosuppressive capability of vitamin D.

T Lymphocytes. Aside from its influence on T lymphocytes via APCs such as monocytes, macrophages, and dendritic cells, $1,25(OH)_2D$ also has a direct effect on T lymphocytes. $1,25(OH)_2D$ has been shown to inhibit T cell proliferation (124, 137) via decreased production of IL-2 and IFNγ.(138) IL-2 is an autocrine growth factor for T cells, and decreased expression induced by $1,25(OH)_2D$ prevents T cell activation and proliferation.(139) INFγ is a major positive feedback signal for APCs, thus inhibition via $1,25(OH)_2D$ prevents antigen presentation to T cells.(140) Furthermore, $1,25(OH)_2D$ has also been shown to decrease the proliferation of all helper T cells and decrease Th production of IFNγ, IL-2, and IL-5.(141) In addition, Th2 cells showed increased production of IL-4 in the presence of $1,25(OH)_2D$ (141), indicating a propensity toward Th2 differentiation. In addition, Boonstra *et al*. also showed that treatment of Th2 cells in vitro with $1,25(OH)_2D$ produced a population of lymphocytes skewed toward Th2 differentiation due to increased IL-4, IL-5, and IL-10 production.(142) A role for $1,25(OH)_2D$ has also been implicated in T cell survival and death. Cippitelli *et al.* showed that $1,25(OH)_2D$ inhibits activation-induced T cell death by preventing FasL expression.(143) Under normal conditions FasL binds to the Fas receptor, also known as the death receptor, resulting in cell death.

A separate group of T cells known as T regulatory cells, or Tregs, have also been implicated as a target for $1,25(OH)_2D$ and a mechanism by which vitamin D might elicit immunomodulation. Gregori *et al.* show that in mice, $1,25(OH)_2D$ treatment in

combination with the immunosuppressant drug mycophenolate mofetril caused an increase in the proportion of Tregs in a lymph node located near an islet allograph.(144) Furthermore, the combination of these two immunosuppressants protected against graft rejection, and when the Tregs were transferred to untreated mice, these mice were also protected from graft rejection. Another study using a $1,25(OH)_2D$ analog revealed that treatment of diabetic mice with the analog decreased disease progression and was accompanied by increased Tregs that were capable of inhibiting T cell responses.(145) More recently, dendritic cells from myeloid lineage were exposed to $1,25(OH)_2D$ and showed increased production of CCL22, a chemoattractant for Tregs, and increased Treg activity.(134) Despite the aforementioned studies, the role of $1,25(OH)_2D$ on Treg modulation and its function in the immune system remains unclear.

Natural Killer Cells. Although the research is limited, some studies do show a potential role of $1,25(OH)_2D$ on NK cell activity. An early report by Merino *et al.*, showed that $1,25(OH)_2D$ inhibits cytotoxic activity of peripheral NK cells and suggests that the inhibition may occur at the level of activation.(146) Leung *et al.*, also investigated the effects of $1,25(OH)_2D$ on human NK cells.(147) Results indicated that vitamin D was capable of inhibiting IFN and IL-2 activation of NK cells. NK cell activity has also been investigated in patients with chronic renal failure who receive hemodialysis.(148) These patients had decreased circulating levels of $1,25(OH)_2D$ and NK activity compared with healthy controls. After 28 days of vitamin D oral supplementation, NK activity was significantly increased. More recently, NK cells isolated from the human uterine lining during the first trimester of pregnancy were treated with $1,25(OH)_2D$ and 25(OH)D, and their cytokine profile was analyzed.(149)

These cells showed decreased synthesis of both TNFα and IL-6. Taken together, these studies indicate a potential, but poorly characterized role of vitamin D in the functioning of NK cells.

B Lymphocytes. The influence of vitamin D on B lymphocytes is also poorly defined. Provvedini *et al.* first claimed discovery of the VDR expression in B cells in 1989 (115); however, in 2000, Veldman *et al.* failed to identify detectable amounts of VDRs in B cells and concluded that they are not likely targets of $1,25(OH)_2D$.(150) Morgan *et al.* suggested VDRs are up-regulated following cellular activation; therefore, reactivity of B cells to $1,25(OH)_2D$ is dependant upon stage of activation and differentiation.(151) In a follow-up study, B cell reactivity to $1,25(OH)_2D$ was characterized by its ability to up-regulate VDR and initiate $1,25(OH)_2D$-mediated gene transcription.(152) Results showed that B cell reactivity to $1,25(OH)_2D$ is present in naïve, germinal center, and memory B cell populations. In addition, activation, as opposed to differentiation, is a more important determinant for reactivity to $1,25(OH)_2D$. Aside from the discrepancies in the literature regarding VDR expression, $1,25(OH)_2D$ has been shown to inhibit Ig production both in vitro and in vivo.(153-157) Nevertheless, it is unclear whether this is the result of a direct influence of $1,25(OH)_2D$ on B lymphocytes, or an indirect effect of either inhibited T cell proliferation (158) or cytokine production by monocytes and macrophages.(159) Similar to that of NK cells, research indicates a potential role for vitamin D in B cell functioning; however, conflicting and limited research makes interpretation difficult.

IV. Disease States

Autoimmune Diseases

During periods of normal immune functioning, Th1 and Th2 cells regulate each other, producing a balance of the 2 types of helper T cells. Th1 cells secrete IFNγ, IL-2, and TNFα, and their activation is required for cell-mediated immune responses to tumors and intracellular pathogens.(111) Th2 cells secrete IL-4, IL-5, IL-6, and IL-10, which are required for proper antibody responses to extracellular pathogens.(160) Despite tight regulation, the Th1 and Th2 balance can become offset, resulting in disease pathogenesis. Autoimmune diseases result when Th1 cells begin attacking self antigens. When Th2 cells begin reacting to environmental conditions, allergies and asthma result. As previously discussed, $1,25(OH)_2D$ decreased purified Th cell production of IL-2 and increased the production of IL-4 by Th2 cells.(141) It has been shown that the secretion of these two cytokines may account for the ability of $1,25(OH)_2D$ to suppress autoimmune diseases in vivo.(161, 162)

Multiple Sclerosis. Multiple sclerosis (MS) is an autoimmune disease characterized by the break down of the myelin sheath surrounding both peripheral and central nerve axons, inflammation, scar formation, and axon destruction. The absence of these myelin sheaths causes abnormal neuron firing, leading to paralysis and possibly death.(81) MS symptoms are associated with increased pro-inflammatory cytokines, IL-2, TNFα, and IFNγ, and decreased anti-inflammatory cytokines including transforming growth factor β1 (TGF-β1) and IL-13.(163, 164)

The role of vitamin D in MS disease onset and progression is currently unclear. However, epidemiological data on MS prevalence reveals an increasing number of cases

as the distance from the equator increases.(165) Because UV radiation is negatively correlated with latitude; it has been hypothesized that the decreased prevalence of MS at lower latitudes may reflect the immunosuppressive capability of vitamin D induced via UVB light.(80, 81, 166, 167) In addition, associations have also been made between season and the number of active magnetic resonance imaging (MRI) lesions in MS patients, which reflect subclinical disease symptoms.(168, 169)

A number of studies have shown that MS patients have insufficient vitamin D status and reduced bone mass.(170, 171) Nieves et al showed that the mean circulating 25(OH)D level in population of MS patients was in the insufficient range, at 43 nmol/L.(171) In addition, 23% of the patients were vitamin D deficient, with a 25(OH)D level < 25 nmol/L. Fracture risk was also increased in this patient population. Goldberg *et al.* studied a group of MS patients who were treated with a dietary supplement containing calcium, magnesium, and vitamin D for 1 to 2 years.(172) When compared to personal history, patients receiving the supplementation experienced less than half of the expected exacerbations during the course of the study. A more recent supplementation study conducted by Mahon *et al.* revealed that providing MS patients with 6 months of 1000 IU vitamin D and 800 mg of calcium supplementation significantly increased both 25(OH)D concentrations and serum TGF-β1.(173)

Experimental autoimmune encephalomyelitis (EAE) is an experimental model of MS induced in mice. EAE is mediated by $CD4^+$ T lymphocytes that target the CNS and induce inflammation and eventually paralysis. Administration of $1,25(OH)_2D$ at the first appearance of EAE symptoms has been shown to completely prevent disease progression in mice.(174) Furthermore, withdrawal of $1,25(OH)_2D$ enabled disease progression to

ensue. Induced vitamin D deficiency in these mice also increased susceptibly to EAE. Although the mechanism by which vitamin D influences EAE progression is unclear, several hypotheses have been put forth. The spinal cord cells of mice induced with EAE followed by administration of $1,25(OH)_2D$ revealed significantly increased gene expression of pro-apoptotic proteins and evidence of increased apoptosis was detected.(175) Thus, increasing apoptosis of inflammatory cells may be one role of $1,25(OH)_2D$ in preventing EAE disease progression. It has also been hypothesized that $1,25(OH)_2D$ may inhibit EAE via the anti-inflammatory cytokine IL-10. Using mice with a disrupted IL-10 and IL-10 receptor genes, Spach *et al.* showed that $1,25(OH)_2D$ was incapable of inhibiting EAE in these mice.(176) Thus, the IL-10 / IL-10 receptor pathway has been implicated as an essential component of $1,25(OH)_2D$'s influence on EAE disease pathogenesis. Although this evidence is significant, the role of vitamin D in EAE in mice cannot be directly translated to MS in humans.(173)

Rheumatoid Arthritis. Rheumatoid arthritis (RA) is an autoimmune disease characterized by infiltration of T cells, macrophages, and B cells into the synovial membrane that lines the joints, causing a chronic state of inflammation.(177) The disease course also involves overproduction of TNFα and IL-6, mediated by a dysfunctional Th1 response.(178)

Unlike MS, RA does not have a significant association between latitude and disease prevalence.(179) Nevertheless, vitamin D insufficiency or deficiency has been documented in RA patients.(82, 83) Aguado *et al.* showed that the prevalence of vitamin D deficiency was 64% in postmenopausal women with RA.(82) In addition, the women with vitamin D deficiency showed an inverse relationship between BMD at the hip and

25(OH)D status. Kroger *et al.* showed that 16% of the 143 female (mean age 50.7) patients with RA had serum 25(OH)D concentrations below 12.5 nmol/L.(83) Furthermore, serum $1,25(OH)_2D$ levels were shown to be negatively correlated to disease activity in RA patients.(180)

Results from the Iowa Women's Health Study, a prospective cohort study of 29,368 women, showed that greater intake of vitamin D via diet or supplementation was inversely associated with RA risk, indicating a preventive role of vitamin D in disease onset.(181) Unfortunately, invention trials supplementing vitamin D and its metabolites have produced varied results in RA patients. Supplementation of 19 RA patients with 2 μg of a vitamin D analog for 3 months produced decreased disease activity in 89% of the patients, with only 2 patients showing no improvement.(182) Additionally, serum C-reactive protein, an indicator of inflammation, was also significantly decreased in these patients. Dottori *et al.* supplemented RA patients with 25(OH)D in combination with standard therapy for as little as 30 days and showed improved pain symptoms in the supplemented group compared with a group receiving only standard therapy.(183) Despite these promising results, two other studies have shown that supplementation for both 8 weeks (184) and 16 weeks(185) with either 1 or 2 μg of vitamin D analogs produces no effects on RA disease activity or symptomology. Thus, the role of vitamin D supplementation on RA disease progression, activity, or symptoms is currently unclear.

Two animal models of RA have been investigated, including Lyme arthritis and collagen-induced arthritis. Mice infected with lyme arthritis were supplemented with a diet containing $1,25(OH)_2D$ and disease symptoms were prevented.(186) Using a vitamin

D analog, Larsson et al showed that the development of arthritis in rats with collagen-induce arthritis could again be effectively suppressed by treatment.(187)

Inflammatory Bowel Diseases. Inflammatory Bowel Disease (IBD), which includes ulcerative colitis (UC) and Crohn's Disease (CD), is caused by an immune-mediated attack against the gastrointestinal tract.(111) Increased Th-1 cell cytokines, including IFNγ and IL-2, have been implicated in chronic intestinal inflammation in patients with CD.(188)

A role of vitamin D in IBD is also supported by geographic data. An evaluation of hospital discharges in the United States revealed that both CD and UC are more prevalent in the northern parts of the US.(189) In addition, prevalence was greater in urban areas than rural. These results again suggest that UVB light is associated with autoimmune disease prevalence.

At the time of diagnosis, patients with either UC or CD had a decreased 25(OH)D concentration compared with standard reference ranges and a control group with irritable bowel syndrome.(85) Twenty-seven percent of patients with CD and 15% of patients with UC were also shown to have deficient levels of 25(OH)D (<30 nmol/L).(84) CD patients also had a significantly lower mean 25(OH)D concentration compared with UC patients. Furthermore, vitamin D deficiency, as well as chronic inflammation, likely contributes to the decreased BMD found in patients with both UC and CD.(190)

Experimental IBD has been investigated in mice, and in accordance with human IBD, increased production of Th1 cytokines was associated with Crohn's disease-like symptoms.(191) Injecting T cells that preferentially produce Th1 cytokines, Frociu *et al.* showed that VDR KO mice had increased severity of IBD compared with WT mice.(192)

The IL-10 KO mouse is another experimental model of IBD that will induce symptoms similar to human IBD. Vitamin D deficient IL-10 KO mice have a significantly increased mortality rate compared to vitamin D sufficient IL-10 knockouts, and vitamin D supplementation in the deficient mice resolves the symptoms.(193) When IL-10/VDR double KO mice were examined, the mortality rate was again significantly increased compared to either VDR or IL-10 single KOs.(192) This research suggests an important role for vitamin D in the regulation of inflammation in the gastrointestinal tract.

Type I Diabetes Mellitus. Type I Diabetes Mellitus (DM) is a disease that is caused by autoimmune destruction of pancreatic beta cells, leading to insulin deficiency.(81) As with several other autoimmune diseases, a latitudinal gradient has also been reported for type I DM. In Australia, a three-fold increase in Type I DM prevalence was seen in the northernmost part of the country compared with the southernmost region.(179) In addition, a study of Type I DM in China also revealed increased disease prevalence in the northern region of the country.(194)

Several studies have examined the association between vitamin D supplementation and Type I DM risk. In a birth cohort study, Hypponen *et al.* examined 10,366 children in Finland and found that vitamin D supplementation was associated with a decreased frequency of Type I DM.(195) Treatment of children with 2000 IU vitamin D per day from 1 year of age decreased their risk of developing the disease by 80% up to the age of 20. Those children who were vitamin D deficient at 1 year of age had a 4-fold increase in developing Type I DM. In addition, Stene *et al.* investigated vitamin D supplement use during the first year of life, and determined that use of cod liver oil was associated with a significantly lower risk of type I DM.(196) Finally, a large multi-center

study of 820 Type I DM patients across Europe focused on early exposure to vitamin D and disease risk.(197) Results showed that vitamin D supplementation was again associated with a decreased risk of Type I DM.

Animal research has also implicated a role of Vitamin D in disease pathogenesis. Zella *et al.* reported that vitamin D deficiency increases the incidence of experimentally induced diabetes in mice.(86) In addition, vitamin D supplementation to deficient mice prevented disease onset for up to 200 days of life. Gregori *et al.* demonstrated that treatment of mice with a $1,25(OH)_2D$ analog inhibited IL-12 production and blocked Th1 cell infiltration into the pancreas.(145) The frequency of Treg cells in the pancreatic lymph nodes were also increased and the progression of Type I DM was decreased. Taken together, this research indicates a potential relationship between vitamin D intake or supplementation and disease progression.

Cardiovascular Disease

Like autoimmune disease, cardiovascular disease incidence and severity may also have a geographical distribution. As distance from the equator increases, there is a subsequent increase in blood pressure and prevalence of hypertension.(198) Race and skin color also provide evidence that vitamin D is associated with cardiovascular disease risk since African Americans are both at greater risk for vitamin D deficiency as well as hypertension and heart disease.(88, 198)

It has been shown that patients with more severe congestive heart failure had significantly lower 25(OH)D levels compared to those with less severe disease, and a diagnosis of osteopenia or osteoporosis was present in nearly half of the severe

patients.(93) Furthermore, Fahrleitner *et al.* investigated the vitamin D status of patients with peripheral artery disease (PAD) and found that patients with the most serious stage of PAD showed significantly lower 25(OH)D concentrations.(88) In addition, patients who reported their disease symptoms as severely restricting daily life showed lower serum 25(OH)D than those reporting only a moderate restriction. In contrast, Sowers *et al.* found a significantly positive association between $1,25(OH)_2D$ and systolic blood pressure using a multiple regression model.(199) However, circulating $1,25(OH)_2D$ concentration is not a reliable marker of status and 25(OH)D concentrations were not reported.

Further support for the hypothesis that vitamin D protects against cardiovascular disease is provided by Krause *et al.*, who exposed hypertensive patients to UVB therapy for 3 months.(200) These patients had significant reductions in systolic and diastolic blood pressure and 162% increase in circulating 25(OH)D compared with a control group receiving only UVA therapy. Thus, vitamin D is implicated as a primary factor contributing to the improved blood pressure. In a double-blind, randomized placebo-controlled trial, Schleithoff *et al.* also showed that vitamin D supplementation (2000 IU per day) for 9 months significantly increased 25(OH)D status, while simultaneously improving cytokine profiles.(87) Patients receiving supplementation had decreased concentrations of the pro-inflammatory cytokine, TNFα, and increased concentrations of the anti-inflammatory cytokine, IL-10, compared to the placebo group.

Vitamin D receptor KO mice provide additional support for the involvement of vitamin D deficiency in cardiovascular disease. VDR KO mice have been shown to develop high blood pressure and cardiac hypertrophy due, in part, to over-stimulation of

the renin-angiotensin system.(201, 202) Nonetheless, the protective effects of vitamin D against cardiovascular disease remain unclear and require further investigation.

Cancers

Like the aforementioned diseases, an inverse correlation also exists between cancer mortality rate and latitude. Grant, W.B. has shown that solar UVB exposure is associated with a reduced risk of breast, colon, ovary, and prostate cancer, in addition to non-Hodgkin lymphoma.(203) Grant, W.B. also identified an inverse relationship between UVB and mortality rates of bladder, esophageal, kidney, lung, pancreatic, rectal, stomach, and uterine cancer. Furthermore, the annual number of premature deaths from cancer due to low UVB exposure was estimated at 21,700. In a follow-up study, Grant, W.B. expanded his study sites to include location across Europe.(204) Again, an inverse correlation was found between UVB radiation and bladder, breast, endometrial, ovarian, prostate, and renal cancer, as well as multiple myeloma. Another study examining breast, colon, and prostate cancer prognosis related to UV radiation found a significant variation in prognosis by season.(205) Just as summer and fall produce the highest circulating 25(OH)D status, diagnoses during these months had the lowest risk of cancer death. This research indicates that high vitamin D status may have a protective effect against some cancers, a hypothesis supported by a recent study conducted by Lappe *et al.* (206) Their analysis of a 4-year, double-blind, placebo-controlled trial of 1179 healthy postmenopausal women receiving either 1400 – 1500 mg of calcium or 1400 – 1500 mg of calcium plus 1100 IU of vitamin D_3 revealed that all cancer incidence was decreased in the group of women receiving vitamin D supplementation. Furthermore, a simple

logistic regression, using cancer as the outcome and 25(OH)D as the predictor, showed a 35% reduced risk of cancer for every 25 nmol/L increase in serum 25(OH)D.

Further support for the association between vitamin D status and cancer risk is evident in the obese and African American populations. Obesity is a known risk factor for the development of some cancers and a relationship between obesity and decreased vitamin D status has been repeatedly outlined.(207-215) Several hypotheses have been proposed to explain the prevalence of low vitamin D status in this population, including elevated negative feedback control on synthesis of 25(OH)D caused by increased production of $1,25(OH)_2D$.(214) It has also been proposed that vitamin D deficiency present in obese subjects is likely caused by increased deposition or sequestration in adipose tissue.(212, 215) In addition, African Americans tend to have lower circulating 25(OH)D and have the highest death rate and shortest survival of any racial and ethnic group in the United States for most cancers.(216) As stated previously, melanin competes with provitamin D_3 for UVB photons, therefore, limiting production of previtamin D_3 in people with high melanin content in the skin, as is the case with African Americans.

It has been suggested that $1,25(OH)_2D$ regulates cell growth by increasing cell differentiation and apoptosis and decreasing angiogenesis, cell proliferation, and metastases, thus preventing cancer progression.(217) Mechanisms of action of vitamin D on cancer likely involve inhibition of the cell cycle leading to down regulation of genes involved in DNA replication and repair, yet the molecular mechanism is complex and currently not well understood.(218)

Years of research have been devoted to implicating vitamin D in the prevention and treatment of various types of cancers. Of the different cancers, prostate, breast, and

colon, in that order, have been the most thoroughly researched, with the most promising outcomes.

Prostate Cancer. In addition to the previously mentioned research on cancer incidence and UVB exposure (203), John *et al.* analyzed NHANES I follow-up data and found a significant inverse association between white men born in regions receiving increased solar radiation and cases of both fatal and non-fatal prostate cancer.(219) Additionally, more frequent sun exposure in adulthood was associated with a decreased risk of fatal prostate cancer. Decreased sun exposure has also been associated with increased risk for prostate cancer in men with specific VDR haplotypes.(220, 221) Unlike research on UVB exposure, dietary intake studies have failed to identify an association between vitamin D intake and prostate cancer risk.(222, 223) Li *et al.* examined the connection between circulating vitamin D and prostate cancer risk in 14,916 men.(92) Men whose 25(OH)D and $1,25(OH)_2D$ levels were below the median had significantly increased risk of aggressive prostate cancer. In addition, men with the VDR FokI genotype and 25(OH)D concentrations below the median had an increased risk of total and aggressive prostate cancer. Conversely, the same genotype present in men with elevated 25(OH)D levels was not associated with increase risk.

A clinical pilot study conducted by Gross *et al.* investigated the effects of $1,25(OH)_2D$ supplementation in patients with recurrent prostate cancer.(224) Subjects received up to 2.5 μg of $1,25(OH)_2D$ daily for 6 to 15 months. The rate of prostate specific antigen rise (PSA), a marker of disease, was significantly slowed in 6 out of the 7 subjects; however, side effects of hypercalcemia limited the dose that could be provided. Recent research has since shown that high dose $1,25(OH)_2D$ is safe when administered

intermittently.(225, 226) Following an intermittent dosing schedule, calcitriol was combined with dexamethasone in the treatment of patients with androgen-independent prostate cancer.(227) Twelve micrograms of $1,25(OH)_2D$ was administered 3 times per week and dexamethasone was administered 4 times per week. Results showed that 80% of patients experienced a slowing of PSA rise and 34% had stable or decreased PSA.

The mechanism of action for vitamin D in the prostate is mediated through the VDR, which is found in both prostate epithelial cells (73) and cancer cells.(74-76) In addition, epithelial prostate cells also express 1α-OH-ase, the enzyme responsible for converting 25(OH)D to $1,25(OH)_2D$, indicating a paracrine function of vitamin D within the prostate.(228) However, Hsu *et al.* found that in primary cultures of cancer cells, 25(OH)D had significantly less antiproliferative capabilities compared with $1,25(OH)_2D$.(229) Accordingly, Chen *et al.* observed a significant decrease in 1α-OH-ase activity in prostate cancer cells compared with normal cells.(230) Thus, supplementation with $1,25(OH)_2D$, as opposed to 25(OH)D has been the primary focus of clinical trials in prostate cancer. In addition, increased expression of 24-OH-ase, the enzyme responsible for converting $1,25(OH)_2D$ to its inactive form, has also been identified 7 prostate cancer cell lines.(231) This research indicates that vitamin D has an important function in normal and cancerous prostate cells.

As with other cancers, the protective role of $1,25(OH)_2D$ in prostate cancer is thought to be mediated through its antiproliferative and apoptotic capabilities. In the several prostate cancer cell lines, $1,25(OH)_2D$ causes cell cycle arrest and apoptosis.(76, 231-233) Additionally, $1,25(OH)_2D$ has also been shown to inhibit prostate cancer cell growth in both androgen-dependent and androgen-independent cell lines.(234)

Breast Cancer. The link between breast cancer and vitamin D extends beyond geographical associations (204). The National Health and Nutrition Examination Survey (NHANES) I data, including the years of 1971 to 1992, indicated that dietary vitamin D intake was association with reduced risk of breast cancer, with the most significant risk reduction present in women living in regions of the United States with the highest solar radiation.(235) Additionally, within the Nurses Health Study, Shin *et al.* found that among premenopausal women, high intake of low fat dairy foods was associated with reduced risk of breast cancer.(236) This is of particular importance because, although minimal compared to sunlight, fortified dairy products are an important dietary source of vitamin D in the United States. Knekt *et al.* also found a protective effect of increased milk consumption on breast cancer risk.(237)

Animal studies have also demonstrated a link between vitamin D status and breast cancer risk. Jacobson *et al.* found that rats fed diets deficient in calcium and vitamin D had increased incidence of mammary lesions compared with rats fed adequate calcium and vitamin D.(238) In addition, the deficient animals also had increased lesion weight. Mehta *et al.* showed that both $1,25(OH)_2D$ and a vitamin D analog inhibited the development of induced preneoplastic lesions in mouse mammary glands.(239) Incubation of mammary glands with both $1,25(OH)_2D$ and its analog also significantly increased VDR and TGFβ (a negative growth regulator) expression. Other vitamin D analogs have also been shown to decrease chemically induced mammary tumor incidence and extend tumor latency in rats.(240, 241) VDR KO mice studies also provide support for a protective role for vitamin D in breast cancer. Zinser *et al.* discovered an increase in chemically induced preneoplastic lesions in mammary glands of VDR KO mice

compared with WT mice.(242) Furthermore, mammary cells from VDR KO mice were resistant to both 1,25$(OH)_2$D and analog mediated cell cycle arrest and apoptosis, unlike cells from WT mice.

Evidence of the relationship between breast cancer and vitamin D in humans includes the expression of VDRs on both normal and neoplastic breast tissues.(71, 72) Furthermore, polymorphisms in the gene responsible for VDR expression have been repeatedly associated with increased breast cancer risk.(243-247) Indicated mechanisms of action by 1,25$(OH)_2$D on breast cancer cells includes cell cycle inhibition, resulting in decreased proliferation, and induced apoptosis. In estrogen dependent human breast cancer cells (MCF-7), 1,25$(OH)_2$D has been shown to induce cell cycle arrest via up-regulation of cell cycle inhibitors, leading to down-regulation of growth promoting signals (IGF-1) and up-regulation of the negative growth regulator TGFβ.(248, 249) 1,25$(OH)_2$D is also capable of inducing anti-proliferative effects on estrogen independent cell lines; however, effectiveness tends to be greater in estrogen dependant cells.(250) Aside from its antiproliferative effects, 1,25$(OH)_2$D also appears to be capable of inducing apoptosis. MCF-7 cells treated with 1,25$(OH)_2$D exhibited morphological and biochemical markers of apoptosis, including cell shrinkage, cytoplasmic condensation, condensed chromatin, nuclear matrix re-organization, and up-regulation of apoptotic proteins.(251) Follow-up studies have also repeated demonstrated the ability of 1,25$(OH)_2$D to mediate apoptosis in breast cancer cells.(252, 253) Finally, vitamin D analogs have been shown to increase the effectiveness of other apoptotic treatments such as, anti-estrogen drugs, TNFα, radiation, and chemotherapy.(254-257) Collectively,

research provides convincing evidence that vitamin D and its receptor may be appropriate targets for breast cancer prevention and treatment.

Colon Cancer. Vitamin D was first hypothesized to have a protective effect against colon cancer in 1980, when Garland *et al.* discovered colon cancer mortality rates in the United States were highest in regions where people were exposed to minimal sunlight.(258) This hypothesis was again confirmed when Emerson *et al.* analyzed data from 9 population-based cancer registries in the United States and found that incidence of colon and rectal cancer among men increased with decreasing solar radiation and incidence of colon cancer alone among women showed a similar trend.(259) Further support for the hypothesis was contributed by Garland *et al.* when they obtained blood samples from 25,620 subjects and divided the subjects into quintiles based on 25(OH)D status.(89) Those in the third (67-80 nmol/L) and fourth (82-102 nmol/L) quintiles had a 75% and 80% reduced risk of colon cancer, respectively. Additionally, subjects with a serum 25(OH)D concentration greater than 52 nmol/L had a 3-fold decreased risk of developing colon cancer compared to subjects with 25(OH)D concentrations less than 52 nmol/L. More recently, a meta analysis revealed a significant dose response between increasing 25(OH)D status and decreasing colorectal cancer risk.(90) Furthermore, serum 25(OH)D concentrations of 85 nmol/L or greater was associated with a 50% lower risk of cancer compared with 25(OH)D levels at or below 31 nmol/L. Holt *et al.* was also able to show that serum levels of 25(OH)D were inversely correlated with the size of the proliferative compartment within the rectal crypt.(91)

A clinical trial conducted by Holt *et al.* examined the effects of 6 months of calcium and vitamin D supplementation on biomarkers of colon cancer risk.(260) A

portion of a polyp, as well as flat mucosal tissue was removed and examined from each of the 19 subjects. Following 6 months of treatment with 1800 mg of elemental calcium and 400 IU of vitamin D, the remaining portion of the polyp was removed and analyzed. Markers of proliferation decreased in both the polyps and flat mucosa in those subjects receiving the treatment.

As can be expected, expression of the VDR was found in colon cancer cell lines (77), as well as both malignant and non-malignant human colon tissue.(78) Park *et al.* genotyped 190 Korean colorectal cancer patients and 318 controls with no history of disease and found that start codon variants in the VDR gene were associated with increased risk of colorectal cancer.(261) Surprisingly, 2 other VDR gene variants were associated with decreased risk. VDR KO mice were used by Kallay *et al.* to demonstrate the ability of $1,25(OH)_2D$ to protect against colonic pathogenesis.(262) They showed that without VDR expression, colonic hyperproliferation ensued. Thus, the proposed mechanism of action of vitamin D in colon cancer is again related to its antiproliferative and apoptotic capabilities.

Other Cancers. Vitamin D has also been implicated in lung cancer risk and disease progression, although the body of literature on this topic is significantly smaller than the aforementioned cancers. As was previously stated, Grant, W.B. found an association between UVB exposure and lung cancer risk.(203) Zhou *et al.* investigated the association between the time of year that early-stage (stages IA, IB, IIA, IIB) non-small cell lung cancer patients had surgery, in addition to vitamin D intake, with recurrence-free survival (RFS) and overall survival.(263) Results showed that patients who had surgery in the summer months had a better RFS compared with those who had

surgery during the winter months. In addition, patients who had surgery during the summer months and had the highest vitamin D intake had a 56% 5-year RTS rate compared with a 23% 5-year RTS rate in patients who had surgery during the winter months and had the lowest vitamin D intake. In a follow-up study, Zhou *et al.* investigated the implications of 25(OH)D concentrations on overall survival and RFS, again in patients with early-stage non-small cell lung cancer.(264) There was a strong association among 25(OH)D status and overall survival among patients with stages IB, IIA, and IIB lung cancer, but interestingly, no association was present among patients with stage IA cancer. It is possible that this lack of association may be partially explained by the increased expression of 1α-OH-ase found in alveolar macrophages, which could potentially contribute to the immunosuppression present in lung cancer.(265) Additionally, 24-OH-ase expression has also been discovered in normal lung tissue and upregulation was present in lung cancer cell lines, causing the degradation of the active form of vitamin D and inhibition its apoptotic capabilities.(70) Mouse models, however, have shown more promising research with both 1,25(OH)2D and its analog inhibiting metastatic growth of lung cancer cells.(266, 267) Thus, the role of vitamin D in lung cancer prevention and treatment is multifaceted and currently not well understood.

Vitamin D has also recently been implicated in ovarian cancer prevention and treatment. As was previously mentioned, UVB radiation is associated with reduced risk for ovarian cancer.(203, 204) A recent study conduced by Garland *et al.* characterized ovarian cancer incidence rates in 175 countries.(268) Again, the data revealed that ovarian cancer rates were the greatest in countries located at higher latitudes. In addition,

stratospheric ozone was measured, which is known to reduce transmission of UVB. It was positively associated with cancer incidence, providing more evidence that UVB, and thus vitamin D, plays a role in ovarian cancer prevention. Nevertheless, Tworoger *et al.* found no associations between circulating 25(OH)D and ovarian cancer, as well as $1,25(OH)_2D$ and ovarian cancer.(269) However, they did find significance among overweight and obese women. Those with adequate status ($\geq$ 80 nmol/L) compared to women with inadequate vitamin D had a modestly decreased risk of serious ovarian cancer. At a cellular level, $1,25(OH)_2D$ has been shown to induce growth suppression of ovarian cancer cells and cause cell death.(270) Additionally, a vitamin D analog has been shown to suppress the growth of implanted ovarian tumors in nude mice via inhibition of cellular proliferation and induction of apoptosis.(271) Taken together, this research provides evidence that vitamin D might have a role in ovarian cancer; however, more research is needed before conclusions can be drawn.

Infectious Diseases

Influenza. In 1981, Hope-Simpson, showed that influenza A epidemics in temperate latitudes peak in the month following the winter solstice.(272) He hypothesized that solar radiation produced a "seasonal stimulus" affecting influenza A; however, the mechanism was not identified at the time. Since then, the association between influenza and vitamin D has been strengthened. A relationship between rickets, a disease caused by vitamin D deficiency, and increased respiratory infection has been appreciated for many years.(273, 274) Furthermore, Wayse *et al.* discovered a significant risk for severe acute lower respiratory infection among Indian children with sub-clinical

25(OH)D deficiency and those who were non-exclusively breastfeeding in the first 4 months of life.(275) Linday *et al.* supplemented children living in New York City with 600-700 IU of vitamin D via cod liver oil and a multivitamin.(276) These children receiving supplementation had significantly fewer number of upper respiratory tract visits to pediatricians' offices compared to controls. Cannell *et al.* proposes that vitamin D's influence on influenza may be related to its ability to stimulate the production of anti-microbial peptides, as well as its ability to suppress cytokine and chemokine production.(277) Nevertheless, more research is needed to strengthen this hypothesis, as well as the association between vitamin D and influenza prevention and/or treatment.

Tuberculosis. The most convincing evidence that vitamin D mediates antimicbrobial activity against Tuberculosis (TB) was published in *Science* in 2006 by Liu *et al.*(278) They reported that activation of macrophages caused up-regulation of both VDR and 1α-OH-ase, which caused the production of the antimicrobial peptide known as cathelicidin, and led to the killing of *Micobacterium tuberculosis.* Additionally, they also showed that African-American individuals, who are known to have increased susceptibility to TB, had decreased 25(OH)D levels and had inefficient up-regulation of cathelicidin. Another proposed mechanism of action involves $1,25(OH)_2D$ inducing a superoxide release via the phosphatidylinositol 3-kinase pathway.(279)

Prior to the advent of antibiotics, pharmacologic doses of vitamin D were commonly used to treat TB; however, this practice became infrequent with the introduction of effective antibiotic therapy against *Micobacterium tuberculosis*. Despite the growing evidence suggesting that vitamin D is capable of inducing antimicrobial activity against TB, results of clinical trials have been discouraging. Martineau *et al.*

reviewed 3 randomized controlled trials and 10 prospective studies in which vitamin D was administered to patients with pulmonary TB.(280) Unfortunately, only 4 studies were conducted in the past 50 years, none of which found statistically significant improvements in the patients' therapeutic response.(281-284) Martineau *et al.* attributes this lack of evidence for clinical relevance to methodologically-flawed studies caused by the absence of control groups, poor reporting of clinical observations, a lack of statistical power, and invalid outcomes. Thus, before conclusions can be drawn regarding the clinical relevance of $1,25(OH)_2D$ on TB disease prevention and progression, new research must be conducted using the aforementioned guidelines.

V. Summary

There is a growing body of evidence implicating a significant role for vitamin D in human health, particularly influencing health beyond improved calcium regulation. Additionally, a mass of evidence has accumulated linking vitamin D deficiency or insufficiency with immune malfunction. The mounting research suggests great potential for vitamin D to have an impact on human health that has yet to be fully recognized or understood. The immunomodulatory capabilities of vitamin D provide plausibility for protection against numerous diseases and conditions, specifically autoimmune diseases and cancers. According to Grant *et al.*:

> "Presently, the role of UVB and vitamin D in reducing the risk of cancer is considered a scientific finding that satisfies most, if not all, the criteria for causality in a biological system . . . The most important criteria appear to be: (1) strength of association; (2) consistency in results for different populations; (3) generally linear dose-response gradients; (4) exclusion of possible confounding factors for explaining the observations; and (5) identification of mechanisms to explain the observations. These criteria

> are generally satisfied for several cancers in particular and many cancers in general."(33)

The link between vitamin D and numerous cancers, autoimmune diseases, and other diseases provides convincing support for the need to examine the relationship between vitamin D status and inflammatory marker status in not only those suffering from disease, but also otherwise healthy individuals.

The overall objective of our laboratory is to determine the relationship between vitamin D status, inflammatory markers, and bone mineral density in healthy pre- and post-menopausal women who regularly use tanning beds. *Our specific hypothesis for the study described herein is that increased vitamin D status in healthy women who regularly use a tanning bed will result in decreased levels of circulating inflammatory markers.*

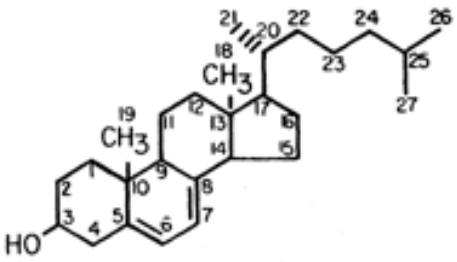
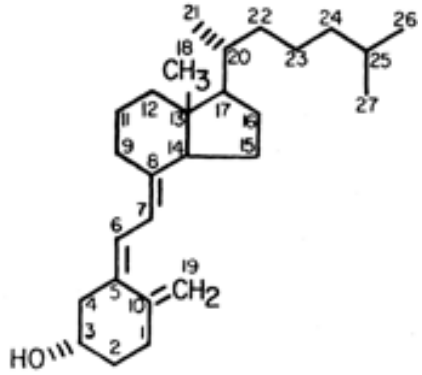
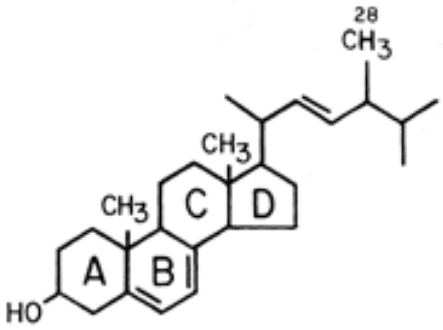
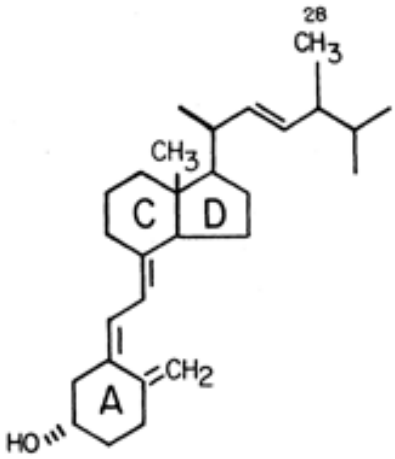
7 – Dehydrocholesterol (Provitamin D_3)	Vitamin D_3
	
Ergosterol (Provitamin D_2)	**Vitamin D_2**
	

Figure 1. Structure of vitamin D_3 and D_2 and their precursors. Adapted from (285).

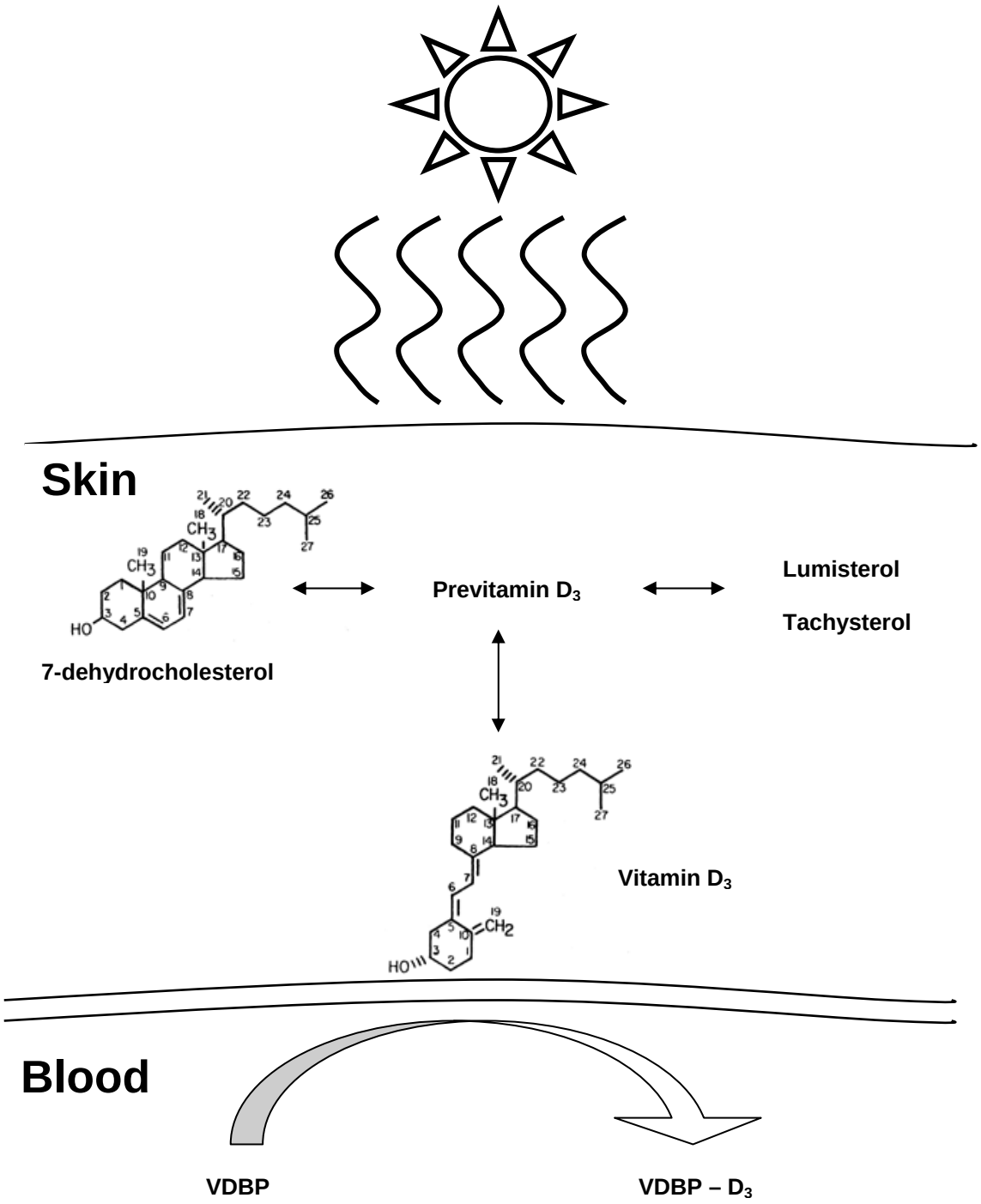


Figure 2. Vitamin D production in the skin. 7-dehyrocholesterol is converted to previtamin D_3, which is then converted to vitamin D_3 in the skin. It is then transferred to the blood where it is complexed with vitamin D binding protein (VDBP). Upon photoactivation, previtamin D_3 can also be converted to biologically inert isomers: lumisterol or tachysterol. Adapted from (286).

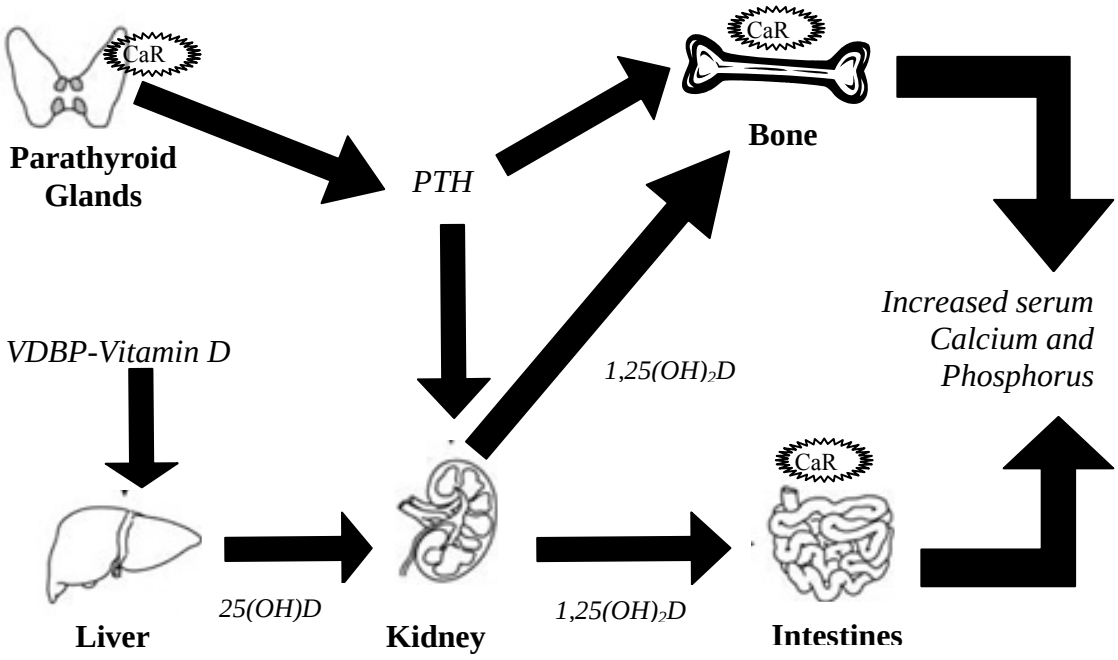


Figure 3. Vitamin D regulation of calcium and phosphorus homeostasis. Vitamin D, bound to the VDBP, circulates in the blood and is converted in the liver to 25(OH)D. 25(OH)D can then be converted to its active form, $1,25(OH)_2D$, in the kidney. Parathyroid glands sense extracellular calcium concentrations that fall less than 2.5 mM via the calcium receptor (CaR) and increase PTH section. PTH can then act on bone to release calcium and phosphorus. PTH can also act on the kidney to increase the formation of $1,25(OH)_2D$, decrease the secretion of calcium, and increase excretion of phosphorus. $1,25(OH)_2D$ can also act on bone to release calcium and phosphorus, as well as bind to its receptor in the small intestines to increase absorption of dietary calcium and phosphorus. The net effect is increased serum calcium and phosphorus. Adapted from (1, 287, 288).

Source	Amount Obtained
Cod liver oil, 1 tablespoon	1,360 IU
Salmon, cooked, 3 ½ ounces	360 IU
Mackerel, cooked, 3 ½ ounces	345 IU
Tuna fish, canned in oil, 3 ounces	200 IU
Milk, nonfat, reduced fat, and whole, vitamin D fortified, 1 cup	98 IU *May vary (11, 12)
Margarine, fortified, 1 tablespoon	60 IU
Pudding, prepared from mix and made with vitamin D fortified milk, ½ cup	50 IU
Ready-to-eat cereals fortified with 10% DV for vitamin D, ¾ cup to 1 cup servings	40 IU
Egg, 1 whole	20 IU
Liver, beef, cooked, 3 ½ ounces	15 IU
Cheese, Swiss, 1 ounce	12 IU
Orange juice	400 IU per quart
Solar UVB	0 IU (during winter in northern latitudes) to 10,000 IU per day
Artificial UVB	2,000 to 4,000 IU (during 10-minute tanning session)
Supplements	200 to 1,000 per pill

Table 1. Sources of Vitamin D. Adapted from (33, 289)

25(OH)D Level (nmol/L)	Health Implications
< 50	Deficiency
50 – 80	Insufficiency
80 – 250	Sufficiency
135 – 225	Normal in Countries Near the Equator
> 250	Excess
> 325	Intoxication

Table 2. Health implications of serum 25(OH)D levels. Adapted from (33).

Baseline (nmol/L)	Daily Oral Intake
20 – 40	2200 IU
40 – 60	1800 IU
60 – 80	1160 IU
> 80	0 IU

Table 3. Estimated daily oral intake needed to reach a serum 25(OH)D status of 80 nmol/L. Adapted from (36).

Age Group (years)	Insufficiency After Winter Season (%)*	Insufficiency After Summer Season (%)*
18 – 29	32	4
30 – 39	25	18
40 – 49	30	20
≥ 50	16	4

Table 4. Percentage of adults (60% Caucasian) with vitamin D insufficiency located in Boston, MA (42°N). *****Vitamin D insufficiency is characterized as a serum 25(OH)D of ≤ 50 nmol/L. Adapted from (25, 290)

Immune Cell	Presence of VDR	Function of 1,25(OH)$_2$D on Immune Cells
Monocytes	Yes	▪ Induces differentiation toward macrophages (123-125) ▪ Diminishes capacity to stimulate T cell activation as APCs (126) ▪ Enhances phagocytosis and chemotaxis ability (126)
Macrophages	Yes	▪ Downregulates TNFα expression when incubated with 25(OH)D (127) ▪ Enhances phagocytosis and chemotaxis ability (126) ▪ Negatively regulates IL-12 production (136)
Dendritic Cells	Yes	▪ Inhibits differentiation and maturation and induces spontaneous apoptosis (128-132) ▪ Decreases antigen-presentation function and ability to activate T cells (130) ▪ Causes decreased IL-12 production (134, 136) and increased IL-10 production (130, 133)
T Lymphocytes	Yes	▪ Inhibits T cell proliferation via decreased production of IL-2 and IFNγ (124, 137, 138) ▪ Decreases proliferation of all Th cells and their production of IFNγ, IL-2, and IL-5 (141) ▪ Increases production of IL-4, IL-5, and IL-10 by Th2 cells, indicating propensity toward Th2 differentiation (141, 142) ▪ Inhibits T cell death by preventing FasL expression (143) ▪ May influence Regulatory T cells (134, 144, 145)
B Lymphocytes	Unclear (115, 150, 152)	▪ Inhibits Ig production (153-157)
NK Cells	Unknown	▪ Inhibits cytotoxic activity (146) ▪ Inhibits IFN and IL-2 activation of NK cells (147)

Table 5. Function of 1,25(OH)$_2$D on immune cells as reported in the literature.

METHODS

I. Study Design

This study was an observational design to explore the relationship between vitamin D status and inflammatory and bone status in women who regularly use a tanning bed ($\geq$ 1x/week for at least 4 months) and women with minimal daily sun exposure. This study was approved by the University of Missouri Health Sciences Institutional Review Board.

II. Subjects

A total of 69 female subjects, ages 25 – 82, were recruited from the University of Missouri-Columbia campus, local tanning salons, and gyms. To be included in the study, participants had to be Caucasian females who were at least 25 years of age. Additionally, to qualify as a "Tanner," women had to regularly use a broad spectrum (UVA and UVB) tanning bed at least once per week for a minimum of 4 months. "Non-Tanners" needed to have minimal daily sunlight exposure, as assessed by a screening questionnaire, and no tanning bed use. Subjects were excluded from the study if they (1) took a vitamin D supplement other than a regular multivitamin; (2) had a current or previous medical condition or took a medication affecting vitamin D status; (3) had a current or previous medical condition or took a medication affecting bone health; (4) had a current or previous medical condition or took medication affecting immune functioning; (5) had implanted metal that would interfere with the determination or bone mineral density; (6) were undergoing ultraviolet radiation as medical therapy; (7) exclusively used high-

pressure (UVA-only) tanning beds; (8) exercised more than 7 hours per week; (9) were pregnant; or (10) smoked cigarettes.

Potential subjects were mailed a copy of the consent form and instructed to read it prior to the initial screening telephone call. During the phone call, the study coordinator reviewed the consent form by providing an oral explanation of the study purpose, protocol, and possible risks and benefits to the subject, and clarified any questions or concerns. Potential subjects then gave oral consent if they wished to participate; at which time the initial screening questions (Appendices A and B) were asked with the subjects' permission. If the subjects qualified, the testing visit was scheduled between 7 am and 11 am to control for diurnal variations in iPTH and inflammatory markers. Subjects were instructed to refrain from exercise and fast for 8 to 10 hours prior to their scheduled visit. On the day of the visit, all subjects within childbearing age took a urine pregnancy test to confirm non-pregnant status. All study visits were conducted between the months of January and June of 2007 so as to obtain serum samples during the seasonal nadir for 25(OH)D.

II. Questionnaire Data

Health History and Medical Questionnaire

The Health History and Medical Questionnaire (Appendix C) is a one-page questionnaire that inquires about current or previous health conditions or diseases, menopausal status, current or previous medication use, and exercise habits. This questionnaire was developed for use in this study.

Sun Exposure Questionnaire

The Sun Exposure Questionnaire (Appendix D) is a one-page questionnaire that assesses tanning bed use, outdoor sun exposure, and sunscreen use. This questionnaire was developed for use in this study.

Fitzpatrick Method of Skin Typing

The Fitzpatrick method of skin typing is a validated tool that was used to assess the skin type of all subjects (Appendix E).(291) Skin type was documented because pigmentation affects the amount of vitamin D synthesized in the skin.

Gail Model Breast Cancer Risk Assessment

The Gail Model Breast Cancer Risk Assessment Tool is a validated online interactive tool for measuring the risk of invasive breast cancer (Appendix F).(292) The questionnaire is only valid for women over the age of 35, thus; only women in this study who were over 35 years of age completed the questionnaire.

Harvard Food Frequency Questionnaire

The Harvard Food Frequency Questionnaire (4-page 88GP) is a validated tool that was used to assess the dietary intake of all subjects (Appendix G).(293) Completed questionnaires were mailed back to Harvard for analysis.

24-Hour Dietary Recall

A Registered Dietitian conducted a 24-hour dietary recall on the day of the study. This information was verified against the Food Frequency Questionnaire data in the case of discrepancies or questionable data.

III. Measurements

Anthropometric Data

Each subjects' weight was determined to the nearest pound and height was measured to the nearest 0.5 inch. Body composition was determined by the whole body DXA (Hologic Delphi A, Waltham, MA) scan.

Bone Measurements

Bone mineral content (BMC) and areal bone mineral density (BMD) was measured by DXA at 3 sites: the lumbar spine, total hip, and whole body average. Areal BMD (g/cm^2) was calculated from bone area (cm^2) and BMC (g). This instrument uses a linear X-ray fan beam with switched-pulse dual-energy and a multi-element detector array. Bone mineral density precision is <1% and calibration uses an internal reference system. Z-scores were calculated by assessing the subjects' bone density compared to expected bone density for age-matched peers. Hip reference data was taken from the NHANES III study (294), spine reference data was based on the U.S. White reference database(295), and the whole body database was base on a Hologic, Inc. proprietary internal study.

Serum Measurements

A trained phlebotomist drew 10 mL of blood from the antecubital vein of participants in the supine position. All blood was drawn between the hours of 7:30 am and 11:30 am. Blood was transferred from the syringe to a serum separator vacutainer tube via a blood transfer device, and allowed to clot at room temperature for 30 minutes. The coagulated blood was then centrifuged at 3000 rpm for 15 minutes at 4°C. The serum was removed, aliquoted into 0.5 mL sterile microcentrifuge tubes, and stored at -80°C.

Intact-PTH (iPTH) was measured using an iPTH (1-84) Enzyme-Linked Immunosorbent Assay (ELISA) (ALPCO Diagnositics, Salem, NH, Intra-assay CV = 2.5%). Estradiol was measured using an Estradiol ELISA (ALPCO Diagnostics, Salem, NH, Intra-assay CV = 7.7%) to characterize each subject's menstrual/menopausal status. Cortisol was measured as a potential confounding variable on inflammatory marker status using a Cortisol EIA (ALPCO Diagnositics, Salem, NH, Intra-assay CV = 5.8%). Five inflammatory markers were measured, including C-Reactive Protein, TNFα, IL-6, IL-10, and IL-1β. An ELISA was also used to measure C-Reactive Protein (R&D Systems Inc., Minneapolis, MN, Intra-assay CV = 5.5%). TNFα, IL-6, and IL-10 were measured using high sensitivity ELISAs (R&D Systems Inc., Minneapolis, MN, Intra-assay CV = 5.3%, 7.4%, and 7.7%, respectively). Finally, IL-1β was measured using an ELISA (BD Biosciences, San Diego, CA, Intra-assay CV = 2.8%).

The overall ELISA protocol is as follows. Antibodies specific for each marker are pre-coated onto a 96-well microtitre plate. Standards and samples are pipetted into

the wells in duplicate and the immobilized antibodies bind the markers present. This is followed by an incubation step and then a wash step to remove any unbound substances. An enzyme-linked antibody specific for the marker is then added to the wells. A second wash step removes any unbound antibody-enzyme reagent, and an acidic substrate solution is then added to the wells. Color develops in proportion to the amount of marker bound in the initial step. Finally, a stop solution is added and the intensity of color is measured at the specified wavelengths. Standards with known concentrations are analyzed and a standard curve is generated. The concentrations of the unknown samples are interpolated from the equation of the curve. All standards and samples are analyzed in duplicate and averaged.

25(OH)D serum levels were measured using a ^{125}I radioimmunoassay (RIA) kit (Diasorin, Stillwater, MN, Intra-assay CV = 10.8%). The 25(OH)D RIA is a two-step procedure. First, 25(OH)D and other hydroxylated metabolites are rapidly extracted from serum using acetonitrile. The extracted sample is then assayed using an antibody with specificity to 25(OH)D. The sample, antibody, and tracer are incubated for 90 minutes at room temperate. An antibody-precipitating complex is added, followed by another 20-minute incubation at room temperature, causing phase separation. A buffer is then added to aid in reducing non-specific binding and the samples are centrifuged. The supernatants are decanted and a gamma scintillation counter is used to count each sample for a minimum of 1 minute. A standard curve is then constructed and the 25(OH)D concentration for each sample is determined.

IV. Statistics

Unpaired 2-tailed t-tests were used to determine differences in subject characteristics and measured outcomes. Linear regression and multiple linear regression models were used to determine the relationship between vitamin D status and all measured outcomes. Potential covariates were controlled for using multiple linear regression models and stepwise selection methods. All statistics were performed using SAS statistical software version 9.1 (SAS Inc, Cary, NC). A *P*-value less than 0.05 was considered significant.

RESULTS

I. Subject Characteristics

Subject Characteristics are presented in Table 6. Sixty-nine women between the ages of 25 and 82 participated in the study. Forty-nine of the women were classified as Non-Tanners and 20 women were classified as Tanners. There were no significant differences in age, height, weight, BMI, % body fat, serum estradiol, cortisol, or hormonal contraceptive use between groups. The skin type of the Tanners was significantly higher than that of the Non-Tanners (P=0.0031).

Characteristic	**Non-Tanners n = 49**	**Tanners n = 20**
Age (years)	39.8 ± 1.75	41.7 ± 3.45
Height (m)	1.68 ± 0.008	1.65 ± 0.012
Weight (kg)	65.87 ± 1.633	67.88 ± 2.658
Body Mass Index (kg/m^2)	23.8 ± 0.52	25.0 ± 1.06
% Body Fat	30.1 ± 0.99	30.6 ± 1.65
Skin Type	2.4 ± 0.10	3.1 ± 0.19*
Estradiol (pg/mL)	158 ± 15.64	151.34 ± 15.25
Cortisol (μg/dL)	8.4 ± 0.46	9.4 ± 1.17
Contraceptive Use (%)	30.6%	20%

Table 6. Subject characteristics. Data are expressed as means ± SEM. *Means are significantly different from Non-Tanners, P<0.05.

II. Pertinent Dietary Data

Selected dietary data are presented in Table 7. There were no differences between groups for the selected nutrient intakes.

Nutrient	Non-Tanners (n = 49)	Tanners (n = 20)
Total Calories (kcal)	2012 ± 101	2121 ± 259
Carbohydrates (g)	248 ± 14	240 ± 28
Protein (g)	96 ± 6	106 ± 11
Total Fat (g)	69 ± 4	80 ± 13
Saturated (g)	24.4 ± 1.5	28.8 ± 4.9
Monounsaturated (g)	25.0 ± 1.7	30.0 ± 5.0
Polyunsaturated (g)	13.3 ± 1.0	14.8 ± 2.2
Total n-3 (g)	1.6 ± 0.1	1.6 ± 0.2
Long Chain n-3 (g)	0.3 ± 0.0	0.3 ± 0.1
Vitamin D (IU)	426 ± 35	396 ± 62
Folate (μg)	765 ± 50	673 ± 88
Calcium (mg)	1242 ± 67	1328 ± 146
Phosphorus (mg)	1556 ± 75	1651 ± 161
Magnesium (mg)	390 ± 19	421 ± 43
Sodium (mg)	2216 ± 136	2374 ± 274
Caffeine (mg)	132 ± 17	160 ± 39
Alcohol (g)	7 ± 1	6 ± 2

Table 7. Selected dietary data for all subjects. Data are expressed as means ± SEM. There were no significant differences between groups.

III. Vitamin D status

Serum 25(OH)D concentrations of all subjects are presented in Figure 4. The mean serum 25(OH)D status (nmol/L) of the Tanners (129.6 ± 10.97 nmol/L) was significantly higher than that of the Non-Tanners (74.4 ± 4.02 nmol/L) (P<0.0001).

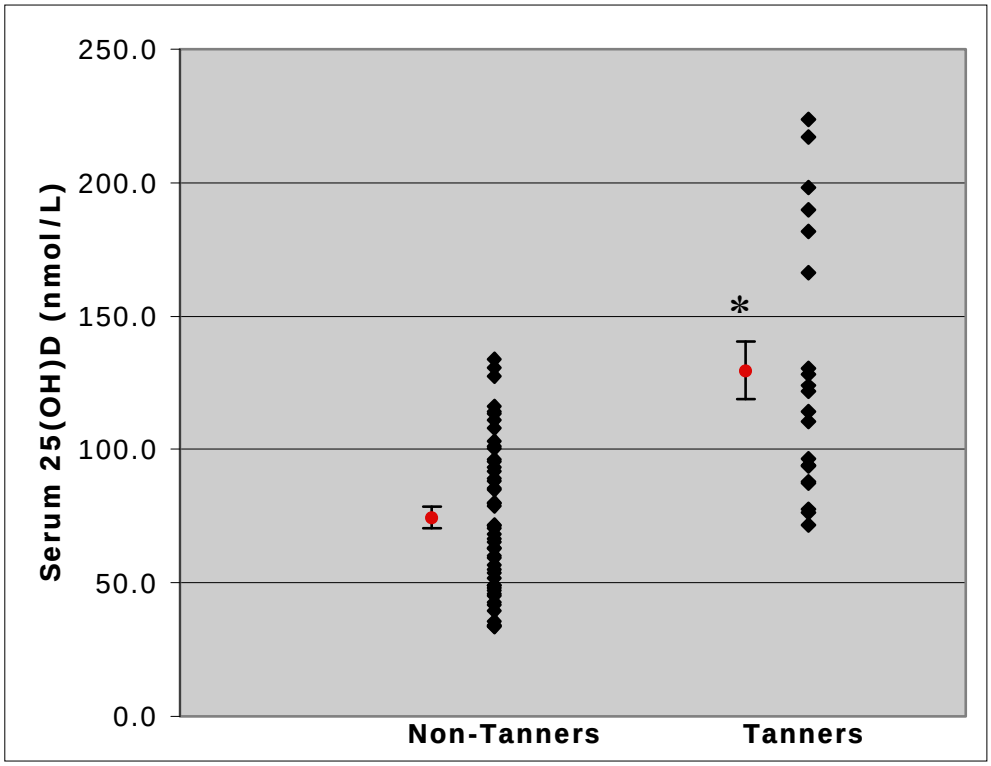


Figure 4. Serum 25(OH)D concentrations of Non-Tanning and Tanning women. Single points for each subject group are means ± SEM. *Means are significantly different from Non-Tanners, P<0.0001.

According to the health implications of 25(OH)D status presented by Grant, W.B. and Holick, M.F. (Table 2) (33), 26.5% of Non-Tanners had deficient levels of serum 25(OH)D, 35.7% were insufficient, and only 38.8% had sufficient serum concentrations (Figure 5). Of the Tanners, no one was classified as deficient, 15% had insufficient levels, and 85% had sufficient serum 25(OH)D concentrations.

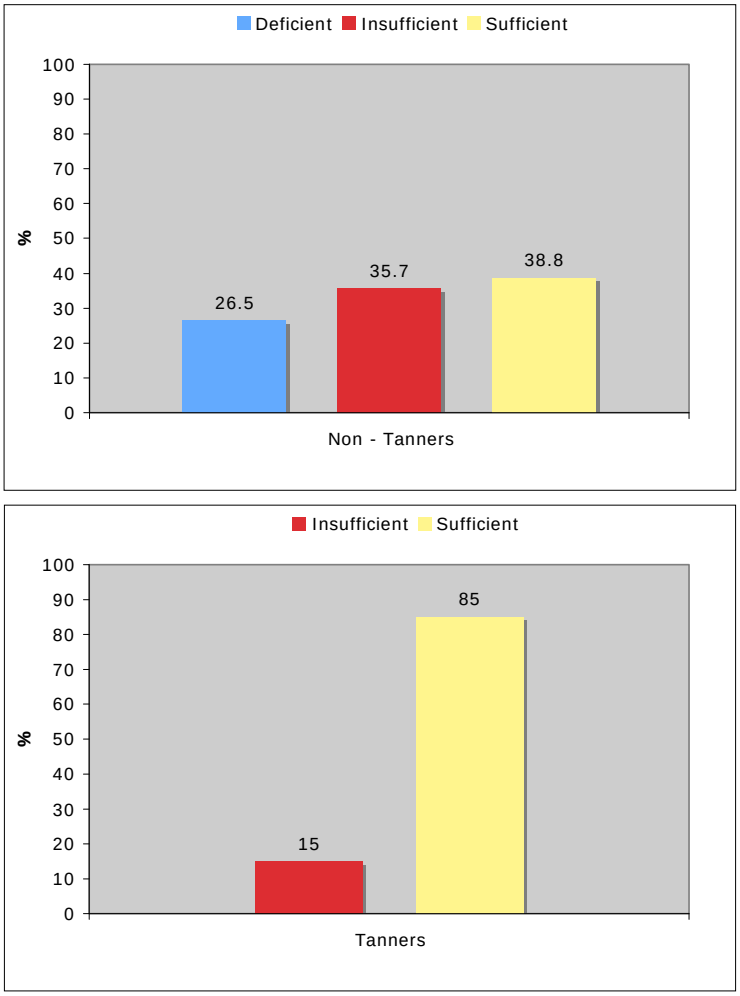


Figure 5. Percent of Non-Tanners and Tanners in categories according to serum 25(OH)D concentrations.

Serum iPTH concentrations of all subjects are presented in Figure 6. The mean iPTH concentration (pg/mL) of the Tanners (26.2 ± 2.57 pg/mL) was significantly lower than that of the Non-Tanners (48.1 ± 3.06) (P<0.0001). The relationship between 25(OH)D concentration and iPTH is presented in Figure 7. As 25(OH)D increases, iPTH decreases and then begins to level off.

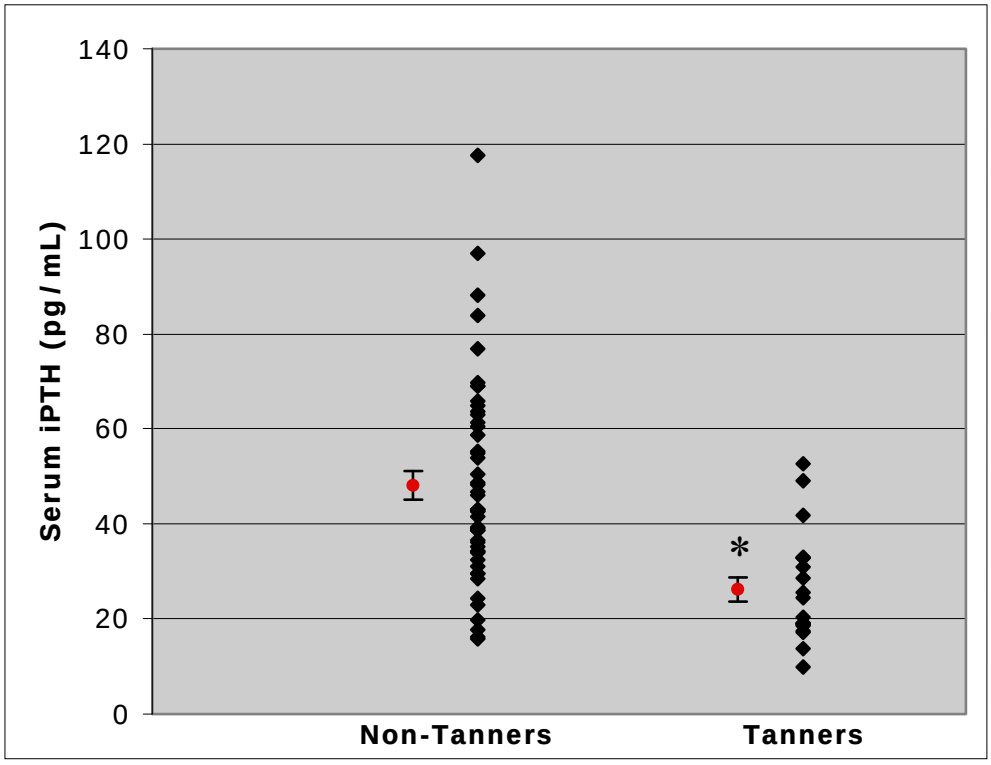


Figure 6. Serum iPTH concentrations of Non-Tanning and Tanning women. Single points for each subject group are means ± SEM. *Means are significantly different from Non-Tanners, P<0.0001.

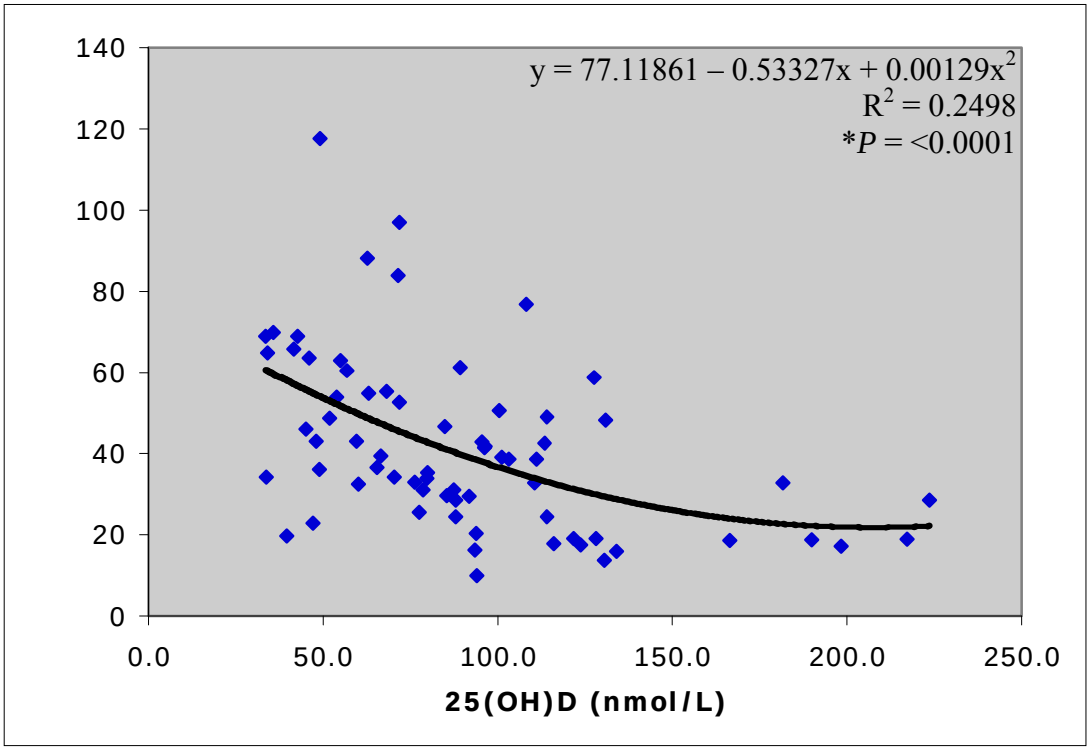


Figure 7. The relationship between 25(OH)D concentration and iPTH. The regression equation and R^2 value is presented. *Regression equation is significant, $P<0.05$.

IV. Bone Outcomes

Whole body BMD, BMC, and Z-scores; spine BMD, BMC, and Z-scores; and hip BMD, BMC, and Z-scores are presented in Table 8. There were no significant differences in any of the bone outcomes between the Non-Tanners and Tanners. Linear regression analyses revealed no significant relationships between 25(OH)D and the bone outcomes (Table 9). Using a stepwise selection method, percent body fat had a significant positive relationship to spine BMD ($R^2 = 0.116$, $P = 0.0087$) and spine Z-score ($R^2 = 0.2326$, $P = 0.0001$).

	Non-Tanners (n = 47 – 49)	**Tanners (n = 17)**	***P* Value**
Whole Body			
BMD (g/cm^2)	1.122 ± 0.011	1.126 ± 0.971	0.8429
BMC (g)	2175.1 ± 38.33	2281.8 ± 53.34	0.1405
Z-Score	0.69 ± 0.14	0.71 ± 0.21	0.9446
Hip			
BMD (g/cm^2)	0.927 ± 0.017	0.950 ± 0.024	0.4617
BMC (g)	30.3 ± 0.75	30.3 ± 1.0	0.9974
Z-Score	0.17 ± 0.14	0.27 ± 0.20	0.7015
Spine			
BMD (g/cm^2)	1.033 ± 0.016	1.035 ± 0.027	0.9487
BMC (g)	62.8 ± 1.5	61.8 ± 2.0	0.7378
Z-Score	0.30 ± 0.17	0.21 ± 0.27	0.7905

Table 8. Bone density measures of Non-Tanning and Tanning women. BMD, bone mineral density; BMC, bone mineral content. Data are presented as means ± SEM.

	Linear Regression		
	Parameter Estimate	R^2 Value	*P* Value
Whole Body			
BMD	0.00003354	0.0004	0.8808
BMC	-0.15484	0.0007	0.8322
Z-Score	0.00013085	0.0000	0.9601
Hip			
BMD	-0.00014480	0.0026	0.6705
BMC	-0.01190	0.0110	0.4010
Z-Score	-0.00184	0.0083	0.5198
Spine			
BMD	-0.02266	0.0118	0.2255
BMC	-0.03918	0.0316	0.1564
Z-Score	-0.00289	0.0155	0.3594

Table 9. The relationship between 25(OH)D concentrations and bone mineral density (BMD), bone mineral content (BMC), and Z-score for the whole body, hip, and spine in Non-Tanning and Tanning women.

V. Inflammatory Marker Outcomes

Mean serum TNFα, IL-6, IL-10, and CRP values for each group are presented in Table 10. Serum TNFα was significantly lower in the Tanners than the Non-Tanners. IL-6, CRP, and IL-10 did not significantly differ between groups. IL-1β concentrations are not reported due to a minimal number of subjects falling within the standard curve (n = 17).

	Non-Tanners (n = 47 – 49)	**Tanners (n = 17 – 20)**	***P* Value**
TNFα (pg/mL)	1.22 ± 0.11	0.79 ± 0.11*	0.0200
IL-6 (pg/mL)	1.20 ± 0.11	1.22 ± 0.15	0.7031
IL-10 (pg/mL)	2.20 ± 0.20	2.88 ± 0.76	0.2328
CRP (mg/L)	0.7 ± 0.1	0.7 ± 0.1	0.8578

Table 10. Serum inflammatory marker concentrations of Non-Tanning and Tanning women. Data are presented as means ± SEM. *Tanners are significantly different from Non-Tanners, $P<0.05$.

Figure 8 reveals the relationships between 25 (OH)D and TNFα, IL-6, IL-10, and CRP. Serum 25(OH)D concentrations had a significant inverse relationship with TNFα ($R^2 = 0.0605$, $P = 0.0463$). Thus, serum 25(OH)D status is capable of explaining 6.05% of the variation in TNFα concentrations. IL-6, IL-10, and CRP concentrations were not significantly associated with 25(OH)D status.

When controlling for percent body fat, menopausal status, age, serum estradiol, serum cortisol, and hormonal contraceptive use, a significant relationship remained between 25(OH)D and TNFα. Controlling for percent body fat, menopausal status, age, serum estradiol, serum cortisol, and hormonal contraceptive use did not change the relationship between 25(OH)D concentrations and IL-6, IL-10, and CRP. Analysis of potential covariates did reveal a significant positive relationship between menopausal status and IL-6 concentrations ($R^2 = 0.0764$, $P = 0.0246$).

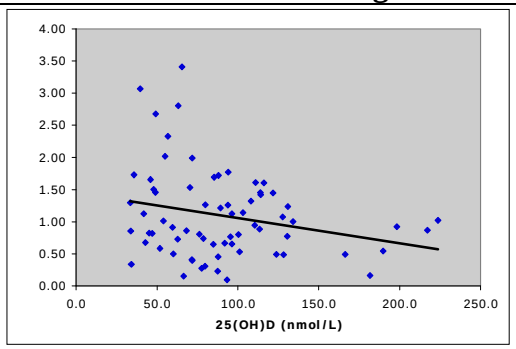
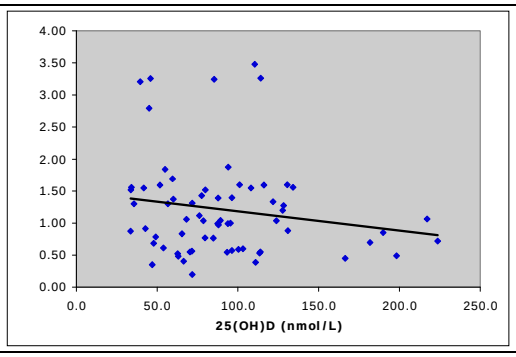
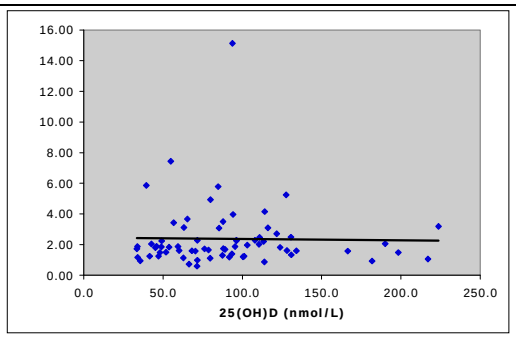
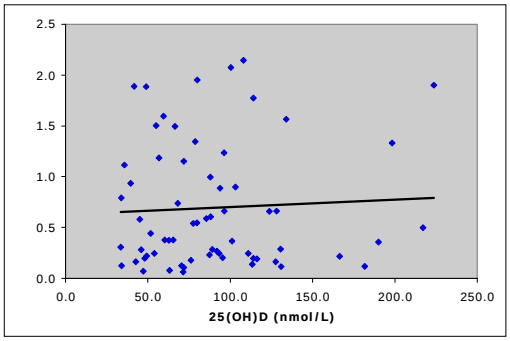
	Linear Regression	
TNFα		$R^2 = 0.0605$ $P = 0.0463$* y = 1.45095 – 0.00393x
IL-6		$R^2 = 0.0440$ $P = 0.0909$ y = 1.48246 – 0.00339x
IL-10		$R^2 = 0.0003$ $P = 0.8906$ y = 2.45715 – 0.00080588x
CRP		$R^2 = 0.0028$ $P = 0.6770$ y = 0.62864 + 0.00072368x

Figure 8. The relationship between 25(OH)D and serum TNFα, IL-6, IL-10, and CRP concentrations in Non-Tanning and Tanning women. Linear regression equations for each graph are presented. *Significant regression equation, $P<0.05$.

DISCUSSION

The objective of the present study was to determine the relationship between 25(OH)D concentrations, inflammatory markers, and bone mineral density in healthy pre- and post-menopausal women who regularly use tanning beds. Furthermore, we hypothesized that increased serum 25(OH)D would result in decreased levels of circulating inflammatory markers. Although IL-6, IL-10, and CRP did not have a statistically significant relationship with 25(OH)D concentrations, Tanners had significantly lower circulating levels of serum TNFα than Non-Tanners, and linear regression models revealed a significant inverse relationship between 25(OH)D and TNFα.

Subject Characteristics. The only significant characteristic difference between the Tanners and Non-Tanners was skin type, as assessed by the Fitzpatrick Method of Skin Typing (Appendix E). This method determines skin type based on ethnic background and ability to burn and/or tan. Thus, it is not surprising that the Tanners had a higher skin type than Non-Tanners because their skin is capable of tanning. Likewise, women with low skin types would not be expected to using a tanning bed, since their skin is less able to tan.

25-Hydroxyvitamin D. Tanners had a significantly higher serum 25(OH)D levels than Non-Tanners. Additionally, 26.5% of Non-Tanners had deficient levels of serum 25(OH)D, 35.7% were insufficient, and only 38.8% of healthy women Non-Tanners could be classified as sufficient according to the categories of 25(OH)D status proposed by Grant, W.B. and Holick, M.F. (Table 2).(33) Conversely, none of the Tanners were deficient, 15% had insufficient levels, and 85% had sufficient serum 25(OH)D

concentrations. These results are in accordance with the overwhelming number of reports documenting vitamin D deficiency and insufficiency in healthy people, (25, 42-51) and provide added support for new recommendations for vitamin D fortification and supplementation, including moderate and responsible sun exposure.

The only other study to examine the 25(OH)D concentrations of individuals who regularly use tanning beds, conducted by Tangpricha *et al.*, showed similar outcomes.(296) In this study, 106 non-tanning control subjects were compared with 50 men and women who regularly used a tanning bed 1 or more times per week for greater than 6 months. Results showed that 41.5% of the non-tanning group was vitamin D deficient, characterized by a 25(OH)D concentration $\leq$ 50 nmol/L. Additionally, 8% of tanners in that study also had 25(OH)D levels that were classified as deficient, in contrast to the present study, in which no tanners could be classified as deficient. This is likely the result of differences in study site locations. Tangpricha *et al.* conducted their study in Boston, MA (42°N) during early March through early June of 2003. Our study was conducted during similar months (late January – early June of 2007); however, the study site was Columbia, MO (39°N). Thus, as compared to those in Boston, MA, subjects in Columbia, MO are located at a lower latitude, and therefore capable of cutaneous synthesis of vitamin D later into the fall months prior to the study and slightly earlier in the spring months while the study was being conducted. Furthermore, mean 25(OH)D concentrations of the Non-Tanners were higher in our study than those in the study conducted by Tangpricha *et al.*, (74.4 $\pm$ 4.02 nmol/L and 60.3 $\pm$ 3.0 nmol/L, respectively). Tanners in Boston, MA, however, had a slightly higher mean 25(OH)D concentration than those in Columbia, MO (115.5 $\pm$ 8.0 versus 129.6 $\pm$ 10.97 nmol/L).

iPTH. Serum concentrations of iPTH in our study were significantly higher in the Non-Tanners than in the Tanners (41.9 $\pm$ 3.06 pg/mL and 26.2 $\pm$ 9.88 pg/mL, respectively). The inverse relationship between 25(OH)D and iPTH seen in this study is in accordance with numerous other reports of iPTH and 25(OH)D concentrations.(26, 28, 36, 296-300) This inverse relationship is also consistent with the findings of Tangpricha *et al.*, who found mean iPTH concentrations in non-tanners to be significantly higher than those of the tanners (25.3 pg/mL versus 21.4 pg/mL).(296) Figure 7 shows the relationship between 25(OH)D concentrations and iPTH concentrations. Although sample size is limited, particularly in the upper range 25(OH)D levels, it appears that iPTH reaches it nadir around a 25(OH)D concentration of 100 nmol/L. This value is similar to the optimal vitamin D status proposed by Heaney R.P. (30, 36), Hollis B.W. (27), Grant W.B and Holick M.F (33) and numerous others (27-31, 33, 35, 299, 300).

Bone Outcomes. We found no significant differences in bone outcomes between groups (Table 7). Whole body and hip measurements were consistently higher in the Tanners than the Non-Tanners; however, these differences did not reach significance. Linear regression analyses also failed to show a significant relationship between 25(OH)D status and BMD, BMC, and Z-scores for the whole body, spine, and hip. Percent body fat was the only independent variable with a significant positive relationship to bone, more specifically, spine BMD and spine Z-score. It is well documented that increased body weight is associated with increased BMD, thus our results are in agreement with the literature.(301, 302)

In contrast, the aforementioned study by Tangpricha *et al.* found that non-tanners (n = 106) had significantly lower total hip BMD and Z-scores than tanners (n = 50).(296)

Additionally, Bischoff-Ferrari *et al.* showed a positive relationship between serum 25(OH)D and total hip bone mineral density in 13,432 subjects using NHANES III data.(31) Two other cross-sectional studies have also shown a positive association between 25(OH)D concentrations and spine bone mineral density.(303, 304) It is important to note that these studies had sample sizes that were more than double the sample size of our study (n = 161 and n = 166). Moreover, subjects in the present study were not excluded based on the type of exercise that they currently participated in, or participated in during peak bone formation years. Since weight-bearing exercise has a known impact on bone mineral density, (305) it is possible that varying exercise routines among subjects may have also contributed the lack of significance in bone measures between groups.

Inflammatory markers. IL-10 concentrations did not significantly differ between Tanners and Non-Tanners. In addition, linear regression models (Figure 8) did not reveal a significant relationship between IL-10 and 25(OH)D concentrations. IL-10 is generally recognized as an anti-inflammatory cytokine and slightly increased levels are considered beneficial. Studies have shown promising effects of vitamin D on IL-10 in vitro (130, 133, 142) and in animal models.(176, 306) In addition, human studies in diseased populations, such as congestive heart failure (87), and in the cord blood of healthy newborn infants (307) have shown positive relationships between 25(OH)D concentrations and IL-10. Seasonal fluctuations in IL-10 have also been observed in isolated humans in the Antarctic.(308) IL-10 is produced at different stages of infection and functions as both an immunosuppressive cytokine and an immune-stimulating cytokine causing the activation of mast cells, promotion of cytotoxic T cells, and

activation of B cells. Additionally, IL-10 is produced in numerous cell types and it is currently unknown whether the IL-10 produced in different cells may be responsible for different levels of immune regulation.(309) Thus, the implications and causes of circulating IL-10 are not completely clear and may play a significant role in the ambiguous outcomes of the aforementioned studies.

There was also not a significant difference between circulating C-Reactive Protein levels in the Non-Tanners and Tanners, and linear regression analyses did not reveal a significant relationship between 25(OH)D and CRP. C-Reactive Protein is a non-specific inflammatory marker that can be used as a general measure of wellness, as it increases with mild chronic infection, aging, and tissue damage.(310) Research in diseased populations, specifically those with diabetes and cardiovascular disease (311), arthritis (183, 312, 313), prolonged chronic illness (94), and clinically deficient 25(OH)D status (<27.5 nmol/L) (314), has shown promising negative associations between vitamin D and CRP levels. Nevertheless, supplementation studies in healthy post-menopausal women (315) and patients with congestive heart failure (87) failed to see changes in CRP levels after supplementation. Seasonal variation in CRP has been noted in older (75+ years) healthy adults (316); however, CRP is know to increase with age (317) causing these subjects to have a higher baseline CRP than those in our study. The relationship between vitamin D and CRP is currently unclear, but the literature appears to indicate a more profound inverse relationship in unhealthy populations.

There were no significant differences in serum IL-6 concentrations between the Non-Tanners and Tanners. A linear regression (Figure 8) did indicate an inverse relationship between 25(OH)D concentrations and serum IL-6, although not statistically

significant. Several in vitro studies have previously revealed that 1,25$(OH)_2$D and several of its analogs are capable of inhibiting the production of IL-6 in various cell types.(149, 158, 318-320) Nevertheless, effects of vitamin D on circulating IL-6 concentrations have failed to show significance in both healthy (321, 322) and unhealthy populations(87, 313). One study did show that both oral and intravenous 1,25$(OH)_2$D supplementation were capable of significantly decreasing serum IL-6 concentrations following 6 months of treatment in patients receiving hemodialysis.(323) However, inclusion criteria consisted of iPTH levels three times higher than the normal upper limits. It has been well documented that parathyroid hormone induces the production of IL-6 by osteoblasts, (324, 325) thus, it is likely that the effects of vitamin D supplementation on serum IL-6 in this population are mediated primarily through the inverse relationship between 25(OH)D and iPTH. A linear regression of iPTH and IL-6 concentrations in our subjects did not reveal a significant relationship. A significant relationship was present between Il-6 and menopausal status: an expected interaction due to several studies reporting IL-6 concentrations increasing with age and contributing to disability and mortality.(326-329) Thus, although our results show a non-significant relationship between increasing 25(OH)D concentrations and decreasing serum IL-6, the appearance of a trend ($P = 0.0909$) warrants further investigation. Low IL-6 levels have the potential to decrease disability and mortality in older populations in addition to helping maintain bone health.

Despite the lack of significance between 25(OH)D concentrations and the previously mentioned inflammatory markers, Tanners had significantly lower TNFα concentrations than Non-Tanners. Additionally, a linear regression revealed a significant

inverse relationship between serum 25(OH)D and TNFα for all subjects (Figure 8). This relationship remained significant after controlling for potential covariates: body fat percentage, menopausal status, age, serum estradiol, serum cortisol, and hormonal contraceptive use. Estradiol was measured to account for the potential of menstrual cycle stage to influence inflammatory marker status. The lack of significance between serum estradiol and any of the inflammatory markers supports previous research indicating that although hormone status may effect cytokine levels, the impact is not detectable.(330) Cortisol is the major glucocorticoid secreted by the adrenal cortex has anti-inflammatory and immunosuppressive functions within the body (331); however, regardless of cortisol levels, a significant inverse association between 25(OH)D and TNFα was present. Hinton *et al*. found that hormonal contraceptive use was also associated with increased TNFα levels in female athletes (332), yet our data did not indicate that a significant relationship was present between hormonal contraceptive use and TNFα ($P = 0.2336$, data not shown), and 25(OH)D was still a significant predictor of TNFα regardless of contraceptive use.

Similar to that of iPTH, it appears that the 25(OH)D concentration at which TNFα reaches its nadir is at or above 100 nmol/L. This again provides even greater evidence to support increasing vitamin D requirements and changing clinical guidelines to reflect an optimal vitamin D status near 80-100 nmol/L.

These results agree with experimental data showing that vitamin D is capable of suppressing TNFα production.(127, 149, 333, 334) Additionally, Zhu *et al.* recently showed that in the colonic tissue of mice with experimental IBD, $1,25(OH)_2D$ was capable of down-regulating several genes associated with TNFα, including proteins

involved in the transcription of TNFα, one of its primary receptors, and TNFα itself.(333) TNFα is produced by activated monocytes and macrophages, mast cells, T cells, B cells, NK cells, fibroblasts, hepatocytes, splenocytes, and ovarian, epidemeral and thymic stromal cells.(110) Thus, vitamin D may be acting upon numerous cell types in order to decrease serum TNFα concentrations.

Human studies have also shown promising effects of vitamin D on TNFα concentrations in diseased populations. Serum levels of TNFα increased in congestive heart failure patients over a period of 9 months, whereas serum TNFα concentrations in patients receiving daily supplementation of vitamin D (2000 IU) remained constant.(87) Calcitriol supplementation for 6 months in post-menopausal women with osteoporosis resulted in a significant reduction in serum TNFα concentrations and increase in bone mineral density.(335) Additionally, six months of calcitriol supplementation in hemodialysis patients also caused significant decreases in serum TNFα.(336) Our study, however, is the first to show a significant inverse relationship between serum 25(OH)D and TNFα concentration in a healthy population.

The mean TNFα concentration of the Non-Tanners and Tanners was 1.22 $\pm$ 0.11 pg/mL and 0.79 $\pm$ 0.11, respectively ($P = 0.0200$), which is approximately a 35% reduction in serum TNFα due to increased vitamin D status. Decreasing levels of circulating TNFα has the potential to positively impact the risk for numerous diseases including multiple sclerosis, inflammatory bowel disease, rheumatoid arthritis, heart disease, osteoporosis, cancer, and numerous others. TNFα levels are increased in the serum and cerebrospinal fluid of multiple sclerosis patients and are significantly correlated with clinical impairment.(337, 338) Giovannoni *et al*. showed that patients

with $\geq$ 2 active brain lesions visible on MRI had serum TNFα concentrations of 13.1 pg/mL, compared to those with less than 2 active brain lesions who had TNFα levels of 11.5 pg/mL ($P = 0.04$).(339) Therefore, a 35% reduction in serum TNFα concentrations induced by vitamin D, which would decrease TNFα levels to 8.5 pg/mL in this population, may be capable of improving or possibly preventing active brain lesions in MS. Additionally, glucocorticoids are commonly utilized in the treatment of this disease, which function in part by reducing serum TNFα levels.(340)

TNFα also plays a central role in inflammatory bowel diseases. Komatsu *et al*. showed that serum TNFα was 9.46 pg/mL in patients with active ulcerative colitis, compared to those with inactive ulcerative colitis at 5.54 pg/mL, which is approximately a 41% difference in TNFα concentrations.(341) Furthermore, those with active Crohn's disease had TNFα concentrations of 14.0 pg/mL, in contrast to patients with inactive Crohn's disease at 11.5 pg/mL. This corresponds to only an 18% difference in circulating TNFα concentrations. Therefore, the potential 35% reduction in TNFα levels induced by increasing vitamin D status may be capable of improving symptoms of IBD and extending periods of inactivity. Anti-TNFα medications have shown clinical significance and progress towards more effetctive management of IBDs.(342) Martinez-Borra *et al*. found that patients with lower TNFα concentrations (14 $\pm$ 25 pg/mL) prior to treatment with the anti-TNF drug infliximab, responded to the treatment, whereas non-responders had significantly higher baseline serum levels (201 $\pm$ 362 pg/mL).(343) Therefore, it is possible that decreasing serum TNFα via vitamin D supplementation may have a synergistic effect with anti-TNF medications.

Disease progression in rheumatoid arthritis is also significantly impacted by TNFα, as it is a primary mediator of leukocyte recruitment to the afflicted joint, as well as a significant contributor to synovial inflammation.(344) Kutukculer, *et al*. showed that plasma TNFα levels increased to 11.5 ± 13.2 pg/mL during active periods of chronic juvenile arthritis, characterized by an increasing number of active joints regardless of drug therapy.(345) If increasing 25(OH)D status can decrease serum TNFα levels by 35%, then these patients could decrease their TNFα concentrations to 7.5 pg/mL, which may improve clinical symptoms and bring TNFα levels closer to those of inactive patients (4.0 ± 3.2 pg/mL). Additionally, it may be possible that long-term vitamin D-induced reductions in TNFα may prevent exacerbations and enable patients to have longer periods of disease inactivity. As expected, anti-TNFα medications and recombinant TNFα receptors are effective treatments for this disease and have also been shown to increase bone mineral density in these patients.(346, 347)

TNFα has also been implicated in the progression of heart disease, as elevated levels of TNFα are present in patients with advancing disease. TNFα levels were 3.2 ± -0.2 pg/mL in patients with New York Heart Association (NYHA) function class II, 4.0 ± -0.3 pg/mL in NYHA function class III patients, and 5.3 ± -0.9 pg/mL in NYHA function class IV patients.(348) As heart failure progressed, indicated by increasing class number, TNFα levels increased, albeit only by ~1 pg/mL with increasing class. Therefore, reductions in TNFα induced via vitamin D supplementation may be capable of slowing disease progression. Additionally, anti-TNFα therapy has been used in heart failure with promising results.(349)

TNFα is both harmful and beneficial in terms of cancer prevention and treatment. Sustained chronic TNFα production causes autocrine growth signals, insensitivity to anti-growth signals, angiogenesis, evasion of apoptosis, invasion and metastasis, and replication potential: all necessary factors in cancer cell survival.(350) Nevertheless, TNFα can also induce apoptosis, resulting in cancer cell death. Despite these contradictory actions, it appears that vitamin D may be capable of modulating TNFα pathways so that only beneficial effects of TNFα are promoted. We have shown that vitamin D may be capable of decreasing systemic TNFα concentrations, whereas other have shown that $1,25(OH)_2D$ and its analogs can increase TNF-induced apoptosis in both breast cancer and prostate cancer cells.(351-353) In addition to its potential role in disease treatment, the numerous epidemiological studies reporting an inverse relationship between UVB light and disease incidence indicates a potential for high vitamin D status, and therefore low serum TNFα, to also play a role in disease prevention.(165, 179, 189, 198, 203, 204, 219, 235, 258, 259)

Limitations. The primary limitation to this study was sample size. Women who tanned regularly and qualified to participate based on our inclusion and exclusion criteria were far more difficult to recruit than non-tanning women. Additionally, women who tan regularly are inherently different from non-tanners, as O'Riordan *et al.* has recently reported.(354) Frequent tanning bed use is associated with health risk behaviors including frequent dieting, using laxatives or vomiting to control weight, smoking cigarettes, binge drinking, and recreational drug use. It is important to mention, however, that we attempted to either control or account for these behaviors through inclusion/exclusion criteria and questionnaire data.

It is likely that we did not see differences in bone measures between the two groups because of the limited sample size, particularly in the tanning group. Furthermore, it is possible that differences in other measured outcomes were not significant due to sample size. Nevertheless, the significant relationship between TNFα and 25(OH)D levels is strengthened due to a limited sample size and a non-homogenous pool of subjects (i.e. Tanners different than Non-Tanners).

Future Research. The implications of this study on future research are immense. First, in reference to optimal vitamin D status, larger studies need to be completed in order to better quantify the 25(OH)D concentration at which TNFα levels reach their nadir. Measuring IL-6 concentrations in a study with a larger sample size may also reveal significance. Additionally, long term studies on the benefits of low circulating levels of TNFα on disease incidence and progression are needed to understand long term effects of increased vitamin D status. Finally, intervention studies need to be conducted in order to evaluate the effects of vitamin D supplementation or UVB phototherapy on any and all of the aforementioned diseases. If proven effective, vitamin D therapy may be utilized in addition to or in place of anti-TNFα medications.

Conclusion. Healthy female women, between the ages of 25 and 82, who regularly visit a tanning salon ($\geq$ 1 time per week for 4 months) had significantly higher serum 25(OH)D, lower iPTH, and lower TNFα concentrations than Non-Tanner controls. Additionally, a linear regression of all subjects' serum 25(OH)D and TNFα levels revealed a significant inverse relationship. We are the first to show this inverse relationship in healthy females. Taken together, this study provides important evidence and support for increasing the current vitamin D requirements and clinical levels of

sufficiency. Additionally, it indicates the potential for vitamin D treatment in numerous diseases and conditions where TNFα plays a significant role in disease severity and progression.

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APPENDIX A. PRE-SCREENING FORM: NON-TANNERS

Name: _____ Phone: _____
Email: _____ Address: _____
Age: _____ _____

1. UV light exposure:

a. Do you make a conscious effort to avoid the sun? **Y / N**
b. Do you wear a daily sunscreen (lotion, makeup, moisturizer)? **Y / N**
c. Do you work outdoors? **Y / N**
d. On average, how many minutes per day do you spend in the sun? _____
e. Are you currently undergoing UV radiation as medical therapy? **Y / N**
f. Do you ever go out in the sun for the purpose of tanning? **Y / N**
   - **If yes**, how often? _____

2. Supplements/ Medications

a. Do you take any dietary supplements other than a general multivitamin? **Y / N**
   - **If yes**, what kind? _____

b. Do you currently take any of the following:
   - anticonvulsant medications *(ex; Dilantin)*? **Y / N**
   - cholesterol-lowering medications (*ex; cholestyramine, colestipol)*? **Y / N**
   - medications to treat the symptoms of stomach/duodenal ulcers or acid reflux *(ex; Cimetidine, Tagamet, Tagamet HB)*? **Y / N**
   - Hormone Replacement Therapy? **Y / N**
   - corticosteroids *(ex; hydrocortisone, prednisone)*? **Y / N**
   - Heparin to prevent blood clots? **Y / N**

Other medications? List:_____
_____
_____

3. Misc. Conditions

a. Do you smoke? **Y / N**
b. Do you have a history of osteoporosis? **Y / N**
c. Do you have any implanted metal in your body? **Y / N**
d. Are you pregnant or might be pregnant? **Y / N**
e. When was your last menstrual period? _____
f. How many menstrual periods do you have per year? _____
g. Are you on a special diet? **Y / N**
   - **If yes,** describe:_____

APPENDIX B. PRE-SCREENING FORM: TANNERS

1. UV light exposure:

a. How often do you use a commercial tanning bed? _____
b. For how long have you been following this tanning routine? _____
c. What type of tanning bed do you use? _____
d. Do you ever go out in the sun for the purpose of tanning? **Y / N**
   - **If yes**, how often? _____

2. Supplements/ Medications

a. Do you take any dietary supplements other than a general multivitamin? **Y / N**
   - **If yes**, what kind? _____

b. Do you currently take any of the following:
   - anticonvulsant medications *(ex; Dilantin)*? **Y / N**
   - cholesterol-lowering medications (*ex; cholestyramine, colestipol)*? **Y / N**
   - medications to treat the symptoms of stomach/duodenal ulcers or acid reflux *(ex; Cimetidine, Tagamet, Tagamet HB)*? **Y / N**
   - Hormone Replacement Therapy? **Y / N**
   - corticosteroids *(ex; hydrocortisone, prednisone)*? **Y / N**
   - Heparin to prevent blood clots? **Y / N**

Other medications? List: _____
_____
_____

3. Misc. Conditions

a. Do you smoke? **Y / N**
b. Do you have a history of osteoporosis? **Y / N**
c. Do you have any implanted metal in your body? **Y / N**
d. Are you pregnant or might be pregnant? **Y / N**
e. When was your last menstrual period? _____
f. How many menstrual periods do you have per year? _____
g. Are you on a special diet? **Y / N**
   - If yes, describe: _____

APPENDIX C. HEALTH HISTORY AND MEDICAL QUESTIONNAIRE

Subject ID Number: _____ Sex: Male Female
Age: _____ Height: _____ Weight: _____
Date: _____
Personal Physician's Name: _____

Have You Ever Had:
Osteoporosis **Y / N** Liver Disease **Y / N** Kidney Disease **Y / N**
Parathyroid Disease **Y / N**
Any Autoimmune Disease **Y / N** If **Yes,** please name: _____
Cancer of any kind **Y / N** If **Yes,** please name: _____
Please list any other current disease(s) or condition(s): _____
Significant weight loss **Y / N** If **Yes,** when & how many lbs: _____
Do you smoke? **Y / N** If Yes, how much? _____
Are you pregnant or might be pregnant? **Y / N**
When was your last menstrual period? _____
How many menstrual periods do you have per year? _____
Do you have any implanted metal in your body? **Y / N**
Are you on a special diet? **Y / N** If **Yes,** please describe: _____
You are an/a African-American _____ Asian _____ Caucasian _____ Hispanic _____
Native American _____ Pacific Islander _____ Other _____

Have You Ever Taken Medication for: Medication & Dosage Date
Y / N Convulsions: _____
Y / N High Cholesterol: _____
Y / N High Blood Pressure: _____
Y / N Stomach/Duodenal Ulcers or Acid Reflux: _____
Y / N Menopausal Symptoms: _____
Y / N Long-term or Chronic Inflammation: _____
Y / N Immune Suppression: _____
Please list any other medications, including dosage you are currently taking or have taken in the past month: _____
_____
_____

Do you exercise regularly? **Y / N** If yes, what activity(ies)?_____
_____
How often?_____

APPENDIX D. SUN EXPOSURE QUESTIONNAIRE

Do you use a tanning bed or capsule? **Y / N**
If yes, how often?
_____ times/week **OR** _____ times/month **OR** _____ times/year
If weekly or monthly, for how long have you been tanning using this schedule?
_____ months **OR** _____ years

What kind of bed/capsule do you use?

How much time do you usually spend outdoors per day? (Includes working outside, walking to and from your car, exercising, gardening, purposeful sun exposure for tanning purposes, etc) _____ min
Is this the same on both weekdays and weekends? If no, please explain.

How often do you usually wear sunscreen or sunblock? (Includes SPF in lotions, moisturizers, tanning lotions/oils, and makeup) e.g. *daily, only when out in the sun for long periods of time, while tanning, never, etc.*

What SPF level do you normally use, if any?

APPENDIX E. FITZPATRICK METHOD OF SKIN TYPING

What skin type are you?

Type 1 I always burn, never tan, and am fair with red or blond hair and freckles (albinos, some redheads)

Type 2 I easily burn, hardly get tan, and am fair skinned (people of northern European origin, such as Scandinavians or Celts)

Type 3 I occasionally burn and gradually tan (people of Mediterranean and Middle East origin)

Type 4 I rarely burn and always tan (people of East Asian origin and some Indians and Pakistanis)

Type 5 I seldom burn, always tan, and have medium-to-dark skin (people of African origin, South East Asians, and some Indians and Pakistanis)

Type 6 I never burn and tan darkly (people with "blue-black" skin, of African origin, and dark-skinned Asians such as Tamils)

APPENDIX F. GAIL MODEL BREAST CANCER RISK ASSESSMENT

This questionnaire can be found online at: http://www.cancer.gov/bcrisktool/

1. Does the woman have a medical history of any breast cancer or of ductal carcinoma in situ (DCIS) or lobular carcinoma in situ (LCIS)?

2. What is the woman's age?
This tool only calculates risk for women 35 years of age or older.

3. What was the woman's age at the time of her first menstrual period?

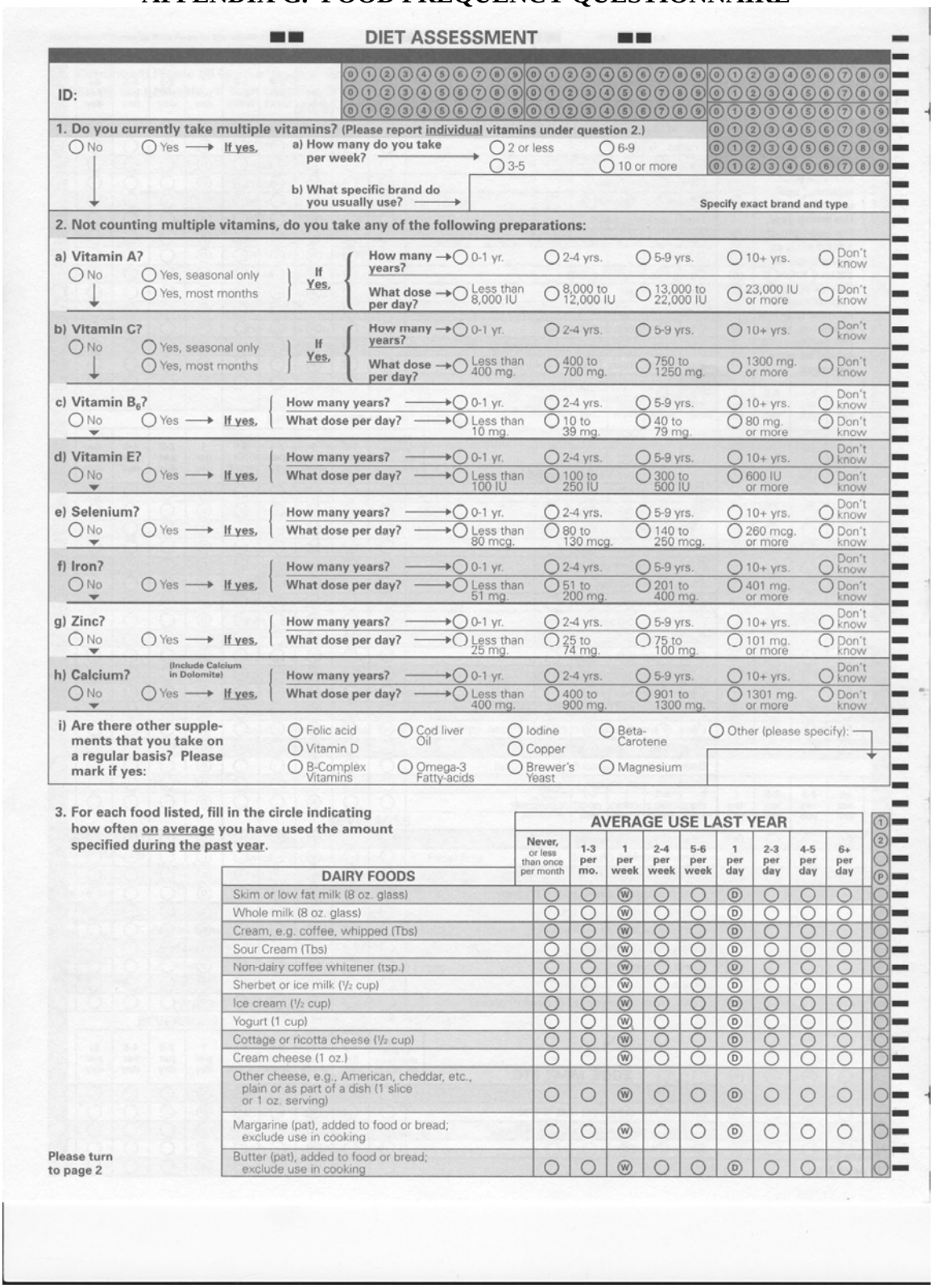
4. What was the woman's age at the time of her first live birth of a child?

5. How many of the woman's first-degree relatives - mother, sisters, daughters - have had breast cancer?

6. Has the woman ever had a breast biopsy?
6a. How many breast biopsies (positive or negative) has the woman had?
6b. Has the woman had at least one breast biopsy with atypical hyperplasia?

7. What is the woman's race/ethnicity?

APPENDIX G. FOOD FREQUENCY QUESTIONNAIRE



DIET ASSESSMENT

ID:

1. Do you currently take multiple vitamins? (Please report individual vitamins under question 2.)

○ No ○ Yes → If yes,
- a) How many do you take per week? → ○ 2 or less ○ 3-5 ○ 6-9 ○ 10 or more
- b) What specific brand do you usually use? → Specify exact brand and type

2. Not counting multiple vitamins, do you take any of the following preparations:

a) Vitamin A? ○ No ○ Yes, seasonal only ○ Yes, most months — If Yes,	How many years?	○ 0-1 yr.	○ 2-4 yrs.	○ 5-9 yrs.	○ 10+ yrs.	○ Don't know
	What dose per day?	○ Less than 8,000 IU	○ 8,000 to 12,000 IU	○ 13,000 to 22,000 IU	○ 23,000 IU or more	○ Don't know
b) Vitamin C? ○ No ○ Yes, seasonal only ○ Yes, most months — If Yes,	How many years?	○ 0-1 yr.	○ 2-4 yrs.	○ 5-9 yrs.	○ 10+ yrs.	○ Don't know
	What dose per day?	○ Less than 400 mg.	○ 400 to 700 mg.	○ 750 to 1250 mg.	○ 1300 mg. or more	○ Don't know
c) Vitamin B_6? ○ No ○ Yes → If yes,	How many years?	○ 0-1 yr.	○ 2-4 yrs.	○ 5-9 yrs.	○ 10+ yrs.	○ Don't know
	What dose per day?	○ Less than 10 mg.	○ 10 to 39 mg.	○ 40 to 79 mg.	○ 80 mg. or more	○ Don't know
d) Vitamin E? ○ No ○ Yes → If yes,	How many years?	○ 0-1 yr.	○ 2-4 yrs.	○ 5-9 yrs.	○ 10+ yrs.	○ Don't know
	What dose per day?	○ Less than 100 IU	○ 100 to 250 IU	○ 300 to 500 IU	○ 600 IU or more	○ Don't know
e) Selenium? ○ No ○ Yes → If yes,	How many years?	○ 0-1 yr.	○ 2-4 yrs.	○ 5-9 yrs.	○ 10+ yrs.	○ Don't know
	What dose per day?	○ Less than 80 mcg.	○ 80 to 130 mcg.	○ 140 to 250 mcg.	○ 260 mcg. or more	○ Don't know
f) Iron? ○ No ○ Yes → If yes,	How many years?	○ 0-1 yr.	○ 2-4 yrs.	○ 5-9 yrs.	○ 10+ yrs.	○ Don't know
	What dose per day?	○ Less than 51 mg.	○ 51 to 200 mg.	○ 201 to 400 mg.	○ 401 mg. or more	○ Don't know
g) Zinc? ○ No ○ Yes → If yes,	How many years?	○ 0-1 yr.	○ 2-4 yrs.	○ 5-9 yrs.	○ 10+ yrs.	○ Don't know
	What dose per day?	○ Less than 25 mg.	○ 25 to 74 mg.	○ 75 to 100 mg.	○ 101 mg. or more	○ Don't know
h) Calcium? (Include Calcium in Dolomite) ○ No ○ Yes → If yes,	How many years?	○ 0-1 yr.	○ 2-4 yrs.	○ 5-9 yrs.	○ 10+ yrs.	○ Don't know
	What dose per day?	○ Less than 400 mg.	○ 400 to 900 mg.	○ 901 to 1300 mg.	○ 1301 mg. or more	○ Don't know

i) Are there other supplements that you take on a regular basis? Please mark if yes:
○ Folic acid ○ Vitamin D ○ B-Complex Vitamins ○ Cod liver Oil ○ Omega-3 Fatty-acids ○ Iodine ○ Copper ○ Brewer's Yeast ○ Beta-Carotene ○ Magnesium ○ Other (please specify):

3. For each food listed, fill in the circle indicating how often on average you have used the amount specified during the past year.

DAIRY FOODS	Never, or less than once per month	1-3 per mo.	1 per week	2-4 per week	5-6 per week	1 per day	2-3 per day	4-5 per day	6+ per day
Skim or low fat milk (8 oz. glass)	○	○	Ⓦ	○	○	Ⓓ	○	○	○
Whole milk (8 oz. glass)	○	○	Ⓦ	○	○	Ⓓ	○	○	○
Cream, e.g. coffee, whipped (Tbs)	○	○	Ⓦ	○	○	Ⓓ	○	○	○
Sour Cream (Tbs)	○	○	Ⓦ	○	○	Ⓓ	○	○	○
Non-dairy coffee whitener (tsp.)	○	○	Ⓦ	○	○	Ⓓ	○	○	○
Sherbet or ice milk (½ cup)	○	○	Ⓦ	○	○	Ⓓ	○	○	○
Ice cream (½ cup)	○	○	Ⓦ	○	○	Ⓓ	○	○	○
Yogurt (1 cup)	○	○	Ⓦ	○	○	Ⓓ	○	○	○
Cottage or ricotta cheese (½ cup)	○	○	Ⓦ	○	○	Ⓓ	○	○	○
Cream cheese (1 oz.)	○	○	Ⓦ	○	○	Ⓓ	○	○	○
Other cheese, e.g., American, cheddar, etc., plain or as part of a dish (1 slice or 1 oz. serving)	○	○	Ⓦ	○	○	Ⓓ	○	○	○
Margarine (pat), added to food or bread; exclude use in cooking	○	○	Ⓦ	○	○	Ⓓ	○	○	○
Butter (pat), added to food or bread; exclude use in cooking	○	○	Ⓦ	○	○	Ⓓ	○	○	○

Please turn to page 2

3. (Continued) Please fill in your average use, during the past year, of each specified food.

Please try to average your seasonal use of foods over the entire year. For example, if a food such as cantaloupe is eaten 4 times a week during the approximate 3 months that it is in season, then the average use would be once per week.

FRUITS	Never, or less than once per month	1-3 per mo.	1 per week	2-4 per week	5-6 per week	1 per day	2-3 per day	4-5 per day	6+ per day
Raisins (1 oz. or small pack) or grapes	○	○	Ⓦ	○	○	Ⓓ	○	○	○
Prunes (½ cup)	○	○	Ⓦ	○	○	Ⓓ	○	○	○
Bananas (1)	○	○	Ⓦ	○	○	Ⓓ	○	○	○
Cantaloupe (¼ melon)	○	○	Ⓦ	○	○	Ⓓ	○	○	○
Watermelon (1 slice)	○	○	Ⓦ	○	○	Ⓓ	○	○	○
Fresh apples or pears (1)	○	○	Ⓦ	○	○	Ⓓ	○	○	○
Apple juice or cider (small glass)	○	○	Ⓦ	○	○	Ⓓ	○	○	○
Oranges (1)	○	○	Ⓦ	○	○	Ⓓ	○	○	○
Orange juice (small glass)	○	○	Ⓦ	○	○	Ⓓ	○	○	○
Grapefruit (½)	○	○	Ⓦ	○	○	Ⓓ	○	○	○
Grapefruit juice (small glass)	○	○	Ⓦ	○	○	Ⓓ	○	○	○
Other fruit juices (small glass)	○	○	Ⓦ	○	○	Ⓓ	○	○	○
Strawberries, fresh, frozen or canned (½ cup)	○	○	Ⓦ	○	○	Ⓓ	○	○	○
Blueberries, fresh, frozen or canned (½ cup)	○	○	Ⓦ	○	○	Ⓓ	○	○	○
Peaches, apricots or plums (1 fresh, or ½ cup canned)	○	○	Ⓦ	○	○	Ⓓ	○	○	○

VEGETABLES	Never, or less than once per month	1-3 per mo.	1 per week	2-4 per week	5-6 per week	1 per day	2-3 per day	4-5 per day	6+ per day
Tomatoes (1)	○	○	Ⓦ	○	○	Ⓓ	○	○	○
Tomato juice (small glass)	○	○	Ⓦ	○	○	Ⓓ	○	○	○
Tomato sauce (½ cup) e.g., spaghetti sauce	○	○	Ⓦ	○	○	Ⓓ	○	○	○
Red chili sauce (1 Tbs)	○	○	Ⓦ	○	○	Ⓓ	○	○	○
Tofu or soybeans (3-4 oz.)	○	○	Ⓦ	○	○	Ⓓ	○	○	○
String beans (½ cup)	○	○	Ⓦ	○	○	Ⓓ	○	○	○
Broccoli (½ cup)	○	○	Ⓦ	○	○	Ⓓ	○	○	○
Cabbage or cole slaw (½ cup)	○	○	Ⓦ	○	○	Ⓓ	○	○	○
Cauliflower (½ cup)	○	○	Ⓦ	○	○	Ⓓ	○	○	○
Brussels sprouts (½ cup)	○	○	Ⓦ	○	○	Ⓓ	○	○	○
Carrots, raw (½ carrot or 2-4 sticks)	○	○	Ⓦ	○	○	Ⓓ	○	○	○
Carrots, cooked (½ cup)	○	○	Ⓦ	○	○	Ⓓ	○	○	○
Corn (1 ear or ½ cup frozen or canned)	○	○	Ⓦ	○	○	Ⓓ	○	○	○
Peas, or lima beans (½ cup fresh, frozen, canned)	○	○	Ⓦ	○	○	Ⓓ	○	○	○
Mixed vegetables (½ cup)	○	○	Ⓦ	○	○	Ⓓ	○	○	○
Beans or lentils, baked or dried (½ cup)	○	○	Ⓦ	○	○	Ⓓ	○	○	○
Yellow (winter) squash (½ cup)	○	○	Ⓦ	○	○	Ⓓ	○	○	○
Eggplant, zucchini, or other summer squash (½ cup)	○	○	Ⓦ	○	○	Ⓓ	○	○	○
Yams or sweet potatoes (½ cup)	○	○	Ⓦ	○	○	Ⓓ	○	○	○
Spinach, cooked (½ cup)	○	○	Ⓦ	○	○	Ⓓ	○	○	○
Spinach, raw as in salad	○	○	Ⓦ	○	○	Ⓓ	○	○	○
Kale, mustard or chard greens (½ cup)	○	○	Ⓦ	○	○	Ⓓ	○	○	○
Iceberg or head lettuce (serving)	○	○	Ⓦ	○	○	Ⓓ	○	○	○
Romaine or leaf lettuce (serving)	○	○	Ⓦ	○	○	Ⓓ	○	○	○
Celery (4" stick)	○	○	Ⓦ	○	○	Ⓓ	○	○	○
Beets (½ cup)	○	○	Ⓦ	○	○	Ⓓ	○	○	○
Alfalfa sprouts (½ cup)	○	○	Ⓦ	○	○	Ⓓ	○	○	○
Garlic, fresh or powdered (1 clove or shake)	○	○	Ⓦ	○	○	Ⓓ	○	○	○

EGGS, MEAT, ETC.	Never, or less than once per month	1-3 per mo.	1 per week	2-4 per week	5-6 per week	1 per day	2-3 per day	4-5 per day	6+ per day
Eggs (1)	○	○	Ⓦ	○	○	Ⓓ	○	○	○
Chicken or turkey, with skin (4-6 oz.)	○	○	Ⓦ	○	○	Ⓓ	○	○	○
Chicken or turkey, without skin (4-6 oz.)	○	○	Ⓦ	○	○	Ⓓ	○	○	○
Bacon (2 slices)	○	○	Ⓦ	○	○	Ⓓ	○	○	○
Hot dogs (1)	○	○	Ⓦ	○	○	Ⓓ	○	○	○

Please go to page 3

3. (Continued) Please fill in your average use, during the past year, of each specified food.

MEATS (CONTINUED)	Never, or less than once per month	1-3 per mo.	1 per week	2-4 per week	5-6 per week	1 per day	2-3 per day	4-5 per day	6+ per day
Processed meats, e.g., sausage, salami, bologna, etc. (piece or slice)	○	○	Ⓦ	○	○	Ⓓ	○	○	○
Liver (3-4 oz.)	○	○	Ⓦ	○	○	Ⓓ	○	○	○
Hamburger (1 patty)	○	○	Ⓦ	○	○	Ⓓ	○	○	○
Beef, pork, or lamb as a sandwich or mixed dish, e.g., stew, casserole, lasagne, etc.	○	○	Ⓦ	○	○	Ⓓ	○	○	○
Beef, pork, or lamb as a main dish, e.g., steak, roast, ham, etc. (4-6 oz.)	○	○	Ⓦ	○	○	Ⓓ	○	○	○
Canned tuna fish (3-4 oz.)	○	○	Ⓦ	○	○	Ⓓ	○	○	○
Dark meat fish, e.g., mackerel, salmon, sardines bluefish, swordfish (3-5 oz.)	○	○	Ⓦ	○	○	Ⓓ	○	○	○
Other fish (3-5 oz.)	○	○	Ⓦ	○	○	Ⓓ	○	○	○
Shrimp, lobster, scallops as a main dish	○	○	Ⓦ	○	○	Ⓓ	○	○	○

BREADS, CEREALS, STARCHES	Never, or less than once per month	1-3 per mo.	1 per week	2-4 per week	5-6 per week	1 per day	2-3 per day	4-5 per day	6+ per day
Cold breakfast cereal (1 cup)	○	○	Ⓦ	○	○	Ⓓ	○	○	○
Cooked oatmeal (1 cup)	○	○	Ⓦ	○	○	Ⓓ	○	○	○
Other cooked breakfast cereal (1 cup)	○	○	Ⓦ	○	○	Ⓓ	○	○	○
White bread (slice), including pita bread	○	○	Ⓦ	○	○	Ⓓ	○	○	○
Dark bread (slice)	○	○	Ⓦ	○	○	Ⓓ	○	○	○
English muffins, bagels, or rolls (1)	○	○	Ⓦ	○	○	Ⓓ	○	○	○
Muffins or biscuits (1)	○	○	Ⓦ	○	○	Ⓓ	○	○	○
Brown rice (1 cup)	○	○	Ⓦ	○	○	Ⓓ	○	○	○
White rice (1 cup)	○	○	Ⓦ	○	○	Ⓓ	○	○	○
Pasta, e.g., spaghetti, noodles, etc. (1 cup)	○	○	Ⓦ	○	○	Ⓓ	○	○	○
Other grains, e.g., bulgar, kasha, couscous, etc. (1 cup)	○	○	Ⓦ	○	○	Ⓓ	○	○	○
Pancakes or waffles (serving)	○	○	Ⓦ	○	○	Ⓓ	○	○	○
French fried potatoes (4 oz.)	○	○	Ⓦ	○	○	Ⓓ	○	○	○
Potatoes, baked, boiled (1) or mashed (1 cup)	○	○	Ⓦ	○	○	Ⓓ	○	○	○
Potato chips or corn chips (small bag or 1 oz.)	○	○	Ⓦ	○	○	Ⓓ	○	○	○
Crackers, Triskets, Wheat Thins (1)	○	○	Ⓦ	○	○	Ⓓ	○	○	○
Pizza (2 slices)	○	○	Ⓦ	○	○	Ⓓ	○	○	○

		BEVERAGES	Never, or less than once per month	1-3 per mo.	1 per week	2-4 per week	5-6 per week	1 per day	2-3 per day	4-5 per day	6+ per day
CARBONATED BEVERAGES	Low Calorie (sugar-free) types	Low calorie cola, e.g., Tab with caffeine	○	○	Ⓦ	○	○	Ⓓ	○	○	○
		Low calorie caffeine-free cola, e.g., Pepsi Free	○	○	Ⓦ	○	○	Ⓓ	○	○	○
Consider the serving size as 1 glass, bottle or can for these carbonated beverages.		Other low calorie carbonated beverage, e.g., Fresca, Diet 7-Up, diet ginger ale	○	○	Ⓦ	○	○	Ⓓ	○	○	○
	Regular types (not sugar-free)	Coke, Pepsi, or other cola with sugar	○	○	Ⓦ	○	○	Ⓓ	○	○	○
		Caffeine Free Coke, Pepsi, or other cola with sugar	○	○	Ⓦ	○	○	Ⓓ	○	○	○
		Other carbonated beverage with sugar, e.g., 7-Up, ginger ale	○	○	Ⓦ	○	○	Ⓓ	○	○	○
OTHER BEVERAGES		Hawaiian Punch, lemonade, or other non-carbonated fruit drinks (1 glass, bottle, can)	○	○	Ⓦ	○	○	Ⓓ	○	○	○
		Decaffeinated coffee (1 cup)	○	○	Ⓦ	○	○	Ⓓ	○	○	○
		Coffee (1 cup)	○	○	Ⓦ	○	○	Ⓓ	○	○	○
		Tea (1 cup), not herbal teas	○	○	Ⓦ	○	○	Ⓓ	○	○	○
		Beer (1 glass, bottle, can)	○	○	Ⓦ	○	○	Ⓓ	○	○	○
		Red wine (4 oz. glass)	○	○	Ⓦ	○	○	Ⓓ	○	○	○
		White wine (4 oz. glass)	○	○	Ⓦ	○	○	Ⓓ	○	○	○
		Liquor, e.g., whiskey, gin, etc. (1 drink or shot)	○	○	Ⓦ	○	○	Ⓓ	○	○	○

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3. (Continued) Please fill in your average use during the past year, of each specified food.

SWEETS, BAKED GOODS, MISCELLANEOUS	Never, or less than once per month	1-3 per mo.	1 per week	2-4 per week	5-6 per week	1 per day	2-3 per day	4-5 per day	6+ per day
Chocolate (bars or pieces) e.g., Hershey's, M & M's	○	○	Ⓦ	○	○	Ⓓ	○	○	○
Candy bars, e.g., Snickers, Milky Way, Reeses	○	○	Ⓦ	○	○	Ⓓ	○	○	○
Candy without chocolate (1 oz.)	○	○	Ⓦ	○	○	Ⓓ	○	○	○
Cookies, home baked (1)	○	○	Ⓦ	○	○	Ⓓ	○	○	○
Cookies, ready made (1)	○	○	Ⓦ	○	○	Ⓓ	○	○	○
Brownies (1)	○	○	Ⓦ	○	○	Ⓓ	○	○	○
Doughnuts (1)	○	○	Ⓦ	○	○	Ⓓ	○	○	○
Cake, home baked (slice)	○	○	Ⓦ	○	○	Ⓓ	○	○	○
Cake, ready made (slice)	○	○	Ⓦ	○	○	Ⓓ	○	○	○
Sweet roll, coffee cake or other pastry, home baked (serving)	○	○	Ⓦ	○	○	Ⓓ	○	○	○
Sweet roll, coffee cake or other pastry, ready made (serving)	○	○	Ⓦ	○	○	Ⓓ	○	○	○
Pie, homemade (slice)	○	○	Ⓦ	○	○	Ⓓ	○	○	○
Pie, ready made (slice)	○	○	Ⓦ	○	○	Ⓓ	○	○	○
Jams, jellies, preserves, syrup, or honey (1 Tbs)	○	○	Ⓦ	○	○	Ⓓ	○	○	○
Peanut butter (Tbs)	○	○	Ⓦ	○	○	Ⓓ	○	○	○
Popcorn (1 cup)	○	○	Ⓦ	○	○	Ⓓ	○	○	○
Nuts (small packet or 1 oz.)	○	○	Ⓦ	○	○	Ⓓ	○	○	○
Bran, added to food (1 Tbs)	○	○	Ⓦ	○	○	Ⓓ	○	○	○
Wheat germ (1 Tbs)	○	○	Ⓦ	○	○	Ⓓ	○	○	○
Chowder or cream soup (1 cup)	○	○	Ⓦ	○	○	Ⓓ	○	○	○
Oil and vinegar dressing, e.g., Italian (1 Tbs)	○	○	Ⓦ	○	○	Ⓓ	○	○	○
Mayonnaise or other creamy salad dressing (1 Tbs)	○	○	Ⓦ	○	○	Ⓓ	○	○	○
Mustard, dry or prepared (1 tsp)	○	○	Ⓦ	○	○	Ⓓ	○	○	○
Pepper (1 shake)	○	○	Ⓦ	○	○	Ⓓ	○	○	○
Salt (1 shake)	○	○	Ⓦ	○	○	Ⓓ	○	○	○

4. How much of the visible fat on your meats do you remove before eating?
○ Remove all visible fat ○ Remove small part of fat
○ Remove majority ○ Remove none
○ (Don't eat meat)

5. What kind of fat do you usually use for frying and sautéing? (Exclude "Pam"-type spray)
○ Real butter ○ Vegetable oil ○ Lard
○ Margarine ○ Vegetable shortening

6. What kind of fat do you usually use for baking?
○ Real butter ○ Vegetable oil ○ Lard
○ Margarine ○ Vegetable shortening

7. What form of margarine do you usually use?
○ None ○ Stick ○ Tub ○ Spread
○ Low-calorie stick ○ Low-calorie tub

8. How often do you eat food that is fried at home? (Exclude the use of "Pam"-type spray)
○ Daily ○ 4–6 times per week
○ 1–3 times per week ○ Less than once a week

9. How often do you eat fried food away from home? (e.g., french fries, fried chicken, fried fish)
○ Daily ○ 4–6 times per week
○ 1–3 times per week ○ Less than once a week

10. How many teaspoons of sugar do you add to your beverages or food each day? → tsp.

11. What type of cooking oil do you usually use? → Specify type and brand

12. What kind of cold breakfast cereal do you usually use? → Specify type and brand

13. Are there any other important foods that you usually eat at least once per week?

Include for example: paté, tortillas, yeast, cream sauce, custard, horseradish, parsnips, rhubarb, radishes, fava beans, carrot juice, coconut, avocado, mango, papaya, dried apricots, dates, figs.

(Do not include dry spices and do not list something that has been listed in the previous sections.)

Other foods that you usually use at least once per week	Usual serving size	Servings per week
(a)		
(b)		
(c)		
(d)		