January 2008

The Relationship between Serum Leptin, 25-hydroxyvitamin D3, and Body Composition

Isabel L. Guenther
University of Massachusetts Amherst

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THE RELATIONSHIP BETWEEN SERUM LEPTIN, 25-HYDROXYVITAMIN D₃, AND BODY COMPOSITION

A Thesis Presented

by

ISABEL GUENTHER

Submitted to the Graduate School of the University of Massachusetts Amherst in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

September 2008

Nutrition
THE RELATIONSHIP BETWEEN SERUM LEPTIN, 25-HYDROXYVITAMIN D₃, AND BODY COMPOSITION

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CHAPTER 1

INTRODUCTION

Two pressing issues of public health concern are plaguing many parts of the world today and are pervasive throughout nearly all age groups, genders, and races. The first, obesity, has long been recognized as an ever-growing epidemic, and extensive prevalence data exist reflecting its gravity. The second, vitamin D deficiency, has only recently entered the public health consciousness, which is why data on its prevalence are still incomplete, but nonetheless compelling. Both represent conditions severe and widespread enough to warrant extensive study accompanied by timely action. Moreover, these conditions may in fact be interrelated.

Table 1.1 Body Mass Index

<table>
<thead>
<tr>
<th>BMI</th>
<th>Weight Classification</th>
</tr>
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<tbody>
<tr>
<td>&lt; 18.5</td>
<td>underweight</td>
</tr>
<tr>
<td>18.5 – 24.9</td>
<td>normal/healthy weight</td>
</tr>
<tr>
<td>25.0 – 29.9</td>
<td>overweight</td>
</tr>
<tr>
<td>≥ 30</td>
<td>obese</td>
</tr>
</tbody>
</table>

\[ BMI = \frac{\text{weight (kg)}}{\text{height (m}^2\text{)}} \]
Obesity is characterized by excess body weight in the form of fat, and is defined by a Body Mass Index (BMI) of $\geq 30$ kg/m$^2$ (Table 1.1). Excess body fat carries with it a considerable risk for developing chronic diseases such as type-2-diabetes and heart disease (Solomon & Manson, 1997).

The past 20 years have seen a very large increase in obesity in the United States. Just looking at recent years reveals the extent of this development. In 1995, less than 20% of the population in each of the 50 states was obese. Only five years later, in 2000, 22, or almost half the states in the U.S. had reached or passed the 20% mark. Another five years later, in 2005, that number had more than doubled to 46 states. Some states fared even worse. In 17 states, the prevalence of obesity had risen to $\geq 25\%$, and to $\geq 30\%$ in three states (Louisiana, Mississippi, West Virginia). In 2006, the only state with $<$20% of its population obese was Colorado. An additional 30% or more of each state’s population is overweight, leaving less than half of the total U.S. population at a normal weight [Centers for Disease Control and Prevention (CDC), 2007].

In Massachusetts, the prevalence of obesity has increased greatly in just the past six years. In the 18-24 age group, it more than doubled from 7.4% in 1998 to 16.5% in 2006. The 25-34 age group experienced an increase from 13.4% to 16.5% in the same time period. Both genders are affected, although the increase has been more pronounced in females than in males. While the prevalence of obesity has increased most dramatically in those who have not completed high
school, all educational levels are affected, including college graduates. Those with some post-high school education as well as college graduates experienced a rise in obesity prevalence from 13.5% and 11.1% in 1998 to 21.6% and 15.5% in 2006, respectively [CDC Behavioral Risk Factor Surveillance System (BRFSS), 2006].

Obesity is caused by an energy imbalance that occurs when more energy is consumed than expended. Ultimately, excessive food intake and/or lack of physical activity lie at the root of almost every case of obesity. However, while the basic cause seems simple, a host of factors influences the two main determinants. These include availability of energy-dilute foods such as fruits and vegetables, opportunity for planned and incidental physical activity, emotional factors governing eating behavior, as well as physical factors determining appetite and energy expenditure.

As a species, humans have been able to maintain a constant body weight over long periods of time without consciously matching energy intake to energy output. Evolution has endowed us with an intricate hormonal system that, under normal circumstances, is able to ensure a state of relative energy balance. One of the major players in this system is leptin (Schwartz et al., 2000).

Leptin, the product of the \textit{ob} gene, belongs to the group of adipokines (fat cell secretory products) that are involved in maintaining energy balance. The
hormone initiates a pathway that induces energy expending processes while suppressing appetite. In the control of food intake, leptin may elicit both short- and long-term responses. It may aid in controlling body weight by informing the brain about the size of the body’s fat stores (Cohen, 2006). Simultaneously, it may elicit a meal-specific satiety response to control short-term food intake (Pico et al. 2003). In addition to influencing food intake, leptin stimulates energy expenditure by inducing thermogenesis (Siegrist-Kaiser et al., 1997). Leptin acts as a gene transcription factor (Zhang & Scarpace, 2006). It may therefore regulate the expression of a number of proteins, both related and unrelated to energy balance. Furthermore, the expression of leptin itself may be influenced by other hormones. Limited evidence suggests that the active form of vitamin D may be one such hormone (Menendez et al., 2001).

Vitamin D is a fat-soluble vitamin, the active form of which regulates the expression of numerous genes, including those involved in serum calcium homeostasis (Lips, 2006). In addition, the active form of vitamin D has been identified as a modulator of cell differentiation and proliferation (Miyaura et al., 1985; Tanaka et al., 1982). The most important source of vitamin D is endogenous cutaneous synthesis as a result of sunlight exposure. Some plant and animal products contain small amounts of the vitamin. However, natural food sources are few, and cutaneous synthesis is affected by so many variables that it by no means produces consistently sufficient quantities of vitamin D in many populations (Holick, 2004a). Research over the past several decades has begun to reveal the high prevalence of vitamin D deficiency that appears to span across
nations and populations (Calvo & Whiting, 2003; Chapuy et al., 1997; Harinarayan et al., 2007; Oliver et al., 2004; Zadshir et al., 2005). In addition, new research into vitamin D’s calcemic and non-calcemic functions has spurred debate surrounding a possible revision of the current Adequate Intake (AI) for vitamin D, with many experts demanding it be increased (Dawson-Hughes et al., 2005; Heaney, 2003; Holick, 2004a; Hollis & Wagner, 2004; Whiting & Calvo, 2005).

Poor vitamin D status often accompanies obesity (Arunabh et al., 2003; Buffington et al., 1993). Conversely, numerous studies have confirmed that serum leptin concentrations increase proportionally to body fat mass (Havel et al., 1996; Considine et al., 1996; Rosenbaum et al., 1996). To date, only three studies have evaluated the relationship between vitamin D and leptin, and only one study has reported an inverse association between serum concentrations of leptin and 25(OH)D₃, the circulating form of vitamin D (Gomez et al., 2004). The present study aims to evaluate this relationship in young women living in Western Massachusetts as well as determine whether BMI and body fat mass modify this relationship.
CHAPTER 2

VITAMIN D

The term vitamin D encompasses a number of metabolites as they occur in animals and plants. Ergocalciferol, or vitamin D$_2$, is the form of the vitamin found in plants. Cholecalciferol, or vitamin D$_3$, is the principal form of the vitamin and is synthesized cutaneously by most animals, including humans, from 7-dehydrocholesterol. Both ergocalciferol and cholecalciferol can undergo two subsequent hydroxylations in the body to yield, respectively, the circulating forms 25-hydroxyvitamin D$_2$ and 25-hydroxyvitamin D$_3$ (25(OH)D$_2$ and 25(OH)D$_3$), and the active forms, 1,25-dihydroxyvitamin D$_2$ and 1,25-dihydroxyvitamin D$_3$ (1,25(OH)$_2$D$_3$ or calcitriol; Fig. 2.1). Although in the United States ergocalciferol is sometimes used in supplements and to fortify foods, it is utilized by the body only 20-40% as efficiently as cholecalciferol (Holick, 2005).

Calcitriol has long been recognized as an indispensable regulator of serum calcium homeostasis (Lips, 2006). Because of its ability to promote intestinal calcium absorption, calcitriol furthermore contributes significantly to the mineralization and maintenance of bone (Lips, 2006). Besides this primary function, calcitriol has been recognized in recent years for its ability to induce cell differentiation and inhibit proliferation in a number of tissues (Trump et al., 2004).
In line with these findings, a number of epidemiologic studies point toward a possible anti-tumorigenic role of vitamin D, possibly in the form of calcitriol (Garland et al., 1989; Garland et al., 1990; Gorham et al., 2005; Grant, 2003). In addition, a growing body of research is beginning to reveal a preventive role for vitamin D in a host of other diseases, such as multiple sclerosis, cardiovascular disease, and both forms of diabetes mellitus (Mathieu et al., 2005; Ponsonby et al., 2002; van der Mei et al., 2003).

![Figure 2.1. Chemical forms of vitamin D](image)

While present in a limited number of foods, the main source of vitamin D is endogenous synthesis triggered by ultraviolet radiation. Subsequent hydroxylations result in synthesis of the active form, calcitriol, which then acts via two different mechanisms to influence biological pathways (Dusso et al., 2005; Lips, 2006). Because endogenously synthesized vitamin D is difficult to quantify, no Recommended Dietary Allowance (RDA) has been established, and current recommendations are based on the estimated AI. Based on new evidence concerning calcitriol’s role in calcium homeostasis as well as its possible additional functions, a group of researchers is currently arguing for a revision of the AI to reflect a growing body of evidence suggesting that physiologic needs
are higher than previously recognized (Dawson-Hughes et al., 2005; Heaney, 2003; Holick, 2004a; Hollis, 2005; Whiting & Calvo, 2005).

2.1 CUTANEOUS SYNTHESIS AND CONVERSION TO ACTIVE FORM

2.1.1 Mechanism
Given sunlight exposure, the human body is capable of synthesizing vitamin D. Triggered by exposure to UV-B radiation at wavelengths between 290-315 nm, 7-dehydrocholesterol (which is naturally present in the skin) is isomerized to previtamin D$_3$. Thermal transformation then causes the non-enzymatic isomerization of previtamin D$_3$ to vitamin D$_3$. In this form, vitamin D$_3$ can enter the blood where it travels to the liver bound to vitamin D binding protein (DBP). In the liver, the P450 enzyme CYP27A1 (25-hydroxylase) catalyzes the hydroxylation of vitamin D$_3$ to 25(OH)D$_3$, the circulating form of the vitamin that possesses very little biological activity. From the liver, 25(OH)D$_3$ is distributed to all the tissues containing vitamin D receptors (VDR), including the kidneys, bones, and reproductive organs. When the need for vitamin D activity arises, 25(OH)D$_3$ undergoes a second hydroxylation, catalyzed by CYP27B1 (1α-hydroxylase), to 1,25(OH)$_2$D$_3$ (calcitriol), a steroid hormone and the active form of the vitamin. Although the second hydroxylation occurs primarily in the kidneys, many other tissues have recently been shown to contain the 1α-hydroxylase necessary for this step (Table 2.1). Following the second hydroxylation, renally hydroxylated
calcitriol acts as an endocrine hormone, while extrarenally hydroxylated calcitriol is thought to act in an autocrine and paracrine fashion, specifically with respect to cell differentiation (Lips, 2006).

### Table 2.1. Selected Tissues and Cells Expressing the VDR and 1α-hydroxylase

<table>
<thead>
<tr>
<th>VDR</th>
<th>1α-hydroxylase</th>
</tr>
</thead>
<tbody>
<tr>
<td>kidneys</td>
<td>immune cells</td>
</tr>
<tr>
<td>bones</td>
<td>colon</td>
</tr>
<tr>
<td>brain</td>
<td>placenta</td>
</tr>
<tr>
<td>heart</td>
<td>breast</td>
</tr>
<tr>
<td>skin</td>
<td>prostate</td>
</tr>
<tr>
<td>pancreatic β-islet cells</td>
<td>pancreas</td>
</tr>
<tr>
<td>breast</td>
<td></td>
</tr>
<tr>
<td>prostate</td>
<td></td>
</tr>
<tr>
<td>colon</td>
<td></td>
</tr>
<tr>
<td>activated T and B lymphocytes</td>
<td></td>
</tr>
</tbody>
</table>

#### 2.1.2 Influences on Cutaneous Synthesis and Vitamin D Status

Food sources of vitamin D include fatty fish (salmon, mackerel, sardines, etc.), cod liver oil, as well as fortified versions of milk, orange juice, cereals, and some other dairy products (Holick, 2004a). Table 2.2 lists the vitamin D content of selected foods. A commonly consumed vitamin-D-containing food, fortified milk, contains 98 IU per 1-cup serving (about half of the current AI for females up to age 50). In contrast, 1 total-body minimal erythemal dose of sun exposure (MED, the amount of sun exposure required to produce a slight pinkness of the skin) results in the cutaneous synthesis of 10,000 – 20,000 IU (Hollis, 2005).
Theoretically, the human body is capable of meeting its vitamin D needs through sun exposure. However, cutaneous synthesis depends on so many variables that vitamin D status is often inadequate, and vitamin D deficiency is a common problem throughout the world. Vitamin D deficiency manifests as rickets in children and as osteomalacia in adults. Both conditions are characterized by a decrease in bone mineralization caused by impaired calcium absorption as a result of insufficient mediation by calcitriol (Holick, 2004a).

Only a narrow range of wavelengths (290-315 nm UV-B) stimulates photosynthesis of vitamin D₃. Consequently, at certain latitudes, no vitamin D₃ is made during the winter months when sunlight meets the atmosphere at an oblique angle causing most of the UV-B radiation to be absorbed by the ozone layer. Any geographical area above a latitude of 37° in the Northern hemisphere (such as Massachusetts) or below 37° in the Southern hemisphere supports

### Table 2.2. Selected Food Sources of Vitamin D (IU)*

<table>
<thead>
<tr>
<th>Food</th>
<th>IU</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cod liver oil, 1 tablespoon</td>
<td>1,360</td>
</tr>
<tr>
<td>Salmon, cooked, 3 ½ ounces</td>
<td>360</td>
</tr>
<tr>
<td>Mackerel, cooked, 3 ½ ounces</td>
<td>345</td>
</tr>
<tr>
<td>Sardines, canned in oil, drained, 1 ¾ ounces</td>
<td>250</td>
</tr>
<tr>
<td>Tuna fish, canned in oil, 3 ounces</td>
<td>200</td>
</tr>
<tr>
<td>Milk, nonfat, reduced fat, and whole,</td>
<td></td>
</tr>
<tr>
<td>vitamin D fortified, 1 cup</td>
<td>98</td>
</tr>
<tr>
<td>Ready-to-eat cereals fortified with 10% of the Daily Value for vitamin D, ¾ cup to 1 cup</td>
<td>40</td>
</tr>
<tr>
<td>Orange juice, vitamin D fortified, 1 cup</td>
<td>100</td>
</tr>
</tbody>
</table>

Adapted from http://ods.od.nih.gov/factsheets/vitamind.asp#h2
*40 IUs = 1 µg vitamin D₃
virtually no vitamin D$_3$ synthesis during the winter months (November through February in the Northern hemisphere; Holick, 2004a).

Other risk factors for vitamin D deficiency include obesity, age, and skin color. The ability to synthesize vitamin D$_3$ decreases with age as 7-dehydrocholesterol levels in the skin decline (MacLaughlin & Holick, 1985). The use of sunscreen with a skin protective factor (SPF) of 8 or more inhibits synthesis by absorbing UV-B radiation, as does the pigment melanin, meaning that cutaneous vitamin D$_3$ synthesis is lower in individuals with dark skin (Holick, 2004a). In fact, very dark-skinned persons need 120 minutes of peak summer UV-B exposure to synthesize as much vitamin D$_3$ (1 MED) as a light-skinned person can make in 10-12 minutes with comparable UV-B exposure (Hollis, 2005). Although 25(OH)D$_3$ has a half-life of only two weeks, its fat-soluble precursor, cholecalciferol, can be sequestered in body fat for use when cutaneous synthesis is insufficient (such as in the winter; Holick, 2004b). However, in obese persons, the excess fat is thought to provide such deep storage depots that cholecalciferol sequestered there is difficult to mobilize, resulting in lower serum 25(OH)D$_3$ concentrations (Wortsman et al., 2000).

Prolonged UV-B exposure does not result in excessive vitamin D$_3$ synthesis, because the pre-vitamin form is catabolized to lumisterol and tachysterol. Furthermore, vitamin D$_3$ is degraded to supersterol I, supersterol II, and 5,6-trans-vitamin D$_3$ in response to excessive UV-B exposure (Holick, 2000).
However, toxicity can occur from excessive supplement intake. There is currently no consensus as to the serum levels that have to be reached to produce toxicity. Rizzoli et al. (1994) reported on seven cases of hypercalcemia that were associated with plasma 25(OH)D$_3$ levels ranging from 221 nmol/L (intake: 600,000 IU/wk or ~86,000 IU/d) to 1692 nmol/L (intake: 300,000 IU/d). Supplemental vitamin D$_3$ intake in these subjects ranged from 10,000 to 300,000 IU per day. The duration of supplement treatment prior to admission for hypercalcemia was highly variable, but had been at least three weeks (Rizzoli et al., 1994).

At high serum concentrations, 25(OH)D$_3$ is capable of eliciting responses mediated by the nuclear vitamin D receptor (VDR$_{nuc}$). Considering that hydroxylation to calcitriol is tightly controlled, toxicity is probably attributable to high plasma levels of 25(OH)D$_3$ rather than calcitriol. Vitamin D toxicity is characterized by hypercalcemia, resulting in soft tissue calcification and kidney stones. Other symptoms of vitamin D toxicity include hypercalciuria, anorexia, nausea and vomiting, thirst, muscular weakness, joint pain, disorientation, and eventual death if not treated (Norman, 2001).
2.2 PATHWAYS OF ACTION

Calcitriol exerts its effects via two different pathways – genomic and nongenomic. The genomic pathway begins with calcitriol binding to the VDR\textsubscript{nucl}. The ensuing complex forms a heterodimer with the retinoic acid X receptor (RXR), and then binds to a vitamin-D-responsive element on specific genes. Subsequently, a number of transcriptional factors such as DRIP (vitamin D receptor-interacting protein) attach to it, initiating or suppressing transcription and translation of the vitamin-D-responsive gene (Lips, 2006).

The nongenomic pathway, or rapid response pathway, causes a much quicker biological response than is achieved by gene transcription. It is thought to occur as a result of calcitriol binding to receptors located on the outer plasma membrane of cells (VDR\textsubscript{mem}). Two receptors – 1,25D\textsubscript{3}-MARRS and anexin II – have been identified to date, although much remains to be learned about these proteins (Dusso et al., 2005). Numerous studies suggest that an intricate interplay, or “cross-talk,” takes place between the genomic and nongenomic actions of calcitriol. Hence, the VDR\textsubscript{nucl} may be needed for nongenomic actions, and nongenomic actions may influence calcitriol-induced gene expression. However, these mechanisms remain to be elucidated (Dusso et al., 2005).
2.3 PHYSIOLOGIC ROLES OF CALCITRIOL

2.3.1 Calcium Homeostasis and Bone Metabolism

Calcium homeostasis is one of the most tightly regulated systems in the human body, relying on multilevel control mechanisms. When serum calcium levels drop below 2.5 mmol/L, parathyroid hormone (PTH) is released by the parathyroid gland, triggering a number of processes that help restore serum calcium levels to normal. PTH results in the activation of renal 1α-hydroxylase, increased bone resorption through activation of osteoclasts, and increased renal calcium reabsorption (Holick, 2004a). Renal 1α-hydroxylase catalyzes the conversion of 25(OH)D₃ to its active form calcitriol (Lips, 2006).

Calcitriol acts on several levels to normalize serum calcium concentrations. It attaches to VDRs on intestinal enterocytes, which results in increased expression of an epithelial calcium channel as well as increased expression of the gene coding for calbindin and other proteins thought to facilitate calcium transport across the enterocyte into the circulation (Holick, 2005). Calcitriol also facilitates intestinal calcium absorption through the nongenomic pathway without mediation by calbindin (Lips, 2006).

Calcitriol works in concert with PTH to cause an increase in bone resorption. Calcitriol binds to VDRs in osteoblasts, triggering the expression of receptor activator of nuclear factor-κB ligand (RANKL), which, in turn, binds to
preosteoclasts, causing them to differentiate into mature osteoclasts. As osteoclasts break down bone by releasing hydrochloric acid and collagenases, calcium is freed from hydroxyapatite, the bone mineral complex, and released into the circulation (Holick, 2004a and 2005).

The combined actions of PTH and calcitriol increase serum calcium levels by enhancing dietary calcium absorption, reducing renal calcium excretion, and mobilizing calcium stored in bone. In the absence of calcitriol, excess PTH is produced, producing a condition known as secondary hyperparathyroidism. In addition to its effects on calcium homeostasis, PTH also promotes urinary phosphorus excretion, and secondary hyperparathyroidism results in excessive urinary phosphorus loss and inadequate hydroxyapatite formation (Holick, 2004a). Over time, the metabolic responses to vitamin D deficiency result in increased bone resorption and skeletal weakening.

### 2.3.2 Nontraditional Roles

Research over the last three decades has revealed the importance of vitamin D in a number of physiologic responses apart from calcium homeostasis. The VDR has been identified in a number of cells and organs, such as activated T and B lymphocytes, β-islet cells, and cells in the prostate, breast, colon, and other organs (Holick, 2004a). In addition, the 25-hydroxyvitamin D-1α-hydroxylase is present in many extrarenal tissues and cells, such as immune cells and colon cells, placenta, breast, prostate, and pancreas (Table 2.1; Martini & Wood,
Discovery of the widespread distribution of both the VDR and the 25-hydroxyvitamin-D-1α-hydroxylase has triggered extensive research into their functions.

**Cell Proliferation and Differentiation in Relation to Cancer**

Investigators found that extrarenally produced calcitriol acts as an autocrine and paracrine hormone that inhibits proliferation and induces differentiation of normal and some types of cancerous cells (Holick, 2004a). The first studies in this area were done on leukemic cells. Tanaka et al. (1982) found that calcitriol inhibited the proliferation of human leukemic cells by 50% and induced their differentiation into mature granulocytes at concentrations of 0.12 nmol/L and above. Miyaura et al. (1985) demonstrated calcitriol’s ability to suppress proliferation and promote differentiation of human promyelocytic leukemia cells into macrophages. Since then, this effect of calcitriol has been confirmed in many other cell types (Trump et al., 2004).

Based on these findings, researchers hypothesized that calcitriol may have anti-tumorigenic properties. In support of this hypothesis, epidemiologic studies have reported associations between geographical latitude and cancer risk. Breast cancer mortality rates varied considerably between the southern and northern United States, and the risk of fatal breast cancer decreased with increasing strength of UV radiation (Garland et al., 1990; Bertone-Johnson et al., 2005). Increasing latitude correlated with increasing prostate cancer mortality in a study
of 3073 counties across the United States (Hanchette & Schwartz, 1992). Serum 25(OH)D₃ concentrations of 20 – 26 ng/mL (~50 – 65 nmol/L) were associated with a 52% lower colon cancer risk in a prospective study conducted in Maryland. Risk decreased even further with increasing 25(OH)D₃ concentrations up to 41 ng/mL (~100 nmol/L; Garland et al., 1989). Vitamin D intake of ≥1000 IU/day or serum 25(OH)D₃ levels of ≥33 ng/mL (~82 nmol/L) resulted in a 50% decrease in risk for colorectal cancer (Gorham et al., 2005). In a European study, Grant (2003) found that exposure to UV-B radiation and mortality rates were inversely correlated for several cancers, including those of the bladder, breast, endometrium, ovaries, prostate, and kidneys.

**Multiple Sclerosis, Diabetes, and Heart Disease**

Cancers are not the only diseases that may be influenced by vitamin D status. Positive associations have been reported between latitude and the risk of developing multiple sclerosis (Ponsonby et al., 2002). Risk of multiple sclerosis was furthermore inversely correlated with amount of sun exposure during childhood and adolescence in one study (van der Mei et al., 2003). Cutaneous vitamin D₃ synthesis may be the mediating factor in these relationships (Ponsonby et al., 2002).

The presence of 1α-hydroxylase in the pancreas and the expression of the VDR in pancreatic β-cells suggest that calcitriol may be involved in the insulin response. In fact, vitamin D deficiency has been associated with the
development of both type 1 and type 2 diabetes mellitus in humans (Mathieu et al., 2005) and decreased insulin secretion in rats (Ayesha et al., 2001). Furthermore, higher vitamin D intake in early childhood may be associated with decreased risk of developing type 1 diabetes mellitus (Holick, 2004a).

Both multiple sclerosis and type 1 diabetes mellitus are autoimmune diseases. The VDR is present on several immune cells, indicating that the binding of calcitriol may elicit certain responses in these cells (Holick, 2005). In fact, calcitriol is thought to exert some control over the immune system by acting as an immunosuppressive agent (specifically by suppressing inflammatory T cells) that is selective for autoimmune effects, but does not interfere with combating opportunistic infections (Deluca & Cantorna, 2001). Calcitriol's protective effects against type 2 diabetes mellitus may be mediated by its proposed ability to enhance pancreatic β-cell function and promote the biosynthesis of insulin (Bourlon et al., 1999).

In 1997, Rostand reported that increasing latitude is correlated with the prevalence of hypertension. To determine if vitamin D may be the mediating factor in this relationship, Krause et al. (1998) evaluated this relationship and showed that treatment with UV-B radiation increased circulating 25(OH)D₃ and decreased systolic and diastolic blood pressure. The mechanism of vitamin D’s possible cardioprotective effect is not yet clear, but may be due to calcitriol’s effect on blood pressure. Calcitriol downregulates renin (Li et al., 2002), an
enzyme that increases blood pressure, and relaxes smooth muscle cells, including those in the vasculature (O'Connell et al., 1994).

Inhibition of Adipogenesis

Cell culture studies over the past decade indicate a role for calcitriol in adipogenesis. Many studies indicate an inhibitory effect (Kawada et al., 1996; Kelly & Gimble, 1998; Sato & Hiragun, 1988), although some suggest that calcitriol may act in a stimulatory fashion (Atmani et al., 2003; Bellows et al., 1994). The molecular changes produced by calcitriol in the differentiating pre-adipocyte have not been extensively studied. The following paragraphs outline the events of the adipogenic program as well as the intermediary steps that have thus far been suggested to be responsible for calcitriol's inhibitory effect.

Adipogenesis consists of a cascade of events that transforms pre-adipocytes to mature adipocytes in a matter of several days. Differentiation factors initiate mitotic clonal expansion (cell division) of the pre-adipocyte and induce compounds such as CCAAT-enhancer-binding proteins beta and delta (C/EBPβ and C/EBPδ). This is followed by the upregulation of the transcriptional regulators C/EBPα and peroxisome proliferators-activated receptor gamma (PPARγ). Subsequently, the cells continue to differentiate until they have reached a terminal, mature state (Kong & Li, 2006).
Calcitriol has been shown to influence the early stages of adipogenesis. When added to a 3T3-L1 pre-adipocyte cell line at 0h, calcitriol inhibited adipogenesis in a dose-dependent manner post clonal expansion by blocking the expression of certain differentiation markers, including C/EBPα and PPARγ (Kong & Li, 2006). In another study, calcitriol down-regulated the expression of C/EBPβ, which is necessary for the induction of C/EBPα and PPARγ, and up-regulated expression of ETO, an inhibitor of C/EBPβ action (Blumberg et al., 2006).

The unliganded VDR itself was capable of inhibiting adipogenesis when overexpressed. With and without the presence of calcitriol, VDR inhibited PPARγ transacting activity by competitively inhibiting heterodimer formation of PPARγ with RXR (Kong & Li, 2006).

Because calcitriol affects compounds that appear early on in the adipogenic program, addition after 48h did not have an effect. In the absence of calcitriol, the VDR, which mediates the action of calcitriol, appears to decline in number after 4 – 8 hours. The window for calcitriol’s inhibitory effect is small, and arrested differentiation of the pre-adipocyte appears to be completely reversible upon removal of calcitriol. (Kong & Li, 2006).

Calcitriol may therefore be able to block some differentiation factors early on in the adipogenic program, hence effectively halting adipogenesis and perhaps the development of adiposity. Since adipocytes are not simply triglyceride-storing
cells, but also secrete a number of hormones, such as leptin, calcitriol-mediated inhibition would indirectly reduce the production of these hormones.

2.4 INTAKE RECOMMENDATIONS

Vitamin D status is assessed using serum levels of the circulating form 25(OH)D$_3$ as an indicator. The traditional cut-off for deficiency is 27.5 nmol/L in children and 37.5 nmol/L in adults (Holick, 2004a). However, recent studies have called these cut-off values into question. Vitamin D deficiency is currently defined as the occurrence of the short-latency diseases rickets (in children) and osteomalacia (in adults). Therefore, the 25(OH)D$_3$ blood level below which these diseases develop has been used to indicate deficiency. However, this definition leaves out a number of long-latency diseases (e.g., osteoporosis and some cancers), whose course is advanced when 25(OH)D$_3$ levels fall below a much higher threshold (Heaney, 2003). For example, an analysis of data from several thousand participants from the third National Health and Nutrition Examination Survey (NHANES III) revealed that bone mineral density (BMD) is positively correlated with circulating 25(OH)D$_3$ over a wide range of concentrations, including concentrations greater than 37.5 nmol/L (Bischoff-Ferrari et al., 2004). The currently accepted “optimal” value of 37.5 nmol/L is therefore by no means associated with optimal BMD (Hollis, 2005).
In search of an alternative standard, investigators now agree that maximal PTH suppression and greatest calcium absorption should be used as indicators for determining optimal circulating 25(OH)D$_3$ values (Dawson-Hughes et al., 2005). Estimates of 25(OH)D$_3$ levels that produce these effects range from 40 to 110 nmol/L (Barger-Lux et al., 1998). Holick (2005) suggests a minimum 25(OH)D$_3$ concentration of 50 nmol/L and an optimal concentration of 78-100 nmol/L. Retention of the traditional deficiency cut-off value along with the development of a new optimal concentration created a grey zone that was named “insufficient” or “sub-optimal” vitamin D status. Eventually, this term should be abolished in favor of a clear distinction between deficiency and sufficiency (Heaney, 2003).

Despite new recommendations, the establishment of an RDA remains a challenging task since it is difficult to experimentally determine physiologic needs given that the vitamin can be produced cutaneously. The current AI is 200 IU (5µg) for children and adults under the age of 51, 400 IU for those aged 50 – 70 years, and 600 IU for those 71 years and older. But current intake recommendations, like deficiency cut-off values, are set to prevent the short-latency diseases rickets and osteomalacia, not long-latency diseases such as osteoporosis, cancers, and autoimmune diseases (Whiting & Calvo, 2005). In addition, meeting the current AI does not necessarily ensure blood levels of the hitherto considered “adequate” value of 37.5 nmol/L (Nesby-O'Dell et al., 2002). Hence, a revision of the recommended intake is clearly needed.
To maintain a minimum serum 25(OH)D$_3$ level of 50 nmol/L, a daily vitamin D intake of 600 IU is needed; a daily intake of 800 – 1000 IU results in an optimal serum level of 75 nmol/L (Holick, 2004b). A weekly dose of 28,000 IU (about twice the average amount of vitamin D produced in response to 1 total-body MED) resulted in mean serum 25(OH)D$_3$ levels of 112±41 nmol/L in those with baseline serum levels of less than 60 nmol/L$^1$ (Vieth et al., 2004). Hence, Holick (2004a) proposes an AI of 800 – 1000 IU for those getting no sunlight exposure. Although a multiple of the current AI, this recommendation still lies far below the Upper Limit (UL) of 2000 IU/d as defined by the Institute of Medicine (Holick, 2004a).

$^1$ It should be noted that the response to these intakes varies according to vitamin D status. Persons with low baseline serum 25(OH)D$_3$ levels experience a more pronounced increase (~1.0 nmol/L per 40 IU) in 25(OH)D$_3$ than those with higher baseline levels of 70 nmol/L (~0.7 nmol/L per 40 IU; Heaney et al., 2003).
2.5 PREVALENCE OF DEFICIENCY

Studies conducted in recent years have begun to reveal the high prevalence of vitamin D deficiency throughout the world and in many different population groups (Calvo & Whiting, 2003; Chapuy et al., 1997; Harinarayan et al., 2007; Lehtonen-Veromaa et al., 1999; Oliveri et al., 2004). Because of the UV-absorbing property of the pigment melanin and an age-related decline in levels of 7-dehydrocholesterol, dark-skinned and older persons would appear to be most at risk. While these populations do experience much vitamin D deficiency, other groups, such as young adult women, are substantially affected as well. Overall, the groups most afflicted with vitamin D deficiency are women, non-white persons, and the elderly (Calvo & Whiting, 2003; Oliveri et al., 2004; Zadshir et al., 2005).

White (30%), Hispanic (42%), and African American (84%) elderly Bostonians were vitamin D deficient at the end of August, a time of year when sunlight is strong enough to support cutaneous vitamin D synthesis (Holick, 2004a). The problem of vitamin D deficiency in the elderly is not limited to the United States. Depending on latitude, 52% to 87% of healthy Argentinian elderly (65 years and older) had 25(OH)D$_3$ levels below 20 ng/mL (~ 50 nmol/L; Oliveri et al., 2004).

An analysis based on data taken from NHANES III, conducted from 1988 to 1994, reports deficiency in 42.4% of African American and 4.2% of white women,
based on a conservative cut-off value of 37.5 nmol/L (Nesby-O'Dell et al., 2002). While the percentage for white women appears low, the prevalence of deficiency would have likely been higher if a more appropriate cut-off value of 80 nmol/L had been used to define deficiency.

In fact, another group of investigators analyzing the same NHANES III data, but with a different cut-off value, found the prevalence of deficiency (25 – 70 nmol/L) to be ~78% in Black, 45% in White, and ~50% in all women 18 years and older. The prevalence in all women was significantly higher than the prevalence in all men (p<0.01; Zadshir et al., 2005). In the same study, fewer more-educated women (more than high school) than less-educated women (less than high school) were deficient (44% vs 59%), although deficiency was quite common in both groups (Zadshir et al., 2005).

A combination of factors might be responsible for the difference in prevalence of vitamin D deficiency between women and men. Women have more body fat, a characteristic that has been associated with lower circulating levels of 25(OH)D$_3$ (Wortsman et al., 2000). More women than men work in indoor professions, limiting women’s exposure to sunlight (Fronczek & Johnson, 2003). Women might be more health-conscious and therefore more likely to regularly use sunscreen, a practice that inhibits the cutaneous synthesis of vitamin D (Abroms et al., 2003). A focus on health and appearance may cause more women to engage in dieting behavior that may manifest as kilocalorie restriction or
avoidance of certain foods (e.g. dairy products; Kruger et al., 2004). Such restrictions can lead to reduced intake of vitamin-D-containing foods.

Although advanced age increases the risk for vitamin D deficiency, youth does not protect from it. Tangpricha et al. (2002) reported vitamin D insufficiency [based on a cut-off value of 50 nmol/L (=20 ng/ml) at the end of winter] in 36% of healthy men and women between the ages of 18 and 29 living in Boston.

Stratifying by gender furthermore reveals the high prevalence of deficiency in young women. In the NHANES III study, roughly 44% of women between the ages of 18 and 39 years were deficient (Zadshir et al., 2005). Summer/higher latitude and winter/lower latitude deficiency (<62.5 nmol/L) occurred in 30% and 55%, respectively, of 20- to 39-year-old women living at latitudes between 25° and 41°N (Looker et al., 2002). Veith et al. (2001) reported that 21.3% of white, 31.9% of non-white, and 25% of black 18 – 35 year old Canadian females living at latitude 43°N were deficient (<40 nmol/L) in the winter.

While men, Caucasians, and younger persons are less likely to be vitamin D deficient, the prevalence of deficiency in these groups is still substantial (Calvo & Whiting, 2003; Zadshir et al., 2005). Roughly 40% of a sample of male NHANES III participants were deficient (25 – 70 nmol/L), 34% of whom were white and 37% of whom were between the ages of 18 and 39 years (Zadshir et al., 2005).
The cut-off values used in these studies are mostly set above the traditional 37.5 nmol/L, but below 80 nmol/L, the level that has begun to emerge as an optimum for preventing all vitamin-D-modified disease. Therefore, the percentages of deficiency in these studies are conservative estimates which will increase once the 80 nmol/L threshold is adopted by the public health community.

Deficiency occurs as a result of inadequate cutaneous synthesis and low dietary intake. In an age of widespread sunscreen use or avoidance of sun exposure, dietary and supplement sources ought to be emphasized to ensure adequate vitamin D status. However, based on an analysis of Continuing Survey of Food Intakes by Individuals (CSFII) and NHANES III data (Moore et al., 2004), dietary vitamin D intake in the U.S. is low (even by the current AI) in those 51 years and older (range of mean intakes: 160 – 236 IU) as well as in teenage and adult females (range of mean intakes: 140 – 180 IU; Moore et al., 2004). Less than 30% of female adults (less than 20% in age group 19 – 30), less than 10% of 51 – 70 year olds, and less than 2% of those older than 70 years met requirements through food alone. Supplement use increased the mean intake range to 210 to 393 IU, however less than a third of subjects in each age groups took a vitamin D supplement (Moore et al., 2004).

Living at latitudes further from the equator increases risk for vitamin D deficiency (Calvo & Whiting, 2003; Oliveri et al., 2004). In addition, intake of vitamin-D-containing foods is low, and less than a third of some high-risk populations meet
the current AI, which itself is thought by many experts to be too low. Vitamin D deficiency is more common in women than men, non-whites than whites, and older than younger persons and is furthermore substantial in other groups, including young adult women.
CHAPTER 3

LEPTIN

Leptin, the product of the ob gene, belongs to the group of adipokines that are involved in maintaining energy balance. It was discovered along with its encoding gene in 1994 by the research group of Jeffrey M. Friedman (Zhang et al., 1994). The name leptin was coined later on based on the Greek word for “thin” – “leptos”. The 16-kDa hormone is produced mainly, but not exclusively, by white adipocytes in proportion to fat mass and binds to receptors in the hypothalamus, and possibly other tissues, to exert its appetite-suppressing and energy-expenditure-enhancing effects (Cohen, 2006; Considine et al., 1996; Maffei et al., 1995).

Other tissues that express leptin include the placenta and stomach. A limited body of studies suggests that gastric leptin may induce a short-term satiety-promoting effect (Pico et al., 2002 and 2003). Ongoing research suggests that leptin may also promote weight loss and maintenance by acting on adipocytes directly, specifically by influencing lipogenic and lipolytic pathways (Siegrist-Kaiser et al., 1997). However, while leptin is part of a hormonal system that helps regulate energy balance, it neither prevents nor cures obesity. In fact, a substantial increase in body fat mass is invariably accompanied by an increase in
serum leptin levels and the physiologic effects of resistance to leptin (Considine et al., 1996; Schwartz et al., 2000). In addition to its traditional role in weight maintenance, leptin may also be involved in other body systems, including the skeleton, the reproductive system, and the insulin response (Cohen, 2006; Wauters et al., 2000).

### 3.1 LEPTIN AND OBESITY

The discovery of leptin was groundbreaking as it provided a possible biologic cause for obesity, leading researchers to discard the long-held belief that obesity is a purely behavioral condition. The first leptin studies were conducted with knockout mice that lacked the ability to produce leptin due to a mutation in the *ob* gene. This mutation led to uncontrolled food intake and obesity. Exogenous leptin given to these mice resulted in decreased food intake and increased energy expenditure leading to weight loss (Halaas et al., 1995; Pelleymounter et al., 1995). This effect was subsequently observed in humans. Heymsfield et al. (1999) showed that administration of exogenous leptin to lean and obese subjects led to decreased energy intake and subsequent weight loss (95% of which was fat loss) in a dose-dependent manner.
3.1.1 Control of Food Intake

In the control of food intake, leptin may elicit two independent responses. Being secreted proportionally to fat mass, it appears that leptin helps to inform the brain about the size of fat stores in the body, thereby allowing for adjustments if fat mass goes out of bounds in either direction (Cohen, 2006). This response is mediated by the hypothalamus and promotes long-term weight regulation. While circulating leptin concentrations are proportional to body fat stores, they are not constant (Maffei et al., 1995), and fluctuations do occur in response to short-term energy imbalance, such as short-term periods of overeating or starvation (Keim et al., 1998; Weigle et al., 1997). The observations suggest that the more sensitive responses to energy imbalance in the form of immediate satiety signals, which are likely peripherally mediated, may help to promote the maintenance of a desired level of body fat rather than substantial deviations from it followed by a restorative period (Schwartz et al., 2000).

Hypothalamic regulation

The binding of leptin to receptors in the hypothalamus sets into motion a cascade of neuronal signals that promote energy homeostasis. While leptin receptors are present in different areas of the hypothalamus, the arcuate nucleus holds the largest number per area, and it appears that this region is instrumental in the leptin response (Schwartz et al., 2000).
Leptin appears to influence the expression of several compounds involved in appetite regulation. For instance, leptin receptors are present on neurons expressing neuropeptide Y (NPY), agouti-related protein (AGRP), pro-opiomelanocortin (POMC), and cocaine- and amphetamine-regulated transcript (CART), and it is thought that these molecules act as downstream mediators in the leptin signaling response. The anabolic effector signaling molecules NPY and AGRP appear to be downregulated by leptin, while their catabolic counterparts POMC (the precursor of the melanocortin family) and CART appear to be upregulated by leptin.

NPY is released in response to fasting and stimulates appetite. Increased secretion of NPY in the paraventricular nucleus of rats was observed after a period of fasting or prior to feeding in rats maintained on a feeding schedule compared to rats fed ad libitum, indicating that NPY is released in response to hunger. Food-deprived rats ate 5.3±4g compared to 1.0±8g (controls) during the feeding period. NPY levels returned to control upon feeding (Kalra et al., 1991). In another group of rats, NPY treatment led to increases in food intake, rate of weight gain, and plasma leptin. Leptin administration repressed NPY production in the arcuate nucleus, the paraventricular nucleus, and the dorsomedial hypothalamus (Wang et al., 1997).

NPY mRNA levels are higher in the hypothalamus of the ob/ob mouse, which does not express leptin (Wilding et al., 1993), indicating that leptin is necessary
for NPY suppression. The double mutant NPY\textsuperscript{-/} \textit{ob/ob} has a less obese phenotype and less body fat than the \textit{ob/ob} mouse, probably as a result of lower food consumption and higher metabolic rate due to the missing NPY gene (Erickson et al., 1996). These observations indicate that NPY mediates some, but not all, of the outcomes associated with absence of leptin. In addition to stimulating food intake and suppressing energy expenditure, the hypothalamic NPY activates hepatic and adipose lipogenic enzymes (Schwartz et al., 2000).

POMC is the precursor molecule for melanocortins, molecules that are implicated in the inhibition of ingestive behavior (Cohen, 2006; Schwartz et al., 1997). The melanocortin system plays an important role in energy homeostasis. Disruption of this system (e.g. by receptor mutation) has been found to lead to obesity and diabetes symptoms in mice (Huszar et al., 1997). This effect might be explained by the overexpression of NPY that results from mutation of melanocortin-4-receptor (MC4R), the main receptor for \(\alpha\)-MSH, one of the melanocortins (Cohen, 2006).

Neurons expressing the POMC gene also express the leptin receptor (Schwartz et al., 1997), and binding of leptin appears to increase POMC expression. \textit{Db/db} mice, which lack the leptin receptor, and \textit{ob/ob} mice express 46% and 70% less POMC mRNA in the arcuate nucleus compared to wild-type mice. Intraperitoneal leptin injection (150[Mu]g) increased POMC mRNA in \textit{ob/ob} mice by 73% compared to controls. No effect was seen in \textit{db/db} mice, indicating that an
interaction between leptin and its receptor (which is absent in db/db mice) was responsible for the effect (Schwartz et al., 1997). Fasting, which downregulates leptin, resulted in 53% less POMC mRNA in the rostral arcuate nucleus of wild-type mice (Schwartz et al., 1997). When wild-type rats were injected with 3.5[Mu]g of leptin during a period of fasting, POMC mRNA expression in the rostral arcuate nucleus increased by 39% (Schwartz et al., 1997). These findings support the idea that POMC expression is upregulated by leptin.

Similarly to POMC, CART is an anorexigenic neuropeptide, whose expression also appears to be upregulated by leptin. Central administration of CART to normal and NPY-treated rats resulted in decreased food intake (Kristensen et al., 1998). Central administration of 1µg and 2µg CART to rats that had been fasting for 24 hours decreased feeding by 59% and 68% compared to controls (Kristensen et al., 1998). Rats injected with anti-CART (CART antiserum) showed increased night-time feeding compared to controls (Kristensen et al., 1998). Normal rats that had been fasted for 24 or 48 hours showed decreased expression of CART mRNA in the arcuate nucleus and, though not significantly, the dorsomedial hypothalamus (Kristensen et al., 1998). Obese Zucker rats (fa/fa) and obese ob/ob mice, which express less CART mRNA than their wild-type counterparts were given daily intraperitoneal leptin injections. Ten days on this regimen lowered food intake in the ob/ob mice (20±3g vs 43±3g in controls), restored CART mRNA levels to normal in the arcuate nucleus and increased levels in the lateral nucleus (Kristensen et al., 1998). These findings lend support
to the theory that CART is an anorexigenic neuropeptide that is upregulated by leptin.

Leptin seems to direct gene expression of these neuropeptides via different pathways, the best characterized of which is the Janus tyrosine kinase 2/cytosolic signal transducer and activator of transcription protein 3 (JAK2/STAT3) pathway. Leptin binding stimulates the phosphorylation of the long form of the leptin receptor (OB-Rb) by JAK2. This recruits STAT3 and prompts JAK2 to activate STAT3 through phosphorylation. STAT3 subsequently splits into two dimers that are translocated to the cell nucleus where they act as gene transcription factors (Zhang & Scarpace, 2006).

_Peripheral Regulation_

In addition to central regulation, leptin may act peripherally to induce short-term regulation of food intake. In a study with women, low levels of leptin were associated with increased hunger, desire to eat, and prospective consumption (Keim et al., 1998). The source of leptin required for this potential short-term response is likely not the adipocyte. While circulating leptin levels decrease almost immediately in response to fasting, they are not responsive to excess food ingestion at a single eating occasion. Excess food ingestion has to occur over a period of several days before leptin expression is upregulated in adipocytes (Pico et al., 2003).
The short-term effects of leptin may be attributable to gastric leptin, which may work in conjunction with other satiety factors, such as cholecystokinin (CCK; Pico et al., 2003). In fact, fasting appears to downregulate gastric leptin mRNA expression in mice, while re-feeding appears to upregulate it (Pico et al., 2002). Assuming that the absence of leptin induces a hunger sensation, blocking its gastric synthesis in a fasted state may serve to prompt the animal to eat. Once sufficient food has been ingested, gastric leptin may be needed to help initiate the cessation of the meal.

The exact mechanism of gastric leptin action is not known. It is thought that it acts via endocrine and paracrine routes. Gastric leptin is secreted by two types of cells in the stomach, the chief cells, which also produce proteolytic enzymes, and special endocrine cells, the P cells (Pico et al., 2003). Leptin could stimulate nerve endings in the stomach and intestine, or it could act in the stomach lumen, interacting with gastric epithelial cells (Pico et al., 2003). Gastric leptin is probably not released into the circulation in sizable amounts and therefore does not act substantially via direct brain receptor stimulation (Pico et al., 2003).

Ideally, the proposed effect of gastric leptin would protect against obesity by preventing a prolonged state of positive energy balance from ever occurring. However, while satiety factors influence meal size, research in mice and rats suggests that they do not influence long-term energy balance when given exogenously due to compensatory mechanisms, such as increased meal
frequency (Pico et al., 2003). Gastric leptin may be subject to these same compensatory responses.

3.1.2 Influence on Adipocytes and Lipolysis

In addition to providing satiety signals, leptin may influence fat mass directly by inducing lipolysis and adipocyte apoptosis. Leptin-treated fat pads from Sprague-Dawley rats exhibited increased glycerol release – an indicator of lipolysis – in a dose- and time-dependent manner, with leptin concentrations as little as 0.1 nmol/L showing an effect. A similar increase in glycerol release was evident in fat pads of lean (Fa/fa) Zucker rats, while fat pads from their obese (fa/fa) counterparts did not respond, implying that the lipolytic effect is mediated by leptin through the long form of the leptin receptor (OB-Rb) which is not functional in fa/faf Zucker rats.

Leptin’s lipolytic action appears to be mediated via the JAK2/STAT3 pathway. Intravenous injection of 500 µg leptin into Fa/fa rats resulted in translocation of STAT1 in brown adipose tissue. Leptin also increased STAT binding to nuclear extracts in brown and white adipose tissue (BAT and WAT) cultures 1.8-fold and 2.7-fold, respectively. No STAT activation was observed in fa/fa derived cultures, which do not express a functional leptin receptor. Since intracerebroventricular (i.c.v.) injection had no effect, it appears that leptin induces lipolysis through peripheral, rather than central, activation (Siegrist-Kaiser et al., 1997).
Leptin may also promote weight loss by inducing adipocyte apoptosis. I.c.v. infusion of 5µg and 2.5µg leptin into male and female Sprague-Dawley rats resulted in apoptosis of white adipocytes (from retroperitoneal, epididymal, and parametrial fat tissues) as evidenced by internucleosomal DNA degradation, abnormal cell morphology, condensed chromatin in fat tissue sections, as well as decreased weight and DNA content in fat pads. DNA degradation was not found in other tissues, indicating that the apoptotic effect is restricted to adipocytes (Qian et al., 1998).

### 3.1.3 Modification of Energy Expenditure

Leptin influences energy balance by stimulating energy expenditure. It induces thermogenesis by causing the uncoupling of oxidative phosphorylation from the electron transport chain (Fietta, 2005). Leptin given intravenously to male rats resulted in a 1.6-fold increase in insulin-stimulated glucose utilization in brown adipose tissue, an indicator of increased energy expenditure. This effect was not observed when leptin was injected i.c.v., indicating that the effect is not mediated via the brain, but is a direct action on the adipose tissue (Siegrist-Kaiser et al., 1997).

### 3.1.4 Leptin Resistance

While early studies in leptin-deficient mice generated hope that obesity might soon be treated pharmacologically, subsequent studies on humans showed less promise. Leptin deficiency is extremely rare in humans. In fact, obese persons
have much higher serum leptin levels (31.3 ± 24.1 ng/mL versus 7.5 ± 9.3 ng/mL; Considine et al., 1996; Havel et al., 1996) and twice the ob mRNA than their lean counterparts (Considine et al., 1996). This is not surprising considering that leptin is made by adipose tissue and its serum concentrations increase proportionally to fat mass (r = 0.85; Considine et al., 1996). It does, however, raise an important question: If leptin helps regulate body weight, why do increased leptin concentrations in obese individuals fail to bring about weight loss? These seemingly contradictory findings led to the proposition that humans may become resistant to leptin as their body weight increases, thereby preventing the weight-reducing effect of leptin (Schwartz et al., 2000). A few different mechanisms explaining this resistance have been proposed.

*Impaired Transport across the Blood Brain Barrier*

Leptin probably gains entry into the brain via leptin transporters. In leptin resistance, transport of leptin across the blood-brain barrier may be impaired. This theory is supported by the fact that the ratio of leptin in the brain to leptin in the blood is lower in obese than in lean humans (Caro et al., 1996). As the brain fails to receive leptin, adipocytes may be prompted to increase production, leading to even higher levels in the blood (Schwartz et al., 2000).

This defect in transport may be exacerbated by a number of factors, including the presence of certain triglycerides, as demonstrated by a series of experiments conducted by Banks et al. (2004). During starvation (long-term food deprivation, 48 hours in this study), which leads to increased serum triglycerides due to
lipolysis of stored fat, brain uptake of leptin was reduced by 44% as demonstrated by a reduced brain/serum ratio of intravenously injected radioactive leptin (I-Lep) in mice.

Injection of bovine whole milk, but not non-fat milk, into the mouse peritoneal cavity resulted in impaired I-Lep transport across the blood-brain barrier, while intralipid (a source of plant triglycerides) had no effect. Three of four different intravenously injected triglycerides inhibited blood-brain barrier transport of I-Lep, while one did not. Intravenously injected free fatty acids had no effect on I-Lep transport. A high-fat diet increased serum triglycerides and decreased leptin transport, while fasting (short-term food deprivation, 16 hours in this study), which is characterized by a decrease in serum triglycerides, achieved the opposite. Administration of Gemfibrozil, a triglyceride-reducing drug, decreased serum triglycerides and increased leptin transport (Banks et al., 2004). Based on these results in mice, it appears that some triglycerides, notably those from foods of animal origin and those naturally released into the blood stream, may block leptin transport across the blood-brain barrier.

To determine whether impaired leptin transport across the blood-brain barrier precedes aging and obesity or is a result of these factors, Banks and Farrell (2003) conducted a series of studies on CD-1 male mice. Aging brought about considerable weight gain in the obese, but not the thin mice. Influx of I-Lep from the blood into the brain dropped significantly with age in obese, but not thin mice,
suggesting that the impairment of leptin transport across the blood-brain barrier is acquired with weight gain. In addition, influx of I-Lep into the brain was negatively correlated with body weight in 5-month old mice. Impaired leptin transport seems to be reversible. In fat mice, weight loss through fasting or leptin injection resulted in greater brain uptake of leptin compared to fat controls (Banks & Farrell, 2003).

To locate the point at which leptin transport across the blood-brain barrier is halted, Banks and Farrell (2003) employed capillary depletion, a technique used to determine whether I-Lep is held in brain capillaries or crosses into the brain parenchyma. Capillary depletion did not differ between thin and fat mice, indicating that leptin was transported across the blood-brain barrier normally once it had been taken up by the brain endothelial cells. Hence, the transport defect must occur before leptin enters the brain endothelial cells.

Impairment of transport across the blood-brain barrier appears to be one mechanism that can explain leptin resistance. Transport of leptin may be blocked by certain triglycerides, notably those from foods of animal origin as well as those naturally released into the blood stream. Mice acquired leptin resistance with weight gain, and normal leptin function was restored with weight loss, indicating that leptin resistance may be acquired with weight gain and reversible with weight loss. The location of impaired transport remains unclear, but seems to occur prior to uptake by the brain endothelial cells.
Excess Central Leptin

According to the theory set forth above, leptin resistance results from leptin deficiency in the brain. Contrary to this idea, another theory proposes that leptin resistance results from excess central leptin. Zhang and Scarpace (2006) artificially introduced the leptin gene into rats. This led to leptin overexpression, decreased food intake, and increased oxygen consumption. However, this effect disappeared toward the end of the observation period, indicating that excess central leptin may lead to leptin resistance.

The attenuation of the effect happens more quickly in old-obese rats. Similarly, 5-month-old F344xBN rats with an artificially implanted leptin gene exhibited reduced food intake and increased oxygen consumption, both of which normalized half-way or two-thirds through the observation period. A 7-day regimen of i.c.v. leptin in these rats did not reduce food intake as it did in controls (Zhang & Scarpace, 2006). In addition, high-fat feeding resulted in markedly higher energy consumption, greater weight gain, and greater visceral fat accumulation in rats with the artificial leptin gene than in control rats, implying that leptin resistance as brought about by excess central leptin mutes leptin's energy-compensatory response in the face of a high-energy diet (Zhang & Scarpace, 2006).
Defects in the Leptin Signaling Pathway

In addition to impaired transport across the blood-brain barrier and excess central leptin, leptin resistance may manifest as a defective leptin signaling pathway. To determine the point in the leptin signaling pathway at which a defect occurs, Zhang and Scarpace (2006) tested events downstream of hypothalamic leptin binding. Leptin increases the expression of POMC, the precursor for melanocortins such as alpha-melanocyte stimulating hormone (α-MSH) that binds to MC3R and MC4R to produce a decrease in food intake and an increase in energy expenditure. Leptin resistance as brought about by age, excess leptin, or diet coincided with impaired POMC expression and therefore decreased melanocortin synthesis. Third ventricle infusion with MTII, a synthetic form of α-MSH, allowed for typical leptin-related outcomes such as decreased food intake and loss of adiposity and body mass by circumventing leptin-activated melanocortin synthesis (Zhang & Scarpace, 2006). Similarly, artificial POMC gene delivery to obese Zucker rats and rats with adult-onset obesity resulted in leptin-related outcomes despite leptin resistance (Zhang & Scarpace, 2006).

These results indicate that leptin resistance occurs upstream of leptin-induced neuropeptide expression. One of these upstream events is the JAK2/STAT3 pathway, as discussed earlier. Post-receptor-binding STAT3 phosphorylation is part of this pathway. While leptin-activated phosphorylation of STAT3 to P-STAT3 was similar in young-lean and old-obese F344xBN rats at low levels of exogenous leptin given i.c.v., doses of 20ng of leptin and above resulted in a
steeper increase in P-STAT3 concentrations in young than in old rats. The maximum phosphorylation was 41% higher in young as compared to old rats. P-STAT3 DNA binding, the final event in leptin-mediated gene expression, was more than 8-fold greater in young-lean and less than 4-fold greater in old-obese rats following 1µg leptin i.c.v. injection (Zhang & Scarpace, 2006). These data suggest that obesity results in defects in the leptin signaling pathway at the points of STAT3 phosphorylation and P-STAT3 DNA binding.

*Diet Induced Leptin Receptor Deficiency*

Experiments conducted by Zhang and Scarpace (2006) suggest that diet can influence leptin receptor expression. High-fat feeding of young-lean F344xBN rats for 100 days reduced leptin receptor expression, but increased basal leptin signaling 3-fold, perhaps as a compensatory response. When these rats were given i.c.v. injections of 2µg of leptin, STAT3 phosphorylation increased less than 2-fold as compared to chow-fed animals that experienced a greater than 6-fold increase, perhaps reflecting the decreased number of leptin receptors or a diet-induced defect in the leptin signaling pathway. A 45-day long calorie restriction followed by administration of 2µg of leptin increased the maximal STAT3 phosphorylation in both groups (Zhang & Scarpace, 2006), suggesting that high-fat feeding had an inhibitory effect on STAT3 phosphorylation that disappeared once the dietary factor had been removed.
In another study, rats on a high-fat diet had a 22% reduced hypothalamic leptin receptor expression compared to chow-fed rats. Caloric restriction increased leptin receptor mRNA by 43% and 58% compared to chow-fed and non-calorie-restricted high-fat fed rats, respectively (Zhang & Scarpace, 2006). High-fat feeding in these studies appears to have blunted the leptin response by reducing the number of leptin receptors or by interfering with STAT3 phosphorylation.

3.2 OTHER POSSIBLE LEPTIN TARGETS

3.2.1 Bone Growth and Maintenance
The idea that leptin may regulate bone mass was born from extensive and conclusive research that positively correlates body weight and bone mineral density (Albala et al., 1996; Kirchengast et al., 2002; Ribot et al., 1987; Seeman et al., 1983). However, animal research in this area has produced somewhat conflicting results that ascribe both inhibitory and stimulatory roles to leptin. Leptin knockout mice have higher bone mass than wild-type mice, and i.c.v. administration of leptin causes bone mass to diminish in these as well as normal mice (Ducy et al., 2000). Conversely, leptin promoted bone growth upon peripheral injection and in in vitro studies (Gordeladze et al., 2002; Steppan et al., 2000). It seems, therefore, that depending on the site of leptin binding - peripheral or central - the hormone elicits opposite responses with respect to bone physiology.
The peripheral effect seems to be mediated directly in bone tissue. A leptin receptor has been identified in human osteoblasts, supporting a direct role of leptin in these cells (Reseland et al., 2001). In fact, leptin stimulated differentiation of marrow stromal cells into osteoblasts (Thomas et al., 1999), inhibited differentiation of osteoclasts (Holloway et al., 2002), stimulated bone mineralization and inhibited apoptosis of osteoblasts (Reseland et al., 2001; Gordeladze et al., 2002).

The central effect appears to be mediated via the hypothalamus. Neurons in the ventromedial nucleus of the hypothalamus have been associated with bone mass regulation via two proposed pathways. Contrary to the evidence put forth by the animal studies cited above, these proposed pathways elicit opposite outcomes. The sympathetic nervous system (SNS) pathway starts with noradrenalin binding to the osteoblastic receptor β2-AR, which then produces the receptor RANK. When nuclear factor kappa beta (NFκB) binds to RANK, osteoclasts mature and begin the resorption of bone. Leptin initiates this pathway, but also activates a less well studied antagonistic pathway, the CART pathway, that leads to inhibition of osteoclast differentiation, and therefore prevents resorption. Absence of CART leads to osteoporosis in mice. NPY2R, one of NPY’s receptors, is postulated to play a role in bone mass as well (Elefteriou et al., 2005; Cohen, 2006). Leptin’s capability to activate both the bone-building SNS and the bone-resorbing CART pathway, indicates that leptin may act as a regulator of bone mass.
In an effort to determine the significance of leptin in human bone metabolism, an epidemiologic look reveals an inconclusive picture. Some studies on adult women have found no association between leptin and BMD (G. Martini et al., 2001; Ruhl & Everhart, 2002) or markers of bone metabolism (Goulding & Taylor, 1998; Martini et al., 2001; Rauch et al., 1998), especially after adjusting for BMI. Others confirm a positive correlation between plasma leptin and BMD in premenopausal (Thomas et al., 2001) and postmenopausal (Thomas et al., 2001; Yamauchi et al., 2001) women, and an inverse correlation in men (Ruhl & Everhart, 2002; Sato et al., 2001). Pasco et al. (2001) found associations only for specific skeletal sites in non-obese women (e.g. lateral spine).

3.2.2 The Insulin Response

In addition to being obese, mice with the ob/ob defect are insulin resistant, and administration of leptin reverses their hyperglycemia, hyperinsulinemia, and insulin resistance (Muzzin et al., 1996; Pelleymounter et al., 1995). Central and peripheral leptin administration increased insulin sensitivity in mice and rats (Sivitz et al., 1997; Kamohara et al., 1997). Increased leptin levels have been observed in humans with type 1 and type 2 diabetes as well as those with artificially induced hyperinsulinemia (Wauters et al., 2000). In addition, serum leptin and insulin have been positively correlated (Widjaja et al., 1997). Leptin expression was induced by insulin in vitro at 72h (Kolaczynski et al., 1996). On the other hand, leptin may initiate SOCS3 action, which inhibits both leptin and
insulin signaling (Myers, 2004; Cohen 2005). The evidence linking leptin and the insulin response is inconclusive, suggesting both an insulin-supporting and an insulin-inhibiting role for leptin.

### 3.2.3 Reproduction

The observation that ob/ob mice are sterile led to the idea that leptin may be instrumental in puberty, menarche, and menstruation. In the few human subjects that have an ob/ob or leptin receptor mutation, puberty is delayed (Wauters et al., 2000). In girls, both menarche and menses require maintenance of a certain level of fat mass. Recognizing that serum leptin rises proportionally to fat mass, it seems plausible that leptin may be involved in the reproductive system. Several studies provide evidence in support of this idea.

For instance, Chehab et al. (1996) reported that leptin treatment is capable of reversing reproductive impairment in ob/ob male and female mice. In girls, a rise in leptin preceded onset of puberty (Garcia-Mayor et al., 1997), indicating that leptin may be involved in mediating the hormonal changes leading to reproductive maturation. In rodents, centrally acting leptin is furthermore capable of triggering the release of follicle stimulating hormone (FSH) and luteinizing hormone (LH), both of which are essential to the reproductive cycle (Wauters et al., 2000). Conversely, estrogens and FSH stimulate leptin secretion in vitro in human female, but not male, tissue samples, while androgens inhibit leptin secretion. In addition, leptin and a soluble form of its receptor are expressed in
the placenta, and leptin levels fluctuate throughout the menstrual cycle and are elevated during pregnancy (Wauters et al., 2000). These findings indicate that leptin may support normal reproductive function, including onset of puberty, menses, and pregnancy, perhaps through bi-directional interaction with sex hormones.
CHAPTER 4

RELATIONSHIP BETWEEN LEPTIN AND VITAMIN D

The study of the interactions between leptin and vitamin D is a new area of interest, and few studies have examined the potential relationship between the two compounds. Results from these studies provide evidence that vitamin D and leptin do regulate each other.

In an attempt to isolate factors that regulate leptin secretion, Menendez et al. (2001) added 3,5,3'-tri-iodothyronine, calcitriol, and retinoic acid concentrations of $10^{-9}$, $10^{-8}$, and $10^{-7}$ M, respectively, to human male and female omental adipose tissue. In addition, a mixture of all-trans-retinoic acid and calcitriol combined at a concentration of $10^{-7}$M was tested. At all concentrations tested, calcitriol inhibited leptin secretion within 96 hours of exposure, especially at 72 h and 96 h at $10^{-7}$ and at 96 h at $10^{-8}$M. Calcitriol and all-trans-retinoic acid together inhibited leptin secretion in a synergistic manner at 72 h.

While Menendez’s results indicate that calcitriol is a regulator of leptin secretion, a study by Matsunuma et al. (2004) suggests that leptin influences calcitriol synthesis. Matsunuma et al. injected obese $ob/ob$ mice with leptin to measure the effect of exogenous leptin on a number of bone-related variables. Because of
their genetic mutation, the mice exhibited several abnormalities. These included elevated concentrations of calcitriol, renal 1α-hydroxylase, 24-hydroxylase mRNA, serum Ca²⁺ and phosphorus, increased excretion of Ca²⁺ and phosphorus, higher activity of serum alkaline phosphatase (a marker of bone formation) and TRAP (a marker of bone resorption), and decreased BMD and femoral length. Injection with recombinant murine leptin at 4 mg/kg every 12h for 2d corrected some of these abnormalities. Elevated calcitriol decreased to the level observed in control mice (table 4.1). In accordance, renal 1α-hydroxylase and 24-hydroxylase mRNA as well as serum Ca²⁺ and phosphorus decreased to normal. Serum PTH concentrations increased. VDR expression did not change with leptin deficiency or administration.

<table>
<thead>
<tr>
<th>Table 4.1. Effect of leptin treatment on elevated calcitriol levels</th>
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<tbody>
<tr>
<td><strong>Treatment condition</strong></td>
</tr>
<tr>
<td>ob/ob mice without leptin injection</td>
</tr>
<tr>
<td>ob/ob mice after leptin injection</td>
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<tr>
<td>control mice (not leptin deficient)</td>
</tr>
</tbody>
</table>

An interaction between calcitriol and leptin is evident from the results of the Menendez (2001) and Matsunuma (2004) studies. While plasma levels of calcitriol and 25(OH)D₃ generally are poorly correlated, 25(OH)D₃, which serves as the biomarker for vitamin D status, is the precursor of calcitriol and therefore necessary for its synthesis. In the only human study to examine the relationship between 25(OH)D₃ and leptin, Gomez et al. (2004) found a negative correlation
between the two compounds in women without vitamin D deficiency. This analysis was part of a study that also looked at the insulin-like growth factor I (IGF-I) system as well as anthropometric and body composition variables as predictors of 25(OH)D$_3$.

Gomez et al. (2004) studied 253 subjects (126 men, mean age 41.6 years; 127 women, mean age 40.8 years) from the Spanish town of L'Hospitalet de Llobregat. Participants were healthy and were not attempting weight loss by means of diet or exercise during the study period. Subjects were excluded from the study if they had a history of thyroid dysfunction or had a history of treatment with thyroid hormone, calcium, vitamin D, steroids, or thiazides. Anthropometric data included BMI, waist and hip circumference, waist-hip ratio, and body composition, as measured by bioelectrical impedance. Radioimmunoassay kits by Linco and DiaSorin were employed to measure serum leptin and 25(OH)D$_3$, respectively. Spearman’s correlation coefficient and multivariate linear regression were used to determine relationships among variables.

Of the 253 subjects, 45 were vitamin D deficient (defined in this study as serum 25(OH)D$_3$ < 38 nmol/L), and 24 were severely deficient (serum 25(OH)D$_3$ < 25 nmol/L). Vitamin D status varied by season. Serum 25(OH)D$_3$ concentrations were 73.9 ± 16.4 nmol/L in the summer, 53.9 ± 18.1 nmol/L in the fall, 39.6 ± 16.7 nmol/L in the winter, and 46.7 ± 15.5 nmol/L in the spring. BMI, hip circumference, and fat mass were significantly lower and IGF-I levels were
significantly higher in non-deficient subjects than in those with vitamin D deficiency. An inverse trend with decade of age was observed in 25(OH)D$_3$ levels in both genders. In men, leptin levels were relatively constant across age groups, while in women serum leptin was twice as high in the oldest compared to the youngest age group.

In women without vitamin D deficiency (serum 25(OH)D$_3$ $\geq$ 38 nmol/L), a negative correlation was observed between 25(OH)D$_3$ and age, BMI, waist and hip circumferences, fat free mass, fat mass, and leptin. In both men and women with vitamin D deficiency, no correlations between serum vitamin D and any of the variables were found. The authors suggest that leptin – among other factors – may play a role in the regulation of 25(OH)D$_3$ concentrations in women. Gomez et al. concluded that an inverse correlation exists between serum leptin and 25(OH)D$_3$ (Gomez et al., 2004). However, because of its cross-sectional design, this study does not provide evidence for causality.

These three studies provide evidence that vitamin D and leptin interact with each other. It appears that calcitriol and leptin may be capable of regulating each other, perhaps depending on initial concentration and type of tissue. Leptin and 25(OH)D$_3$ were correlated in women with adequate vitamin D status, but not in men, suggesting a gender difference with respect to the interaction between the compounds.
CHAPTER 5

PURPOSE OF THE STUDY

Obesity continues to be on the rise worldwide. Any factor that contributes to the obesity epidemic needs to be identified. Considering the call for increased recommended intake levels of vitamin D, it is important to understand how such revisions might influence other factors, such as leptin expression, not traditionally associated with the vitamin. While research on leptin has been extensive, much remains to be learned about the variables influencing its expression.

Tissue culture and animal studies by Menendez et al. (2001) and Matsunuma et al. (2004) suggest interplay between leptin and calcitriol. In addition, leptin could influence the expression of the hydroxylating enzymes responsible for converting vitamin D into its different forms. Furthermore, the inhibitory influence of calcitriol on adipogenesis could indirectly lower leptin expression. Calcitriol synthesis depends on the presence of its precursor, 25(OH)D₃. Gomez et al. (2004) showed an inverse correlation between serum leptin and serum 25(OH)D₃ in women without vitamin D deficiency.

The objective of the current study is to examine the relationship between serum concentrations of leptin and 25(OH)D₃ in healthy 18- to 30-year-old women at the
University of Massachusetts. More specifically, this study will determine whether serum leptin is a significant predictor of serum 25(OH)D$_3$. In addition, our analysis will determine whether this relationship is independent of BMI and body composition. The results from this study will lead to a more complete understanding of the factors that influence adiposity and will help to inform discussions concerning revision of dietary vitamin D recommendations.

5.1 HYPOTHESES AND SPECIFIC AIMS

**Hypotheses:**

- Leptin is positively correlated with BMI and body fat mass.
- 25(OH)D$_3$ is inversely correlated with BMI and body fat mass.
- Leptin and 25(OH)D$_3$ are inversely correlated.
- The relationship between leptin and 25(OH)D$_3$ depends on or is modified by BMI and/or body fat mass.

**Specific Aims:**

1. Determine the relationship between serum concentrations of leptin and 25(OH)D$_3$. 
2. Determine the relationship between the aforementioned biomarkers and BMI.
   a) Determine the relationship between serum leptin concentrations and BMI.
   b) Determine the relationship between serum $25(\text{OH})\text{D}_3$ concentrations and BMI.

3. Determine the relationship between the aforementioned biomarkers and body fat mass.
   a) Determine the relationship between serum leptin concentrations and body fat mass.
   b) Determine the relationship between serum $25(\text{OH})\text{D}_3$ concentrations and body fat mass.

4. Statistically determine whether body fat mass or BMI significantly confound or modify the relationship between leptin and $25(\text{OH})\text{D}_3$. 
CHAPTER 6

MATERIALS AND METHODS

6.1 SAMPLE

As part of the University of Massachusetts Vitamin D Study, female subjects between the ages of 18 and 30 were recruited from the Western Massachusetts population (mostly from the UMass Amherst campus) during the spring of 2006 and the 2006-2007 school year. Women were excluded from the main study if they were pregnant and/or had any of the following conditions: high blood pressure, kidney diseases, liver diseases, bone diseases (e.g. osteopenia, osteomalacia), digestive disorders (e.g. celiac disease, Crohn’s disease, ulcerative colitis), rheumatologic diseases (e.g. rheumatoid arthritis), multiple sclerosis, thyroid diseases (e.g. Grave’s disease, hyperthyroidism, hypothyroidism, benign thyroid nodules), cancer, type 1 or type 2 diabetes, depression, hyperparathyroidism, elevated cholesterol or hyperlipidemia, and polycystic ovaries or polycystic ovarian syndrome. Women who took the following medications were also excluded from the study: corticosteroids, anabolic steroids, anticonvulsants, Propranolol, Tagamet and Cimetidine. The current study included the first 46 subjects for whom both biomarker and Dual Energy X-ray Absorptiometry (DXA) measurements were available.
6.2 DATA COLLECTION

6.2.1 Collection of Dietary and Demographic Data

Each woman was required to complete one study visit, which was scheduled during the late luteal phase of her menstrual cycle, three to five days before the expected start of the next menstrual period. At that visit, women completed the Harvard food frequency questionnaire (FFQ) and a questionnaire designed specifically for this study to elicit demographic as well as health-related data.

The Harvard FFQ has been validated and was found to correlate well with multiple one-week diet records with respect to its ability to quantify nutrient intake, especially after adjustment for total caloric intake ($r = 0.36$ to $0.75$ depending on nutrient). Repeat administration was shown to result in reasonable reproducibility ($r = 0.49$ to $0.71$ depending on nutrient; Willett et al., 1985). With respect to the assessment of intake of individual foods, the FFQ also produces reproducible ($r = 0.31$ to $0.92$ depending on food item, mean $r = 0.59$) and valid ($r = 0.17$ to $0.95$, mean $r = 0.63$) results as compared to two one-week diet records (Feskanich et al., 1993).

The FFQ queries about common food sources of vitamin D (milk and fatty fish) as well as foods that may or may not contain the vitamin, depending on the manufacturer’s fortification practices (yogurt, cheese, breakfast cereals). An open-ended question about the type and brand of the respondent’s breakfast
cereal allows for the quantification of vitamin D intake from this frequently consumed food category.

There are nine response options for foods ranging from never or less than once per month to six or more times per day. The FFQ contains a section on vitamin supplements, including multiple vitamin preparations. The response options for multiple vitamins range from two or less to 10 or more per week. Participants are furthermore asked to indicate brand and type of multivitamin used. Although a vitamin D supplement is not listed in a category of its own, it is listed as a choice under other regularly consumed supplements. The Harvard FFQ was adapted for this study to inquire about the previous two months instead of one year and to include additional foods (vitamin-D-fortified orange juice, soy products).

6.2.2 Measurement of BMI

Height was measured to the nearest 0.5 centimeter using a wall-mounted stadiometer. Weight was measured two ways, using the Detecto beam balance scale and using the DXA scanner. With either method, weight was measured to the nearest 0.1 kilogram. Since all body composition data are based on DXA measurements, DXA measurements of weight were also used to calculate BMI. Waist circumference to the nearest 0.5 inch was measured using a measuring tape. Measurements were performed fasting and without shoes. Subjects were allowed to have water the morning of the visit.
6.2.3 Measurement of Body Composition

Body composition was obtained either on the same day or at a second visit using the General Electric Prodigy DXA scanner. In addition to the yearly preventive maintenance check, a calibration scan is run daily on the machine. All DXA scans was performed by trained study personnel. DXA’s main advantage in estimating body composition is its ability to measure the three main body components (bone, soft lean tissue, and fat) directly. Other methods measure only one and use extrapolation to obtain results for the others. Compared to similar methods, such as dual photon absorptiometry (DPA), DXA uses a more stable source of radiation, thought to decrease precision error (Haarbo et al., 1991). Studies by both Haarbo et al. (1991) and Mazess et al. (1990) report reasonable precision errors for percent fat (SD 1.6% and 1.4%) and fat mass (SD 1.1 kg and 1.0 kg). Using ox inner thigh, porcine lard, and mixtures of the two, Haarbo et al. (1991) detected an accuracy error of 4.9% when comparing DXA measurements of percent fat with results from chemical fat extraction. The authors regard this error as satisfactory for both research purposes and diagnosis of the individual patient.

6.2.4 Specimen Collection and Biomarker Assay

Fasting venous blood was collected into serum separator vacutainers (SST™ Plus Blood Collection Tubes by Becton Dickinson) and kept on ice until serum was separated. Blood samples were centrifuged at 3000 x g for 15 minutes, and the serum was aliquoted into multiple cryotubes and stored at -80°C until
biomarker assessment. Serum 25(OH)D₃ concentrations were determined using a radioimmunoassay (RIA) kit (DiaSorin, Minnesota, USA). This assay has been validated by numerous investigators (Hollis, 2000). Serum leptin concentrations were similarly assayed using a RIA kit (Linco, St. Charles, MO; Ma et al., 1996). Gamma irradiation (in counts per minute) was quantified using a Beckman “Gamma 4000” gamma counter (Beckman Coulter, California, USA). RIA analysis involves extraction and preparation of the sample followed by measurement of gamma counts per time unit for each sample, which are plotted against a standard curve to determine biomarker concentrations. The calculations are outlined in table 6.1. The study was approved by the Human Subjects Committee of the University of Massachusetts, and each subject provided written informed consent.

Table 6.1. RIA Calculations

1. Average duplicate counts.
2. Subtract non-specific-binding counts from each average count (except for Total Counts).
3. Calculate the percentage of tracer bound:
   % tracer bound = (total binding counts/total counts) x 100
4. Calculate the percentage of total binding:
   %B/Bo = (sample or standard/total binding) x 100
5. Plot %B/Bo for each standard on the y-axis and the known concentration on the x-axis using log-log graph paper.
6. Construct a reference curve from these points.
7. Interpolate the reference curve to determine concentrations of the unknown samples.

Adapted from LINCO Research RIA Kit instruction brochure
6.3 STATISTICAL ANALYSIS

Data were summarized using mean ± SD for continuous and percentages for categorical variables. Leptin was logarithmically-transformed using the natural logarithm (ln), because it was not normally distributed. Dietary variables were dichotomized to show how many subjects did not meet the RDA. Serum 25(OH)D$_3$ concentration was divided into three categories to determine prevalence of deficient and suboptimal vitamin D status. Mean 25(OH)D$_3$ was stratified by month and season and compared using the Welch statistic. Tamhane’s test was used for post hoc analyses. Welch and Tamhane were used because the groups did not have equal variances.

Characteristics of study subjects were stratified by vitamin D status and differences between strata were determined with ANOVA. The Welch statistic was used for variables with unequal variances as determined by Levene’s test. Contrast tests did not reveal any differences between the “deficient” and “suboptimal” groups, so these groups were collapsed into one “insufficient” group, and differences were again determined with regular ANOVA or Welch. Chi-square statistics were computed for several sun exposure variables to determine whether differences in the proportion of these variables between vitamin D status strata were likely due to chance. Serum 25(OH)D$_3$ was stratified by several sun exposure variables and differences between strata were determined using regular ANOVA or Welch.
To determine correlations, variables that were not normally distributed were ln-transformed. Pearson’s rho was used for all dietary variables, except for total vitamin D, vitamin D from food, and vitamin B6. Correlations for the latter three were assessed using Spearman’s rho. For Pearson’s correlations, ln-transformed values were used for all variables except energy, vitamin A, and 25(OH)D$_3$, which were normally distributed.

The associations between body composition measures, 25(OH)D$_3$, and leptin were assessed using correlation analysis (Pearson’s rho), followed by further analyses using bivariable and multivariable linear regression. Covariates included in the regression models were BMI, FM, %BF, age, smoking status, and sunscreen use. The leptin-25(OH)D$_3$ relationship was further evaluated for effect modification by %BF and BMI. The data were stratified by %BF (cut-off 30%). Beta coefficients were calculated for each stratum using the same multivariable linear regression model as for the un-stratified analysis. Any differences in the beta coefficients were evaluated for statistical significance by re-running the regression model with a multiplicative interaction term. The multiplicative interaction term was created by multiplying the continuous variable ln(leptin) with the dichotomous stratification variable that identified women as having normal or high body fat. The significance level was set at p=0.05 for all analyses. The Statistical Package for Social Sciences (SPSS, Windows version 15.0, SPSS Inc. Chicago IL, USA) was used for all analyses.
Characteristics of the study sample are shown in Table 7.1. Most participants had waist circumferences of 88 cm or less (n=42) and BMIs between 18.5 kg/m² and 25 kg/m², with a mean of 21.9 kg/m² (n=35). Four women (8.7%) had BMIs below 18.5 kg/m², and seven women (15.2%) had BMIs above 25 kg/m². However, the sample shows considerable variability with respect to body fat percentage (%BF). Women were about equally divided into high (%BF>30, n=26) and normal (%BF≤30, n=20) body fat groups with values ranging from 14.5 to 45.2%. Mean serum leptin was 10.1 ± 6.8 ng/mL. Mean serum 25(OH)D₃ was 105.4 ± 38.0 nmol/L. About half of women (n=25) reported using sunscreen. One participant indicated that she was currently a smoker, and seven participants said they used to smoke (data not shown).

Mean intakes of energy, macronutrients and select micronutrients are shown in Table 7.2. Almost all women (≥95%) consumed at least the RDA for phosphorus, niacin, riboflavin, and vitamins A, B6, and B12. Vitamin D intake from food and supplements ranged from 22 IU to 1446 IU. Mean vitamin D intake was 367 ± 242 IU, but almost one fourth (22%) of participants did not meet the current AI for vitamin D (200 IU), and 96% of women consumed < 800 IU, the lower limit of the
vitamin D requirement proposed by Holick (2004b; data not shown). Calcium requirements were not met by 35% of the study sample.

Table 7.1. Characteristics of Women in the Study Sample

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>21.6 ± 3.2</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>61.4 ± 10.0</td>
</tr>
<tr>
<td>Weight (kg) based on DXA</td>
<td>60.6 ± 9.9</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>166.0 ± 6.7</td>
</tr>
<tr>
<td>Body Mass Index, BMI (kg/m²)</td>
<td>21.9 ± 2.9</td>
</tr>
<tr>
<td>Waist Circumference (cm)</td>
<td>77.2 ± 7.7</td>
</tr>
<tr>
<td>Fat Mass (kg)</td>
<td>18.4 ± 6.8</td>
</tr>
<tr>
<td>Body Fat Percentage</td>
<td>30.8 ± 7.8</td>
</tr>
<tr>
<td>Bone Mineral Density (g/cm²)</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td>Leptin (ng/mL)</td>
<td>10.1 ± 6.8</td>
</tr>
<tr>
<td>25(OH)D₃ (nmol/L)</td>
<td>105.4 ± 38.0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Percent (N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Race²</td>
</tr>
<tr>
<td>White</td>
</tr>
<tr>
<td>Asian</td>
</tr>
<tr>
<td>Other</td>
</tr>
<tr>
<td>Latina³</td>
</tr>
<tr>
<td>Education</td>
</tr>
<tr>
<td>Some college/currently in college</td>
</tr>
<tr>
<td>College degree</td>
</tr>
<tr>
<td>Some graduate school</td>
</tr>
<tr>
<td>Graduate degree</td>
</tr>
<tr>
<td>Sunscreen user</td>
</tr>
<tr>
<td>Tanning bed/booth user</td>
</tr>
</tbody>
</table>

¹N = 46 for all variables except tanning bed use (N = 27)
²“Other” includes those that marked two race categories
³“Latina” is a category of its own. A subject can therefore identify herself as Latina in addition to her race.
Table 7.2. Estimated Dietary Intake of Selected Nutrients

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>Mean ± SD</th>
<th>DRI</th>
<th>Percent with Intakes Below RDA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy (kcal)</td>
<td>2158 ± 781</td>
<td>---</td>
<td>n/a</td>
</tr>
<tr>
<td>Carbohydrate (g)</td>
<td>281 ± 117</td>
<td>---</td>
<td>n/a</td>
</tr>
<tr>
<td>Protein (g)</td>
<td>101 ± 39</td>
<td>---</td>
<td>n/a</td>
</tr>
<tr>
<td>Total fat (g)</td>
<td>69 ± 30</td>
<td>---</td>
<td>n/a</td>
</tr>
<tr>
<td>Vitamin D (IU)</td>
<td>367 ± 242</td>
<td>200</td>
<td>22</td>
</tr>
<tr>
<td>Calcium (mg)</td>
<td>1239 ± 606</td>
<td>1000</td>
<td>35</td>
</tr>
<tr>
<td>Phosphorus (mg)</td>
<td>1649 ± 610</td>
<td>700</td>
<td>2.2</td>
</tr>
<tr>
<td>Niacin (mg)</td>
<td>33 ± 14</td>
<td>14</td>
<td>4.3</td>
</tr>
<tr>
<td>Riboflavin (mg)</td>
<td>3.2 ± 1.7</td>
<td>1.1</td>
<td>0</td>
</tr>
<tr>
<td>Vitamin B6 (mg)</td>
<td>3.7 ± 3.8</td>
<td>1.3</td>
<td>4.3</td>
</tr>
<tr>
<td>Vitamin B12 (µg)</td>
<td>9.1 ± 6.1</td>
<td>2.4</td>
<td>2.2</td>
</tr>
<tr>
<td>Vitamin A (µg RAE)</td>
<td>4585 ± 1983</td>
<td>700</td>
<td>0</td>
</tr>
</tbody>
</table>

1 Daily intake was estimated from a modified version of the Harvard FFQ (Willett et al., 1988).
2 Only energy and vitamin A are normally distributed.
3 Recommended intakes (DRIs) for women this age

Twenty-four percent of participants had serum vitamin D levels below 80 nmol/L (Table 7.3), indicative of suboptimal vitamin D status, although only one subject fell below the traditional deficiency cut-off of 37.5 nmol/L. The characteristics of the study sample varied across strata of vitamin D status only with respect to sunscreen use (Table 7.4). Of the 11 women who had insufficient levels of 25(OH)D₃, 10 (91%) reported regular use of sunscreens. Of the 35 women who had sufficient levels of 25(OH)D₃, 15 (43%) reported regular use of sunscreen. Mean 25(OH)D₃ was significantly different between sunscreen users (95.4 ± 35.9 nmol/L) and non-sunscreen users (117.3 ± 37.8 nmol/L, p=0.05). All tanning bed users (N=6) had sufficient vitamin D status. Mean 25(OH)D₃ did not differ between strata of other sun exposure variables (travel to a sunny location, average daily hours spent outside in minimal clothing in the week prior to blood
draw, average daily hours spent outside in minimal clothing during the summer prior to blood draw, SPF).

Table 7.3. Vitamin D Status\(^1\) of Women in Study Sample (N = 46)

<table>
<thead>
<tr>
<th>Status</th>
<th>% (n)</th>
<th>Mean (± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deficient, &lt; 50 nmol/L</td>
<td>6.5 (3)</td>
<td>41.2 ± 12.6</td>
</tr>
<tr>
<td>Suboptimal, 50 – 79.9 nmol/L</td>
<td>17.4 (8)</td>
<td>66.6 ± 8.1</td>
</tr>
<tr>
<td>Sufficient, ≥ 80 nmol/L</td>
<td>76.1 (35)</td>
<td>119.8 ± 31.0</td>
</tr>
</tbody>
</table>

\(^1\) Based on serum concentrations of 25(OH)D\(_3\).

Table 7.4. Characteristics of Study Subjects, Stratified by Vitamin D Status

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Vitamin D Status (nmol/L)</th>
<th>P Value(^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Insufficient(^3) 25(OH)D(_3) &lt; 80 N= 11</td>
<td>Sufficient 25(OH)D(_3) ≥ 80 N= 35</td>
</tr>
<tr>
<td>Mean (± SD)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>25(OH)D(_3) (nmol/L)</td>
<td>60.0 ± 14.8</td>
<td>119.8 ± 31.0</td>
</tr>
<tr>
<td>Leptin (ng/mL)</td>
<td>7.8 ± 6.0</td>
<td>10.8 ± 7.0</td>
</tr>
<tr>
<td>Vitamin D Intake (IU)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Food</td>
<td>232 ± 181</td>
<td>234 ± 146</td>
</tr>
<tr>
<td>Supplements</td>
<td>120 ± 165</td>
<td>138 ± 255</td>
</tr>
<tr>
<td>Total</td>
<td>351 ± 234</td>
<td>372 ± 247</td>
</tr>
<tr>
<td>Calcium Intake (mg)</td>
<td>1381 ± 904</td>
<td>1194 ± 486</td>
</tr>
<tr>
<td>Age (years)</td>
<td>23.6 ± 3.9</td>
<td>21.0 ± 2.7</td>
</tr>
<tr>
<td>BMI (kg/m(^2))</td>
<td>21.5 ± 3.7</td>
<td>22.0 ± 2.6</td>
</tr>
<tr>
<td>Fat Mass (kg)</td>
<td>19.0 ± 7.6</td>
<td>18.2 ± 6.7</td>
</tr>
<tr>
<td>Body Fat (%)</td>
<td>31.9 ± 7.9</td>
<td>30.4 ± 7.8</td>
</tr>
<tr>
<td>Bone Mineral Density (g/cm(^2))</td>
<td>1.14 ± 0.07</td>
<td>1.14 ± 0.07</td>
</tr>
<tr>
<td>Percent</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sunscreen User</td>
<td>91.0</td>
<td>42.9</td>
</tr>
<tr>
<td>BMI &lt; 18.5 kg/m(^2)</td>
<td>18.2</td>
<td>5.7</td>
</tr>
</tbody>
</table>

\(^1\) N = 46 for all variables.
\(^2\) Statistical significance assessed using ANOVA for all variables except “sunscreen user”, which used chi-square analyses.
\(^3\) The column titled “insufficient” includes subjects from the deficient and suboptimal groups. These two groups were collapsed into one because there was no significant difference between them with respect to the variables in this table and because sample sizes in these groups were very small.
Table 7.5. Mean Serum 25(OH)D$_3$ by Month of Blood Draw$^1$

<table>
<thead>
<tr>
<th>Month of Blood Draw</th>
<th>% (n)</th>
<th>Mean (± SD) 25(OH)D$_3$ (nmol/L)$^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>March 2006/2007</td>
<td>34.8 (16)</td>
<td>105.6 ± 49.1$^{a,b}$</td>
</tr>
<tr>
<td>April 2006</td>
<td>32.6 (15)</td>
<td>102.1 ± 30.8$^a$</td>
</tr>
<tr>
<td>May 2006</td>
<td>4.3 (2)</td>
<td>143.2 ± 14.9$^{a,b}$</td>
</tr>
<tr>
<td>October/November 2006</td>
<td>4.3 (2)</td>
<td>138.8 ± 2.0$^b$</td>
</tr>
<tr>
<td>December 2006</td>
<td>4.3 (2)</td>
<td>111.6 ± 66.0$^{a,b}$</td>
</tr>
<tr>
<td>January 2007</td>
<td>4.3 (2)</td>
<td>61.8 ± 16.9$^{a,b}$</td>
</tr>
<tr>
<td>February 2007</td>
<td>15.2 (7)</td>
<td>102.3 ± 15.5$^a$</td>
</tr>
</tbody>
</table>

October and November 2006 were combined because of low participant number in each month. March 2006 and 2007 were also combined. Mean 25(OH)D$_3$ was not significantly different between these two months (90.0 ± 7.7 vs. 109.2 ± 54.1, p>0.05).

$^2$Month categories with different superscripts are significantly different as determined by Tamhane’s test.

Figure 7.1. Mean Serum 25(OH)D$_3$ by Month of Blood Draw
Figure 7.2. Average daily hours spent outside with minimal clothing in week prior to blood draw

Figure 7.3. Average daily hours spent outside with minimal clothing during summer prior to blood draw
Most women participated in the study during the months of March 2006/2007 and April 2006. The three deficient subjects were seen in January and March. Although mean serum 25(OH)D$_3$ level was lowest in January, this level did not differ significantly from other months. However, mean serum 25(OH)D$_3$ concentration was significantly higher in October/November 2006 than in April 2006 or February 2007 (Table 7.5, Fig 7.1). When combining months into seasons, no significant differences in serum 25(OH)D$_3$ levels were observed (data not shown).

Most participants (71.7%) did not spend any time outside in minimal clothing during the week prior to blood draw (Figure 7.2). Those who did, did so in March, April, and May. All participants but one spent a daily average of one or more hours outside during the summer prior to blood draw (Figure 7.3). The non-Caucasian and all but one of the Latina subjects spent three or more hours outside.

When assessing the relationship between dietary intake of vitamin D and serum levels of 25(OH)D$_3$, we found that mean serum 25(OH)D$_3$ was significantly lower in women whose total dietary vitamin D intake fell below the AI (200 IU) than in women who consumed 200 IU/d or more of vitamin D (83.1±27.7 vs 111.6±38.4 nmol/L, p=0.03). When vitamin D intakes from food and supplements – the two components of total dietary vitamin D intake – were considered separately, no difference in mean serum 25(OH)D$_3$ between women who consumed less than
200 IU/d and women who consumed 200 IU/d or more was observed for either intake from food (100.1±32.0 vs 110.7±43.3 nmol/L, p=0.35) or intake from supplements (102.0±40.0 vs 112.5±34.8 nmol/L, p=0.38). Mean serum 25(OH)D₃ furthermore did not differ between women consuming less and women consuming more than 800 IU of vitamin D (105.9±37.6 vs 95.1±63.8 nmol/L, p=0.70). In addition, serum 25(OH)D₃ was not significantly correlated with vitamin D intake from food, vitamin D intake from supplements, or total vitamin D intake (r=0.04, p=0.79; r=0.14, p=0.34; r=0.17, p=0.26). In fact, neither serum 25(OH)D₃ nor leptin was significantly correlated with intake of any of the macro- and micronutrients evaluated (data not shown). Serum 25(OH)D₃ was positively correlated with intake of cold cereal (r=0.37, p=0.01), and serum leptin was positively correlated with intake of cold and cooked cereals (r=0.36, p=0.01, and r=0.31, p=0.04, respectively).

The correlations between serum 25(OH)D₃, leptin, and measures of adiposity are shown in Tables 7.6 and 7.7. In unstratified analyses (Table 7.6), serum leptin concentration was significantly positively correlated with all measures of adiposity, with the strongest correlation (r=0.82, p<0.001) observed for %BF. Serum leptin was also positively correlated with BMD (r=0.44, p=0.002). Serum 25(OH)D₃ was not significantly correlated with serum leptin, BMD, or any of the adiposity measures.
Table 7.6. Association Between Leptin, 25(OH)D₃ and Measures of Body Composition, Unstratified

<table>
<thead>
<tr>
<th></th>
<th>25(OH)D₃</th>
<th>ln(Leptin)</th>
<th>BMI</th>
<th>FM</th>
<th>% Body Fat</th>
<th>BMD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>r</td>
<td>p</td>
<td>r</td>
<td>p</td>
<td></td>
<td>p</td>
</tr>
<tr>
<td>25(OH)D₃</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>r</td>
<td>1.00</td>
<td>0.20</td>
<td>0.05</td>
<td>-0.02</td>
<td>-0.004</td>
<td>-0.03</td>
</tr>
<tr>
<td>p</td>
<td>0.19</td>
<td>0.75</td>
<td>0.89</td>
<td>0.98</td>
<td>0.98</td>
<td>0.84</td>
</tr>
<tr>
<td>ln(Leptin)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>r</td>
<td>1.00</td>
<td>0.55</td>
<td>0.77</td>
<td>0.82</td>
<td>0.82</td>
<td>0.44</td>
</tr>
<tr>
<td>p</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>BMI</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>r</td>
<td>1.00</td>
<td>0.84</td>
<td>0.68</td>
<td>0.53</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>FM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>r</td>
<td>1.00</td>
<td>0.93</td>
<td>0.53</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p</td>
<td>&lt;0.001</td>
<td></td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>% Body Fat</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>r</td>
<td>1.00</td>
<td></td>
<td>0.41</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p</td>
<td></td>
<td></td>
<td>0.01</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

P-values are 2-tailed.

Analyses stratified by %BF reveal that the relationships between serum 25(OH)D₃, leptin, and the body composition variables were modified by %BF. In stratified analyses (Table 7.7), serum 25(OH)D₃ was significantly, positively correlated with leptin, BMI, and FM in women with normal body fat (%BF ≤ 30). The significant, positive correlation of serum leptin with BMD and all adiposity measures remained in stratified analyses for women with normal body fat. In women with high body fat (%BF > 30), serum leptin was significantly, positively correlated only with FM and %BF, but not with BMI and BMD. Serum leptin differed between normal and high %BF categories (5.2±2.9 and 13.8±6.7 ng/mL, respectively, p<0.001), but serum 25(OH)D₃ did not (107.1±39.9 and 104.1±37.2 nmol/L, respectively, p=0.79). However, serum 25(OH)D₃ differed between the
### Table 7.7. Association Between Leptin, 25(OH)D<sub>3</sub> and Measures of Body Composition, Stratified by Category of %BF<sup>1</sup>

<table>
<thead>
<tr>
<th></th>
<th>25(OH)D&lt;sub&gt;3&lt;/sub&gt;</th>
<th>ln(Leptin)</th>
<th>BMI</th>
<th>FM</th>
<th>% Body Fat</th>
<th>BMD</th>
</tr>
</thead>
<tbody>
<tr>
<td>25(OH)D&lt;sub&gt;3&lt;/sub&gt; ≤30 %BF</td>
<td>r 1.00</td>
<td>0.55</td>
<td>0.47</td>
<td>0.42</td>
<td>0.35</td>
<td>0.27</td>
</tr>
<tr>
<td></td>
<td>p 0.01</td>
<td>0.04</td>
<td>0.06</td>
<td>0.13</td>
<td>0.25</td>
<td></td>
</tr>
<tr>
<td>25(OH)D&lt;sub&gt;3&lt;/sub&gt; &gt;30 %BF</td>
<td>r 1.00</td>
<td>0.12</td>
<td>-0.08</td>
<td>-0.10</td>
<td>-0.09</td>
<td>-0.14</td>
</tr>
<tr>
<td></td>
<td>p 0.56</td>
<td>0.69</td>
<td>0.61</td>
<td>0.67</td>
<td>0.51</td>
<td></td>
</tr>
<tr>
<td>ln(Leptin) ≤30 %BF</td>
<td>r 1.00</td>
<td>0.46</td>
<td>0.84</td>
<td>0.84</td>
<td>0.49</td>
<td></td>
</tr>
<tr>
<td></td>
<td>p 0.04</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.03</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ln(Leptin) &gt;30 %BF</td>
<td>r 1.00</td>
<td>0.20</td>
<td>0.35</td>
<td>0.42</td>
<td>0.15</td>
<td></td>
</tr>
<tr>
<td></td>
<td>p 0.32</td>
<td>0.08</td>
<td>0.03</td>
<td>0.46</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMI ≤30 %BF</td>
<td>r 1.00</td>
<td>0.75</td>
<td>0.40</td>
<td>0.70</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>p &lt;0.001</td>
<td>0.08</td>
<td>0.001</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMI &gt;30 %BF</td>
<td>r 1.00</td>
<td>0.77</td>
<td>0.61</td>
<td>0.29</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>p &lt;0.001</td>
<td>0.001</td>
<td>0.15</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% Body Fat ≤30 %BF</td>
<td>r 1.00</td>
<td>0.87</td>
<td>0.73</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>p &lt;0.001</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% Body Fat &gt;30 %BF</td>
<td>r 1.00</td>
<td>0.79</td>
<td>0.35</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>p &lt;0.001</td>
<td>0.08</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>1</sup>P-values are 2-tailed.
<sup>2</sup>Spearman’s correlation coefficient is given for 25(OH)D<sub>3</sub> and FM.

Low (<18.5 kg/m<sup>2</sup>) and normal (18.5-24.99 kg/m<sup>2</sup>) BMI categories (66.8±37.6 and 113.4±37.4 nmol/L, respectively, p=0.04). Body fat percentage was ≤30% in all “underweight” women (BMI < 18.5 kg/m<sup>2</sup>) and >30% in all “overweight” (BMI 25.0-29.9 kg/m<sup>2</sup>) and “obese” (BMI ≥ 30.0 kg/m<sup>2</sup>) women. However, within the normal BMI range (BMI 18.5 – 24.9 kg/m<sup>2</sup>), 16 women had ≤30% BF and 19 had
>30% BF. BMI and %BF were positively correlated ($r=0.35$, $p=0.01$) within the normal BMI category (data not shown).

### Table 7.8. Association Between 25(OH)D$_3$ and ln(Leptin), Unstratified$^1$

<table>
<thead>
<tr>
<th></th>
<th>$B$</th>
<th>$SE$</th>
<th>$p$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unadjusted</td>
<td>9.8</td>
<td>7.3</td>
<td>0.188</td>
</tr>
<tr>
<td>BMI-adjusted</td>
<td>12.1</td>
<td>8.8</td>
<td>0.178</td>
</tr>
<tr>
<td>FM-adjusted</td>
<td>25.7</td>
<td>11.1</td>
<td>0.025</td>
</tr>
<tr>
<td>%BF-adjusted</td>
<td>30.8</td>
<td>12.4</td>
<td>0.017</td>
</tr>
<tr>
<td>Multivariable-adjusted$^2$</td>
<td>22.8</td>
<td>14.5</td>
<td>0.124</td>
</tr>
</tbody>
</table>

$^1$ Dependent variable: 25(OH)D$_3$

$^2$ Adjusted for age, current and former smoking status, sunscreen use, and %body fat.

In simple linear regression analyses, serum leptin was not significantly associated with serum 25(OH)D$_3$ (Table 7.8). Controlling for BMI did not alter the association. However, in bivariant models that included either fat mass or percent body fat, serum leptin was significantly positively associated with 25(OH)D$_3$ ($B=25.7$, $p=0.03$ and $B=30.8$, $p=0.02$). When fat mass is held constant, serum 25(OH)D$_3$ increased 25.7 nmol/L for every one unit increase in serum ln(leptin). At constant percent body fat, serum 25(OH)D$_3$ increased 30.8 units for every one unit increase in serum ln(leptin).

We observed some evidence of effect modification of the leptin-25(OH)D$_3$ relationship by %BF, although results were not statistically significant. In a multivariable regression analysis adjusting for age, sunscreen use, %BF, and current and former smoking status, leptin was significantly and positively
associated with 25(OH)D$_3$ (B=63.1, p=0.007) only in women with normal %BF, but was not associated with 25(OH)D$_3$ in women with %BF >30 (Table 7.9). We observed no effect modification of the leptin-25(OH)D$_3$ relationship by BMI.

Table 7.9. Association Between 25(OH)D$_3$ and ln(Leptin), Stratified by %BF

<table>
<thead>
<tr>
<th></th>
<th>%BF ≤ 30$^2$</th>
<th>%BF &gt; 30</th>
<th>p for interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$B$</td>
<td>SE</td>
<td>p-value</td>
</tr>
<tr>
<td>Multivariable-adjusted$^1$</td>
<td>63.1</td>
<td>19.8</td>
<td>0.01</td>
</tr>
</tbody>
</table>

$^1$ Adjusted for age, current and former smoking status, sunscreen use, and %body fat.

$^2$ This group included no current smokers.
CHAPTER 8

DISCUSSION

Results from this study provide some evidence for a positive association between 25(OH)D$_3$ and leptin in models controlling for %BF or FM, indicating that the relationship is confounded by adiposity. In contrast to the findings of Gomez et al. (2004), we did not find a relationship between leptin and 25(OH)D$_3$ in unadjusted analyses. The leptin-25(OH)D$_3$ relationship differed between women with normal (≤30% BF) and women with high (>30% BF) %BF, however this difference was not significant. In multivariable regression analysis, leptin was associated with 25(OH)D$_3$ only in women with normal body fat (B=63.1, p=0.007), but not in women with high body fat. Considering that leptin and 25(OH)D$_3$ are processed differently in obese than in lean persons because of high body fat content and leptin resistance, a difference in the relationship between the two compounds according to weight status is plausible. Previous findings by Gomez et al. (2004) indicating an inverse correlation between serum leptin and 25(OH)D$_3$ are not supported by the current study.

Mean serum leptin was 10.1±6.8 ng/mL in the current study, and mean BMI was 21.9±2.9 kg/m$^2$. Ruhl and Everhart (2002) reported mean serum leptin levels of 15.2±0.4 ng/mL and a mean BMI of 25.8±0.2 kg/m$^2$ in a sample of 1906
premenopausal women drawn from NHANES III. The differences in leptin levels are consistent with the differences in BMI, since both measures have been shown to be positively correlated (Considine et al., 1996; Havel et al., 1996; Maffei et al., 1995). Healthy, premenopausal, normal-weight women studied by Krzyzanowska-Swiniarska et al. (2007) had lower mean leptin levels than women in the current study (7.8±3.9 ng/mL vs 10.1±6.8 ng/mL), although the mean BMI in both studies was almost equal (21.9±2.9 vs 21.3±1.7 kg/m\(^2\)). However, %BF in the current study was higher (30.8±7.8 vs 24.5±5.4), and was likely the reason for the higher mean leptin values. Although leptin has been shown to positively correlate with BMI, its physiologic relationship is with the amount of adipose tissue, not muscle mass, which also contributes to BMI.

Leptin was positively correlated with all measures of adiposity in women with normal %BF. The positive correlation with BMI, FM, and %BF is consistent with the results from previous correlation studies and underscores the fact that most serum leptin originates in the adipocyte (Considine et al., 1996, Havel et al., 1996, Maffei et al., 1995). Leptin was furthermore positively correlated with BMD in women with normal %BF and not correlated with BMD in women with high %BF. Some studies have reported a positive association between leptin and BMD (Thomas et al., 2001; Yamauchi et al., 2001), but this relationship has not been observed consistently (Martini et al., 2001; Ruhl & Everhart, 2002). Results from this study provide support for a positive relationship between leptin and BMD in women with normal %BF.
Serum 25(OH)D₃ was not correlated with any of the body composition variables in women with high %BF, and positively correlated with ln(leptin), BMI, and FM in women with normal %BF. Considering 25(OH)D₃’s well-established impact on BMD (Lips 2006), it is somewhat surprising that no correlation between these variables was observed. However, there was little variation in BMD (1.1±0.1 g/cm²) in this cohort, which may explain the lack of correlation with 25(OH)D₃.

Considering evidence of 25(OH)D₃’s inverse relationship with body fat content (Wortsman et al., 2000), it is furthermore surprising that no correlation between 25(OH)D₃ and measures of adiposity was observed in women with high %BF, that correlations were positive in women with normal %BF, and that body composition variables did not differ between vitamin D sufficient and insufficient groups. However, there was little variation in BMI (23.3±2.7 kg/m²), %BF (36.4±3.9), and FM (22.9±4.8 kg) in women with high %BF, which may explain the lack of correlation with 25(OH)D₃.

Although both FM and %BF were strongly positively correlated with BMI (r=0.84 and 0.68, respectively, p<0.001), only FM and %BF, but not BMI, were significant confounders of the relationship between serum levels of 25(OH)D₃ and leptin. The study sample yielded a wide range of FM and %BF values, while BMI had only a small range. There may not have been enough variability in BMI to produce a confounding effect on the relationship between serum levels of 25(OH)D₃ and leptin.
In addition to the strong correlation with BMI, %BF reflected the weight status of women with extreme BMIs: in underweight women (BMI < 18.5 kg/m²), %BF was ≤30%; in overweight (BMI 25.0-29.9 kg/m²) and obese (BMI ≥ 30.0 kg/m²) women, it was >30%. However, within the "healthy" BMI range, women divided about evenly into those with normal (n=16) and those with high (n=19) %BF, indicating that BMI was not a good indicator of body composition in this category. Nonetheless, %BF was positively correlated with BMI within the normal BMI category. Hence, BMI may be associated with FM and %BF at all levels of BMI, but a normal BMI may not necessarily coincide with a healthy body composition.

Although mean serum 25(OH)D₃ in this study was 105.4 ± 38.0 nmol/L, 24% of subjects had biochemical evidence of insufficient vitamin D status (25(OH)D₃ < 80 nmol/L). Many participants in this study were nutrition students and may therefore have had better vitamin D status than the typical young woman. This suggests that the actual prevalence of suboptimal vitamin D status in the general population of young women may be even higher. Serum 25(OH)D₃ levels were expected to be low in the late winter and early spring months and higher in late spring, summer, and early fall. Although this general pattern was observed, significant differences were found only when comparing October/November with April and February. Due to the variability in sample size between months and the small sample size overall, mean 25(OH)D₃ values by month and differences between months should be interpreted carefully.
Although total vitamin D intake from diet and supplements varied widely among subjects (22 – 1446 IU), nearly 80% of participants consumed at least the AI of 200 IU, and the mean intake was 367 IU. However, the inconsistencies in vitamin D intake were not reflected in serum 25(OH)D₃ levels: vitamin D intake from food and supplements did not differ between insufficient and sufficient subjects, and vitamin D intake was not a significant predictor of 25(OH)D₃ levels. The lack of correlation between vitamin D intake and serum 25(OH)D₃ underscores the importance of using biomarker measurements to assess vitamin D status since serum levels of 25(OH)D₃ are related to many nondietary factors.

The current vitamin D AI for women under age 51 (200 IU) is set to produce serum levels of just above 37.5 nmol/L to prevent the deficiency diseases rickets and osteomalacia. However, newer evidence suggests that optimal serum levels should be at least 80 nmol/L (Holick, 2005). In order to achieve 25(OH)D₃ levels of 80 nmol/L and above in the absence of adequate UV-B radiation (such as in January), Holick (2004b) has proposed a daily intake of 800 – 1000 IU. Vitamin D intake observed in the current study ranged from 22 IU to 1446 IU, but only 4.3% of women consumed ≥800 IU. Along with the insignificant relationship between dietary vitamin D and 25(OH)D₃ levels, this observation supports the idea that the subjects with sufficient levels of vitamin D achieved those through sun exposure rather than diet.
Although a single sun exposure variable was not created for this study, we can speculate about the relationship between sun exposure and 25(OH)D$_3$ in this sample by drawing some conclusions from the battery of sun exposure variables. During the week prior to blood draw, most participants (71.7%) did not report spending any time outside in minimal clothing, so the sufficient 25(OH)D$_3$ levels were probably not caused by recent sun exposure. Furthermore, substantial exposure to UV-B radiation in the week prior to blood draw likely would have resulted in much higher individual 25(OH)D$_3$ levels than observed in this study. In the summer prior to blood draw, all but one participant spent a daily average of one or more hours outside wearing minimal clothing. The non-Caucasian subjects, who require a longer sun exposure to achieve a MED, spent three or more hours outside. Hence, nearly all of our subjects spent enough time outside in the summer prior to blood draw to get at least one daily MED (= 10,000 – 20,000 IU), provided they did not use sunscreen.

Serum 25(OH)D$_3$ levels did not differ by the number of hours spent outside wearing minimal clothing in the week and the summer prior to blood draw, suggesting that other factors, such as sunscreen use, may have played a role in the extent of vitamin D synthesis. It may also have been difficult for subjects to accurately estimate average hours spent outdoors. Minimal sun exposure (e.g. 10-15 minutes on one day of the week for light-skinned women) may have been interpreted and indicated on the questionnaire as “no sun exposure,” when in fact
this minimal sun exposure may have sufficed to produce at least some vitamin D in those women who participated in the study during the spring and fall.

Slightly more than half of subjects in this study reported using sunscreen. Of those with insufficient 25(OH)D$_3$ levels, 91% reported using sunscreen, suggesting that sunscreen use was an effective block against vitamin D synthesis in this group. Sunscreen use was also common among women with sufficient 25(OH)D$_3$ levels. Almost half (43%) of women in this group reported using sunscreen, yet that practice did not appear to substantially interfere with vitamin D synthesis. Sunscreen only provides protection from UV-B radiation when applied properly and in sufficient amounts, but most people apply the product improperly and therefore do not reap the full benefits of the stated SPF (Gaughan & Padilla, 1998; Grencis & Stokes, 1999; Stenberg & Larko, 1985).

The current study has several important strengths. Vitamin D deficiency in the Gomez et al. (2004) study was defined by old standards, using 38 nmol/L as the cut-off value. The present study used a more appropriate cut-off value of 80 nmol/L (Barger-Lux et al, 1998; Holick, 2005). Use of this higher cut-off value provided a more sensitive estimate of the prevalence of suboptimal vitamin D levels in this population.

To determine body composition, Gomez et al. (2004) used the Holtain BC bioelectrical impedance analyzer. Bioelectrical impedance has some
shortcomings. Abnormal hydration, such as from excessive sweating or intake of large amounts of water, leads to distorted estimates of fat mass. Furthermore, bioelectrical impedance is an indirect method that employs population-specific regression equations to obtain measurements of fat and lean mass. Thus, the quality and choice of the equation determines the accuracy of the final data (Kyle et al., 2004). In the current study, DXA was used instead. DXA measures fat and lean mass directly, provides good reproducibility and validity, and yields more reliable results. Serum leptin and serum 25(OH)D$_3$ were measured with the same assay kits Gomez et al. used, resulting in an improved ability to compare results.

In addition to differences in methodology, the population of interest varies considerably between the two studies. Gomez et al. investigated a heterogeneous population consisting of men and women and spanning six age decades. The current study focused on a relatively homogeneous group of healthy young women.

The limitations of this study include its cross-sectional design, which does not allow for any conclusions about causality in the relationship between serum leptin and 25(OH)D$_3$. Because this was a preliminary analysis, the sample size was small, limiting statistical power. Furthermore, we recruited only healthy, young women, all of whom were self-selected and many of whom were nutrition majors. These factors may have resulted in recruitment of women who were generally more health- and diet-conscious, which could have resulted in selection bias.
However, dietary factors were not confounders of the leptin-25(OH)D$_3$ relationship, suggesting that women eating a healthy diet likely exhibit the same association between leptin and 25(OH)D$_3$ as women who are not concerned with their diet. There may be other health-related factors (e.g. exercise) not accounted for in this study that may distinguish our sample from healthy, young women in general and that may confound the leptin-25(OH)D$_3$ relationship. In this case, selection bias would limit the generalizability of our results, but could also have made our group more homogenous, which could be an advantage considering our small sample size.

The lack of quality control data and the method of computation used in the RIAs leave some doubt as to the accuracy of the leptin and 25(OH)D$_3$ data. Possible measurement error in the assays could not be estimated because quality control data were not available. The assays furthermore rely on a regression equation to derive concentrations of the analyte from radiation counts. The quality of the equation influences the accuracy of the final values, and there may be equations yielding better results than the one used in this study.
CHAPTER 9

CONCLUSIONS

Results from the current study confirm the positive association of leptin with %BF, underscoring the fact that most serum leptin originates in the adipocyte. Our results furthermore suggest that, after controlling for adiposity, 25(OH)D₃ is positively associated with leptin, and that this relationship may be modified by %BF. In addition, we observed that %BF did not reflect the weight status of women with healthy BMIs, indicating that BMI was not a good indicator of body composition in these women. Vitamin D intake was not a good predictor of 25(OH)D₃ levels, indicating that the assessment of vitamin D status may be more reliable when biomarker instead of dietary measurements are used. Our findings furthermore provide additional evidence to confirm that sunscreen use is an effective block against vitamin D synthesis.

Because of the small sample size of the current study, further studies using larger cohorts are needed to confirm the association between 25(OH)D₃ and leptin and to explore possible effect modification by %BF. Additional studies are also needed to elucidate the physiologic significance of this association and to determine whether manipulation of either serum levels of leptin or 25(OH)D₃ through intervention influences adiposity.
APPENDIX A:

RECRUITMENT FLYERS
(created by Sofija Zagarins)

School of
Public Health
and Health Sciences

got PMS?

We are looking for interested women between the ages of **18 and 30** to participate in a nutrition study designed to examine the relationship between diet and PMS.

Participating will take about an hour and a half of your time on a morning that is convenient.

* Compensation provided *
* Free diet and nutrient analysis results *
* Free bone density scan *

For more information, please e-mail the study center at:
- vitamind@nutrition.umass.edu

Or contact the study investigators:
- Alayne Ronnenberg, ScD: 413-545-1076; alayner@nutrition.umass.edu
- Elizabeth Bertone-Johnson, ScD: ebertone@schoolph.umass.edu

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</tbody>
</table>
Curious about your BODY COMPOSITION?

We are looking for interested women between the ages of **18 and 30** to participate in a nutrition study designed to examine the relationship between vitamin D and body composition.

Participating will take about an hour and a half of your time on a morning that is convenient.

* Compensation provided *
* Free diet and nutrient analysis results *
* Free bone density scan *

For more information, please e-mail the study center at:
- vitaminD@nutrition.umass.edu

Or contact the study investigators:
- Alayne Ronnenberg, ScD: (413) 545-1076; alayner@nutrition.umass.edu
- Elizabeth Bertone-Johnson, ScD: ebertone@schoolph.umass.edu
Are you at risk for OSTEOPOROSIS?

We are looking for interested women between the ages of 18 and 30 to participate in a nutrition study designed to examine the relationship between diet and bone density.

Participating will take about an hour and a half of your time on a morning that is convenient.

* Compensation provided *
* Free diet and nutrient analysis results *
* Free bone density scan *

For more information, please e-mail the study center at:
• vitaminD@nutrition.umass.edu

Or contact the study investigators:
• Alayne Ronnenberg, ScD: (413) 545-1076; alayner@nutrition.umass.edu
• Elizabeth Bertone-Johnson, ScD: ebertone@schoolph.umass.edu
APPENDIX B:

INFORMED CONSENT FORM

Project Title: UMass Vitamin D Status Study
Principal Investigators: Alayne Ronnenberg, ScD, Department of Nutrition and
Elizabeth Bertone-Johnson, ScD, Department of Public Health

INFORMED CONSENT DOCUMENT

Your written consent is required before you can participate in this study. Please read this document carefully, initialing each page as you read it, and then sign your name on the last page. If you have difficulty reading or understanding any sections of this document, please do not hesitate to ask the investigators to clarify and/or read these sections to you. This document is in accordance with the University of Massachusetts Human Subjects Review Committee.

Project Title: UMass Vitamin D Status Study

Principal Investigators: Alayne Ronnenberg, ScD
Department of Nutrition
209 Chenoweth Lab
University of Massachusetts
Amherst, MA 01003

Elizabeth Bertone-Johnson, ScD
Department of Public Health
409 Arnold House
University of Massachusetts
Amherst, MA 01003

Sponsors: UMass faculty start-up funds

Invitation to Participate:
You are being invited to participate in this research study, which will be carried out in Arnold House and University Health Services on the University of Massachusetts Amherst campus. As a participant in the study, you will be asked to take part in a testing session lasting approximately 2 hours. More detailed information is included in the remainder of this document.

Subject’s Initials: _______  Date: _______

University of Massachusetts Amherst IRB
(410) 545-9428

Approval Date
03-18-08

Valid Through
06-18-08

IRB Signature
[Signature]
Project Title: UMass Vitamin D Status Study
Principal Investigators: Alysa Ronnenberg, ScD, Department of Nutrition and
Elizabeth Bertone-Johnson, ScD, Department of Public Health

Purpose of the Study:
The main purpose of this study is to measure blood levels of the nutrient vitamin D in young
women on the University of Massachusetts campus. In addition to vitamin D, the study will
also measure blood levels of other nutrients, including calcium, folate, vitamins B6 and B12,
and hormones, such as parathyroid hormone (which works with vitamin D and calcium to
maintain healthy bones). The study will also use blood samples to identify genetic factors,
called polymorphisms, that commonly occur and that can affect the way vitamin D and other
nutrients work within the body. To determine whether your diet and activities have influenced
the amount of minerals in your bones, you will also be asked to undergo a painless bone scan
procedure, called dual x-ray absorptiometry (DXA). This scan will also provide information on
your body composition, including the portion of your body that is made up of fat. Through the
use of a questionnaire, the study also hopes to collect information about diet, activity, lifestyle
and menstrual factors that may be related to blood levels of nutrients.

Procedures:
If you agree to participate in this study, we will obtain 45 mL (about 3 tablespoons) of blood
from your arm. The blood will be drawn by a trained study research assistant in our facility or
at University Health Services before you have eaten on a morning that is convenient for you,
about 3 to 5 days before your next menstrual period is expected. The fasting blood sample will
later be tested for vitamin, mineral, and hormones levels. At this visit, a research assistant will
also weigh and measure you and obtain your waist measurement and blood pressure. You will
also be asked to provide a small droplet of blood from your fingertip to measure the amount of
glucose (sugar) in your blood. This procedure uses a very fine sterile needle, called a lancet.
The results of the blood sugar testing will be available immediately. You will also be asked to
provide a urine sample, which may be tested for other nutrient and hormone levels not
measurable in blood. After approximately 2 weeks, you will be contacted by a member of the
study staff via email to confirm the start date of your next menstrual period.

A small amount of blood will also be used to obtain a sample of DNA, which will be used to
analyze how specific genetic factors, called polymorphisms, affect the way nutrients are
handled by the body. Polymorphisms are different types of these genes that are normal and
common in human populations and are generally not associated with specific diseases. We
will not analyze your blood for genes that are associated with high risk of disease (such as
BRCA1). The specimens collected from you will be stored for 3 years or more and may be
used by Drs. Ronnenberg and Bertone-Johnson for additional research during this period. By
signing this document, you consent to these future potential uses. If you decide later that you
do not want the specimens collected from you to be used for these DNA studies, you may
notify Drs. Ronnenberg or Bertone-Johnson, who will ensure that your samples are discarded
after nutrient and hormone levels have been assessed.

Subject's Initials: Date: __________________________________________

University of Massachusetts Amherst-IRB

[Signature] 10-04-07

IRB Signature 10-04-07
We will ask that you complete a questionnaire to provide information about your health and lifestyle, characteristics of your menstrual cycle, and your diet. We will also ask that you have a DXA bone scan completed at University Health Services. This scan is usually completed the same day as your main study visit. The DXA scan will estimate your body composition and bone mineral density. If, during the course of the study, we become aware of a need for you to be referred for a medical consultation, we reserve the option to contact you.

Costs:
There will be no cost to participate. We will provide all necessary collection materials.

Risks and Discomforts:
The risks to participating in this study are those associated with having blood pressure taken, blood drawn, urine collected, undergoing studies of genetic factors, and undergoing a DXA scan.

For having blood pressure taken, the procedure may cause some mild discomfort as the blood pressure cuff is inflated. For having blood drawn, risks include pain at the site of needle entry, occasional bruising at the site, and rarely, fainting. Risk of infection is minimal since only sterile one-time-use equipment will be used. There are minimal risks associated with providing a urine sample. The collection of a drop of blood with a lancet may cause minimal pain and bleeding.

For the genetic studies, some people involved in these studies have felt anxious about the possibility of carrying an altered gene that they could possibly pass on to their children or which might put them at high risk for developing a disease. We will not be analyzing these types of genes in our study. The genetic factors we are studying, called polymorphisms, are different types of genes that are normal and common in human populations and are generally not associated with specific diseases.

For the DXA scan, the risk from exposure to low-dose radiation is very small and is about the same as would occur in a flight between Boston and Los Angeles.

Benefits:
You will be provided with information concerning your blood sugar levels when you complete the study questionnaires. At a later time, you will also be provided a written copy of the results of the analyzed diet questionnaire, which will summarize the nutrient content of your current diet, and identify the strengths and weaknesses in your diet. Also at a later time, information regarding your blood and urine levels of nutrients will be available, and you will have the opportunity to receive dietary counseling from a senior or graduate nutrition student, if you desire. If you have undergone a DXA bone scan, you will also be provided with a copy of the DXA results, which will indicate your body composition (percent body fat) and your bone mineral density, which may provide some information on your risk of osteoporosis later in life.

There will be no direct benefit to you from the genetic

Subject’s Initials: _____  Date: _____
Project Title: UMass Vitamin D Status Study
Principal Investigators: Alayne Ronnenberg, ScD, Department of Nutrition and
Elizabeth Bertone-Johnson, ScD, Department of Public Health

(DNA) aspects of this study since you will not be provided with any results or information
regarding your DNA test. The investigators, however, may learn more about the way that
nutrient requirements vary among persons based on their individual genetic makeup. Upon
completion of all testing sessions, you will receive $10.00.

Alternatives:
You are under no obligation to participate in this project and may withdraw from participation
at any time.

Confidentiality:
All information provided will be kept confidential and will not be sold or shared with anyone not
associated with the study. The information obtained from this study will not become a part of
your medical record. If data from the study is published, no names or identifying information
will be used. To help ensure your privacy, all of the information you provide will be coded using
a system that does not identify your name. Your identifying information will be stored in
locked file cabinets and kept separate from your study data to prevent an inadvertent breach
in confidentiality. Under normal research circumstances, the researchers and their assistants
will not routinely link your name to the information you provide, although that information will
be available so that you can be informed of the results of the various tests.

Injuries:
If you are injured as a result of this study, you will be responsible for your own medical care.
The researchers will not supply medical care due to injury that may occur during participation
in this study. However, the risk of injury in this study is very low.

Questions:
Should you have any questions about your treatment or any other matter related to your
participation in this project, please contact either of the study's principal investigators, Alayne
Ronenberg, ScD, by phone at 413-545-1076 or via email at ronnenberg@comcast.net, or
Elizabeth Bertone-Johnson, ScD, by phone at 413-577-1672 or via email at
ebertone@sooholh.umass.edu.

The Institutional Review Board at the University of Massachusetts Amherst has approved this
study. If you would like to speak with someone not directly involved in the research study
about your rights as a research study participant, you may contact the Office of Research
Affairs at the University of Massachusetts by phone at 413-545-5428 or via email at
humansubjects@ora.umass.edu.
Project Title: UMass Vitamin D Status Study
Principal Investigators: Alayne Ronnenberg, ScD, Department of Nutrition and
Elizabeth Bertone-Johnson, ScD, Department of Public Health

Subject Statement of Voluntary Consent:
I have read this form or it has been read to me and I understand its contents. By signing the
consent statement below, I agree to participate fully in this study. I understand that the
research may involve genetic studies. Any questions concerning this study and my rights as a
participant have been answered by the study staff. A second copy of this consent form has
been given to me for my own records. My signature below means that I freely agree to
participate in this study.

Subject (Print Name)

Signature

Date

Study Representative Statement:
I have explained the purpose of the research, the study procedures, the possible risks and
discomforts, the possible benefits, and have answered any questions to the best of my ability.

Study Representative Name (Print)

Date

Signature

Subject's Initials:

Date:

University of Massachusetts Amherst IRB
(413) 545-4668
Approval Date
Valid Through
IRB Signature

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APPENDIX C:

CLINIC FORM

Today's date:

Month - Day - Year

Height: ________ inches

Weight: ________ pounds

Waist circumference: ________ inches

_______ centimeters

Blood Pressure:
Systolic: ________ mmHg
Diastolic: ________ mmHg

Glucose level: ________ mg/dL
Hemoglobin level: ________ g/dL

Provided blood sample?  □ yes  □ no

Provided urine sample?  □ yes  □ no

Would consent to/interest in DXA scan:  □ yes  □ no

Date of last menstrual period:

Month - Day - Year

Anticipated start date of next menstrual period:

Month - Day - Year

Ask at follow-up:

Actual date of start of next menstrual period:

Month - Day - Year
## APPENDIX D:

### FOOD FREQUENCY QUESTIONNAIRE

**DIET ASSESSMENT**

1. Do you currently take multiple vitamins? Please report individual vitamins unless question 2.
   - [ ] No
   - [ ] Yes
     - How many per week?
       - [ ] 2 or less
       - [ ] 3-5
       - [ ] 6 or more
   - [ ] Ask/Refer

2. Not counting multiple vitamins, do you take any of the following preparations?
   - [ ] Vitamin A?
     - [ ] No
     - [ ] Yes, seasonal only
     - [ ] Yes, most months
     - How many years?
       - [ ] 0-1 yr
       - [ ] 2-4 yrs
       - [ ] 5-8 yrs
       - [ ] 9-10 yrs
       - [ ] 10 yrs or more
     - What dose per day?
       - [ ] Less than 500 IU
       - [ ] 500 to 1,250 IU
       - [ ] 1,250 to 2,500 IU
       - [ ] 2,500 IU or more
       - [ ] Don’t know
   - [ ] Vitamin C?
     - [ ] No
     - [ ] Yes, seasonal only
     - [ ] Yes, most months
     - How many years?
       - [ ] 0-1 yr
       - [ ] 2-4 yrs
       - [ ] 5-8 yrs
       - [ ] 9-10 yrs
       - [ ] 10 yrs or more
     - What dose per day?
       - [ ] Less than 100 mg
       - [ ] 100 mg to 250 mg
       - [ ] 250 mg to 500 mg
       - [ ] 500 mg or more
       - [ ] Don’t know
   - [ ] Vitamin B6?
     - [ ] No
     - [ ] Yes
     - How many years?
       - [ ] 0-1 yr
       - [ ] 2-4 yrs
       - [ ] 5-8 yrs
       - [ ] 9-10 yrs
       - [ ] 10 yrs or more
     - What dose per day?
       - [ ] Less than 10 mg
       - [ ] 10 mg to 19 mg
       - [ ] 20 mg or more
       - [ ] Don’t know
   - [ ] Vitamin E?
     - [ ] No
     - [ ] Yes
     - How many years?
       - [ ] 0-1 yr
       - [ ] 2-4 yrs
       - [ ] 5-8 yrs
       - [ ] 9-10 yrs
       - [ ] 10 yrs or more
     - What dose per day?
       - [ ] Less than 100 IU
       - [ ] 100 IU to 200 IU
       - [ ] 200 IU to 400 IU
       - [ ] 400 IU or more
       - [ ] Don’t know
   - [ ] Selenium?
     - [ ] No
     - [ ] Yes
     - How many years?
       - [ ] 0-1 yr
       - [ ] 2-4 yrs
       - [ ] 5-8 yrs
       - [ ] 9-10 yrs
       - [ ] 10 yrs or more
     - What dose per day?
       - [ ] Less than 80 mcg
       - [ ] 81 mcg to 120 mcg
       - [ ] 121 mcg or more
       - [ ] Don’t know
   - [ ] Iron?
     - [ ] No
     - [ ] Yes
     - How many years?
       - [ ] 0-1 yr
       - [ ] 2-4 yrs
       - [ ] 5-8 yrs
       - [ ] 9-10 yrs
       - [ ] 10 yrs or more
     - What dose per day?
       - [ ] Less than 10 mg
       - [ ] 10 mg or more
       - [ ] Don’t know
   - [ ] Zinc?
     - [ ] No
     - [ ] Yes
     - How many years?
       - [ ] 0-1 yr
       - [ ] 2-4 yrs
       - [ ] 5-8 yrs
       - [ ] 9-10 yrs
       - [ ] 10 yrs or more
     - What dose per day?
       - [ ] Less than 15 mg
       - [ ] 15 mg or more
       - [ ] Don’t know
   - [ ] Calcium?
     - [ ] No
     - [ ] Yes
     - How many years?
       - [ ] 0-1 yr
       - [ ] 2-4 yrs
       - [ ] 5-8 yrs
       - [ ] 9-10 yrs
       - [ ] 10 yrs or more
     - What dose per day?
       - [ ] Less than 400 mg
       - [ ] 400 mg or more
       - [ ] Don’t know
   - Are there other supplements that you take on a regular basis? Please mark if yes:
     - [ ] Fish oil
     - [ ] Cod liver oil
     - [ ] Vitamin D
     - [ ] B Complex
     - [ ] Omega 3
     - [ ] Antioxidants
     - [ ] 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 three months.

<table>
<thead>
<tr>
<th>DAIRY FOODS</th>
<th>Never</th>
<th>1-3 per week</th>
<th>1 per week</th>
<th>2-4 per week</th>
<th>5-6 per week</th>
<th>1 per day</th>
<th>2-3 per day</th>
<th>4-6 per day</th>
<th>6+ per day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skim or low-fat milk (8 oz glass)</td>
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<tr>
<td>Whole milk (8 oz glass)</td>
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<tr>
<td>Cream, e.g., coffee, whip cream (8 oz)</td>
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<tr>
<td>Sour cream (8 oz)</td>
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<tr>
<td>Other dairy foods, etc</td>
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<tr>
<td>Ice cream (1/2 cup)</td>
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<tr>
<td>Yogurt (1 cup)</td>
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<tr>
<td>Cottage cheese (1/2 cup)</td>
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<tr>
<td>Cheese (1 oz)</td>
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</tbody>
</table>

Please turn to page 2
### PRUITS

<table>
<thead>
<tr>
<th>Food Description</th>
<th>1 per month</th>
<th>1 per week</th>
<th>2-4 per week</th>
<th>5-6 per week</th>
<th>1 per day</th>
<th>2-3 per day</th>
<th>4-5 per day</th>
<th>6+ per day</th>
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</thead>
<tbody>
<tr>
<td>Raisins (1 oz. or small pack) or grapes</td>
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<tr>
<td>Prunes (1/4 cup)</td>
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<tr>
<td>Bananas (1)</td>
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<tr>
<td>Cantaloupe (1/4 melon)</td>
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<tr>
<td>Watermelon (1 slice)</td>
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<tr>
<td>Fresh apples or pears (1)</td>
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<tr>
<td>Apple juice or cider (small glass)</td>
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<tr>
<td>Oranges (1)</td>
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<tr>
<td>Orange juice (small glass)</td>
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<tr>
<td>Grapefruit (1/4)</td>
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<tr>
<td>Grapefruit juice (small glass)</td>
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<tr>
<td>Other fruit juices (small glass)</td>
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<tr>
<td>Strawberries, fresh, frozen or canned (1/3 cup)</td>
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<td>Blueberries, fresh, frozen or canned (1/3 cup)</td>
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<tr>
<td>Peaches, apricots or plums (1 fresh; or 1/2 cup canned)</td>
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</tbody>
</table>

### VEGETABLES

<table>
<thead>
<tr>
<th>Food Description</th>
<th>1 per month</th>
<th>1 per week</th>
<th>2-4 per week</th>
<th>5-6 per week</th>
<th>1 per day</th>
<th>2-3 per day</th>
<th>4-5 per day</th>
<th>6+ per day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tomatoes (1)</td>
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<tr>
<td>Tomato juice (small glass)</td>
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<tr>
<td>Tomato sauce (1/3 cup; e.g., spaghetti sauce)</td>
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<tr>
<td>Red chili sauce (1 Tbsp)</td>
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<tr>
<td>Tofu or soybeans (3-4 oz.)</td>
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<tr>
<td>String beans (1/3 cup)</td>
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<tr>
<td>Broccoli (1/2 cup)</td>
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<tr>
<td>Cabbage or cole slaw (1/4 cup)</td>
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<tr>
<td>Cauliflower (1 cup)</td>
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<tr>
<td>Brussels sprouts (1/2 cup)</td>
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<tr>
<td>Carrots, raw (1/2 carrot or 2-4 sticks)</td>
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<tr>
<td>Carrots, cooked (1/2 cup)</td>
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<tr>
<td>Corn (1 ear or 1/2 cup frozen or canned)</td>
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<tr>
<td>Peas, or lima beans (1/2 cup fresh; frozen, canned)</td>
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<tr>
<td>Mixed vegetables (1/2 cup)</td>
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<tr>
<td>Beans or lentils, baked or dried (1/2 cup)</td>
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<tr>
<td>Butternut (winter) squash (1/2 cup)</td>
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<tr>
<td>Eggplant, zucchini, or other summer squash (1/2 cup)</td>
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<tr>
<td>Yams or sweet potatoes (1/2 cup)</td>
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<tr>
<td>Spinach, cooked (1/4 cup)</td>
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<tr>
<td>Spinach, raw as in salad</td>
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<tr>
<td>Kale, mustard or chard greens (1/4 cup)</td>
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<tr>
<td>Iceberg or head lettuce (servings)</td>
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<tr>
<td>Romaine or leaf lettuce (servings)</td>
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<tr>
<td>Celery (4&quot; stick)</td>
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<tr>
<td>Beets (1/4 cup)</td>
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<tr>
<td>Alfalfa sprouts (1/4 cup)</td>
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<tr>
<td>Garlic, fresh or powdered (1 clove or shake)</td>
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</tbody>
</table>

### EGGS, MEAT, ETC.

<table>
<thead>
<tr>
<th>Food Description</th>
<th>1 per month</th>
<th>1 per week</th>
<th>2-4 per week</th>
<th>5-6 per week</th>
<th>1 per day</th>
<th>2-3 per day</th>
<th>4-5 per day</th>
<th>6+ per day</th>
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</thead>
<tbody>
<tr>
<td>Eggs (1)</td>
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<tr>
<td>Chicken or turkey, with skin (4-6 oz.)</td>
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<tr>
<td>Chicken or turkey, without skin (4-6 oz.)</td>
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<tr>
<td>Bacon (2 slices)</td>
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<tr>
<td>Hot dogs (1)</td>
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</table>

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

<table>
<thead>
<tr>
<th>MCATS (CONTINUED)</th>
<th>Never, or less, than once per month</th>
<th>1-3 per mo</th>
<th>1 per week</th>
<th>2-4 per week</th>
<th>5-6 per week</th>
<th>1 per day</th>
<th>2-3 per day</th>
<th>4-5 per day</th>
<th>6+ per day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pouched meat, e.g., sausage, salami, bologna, etc. (piece or slice)</td>
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<tr>
<td>Liver (3-4 oz.)</td>
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<tr>
<td>Hamburger (1 patty)</td>
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<tr>
<td>Beef, pork, or lamb as a sandwich or mixed dish, e.g., stew, casserole, lasagne, etc.</td>
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<tr>
<td>Beef, pork, or lamb as a main dish, e.g., steak, roast, ham, etc. (4-6 oz.)</td>
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<tr>
<td>Canned tuna fish (C-4 info)</td>
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<tr>
<td>Dark meat fish, e.g., mackerel, salmon, sardines bluefish, swordfish (3-5 oz.)</td>
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<tr>
<td>Other fish (3-5 oz.)</td>
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<tr>
<td>Shrimp, lobster, scallops as a main dish</td>
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</tbody>
</table>

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

<table>
<thead>
<tr>
<th>CARBONATED BEVERAGES</th>
<th>Never, or less, than once per month</th>
<th>1-3 per mo</th>
<th>1 per week</th>
<th>2-4 per week</th>
<th>5-6 per week</th>
<th>1 per day</th>
<th>2-3 per day</th>
<th>4-5 per day</th>
<th>6+ per day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low Calorie (sugar-free) types</td>
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<tr>
<td>Low calorie cola, e.g., 7-Up, diet 7-Up</td>
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<tr>
<td>Low calorie caffeine-free cola, e.g., Pepsi Free</td>
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<tr>
<td>Other low calorie carbonated beverage, e.g., Fresca, Diet 7-Up, diet ginger ale</td>
<td></td>
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<tr>
<td>Coke, Pepsi, or other cola with sugar</td>
<td></td>
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<tr>
<td>Caffeine Free Coke, Pepsi, or other cola with sugar</td>
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<tr>
<td>Other carbonated beverage with sugar, e.g., 7-Up, ginger ale</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>OTHER BEVERAGES</th>
<th>Never, or less, than once per month</th>
<th>1-3 per mo</th>
<th>1 per week</th>
<th>2-4 per week</th>
<th>5-6 per week</th>
<th>1 per day</th>
<th>2-3 per day</th>
<th>4-5 per day</th>
<th>6+ per day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hawaiian Punch, lemonade, or other non-carbonated fruit drinks (1 glass, bottle, can)</td>
<td></td>
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<tr>
<td>Decaffeinated coffee (1 cup)</td>
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<tr>
<td>Coffee (1 cup)</td>
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<td></td>
<td></td>
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<tr>
<td>Tea (1 cup), not herbal teas</td>
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<td>Beer (1 glass, bottle, can)</td>
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<tr>
<td>Red wine (4 oz. glass)</td>
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<tr>
<td>White wine (4 oz. glass)</td>
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<tr>
<td>Liquor, e.g., whiskey, gin, etc. (1 drink or shot)</td>
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</tbody>
</table>

Please turn to page 4
ID:

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

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

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 none
- (Don't eat meat)

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

6. What kind of fat do you usually use for baking?
- Real butter
- Margarine

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
- 1-3 times per week
- 4-6 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
- 1-3 times per week
- 4-6 times per week
- Less than once a week

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

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.

<table>
<thead>
<tr>
<th>Other foods that you usually eat at least once per week</th>
<th>Usual serving size</th>
<th>Servings per week</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin D-fortified orange juice</td>
<td></td>
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<tr>
<td>Soy products</td>
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</tbody>
</table>
Thank you for participating in the UMass Vitamin D Study!

Please provide the requested information. Please write clearly and fill in the bubbles completely. If you have questions please ask the study staff for assistance.

NOTE: All responses will be kept completely confidential, and your name will not be associated with the information you provide in this questionnaire.

1. What is your date of birth?
   Month:   Day:   Year:
   [ ] - [ ] - [19] [ ]

2. Do you consider yourself to be Spanish/Hispanic/Latina?  [ ] Yes  [ ] No

3. Which category(ies) best describes your race? (Mark one or more as appropriate)
   [ ] white
   [ ] black or African American
   [ ] Asian
   [ ] American Indian/Native American
   [ ] Native Hawaiian or Pacific Islander
   [ ] other

4. What is your highest level of education?  [ ] some high school
   [ ] high school diploma
   [ ] some college/currently enrolled in college
   [ ] college degree
   [ ] some graduate school
   [ ] graduate degree
5. Which of the following best describes your father/guardian’s occupation when you were in middle/high school?
   - lawyer, medical doctor, scientist, engineer, college professor or teacher
   - executive, manager or administrator
   - sales or clerical work
   - mechanic, electrician, repairer or craft worker (e.g., carpenter)
   - service worker (e.g., janitor, guard)
   - laborer, handler, equipment cleaner or helper
   - farming
   - military
   - homemaker, stay at home parent
   - did not work
   - don’t know/not in contact with father
   - other →

6. Which of the following best describes your mother/guardian’s occupation when you were in middle/high school?
   - lawyer, medical doctor, scientist, engineer, college professor or teacher
   - executive, manager or administrator
   - sales or clerical work
   - mechanic, electrician, repairer or craft worker (e.g., carpenter)
   - service worker (e.g., janitor, guard)
   - laborer, handler, equipment cleaner or helper
   - farming
   - military
   - homemaker, stay at home parent
   - did not work
   - don’t know/not in contact with mother
   - other →

7. What time did you go to bed last night?  
   Hour:  
   Minutes  
   ○ AM  ○ PM

8. What time did you get out of bed this morning?  
   Hour:  
   Minutes

9. Have you had anything to eat today, not counting water or coffee?  
   ○ yes  ○ no

10. When did you last eat?  
    Hour:  
    Minutes  
    ○ AM  ○ PM
11. At what age did your menstrual periods begin?
   - <= 9 years old
   - 10 years old
   - 11 years old
   - 12 years old
   - 13 years old
   - 14 years old
   - 15 years old
   - 16 years old
   - 17 years old
   - >= 17 years old

12. How many years after the onset of your menstrual periods did your cycles become regular?
   - < 1 year
   - 1-2 years
   - 3-4 years
   - >= 5 years
   - never

13. What is the current usual length of your menstrual cycle? (i.e., interval from first day of period to first day of next period)
   - < 21 days
   - 21-25 days
   - 26-30 days
   - 31-35 days
   - 36-39 days
   - 40-45 days
   - 46-50 days
   - > 50 days/too irregular to estimate

14. During your period, how many days do you generally have bleeding?
   - <= 3 days
   - 4-5 days
   - 6-7 days
   - >= 8 days

15. What is the current usual pattern of your menstrual cycles?
   - Extremely regular (no more than 1-2 days before or after expected)
   - Very regular (within 3-4 days)
   - Regular (within 5-7 days)
   - Usually irregular
   - Always irregular

16. Have you ever been pregnant? Yes No

17. a. Have you ever used oral contraceptives? Yes No
   - Yes No

18. Are you currently using any of the following? (Mark all that apply)
   - Contraceptive implants
   - Depo-Provera/Injectible contraceptives
   - Intrauterine device/IUD
   - None of the above
19. For each symptom listed below, please indicate whether you experience it most months of the year, for at least several days before your menstrual period begins. Don’t include symptoms that you experience throughout your entire menstrual cycle, or symptoms that start when your period starts. For symptoms you do experience, please indicate the usual severity of each (i.e., mild, moderate or severe).

<table>
<thead>
<tr>
<th>Symptom</th>
<th>Not at all</th>
<th>Mild</th>
<th>Moderate</th>
<th>Severe</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abdominal bloating</td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
</tr>
<tr>
<td>Breast tenderness</td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
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<tr>
<td>Distress</td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
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<tr>
<td>Headache</td>
<td>○</td>
<td>○</td>
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<tr>
<td>Hot flashes</td>
<td>○</td>
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<tr>
<td>Nausea</td>
<td>○</td>
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<td>○</td>
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<tr>
<td>Swelling in extremities</td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
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<tr>
<td>Acne</td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
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<tr>
<td>Diarrhea/constipation</td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
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<tr>
<td>Food cravings</td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
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<tr>
<td>Palpitations</td>
<td>○</td>
<td>○</td>
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<td>○</td>
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<tr>
<td>Anxiety/nervousness</td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
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<tr>
<td>Increased/decreased appetite</td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
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<tr>
<td>Irritability</td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
</tr>
<tr>
<td>Emotional hypersensitivity</td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
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<tr>
<td>Fatigue</td>
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<tr>
<td>Mood swings</td>
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<tr>
<td>Tendency to cry easily</td>
<td>○</td>
<td>○</td>
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<tr>
<td>Insomnia</td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
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<tr>
<td>Angry outbursts</td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
</tr>
<tr>
<td>Desire to be alone</td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
</tr>
<tr>
<td>Depression</td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
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<tr>
<td>Confusion</td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
</tr>
<tr>
<td>Forgetfulness</td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
</tr>
<tr>
<td>Abdominal cramping</td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
</tr>
<tr>
<td>Lower back pain</td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
</tr>
</tbody>
</table>

Other:。

Other:。

Severity of “other” symptom(s): ○ ○ ○ ○

If you do not experience any of these symptoms (i.e., you marked “not at all” for each) skip to question 27 on page 6.
20. How would you describe the overall severity of your symptoms together?
   - ○ minimal (no effect on my normal activities)
   - ○ mild (noticeable, but not troublesome)
   - ○ moderate (interferes with my normal activities)
   - ○ severe (intolerable, prevents my normal activities)

21. How many days before the first day of your period do your symptoms usually begin?
   Please write in number of days (ex. 05): [ ]

22. How many days do your symptoms last after your period begins?
   Please write in number of days (ex. 05): [ ]

23. In the week after your menstrual period had stopped, which of the following statements best described your symptoms?
   - ○ My symptoms are completely absent
   - ○ My symptoms are still present but are less severe than before my period
   - ○ My symptoms are present and are as severe as before my period

24. At approximately what age did most of these symptoms begin?
   Please write in age: [ ]

25. Have you seen a health care provider because of these symptoms?
   - ○ yes  ○ no

26. Do you experience any of the following because of your menstrual symptoms? For problems you experience, please indicate the severity (i.e., mild, moderate or severe).

<table>
<thead>
<tr>
<th>Problem</th>
<th>Not a problem</th>
<th>Mild</th>
<th>Moderate</th>
<th>Severe</th>
</tr>
</thead>
<tbody>
<tr>
<td>Relationship discord with family or partner</td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
</tr>
<tr>
<td>Relationship discord with friends or coworkers</td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
</tr>
<tr>
<td>Poor work performance/attendance</td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
</tr>
<tr>
<td>Social isolation</td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
</tr>
<tr>
<td>Suicidal thoughts</td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
</tr>
</tbody>
</table>
27. Please indicate if you are experiencing any of the following symptoms today. If so, please indicate how severe the symptom is today.

<table>
<thead>
<tr>
<th>Symptom</th>
<th>Not at all</th>
<th>Mild</th>
<th>Moderate</th>
<th>Severe</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abdominal bloating</td>
<td>o</td>
<td>o</td>
<td>o</td>
<td>o</td>
</tr>
<tr>
<td>Breast tenderness</td>
<td>o</td>
<td>o</td>
<td>o</td>
<td>o</td>
</tr>
<tr>
<td>Dizziness</td>
<td>o</td>
<td>o</td>
<td>o</td>
<td>o</td>
</tr>
<tr>
<td>Headache</td>
<td>o</td>
<td>o</td>
<td>o</td>
<td>o</td>
</tr>
<tr>
<td>Hot flashes</td>
<td>o</td>
<td>o</td>
<td>o</td>
<td>o</td>
</tr>
<tr>
<td>Nausea</td>
<td>o</td>
<td>o</td>
<td>o</td>
<td>o</td>
</tr>
<tr>
<td>Swelling in extremities</td>
<td>o</td>
<td>o</td>
<td>o</td>
<td>o</td>
</tr>
<tr>
<td>Acne</td>
<td>o</td>
<td>o</td>
<td>o</td>
<td>o</td>
</tr>
<tr>
<td>Diarrhea/constipation</td>
<td>o</td>
<td>o</td>
<td>o</td>
<td>o</td>
</tr>
<tr>
<td>Food cravings</td>
<td>o</td>
<td>o</td>
<td>o</td>
<td>o</td>
</tr>
<tr>
<td>Palpitations</td>
<td>o</td>
<td>o</td>
<td>o</td>
<td>o</td>
</tr>
<tr>
<td>Anxiety/nervousness</td>
<td>o</td>
<td>o</td>
<td>o</td>
<td>o</td>
</tr>
<tr>
<td>Increased/decreased appetite</td>
<td>o</td>
<td>o</td>
<td>o</td>
<td>o</td>
</tr>
<tr>
<td>Irritability</td>
<td>o</td>
<td>o</td>
<td>o</td>
<td>o</td>
</tr>
<tr>
<td>Emotional hypersensitivity</td>
<td>o</td>
<td>o</td>
<td>o</td>
<td>o</td>
</tr>
<tr>
<td>Fatigue</td>
<td>o</td>
<td>o</td>
<td>o</td>
<td>o</td>
</tr>
<tr>
<td>Mood swings</td>
<td>o</td>
<td>o</td>
<td>o</td>
<td>o</td>
</tr>
<tr>
<td>Tendency to cry easily</td>
<td>o</td>
<td>o</td>
<td>o</td>
<td>o</td>
</tr>
<tr>
<td>Insomnia</td>
<td>o</td>
<td>o</td>
<td>o</td>
<td>o</td>
</tr>
<tr>
<td>Angry outbursts</td>
<td>o</td>
<td>o</td>
<td>o</td>
<td>o</td>
</tr>
<tr>
<td>Desire to be alone</td>
<td>o</td>
<td>o</td>
<td>o</td>
<td>o</td>
</tr>
<tr>
<td>Depression</td>
<td>o</td>
<td>o</td>
<td>o</td>
<td>o</td>
</tr>
<tr>
<td>Confusion</td>
<td>o</td>
<td>o</td>
<td>o</td>
<td>o</td>
</tr>
<tr>
<td>Forgetfulness</td>
<td>o</td>
<td>o</td>
<td>o</td>
<td>o</td>
</tr>
<tr>
<td>Abdominal cramping</td>
<td>o</td>
<td>o</td>
<td>o</td>
<td>o</td>
</tr>
<tr>
<td>Lower back pain</td>
<td>o</td>
<td>o</td>
<td>o</td>
<td>o</td>
</tr>
</tbody>
</table>

Other:

Severity of "other" symptom(s): o o o o o

If you do not experience any of these symptoms (i.e., you marked "not at all" for each) skip to question 29 on page 7.
28. How would you describe the overall severity of all your symptoms together today?
   ○ minimal (no effect on normal activities)
   ○ mild (noticeable, but not troublesome)
   ○ moderate (interferes with normal activities)
   ○ severe (intolerable, prevents normal activities)

29. a. Have you ever been diagnosed by a clinician with Premenstrual Syndrome (PMS)?
   ○ no    ○ yes

   b. If yes, did your clinician have you keep a prospective record of your symptoms for at least one menstrual cycle (i.e., a “chart,” calendar or daily record?)
   ○ no    ○ yes → If yes, how many cycles were recorded? □ cycles

30. Do you currently do any one of the following to prevent or treat your symptoms? (mark all that apply)
   ○ no symptoms    ○ do yoga/meditation
   ○ take hot baths    ○ increase exercise level
   ○ drink alcohol    ○ sleep more
   ○ change your diet    ○ take medication

   If you take medication, please indicate what type(s) you currently use:

31. During the past month, what was your average time per week spent at each of the following recreational activities?

<table>
<thead>
<tr>
<th>Time per week</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zero</td>
</tr>
<tr>
<td>Walking or hiking outdoors</td>
</tr>
<tr>
<td>Jogging outdoors or on a treadmill</td>
</tr>
<tr>
<td>Running outdoors or on a treadmill</td>
</tr>
<tr>
<td>Bicycling/using a stationary bike</td>
</tr>
<tr>
<td>Aerobics/dance/moving machine</td>
</tr>
<tr>
<td>Tennis, squash or racket sports</td>
</tr>
<tr>
<td>Lap swimming</td>
</tr>
<tr>
<td>Other aerobic activity such as martial arts or lawn moving</td>
</tr>
<tr>
<td>Yoga or pilates</td>
</tr>
<tr>
<td>Weight training or resistance exercises</td>
</tr>
</tbody>
</table>
32. What is your usual walking pace outdoors?
   ○ easy, casual (less than 2 miles per hour)
   ○ normal (2 to 2.5 miles per hour)
   ○ brisk pace (2 to 3.5 miles per hour)
   ○ very brisk/striding (4 miles per hour or faster)
   ○ unable to walk

33. How many flights of stairs (not individual steps) do you climb daily?
   ○ 2 flights or less   ○ 10-14 flights
   ○ 3-4 flights        ○ 15 or more flights
   ○ 5-8 flights        ○ unable to walk

34. On average, how much time per week do you spend at the following?

<table>
<thead>
<tr>
<th>Time per week</th>
<th>ZERO</th>
<th>ONE</th>
<th>2-5</th>
<th>6-10</th>
<th>11-20</th>
<th>21-40</th>
<th>41-60</th>
<th>61-80</th>
<th>Over 90</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standing or walking around at school or work</td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
</tr>
<tr>
<td>Standing or walking around at home</td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
</tr>
<tr>
<td>Sitting while at the computer, in class, work or driving</td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
</tr>
<tr>
<td>Sitting while reading, talking or eating</td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
</tr>
<tr>
<td>Sitting watching TV</td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
</tr>
</tbody>
</table>

35. Have you smoked 20 or more packs of cigarettes in your lifetime?   ○ yes   ○ no

36. Do you currently smoke?   ○ yes   ○ no

37. If you currently smoke:
   What specific brand and type of cigarettes do you smoke? (e.g., Marlboro Lights 100’s)
   Please enter the brand and type below (leave blank if not a current smoker):

38. If you currently smoke, how many cigarettes do you smoke per day?
   ○ 1-10   ○ 21-40
   ○ 11-20   ○ 41 or more
   ○ 21-30

39. If you ever have smoked (current smoker or quit), at what age did you start smoking?
   Enter age:  □  □

40. If you have quit smoking, at what age did you quit?
   Enter age:  □  □
41. Does anyone smoke in the household where you currently live?  ○ yes  ○ no
   If yes, approximately how many cigarettes are smoked by each member of your household?
   Smoker 1:  ○ 1-10  ○ 11-20  ○ 21-30  ○ 31-40  ○ 41 or more
   Smoker 2:  ○ 1-10  ○ 11-20  ○ 21-30  ○ 31-40  ○ 41 or more
   Smoker 3:  ○ 1-10  ○ 11-20  ○ 21-30  ○ 31-40  ○ 41 or more

42. How often are you exposed to cigarette smoke for 1 or more hours at a time at places other than home (i.e., work, social situations)?
   ○ never
   ○ less than once per week
   ○ 1-3 times per week
   ○ 4-6 times per week
   ○ daily

43. Do you smoke marijuana?  ○ no  ○ yes
   If yes, how often?
   ○ less than once per month
   ○ 1-3 times per month
   ○ 4-6 times per week
   ○ daily

NOTE: All responses will be kept completely confidential.

44. Do you use any of the following once per month or more? (Indicate all that apply)
   ○ ecstasy  ○ heroin
   ○ crystal meth  ○ mushrooms
   ○ cocaine  ○ None of the above
   ○ LSD

The next questions are about drinking alcoholic beverages. Included are liquor (such as whiskey or gin), beer, wine, wine coolers, and any other type of alcoholic beverage. One drink is equal to a 12oz. beer, a 5oz. glass of wine, or one and a half ounces of liquor.

45. In your entire life, have you had at least 12 drinks of any type of alcoholic beverage?
   ○ Yes  ○ No  SKIP TO QUESTION 81

46. In any one year, have you had at least 12 drinks of any type of alcoholic beverages?
   ○ Yes  ○ No

47. In the past 6 months, how often did you drink any type of alcoholic beverage?
   Example: If you drink 3 days a week, please write in the number "3" and
   bubble in "per week"
   ○ Per week
   ○ Per month
   ○ Per year

48. In the past 6 months, on those days that you drank alcoholic beverages, on the average, how many drinks did you have? If you drank less than 1 drink, enter a 1.
   ○ drinks
43. In the past 6 months, on how many days did you have 5 or more drinks of any alcoholic beverage? Example: If you have 5 or more drinks/day twice a month, please write in the number 2 and bubble in "per month"

○ Per week
○ Per times
○ Per month
○ Per year

50. Was there ever a time in your life when you drank 5 or more drinks of any kind of alcoholic beverage almost every day?

○ Yes  ○ No

51. Have you ever had any of the following clinician-diagnosed illnesses?
(Mark all that apply)

○ Lactose intolerance
○ Depression (unipolar depression)
○ Bipolar disorder (manic depressive illness)
○ Endometriosis
○ Uterine fibroids
○ None of the above

52. Are you currently taking any of the following medications? (Mark all that apply)

○ Not taking any medications
○ Selective serotonin reuptake inhibitors/SSRIs (Prozac, Zoloft, Paxil, Effexor, etc.)
○ Other antidepressants (Elavil, Wellbutrin, MAOIs such as Parnate and Nardil, etc.)
○ Tranquilizers (Valium, Thorazine, Xanax, BuSpar, etc.)
○ Lithium
○ Migraine prevention (Imitrex, etc.)
○ Antacids (Tums, Rolaid's, etc.)
○ Other medications (please specify below)

53. Have you ever had wheezing or whistling in the chest at any time in the past?

○ Yes  ○ No  If you answered "No" please skip to question 55

54. a. Have you had wheezing or whistling in the chest in the last 12 months?

○ Yes  ○ No  If you answered "No" please skip to question 55

b. How many attacks of wheezing have you had in the last 12 months?

○ None  ○ 1 to 11
○ 1 to 3  ○ More than 12

c. In the last 12 months, how often, on average, has your sleep been disturbed due to wheezing?

○ Never woken with wheezing
○ Less than one night a week
○ One or more nights per week

54. d. In the last 12 months, has wheezing ever been severe enough to limit your speech to only one or two words between breaths?

○ Yes  ○ No
55. Have you ever had asthma?
   ○ yes  ○ no

56. In the last 12 months, has your chest sounded wheezy during or after exercise?
   ○ yes  ○ no

57. In the last 12 months, have you had a dry cough at night, apart from a cough associated with a cold or chest infection?
   ○ yes  ○ no

58. a. Have you ever had an itchy rash which was coming and going for at least 6 months?
   ○ yes  ○ no  If you answered "no" please skip to question 59

   b. Have you had this itchy rash at any time in the last 12 months?
   ○ yes  ○ no  If you answered "no" please skip to question 59

   c. Has this itchy rash at any time affected any of the following places: the folds of the elbows, behind the knees, in front of the ankles, under the buttocks, or around the neck, ears or eyes?
   ○ yes  ○ no

   d. Has this rash cleared up completely at any time during the last 12 months?
   ○ yes  ○ no

   e. In the last 12 months, how often, on average, have you been kept awake at night by this itchy rash?
   ○ Never in the last 12 months
   ○ Less than one night a week
   ○ One or more nights per week

59. Have you ever had eczema?
   ○ yes  ○ no

60. Have you lost or gained more than 10 pounds in the last 2 months?
   ○ no
   ○ yes, lost >= 10 pounds
   ○ yes, gained >= 10 pounds

61. How often do you eat organic foods such as fruits, vegetables, meats and/or dairy products?
   ○ never  ○ rarely  ○ occasionally  ○ often  ○ always

62. In the past week, how many hours per day on average have you spent outdoors wearing minimal clothing? (i.e., shorts and a T-shirt/tank top)
   ○ 0 hours  ○ 3-4 hours
   ○ 1-2 hours  ○ >= 5 hours

63. Over the past summer, how many hours per day on average did you spend outdoors wearing minimal clothing?
   ○ 0 hours  ○ 3-4 hours
   ○ 1-2 hours  ○ >= 5 hours
64. Do you regularly wear sunscreen? ○ no ○ yes
   If yes, what SPF do you usually use?
   ○ less than 15 ○ 30 to 39
   ○ 15 to 19 ○ 40 or higher
   ○ 20 to 29

65. In the past 3 months, have you traveled to a “sunny” location? ○ no ○ yes
   If yes, where?
   If yes, how many days were you there? ____________ days

66. In the past 6 months, have you used a tanning bed or tanning booth? ○ no ○ yes
   If yes, how often?
   ○ more than once a week
   ○ weekly
   ○ bi-weekly (every two weeks)
   ○ monthly
   ○ bi-monthly (every two months)
   ○ only once or twice

67. On an average weekday, how many hours do you sleep per night?
   ○ <= 3 hours ○ 4-5 hours ○ >= 10 hours ○ 6-7 hours

68. On an average weekend day, how many hours do you sleep per night?
   ○ <= 3 hours ○ 4-5 hours ○ >= 10 hours ○ 6-7 hours

The following questions regard YOUR infancy (feel free to call a parent if you need to):

69. Were you breastfed as a baby?
   ○ Yes ____________ If yes, for how many months were you breastfed?
   ○ unknown ○ 3 months or less
   ○ Not sure ○ 4 to 6 months
   ○ 6 months or more

70. What was your birth weight in pounds?
   ○ not sure ○ 7.0 to 8.4 pounds
   ○ less than 5.5 pounds ○ 8.5 to 9.9 pounds
   ○ 5.5 to 6.9 pounds ○ 10 pounds or more

71. Were you (please answer all that apply):
   ○ full term (not premature)
   ○ 2 or more weeks premature
   ○ a twin, triplet, etc.
72. In the past six months, have you been treated by a healthcare provider or have you treated yourself for any of the following reproductive tract infections? Please mark all that apply:

- Bacterial vaginosis (BV)
- Genital warts
- Yeast
- Genital herpes
- Chlamydia
- Trichomoniasis
- Gonorrhea
- Not sure
- Syphilis
- None of the above (skip to question 73)

If you received treatment, what medication(s) did you receive? Mark all that apply:

- Over the counter creams or suppositories (examples: Monistat-7, Vagistat, Femstat)
- Metronidazole (Flagyl)
- Clindamycin (Cleocin)
- Penicillin pills
- Penicillin shot
- Doxycycline
- Zovirax
- Don’t know/ can’t remember
- None of the above

If you received treatment, when were you treated?

- One month ago or less
- 2 to 3 months ago
- 4 to 6 months ago

73. If you have not received treatment for a reproductive tract infection, please indicate whether you have experienced any of the following symptoms within the past six months:

- Vaginal itching
- Vaginal burning
- Vaginal ulcer
- Out of the ordinary (or unusual) vaginal discharge
- None of the above

If yes, was the discharge:

- Gray-white, bad-smelling
- Yellow-green, frothy
- White, no odor

74. In the past 6 months, have you been treated for a urinary tract infection (UTI)?

- No
- Yes

If yes, how many urinary tract infections did you have in the last 6 months?

- One
- Two
- Three or more

If yes, when were you last treated?

- One month ago or less
- 2 to 3 months ago
- 4 to 6 months ago

Thank you! Please return questionnaire to study staff.
UNIVERSITY OF MASSACHUSETTS
UNIVERSITY HEALTH SERVICES
150 INFIRMARY WAY AMHERST, MA 01003

Patient: [Redacted] 23.4 years Patient ID: 0406027
Birth Date: 06/16/2006 3:23:54 PM (6.80)
Height / Weight: 70.0 in. 150.0 lbs. Measured: 06/16/2006 3:25:37 PM (6.80)
Sex / Ethnic: Female White Analyzed:

ANCILLARY RESULTS [Total Body]

<table>
<thead>
<tr>
<th>Region</th>
<th>BMD (g/cm²)</th>
<th>Young-Adult (%)</th>
<th>Age-Matched (%)</th>
<th>BMC (g)</th>
<th>Area (cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Head</td>
<td>3.158</td>
<td>-</td>
<td>-</td>
<td>716</td>
<td>227</td>
</tr>
<tr>
<td>Arms</td>
<td>0.863</td>
<td>-</td>
<td>-</td>
<td>378</td>
<td>436</td>
</tr>
<tr>
<td>Legs</td>
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<tr>
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<tr>
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</table>

BODY COMPOSITION

<table>
<thead>
<tr>
<th>Region</th>
<th>Tissue (%Fat)</th>
<th>Region (%Fat)</th>
<th>Tissue (g)</th>
<th>Fat (g)</th>
<th>Lean (g)</th>
<th>BMC (g)</th>
<th>Total Mass (kg)</th>
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<tbody>
<tr>
<td>Left Arm</td>
<td>24.4</td>
<td>23.1</td>
<td>3,203</td>
<td>782</td>
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<td>37.8</td>
<td>12,506</td>
<td>4,934</td>
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<tr>
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<td>28.1</td>
<td>14,144</td>
<td>4,107</td>
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<td>30.0</td>
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<tr>
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<tr>
<td>Right Leg</td>
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<tr>
<td>Right Trunk</td>
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<td>28.1</td>
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</tr>
<tr>
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<td>26.1</td>
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<tr>
<td>Total</td>
<td>31.9</td>
<td>30.4</td>
<td>63,901</td>
<td>20,379</td>
<td>43,522</td>
<td>3,147</td>
<td>67.0</td>
</tr>
</tbody>
</table>

1 - Statistically 98% of repeat scans fall within 1SD (± 0.010 g/cm² for Total Body Total)
2 - USA, Total Body Reference Population, Ages 20-40
3 - Matched for Age, Weight (females 25-100 kg), Ethnic
Filename: zazarm, o0wes0h5, dfb

GE Medical Systems
LUNAR

Prodigy
DF=12467
**UNIVERSITY OF MASSACHUSETTS**
**UNIVERSITY HEALTH SERVICES**
150 INFIRMIARY WAY AMHERST, MA 01003

**Patient ID:** 0406027  
**Physician:** Sofija  
**Measured:** 06/16/2006 3:23:54 PM (6.80)  
**Analyzed:** 06/16/2006 3:25:37 PM (6.80)

---

**Total Body Tissue Quantitation**

![Diagram of human body with tissue quantitation]

**Composition Reference: Total**

<table>
<thead>
<tr>
<th>Tissue (%Fat)</th>
<th>Centile</th>
<th>Weight (kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td>74</td>
</tr>
<tr>
<td>15%</td>
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<tr>
<td>20%</td>
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</tr>
<tr>
<td>25%</td>
<td>10</td>
<td>68</td>
</tr>
<tr>
<td>30%</td>
<td>20</td>
<td>66</td>
</tr>
<tr>
<td>35%</td>
<td>30</td>
<td>68</td>
</tr>
<tr>
<td>40%</td>
<td>40</td>
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<tr>
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<tr>
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<td>90%</td>
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<td>67</td>
</tr>
<tr>
<td>95%</td>
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<td>67</td>
</tr>
<tr>
<td>100%</td>
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<td>67</td>
</tr>
</tbody>
</table>

---

**Region**  
**Tissue (%Fat)**  
**Centile**  
**T.Mass (kg)**  
**Fat (g)**  
**Lean (g)**  
**BMC (g)**

<table>
<thead>
<tr>
<th>Region</th>
<th>Tissue (%Fat)</th>
<th>Centile</th>
<th>T.Mass (kg)</th>
<th>Fat (g)</th>
<th>Lean (g)</th>
<th>BMC (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>31.9</td>
<td>68</td>
<td>67.0</td>
<td>20,379</td>
<td>43,522</td>
<td>3,147</td>
</tr>
</tbody>
</table>

---

**Measured Date**  
**Age (years)**  
**Tissue (%Fat)**  
**Centile**  
**T.Mass (kg)**  
**Fat (g)**  
**Lean (g)**

<table>
<thead>
<tr>
<th>Measured Date</th>
<th>Age (years)</th>
<th>Tissue (%Fat)</th>
<th>Centile</th>
<th>T.Mass (kg)</th>
<th>Fat (g)</th>
<th>Lean (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>06/16/2006</td>
<td>23.3</td>
<td>31.9</td>
<td>68</td>
<td>67.0</td>
<td>20,379</td>
<td>43,522</td>
</tr>
</tbody>
</table>

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Image not for diagnosis
Printed: 05/02/2006 9:50:28 AM (6.80) 76 0.15:153.85:31.2 0.00·1.00  
4.80x13.00 10.4%Fat=31.9%  
0.00·0.00·0.00·0.00  
Filename: zagamem_Hywac6ntk.db  
Scan Mode: Standard

---

**GE Medical Systems**  
**LUNAR**

---

Prodigy  
DF-12497


Rauch, F., Blum, W. F., Klein, K., Alloio, B., & Schonau, E. (1998). Does leptin have an effect on bone in adult women? *Calcified tissue international, 63*(6), 453-455.


