Vitamin D Metabolites in Young Adults of Diverse Ancestry Living in the Greater Toronto Area

by

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Abstract

Vitamin D plays a critical role in bone metabolism and many cellular and immunological processes, and low vitamin D levels have been associated with several chronic and infectious diseases. Previous studies have reported that many otherwise healthy adults of European ancestry living in Canada have low vitamin D concentrations during the wintertime. However, individuals of non-European ancestry are at a higher risk of having low vitamin D levels. This thesis examined vitamin D status in a sample of young adults of diverse ancestry living in the Greater Toronto Area. In my research I found that: 1) vitamin D levels (measured as 25(OH)D concentrations) are low in Canadian young adults, particularly in those of non-European ancestry; 2) vitamin D intakes, which were estimated to be on average higher than current Health Canada recommendations of 200 International Units (IU) per day, were inadequate to maintain optimal vitamin D levels year-round; 3) vitamin D levels undergo large seasonal changes. Winter 25(OH)D concentrations are substantially lower than those observed during the fall; 4) vitamin D intake is an important year-round predictor of 25(OH)D concentrations, but skin pigmentation and sun exposure are also important predictors during the times when UVB is adequate for cutaneous synthesis; and 5) vitamin D binding protein (VDBP) polymorphisms are significant predictors of 25(OH)D concentrations, but their effects vary by ancestry and season, indicating

gene-environment interaction effects. My research shows that higher vitamin D intakes are needed to offset the seasonal drop in vitamin D levels and to ensure adequate vitamin D levels year-round for those at higher risk of insufficiency.

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List of Abbreviations

$1,25(OH)_2D_3$	1,25-dihydroxyvitamin D_3 or Calciferol
1αOHase	$25(OH)D$-1α-hydroxylase or CYP27B1
25OHase	Vitamin D-hydroxylase (includes CYP2R1)
$25(OH)D$	25-hydroxyvitamin D_3 or cholecalciferol
7DHC	7-dehydrocholesterol
AI	Adequate intake
AIM	Antimicrobial peptide
ANOVA	Analysis of variance
Asp	Aspartic acid
BMD	Bone mass density
BMI	Body mass index
CHMS	Canadian Health Measures Survey
CV	Coefficient of Variation
CYP27B1	25-hydroxyvitamin D 1α-hydroxylase
CYP2R1	Vitamin D 25- hydroxylase
EAR	Estimated adequate requirement
FFQ	Food frequency questionnaire
Gc	Group specific component
Glu	Glutamine
IOM	Institute of Medicine
IU	International unit (1 IU vitamin D = 0.025 µg)
LL37	Cathelicidin
Lys	Lysine
MS	Multiple sclerosis

NHANES	National Health and Nutrition Examination Survey
PTH	Parathyroid hormone
RAI	Recommended adequate intake
RDI	Recommended daily intake
RIA	Radioimmunoassay
RXR	Retinoid X receptor
SD	Standard deviation
SE or SEM	Standard error of mean
SNP	Single nucleotide polymorphism
TB	Tuberculosis
Thr	Threonine
TLR	Toll like receptor
UV	Ultraviolet
UVA	Ultraviolet radiation A
UVB	Ultraviolet radiation B
UVR	Ultraviolet radiation
VDBP	Vitamin D binding protein
VDBP	Vitamin D binding protein gene, NCBI gene ID# 2638
VDR	Vitamin D receptor
VDRE	Vitamin D response element

Preface: Thesis Organization

This thesis comprises five chapters; the first chapter is a general introduction, and is followed by three chapters that are written in publication format and a final discussion chapter.

Chapter 1 is a general introduction to vitamin D, including a description of its discovery and potential role in human evolution, a discussion of vitamin D metabolism and assessment of vitamin D levels, an overview of the importance of vitamin D to human health and a summary of vitamin D status in population groups relevant to the research described in this thesis.

Chapter 2 focuses on wintertime vitamin D levels in a sample of young Canadian adults of diverse ancestry living in the Greater Toronto Area. The results are compared with previous studies of vitamin D levels in Canadian adults. I also discuss the major variables affecting wintertime vitamin D levels in this sample. This work has previously been published in *BMC Public Health* (Gozdzik et al. 2008).

Chapter 3 evaluates seasonal variation in vitamin D levels in young adults of East Asian, European and South Asian ancestry. This chapter also reports the relationship between several factors (including skin pigmentation, vitamin D intake, sun exposure, etc.) and vitamin D concentrations. This work has been previously published in the *Journal of Nutrition* (Gozdzik et al., 2010).

Chapter 4 is an examination of two vitamin D binding protein (*VDBP*) genetic polymorphisms and their associations with vitamin D concentrations in a sample of diverse ancestry living in the Greater Toronto Area. The relative contribution of each polymorphism to vitamin D concentration is evaluated in context of the other relevant variables examined. This chapter has been submitted for review.

Finally, Chapter 5 summarizes the general conclusions of this research and its relevance to our knowledge of vitamin D.

Chapter 1

1   Vitamin D: Background and Introduction

1.1 Vitamin D: History and Evolutionary Perspective

1.1.1 The Discovery of Vitamin D

Severe vitamin D deficiency causes rickets in children and osteomalacia in adults (Holick, 2003). It is not surprising, then, that the discovery of vitamin D was tightly linked to the discovery of the causes of the disease rickets. Rickets is a bone disorder in children characterized by low mineralization of the skeleton and results in growth retardation, deformities of the skeleton, bony projection on the rib cages (sometimes called a "rachitic rosary"), and either bowed legs or knocked knees (Carpenter, 2008; Holick, 2004a).

Rickets was recognized as a distinct disease by the middle of the 17th century: two dissertations on the disease where published within years of each other: one by Daniel Whistler in 1645 and another Francis Glisson in 1650 (Rajakumar et al., 2007). During the Industrial Revolution, rickets became an extremely common disease in children living in large cities in northern Europe and North America. Evidence from autopsies carried out in Leiden, Netherlands, at the end of the 19th century found signs of rickets in 96% of infants less than 18 months old (Rajakumar, 2003; Schmorl, 1909). By 1900, 80% of children in Boston were also suffering from rickets (Holick, 2004a).

The causes of rickets, however, were elusive, and several treatments were suggested, one of which was cod liver oil. Cod liver oil had been used as a medicine for rheumatism and as a folk treatment for rickets in Scandinavia and the Netherlands (Carpenter, 2008). The first reported use of cod liver oil as a treatment for rickets was published in 1824 by Schutte (Rajakumar et al., 2007). However, by the beginning of the 20th century, cod liver oil had fallen out of favour, until animal experimentation by Mellanby and McColumm showed the ability of cod liver oil to treat rickets (Carpenter, 2008; Rajakumar et al., 2007). Mellanby proved that he could cure rickets in dogs by feeding them cod liver oil (Holick, 2006b; Mellanby, 1918). However, cod liver oil was already known for preventing night blindness (attributed to vitamin A) and it was not known if this same compound was also preventing rickets. McCollum found that when oil was heated and oxidized, its ability to prevent night blindness was removed; however it still maintained its anti-rachitic properties (Holick, 2006b; McCollum et al., 1922; Mohr, 2009). Since this anti-rachitic compound was not the same as the one responsible for the prevention of night blindness (vitamin

A), it was dubbed a new vitamin, vitamin D (Holick, 2006b; McCollum et al., 1922; Mohr, 2009; Rajakumar et al., 2007).

Concurrently, the role of sunlight in the treatment of rickets was also being observed and described. In 1822, Sniadecki, a Polish physician noted that the rates of rickets were much higher in children who lived in urban Warsaw than in those who lived in rural areas (Lin and White, 2004; Mozolowski and Sniadecki, 1939). Sniadecki recommended the exposure of children to sunlight as a cure for rickets, but unfortunately, his recommendations were largely ignored by the medical community (Mohr, 2009). In 1890, Palm observed that while rickets was common in Great Britain, it was unheard of in China and India, although children were living in poverty and had poor nutrition, and he postulated that sunlight may be a causal factor (Holick, 2004a; Rajakumar et al., 2007). In 1919, Huldschinsky demonstrated that exposure to artificial ultraviolet light from a mercury vapour arc lamp healed rickets in children in Germany (Huldschinsky, 1919; Rajakumar et al., 2007). In the U.S., in 1921, Hess and Unger were able to treat rickets by exposing children to sunlight on top of the hospital roof (Hess and Unger, 1921).

In 1922, Chick and coworkers published the results of a study conducted in Vienna after World War I that found that the same antirachitic effects could be obtained by either sunlight or cod-liver oil (Carpenter and Zhao, 1999; Chick et al., 1922; Holick, 2006b; Huldschinsky, 1919). These two methods of treatment of rickets appeared irreconcilable until the mid 1920s. In 1924, it was independently discovered by two separate groups that foods irradiated with ultraviolet (UV) light possessed anti-rachitic properties (Hess and Weinstock, 1924; Steenbock, 1924). The ability to synthesize vitamin D by irradiation resulted in tremendous public health changes in the treatment of rickets. Fortification programs in several countries reduced the incidence of rickets, and particularly in the U.S., rickets had nearly disappeared by the 1960s (Rajakumar and Thomas, 2005)

1.1.2   Evolutionary Development of Vitamin D in Non-vertebrates and Vertebrates

The compound known as vitamin D has been found in organisms that are more than 500 million years old, including phytoplankton (*Emiliania huxleyi)*, and diatoms (*Skeletonema menxelii)*, which can endogenously synthesize vitamin D (ergosterol, or vitamin D_2) (Holick, 1989; Holick, 2006b). Although the original function and the evolution of vitamin D are not known in detail, Holick (1989) has proposed that vitamin D and its precursors in these early life forms may have had roles as a natural sunblock or as a photochemical signal indicator (to ascertain how much UVR was present in the environment) (Holick, 1989; Holick, 2006b).

Vitamin D was essential in the evolution of vertebrates because the formation of a bony skeleton requires control of mineral metabolism, a function that in vertebrates is performed by vitamin D. Although the evolutionary process is not well understood, in vertebrates vitamin D began to maintain intracellular and extracellular calcium and phosphorous concentrations within ranges that were optimal for proper bone ossification and other metabolic functions (Holick, 2006b).

1.1.3   The Role of Vitamin D in Human Evolution

Vitamin D has been widely accepted to be a major selective factor in the evolution of variable skin pigmentation in human populations (Harrison, 1973; Loomis, 1967). Skin pigmentation is one of the most variable phenotypes in humans. As a continuous trait, human skin colour shows a distribution that ranges from the very dark to the very light. However, the geographic distribution of skin colour is not random; rather, it shows a strong relationship with latitude and particularly with distance from the equator (Figure 1-1). Individuals from populations closer to the equator have generally more pigmented skin, while those who live farther from the equator have less pigmented skin (Jablonski and Chaplin, 2000). Unlike most genetic and other physical human traits, the variation in skin pigmentation is largely attributable to differences between major geographic groups, and not to the variation within populations. Using the F_{ST} statistic, Relethford (2002) found that 88% of the variation in skin colour could be explained by differences among geographic regions. This value is in sharp contrast to the variation observed between major geographic groups for an "average" genetic marker, which accounts for only 10-15% of the total variation; similarly, for other qualitative traits, such as craniometric traits, the

Figure 1-1: Map of worldwide skin distribution for local populations.
Data collected by Renaldo Biasutti prior to 1940. Taken from: http://anthro. palomar.edu/adapt/adapt_4.htm.
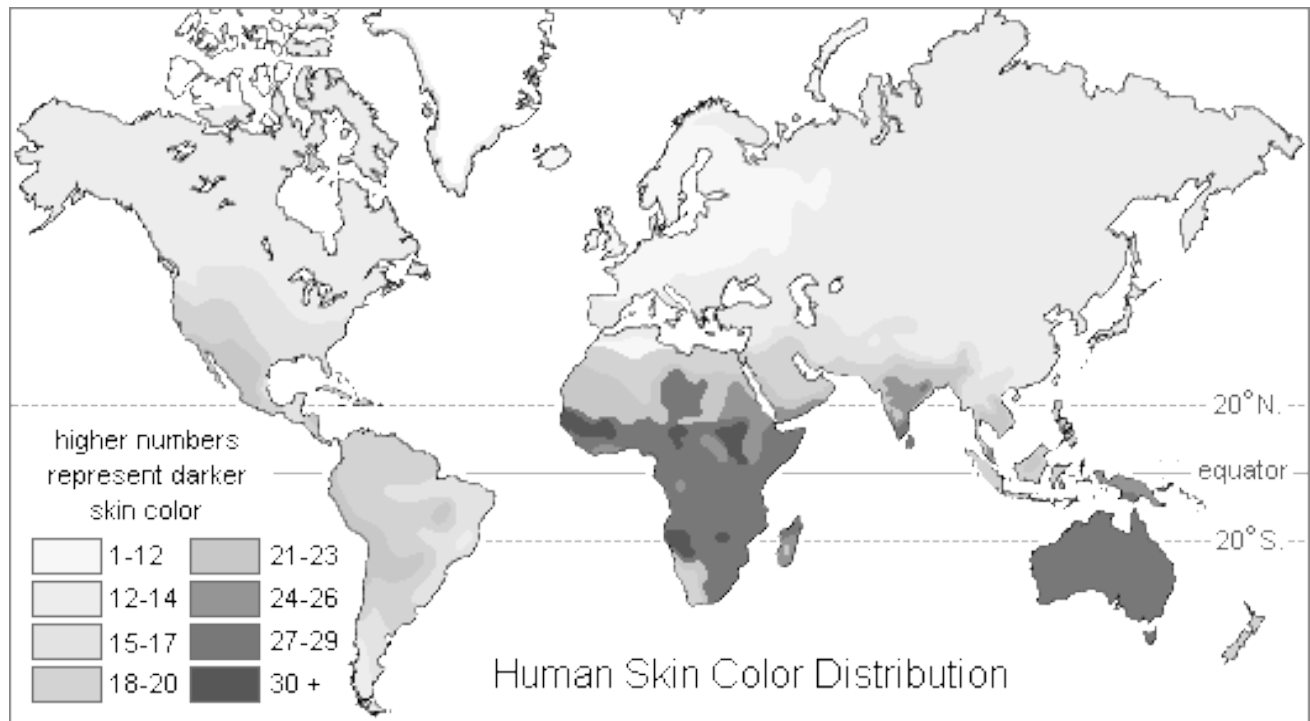
differences between major geographic groups account for only 15% of the total variation (Relethford, 2002; Tishkoff and Kidd, 2004).

Although a number of hypotheses have been suggested to explain the pattern of human skin pigmentation (sexual selection, cold protection, etc), the "vitamin D hypothesis" is the most widely accepted by anthropologists. The strong clinal relationship between skin pigmentation and latitude suggests the action of natural selection due to some environmental factors that show a similar latitudinal gradient. In tropical and equatorial regions, natural selection would favour dark skin to prevent the damaging effects of ultraviolet radiation (UVR). The "vitamin D hypothesis" explains the lighter skin observed at latitudes far from the equator as a result of natural selection favouring lower melanin levels, in order to allow more efficient cutaneous synthesis of vitamin D in regions with reduced UVB incidence (Loomis, 1967).

1.1.3.1   Human Skin Pigmentation

In human populations, the observable differences in skin colour result in large part from variation in melanin levels. Melanin is a mixture of biopolymers that are synthesized in specialized cells of our skin, known as melanocytes, which are located in the basal layer of the epidermis (Parra, 2007). Within the melanocytes, melanin is synthesized in specialized organelles named melanosomes, and the amino acid tyrosine is the main substrate in melanin synthesis (Parra, 2007). Two major types of melanin are found in human melanosomes – the dark brown eumelanin and the lighter red-yellow pheomelanin (Barsh, 2003). The variation in skin pigmentation does not seem to be directly related to the number of melanocytes found in a body site, since the number of melanocytes appears to be about the same in all individuals. Skin pigmentation varies due to the type of melanin synthesized in the melanocytes (the ratio of eumelanin to pheomelanin) and also due to the shape and distribution of the melanosomes within the melanocytes (Barsh, 2003) (see Figure 1-2). In dark pigmented skin, the melanosomes are more highly pigmented, larger, and distributed as single units. In lightly pigmented skin, the melanosomes tend to be less pigmented, smaller in shape and packed into groups (Alaluf et al., 2002).

Figure 1-2: Population differences in the pattern of melanosome size and distribution in the skin.
The number of melanocytes does not differ between population groups, however, the number, size and distribution of the melanosomes does differ. Taken from: (Juzeniene et al., 2009).
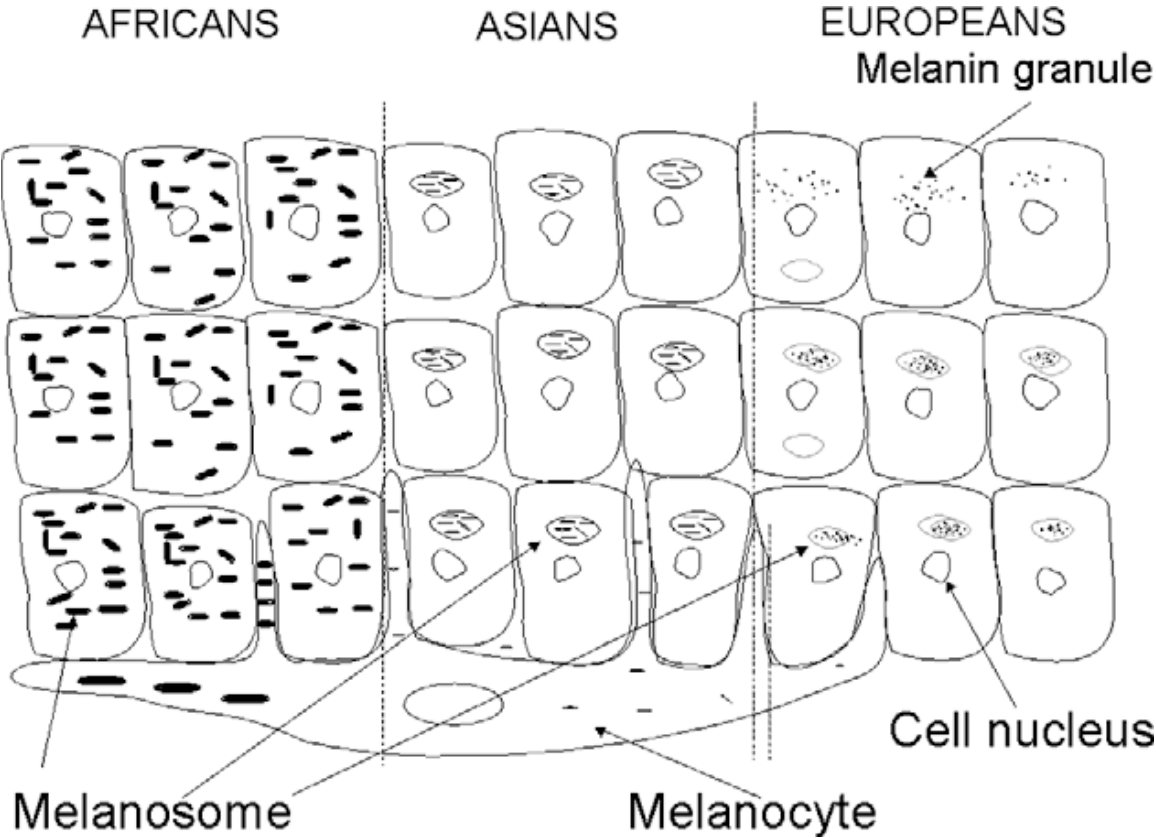
Melanin acts as an optical and chemical photo-protective filter in the skin, thereby reducing the penetration of light of all wavelengths into the epidermal tissues (Jablonski and Chaplin, 2000). Solar radiation that is able to bypass the protective layer of the earth's atmosphere includes UVB (280-320 nm), UVA (320-400 nm), visible wavelength (400-750 nm) and infrared (>750 nm) wavelengths, of which UVB is the most damaging to human skin (Beall and Steegmann Jr., 2000; Jablonski and Chaplin, 2000). Melanin works as an optical filter to attenuate solar radiation by scattering and also as a chemical filter that can absorb compounds produced by photochemical actions which would otherwise be toxic or carcinogenic (Jablonski and Chaplin, 2000).

1.1.3.2   The Vitamin D Hypothesis for Human Skin Pigmentation

Although for the most part the effects of UVR on the skin are harmful, there is one important exception: UVB radiation is essential for the synthesis of vitamin D in the skin. Although it is available from food, particularly fish oils, egg yolk, liver and milk products, synthesis of vitamin D via exposure of the skin to the sun is the most abundant source of vitamin D for most people (Holick, 2003; Holick, 2005b). Low levels of vitamin D during childhood and in utero can cause growth retardation and skeletal deformities in children and can increase the risk of hip fracture later in life (Holick, 2003). In adults, vitamin D deficiency can lead to osteopenia and osteoporosis and can cause osteomalacia and increased risk of bone fracture (Holick, 2007). In recent years, other functions of vitamin D nutrition have been recognized, including immunoregulation and regulation of cell differentiation and proliferation (Holick, 2004a) and vitamin D deficiency has been linked to several forms of cancer and other disorders, including multiple sclerosis, diabetes and hypertension (Holick, 2003; Vieth, 1999). The health effects of vitamin D deficiency will be addressed in detail later in this chapter.

Loomis's (1967) original vitamin D hypothesis had two components: 1) that highly melanized skin pigmentation evolved in peoples living in equatorial regions of the world, since high concentrations of vitamin D are known to be toxic and, 2) that skin pigmentation had become less melanized outside of the tropical regions of the world to allow for adequate vitamin D synthesis (Jablonski, 2004; Loomis, 1967). While most researchers would agree with the second tenet of Loomis' theory, the first tenet (indicating that dark skin evolved to prevent vitamin D toxicity resulting from too much cutaneous synthesis in the tropics) has been disproved

(Jablonski, 2004). The revised vitamin D hypothesis now explains the modern distribution of human pigmentation as the result of a balance between natural selection favouring dark skin pigmentation in equatorial and tropical areas where UVB exposure is high, and favouring lighter pigmentation in areas far from the equator to facilitate and maximize the ability to synthesize vitamin D in the presence of reduced UVB incidence (Jablonski and Chaplin, 2000). This hypothesis is well supported by the evidence of the harmful effects of UVR on human skin, and by the evidence that those with highly melanized skin require longer times to synthesize the same amount of vitamin D as those with lightly melanized skin. Lighter pigmented skin takes 30-34 minutes to reach a maximal level of previtamin D synthesis (a vitamin D precursor) when exposed to equatorial amounts of UVB, but it takes 180-210 minutes for darker skin to reach the same level (Holick et al., 1981). This supports the hypothesis that individuals with darker skin would have been at a selective disadvantage when humans migrated outside of the tropics, due to their limited potential to synthesize vitamin D in areas of reduced UVB availability.

As further evidence of the selective disadvantage of low vitamin D levels, severe vitamin D deficiency can result in a decrease in reproductive output by altering the shape of a woman's pelvis so that natural childbirth becomes impossible and can even lead to death (Beall and Steegmann Jr., 2000). Prior to vitamin supplementation in the U.S., African-American women had a much higher incidence of deformed pelvis (15%) than European-American women (2%) (Beall and Steegmann Jr., 2000). In fact, the development and increased use of the caesarian section coincided with the high rates of rickets at the turn of the 20th century, due to the high incidence of mortality during childbirth as a result of a rachitic pelvis (Merewood et al., 2009). As it was stated above, vitamin D provides many other health benefits beyond bone health (e.g. defense against microbial infections), suggesting that selection favouring optimal synthesis of vitamin D likely played a large role in the distribution of human skin colour.

1.2   Vitamin D: General Information

1.2.1   Vitamin D Sources

For most people, the synthesis of vitamin D via exposure of the skin to the sun's ultraviolet B (UVB) light is the most abundant source of their vitamin D (a process which is described in the following section) (Holick, 2007). In addition to the endogenous production of vitamin D in the

skin, there are also dietary sources of vitamin D that originate either from plants (vitamin D_2 or ergocalciferol) or animals (vitamin D_3 or cholecalciferol) but these naturally occurring sources are rather limited. Among some of the best natural sources of vitamin D_3 are fatty fish, such as fresh wild salmon (which can provide about 600-1000 IU of vitamin D_3 per 3.5 oz serving) and fish oils, such as cod liver oil (provides between 400-1000 IU of vitamin D_3 per 1 tablespoon) (Holick, 2007). Among plants, sun-dried mushrooms are an excellent source of vitamin D_2 (about 1600 IU of vitamin D_2 per 3.5 ounces of sun dried shiitake mushrooms) (Holick, 2007).

Because of the limited number of natural food sources of vitamin D and their lack in our diets, several countries, including Canada, routinely fortify certain food sources with vitamin D (Calvo et al., 2004). In Canada, fluid milk products and margarine are both fortified with vitamin D_3. All fluid milks (skim, 1%, 2% and homogenized) contain about 100 IU per 250 mL, while margarine is fortified at 530 IU per 100 g (Calvo et al., 2004). Some orange juice products and plant milk products (particularly soy milk) are also fortified with vitamin D at about the same concentration as milk (Holick, 2007). Infant formula can have a maximum level of vitamin D of 100 IU per 100 kcal (Gorman, 2003). Vitamin D_3 supplements are also readily available in doses of 200-1000 IU without a prescription in Canada. In recent years, liquid solution supplements have also become available, which is of particular importance to infant health since breast milk has very low vitamin D content (unless the mother has a very high vitamin D intake) and liquid supplements can be easily added to breast milk (Henderson, 2005; Wagner et al., 2008). Formula fed babies do not have this same concern since formula is also fortified at the same level as fluid milk (Canadian Pediatric Society, 2007).

1.2.2   Photobiology of Vitamin D

Vitamin D can be synthesized upon exposure of ultraviolet B (UVB) photons in both animals and plants, through UVB photoisomerization of provitamins. In animals, vitamin D is synthesized from cholesterol – more specifically, from provitamin D_3 (also called 7-dehydrocholesterol (7DHC)) which is found in large quantities in the skin of many vertebrate animals (including humans, although exceptions do exist, including some bat species, mole rats, cats and dogs, all of which are unable to synthesize adequate amounts of vitamin D endogenously) (Holick, 2004b) (see Figure 1-3). During exposure to sunlight, high-energy ultraviolet B (UVB) photons penetrate the epidermal and dermal cells of the skin and are

absorbed by 7DHC and this reaction results in the creation of previtamin D_3 (from the precursor 7DHC) (Holick et al., 1980; Holick et al., 1981; Webb et al., 1988). Because previtamin D_3 is thermodynamically unstable, there is a rearrangement of the compound to form the more thermodynamically stable vitamin D_3 (cholecalciferol) structure. Vitamin D_3 is drawn into the dermal capillary bed by the vitamin D-binding protein (VDBP) and makes its way into the circulation (Deeb et al., 2007). In the presence of biologically sufficient amounts of $1,25(OH)_2D_3$ in the circulation, previtamin D_3 and vitamin D_3 are transformed by solar UVA or UVB to a variety of inert byproducts, therefore excessive sunlight exposure cannot lead to overproduction of the biologically active $1,25(OH)_2D_3,$ which is toxic at high concentrations (Jablonski, 2004).

In plants and fungi, synthesis occurs in essentially the same manner upon exposure to UVB, although provitamin D_2 or ergosterol (and not 7DHC) is the precursor of vitamin D synthesis in plants and fungi, and the resulting vitamin D_2 is ergocalciferol (Holick, 2004b).

1.2.3   Metabolism of Vitamin D

Once in the circulation, vitamin D must undergo two hydroxylations to become the physiologically active hormone, $1,25(OH)_2D_3$ (Bikle, 2009). These reactions can occur via either an endocrine pathway or a more recently proposed autocrine pathway (Heaney, 2008).

In the well-known endocrine pathway, circulating vitamin D from all sources is transported to the liver, where it undergoes a 25-hydroxylation (via the action of a vitamin D 25-hydroxylase, most likely CYP27R1) to form 25-hydroxyvitamin [25(OH)D], which is released into the circulation (Cheng et al., 2004; Strushkevich et al., 2008). A second hydroxylation must take place to convert 25(OH)D into the active vitamin D hormone, 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$ or calcitriol] which takes place in the kindney via the action of 25-hydroxyvitamin D-1αhydroxylase (CYP27B1 or 1αOHase) (Anderson et al., 2003; Zittermann, 2003) (see Figure 1-3 for a diagram depicting vitamin D synthesis). In the endocrine pathway, the renally produced $1,25(OH)_2D_3$ then tightly controls calcium absorption in the gut tissues (Peterlik and Cross, 2009). Even if serum 25(OH)D concentrations are low, the renal vitamin D pathway tightly controls the concentration of serum $1,25(OH)_2D_3$ within a normal range due to the function of parathyroid hormone (PTH) and serum calcium on the synthesis of 1αOHase (Peterlik and Cross, 2009).

Figure 1-3: Synthesis of vitamin D, either from endogenously made vitamin D3 or from dietary supplementation of vitamin D2/D3.
Two hydroxylations, one in the liver and another in the liver, produce the active hormone, $1,25(OH)_2D_3$. Taken from:(Deeb et al., 2007).

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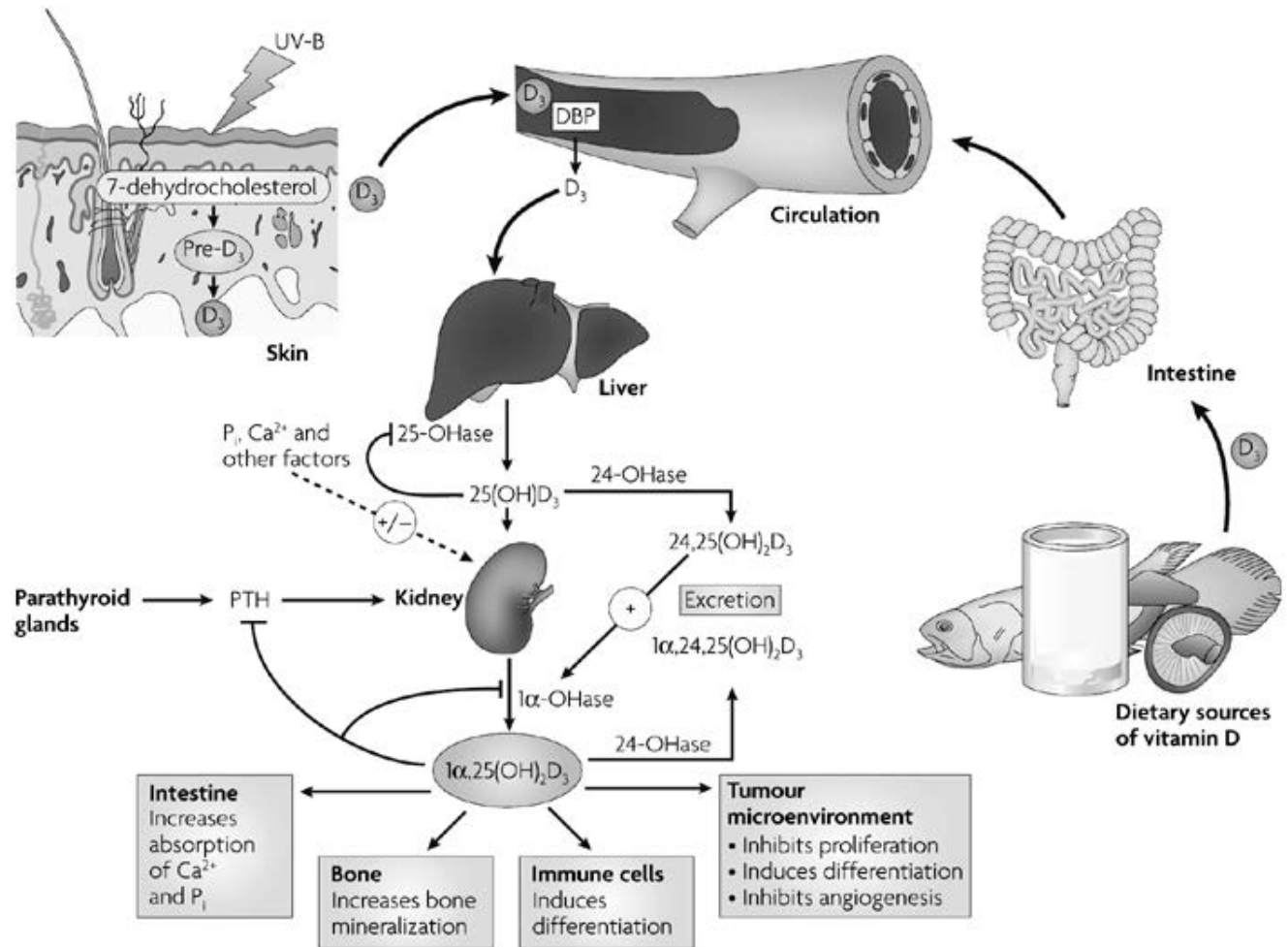
The more recently proposed autocrine pathway also involves the same two hydroxylations, but the location of the second hydroxylation reaction differs from the endocrine pathway. In the autocrine pathway, vitamin D still undergoes hydroxylation to serum 25(OH)D in the liver, but the secondary hydroxylation instead takes place in non-renal tissues found throughout the body, where $1,25(OH)_2D_3$ is locally synthesized. Extra-renal tissues that contain the 1αOHase include immune cells (including macrophages), epithelia of many tissues, parathyroid glands, brain, testis, placenta and bone (Anderson et al., 2003; Bikle, 2009). This locally produced $1,25(OH)_2D_3$ is then able to exert effects on a wide variety of tissues. Unlike the endocrine synthesis, the autocrine vitamin D pathway is tightly linked to the amount of available 25(OH)D as a substrate (Chun et al., 2008), because extra-renal 1αOHase is not sensitive to PTH and extracellular calcium (Peterlik and Cross, 2009), likely due to the fact that they lack their cognate receptors (Bikle, 2009). Therefore, at low serum 25(OH)D concentrations, the activity of 1αOHase will be reduced in non-renal tissues, which will be unable to synthesize the necessary amounts of $1,25(OH)_2D_3$ to allow for the non-renal effects of vitamin D, such as cellular growth, differentiation and immune function (Peterlik and Cross, 2009). This may explain why low levels of 25(OH)D are associated with a multitude of chronic, inflammatory, autoimmune, metabolic diseases (Peterlik and Cross, 2009).

The compound resulting from either metabolic pathway is the same: $1,25(OH)_2D_3$ or calcitriol, a hormone that appears to be pluripotent in nature, and can affect the expression of a multitude of genes (Chun et al., 2008). The synthesized $1,25(OH)_2D_3$ moves into the nucleus where it binds with the vitamin D receptor (VDR) and then forms a heterodimer with the retinoid X receptor (RXR). This heterodimer (VDR-RXR) is able to bind with the vitamin D response elements located in the promoters of target genes (Chun et al., 2008). VDRs have been found in a broad range of tissues, including brain, gut, heart, skeletal muscle, liver, pancreas and cells of the immune system (Reschly et al., 2007). It is estimated that the extra-renal vitamin D pathway can regulate hundreds of genes that are responsible for a wide array of functions, including cell growth, immunity, and inflammatory reactions (Holick, 2009a).

Figure 1-4: Organs and tissues capable of synthesis 1,25(OH2)D3.
Previously it was believed that the kidney was the only place where this second hydroxylation took place, but local synthesis at various tissues and cells is now known. Taken from: (Hollis and Wagner, 2006)
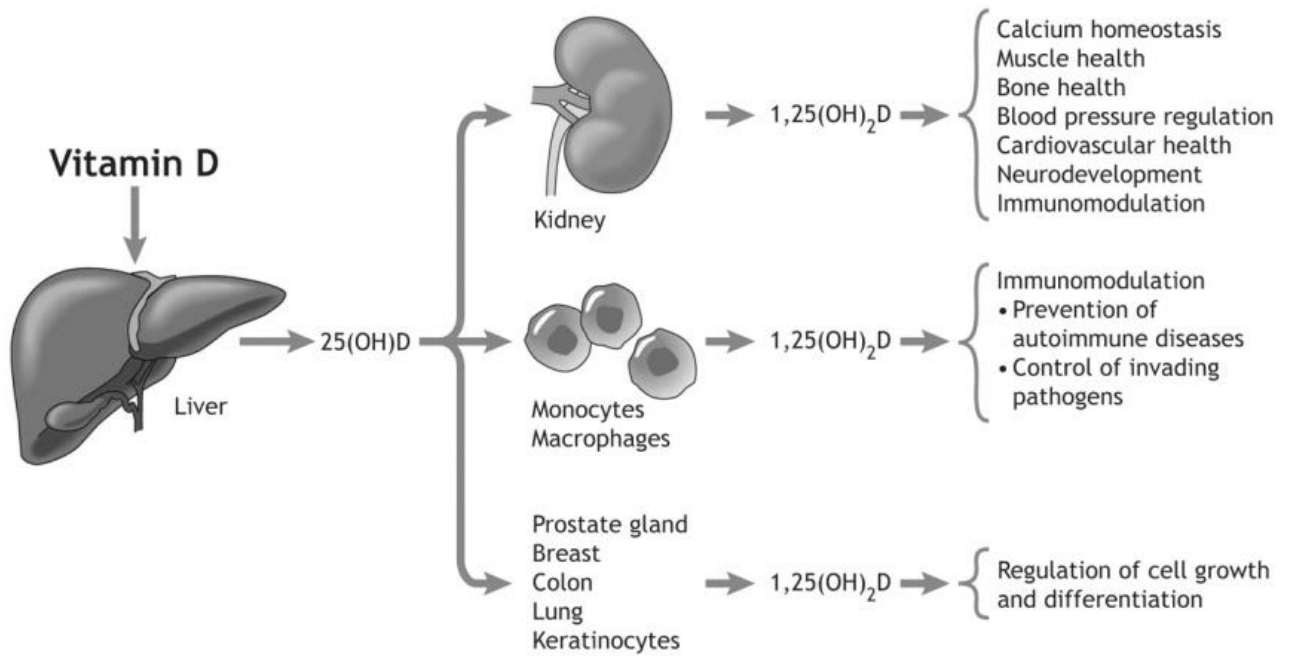
1.2.4   Factors Influencing Vitamin D Levels

There are many factors that can influence vitamin D levels and particularly endogenous synthesis including:

1) Skin pigmentation. Darkly pigmented skin needs far more UVB exposure time to produce the same amount of vitamin D than lightly pigmented skin, because melanin is a highly effective natural sunscreen and interferes with the production of vitamin D (Clemens et al., 1982).

2) Latitude. At high latitudes there is less available UVB year-round. In particular, at latitudes above 35°N there is not enough UVB for cutaneous synthesis for some months of the year, due to the zenith angle of the sun (Webb et al., 1988).

3) Deliberate avoidance of sun exposure and/or extensive use of sunscreen. It is important to note that a sunscreen of SPF 8 can reduce cutaneous synthesis up to 95% (Holick et al., 2007).

4) Increased age. The skin loses the ability to synthesize vitamin D with age, particularly in adults older than 70 years (Holick, 2004b; MacLaughlin and Holick, 1985).

5) Weight. Higher adiposity has been associated with lower vitamin D levels which may result from adipose tissue being used as a storage site for vitamin D (Blum et al., 2008; Mawer et al., 1972), or may be associated with reduced sun exposure in obsese individuals.

6) Low dietary intake of vitamin D rich foods and supplements, particularly in parts of the world were UVB incidence is not constant year-round (Rucker et al., 2002; Vieth et al., 2001; Webb et al., 1988);

7) Malabsorption disorders that affect the body's ability to absorb vitamin D (including celiac disease, Crohn's disease, cystic fibrosis) (Lo et al., 1985);

8) Diseases and disorders of the kidneys and/or liver that affect vitamin D metabolism (Holick, 2007).

9) Certain medications, including anticonvulsants, anti-rejection medications, corticosteroids (Holick, 2007; Zhou et al., 2006).

1.2.5   Vitamin D Binding Protein

Once in the circulation, vitamin D and its metabolites are bound to protein carriers in human plasma, of which the vitamin D binding protein (VDBP), also known as the group-specific component (or Gc), is the most common (Speeckaert et al., 2006). VDBP binds with particularly high affinity to 25-hydroxyvitamin D_3, with up to 99% of circulating 25(OH)D being bound to the VDBP (Nykjaer et al., 1999; Rowling et al., 2006). VDBP has a short half-life ranging from 2.5 to 3 days, while the half-life of 25(OH)D ranges from one to two months (Safadi et al., 1999; Sinotte et al., 2009). The *VDBP* gene (*GC*, NCBI Gene ID#2638) is located on chromosome 4 and it is highly variable, with more than 120 different alleles described in human populations (Arnaud and Constans, 1993). There are three major electrophoretic protein variants (or isoforms), Gc1f, Gc1s and Gc2, that are determined by two non-synonymous single nucleotide polymorphisms (SNPs): T436K (rs4588; which results in a C-to-A transversion [threonine (ACG) to lysine (AAG)] and D432E (rs7041, which results in a T-to-G transversion [aspartic acid (GAT) to glutamic acid (GAG)] (Abbas et al., 2008; Lauridsen et al., 2001). The three major isoforms carry different amino acid combinations at these two SNPs: Gc-1f has a combination of threonine and aspartic acid (Thr-Asp); Gc1s has a combination of threonine and glutamic acid (Thr-Glu) and finally Gc2 has a combination of lysine and aspartic acid (Lys-Asp). The three major isoforms combine to form six possible diplotypes: 1f-1f, 1f-1s, 1s-1s, 1s-2, 1f-2, and 2-2 (Lauridsen et al., 2004).

Studies have shown that there are differences in binding affinity for 25(OH)D among the three Gc variants, with Gc1f having the highest affinity for 25(OH)D, Gc2 having the lowest affinity and Gc1f having intermediate affinity (Chun et al., 2008; Engelman et al., 2008; Lauridsen et al., 2001). Arnaud and Constans (1993) demonstrated that the affinity of the Gc1f allele was four times higher than that of the Gc2 allele and double that of the Gc1s allele (Arnaud and Constans, 1993). It has been suggested that the differences in affinity of the DBP alleles are due to differential glycosylation. Borges et al. (2008) reported that the Gc-1f and Gc-1s alleles are modified with a trisaccharide and a disaccharide at threonine 436, while the Gc-2 allele (with no acceptor threonine residue) is only modified with the disaccharide at an adjacent threonine and does not contain the trisaccharide. A more recent study by Ravnsborg et al. (2010) has confirmed the structure of the O-linked glycan. However, these authors reported no indication of an

independent disaccharide in addition to the well-confirmed trisaccharide in the VDBP peptide, suggesting that the glycosylation difference amongst the VDBP isoforms may be due to the presence (Gc-1f and Gc-1s) or absence (Gc-2) of the trisaccharide alone at T436.

The genetic variation at the *VDBP* locus and its effect on 25(OH)D concentrations in sample of young adults of different ancestry will be discussed in detail in chapter 4.

1.3   Assessing Vitamin D Levels

By consensus, vitamin D status is assessed by the circulating levels of 25(OH)D (the precursor to $1,25(OH)_2D_3$), which is an indicator of the amount of vitamin D coming into the body either via endogenous synthesis and/or via diet and/or supplements (Holick, 2005a; Vieth et al., 2001). 25(OH)D is relatively stable (has a half life of one to two months) and is found at high concentrations in the blood. Current measurements methods of vitamin D typically include both the D_2 and D_3 forms in the final measurement and do not distinguish between the protein-bound and free 25(OH)D in the circulation (Lai et al., 2010). While it is unusual for the inactive metabolite to be considered the biomarker for nutritional status, previous studies have shown that the concentrations of the active vitamin d compound, $1,25(OH)_2D_3$, are not good indicators of vitamin D health because they are tightly regulated and they are often normal, or even elevated, in vitamin D deficient patients (Holick, 2005a; Rucker et al., 2002). Some have argued that 25(OH)D is inadequate to define dietary requirements of vitamin D due to the fact that optimal concentrations remain unknown or much contested (see below for details), and have suggested that functional outcomes be used instead, such as the concentrations of PTH, calcium absorption, bone markers [such as bone resorption or body mass density (BMD)] (Seamans and Cashman, 2009; Weaver and Fleet, 2004). However, a recent study of the existing and potential indicators of vitamin D health concluded that 25(OH)D concentrations are a robust and reliable measure of vitamin D status (Seamans and Cashman, 2009).

1.3.1   Normal Vitamin D Status

While 25(OH)D has been almost unanimously agreed upon as the best indicator of vitamin D status, the discussion regarding what constitutes a "normal" or "adequate" 25(OH)D concentrations has been fraught with considerable debate, primarily due to the lack of consensus of what constitutes an "adequate" vitamin D level. This problem arises because different health

outcomes of vitamin D will have different optimal 25(OH)D concentrations. For example, to prevent rickets or osteomalacia the 25(OH)D concentrations must be higher than 25 nmol/L. However, if we look at optimal dietary calcium absorption, concentrations in excess of 80 nmol/L will be required (optimal concentrations for different health outcomes will be discussed in a later section), and for the prevention of cancer, concentrations of 90-120 nmol/L are required (Bischoff-Ferrari, 2008). This matter is further complicated by the lack of a standardized test for measuring 25(OH)D and the issue of inter-laboratory variation. Therefore, it is not surprising that several 25(OH)D cutoff levels have been described to indicate vitamin D deficiency or insufficiency, and there is no universal agreement on the "adequate" or "optimal" level of serum 25(OH)D (Bischoff-Ferrari, 2008; Bischoff-Ferrari et al., 2006; Henry et al., 2010; Vieth, 2009). A recent review (Borradale and Kimlin, 2009) defined the following cutpoints: i) severe deficiency, <12.5 nmol/L; ii) moderate deficiency, 12.5-25 nmol/L; iii) mild deficiency, 25-50 nmol/L, iv) insufficiency, 50-75 nmol/L and v) sufficiency, >75 nmol/L. It should be noted, however, that not all vitamin D researchers agree on these cutpoints, and within the vitamin D research community there is strong disagreement as to the minimum levels of 25(OH)D that should be termed "optimal". The nutritional guidelines from the 14th Vitamin D Workshop summarize two distinct views held amongst the vitamin D research community of supporting either 1) using 50 nmol/L as the absolute minimum vitamin D level for all individuals in order to maintain the classical actions of vitamin D on bone and mineral metabolism (which are optimized at 50 nmol/L), or 2) a minimum level of 75 – 100 nmol/L is necessary in all individuals due to the associations between vitamin D levels and a multitude of diseases, including certain cancer, multiple sclerosis, hypertension, cardiovascular disease and immune function (Henry et al., 2010). The associations between certain vitamin D associated disorders and vitamin D levels will be discussed more thoroughly in a further section. Throughout this study, I will report the proportion of the participants in our sample under three widely used thresholds -25 nmol/L, 50 nmol/L and 75 nmol/L - and I will consider 25(OH)D levels >75 nmol/L as optimal.

1.3.2   Official Vitamin D Recommendations

The Institute of Medicine (IOM), the governing body on nutrient requirements in the United States and Canada, last defined a reference serum 25(OH)D concentration of less than <27.5

nmol/L as deficiency in their 1997 report on vitamin D (Standing Committee on the Scientific Evaluation of Dietary Reference Intakes, 1997). This value was chosen because concentrations below this level were consistent with vitamin D deficiency in infants, neonates, and young children (Standing Committee on the Scientific Evaluation of Dietary Reference Intakes, 1997). Because little information was known about the levels of serum 25(OH)D needed to maintain normal serum calcium metabolism and peak bone mass in older children and young and middle-aged adults, this value was extended to the other age groups, with the exception of the elderly, for which there was considerable evidence that a higher level is needed (Standing Committee on the Scientific Evaluation of Dietary Reference Intakes, 1997). The fact that vitamin D levels are affected by so many factors, particularly extent of sun exposure, skin pigmentation, latitude, time of day, season, weather conditions and sunscreen made it difficult for the IOM committee to determine an estimated adequate requirement (EAR) for vitamin D (this measure would ensure that 97.5% of the population met their nutritional requirements) (Standing Committee on the Scientific Evaluation of Dietary Reference Intakes, 1997). Instead, the IOM determined an adequate intake (AI) for vitamin D which should prevent clinical signs of vitamin D deficiency (e.g. prevent 25(OH)D dipping below 27.5 nmol/l) (Standing Committee on the Scientific Evaluation of Dietary Reference Intakes, 1997). The IOM recommended an AI for vitamin D ranging from 200 to 600 International Units (IU), depending on age (Standing Committee on the Scientific Evaluation of Dietary Reference Intakes, 1997). For adults aged 19-49, the AI was 200 IU, a value that was based primarily on a study of women in Nebraska (41°N) who had received treatment for their osteomalacia (Kinyamu et al., 1997; Standing Committee on the Scientific Evaluation of Dietary Reference Intakes, 1997). In this study, Kinyamu *et al* reported that all but 6% of 52 women aged 25-35 years of age maintained 25(OH)D concentrations above 30 nmol/L when their intake was 131-135 IU/day (Kinyamu et al., 1997).

Many studies have examined in greater detail the dose-response effects of vitamin D supplementation and have challenged the IOM recommendations in recent years. Barger-Lux et al., (1998) showed that supplements of 400 IU per day only have a modest effect on 25(OH)D levels, only raising it by 11 nmol after 8 weeks of treatment (Barger-Lux et al., 1998). In fact, 400 IU appear to increase serum concentrations by just 7-12 nmol/L, depending on the starting levels (Heaney et al., 2003; Vieth et al., 2007). Heaney et al., (2003) studied a sample of men living in Omaha, United States (41°N) and found that a man with a starting concentration of 50

nmol/L would require an intake of 1170 IU/day to achieve 80 nmol/L (dose-slope – 0.70 nmol/L/µg) (Heaney et al., 2003). Aloia et al. (2008) performed a randomized double blind supplemental study in 262 healthy white and African American men and women living in Mineola, New York and surrounding Long Island areas (40°N) and determined what doses would be needed to raise 25(OH)D concentrations between 80 to 140 nmol/L (Aloia et al., 2008). Aloia et al (2008) found that both African American and white subjects were able to achieve 25(OH)D concentrations in excess of 75 nmol/L at 18 weeks of intervention. However, the doses required in most (23/24) African American patients were 50% higher (4000 IU/day) compared to the white patients, the majority (18/31) of whom were taking 2000 IU/day (Aloia et al., 2008). However, compliance was low in this previous study. A recent randomized double blind intervention study was carried out during the winter in men and women aged 20-40 years living in Ireland (50°N) using different doses of vitamin D supplements (0, 200, 400, or 800 IU/day) (Cashman et al., 2008). Their findings suggest that to maintain 25(OH)D concentrations above 25 nmol/L, a daily intake of 348 IU/day is required, while maintaining concentrations above 37.5, 50 or 80 nmol/L would require 796, 1120 or 1644 IU/day, respectively (Cashman et al., 2008). Their slope calculation indicates that for every 1 µg of vitamin D intake (40 IU), 25(OH)D concentrations will rise by only 1.96 nmol/L (Cashman et al., 2008).

The Institute of Medicine (IOM) is currently conducting a review of the Dietary Reference Intakes for vitamin D, funded jointly by the U.S. and Canadian governments, with a final report expected later in 2010 (Health Canada, 2009).

1.3.3   "Normal" Vitamin D Status in Human Evolution

Before considering the vitamin D levels that are presently observed in modern human populations, it is important to first consider what these concentrations may have been earlier in human evolution. Natural selection would have adapted early *Homo sapiens* to the environment of tropical/equatorial Africa. Given this, we would expect that early *Homo sapiens* were darkly pigmented to protect the skin from the damage of UVB radiation (sun burn, skin cancer, sweat gland damage, as was discussed above) and to protect against photodegradation of nutrients (especially folate) (Jablonski, 2004; Jablonski and Chaplin, 2000). Because humans evolved in an area of the world with year-round high UVB incidence, we can assume that their skin's ability

to synthesize vitamin D would have been maximized even in the presence of their darkly melanized skin.

While it is clearly impossible to gauge the circulating vitamin D levels of our early human ancestors, we can estimate these values by examining vitamin D concentrations in individuals who currently live in regions with high UVR incidence and/or those who expose most of their bodies to the sun, including lifeguards, farmers and other outdoor workers. Although such studies are limited, they do support that serum concentrations of 25(OH)D, the main circulating vitamin D metabolite, can exceed 100 nmol/L in individuals who do not limit their UVB exposure (Vieth, 2003). Haddad and Chuy (1971) studied several lifeguards (n=9) from St. Louis, Missouri (38°N), and observed that 25(OH)D serum concentrations reached a maximum of 161 nmol/L during the summer months, compared to mean concentration of 68 nmol/L for the total study sample (many of whom were not outdoor workers) (Haddad and Chyu, 1971). Better et al. (1980) examined 25(OH)D in a small sample of lifeguards (n=34) living in Israel, and reported a mean concentration of 148 nmol/L (Better et al., 1980). Devgun et al. (1981) similarly found that serum 25(OH)D concentrations were much higher (exceeding 80 nmol/L in late fall) in outdoor workers living at high latitudes in Dundee, Scotland (56°N), compared to indoor workers and in patients at a hospital (this last group did not have any sun exposure) (Devgun et al., 1981). Haddad et al. (1982) found that mean 25(OH)D concentrations in Puerto Rican hospital personal (n=26) were 105 nmol/L while those observed in Puerto Rican (18°N) farmers (n=18) were 135 nmol/L (Haddad et al., 1982). The highest 25(OH)D concentration from this previous study was observed in a Puerto Rican farmer and was found to be 225 nmol/L (Haddad et al., 1982). Barger-Lux and Heaney (2002) examined seasonal variation in 25(OH)D concentrations in 30 outdoor workers and they found that the mean summer 25(OH)D concentration was 122 nmol/L, and three of their participants had 25(OH)D concentrations in excess of 200 nmol/L (203-211 nmol/L) (Barger-Lux and Heaney, 2002). Binkley et al (2007) examined 25(OH)D concentrations in 93 young adults living in Honolulu, Hawaii (21° north) and found that the highest 25(OH)D concentration was 155 nmol/L (Binkley et al., 2007). A recent study by Azizi et al (2009) examined 25(OH)D concentrations and their seasonal changes in both outdoor and indoor workers living in Israel and report a summertime mean of 74 nmol/L in the outdoor workers (Azizi et al., 2009).

Although outdoor workers might appear to receive an extreme amount of sun exposure, studies of "normal" healthy individuals have also provided evidence that sun exposure can produce 25(OH)D concentrations in excess of 80-100 nmol/L. Dawson-Hughes et al (1997) examined 25(OH)D levels in healthy elderly men and women (n=391) with average intakes not exceeding 200 IU/day in Boston and found that mean summer (June to September) 25(OH)D concentrations were 101 nmol/L in men and 78 nmol/L in women (Dawson-Hughes et al., 1997). Furthermore, 19 of the men and 14 of the women had at one point reached 25(OH)D levels in excess of 125 nmol/L (Dawson-Hughes et al., 1997). A recent study by Maeda et al (2010) examined vitamin D levels in 97 older adults (mean age 67 years old) who remain outdoor physically active living in San Paolo, Brazil (23°N) and observed that summer mean 25(OH)D concentrations were 92 nmol/L (Maeda et al., 2010).

An alternative method to assess what the "normal" vitamin D concentration may have been for humans is via experimental studies that expose individuals to artificial UVB radiation that simulates sun exposure. Several such studies have been carried out over the years with varying results. In this section, I will focus on studies involving younger adults (<70 yrs old; since age is a known confounding factor as the amount of 7-dehydrocholesterol decreases in the skin as we age and therefore reduces the skin's ability to produce 25(OH)D endogenously (MacLaughlin and Holick, 1985). Stamp et al. (1977) observed 25(OH)D concentrations in seven subjects who were exposed to artificial UVR and found they exceeded 100 nmol/L after 3 weeks (Stamp et al., 1977). Brazerol et al (1984) exposed black and white young adults (20 to 35 years) with sequential sub-erythemal doses of UVB and saw an increase in their 25(OH)D from baseline of 53 nmol/L to 118 nmol/L (Brazerol et al., 1985). Varghese et al (1989) exposed 7 males to 10 exposures of UVB over two weeks and was able to increase the 25(OH)D concentrations from 53 nmol/L to 124 nmol/L (Varghese et al., 1989).

The studies discussed above demonstrate that humans can naturally attain high 25(OH)D concentrations from endogenous synthesis via exposure of their skin to UVB, provided sufficient sun exposure is allowed to take place. However, for many individuals and populations, many things have changed (including our culture and natural surroundings) since our ancestors first evolved in tropical/equatorial Africa. Not only have we moved away from the diet of our ancestors, particularly in recent times, we also do not have the same behaviours and attitudes

towards sun exposure. Sun exposure tends to be limited to face, hands and arms for most people, in order to limit the damaging effects of UVR, or due to cultural reasons in some populations that do not favour melanized skin. Furthermore, dermatological recommendations warn against sun exposure due to the increased risk of melanoma that comes with excess of UVR exposure.

1.4   Health Effects of Vitamin D

Traditionally, vitamin D has been associated with bone health and as described above, it was in fact first discovered in relation to its ability to cure the bone disorder rickets in children (Lee et al., 2008). The role of vitamin D in the regulation of bone metabolism in the human skeleton has been well studied and is well understood. However, it is becoming increasingly obvious that the role of vitamin D goes far beyond skeletal health, and vitamin D appears to play a significant role in protection against numerous chronic conditions, which will be discussed later in this chapter (Bischoff-Ferrari, 2008; Holick, 2007). Recent research indicates that many tissues other than the liver and kidney have vitamin D receptors, and are capable of locally synthesizing $1,25(OH)_2D_3$ (Hollis and Wagner, 2006; Peterlik and Cross, 2005). Among the currently identified non-renal cells and tissues that contain vitamin D receptors are keratinocytes, osteoblasts, activated T and B lymphocytes, β-islet cells, small intestine, prostate, colon, and most organs in the body, including brain, heart, skin, gonads, prostate and breast (Hansen et al., 2001; Holick, 2003; Holick, 2004b; MacDonald, 1999; Omdahl et al., 2002; Schwartz, 2005; Stumpf et al., 1979). $1,25(OH)_2D_3$ can also be synthesized by macrophages and hematopoietic target cells at inflammation sites (Reichel and Norman, 1989).

Epidemiological studies have shown strong associations between seasonal fluctuations in vitamin D levels and the incidence of various chronic and infectious diseases. Many researchers now believe that reduced local synthesis of $1,25(OH)_2D$ may be responsible for several chronic diseases, including autoimmune diseases, cancer, congestive heart failure, diabetes, hypertension and metabolic syndrome (Holick, 2007; Lee et al., 2008). However, to date most studies have been observational, and few randomized controlled trials have been conducted. The following section will examine our current understanding of the association of vitamin D levels with health outcomes.

1.4.1   Skeletal Diseases

Bone integrity and structure is maintained by $1,25(OH)_2D_3$, and vitamin D deficiency can cause rickets in children and osteomalacia in adults (Holick, 2007). Both conditions produce a poorly mineralized skeleton, and result in soft and distorted bones, particularly in the weight-bearing parts of the skeleton, such as the legs and pelvis (Beall and Steegmann Jr., 2000). Rickets causes many physical deformities including stunted growth, a protruding abdomen, muscle weakness, enlarged ends of long bones and ribs, an abnormally shaped thorax, and legs that are bowlegged or knock-kneed (Henderson, 2005). Despite widespread belief that rickets has been eradicated in Canada, according to the Canadian Pediatric Society, more than 79 cases were reported from 2002 and 2004, and intermediate and dark-skinned children are at higher risk of this disease (Canadian Pediatric Surveillance Program, 2004). The increase in breastfeeding has also contributed to the return of rickets, as breast milk contains very small amounts of vitamin D and many mothers are not informed they may need vitamin D supplements (Henderson, 2005).

Low levels of vitamin D promote an increased production and secretion of parathyroid hormone (PTH), which acts to further induce bone loss and increase the chances of osteoporosis (Holick, 2005a). Osteoporosis is linked to vitamin D deficiency and it is a serious problem amongst adults, particularly postmenopausal women, because it greatly increases the risk of fractures, some of which can be fatal. Osteoporosis is the suspected cause of over 300,000 hip fractures in the United States, and 24,000 hip fractures in Canada every year (Wiktorowicz et al., 2001). Long term care and health costs associated with osteoporosis-related fractures have been estimated at $13.8 billion in the United States in 1994 and $1.3 billion in Canada in 1993, hip fractures being the largest component of that cost (Wiktorowicz et al., 2001).

Results from the NHANES III survey indicate that higher bone mass density (BMD) is associated with higher 25(OH)D concentrations in both young and older adults (Bischoff-Ferrari et al., 2004). A recent meta-analysis examined the role of vitamin D in fracture risk. The meta-analysis included 12 double-blind randomized controlled trials for non-vertebral fractures (n=42,279) and 8 randomized control trials for hip fractures (n=40,886) (Bischoff-Ferrari et al., 2009). The authors found that the efficacy of vitamin D in reducing fractures was dose dependent and was higher at concentrations in excess of 75 nmol/L. While a dose of 400 IU/day did not

reduce fractures, a dose between 482-770 IU/day reduced 20% of non-vertebral fractures and 18% of hip fractures (Bischoff-Ferrari et al., 2009).

1.4.2 Cancer

It has been observed that cancer mortality increases in regions with lower UVB incidence and also shows higher incidence among African-Americans and overweight and obese people, each of which is associated with lower levels of circulating vitamin D (Giovannucci, 2005). Both colon cancer and breast cancer show higher incidence at higher latitudes in the United States (Garland et al., 1989; Garland et al., 1990). The Nurses' Health study found that nurses that ingested greater than 500 IU of vitamin D daily showed a significantly reduced risk for developing breast cancer when compared to those who ingested 150 IU or less (Peterlik and Cross, 2005). Prostate (Schwartz, 2005) and other cancers (Holick, 2004a) appear to follow a similar latitudinal trend. Feskanich et al., 2004 examined the risk of colorectal cancer in women in the Nurses' Health Study and found that serum 25(OH)D concentrations were inversely related to cancer risk (Feskanich et al., 2004). Women that were in quintile 5 (median 25(OH)D: 88 nmol/L) had a relative risk of 0.53 compared to quintile 1 (median 25(OH)D: 38 nmol/L) (Feskanich et al., 2004). A prospective study by Garland et al. (1989) found that patients with 25(OH)D concentrations <50nmol/l had a 2-fold increased risk of developing colon cancer (Garland et al., 1989). Grant (2002) found that increased exposure to the sun resulted in lowered incidence of premature death as a result of cancer in both women and men (Grant, 2002). It is estimated that 10% of deaths attributed to breast cancer could have been prevented with sufficient exposure to UVB radiation (Peterlik and Cross, 2005). Giovannucci et al (2006) examined vitamin D levels and cancer incidence in American men from the Health Professionals Follow-Up Study and from their models, they predicted that an increase of 25 nmol/L in 25(OH)D concentrations was associated with a 17% reduction in total cancer incidence, a 29% reduction in total cancer mortality and a 45% reduction in mortality from digestive system cancers (Giovannucci et al., 2006). Holick et al (2006) observed that women who had 25(OH)D concentrations lower than 30 nmol/L who were followed for 8 years, had a 253% higher risk for developing colorectal cancer (Holick, 2006a).

Few randomized controlled trials have examined the role of vitamin D in cancer incidence and the findings have not been consistent. A fracture study by Lappe et al. (2007) examined cancer

incidence as a secondary outcome in postmenopausal women (n=1179) in a 4-year, population-based, double blind, randomized-control trial. Women were given one of three treatments: a placebo, calcium (1400-1500 mg/day) alone or vitamin D (1100 IU/day) and calcium together. After four years, women who were on either treatment had lower cancer incidence than those taking the placebo, and reduction of cancer of 60% and 47% was observed in women who took the vitamin D-calcium and calcium alone treatments, respectively (Lappe et al., 2007). However, this study was not designed to examine cancer risk as a primary outcome and there the incidence of cancer in the placebo group was low. On the other hand, a randomized double-blind placebo controlled trial in 36,282 postmenopausal women from the Women's Health Initiative (WHI) was a longer study (average 7 years) that observed no difference in colorectal cancer risk among those supplementing with vitamin D and calcium compared to those taking the placebo (OR=1.08, p=0.51) (Wactawski-Wende et al., 2006). However, the doses in the WHI may not have been large enough to produce an effect (only a total of 400 IU/day) (Wactawski-Wende et al., 2006). Clearly, more research is necessary in this area to characterize the role vitamin D may play in cancer incidence.

The mechanism of vitamin D suppression of cancer is thought to act via $1,25(OH)_2D_3$ reducing the proliferation of cells, and promoting differentiation when vitamin D receptors are activated in tissues (Giovannucci, 2005). High circulating concentrations of $25(OH)D$ may also be beneficial since many cell types, including cancerous ones, express 1-hydroxylase, which is able to convert $25(OH)D$ into the active $1,25(OH)_2D_3$ (Giovannucci, 2005).

1.4.3   Autoimmune Conditions

The incidence of several autoimmune diseases shows a strong relationship with latitude and reduced vitamin D levels. Compromised vitamin D status increases the risk for Th-1cytokine-mediated autoimmune diseases, such as inflammatory bowel disease, rheumatoid arthritis, systemic lupus erythematosus, multiple sclerosis and type 1 diabetes mellitus (Peterlik and Cross, 2005). In particular, the latitudinal relationship with the prevalence of multiple sclerosis is quite striking: people who were born below 35°N latitude and lived at or below that latitude for the first 10 years of their lives had decreased lifetime risks of developing multiple sclerosis, compared with those who were born above 35°N latitude (Embry et al., 2000; Hernan et al., 1999; Ponsonby et al., 2002). A recent study of the major histocompatability complex (MHC)

locus HLA-DRB1 revealed that a dominant haplotype found in Northern Europe (HLA-DRB1*1501) increases the risk of MS by 3 fold (Ramagopalan and Ebers, 2008). Ramagopalan et al (2009) observed that vitamin D interacts with HLA-DRB1*1501 to influence its expression, providing more direct genetic evidence for the observational studies indicating a causal link between sun exposure and vitamin D levels in MS risk (Ramagopalan et al., 2009).

Similarly to MS, type 1 diabetes mellitus also shows higher prevalence at latitudes north of the tropics and subtropics, where UVB levels are lower. In addition, there is seasonal variation in the diagnosis of type 1 diabetes, with the largest proportion of cases diagnosed during fall and winter and the lowest during the summer (Luong et al., 2005). Several studies in both rats and humans have demonstrated that vitamin D deficiency causes reduced insulin secretion, and that $1,25(OH)_2D_3$ improves β-cell function and consequently glucose tolerance (Luong et al., 2005). In vitamin D deficient rats, glucose tolerance and insulin secretion were improved with $1,25(OH)_2D_3$ treatment (Luong et al., 2005). During the 1960's, more than 10,000 Finnish children received 2000 IU/day for the first year of life and were followed for the next 31 years; compared to their counterparts who did not receive a supplement (n=32), they had a 78% reduced risk of developing type 1 diabetes (Hypponen et al., 2001). A study of 126 healthy glucose-tolerant adults from California found a positive correlation (partial r=0.25, p=0.007) between 25(OH)D concentrations and insulin sensitivity (measured via a hyperglycemic clamp), and observed that participants with 25(OH)D concentrations less than 50 nmol/L had a higher prevalence of metabolic syndrome features than those who had levels >50 nmol/L (30% vs. 11%, p=0.008) (Chiu et al., 2004).

A large prospective nested case-controlled study of U.S. military personnel (>7 million individuals) showed that among those who were white (n=146 cases, 296 controls), the risk of multiple sclerosis decreased significantly with increasing levels of 25(OH)D concentrations (Munger et al., 2006). Every increase in 25(OH)D of 50 nmol/L reduced the risk of MS by 41% (odds ratio=0.59) (Munger et al., 2006). This inverse relationship between risk of MS and 25(OH)D concentrations was particularly strong in whites when 25(OH)D levels were measured before the age of 20 years. Interestingly, no significant associations between 25(OH)D concentrations and MS risk were found in blacks and Hispanics (n=109 cases, 218 controls), although they had lower 25(OH)D levels than whites (Munger et al., 2006).

The mechanism via which vitamin D affects adaptive immunity appears to be the suppression of proliferation and immunoglobulin production by 1,25(OH)$_2$D, which also slows the differentiation of precursor β cells into plasma cells (Chen et al., 2007a). 1,25(OH)$_2$D also inhibits T cell proliferation, particularly of the T helper (Th)-1 cells that are able to activate macrophages (Lemire et al., 1995; Rigby et al., 1984). The suppression of adaptive immunity may be the reason why vitamin D levels are associated with many auto-immune diseases in which the body detects itself as a target (Bikle, 2009).

1.4.4   Cardiovascular Disease

Epidemiological studies suggest that conditions that decrease vitamin D synthesis in the skin, such as having dark skin and living in temperate latitudes, are associated with increased prevalence of hypertension (Rostand, 1997). There appears to be an inverse relationship between 25(OH)D and 1,25(OH)$_2$D$_3$ levels and blood pressure. Recent research in mice lacking the gene encoding the vitamin D-receptor, indicates that 1,25(OH)$_2$D$_3$ decreases the expression of the gene encoding renin through its interaction with the vitamin D-receptor (Li et al., 2004). The renin-angiotensin system plays an important role in the regulation of blood pressure. Inappropriate activation of the renin-angiotensin system is thought to play a role in some forms of human hypertension and increasing vitamin D levels may be important for improving cardiovascular health (Li et al., 2004).

A trial of elderly women (n=148 women, mean age=74) that had baseline 25(OH)D concentrations below 25(OH)D underwent intervention for only two months with either 800 IU/day of vitamin D and 1200 mg/day of calcium or the calcium alone and blood pressure to test which treatment would best improve blood pressure (Pfeifer et al., 2001). The study found that in the calcium and vitamin D group, systolic blood pressure decreased by 13 mmHg (p=0.02), diastolic blood pressure decreased by 7 mmHg (p=0.10) and heart rate slowed by 4 beats per minute (p=0.02) (Pfeifer et al., 2001).

1.4.5   Innate Immunity and Infections

The role of sunlight in preventing microbial infections has been investigated for over a century. In 1895, Niels Finsen found that tuberculosis (TB) infected patients were cured or improved with exposure to sunlight (phototherapy) (Zasloff, 2006). The incidence of TB continues to be an

important problem among some population groups. For example, the incidence of TB among some immigrant groups is higher in England than it is in their country of origin (Ustianowski et al., 2005). Although the causes of such increased incidence are probably multifactorial, a likely contributor is vitamin D deficiency, as 1,25(OH)2D is able to induce the differentiation of monocytes into macrophages and increase their activity (Zittermann and Gummert)

In the U.K., Gujarati Asians in west London showed a high prevalence of 25(OH)D deficiency, and patients with active TB had the lowest 25(OH)D concentrations (Wilkinson et al., 2000). Lack of sufficient sunlight is likely the cause of this deficiency, however, a vegetarian diet that lacks vitamin D supplementation may also play a role in some groups (Hindus) (Ustianowski et al., 2005). Interestingly, polymorphisms in the vitamin D receptor have also been associated with susceptibility to TB in the Gujarati Asians in London and other populations (Freidin et al., 2006; Wilkinson et al., 2000).

A randomized control trial (RECORD) with 5,292 elderly participants in England and Scotland examined incidence of infections after receiving either 800 IU/day vitamin D or a placebo and observed that those who received vitamin D had reduced incidence of infections and antibiotic use in March compared to the placebo (Avenell et al., 2007). Aloia and Li-Ng (2007) conducted a randomized control trial in 280 black healthy postmenopausal women who received a placebo or 800 IU/day for two years, which was increased to 2000 IU for the third year in the treatment group. The results of this study found that women receiving the vitamin D treatment had a 93% reduction in reported upper respiratory tract infections (Aloia and Li-Ng, 2007).

Recent studies have revealed the potential molecular mechanisms behind the antimicrobial action of vitamin D. The human immune system, like that of other multicellular organisms, produces antimicrobial peptides (AIM's) and proteins which can kill virus, fungi, protozoa, bacteria and other microbes (Zasloff, 2006). One of the better studied AIM's is LL-37 (also known as cathelicidin) (Zanetti, 2004), which recent studies have revealed contains sites for the vitamin D receptor (Gombart et al., 2005). The expression of cathelicidin is induced by $1,25(OH)_2D$ in macrophages and epithelial cells, both of which have the VDR and CYP27B1 (68,69). Work by Liu et al. (2006) helped elucidate how the toll-like immune receptor (TLR) activates macrophages, which induce the conversion of 25(OH)D to $1,25(OH)_2D_3$, and the expression of

the VDR and its relevant downstream targets, including LL-37 (Liu et al., 2006). Since 25(OH)D is the substrate for this reaction, low levels of 25(OH)D will reduce the ability of the cells to respond with cathelicidin production (Liu et al., 2006; Wang et al., 2004). Therefore, 25(OH)D concentrations can greatly influence an individual's microbial response. Interestingly, monocytes incubated with the 25(OH)D of subjects of European descent had more than twice the amount of LL-37 than the same monocytes incubated with 25(OH)D from subjects of African descent (who are often vitamin D deficient with respect to their European counterparts). However, supplementation with 25(OH) to levels observed in the subjects of European descent produced similar levels of monocytes in the subjects of African descent (Liu et al., 2006).

1.4.6   Summary of Health Effects

While new research is dramatically improving our knowledge about the multiple health effects of vitamin D, it is not totally clear what levels of vitamin D, particularly 25(OH)D, the substrate, are optimal to maximize all health outcomes. What has become increasingly apparent is that the optimal levels may differ depending on the outcome and the system of focus, but in many cases, desirable outcomes are not reached at concentrations below <75 nmol/L. For example, when considering the effects of vitamin D on bone calcium and phosphate homeostasis, a level of 25(OH)D in excess of 25 nmol/L is sufficient to prevent rickets or osteomalacia in most individuals (Standing Committee on the Scientific Evaluation of Dietary Reference Intakes, 1997). However, evidence shows that calcium absorption is not maximized until 25(OH)D concentrations reach ~75 nmol/L (Bischoff-Ferrari, 2008). Fall prevention occurred when 25(OH)D concentrations were at least 60 nmol/L to 95 nmol/L, and non-vertebral fracture prevention was not observed until ranges of 75-112 nmol/L were attained (Bischoff-Ferrari et al., 2009). Current findings about the effects of vitamin D on prevention of immune diseases and cancer also indicated that 25(OH)D concentrations >75 nmol/L are needed (Bischoff-Ferrari et al., 2006). Although vitamin D toxicity is a possibility, published studies of vitamin D toxicity indicate that the vitamin D intakes necessary to cause toxicity were greater than 40,000 IU/day (Vieth, 1999), which is a dose much higher than that required to achieve 25(OH)D concentrations that would optimize health effects.

1.5   Summary of Vitamin D Status in the Northern Hemisphere

1.5.1   Current Vitamin D Status in Canada

Several studies have reported that vitamin D concentrations are low in many otherwise healthy Canadian adults, particularly during the winter months, and that these concentrations show considerable seasonal variation (Vieth et al., 2001). Lebrun et al (1993) studied native Canadian mothers (n=160) living in two Manitoba communities and found that mean 25(OH)D concentration was 20 nmol/L and 76% had 25(OH)D levels below 25 nmol/L (Lebrun et al., 1993). Liu et al (1997) examined vitamin D in elderly adults living in long term residences in Toronto (43 N), where 38% had serum 25(OH)D levels below 40 nmol/L in the fall compared to 60% in the spring. In Liu's study, 9% and 18% of the participants had 25(OH)D concentrations below 25 nmol/L in the fall and in the winter, respectively (Liu et al., 1997). In 1999, Watiers et al. examined vitamin D levels in 121 women living in the Inuvik zone of the Northwest Territories, and observed average 25(OH)D concentrations of 60, 52, and 49 nmol/L in women of Caucasian, Inuit and Native Canadian ancestry, respectively (Waiters et al., 1998). Vieth *et al*. (2001) examined 25(OH)D concentrations in a cross-sectional sample of young women (n=796, aged 18-35 years old) in Toronto and found that 7% of women of European ancestry and 17% of women of Asian and Native American ancestry had 25(OH)D concentrations below 40 nmol/L during the summer months, while these numbers increased to 21% and 32%, respectively, during the winter months. Rucker *et al*. (2002) examined a group of men and women (n=188) of mostly European ancestry living in western Canada and observed that 9% had 25(OH)D concentrations below 40 nmol/L, 14% had 25(OH)D concentrations below 50 nmol/L and 65% had 25(OH)D concentrations below 80 nmol/L during the summertime, but these numbers increased to 20% (<40 nmol/L), 39% (<50 nmol/L) and 86% (<80 nmol/L) for the wintertime (Rucker et al., 2002).

More recently, Weiler et al (2005) studied 50 healthy mother infant pairs living in Winnipeg, Manitoba (49°N) and found that 47% of the women had 25(OH)D concentrations below 37.5 nmol/L, and among these women, the overall mean concentration was 29 nmol/L (Weiler et al., 2005). A study by Genius et al. (2009) of three clinical practices in Edmonton looked at 25(OH)D concentrations of almost 1500 patients between January 2001 and March 2007 (Genuis et al., 2009). The overall mean 25(OH)D concentration in this sample was 68 nmol/L, however

17% had 25(OH)D below 40 nmol/L, 52% had 25(OH)D between 40-80, and only 32% had 25(OH)D above 80 nmol/L (Genuis et al., 2009). Sloka et al (2009) studied pregnant women across 79 subdivisions in Newfoundland and Labrador and found that mean 25(OH)D concentrations showed significant seasonal fluctuations (p<0.001) and were 52 nmol/L in winter (January to March) and 69 nmol/L in summer (July to September) (Sloka et al., 2009).

The most recent Canadian Health Measures Survey (CHMS) examined vitamin D levels in a representative sample of Canadians aged 6 to 79 years of age in 2007 to 2009 (Langlois et al., 2010a). This study reported that the lowest 25(OH)D concentrations corresponded to the 20 to 39 age group (514 males and 650 females), which had an overall mean 25(OH)D concentrations of 65 nmol/L (males=61 nmol/L, females =70 nmol/L). This study also observed a statistically significant seasonal difference in 25(OH)D concentrations in this age group, from 70 nmol/L measured in the months of April to October to a drop to 60 nmol/L measured in the months of November to March (Langlois et al., 2010a). The CHMS also examined ethnicity and reported the mean 25(OH)D concentrations among white Canadians aged 20-39 years as 70 nmol/L compared to 48 nmol/L for Canadians of Other ancestry (a group that included anyone who did not report their ethnicity as "White" due to small sample size of other ethnicities, including Chinese, South Asian, Black, Filipino, Latin American, Southeast Asian, Arab, West Asian, Japanese, Korean, Aboriginal, and Other) (Langlois et al., 2010a). However, there were several potential confounders in this survey, including that season was not clearly defined, participants who used tanning beds or who travelled to sunny locales were not excluded and seasonal changes were not measured in the same individuals.

1.5.2   Current Vitamin D Status in the United States

In the United States, 25(OH)D concentrations were measured as part of the National Health and Nutrition Examination Survey (NHANES) since the third round of NHANES (between 1988 to 1994) and have become a part of regular NHANES testing since 2000 (Looker et al., 2008). The NHANES study included data for 20,000 men and 18,000 women and the results from the 2000-2004 survey indicated that the mean 25(OH)D concentration for all the participants in the 20-49 years old group (n=5454) was 62 nmol/L (Looker et al., 2008). Looker et al (2008) observed significant differences in 25(OH)D concentrations by sex and ethnicity (Looker et al., 2008). Examination of the NHANES III data in the 20-39 years old category showed that the mean

25(OH)D concentrations among the non-Hispanic white, non-Hispanic black and Mexican-American males were 88, 50 and 70 nmol/L, respectively. Among women in NHANES III, serum 25(OH)D concentrations in the 20-39 age group were 85, 45, and 60 nmol/L in the non-Hispanic white, non-Hispanic black and Mexican-American females, respectively (Ginde et al., 2009). A comparison between NHANES III (1988-1994) and the 2001-2004 sampling showed that there has been an overall decrease of 15 nmol/L in vitamin D levels in the American population between these two sampling times (Ginde et al., 2009). In the 2001-2004 NHANES survey, mean 25(OH)D concentrations in men aged 20-39 years were 65, 38, 55 nmol/L, among the non-Hispanic white, non-Hispanic black and Mexican-American males, respectively. Among women in NHANES 2001-2004, serum 25(OH)D concentrations in the 20-39 age group were 70, 35, and 50 nmol/L in the non-Hispanic white, non-Hispanic black and Mexican-American females, respectively (Ginde et al., 2009). Seasonality was also observed in the NHANES 2000-2004 data with 25(OH)D concentrations being lower when measured November to March (adjusted mean =59 nmol/L) compared to April to October (adjusted mean=64 nmol/L) (Looker et al., 2008). As in all such large studies, NHANES does have several shortcomings: individuals were not measured twice to explore seasonal fluctuations, the sampling took place throughout the U.S., which includes regions with vastly different UVR availability, and measurements from several months were grouped together. Furthermore, since NHANES primarily focuses on three ethnic groups, our knowledge of vitamin D levels in other ethnic groups in the U.S. is severely lacking.

Several other studies have examined vitamin D levels in US samples. Harris and Dawson-Hughes (1998) observed seasonal variation in 25(OH)D concentrations in young American black and white women living in Boston (42°N) and saw values drop from a summer mean of 41 nmol/L in the black women and 85 nmol/L in the white women to 30 nmol/L and 60 nmol/L in the wintertime for the black and white women, respectively(Harris and Dawson-Hughes, 1998). Levis et al (2005) studied 212 men and women in south Florida (18-88 years old, mean 55 yrs) during the wintertime and they observed a 25(OH)D concentration of 63 nmol/L in men and 55 nmol/L in women, with 38% of men and 40% of women having concentration below 50 nmol/L (Levis et al., 2005). Bodnar et al., (2007) examined vitamin D levels at 4-21 weeks of gestation in 200 white and 200 black pregnant women living in Pittsburg (40°N) and found that the mean

25(OH)D concentrations were higher in white women (73 nmol/L) than in black women (40 nmol/L). In this study only 2% of white women had serum 25(OH)D concentrations below 37.5 nmol/L, compared to 45% of black women (Bodnar et al., 2007).

1.5.3   Vitamin D Status in East Asia, Europe and South Asia

One of the main goals of my study has been to evaluate the seasonal variation of vitamin D levels in individuals of East Asian, European and South Asian ancestry living in Toronto. For this reason, it is important to discuss the vitamin D status of the groups analyzed in this project in their ancestral regions.

1.5.4   East Asia

Although some of the most populated parts of East Asia lie further south than their European counterparts (and are therefore exposed to more UVB), vitamin D deficiency appears to be common in this part of the globe. Wat et al. (2007) looked at vitamin D status in a large sample of adults over the age of 50 years (mean age =69) living in Hong Kong (22˚N) and found that mean 25(OH)D was 73 nmol/L, with 23% of individuals having 25(OH)D levels below 50 nmol/L (Wat et al., 2007). Woo et al. (2008) compared baseline vitamin D levels in women of childbearing age living in Hong Kong (22˚N; n=221) vs. those living in Beijing (39˚N; n=220) prior to their intervention study. Not surprisingly, they found that mean 25(OH)D concentrations were significantly higher in Hong Kong (34 nmol/L) than in Beijing (29 nmol/L), and that a smaller proportion of Hong Kong adults (18%) vs. Beijing adults (40%) had 25(OH)D concentrations below 25 nmol/L (Woo et al., 2008). A study of 57 pregnant Mongolian women found that mean 25(OH)D was 60-80 nmol/L in the 6th month of their pregnancy (in September) but dropped to 20-30 nmol/L postpartum (December) (Fraser, 2004).

In general, vitamin D levels appear to be higher in Japan than in the rest of East Asia, perhaps due to the high dependency on fish as a large constituent of the Japanese diet. However, there is still evidence of lower than optimal (<80 nmol/L) 25(OH)D concentrations in studies of the Japanese population. Nakamura et al (2006) looked at vitamin D concentrations in adults (mean age=66 years) in Toyosaka City (34˚N) and observed mean summer 25(OH)D concentrations of 79 nmol/L, with a drop to 60 nmol/L in the winter (Nakamura, 2006). Only 4% of this population had 25(OH)D concentrations less than 30 nmol/L in the winter (none had levels this

low in the summer) (Nakamura, 2006). Ono et al. (2005) examined vitamin D levels in a sample of 197 subjects from Tokai area (35˚N) and found that the mean 25(OH)D concentration was 57 nmol/L. A strong seasonal variation in mean 25(OH)D concentrations was observed in this study, ranging from a high of 79 nmol/L in September to a low of 38 nmol/L in March, suggesting that seasonality was important at this latitude (35°N). Nakamura et al. (2001) examined 77 healthy young adult women workers at nursing homes in Niligata area (37˚N to 57˚N). Mean 25(OH)D concentration was 42 nmol/L, but among those that were younger than 30 years old, 25(OH)D concentrations were significantly lower (34 nmol/L) than in any of the older age groups (30s=51 nmol/L, 40s=47 nmol/L and 50s=55 nmol/L) (Nakamura et al., 2001). These differences were attributed to the lower intake of fish among the younger women, coupled with less time of sun exposure (a very large proportion of the younger workers had night shift duty) (Nakamura et al., 2001). A study of elderly Japanese long term care users (n=80) with a mean age of 82 years old showed that 25(OH)D concentrations in this age group had a mean of 55 nmol/L (median=49.5 nmol/L) but at a follow-up two years later had dropped to a mean of 45 nmol/L (median=43) (Nakamura et al., 2007).

1.5.5   Europe

Vitamin D status in Europe is highly variable and is unique in that it does not show the expected latitudinal association. Instead, it appears that 25(OH)D concentrations are generally higher at higher latitudes and lower at lower latitudes in Europe. This phenomenon may be due to the high intake of cod liver oil supplements, dietary reliance on fatty fish and/or frequent vacationing in sunny locales reported in individuals from Nordic countries (Norway, Sweden) (Lips, 2007).

A study of postmenopausal women in Milan, Italy (45˚N) reported a mean 25(OH)D concentration of 46 nmol/L, with 28% of the women having concentrations below 30 nmol/L (Bettica et al., 1999). Chapuy et al. (1997) examined the vitamin D status of a healthy adult urban sample of 1569 subjects from 20 French cities in nine geographical regions that ranged from 43˚N to 51˚N. Mean 25(OH)D concentrations were different between regions, with the lowest values found in the North (43 nmol/L) and the highest in the Southwest (94 nmol/L) (Chapuy et al., 1997). Overall, 14% of the population studied had 25(OH)D values less than 30 nmol/L (Chapuy et al., 1997). A study of 3276 adult Swiss men and women (25 to 74 years old) found a median 25(OH)D concentration of 46 nmol/L, and no differences were observed

between males and females (Burnand et al., 1992). Six percent of this Swiss sample had 25(OH)D levels below 20 nmol/L, although sampling did not take place year round but only between the months of October to June of 1989 (Burnand et al., 1992).

A study of 308 middle aged women from northern Norway (65-71°N) that took place between November and June of 2001 had an overall mean 25(OH)D concentration of 55 nmol/L (with a range of 8-143 nmol/L) (Brustad et al., 2004a). However, individuals tested during the summer months (May-June) had the highest mean 25(OH)D concentrations (62 nmol/L), while those tested during the winter (January-February) had the lowest (50 nmol/L) (Brustad et al., 2004a). A sample of subjects (n=32) with a high consumption of cod liver and cod liver oil living in Skjervoy, Norway (70 °N) had a baseline 25(OH)D concentration of 67 nmol/L , with males having a higher mean 25(OH)D concentration (69 nmol/L) than females (64 nmol/L) (Brustad et al., 2004b).

The Euronut SENECA study was important because it was the first study to use a centralized laboratory facility to measure 25(OH)D concentrations in different European countries. This made it possible to avoid problems related to inter-laboratory variation and the use of different methods to measure 25(OH)D concentrations. The Euronut SENECA study evaluated 25(OH)D concentrations in 824 elderly adults (70+ years) from 12 European countries ranging from latitude 35°N (Greece) to 61°N (Norway) (van der Wielen et al., 1995). This study confirmed what other research had previously suggested; the lowest 25(OH)D concentrations were generally found in southern Europe (Anogia, Greece had the lowest 25(OH)D concentrations in both males, 25 nmol/L and females, 21 nmol/L), whereas the highest concentrations were observed further north (Yverdon, Switzerland had the highest mean 25(OH)D levels for males, 59 nmol/L, and Elverun, Norway for females, 48 nmol/L). While elderly adults have a reduced ability to synthesize vitamin D, this study still demonstrates the presence of a trend towards lower vitamin D levels in relatively sunny European countries compared to their northern counterparts. The MORE study also examined vitamin D levels in Europe, although this time exclusively in postmenopausal women living in countries throughout Europe and other worldwide locations (Lips, 2010). The MORE study had more than 7500 participants and confirmed the existence of an association between latitude and vitamin D status; as latitude increased in Europe, there was a trend towards higher 25(OH)D concentrations (r^2=0.42) (Lips et

al., 2001). However, it should be noted that 25(OH)D varied greatly by country and region in Europe. In the MORE study, very low concentrations of 25(OH)D (<25 nmol/L) were observed more frequently in Southern Europe and some Central European countries (Poland, Slovakia and Slovenia), and this lower 25(OH)D levels could not be accounted for by season or participant age (Lips et al., 2001).

While vitamin D levels may vary greatly within Europe, an observation that seems to be consistent across studies is that vitamin D levels are low among immigrants to European countries who originate from more southern locales. In The Hauge, Netherlands (52˚N), a study of pregnant women of Western, Turkish, Moroccan or other non-Western backgrounds examined their 25(OH)D concentrations and found that among the Western women, the mean 25(OH)D concentration was 53 nmol/L, compared to 15 nmol/L, 20 nmol/L and 26 nmol/L among the women from Turkish, Moroccan and other non-Western ethnicities, respectively (van der Meer et al., 2006). In a larger four city study from the Netherlands, van der Meer et al (2008) examined 25(OH)D concentrations in a sample of adult men and women (18-65 years old) and found that while the median 25(OH)D concentration for the indigenous Dutch sample was 67 nmol/L, it was much lower amongst the Turkish (27 nmol/L), Moroccan (30 nmol/L), Surinami South Asian (24 nmol/L), Surinami Creole (27 nmol/L) and sub-Saharan African (33 nmol/L) samples. In this latter study, the percentage of individuals who were vitamin D deficient (25(OH)D <25 nmol/L) also varied between groups: Dutch (6%), Turkish (41%), Moroccan (37%), Surinami South Asian (51%), Surinami Creole (45%) and sub-Saharan African (19%) (van der Meer et al., 2008).

In the city of Leicester, United Kingdom, a study compared the vitamin D status of 201 adult white and Gujarati (South Asian) residents between the ages of 20-40 years old (Hamson et al., 2003). Among the Gujarati males (n=42), mean 25(OH)D value was 26 nmol/L and among the Gujarati females (n=7), 24 nmol/L, compared to a mean of 37 nmol/L among white males (n=37) and 33 nmol/L among the white females (n=51). One of the most striking findings of this study was that 60% of the Gujarati males and 51% of females had undetected values of 25(OH)D below 13 nmol/L (Hamson et al., 2003). Roy et al. (2007) examined vitamin D in South Asian women of Pakistani origin aged 18 to 36 from the Manchester area (53˚N) (Roy et al., 2007) . The mean age of the sample was 29 years old and the mean 25(OH)D concentration was 20

nmol/L. Almost all of the women (94%) had 25(OH)D values less than 38 nmol/L, and 26% had values below 13 nmol/L (Roy et al., 2007).

1.5.6   South Asia

Recent studies across South and Southeast Asia have brought to light that concentrations of 25(OH)D in adults are low in these predominantly sunny countries.

Rashid (1983) examined the median 25(OH)D concentration of Pakistani adults and found that mean 25(OH)D concentrations were 74 nmol/L (range from 8 to 158 nmol/L), with females having a lower concentration that males (67 vs. 82 nmol/L). The lowest value in the sample (7.5 nmol/L) belonged to a housewife who strictly observed purdah (Rashid et al., 1983).

In Lucknow, Northern India (27˚N), a study examined 92 healthy volunteer staff at a hospital (mean age was 34) and found a mean 25(OH)D concentration of 31 nmol/L (range: <13 nmol/L to 160 nmol/L) (Arya et al., 2004). Almost half (48%) of the study group had 25(OH)D concentrations less than 25 nmol/L (Arya et al., 2004). Sachan et al., (2005) examined 25(OH)D levels in a sample of pregnant women, also from Lucknow (27˚N), and observed mean 25(OH)D concentrations of 36 nmol/L, with 84% of women having 25(OH)D less than 56 nmol/L, and 43% having 25(OH)D less than 26 nmol/L (Sachan et al., 2005). Vupputuri et al. (2006), studied 105 healthy adults (mean age 43) in New Delhi (28˚N) and observed that 94% had 25(OH)D levels below 50 nmol/L, with 59% of the sample having 25(OH)D concentrations below 23 nmol/L. In concordance to previous studies, more females (69%) than males (53%) had 25(OH)D concentrations below 23 nmol/L (Vupputuri et al., 2006). Another study of 100 females between the ages of 20-40 years old in Delhi, India (28˚N) reported that mean baseline 25(OH)D concentrations were 11 nmol/L before they began an intervention study (Malhotra et al., 2008).

Harinarayan et al (2007) examined 25(OH)D levels in both urban (n=943) and rural living adults (n=205) near Tirupati, India (13˚N) and they observed that mean 25(OH)D in urban males was 48 nmol/L while in rural males it was 60 nmol/L (Harinarayan et al., 2007). This same study found that urban females had a mean 25(OH)D concentration of 16 nmol/L, and rural females of 19 nmol/L (Harinarayan et al., 2007). Among both males and females, there was a significant

difference in 25(OH)D concentrations between those living in the urban vs. the rural setting (Harinarayan et al., 2007).

1.6   Rationale for Study and Study Goals

Human skin pigmentation varies among geographic groups due to the action of natural selection (Jablonski, 2004; Jablonski and Chaplin, 2000). Because of the role pigmentation plays in vitamin D synthesis, it influences the risk different populations have of developing deficiency and insufficiency. As long as people remained in the geographical region of their ancestors, these differences were not as clearly observable. However, with the mass global migrations that have occurred within the last century, these differences are becoming more apparent. Canada, where for a substantial part of the year there is an insufficient amount of UVR for cutaneous vitamin D synthesis and where there has been a large influx of long-range migrants, is in the midst of a vitamin D epidemic.

Due to the geographic location of Canada, the current dietary vitamin D recommendations may be insufficient to achieve optimal vitamin D levels. At latitudes of 41°N (Boston), there is insufficient UVB radiation available for vitamin D synthesis from November to early March, while at latitudes farther north, like Edmonton, Canada (52°N), a "vitamin D winter" extends from mid October to mid March (Webb et al., 1988). Several recent studies indicate that, unsurprisingly, there is a high prevalence of vitamin D insufficiency in otherwise healthy adults living in Canada and the United States (Calvo and Whiting, 2003; Looker et al., 2002; Nesby-O'Dell et al., 2002; Rucker et al., 2002; Tangpricha et al., 2002; Vieth et al., 2001). As expected, at high latitudes, 25(OH)D levels are lowest during the winter when there is not enough UVR to sustain vitamin D synthesis in the skin (Rucker et al., 2002; Tangpricha et al., 2002; Vieth et al., 2001). Unfortunately, there have been few studies evaluating the effect of seasonality on vitamin D status in healthy adults, and those that have been conducted have focused almost exclusively on people of European descent (Rucker et al., 2002; Vieth et al., 2001). However, non-European individuals are at higher risk for vitamin D insufficiency in Canada (Looker et al., 2002; Nesby-O'Dell et al., 2002). A large percentage of the Canadian population, particularly in provinces receiving a large influx of immigrants, such as Ontario, comes from areas of the world with higher UVR incidence throughout the year (vs. Canada). The 2006 Canadian census found that visible minorities represent 43% of the population of metropolitan Toronto and that the two

largest visible minority groups in Toronto in the 2006 census were those of South Asian and Chinese ancestry (Chui et al., 2008). More than half (54%) of all the South Asians resident in Canada live in the Toronto area, where they represent nearly one-third (32%) of all the visible minorities, and comprise 14% of the total of Toronto's population (Chui et al., 2008). In 2006, individuals of Chinese ancestry comprised almost a quarter (22%) of all visible minorities and 10% of the total population of the city of Toronto (Chui et al., 2008). Therefore, it is critical to explore how the climatic conditions characteristic of a high latitude country like Canada, where there is insufficient UVR from October to March to synthesize vitamin D, are influencing vitamin D levels in individuals of different ancestry and pigmentation levels.

The goal of my doctoral research was to further clarify the factors affecting vitamin D status in a group of healthy young adults of diverse ancestry living in the Greater Toronto Area. Specifically, in this study I:

1) examined 25(OH)D concentrations in a sample of young adults of diverse ancestry living in the GTA,

2) investigated vitamin D intake patterns in a sample of young adults of diverse ancestry,

3) assessed seasonal trends in vitamin D status stratified by ancestry,

4) evaluated the impact of skin pigmentation (measured quantitatively by skin reflectometry), vitamin D intake (measured via a food frequency questionnaire), UVB exposure, sex and BMI on seasonal vitamin D levels,

5) studied the effect of genetic variants within the vitamin D binding protein gene on vitamin D levels,

This project aimed to address the following questions: Are there significant differences in vitamin D levels among ancestral groups? What is the extent of the drop in vitamin D concentrations from the early fall, when cutaneous vitamin synthesis is possible, to the winter, when there is not enough UVB to synthesize vitamin D in the skin? Does the magnitude of the decrease differ across ancestral groups? Which are the most important factors influencing

vitamin D levels in the early fall and winter? Answering these questions is important for devising strategies to ensure optimal vitamin D levels in the diverse Canadian population.

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Chapter 2

2 Low Wintertime Vitamin D Levels in a Sample of Healthy Young Adults of Diverse Ancestry Living in the Toronto area: Associations with Vitamin D Intake and Skin Pigmentation.

2.1 Abstract

Background: Vitamin D plays a critical role in bone metabolism and many cellular and immunological processes. Recent research indicates that concentrations of serum 25-hydroxyvitamin D, the main indicator of vitamin D status, should be in excess of 75 nmol/L. Low levels of 25(OH)D have been associated with several chronic and infectious diseases. Previous studies have reported that many otherwise healthy adults of European ancestry living in Canada have low vitamin D concentrations during the wintertime. However, those of non-European ancestry are at a higher risk of having low vitamin D levels. The main goal of this study was to examine the vitamin D status and vitamin D intake of young Canadian adults of diverse ancestry during the winter months.

Methods: One hundred and seven (107) healthy young adults self-reporting their ancestry were recruited for this study. Each participant was tested for serum 25(OH)D concentrations and related biochemistry, skin pigmentation indices and basic anthropometric measures. A seven-day food diary was used to assess their vitamin D intake. An ANOVA was used to test for significant differences in the variables among groups of different ancestry. Linear regression was employed to assess the impact of relevant variables on serum 25(OH)D concentrations.

Results: More than 93% of the total sample had concentrations below 75 nmol/L. Almost three-quarters of the subjects had concentrations below 50 nmol/L. There were significant differences in serum 25(OH)D levels ($p<0.001$) and vitamin D intake ($p=0.034$) between population groups. Only the European group had a mean vitamin D intake exceeding the current Recommended Adequate Intake (RAI = 200 IU/day). Total vitamin D intake (from diet and supplements) was significantly associated with 25(OH)D levels ($p<0.001$). Skin pigmentation, assessed by measuring skin melanin content, showed an inverse relationship with serum 25(OH)D ($p=0.033$).

Conclusions: We observe that low vitamin D levels are more prevalent in our sample of young healthy adults than previously reported, particularly amongst those of non-European ancestry. Major factors influencing 25(OH)D levels were vitamin D intake and skin pigmentation. These data suggest a need to increase vitamin D intake either through improved fortification and/or supplementation.

2.2 Introduction

Vitamin D plays a critical role in bone metabolism and many cellular and immunological processes (Holick, 2003; MacDonald, 1999; Reichel and Norman, 1989; Stumpf et al., 1979). Low levels of vitamin D have been associated with various chronic and infectious diseases including cancer, multiple sclerosis, diabetes, rheumatoid arthritis, osteoporosis, cardiovascular disease, and microbial infections (Garland et al., 2006; Giovannucci, 2005; Giovannucci et al., 2008; Holick, 2005a; Holick, 2005b; Holick, 2007; Lappe et al., 2007; Liu et al., 2006; Zasloff, 2006). Vitamin D is synthesized in the skin upon exposure to the sun's ultraviolet B radiation (UVB) (Holick, 2007). Vitamin D can also be acquired from the diet from sources where it occurs naturally (such as fatty fish, fish oil and eggs), from fortified products (such as milk and orange juice) and from supplements (Holick, 2007). For many people, exposure of their skin to UVB is the primary source of their vitamin D (Holick, 2003; Holick, 2004b). However, at latitudes far from the equator, such as Canada, the amount of UVB available from sunlight during the winter months is inadequate to allow cutaneous vitamin D synthesis (Holick, 2003; Webb et al., 1988).

Several other factors can affect vitamin D concentrations, including: skin pigmentation (melanin, the major natural pigment in the skin, interferes with cutaneous production of vitamin D) (Clemens et al., 1982; Matsuoka et al., 1995); age (the skin loses the ability to synthesize vitamin D with increased age) (Holick, 2004b; MacLaughlin and Holick, 1985); weight (higher adiposity has been associated with lower vitamin D levels) (Arunabh et al., 2003; Compston et al., 1981; Parikh et al., 2004; Wortsman et al., 2000); deliberate avoidance of sun exposure and/or use of sunblock (due to sun safety or cultural reasons) (Holick, 2004a; Holick, 2007); malabsorption disorders which affect the body's ability to absorb vitamin D (including celiac disease, Crohn's disease, cystic fibrosis) (Lo et al., 1985); diseases and disorders of the kidneys and/or liver that affect vitamin D metabolism (Holick, 2007) and use of certain medications (including anticonvulsants, anti-rejection medications, corticosteroids) (Holick, 2007; Zhou et al., 2006).

The standard method for the determination of vitamin D status tests the circulating concentration of serum 25-hydroxyvitamin D [25(OH)D], which measures the amount of vitamin D coming into the body from all sources (cutaneous synthesis, diet or supplements) (Holick, 2008). Previous efforts to assess optimal serum 25(OH)D levels focused on the role of vitamin D in

bone health, and the optimal 25(OH)D concentration was defined as the concentration that maximally suppressed serum parathyroid hormone (PTH) and promoted maximum calcium absorption (Bischoff-Ferrari et al., 2006). In general, vitamin D "deficiency" was classified as concentrations below 25-27.5 nmol/L (Standing Committee on the Scientific Evaluation of Dietary Reference Intakes, 1997; Vieth et al., 2001). Levels below these cutoffs are associated with calcium malabsorption, severe hyperparathyroidism and vitamin D rickets or osteomalacia (Zittermann, 2006). Some past studies have considered serum 25(OH)D levels of 40-50 nmol/L as the low end of the normal range (Malabanan et al., 1998; Need et al., 2000). However, other studies have shown that PTH levels (Chapuy et al., 1997; Thomas et al., 1998) and calcium absorption (Heaney et al., 2003) are not optimized until serum 25(OH)D levels reach approximately 80 nmol/L. Most vitamin D researchers now recognize that concentrations of serum 25(OH)D should be in excess of 75 nmol/L for multiple health outcomes, not only bone health (Bischoff-Ferrari et al., 2006; Vieth et al., 2007). Accordingly, recent reports refer to serum 25(OH)D levels >75 nmol/L as "optimal", between 75 nmol/L and 50 nmol/L as "insufficient" and <50 nmol/L as "deficient" (Holick and Chen, 2008). In our study, we report the percentage of the individuals in our sample under three widely used cutoffs, 25 nmol/L, 50 nmol/L and 75 nmol/L, and we consider 25(OH)D levels >75 nmol/L as optimal.

Previous research indicates that vitamin D concentrations are low in many otherwise healthy Canadian adults, particularly during the winter months (Rucker et al., 2002; Vieth et al., 2001). Vieth *et al*. (Vieth et al., 2001) studied a sample of young women (18-35 years old) in Toronto and found that 21% of white women, 31.9% of non-white women (a group which combined women of First Nations, South Asian, Indo-Asian and East Asian ancestries) and 25% of black women had serum concentrations below 40 nmol/L during the winter months. Rucker *et al*. (Rucker et al., 2002) examined a group of men and women of mostly European ancestry living in western Canada and observed that 20% had serum concentrations below 40 nmol/L, 39% had serum concentrations below 50 nmol/L and 86% had serum concentrations below 80 nmol/L.

Past studies examining the vitamin D status of Canadians have focused primarily on individuals of European ancestry and have included few or no individuals of other ancestries, who constitute a large proportion of the population of Canadian metropolitan areas (Chui et al., 2008). Individuals of European ancestry have a lower risk of vitamin D insufficiency because they have

low cutaneous melanin levels. It is well known that melanin interferes with the production of vitamin D in the skin and that individuals with darker skin pigmentation are at increased risk of vitamin D insufficiency (Chen et al., 2007). Therefore, it is likely that the prevalence of insufficiency among all Canadians exceeds currently reported estimates (Rucker et al., 2002; Vieth et al., 2001). Additionally, previous studies in Canada have failed to measure pigmentation quantitatively. Therefore, it is critical to expand the existing research to explore how differences in skin pigmentation (Jablonski and Chaplin, 2000) or other factors potentially associated with vitamin D levels, such as vitamin D dietary intake, supplementation or sun exposure, affect the vitamin D status of broadly defined population groups. Results of such studies will be important to inform public health policies regarding fortification and recommendation of intakes in order to ensure that all Canadians have sufficient vitamin D levels.

The aim of this study is to evaluate the wintertime vitamin D status and dietary vitamin D intake of young adults of diverse ancestry in Canada, and to assess the impact of quantitatively measured skin pigmentation and dietary intake on serum 25(OH)D levels.

2.3 Materials and Methods

2.3.1 Study Population and Recruitment

Study recruitment took place at the University of Toronto at Mississauga (43°N, Ontario, Canada) during the winter of 2007. The study was advertised to the University of Toronto community online, and also via the use of advertisements at the University of Toronto at Mississauga campus. The sample was a convenience sample and most of the participants were either students or employees of the university.

Participant eligibility for the study was assessed using a questionnaire that was completed prior to study enrollment. The following were exclusion criteria: age (only participants between the ages of 18 and 30 were recruited for this study), diagnosis of kidney/liver damage or other disorders or diseases that may affect vitamin D metabolism or absorption (including osteomalacia, osteopenia, Crohn's disease, etc.), use of medications that affect vitamin D metabolism (steroids, anticonvulsants, etc.) and recent exposure to UVB (such as visits to tanning salons or trips to sunny destinations less than three months before recruitment). Use of vitamin D supplements was not an exclusionary variable because we were interested in

evaluating how many participants take vitamin D supplements, and the effect of supplementation on 25(OH)D levels. Participant ancestry was assessed based on responses to a personal questionnaire, which asked questions pertaining to the birthplace, migration history, native languages and self-reported ethnicity of the participants, their parents and grandparents.

In total, one hundred and seven subjects (58 females, 49 males) were eligible and agreed to participate. This study was approved by the University of Toronto Health Sciences Research Ethics Board, and all participants provided written informed consent.

2.3.2 Data Collection

Participants met with the researchers twice during the study. During the initial visit, which took place between February 14 and March 16, the participants completed a personal questionnaire that assessed ancestry (personal, parental, and grandparental places of birth, ethnicity, language, migration history, and present residence). Anthropometric measurements (weight and height) were also taken, from which body mass index (BMI) was calculated for each participant. All participants were instructed to complete a 7-day food diary, which recorded all beverages and food items consumed over a 7-day period, and a blood sample was drawn. During the second visit, which in most cases took place within two weeks of the first visit, participants returned the completed food diaries and were reimbursed for their participation.

2.3.3 Measuring Pigmentation using Reflectometry

Melanin content was measured in the inner upper arm using a narrow band reflectometer during the initial visit (Dermaspectrometer, Cortex Technology, Hadsund, Denmark) (Shriver and Parra, 2000). Measurements taken on the upper inner arm represent constitutive skin pigmentation (pigmentation in unexposed areas of the skin). The Dermaspectrometer estimates the amount of melanin in the skin from the amount of light reflected back to the machine in the red and green wavelengths of the light spectrum (Shriver and Parra, 2000). Skin color is primarily influenced by two pigments: hemoglobin and melanin, with hemoglobin showing a large optical absorption peak in the green wavelengths and a sharp drop off in the red wavelengths (this is why blood appears red), while melanin shows absorption of light at all wavelengths (Shriver and Parra, 2000). Based on the differences in the spectral curves of the two pigments, Diffey *et al*. (Diffey et al., 1984) suggested that the reflectance of light in the red spectrum would generate an

estimate of the melanin content of human skin, following the equation, Melanin $= \log_{10}$ (1% red reflectance). Melanin Index values calculated using the Dermaspectrometer range from the low 20s to more than 100, with individuals with the lightest skin pigmentation having the lowest values and those with the darkest pigmentation having the highest (Shriver and Parra, 2000).

2.3.4   Biochemical Analyses

An aliquot of whole blood was centrifuged and the serum fraction was removed after clotting and stored at -80°C. Serum parathyroid hormone (intact PTH), calcium, phosphate, and creatinine, were measured on the automated Modular Analytics Serum Work Area (Roche, Basel, Switzerland). Serum 25-hydroxyvitamin D [25(OH)D] concentrations were determined by the DiaSorin "25-OH Vitamin D TOTAL" competitive chemiluminescence immunoassay on the automated LIAISON$^{®}$ analyzer (Stillwater, MN). This method has 100% specificity for both 25(OH) vitamin D_2 and 25(OH) vitamin D_3. This assay has a limit of detection of 10 nmol/L, an intra-assay coefficient of variation (CV) of 5%, and an inter-assay CV of 7%. Samples were analyzed in one continuous batch with quality control samples inserted at periodic intervals.

This 25(OH)D "total" method was previously validated with a different sample set in which serum 25(OH)D was measured using both the "total" method and the DiaSorin radioimmunoassay (RIA) (DiaSorin, Stillwater, MN). A comparison showed a strong correlation between the methods ($r^2 = 0.814$). Statistically, serum 25(OH)D concentrations determined by both methods were indistinguishable from one another ($p = 0.17$, paired t-test).

2.3.5   Nutritional Analyses

Daily intake of vitamin D from dietary and supplemental sources was estimated using a 7-day food diary. Subjects were provided with portion size aids and recorded their food, beverage and supplement intake for seven consecutive days. Vitamin D intake was analyzed with the computer program Food Processor (version 8.0 and its revisions, ESHA Research Inc., Salem OR, which included the 1997 Canadian Nutrient File from Health Canada); Canadian foods were always chosen where fortification was different from USA, e.g., margarine, breakfast cereals.

2.3.6   Statistical Analyses

Differences between population groups in serum 25(OH)D levels and vitamin D intake were evaluated using ANOVA. For these analyses, serum 25(OH)D was log transformed and vitamin D intake was transformed using the square-root transformation. The effects of age, sex, BMI, total vitamin D intake and skin pigmentation (melanin content) on log serum 25(OH)D levels were explored using multiple linear regression. All statistical tests were performed with SPSS (Version 15.0, SPSS Inc., 2006). A power analysis using the software G*Power (Version 3) (Faul et al., 2007) indicated that, using a significance level of $\alpha=0.05$, our study has approximately 87% power to detect a large effect size (f=0.40) in an ANOVA analysis (with a sample size of 75 individuals in three groups) and approximately 87% power to detect a medium effect size ($f^2=0.15$) in a multiple regression analysis (with a sample size of 107 individuals with five predictors).

2.4 Results

2.4.1   Sample Characteristics

Participants were divided into broadly defined subsets based on self-reported geographic origin gathered in the personal questionnaire. Most of the participants self-identified as being of African, East Asian, European or South Asian ancestry. Individuals who reported being of other ancestries or of multiple ancestries were placed into another subgroup designated as "Other". Table 2-1 summarizes the clinical and biochemical characteristics for the total sample and the three population groups well represented in the sample (East Asian, European and South Asian). Because of the small sample of size of individuals in the African and "Other" subgroups, these subgroups were not included in the subgroup statistical analyses. The following variables showed significant differences between the sexes: age (mean male=21.5, female mean=20.1 p=0.002), BMI (male mean=21.2 female mean=18.7, p=0.002), creatinine (male mean=81.4, female mean=59.4, p<0.001) and calcium (male mean=2.43, female mean=2.38, p=0.003). An ANOVA showed that, after controlling for age and sex, there were no significant differences in PTH, calcium, phosphate, and creatinine concentrations between the three ancestral groups (European, East Asian and South Asian). However, there were significant differences in serum 25(OH)D concentrations among the three groups (p<0.001) (see below). The mean melanin

index for the total sample was 33.0, and ranged from 22.4-53.5. Mean melanin index values (and ranges) for the different groups were as follow: East Asian=32.0 (range 26.7-40.4); European=28.6 (range 22.4-32.3); and South Asian=38.3 (range 29.8-53.5). An ANOVA showed that that there was a significant difference in pigmentation among the groups, even after controlling for sex and age (p<0.001).

2.4.2   Vitamin D Status and Ancestry

Figure 2-1 shows the distribution of serum 25(OH)D concentrations according to ancestry. Only one individual had serum measurements below the 10 nmol/L limit of detection. Table 2-2 reports vitamin D status for all participants, and stratified according to ancestry using three widely used cutoffs: <25 nmol/L, <50 nmol/L, <75 nmol/L and finally, optimal vitamin D levels (≥75 nmol/L).

The mean serum 25(OH)D concentration in the global sample was 39.4 ± 21 nmol/L (range: 10-111 nmol/L). The mean was highest in the Europeans (55.9, range 16.4-110.0 nmol/L), followed by that of the East Asians (34.5, range 10.9-111.0 nmol/L) and lowest in the South Asians (30.5, range 10-57.8 nmol/L). Only 6.6% of the total sample had optimal 25(OH)D concentrations, defined as >75 nmol/L. Almost three-quarters (74%) of the sample had concentrations below 50 nmol/L. More importantly, vitamin D levels showed substantial variation according to ancestry: 34.4% of the subjects of European ancestry had concentrations <50 nmol/L, while the prevalence was significantly higher for East Asians (85.2%) and South Asians (93.5%) (Fisher's exact test, p<0.001).

Analysis of the ancestry-specific group means for log serum 25(OH)D concentrations by ANOVA showed significant differences for the European, East Asian and South Asian samples (p<0.001). Post-hoc tests (Tukey HSD) revealed that these results were driven by the significantly higher serum 25(OH)D concentrations in the European group with respect to the other two groups: East Asian and European (p<0.001), and South Asian and European (p<0.001). No significant pairwise differences in mean serum 25(OH)D were found between East Asians and South Asians (p=0.775).

2.4.3   Vitamin D Intake and Ancestry

Mean vitamin D intake in the total sample and stratified by ancestry is reported in Table 2-3. Mean daily total vitamin D intake was substantially higher in the European sample (231.0 ± 173.5 International Units-IU) than in the East Asian (133.4 ± 101.7 IU) and South Asian (164.3 ± 144.3 IU) samples. In all groups, mean daily dietary intake of vitamin D was greater than mean daily intake from supplements. Only 22.9% of the participants reported the use of supplements. Vitamin D intake from food sources was highest in the European group as was vitamin D intake from supplements.

Total daily vitamin D intake (from diet and supplements, transformed using the square-root transformation) differed significantly among groups (ANOVA for East Asian, European and South Asian samples; p=0.034). No significant differences in vitamin D intake were observed between the sexes.

The current recommendation for Adequate Intake (AI) of vitamin D is 200 IU/day for individuals between the ages of 19-50 (Standing Committee on the Scientific Evaluation of Dietary Reference Intakes, 1997). The availability of vitamin D intake data in our study allowed us to further evaluate 25(OH)D levels in individuals with a vitamin D daily intake higher than 200 IU. When the sample was stratified according to total vitamin D intake and we analyzed only the 25(OH)D levels of the individuals with intakes higher than 200 IU/day, 84.4% of the individuals had serum 25(OH)D concentrations <75 nmol/L and 40.6% of the individuals showed 25(OH)D levels <50 nmol/L.

2.4.4   Factors Affecting Vitamin D Status

Several variables are known to affect vitamin D status and these were assessed for their influence on serum 25(OH)D: age (Holick, 2004b; MacLaughlin and Holick, 1985) , BMI (Arunabh et al., 2003; Compston et al., 1981; Parikh et al., 2004; Wortsman et al., 2000), vitamin D intake (Burgaz et al., 2007; Holick and Chen, 2008), and constitutive skin pigmentation (Armas et al., 2007; Clemens et al., 1982; Matsuoka et al., 1995). A linear regression analysis was performed with log serum 25(OH)D as the dependent variable and age, sex, BMI, total vitamin D intake and skin pigmentation as the independent variables. The regression analysis revealed that almost 35% of the variation in log serum 25(OH)D concentrations was explained by the linear

combination of the variables tested (r^2=0.339 , $F_{(5,98)}$=10.049, p<0.001). Table 2-4 shows the bivariate and partial correlations for each of the variables tested in the model. Only two of the five tested variables had a statistically significant relationship with serum 25(OH)D concentrations: total vitamin D intake (p<0.001) and skin pigmentation (p=0.033). On the basis of this analysis, we can infer that both total vitamin D intake and constitutive skin pigmentation are predictors of serum 25(OH)D in this sample. Total vitamin D intake showed a positive correlation with total vitamin D intake and it alone explained 30.4% of the variance in serum 25(OH)D concentrations. Controlling for all the other variables in the analysis (age, BMI, sex and total vitamin D intake), total vitamin D intake explained 28.9% of the variance in serum 25(OH)D (see partial correlations in Table 2-4). Constitutive skin pigmentation shows a negative correlation with serum 25(OH)D and explained 6.5% of the variation in serum 25(OH)D. When controlling for all the other variables, skin pigmentation explained 4.5% of the variance in serum 25(OH)D.

Examination of the partial regression plots suggested the presence of an outlier and the dataset was checked for outliers by examining casewise diagnostics, leverage statistic (h) and Mahalanobis distance. The following criteria were used: casewise diagnostics set at > 3 standard deviations, h>0.2 and Mahalanobis distance >20.52 (χ^2=20.52, with df=5 and α=0.001). One outlier was identified by both leverage statistics and Mahalanobis distance. This case was investigated and it was observed that this participants' pigmentation was the darkest in the sample (their Melanin Index was 15 points higher than the second darkest person). The outlier was removed and the regression was performed again with no report of other outliers.

With the removal of the outlier, the strength of the multiple regression model improved (r^2=0.374, $F_{(5,97)}$=11.597, p<0.001). Once again, only total vitamin D intake (p<0.001) and skin pigmentation (p=0.003) had a significant effect on serum 25(OH)D concentrations. With the removal of the outlier, total vitamin D intake explained 30.9% of the variation in serum 25(OH)D. The relationship between serum 25(OH)D and skin pigmentation also increased with the removal of the outlier and skin pigmentation accounted for 9.2% of the variation in serum 25(OH)D (compared to 6.5% when the outlier was present in the dataset).

2.4.5   Figures

Figure 2-1: Boxplot showing serum 25(OH)D concentrations by ancestry.
The boxplot presents five statistics: the top of the box represents the 75th percentile, the line within the box represents the median and the bottom of the box represents the 25th percentile, while the whiskers correspond to the minimum and maximum values that are not outliers. The points above or below the whiskers represent outliers. The asterisk (*) on the plot corresponds to an outlier whose serum levels were the highest reported in this study (110 nmol/L).
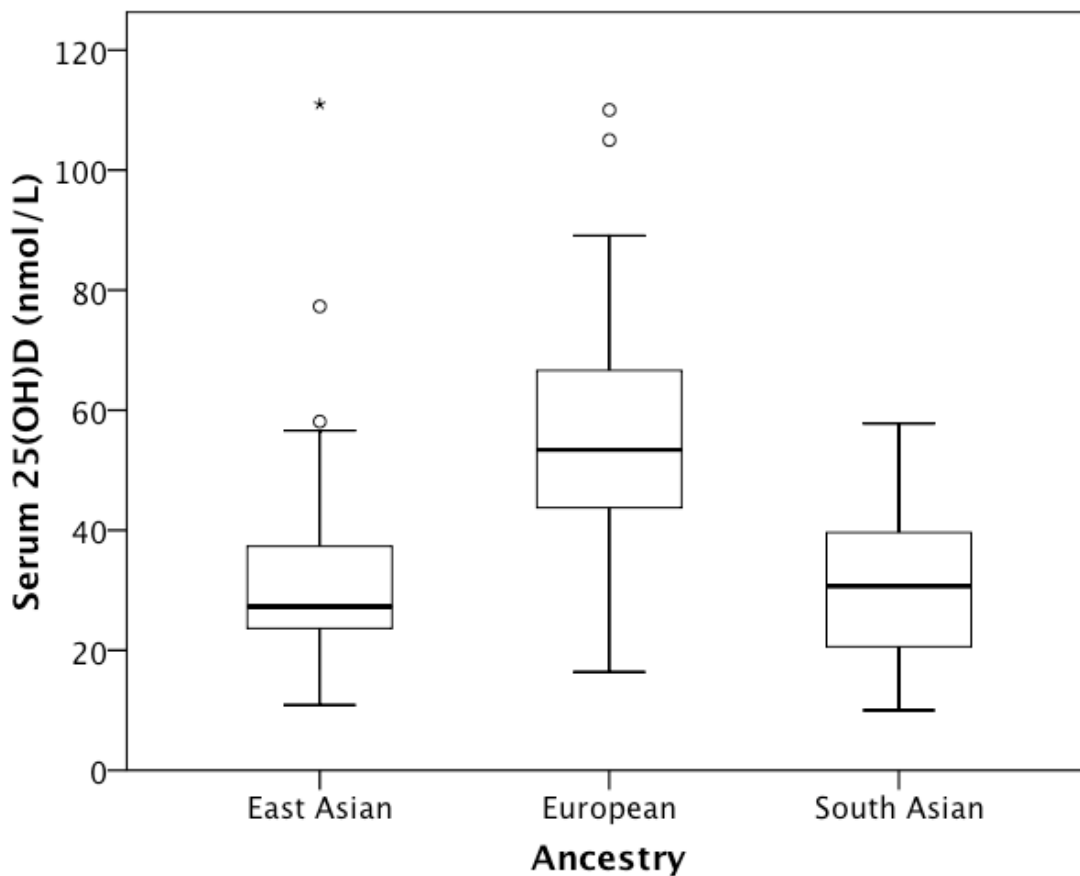
2.4.6   Tables

Table 2-1: Description of the variables collected in the global sample, and stratified by ancestry.
Mean values and 5th and 95th percentiles (in parentheses) are reported. Total sample comprises individuals of African ancestry (n=7), East Asian ancestry (n=27), European ancestry (n=32), Other ancestry (n=9), and South Asian ancestry (n=32). *25(OH)D measurements were not available for one individual of South Asian ancestry, hence n=106 for this variable.

	Total Sample	East Asian	European	South Asian
N (Females, Males)	107 (57, 50)	27 (17, 10)	32 (16, 16)	32 (19, 13)
Age	21 (18, 25)	21 (18, 24)	21 (18, 26)	21 (18, 25)
BMI	19.9 (15.0, 26.6)	18.6 (14.9, 27.4)	20.7 (16.5,28.0)	19.9 (14.4, 25.8)
Melanin Index	35.1 (26.3, 52.7)	32.0 (27.5, 36.8)	28.6 (25.2, 32.0)	38.3 (31.8, 46.8)
25(OH)D (nmol/L)	39.4 (15.3, 77.1)	34.5 (15.1, 71.5)	55.9 (26.7, 96.3)	30.5 (13.3, 51.6)*
PTH (pmol/L)	3.4 (1.8, 5.4)	3.1 (1.9, 4.7)	3.1 (1.8, 5.2)	3.5 (1.7, 5.3)
Calcium (nmol/L)	2.4 (2.3, 2.5)	2.4 (2.2, 2.6)	2.4 (2.3, 2.5)	2.4 (2.3, 2.5)
Phosphate (nmol/L)	1.1 (0.8, 1.3)	1.1 (0.9, 1.3)	1.1 (0.8, 1.3)	1.1 (0.8, 1.4)
Creatinine (μmol/L)	69.4 (48.0, 92.7)	66.5 (45.2, 92.7)	72.9 (56.6, 90.9)	66.8 (48.0, 91.0)

Table 2-2. Wintertime vitamin D status in the global sample, and stratified by ancestry.
*Total sample comprises individuals of African ancestry (n=7), East Asian ancestry (n=27), European ancestry (n=32), Other ancestry (n=9), and South Asian ancestry (n=31). 25(OH)D measurements were not available for one individual of South Asian ancestry.

Vitamin D Status	Total Sample* (n=106)	East Asian (n=27)	European (n=32)	South Asian (n=31)
% <25 nmol/L	25.5	29.6	6.2	35.5
% <50 nmol/L	73.6	85.2	34.4	93.5
% <75 nmol/L	93.4	92.6	84.4	100.0
% >75 nmol/L	6.6	7.4	15.6	0.0

Table 2-3: Mean dietary, supplemental and total vitamin D intake (reported as International Units, IU, per day) in the global sample, and stratified by ancestry.
The 5th and 95th percentiles are indicated in parentheses. *Total sample comprises individuals of African ancestry (n=7), East Asian ancestry (n=27), European ancestry (n=31), Other ancestry (n=8), and South Asian ancestry (n=32). Vitamin D intake data were not available for one individual of European ancestry and one individual of Other ancestry.

Sources (IU/day)	Total Sample* (*n=105*)	East Asian (*n=27*)	European (*n=31*)	South Asian (*n=32*)
Dietary	121.0 (17.1, 309.5)	96.6 (6.71, 213.1)	141.6 (29.1, 321.4)	129.9 (21.9, 355.3)
Supplemental	50.7 (0, 388.5)	36.8 (0, 171.43)	89.4 (0, 400)	34.4 (0, 214.1)
Total	171.7 (19.7, 464.3)	133.4 (8.0, 311.5)	231.0 (34.03, 582.67)	164.3 (27.7, 391.7)

Table 2-4: Bivariate and partial correlations of serum 25(OH)D with predictor variables from the regression analysis.
Bivariate correlations represent the correlation between serum 25(OH)D and each predictor in the regression model. Partial correlations represent the correlation between serum 25(OH)D and each predictor, controlling for all other predictors.

Predictor	Correlations		p
	Bivariate	Partial	
Age	0.024	0.081	0.425
BMI	-0.051	0.000	0.997
Sex	-0.080	0.003	0.977
Skin Pigmentation	-0.254	-0.213	0.033
Total Vitamin D Intake	0.551	0.538	<0.001

2.5 Discussion

Our findings indicate that vitamin D levels are very low in a cohort of healthy young adults living in Southern Ontario, particularly among those of non-European ancestry. Two previous studies examined vitamin D status in the Canadian population, but both primarily sampled individuals of European ancestry (Rucker et al., 2002; Vieth et al., 2001). Our study is consistent with Rucker *et al.* (Rucker et al., 2002) in that most individuals had serum 25(OH)D concentrations below the levels considered optimal by most vitamin D experts (86% had serum 25(OH)D concentrations below 80 nmol/L in Rucker *et al.* compared to 93% of all participants in our study having serum 25(OH)D <75 nmol/L). However, a key finding of our study is that there were significant differences in vitamin D levels among broadly defined ancestral groups living in Canada. Two previous Canadian studies defined vitamin D insufficiency as serum 25(OH)D levels <40 nmol/L, and in these studies 21% and 20% of individuals of European ancestry had concentrations lower than this cutoff (Rucker et al., 2002; Vieth et al., 2001). In our sample, 22% of individuals of European ancestry had 25(OH)D levels less than the 40 nmol/L cutoff, which is comparable to the values observed in previous studies (Rucker et al., 2002; Vieth et al., 2001). However, in our study, 78% of individuals of East Asian ancestry and 77% of individuals of South Asian ancestry had 25(OH)D concentrations lower than 40 nmol/L. These findings demonstrate that while low concentrations of vitamin D were common during wintertime in young adults living in Canada, those of East Asian and South Asian ancestry had vitamin D concentrations that were much lower than their European counterparts.

In our sample, wintertime vitamin D status appears to be affected by both total vitamin D intake and skin pigmentation. The observation that wintertime 25(OH)D levels were primarily influenced by total vitamin D intake is not surprising, given that there is insufficient UVB for cutaneous vitamin D synthesis during the winter months in Canada (Webb et al., 1988). Our finding that there was a significant inverse relationship between skin pigmentation and wintertime serum 25(OH)D concentrations seems to suggest that, when there is sufficient UVB for vitamin D synthesis (late spring, summer and early fall), melanin interferes with the production of vitamin D and this differential cutaneous production of vitamin D is reflected in wintertime 25(OH)D levels (Armas et al., 2007; Mawer et al., 1972; Rosenstreich et al., 1971). Our results are consistent with those of a recently published study (Armas et al., 2007) which

showed that skin pigmentation (measured quantitatively using a reflectometer) had a significant effect on both basal 25(OH)D levels and the rates of increase of 25(OH)D after UVB exposure. Although other studies have noticed the effects of age (MacLaughlin and Holick, 1985) and obesity (Liel et al., 1988; Wortsman et al., 2000) on serum 25(OH)D, these relationships were not observed in our study, likely because our participants were exclusively young adults (18-30 years) who showed more limited variation in BMI and WHR (Table 2-1) than earlier studies (Arunabh et al., 2003; Liel et al., 1988; MacLaughlin and Holick, 1985; Parikh et al., 2004).

Little is known about the vitamin D intake of non-First Nations Canadians who self-report non-European ancestry (Calvo et al., 2004). Our results indicate that the mean total vitamin D intake from food and supplements in these groups was lower than the current Health Canada recommendation for young adults of 200 IU/day (Standing Committee on the Scientific Evaluation of Dietary Reference Intakes, 1997). Our study suggests that those at greatest risk of vitamin D insufficiency are consuming the lowest amounts of vitamin D in their diet and/or supplemental sources. Even consuming the amount of vitamin D currently recommended by Health Canada does not prevent vitamin D insufficiency during the winter in samples consisting primarily of Canadians of European ancestry (Vieth et al., 2001).

Our study has a number of limitations. The sample was primarily comprised of young adults recruited at the University of Toronto, and may not reflect the general population of young people in Canada. Additionally, we did not explore the seasonal variation in vitamin D levels. The sample only featured three well represented population groups, and obviously does not encompass the great population diversity found in Canada, and more particularly, in Canadian metropolitan areas (Chui et al., 2008). However, it should be noted that our sample better represented the population diversity of the Greater Toronto Area than previous studies. The 2006 Canadian census found that visible minorities represent 42.9% of the population of Toronto and 49% of the population of Mississauga (Chui et al., 2008). Individuals of South Asian, Chinese and African Canadian ancestry make up the three largest visible minorities of the Toronto area and represent 31% of all the visible minorities in the city of Toronto. In the city of Mississauga, 41% of all visible minorities are of South Asian ancestry (Chui et al., 2008). We are currently working on a study that will examine the seasonal variation in vitamin D status in a much larger sample that will better reflect the diverse demographic makeup of Canada.

2.6 Conclusion

Our study suggests that the prevalence of low vitamin D levels in young adults living in Canada (Southern Ontario) may be higher than previously described. Our sample included individuals of diverse ancestry, and as such provides a better representation of the multi-ethnic composition of Canadian metropolitan areas than previous studies. Our research also indicates that there are differences in serum 25(OH)D levels and vitamin D intake between population groups and that the currently Recommended Adequate Intake of vitamin D (RAI = 200 IU/day) may not be met by a large proportion of the young adults. Vitamin D intake was particularly low amongst those young Canadians at greatest risk of vitamin D insufficiency. Furthermore, our study suggests that the current vitamin D recommendations in the US and Canada (200 IU/day) are insufficient to ensure optimal circulating 25(OH)D levels, which are defined by most vitamin D experts as 75 nmol/L (Bischoff-Ferrari et al., 2006; Vieth et al., 2007).

The Canadian Cancer Society has recently recommended that a vitamin D supplement of 1000 IU/day be taken by all Canadian adults during the fall and winter, and that those at increased risk should consider year round supplementation (Canadian Cancer Society, 2007). Other Canadian organizations have also recommended higher intakes (800-2000 IU) for adults (Canadian Cancer Society, 2007; Canadian Dermatology Association, 2007; Osteoporosis Canada, 2007). Although further research is needed to determine the vitamin D requirements of individuals of diverse ancestry living in Canada, the results of our study support the need for higher vitamin D intakes to improve the overall health of young Canadians, and the need for food fortification strategies to meet these requirements.

2.7 Competing Interests

RV and DEC have received funding from the Dairy Farmers of Canada. SW has received honoraria from the Dairy Farmers and the Dairy Council. RV has served as a consultant to, or has received honoraria from Cytochroma, Ddrops Company, Merck, Novartis, and Wyeth.

2.8 Author's Contributions

AG participated in the design and coordination of the study, carried out recruitment, collected the anthropological and pigmentation measurements, helped to design the personal and UVR

questionnaires, performed the statistical analyses, and drafted the manuscript. JLB helped in the recruitment and implementation of the study, and helped to draft the manuscript. HW carried out the nutritional analyses of the 7-day food diary. DW performed the serum 25(OH)D biochemical analyses and helped to draft the manuscript. DEC helped in the design of the study and helped draft the manuscript. RV supervised the biochemical analyses, helped with the design of the UVR questionnaire and helped to draft the manuscript. SW designed the 7-day food diary, supervised the nutritional analyses, and helped to draft the manuscript. EP conceived of the study, and participated in its design and coordination, helped to design the personal and UVR questionnaires helped with statistical analysis and helped to draft the manuscript. *All authors read and approved the final manuscript.*

2.9 Acknowledgments

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Chapter 3

3 Serum 25-hydroxyvitamin D Concentrations Fluctuate Seasonally in Young Adults of Diverse Ancestry Living in Toronto

3.1 Abstract

Previous research indicates that circulating vitamin D levels are low in many otherwise healthy adults and that there is considerable seasonal variation in 25-hydroxyvitamin D [25(OH)D] concentrations at high latitudes. We examined seasonal variation in 25(OH)D levels in a sample of young adults of diverse ancestry living in the Greater Toronto Area (GTA). Three hundred and fifty-one (351) healthy young adults completed both a fall and a winter visit during this study. The study was conducted over two years (Year 1: fall 2007/winter 2008 and Year 2: fall 2008/winter 2009). At both visits, each participant's serum 25(OH)D concentration was measured. Information was also obtained on skin pigmentation (measured via reflectometer), vitamin D intake and extent of sun exposure. Overall, the serum 25(OH)D concentration was 54.4 ± 1.3 nmol/L in the fall and 38.4 ± 1.1 nmol/L in the winter. Concentrations differed among ancestral groups at both visits (P<0.001), with South Asians and East Asians having substantially lower concentrations than Europeans. Skin pigmentation (r^2=0.14, P<0.001), supplemental vitamin D intake (r^2=0.09, P<0.001), sun exposure (r^2=0.04, P<0.001) and study year (r^2=0.02, P=0.017) were predictors of fall 25(OH)D concentrations. During the wintertime, serum 25(OH)D concentrations were significantly associated with concentrations taken in the fall (r^2=0.45; P<0.001), with supplemental (r^2=0.15, P<0.001) and dietary vitamin D intake (r^2=0.06, P<0.001) and with study year (r^2=0.02; P=0.009). Our study confirms that serum 25(OH)D concentrations undergo strong seasonal variation at high latitudes and are influenced by vitamin D intake, skin pigmentation, and sun exposure.

3.2 Introduction

Previous research has shown that vitamin D concentrations are low in the general population, particularly at high latitudes, where marked seasonal fluctuations have also been observed (Bodnar et al., 2009; Gozdzik et al., 2008; Harris and Dawson-Hughes, 1998; Rucker et al., 2002; Sloka et al., 2009; Vieth et al., 2001; Zadshir et al., 2005). Such findings are important because low vitamin D levels have been found to be associated with many chronic diseases, including osteoporosis, cancer, diabetes, cardiovascular disease, rheumatoid arthritis, multiple sclerosis (MS), and microbial infections (Garland et al., 2006; Giovannucci, 2005; Giovannucci et al., 2008; Holick, 2005a; Holick, 2005b; Holick, 2007; Lappe et al., 2007; Liu et al., 2006; Zasloff, 2006). Additionally, it has been reported that at higher latitudes individuals of non-European ancestry are more likely to have vitamin D insufficiency than those of European

ancestry (Gozdzik et al., 2008; Moreno-Reyes et al., 2009; Ustianowski et al., 2005; van der Meer et al., 2006; Zadshir et al., 2005).

The circulating concentration of serum 25-hydroxyvitamin D [25(OH)D] is the primary indicator of vitamin D status because it measures the vitamin D from all available sources (cutaneous synthesis, diet or supplements) (Holick, 2009b). Vitamin D experts have recommended that concentrations of serum 25(OH)D in adults should be in excess of 75 nmol/L for multiple health outcomes, including bone health (Bischoff-Ferrari et al., 2006; Vieth et al., 2007). Accordingly, recent reports refer to serum 25(OH)D levels >75 nmol/L as "optimal", between 75 nmol/L and 50 nmol/L as "insufficient" and <50 nmol/L as "deficient" (Dawson-Hughes et al., 2005). In our study, we report the proportion of the participants in our sample under three widely used thresholds - 25 nmol/L, 50 nmol/L and 75 nmol/L - and we consider 25(OH)D levels ≥ 75 nmol/L as optimal.

Several studies have reported that many Canadians have suboptimal 25(OH)D levels, particularly during the winter (Genuis et al., 2009; Newhook et al., 2009; Rucker et al., 2002; Sloka et al., 2009; Vieth et al., 2001; Weiler et al., 2005; Weiler et al., 2007). However, estimates of vitamin D insufficiency among Canadians may actually be higher than previously reported because past studies have focused almost exclusively on individuals of European ancestry (Rucker et al., 2002; Vieth et al., 2001) and it is known that individuals with darker skin have a higher risk of vitamin D insufficiency. Furthermore, few studies have collected information on all of the major factors influencing vitamin D levels, such as seasonality, skin pigmentation, vitamin D intake, BMI and sun exposure.

In a previous study, we reported that wintertime vitamin D levels in East Asian and South Asian adults living in the Greater Toronto Area (GTA) were much lower than in their European counterparts (Gozdzik et al., 2008). However, this previous study did not evaluate seasonal variation in vitamin D levels and had a relatively small sample size. In the present paper, we expanded our previous work by: (1) examining 25(OH)D concentrations and vitamin D intake in a larger sample of young adults of diverse ancestry living in the Greater Toronto Area (GTA), (2) assessing seasonal trends in vitamin D status stratified by ancestry, and (3) evaluating the impact of skin pigmentation (measured quantitatively by skin reflectometry), vitamin D intake (measured via a food frequency questionnaire), UVB exposure, sex and BMI on seasonal vitamin D levels. The present study addressed the following questions: Are there significant differences

in vitamin D levels among ancestral groups? What is the extent of the drop in vitamin D concentrations from the early fall, when cutaneous vitamin synthesis is possible, to the winter, when there is not enough UVB to synthesize vitamin D in the skin? Does the magnitude of the decrease differ across ancestral groups? Which are the most important factors influencing vitamin D levels in early fall and winter? Answering these questions is important for devising strategies to ensure optimal vitamin D levels in the diverse Canadian population.

3.3   Participants and Methods

3.3.1   Study Population and Recruitment

Participants were recruited at the University of Toronto Mississauga (Ontario, Canada) campus (43°N) during the fall of both 2007 and 2008. Our study was advertised to the University of Toronto community online, and we also used hard-copy advertisements at the University of Toronto Mississauga campus. Most of the participants were either students or employees of the university.

Eligibility for the study was assessed using a questionnaire that was completed prior to study enrollment. The exclusion criteria were: age (subjects had to be between 18 and 35 years old); diagnosis of kidney/liver disease or other active or chronic diseases potentially affecting vitamin D metabolism or absorption (such as inflammatory bowel disease, cystic fibrosis) (Lo et al., 1985); use of medications that affect vitamin D metabolism (including corticosteroids and anticonvulsants) (Holick, 2007; Zhou et al., 2006). Consumption of vitamin D supplements was not a criterion for exclusion, since we were interested in evaluating supplement use and its effect on serum 25(OH)D. In this initial questionnaire, potential participants also provided information about ancestry (personal, parental, and grandparental places of birth, ethnicity, language, and current residence). Based on this information, a stratified sampling strategy was used in order to ensure adequate representation of participants of diverse ancestry. The recruitment target was 100 participants per ancestral group. This study was approved by the University of Toronto Health Sciences Research Ethics Board, and all participants provided written informed consent.

3.3.2   Data Collection

Participants met with the researchers twice during the study. The first visit was arranged for the early fall (beginning of September to the middle of October). The second visit was scheduled for the winter (beginning of January until the end of February) (Webb et al., 1988). The study was

conducted over two calendar years (Year 1: fall 2007 and winter 2008, Year 2: fall 2008 and winter 2009).

During the first visit, participants completed a personal questionnaire that assessed ancestry (as described above), and also included general questions about health (with particular emphasis on history of vitamin-D related disorders) and diet (for example, if the participant was vegetarian or lactose intolerant). Study staff measured weight and height, and body mass index (BMI=kg/m^2) was calculated. Participants were asked to complete a Food Frequency Questionnaire, a UVR Exposure Questionnaire and provide a blood sample. During the second visit, participants repeated the protocol (except the personal questionnaire) and were reimbursed for their participation. Participants reporting that they had travelled to a sunny destination between the two visits (N=6) or had used a tanning salon (N=12) continued to participate, but their winter values were excluded from our analyses. Additionally, 3 participants in Year 1 and 6 participants in Year 2 of the study attended the fall visit but did not attend the winter visit. Overall, a total of 351 participants attended the first visit and 322 met the criteria for having their second visit data included in the study.

For data analysis, participants were grouped into broad geographic regions: East Asia, Europe, Middle East, Africa and South Asia. For example, individuals who stated that their ancestors were from China, Japan, and Korea, were grouped as East Asian, while those who reported ancestors from India and Pakistan were grouped as South Asian. Individuals who reported being of multiple ancestries were placed into a subgroup designated as "Other".

3.3.3   Sun Exposure

Extent of sun exposure was calculated from a UVR exposure questionnaire. The questionnaire asked the participants to estimate the amount of time spent outdoors during the previous three months. Questions included "During the summer, how much time on average per day do you spend in the sun between 9 a.m. to 5 p.m.?" and the options provided included <5 min, 5-30 min, <1 h, 1-2 h and >2 h.

3.3.4   Measuring Pigmentation Using Reflectometry

Constitutive skin pigmentation (pigmentation in unexposed areas of the skin) was measured in the inner upper arm using a narrow-band reflectometer (Dermaspectrometer, Cortex Technology,

Hadsund, Denmark) at both visits (Shriver and Parra, 2000). A detailed description of the method can be found elsewhere (Gozdzik et al., 2008).

3.3.5   Biochemical Analyses

An aliquot of whole blood was centrifuged and the serum fraction was removed after clotting and stored at -80°C. Serum parathyroid hormone (PTH), calcium, and phosphate were measured on the automated Modular Analytics Serum Work Area (Roche, Basel, Switzerland).

Vitamin D concentrations were measured using liquid-chromatography tandem mass spectrometry (LC-MS/MS). Serum/plasma spiked with an internal standard was extracted with 1 mL of MTBE. The MTBE phase was evaporated and re-dissolved in 1 mL of 80% methanol and extracted with 1 mL of heptane. The methanol phase was evaporated to dryness and the residue dissolved in 100 μL of 50% methanol and analysed by LC-MS/MS. Chromatographic separation was achieved using linear gradient HPLC (Agilent 1200) on a 1.8 μm column (Agilent XDB-C8 Eclipse) starting at 63% methanol to 100% methanol during 4 min. Mass spectrometric analysis was performed using atmospheric pressure chemical ionization (APCI) on an API5000 LC-MS/MS (Applied Biosystems/Sciex). The ion transitions monitored were $401.4 \rightarrow 383.4$ (25-hydroxycholecalciferol), $417.4 \rightarrow 399.4$ (24,25-dihydroxycholecalciferol), $413.4 \rightarrow 395.4$ (25-hydroxyergocalciferol) and $407.5 \rightarrow 389.4$ (d_6-25-hydroxycholecalciferol). Analyst software (version 1.4.2) mediated data acquisition, peak-area integration and comparison against the standard curve to calculate the concentration of unknowns. The standard curve was derived from calibrators analyzed within the same analytical run.

The LC-MS/MS method used in this study has not been previously published, and was extensively validated. The between-day coefficient of variation (CV) ranged from 3% to 6.9% for 25-hydroxycholecalciferol [25(OH)D3] (depending on the mean serum concentration of the samples), and from 3.1% to 10.4% for 25-hydroxyergocalciferol [25(OH)D2]. The within-day CV was 5.1% for 25(OH)D3 and 7.2 % for 25(OH)D2. The r^2 values for the comparison of the LC-MS/MS estimates and the certified concentration values of the National Institute of Standards and Technology (NIST) Standard Reference Material 972 were > 99.9% for both 25(OH)D3 and 25(OH)D2. Similarly, the r^2 value for the comparison of the concentrations of the Vitamin **D** **E**xternal **Q**uality **A**ssessment **S**cheme (DEQAS) samples for the LC-MS/MS method used in this study and the mean of the DEQAS LC-MS results was 97.1%. Finally, the r^2 values for the comparison of estimates obtained with this LC-MS/MS method and estimates using the

DiaSorin radioimmunoassay (RIA) method (N=50) and the DiaSorin Liaison method (N=45) were 89.8% and 90.6%, respectively. A subset of samples (n=10) were assayed separately and the coefficient of variation was 2.3% for total serum 25(OH)D [arithmetic sum of 25(OH)D3 and 25(OH)D2]. Elsewhere, the term 25(OH)D refers to the summed total of the two vitamers.

3.3.6   Nutritional Analyses

Daily intake of vitamin D from dietary and supplemental sources was estimated using a Food Frequency Questionnaire (FFQ). We have previously validated this FFQ for assessment of vitamin D and calcium intake in young adults of diverse ancestry (Wu et al., 2009). In the validation study, the FFQ results were significantly correlated with 7-day food diary results (r=0.602, P<0.001) and the vitamin D intakes estimated from the FFQ were significantly associated with serum 25(OH)D concentrations (r=0.520, P<0.001). Subjects were provided with portion size aids and recorded their food, beverage and supplement intake. The FFQs were analyzed with the computer program Food Processor (version 8.0 and its revisions, ESHA Research Inc., Salem OR, using the Canadian Nutrient File 2007b values from Health Canada); Canadian foods were always chosen where Canadian fortification was different from elsewhere (for example, margarine and breakfast cereals).

3.3.7   Statistical Analyses

Analysis of Variance (ANOVA) was used to determine if there were significant differences in serum 25(OH)D and vitamin D intake among the three ancestral groups. Post-hoc analyses were conducted with Bonferroni's method. Repeated Measures ANOVA was used to assess the seasonal variation of serum 25(OH)D concentrations and vitamin D intake.

Multiple linear regression was used to examine how well the measured predictors (sex, BMI, vitamin D intake and skin pigmentation) were able to explain variation in serum 25(OH)D levels in the fall and in the winter. In the fall visit, serum 25(OH)D was the dependent variable and the predictors were BMI, sex, skin pigmentation, sun exposure, reported dietary vitamin D intake, reported supplemental vitamin D intake and study year. For the winter visit, BMI, sex, skin pigmentation, reported dietary vitamin D intake, reported supplemental vitamin D intake and calendar year were inserted as possible predictive variables of vitamin D status in the first model of serum 25(OH)D. To assess the effect of fall vitamin D levels on wintertime levels, we introduced fall serum 25(OH)D as another independent variable in a second regression model for

the wintertime sample. Each analysis controlled for sex and study year since significant sex and study year differences were found for 25(OH)D and vitamin D intake (one way ANOVAs, data not shown). Outliers and influential points were assessed using normality tests (Shapiro-Wilk and Anderson-Darling) and Cook's distance, respectively. No outliers were identified in the analyses of 25(OH)D and vitamin D intake.

Models for dependent variables 25(OH)D and total vitamin D intake displayed non-constant error variance. This was corrected by applying natural log transformation to the responses. Results are presented for the log transformed data. All statistical tests were performed with SPSS (Version 15.0, SPSS Inc., 2006) and Minitab (Version 15.1.1, Minitab Inc., 2007).

Power analysis was conducted using the software G*Power (Version 3) (Faul et al., 2007). At a significance level of $\alpha=0.05$, this study had >97% power for detecting a medium effect (Cohen $f=0.25$) for the ANOVA models, >98% power to detect a medium within-subject effect ($f=0.25$) in the repeated measures ANOVA models and >99% power to detect a medium effect ($f^2=0.15$) in the fall and winter regression models.

3.4   Results

3.4.1   Sample Characteristics

351 participants (225 females, 126 males) were analyzed in the fall and 322 (202 females, 120 males) in the winter. The response rate was 92%, due to study drop-out and exclusion of individuals who had visited tanning salons and or/ travelled to sunny locales in the winter. The majority of the participants were of African, East Asian, European, Middle Eastern or South Asian ancestry. Because of the small sample size of the African (N=12), Middle Eastern (N=16) and "Other" (N=13) groups, they were not included in subsequent analyses.

3.4.2   Serum 25(OH)D Concentrations Stratified by Ancestry

Mean 25(OH)D in the fall visit for the full sample was 54.4 ± 1.3 nmol/L. The highest 25(OH)D concentrations were observed in the European sample and the lowest in the South Asian sample, while the East Asian sample had intermediate value. All three groups differed significantly from each other (univariate ANOVA, P<0.001) (Table 3-1). During the winter, the serum 25(OH)D concentrations were substantially lower, with a mean value of 38.4 ± 1.1 nmol/L. Again, the European sample had the highest 25(OH)D concentrations, followed by the East Asian and South

Asian samples, respectively. While the European sample differed significantly from the other two groups (univariate ANOVA, P<0.001), the East Asian and South Asian did not show significant differences in the wintertime.

Serum 25(OH)D concentrations were categorized using thresholds of 25, 50 and 75 nmol/L (Table 3-2). We observed that a higher proportion of non-Europeans had serum 25(OH)D concentrations below 50 nmol/L and 25 nmol/L in both visits. However, the proportion of individuals with 25(OH)D below 25 or 50 nmol/L increased substantially in all groups in the winter visit. Cohen's Kappa between the categories for the fall and winter visits was 0.16 (P<0.001), suggesting a lack of agreement between the categories at both visits, likely due to the strong seasonal drop in 25(OH)D concentrations, which is reported below.

3.4.3   Seasonal Variation in 25(OH)D

We observed a mean decrease in paired serum 25(OH)D levels of 16 nmol/L (from 54.4 to 38.4 nmol/L) from fall to winter (Figure 3-1). Participants of European ancestry experienced the largest absolute decline in mean serum 25(OH)D concentrations (24.5 nmol/L), followed by East Asians (14.9 nmol/L) and South Asians (8.1 nmol/L). The absolute decrease in 25(OH)D was correlated to baseline 25(OH)D concentrations; that is, individuals with higher baseline 25(OH)D concentrations showed the largest absolute drop in 25(OH)D levels, both in the total sample and in each of the subgroups (see Figure 3-2). When considering the relative decline in 25(OH)D, measured as the ratio of winter/fall 25(OH)D, the winter 25(OH)D means were 69%, 68% and 78% of the baseline fall values, for the East Asian, European and South Asian samples, respectively.

Repeated Measures ANOVA revealed significant seasonal differences (within-subject effects) for 25(OH)D concentrations (P<0.001). Season had a significant within-subject interaction with year for 25(OH)D concentrations (P=0.010).

In the repeated measures ANOVA analysis, ancestry had a significant between-subject effect on 25(OH)D (P<0.001), which is in agreement with the ANOVA results given above.

3.4.4   Vitamin D Intake

Fall vitamin D intake was 7.60 ± 0.40 μg/d (304 IU/d) for the total sample, with no significant differences observed among the three groups. During the winter, vitamin D intake was 7.24 ±

0.44 µg/d (290 IU/d), and again no significant differences were observed among groups (Table 3-1). Total vitamin D intake was comprised of dietary intake and intake from vitamin D supplements. Significant differences in dietary intake were observed among the three groups in the fall (P=0.030), South Asians having the highest intake and East Asians having the lowest. No significant differences were observed in fall vitamin D supplement intake. During the winter visit, neither dietary intake nor supplement intake showed differences among the three samples (Table 3-1).

We also examined the proportion of vitamin D intake coming from the diet vs. supplements at fall vs. winter visits. In both seasons, the majority of vitamin D intake came from dietary sources (Table 3-1). However, the proportion of supplement vitamin D intake was higher in the winter (39%) than in the fall (31%) (p<0.001; paired t-test). This was primarily due to an absolute decrease in dietary vitamin D intake and a slight increase in vitamin D supplements during the winter (see Repeated Measures ANOVA below). The overall increase in supplement vitamin D intake was traced to a substantial rise in winter supplement intake in a few participants, since the fraction of all individuals taking vitamin D supplements was similar in the fall and winter (22% in the fall vs. 23% in winter). Furthermore, some differences were observed in the pattern of supplement use between ancestry groups: the percentage of East Asians who took vitamin D supplements dropped from 20% in the fall to 14% in winter, while among Europeans there was an increase from 27% to 29%, and in the South Asians group, there was an even more pronounced increase, from 21% in the fall to 27% in winter.

Repeated Measures ANOVA results showed a trend towards seasonal differences in total vitamin D intake (P=0.060). This was primarily because of lower dietary vitamin D intake during the winter (P=0.032). Supplemental vitamin D intake did not show similar seasonal patterns (P=0.734).

3.4.5   Factors Affecting Vitamin D Status

3.4.5.1   Fall

Approximately 23% of the variation in serum 25(OH)D concentrations was explained by the linear combination of the variables tested (r^2=0.23; P<0.001) (Table 3-3). Skin pigmentation (P<0.001), sun exposure (P<0.001), vitamin D supplements (P<0.001), and study year (P=0.010) were significant predictors of 25(OH)D levels. Controlling for all the other variables in the

model, reported supplemental vitamin D intake showed a positive correlation with serum 25(OH)D and it alone explained approximately 9% of the variance in 25(OH)D concentrations, while skin pigmentation explained approximately 14%, reported sun exposure 4%, and study year 2% (Table 3-3).

3.4.5.2   Winter

In winter, 27% of the variation in serum 25(OH)D was explained by the variables included in the regression (r^2=0.27, P<0.001). Dietary vitamin D intake (P<0.001), supplement vitamin D intake (P<0.001) and skin pigmentation (P<0.001) were significant predictors in this model. Reported supplement intake alone explained 18% of the variation in wintertime 25(OH)D, dietary vitamin D intake explained 6%, and skin pigmentation accounted for 10% (Table 3-3).

A second winter model that included fall 25(OH)D concentrations (Table 3-3) explained approximately 60% of the variation in winter 25(OH)D concentrations (r^2=0.60, P<0.001). When controlling for the other variables in this model, fall 25(OH)D concentrations explained a hefty 45% of the variation in winter 25(OH)D (P<0.001), with winter vitamin D supplements explaining 15% (P<0.001), dietary intake 6% (P<0.0010) and study year 2% of the variation (P=0.009). After introducing fall (baseline) 25(OH)D into the model, pigmentation was no longer significant.

3.4.6   Figures

Figure 3-1: **Serum 25(OH)D concentrations during the fall and winter in East Asian, European and South Asian young adults living in Toronto.**
Values are means ± SE, n=92 (East Asian), n=97 (European) and n=93 (South Asian). Group means without a common letter differ, P<0.05.
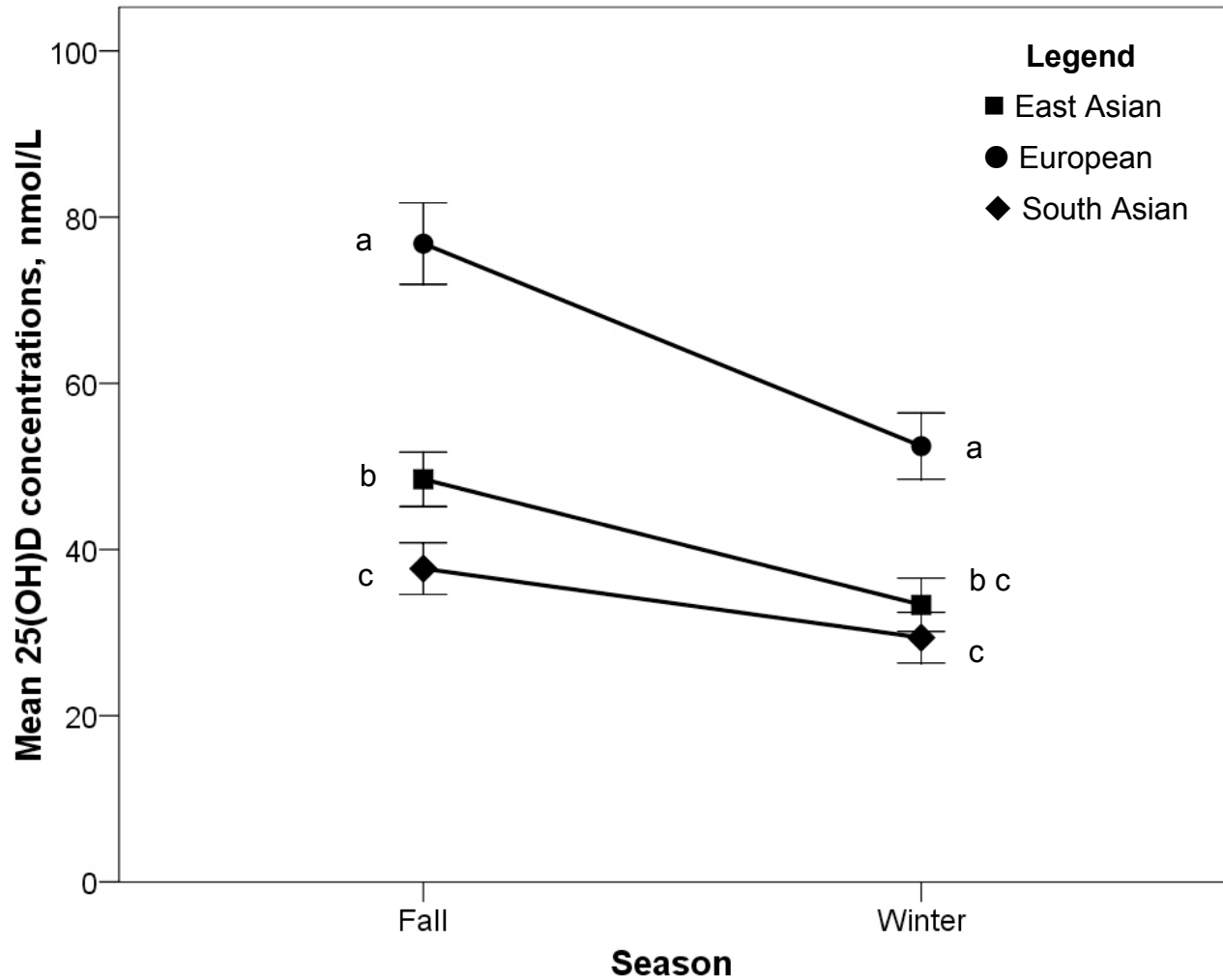
Figure 3-2: Association between the seasonal decresase in serum 25(OH)D concentrations from fall to winter and the baseline fall 25(OH)D concentration.
Data are individual values, *n*=321. Lines above and below the linear regression line are 95% confidence intervals.
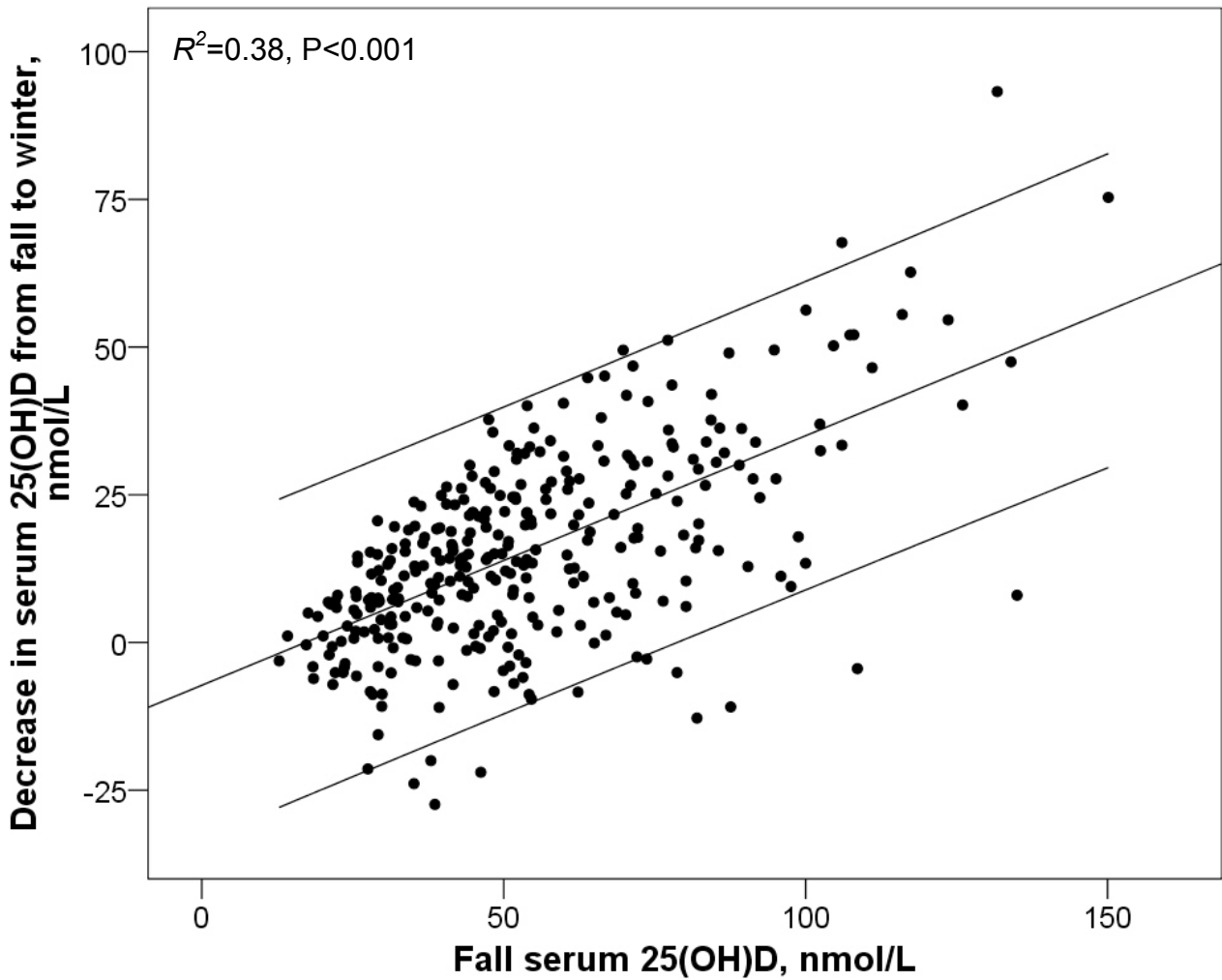
3.4.7 Tables

Table 3-1: Characteristics of the total sample, and by ancestral subgroup.[1,2]

Visit	Variables	Total Sample[2]	East Asian	European	South Asian
Fall	*N*	351	104	110	95
	Age, *yrs*	21.1 ± 0.16	20.4 ± 0.23	22.1 ± 0.38	20.7 ± 0.19
	BMI, *kg/m²*	23.2 ± 0.23	22.3 ± 0.36	23.6 ± 0.47	23.3 ± 0.38
	Skin Pigmentation, *Melanin Index*	34.6 ± 0.39	32.4 ± 0.27[b]	30.4 ± 0.28[c]	39.2 ± 0.62[a]
	Serum 25(OH)D, *nmol/L*	54.4 ± 1.32	48.2 ± 1.50[b]	76.9 ± 2.32[a]	37.5 ± 1.55[c]
	Serum Calcium, *mmol/L*	2.42 ± 0.01	2.41 ± 0.01	2.43 ± 0.02	2.41 ± 0.02
	Serum Phosphate, *mmol/L*	1.20 ± 0.01	1.22 ± 0.02	1.19 ± 0.02	1.21 ± 0.02
	Serum PTH, *pmol/L*	3.66 ± 0.08	3.33 ± 0.11	3.43 ± 0.13	4.26 ± 0.15
	Total Vitamin D Intake, *µg/d*	7.60 ± 0.40	6.31 ± 0.55	8.39 ± 0.80	7.77 ± 0.65
	Diet, *µg/d*	5.21 ± 0.19	4.74 ± 0.34[b]	5.21 ± 0.32[ab]	5.64 ± 0.34[a]
	Supplements, *µg/d*	2.38 ± 0.34	1.57 ± 0.39	3.18 ± 0.73	2.14 ± 0.50
Winter	*N*	321	92	97	93
	Age, *yrs*	21.4 ± 0.17	20.7 ± 0.24	22.6 ± 0.41	21.0 ± 0.20
	BMI, *kg/m²*	23.5 ± 0.24	22.6 ± 0.41	23.8 ± 0.52	23.6 ± 0.41
	Skin Pigmentation, *Melanin Index*	33.1 ± 0.41*	30.9 ± 0.31[b]	28.1 ± 0.27[c]	37.8 ± 0.57[a]
	Serum 25(OH)D, *nmol/L*	38.4 ± 1.08*	33.3 ± 1.60[b]	52.4 ± 2.00[a]	29.4 ± 1.53[c]
	Serum Calcium, *mmol/L*	2.40 ± 0.01*	2.39 ± 0.01[ab]	2.38 ± 0.01[b]	2.42 ± 0.01[a]
	Serum Phosphate, *mmol/L*	1.21 ± 0.01*	1.26 ± 0.02	1.17 ± 0.02	1.22 ± 0.02
	Serum PTH, *pmol/L*	4.23 ± 0.11	3.96 ± 0.17	4.14 ± 0.20	4.66 ± 0.21
	Total Vitamin D Intake, *µg/d*	7.24 ± 0.44	5.63 ± 0.51	7.48 ± 0.76	7.59 ± 0.76
	Diet, *µg/d*	4.39 ± 0.16	4.18 ± 0.29	4.29 ± 0.23	4.59 ± 0.31
	Supplements, *µg/d*	2.85 ± 0.41	1.45 ± 0.41	3.20 ± 0.72	3.01 ± 0.66

[1]Values are means ± SE. Means in a row with superscripts without a common letter differ, P<0.05. *Different from fall, P<0.05.
[2]Total sample includes participants of East Asian, European and South Asian ancestry, as well as individuals of other ancestry (see text).

Table 3-2: Proportion of individuals at different vitamin D concentration thresholds for both the fall and winter visits, stratified by ancestry.

Visit	nmol/L	East Asian	European	South Asian	Total Sample
		n (%)	*n* (%)	*n* (%)	*n* (%)
Fall	<25	2 (2)	0 (0)	17 (18)	23 (6)
	≥25<50	63 (61)	12 (11)	62 (65)	153 (44)
	≥50<75	30 (29)	48 (44)	13 (14)	109 (31)
	≥75	9 (8)	50 (45)	3 (3)	66 (19)
Winter	<25	29 (33)	6 (7)	46 (50)	94 (29)
	≥25<50	52 (57)	40 (41)	40 (43)	146 (46)
	≥50<75	9 (10)	42 (43)	5 (5)	68 (21)
	≥75	2 (2)	9 (9)	2 (2)	13 (4)

Table 3-3: Multiple linear regression analysis evaluating the effect of relevant predictors on 25(OH)D concentrations during the fall and the winter.[1,2]

Models and Predictors	Unstandardized Coefficients		Standardized Coefficients	P-values	Correlations	
	b	St. error	beta		bivariate	partial
Fall I BMI, kg/m^2	-0.0029	0.0023	-0.0629	0.202	-0.029	-0.069
Sex, *M or F*	0.0199	0.0202	0.049	0.326	0.106	0.053
Skin Pigmentation, *Melanin Index*	-0.0095	0.0013	-0.3561	<0.001	-0.319	-0.37
Sun Exposure, *>30 minutes/d*	0.0705	0.0199	0.1791	<0.001	0.16	0.189
Dietary Vitamin D, *μg/d*	0.0036	0.0028	0.0643	0.191	0.131	0.071
Supplemental Vitamin D, *μg/d*	0.0087	0.0015	0.2782	<0.001	0.226	0.296
Year, *1 or 2*	-0.0544	0.021	-0.1299	0.010	-0.051	-0.139
Winter I BMI, kg/m^2	-0.003	0.0025	-0.061	0.222	-0.036	-0.069
Sex, *M or F*	-0.0111	0.0229	-0.0247	0.628	0.066	-0.028
Skin Pigmentation, *Melanin Index*	-0.0085	0.0015	-0.2876	<0.001	-0.229	-0.311
Dietary Vitamin D, *μg/d*	0.0162	0.0038	0.2103	<0.001	0.232	0.235
Supplemental Vitamin D, *μg/d*	0.0122	0.0015	0.4162	<0.001	0.363	0.427
Year, *1 or 2*	0.0214	0.0232	0.0449	0.358	0.069	0.052
Winter II BMI, kg/m^2	-0.0006	0.0019	-0.0116	0.755	-0.036	-0.018
Fall Serum 25(OH)D, *nmol/L*	0.0057	0.0004	0.6383	<0.001	0.7	0.671
Sex, *M or F*	-0.017	0.017	-0.0376	0.320	0.066	-0.057
Skin Pigmentation, *Melanin Index*	-0.0011	0.0012	-0.0373	0.355	-0.229	-0.053
Dietary Vitamin D, *μg/d*	0.0122	0.0028	0.1588	<0.001	0.232	0.238
Supplemental Vitamin D, *μg/d*	0.0082	0.0011	0.279	<0.001	0.363	0.384
Year, *1 or 2*	0.0465	0.0173	0.0974	0.008	0.069	0.151

[1] For the winter, 2 models are presented, with the latter showing the effect of adding the baseline fall serum 25(OH)D concentrations to the model.

[2] 25(OH)D concentrations were log-transformed prior to the analysis.

3.5 Discussion

Our study found that there are large seasonal changes in 25(OH)D concentrations in young adults of diverse ancestry living in the Toronto area. We observed the largest decrease in Europeans (24.5 nmol/L), followed East Asians (14.9 nmol/L) and South Asians (8.1 nmol/L). The decline in 25(OH)D can be explained by the lack of UVR for cutaneous vitamin D synthesis during the winter months. The absence of endogenous vitamin D synthesis during the winter was apparently not compensated by increased vitamin D intake. In fact, we found that vitamin D intake was higher in the fall than in winter, and this was mainly driven by the lower dietary vitamin D intakes during the winter (Table 3-1). In contrast, the vitamin D intake from supplements increased slightly during the winter (except in the East Asian group), but it did not appear to make up for the lack of cutaneous synthesis. We also observed that the wintertime decrease in 25(OH)D levels was correlated with fall 25(OH)D concentrations (Figure 2). This was not surprising, given the absence of endogenous vitamin D synthesis, the overall decrease in vitamin D intake, and the half-life characteristics of 25(OH)D (Jones, 2008; Vieth, 1999).

Our results are consistent with previous studies reporting seasonal changes in serum 25(OH)D in Canadians (Liu et al., 1997; Rucker et al., 2002; Sloka et al., 2009; Vieth et al., 2001). Liu *et al.* (1997) examined 25(OH)D concentrations in adults living in long-term residences in Toronto, finding that 38% had 25(OH)D below 40 nmol/L in the fall compared to 60% in the spring (Liu et al., 1997). Vieth *et al.* (Vieth et al., 2001) observed seasonal differences in their sample of young white women from Toronto: in that study, mean summer 25(OH)D was 76 nmol/L, with a decrease to 58 nmol/L during the winter. Rucker *et al.* (Rucker et al., 2002) also observed seasonal fluctuations in serum 25(OH)D in western Canadians, with a drop in 25(OH)D from 71.6 nmol/L in the summer to 57.3 nmol/L in winter. Sloka *et al.* (2009) studied pregnant women in Newfoundland and Labrador and found that mean 25(OH)D concentrations were 52.1 nmol/L in winter (January to March), compared to 68.6 nmol/L in summer (July to September) (Sloka et al., 2009). Vieth recently proposed that the surprising increased risk of prostate and pancreatic cancers reported in people with higher serum 25(OH)D at higher latitudes is attributable to a greater seasonal amplitude of serum 25(OH)D with higher latitude (Vieth, 2009). The present results are consistent with that perspective, because the greatest seasonal variability in both

relative and absolute fluctuations in 25(OH)D occurred in the participants with the highest summertime levels.

The recent Canadian Health Measures Survey (CHMS) examined 25(OH)D levels in a representative sample of Canadians aged 6 to 79 years from 2007 to 2009 (Langlois et al., 2010b). For those aged 20-39 years, there was a small but statistically significant seasonal effect on 25(OH)D concentrations, from a mean of 69 nmol/L measured during the months with UVB (April to May) to a mean of 60 nmol/L in the months lacking UVB (November to March) (Langlois et al., 2010b). It is important to note that the CHMS survey did not measure seasonal changes in 25(OH)D in the *same* individuals. The CHMS also examined ethnicity and reported that the mean 25(OH)D concentration among white Canadians aged 20-39 years was 70 nmol/L, compared to 48 nmol/L for Canadians of Other ancestry (a group that included anyone who did not report their ethnicity as "White" due to small sample size of other ethnicities) (Langlois et al., 2010b). The CHMS survey reported that the age group with the lowest 25(OH)D concentrations was adults aged 20 to 39 years, indicating that young adults are at greatest risk for vitamin D inadequacy.

We also explored in detail the main factors influencing serum 25(OH)D levels during the fall and the winter. Fall levels of 25(OH)D were primarily influenced by skin pigmentation and supplemental vitamin D intake. Our data indicate that for the fall, one unit increase in melanin index is associated, on average, with a 1% decrease in 25(OH)D concentrations. Similarly, an increase of 5 µg/d (200 IU/d) in vitamin D intake from diet and supplements was associated with increases in serum 25(OH)D of 1.8% and 4.35%, respectively. Wintertime 25(OH)D levels were primarily determined by vitamin D intake and skin pigmentation. The significant association of pigmentation with winter 25(OH)D can be explained by the increased cutaneous synthesis in individuals with lower skin melanin levels during the fall. When baseline fall 25(OH)D concentrations were included in the winter linear regression model, fall serum concentration was the best predictor for the winter variance, and skin pigmentation was no longer significant. When taking into account fall 25(OH)D levels, an increase of 5 µg/d (200 IU/d) in wintertime vitamin D intake from diet and supplements was associated with increases in 25(OH)D levels of 6.1% and 4.1%, respectively. We did not observe significant associations for sex and BMI. Previous studies have shown that BMI is inversely associated with 25(OH)D levels (Arunabh et al., 2003;

Wortsman et al., 2000). However, our sample of young adults had a relatively narrow range of BMI values (mean= 23.2, SD=4.2), so it is not so surprising that we failed to identify a significant effect.

In the present study, vitamin D intake was a major predictor of 25(OH)D concentrations in both seasons, which is consistent with previous studies. Brock et al. studied predictors of 25(OH)D in a large cohort of elderly men (n=1357) and women (n=1264) from the U.S., and they observed that one of the major modifiable predictors of low 25(OH)D concentrations was vitamin D intake from diet and supplements (Brock et al., 2010a). A recent study of Finnish middle aged male smokers (n=2271) found that 25(OH)D concentrations were associated with vitamin D intake in summer and winter (Brock et al., 2010b). Studies of other European and high latitude populations also found dietary vitamin D intake to be a robust predictor of 25(OH)D levels (Burgaz et al., 2007; Chapuy et al., 1997; Huotari and Herzig, 2008). Although direct vitamin D intake measurements were not conducted in the National Health and Nutrition Examination Survey (NHANES) surveys in the U.S., milk consumption was found to be a significant predictor of 25(OH)D (Looker et al., 2008). In the present study, vitamin D intake was a significant predictor of 25(OH)D during both fall (supplemental vitamin D intake only) and winter (both supplemental and dietary vitamin D intake) visits, further indicating the key role that vitamin D intake plays in maintaining circulating 25(OH)D concentrations at high latitudes.

In our sample, mean vitamin D intake met or exceeded the recommended Adequate Intake (AI) of 5 µg/d (200 IU/d) for this age group (Standing Committee on the Scientific Evaluation of Dietary Reference Intakes, 1997) in the total sample and each ancestral group at both visits. However, we observed low 25(OH)D concentrations in many individuals, particularly during the winter. Of the 183 individuals who reported an intake in excess of 5 µg/d in the fall, 78% had serum 25(OH)D below 75 nmol/L, 45% had concentrations below 50 nmol/L and 4% had concentrations below 25 nmol/L. At the winter visit, 92%, 62%, and 10% of the 146 individuals who reported vitamin D intake greater than 5 µg/d had serum 25(OH)D concentrations below 75, 50 and 25 nmol/L, respectively. In agreement with previous studies, our findings show that current Health Canada recommendations are insufficient for maintenance of optimal serum 25(OH)D, particularly in winter (Gozdzik et al., 2008; Vieth et al., 2001; Whiting et al., 2007). The Institute of Medicine (IOM) is currently conducting a review of the Dietary References

Intakes for vitamin D, funded jointly by the U.S. and Canadian governments, with a final report expected by the end of 2010 (Health Canada, 2009).

In 2007, the Canadian Cancer Society announced new vitamin D guidelines, recommending that "adults living in Canada should consider taking vitamin D supplementation of 1,000 international units (IU) a day (25 µg/d) during the fall and winter" and that "adults at higher risk of having lower vitamin D levels should consider taking vitamin D supplementation of 1,000 IU/day all year round. This includes people who are older, with dark skin, who don't go outside often, and who wear clothing that covers most of the skin" (Canadian Cancer Society, 2007). Our study lends support to the recommendations of the Canadian Cancer Society, which take into account risk factors for vitamin D insufficiency (seasonality, UVR exposure and skin pigmentation). Our results indicate that higher vitamin D intakes are necessary to maintain adequate 25(OH)D concentrations year-round in young adults, particularly among those of non-European ancestry. It is important to note that mean total vitamin D intake in South Asians was higher than in East Asians at both seasons, and higher than Europeans during the winter. However, South Asians had the lowest mean 25(OH)D concentrations during both seasons (37.5 ± 1.5 nmol/L in the fall and 29.4 ± 1.5 nmol/L in the winter), most probably due to the fact that participants of South Asian ancestry had, on average, darker skin pigmentation (Table 3-1), which negatively influences endogenous vitamin D synthesis during the summer months. Given the very low 25(OH)D concentrations observed in some young adults in this study, it is possible that intakes in excess of 25 µg/d (1,000 IU/d) may be needed to raise and maintain their 25(OH)D above 75 nmol/L year-round. Further work is required to elucidate the vitamin D intake needed to achieve and maintain optimal vitamin D levels in individuals of different ancestry.

Interestingly, we found study year to be a modest but significant predictor of 25(OH)D concentrations. This association can be explained in part by differences in incident solar radiation in the Toronto area over the 2007 and 2008 summers. Based on preliminary data from the UVR monitoring station at Toronto (Station 65), the average daily spectral irradiation (KJ·m^{-2}·nm^{-1}) was higher for almost all measured wavelengths in 2007 compared to 2008 for the months of March to October (300 nm, 305nm, 305 nm, 310nm, 315 nm and 325 nm; the amount measured at 295 nm was higher in 2008 than 2007). The sum of the average daily spectral

irradiation between the months of March and October for the UVB spectrum (290-320 nm) was higher in 2007 than 2008 (11.7 vs. 11.1 $kJ \cdot m^{-2} \cdot nm^{-1}$) (Environment Canada, 2010b). Furthermore, during the summer of 2008, there was far more precipitation and far fewer sunny days. Precipitation between the months of March to September of 2007 was only 245 mm while during the same time in 2008, the precipitation was 410 mm (Environment Canada, 2010a).

This study has a number of limitations. The sample consisted mostly of young adults that were recruited at a university setting, and may not reflect the general population of young people either in the Toronto area or elsewhere in Canada. However, while this study featured only three well-represented ancestral groups, it is more representative of the population diversity in the Greater Toronto Area than previous studies. The 2006 Canadian census found that visible minorities represent 43% of the population of metropolitan Toronto, the two largest groups being those of South Asian and Chinese ancestry (Chui et al., 2008). More than half (54%) of all the South Asians resident in Canada live in the Toronto area, where they represent nearly one-third (32%) of all visible minorities, and comprise 14% of Toronto's population (Chui et al., 2008). In 2006, individuals of Chinese ancestry comprised almost a quarter (22%) of all visible minorities and 10% of the total population of the city of Toronto. Nevertheless, our sample only encompassed a subset of the population diversity found in Canada, and more particularly, in Canadian metropolitan areas (Chui et al., 2008). Although in our study we considered many of the factors known to affect vitamin D levels (vitamin D intake, skin pigmentation, seasonality, UVR exposure, BMI), we did not include some predictors that have been associated with vitamin D levels in previous studies (for example, smoking).

In conclusion, we report that low levels of serum 25(OH)D, the main indicator of vitamin D status, are widespread in healthy young adults of diverse ancestry living in the GTA, particularly in individuals of non-European ancestry. The main predictors of vitamin D status are vitamin D intake (particularly from supplements) and skin pigmentation. Additionally, there is a substantial decline in vitamin D levels from the fall to the winter, and this drop is proportional to baseline vitamin D levels.

3.6 Acknowledgements

3.7 References

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4   Association of Vitamin D Binding Protein (VDBP) Polymorphisms and Serum 25(OH)D Concentrations in a Sample of Young Canadian Adults of Different Ancestry

4.1   Abstract

Variants of the vitamin D binding protein (*VDBP*) gene appear to be associated with levels of the main circulating vitamin D metabolite, 25-hydroxyvitamin D [(25(OH)D]. We examined the associations between the common variants of the *VDBP* (*GC*) gene and concentrations of 25(OH)D in a sample of young Canadian adults of East Asian, European and South Asian ancestry, taking also into account the effect of vitamin D intake, skin pigmentation, sex, BMI, sun exposure and seasonality. Three hundred and fifty-one (351) healthy young adults were genotyped for two single nucleotide polymorphisms (SNPs), T436K (rs4588) and D432E (rs7041). After controlling for relevant predictor variables in multiple regression models, both T436K and D432E showed significant associations with 25(OH)D concentrations in the total sample. However, the strength of association differed by ancestry and season, strongly pointing to the presence of gene-environment interaction effects. Overall, the most important predictors of 25(OH)D concentrations are skin pigmentation (particularly in the fall, when it explains approximately 14% of the variation in 25(OH)D levels), and vitamin D intake (particularly in the winter, when it explains approximately 22% of the variation in 25(OH)D levels). In contrast, the two *GC* polymorphisms have substantially smaller, although significant, effects on 25(OH)D concentrations (each explaining approximately 2% of the variation).

4.2   Introduction

Low levels of vitamin D have been linked to various common chronic, autoimmune and infectious diseases, including cancer, cardiovascular disease, diabetes, multiple sclerosis, rheumatoid arthritis and tuberculosis (Garland et al., 2006; Giovannucci, 2005; Giovannucci et al., 2008; Holick, 2005a; Holick, 2005b; Holick, 2007; Lappe et al., 2007; Liu et al., 2006; Zasloff, 2006). The main circulating metabolite of vitamin D, 25-hydroxyvitamin D [25(OH)D], is the primary biomarker of vitamin D status because it gives a measure of vitamin D from all available sources, including diet, sun exposure and supplements (Holick, 2007). Once in the circulation, vitamin D and its metabolites are bound to vitamin D binding protein (VDBP), also known as the group-specific component (or GC), which is the main protein involved in vitamin D transport (Speeckaert et al., 2006). VDBP binds with particularly high affinity to 25-hydroxyvitamin D_3 [25(OH)D], with ~99% of circulating 25(OH)D being bound to VDBP (Nykjaer et al., 1999; Rowling et al., 2006). VDBP has a short half-life ranging from 2.5 to 3

days, while the half-life of 25(OH)D ranges from one to two months (Safadi et al., 1999; Sinotte et al., 2009), suggesting that the protein and its ligand are differentially regulated.

The *GC* gene (*VDBP,* NCBI Gene ID#2638*)* is located on chromosome 4 (4q11-13) and encodes a single chain polypeptide of 474 amino acid residues that consists of 3 domains and is part of the albumin family (Christiansen et al., 2007). Cleavage of a 16-residue signal peptide yields a protein of 458 amino acids that is secreted by the liver in much larger molar amounts than required for release of 25(OH)D. The size difference between the nascent peptide and the secreted protein accounts for the discordance between current standardized molecular numbering of genetic variants and historical amino acid numbering of peptide polymorphisms. The *GC* gene is highly variable in human populations (Arnaud and Constans, 1993). There are two well-studied non-synonymous single nucleotide polymorphisms (SNPs) in the *GC* gene, p.T436K (formerly T420K, NCBI rs# rs4588), which results in a C-to-A transversion [threonine (ACG) to a lysine (AAG)], and p.D432E (formerly D416E, NCBI rs# rs7041), which results in a T-to-G transversion [an aspartic acid (GAT) to a glutamic acid (GAG)] (Abbas et al., 2008; Lauridsen et al., 2001). The combination of these two polymorphisms gives rise to three major electrophoretic variants (or isoforms) that are distinguished based on their amino acid composition and glycosylation patterns: GC-1f, GC-1s and GC-2 (Lauridsen et al., 2004). GC-1f is the wild-type allele and is a combination of aspartic acid at position 432 and threonine at 436; GC-1s has a combination of glutamic acid at position 432 and threonine at 436, and GC-2 has a combination of aspartic acid at position 432 and lysine at 436. The two variants at positions 432 and 436 are in strong Linkage Disequilibrium (LD), and the combination of glutamic acid at position 432 and lysine at 436 is typically not observed in human populations. The three common electrophoretic variants (GC-1f, GC-1s and GC-2) combine to form six possible diplotypes: 1f-1f, 1f-1s, 1s-1s, 1s-2, 1f-2, and 2-2 (Lauridsen et al., 2004).

Previous studies have shown that there are differences in binding affinity to 25(OH)D among the three GC variants, with GC-1f having the highest affinity for 25(OH)D, GC-2 having the lowest affinity and GC-1s having intermediate affinity (Chun et al., 2008; Engelman et al., 2008; Lauridsen et al., 2001). Arnaud and Constans (Arnaud and Constans, 1993) demonstrated that the affinity of the GC-1f allele was four times higher than that of the GC-2 allele and double that of the GC-1s allele. It has been suggested that these differences in affinity are due to differential

glycosylation in the three GC alleles (Borges et al., 2008; Lauridsen et al., 2001; Ravnsborg et al.), but the biologic importance of the binding affinity is uncertain, since there is a huge excess of binding sites in relation to physiologic 25(OH)D concentrations. Borges *et al*., (2008) reported that the GC-1f and GC-1s alleles are modified with a trisaccharide and a disaccharide at threonine 436, while the GC-2 allele (with no acceptor threonine residue) is only modified with the disaccharide at an adjacent threonine and does not contain the trisaccharide (Borges et al., 2008). A more recent study by Ravnsborg *et al*., (2010) has confirmed the structure of the O-linked glycan. However, these authors reported no indication of an independent disaccharide in addition to the well confirmed trisaccharide in the VDBP peptide, suggesting that the glycosylation difference amongst the VDBP isoforms may be due to the presence (GC-1f and GC-1s) or absence (GC-2) of the trisaccharide alone at T436 (Ravnsborg et al. 2010). From the glycosylation differences between the alleles, it has been suggested that the GC-2 allele would be metabolized faster. This was supported by Kawakami *et al* (1981), who injected radioactively labelled VDBP into healthy young men and observed that the metabolic rate was higher in GC2-2 than in GC1-1 individuals (Kawakami et al., 1981). The more rapid metabolism of the GC-2 variant was further supported by studies showing that, while the concentration of VDBP in plasma ranges between 200 to 500 mg/L, GC1-1 individuals have higher concentrations of VDBP than GC1-2 individuals, and the lowest concentrations are found in GC2-2 individuals (Lauridsen et al., 2001).

GC polymorphisms show substantial allele frequency variation in groups of different ancestry. Kamboh and Ferrell (1986) reported a geographical cline in GC-1f and GC-1s allele frequency (Kamboh and Ferrell, 1986), with the wild-type GC-1f allele found at higher frequencies in African and East Asian populations compared to their European, Middle Eastern and South Asian counterparts (Engelman et al., 2008; Kamboh and Ferrell, 1986). Conversely, the GC-1s variant is found in the highest frequencies amongst the European, Middle Eastern and South Asian populations and has the lowest frequencies in African populations, particularly those of equatorial, west and south Africa (Kamboh and Ferrell, 1986). The GC-2 variant is found at relatively lower allele frequencies throughout the studied populations in comparison to the GC-1f and GC-1s alleles (Speeckaert et al., 2006). It has been suggested that the frequency of the GC alleles is associated with skin pigmentation and intensity of sun exposure, and that the higher

frequency of the GC-1f allele amongst more darkly pigmented individuals may be explained by its altered vitamin D metabolism (Kamboh and Ferrell, 1986; Speeckaert et al., 2006).

A number of recent studies have reported associations between *GC* genotypes and serum 25(OH)D concentrations (Engelman et al., 2008; Lauridsen et al., 2005; Sinotte et al., 2009). The goal of this study was to examine the association between the common genetic variation of the VDBP protein and serum 25(OH)D concentrations in a sample of young Canadian adults of diverse ancestry and to assess the relative contributions of these markers and other factors (vitamin D intake, skin pigmentation, UV exposure) relevant to vitamin D status.

4.3   Materials and Methods

4.3.1   Participants

Participants for this study were recruited from the Greater Toronto Area. Recruitment took place at the University of Toronto Mississauga (Ontario, Canada) campus (43°N) during the fall of both 2007 and 2008. Detailed description of the study population and data collection are described elsewhere (Gozdzik et al., 2010). This study was approved by the University of Toronto Health Sciences Research Ethics Board, and all participants provided written informed consent.

The exclusion criteria for the present study were: age (<18 and >35 years old); diagnosis of kidney/liver disease or other active or chronic diseases potentially affecting vitamin D metabolism or absorption (e.g., inflammatory bowel disease, cystic fibrosis etc.) (Lo et al., 1985); use of medications that affect vitamin D metabolism (including corticosteroids and anticonvulsants, etc.) (Holick, 2007; Zhou et al., 2006). Use of vitamin D supplements was not a criterion for exclusion, since we were interested in evaluating the use of vitamin D supplements and the effect of supplementation on 25(OH)D levels.

4.3.2   25(OH)D Measurement

An aliquot of whole blood was centrifuged and the serum fraction decanted and stored at -80°C. Serum 25-hydroxyvitamin D [25(OH)D] was measured by liquid-chromatography tandem mass spectrometry (LC-MS/MS). A 200 μL aliquot of serum was spiked with 50 μL of d_6-25-hydroxyvitamin D_3 internal standard and extracted with 1 mL of methyl-t-butyl ether. The upper

ether phase was transferred to a clean borosilicate tube and the solvent evaporated under a stream of nitrogen gas at 40°C. The residue was dissolved in 1 mL of 80% methanol and 1 mL of heptane was added. The methanol phase was transferred into clean borosilicate tubes and evaporated to dryness under a stream of nitrogen gas at 40°C. The residue was dissolved in 100 μL of 50% methanol and transferred into a high-performance liquid chromatography (HPLC) auto-sampler vial. A 20 μL aliquot was then directly injected into the LC-MS/MS instrument.

The HPLC was carried out using an Agilent Technologies 1200 series chromatographic system in linear gradient mode at a flow rate of 0.80 mL/min with an Eclipse C8 column employing a mobile phase consisting of methanol-water (37/63 v/v) increasing to 100% methanol over four min and maintained at 100% methanol for one min. The column was re-equilibrated with methanol-water (37:63) for 1 minute. The column temperature was maintained at 50°C.

The API 5000 (Applied Biosystems/Sciex, Concord, ON, Canada) mass spectrometer was equipped with an atmospheric pressure chemical ionization (APCI) source and operated in the positive mode. The m/z 401.4 → 383.4 ion-transition was monitored for 25-hydroxyvitamin D_3 estimation, m/z 417.4→ 399.4 for 24,25-dihydroxyvitamin D_3, m/z 413.4 → 395.4 for 25-hydroxyvitamin D_2, and m/z 407.5 → 389.4 for d_6-25-hydroxyvitamin D_3 internal standard. The dwell time per transition is set to 50 ms.

Analyst software (version 1.4.2) was used for data acquisition, peak-area integration and comparison against the standard curve used to calculate concentration of unknowns. The standard curve was derived from calibrators analyzed within the same analytical run.

The LC-MS/MS method used in this study was extensively validated. The between-day coefficient of variation (CV) ranged from 3% to 6.9% for 25(OH)D_3 (depending on the mean serum concentration of the samples), and from 3.1% to 10.4% for 25(OH)D_2. The within-day CV was 5.11% for 25(OH)D_3 and 7.19 for 25(OH)D_2. The Pearson (r^2) correlations comparing the LC-MS/MS estimates with the certified concentrations of the National Institute of Standards and Technology (NIST) Standard Reference Material 972 were > 99.9% for both 25(OH)D_3 and 25(OH)D_2. Similarly, the r^2 value for the comparison of the 25(OH)D_3 concentrations of the Vitamin **D E**xternal **Q**uality **A**ssessment **S**cheme (DEQAS) samples for the LC-MS/MS method used in this study and the mean of the DEQAS LC-MS results was 97.12%. Finally, the r^2

values for the comparison of estimates obtained with this LC-MS/MS method and estimates using the DiaSorin radioimmunoassay (RIA) method (N=50) and the DiaSorin Liaison method (N=45) were 89.8% and 90.6%, respectively.

A subset of samples (n=10) were assayed as blind duplicates. The coefficient of variation was 2.3% for total serum 25(OH)D (arithmetic sum of $25(OH)D_3$ and $25(OH)D_2$ concentrations). Elsewhere, the term 25(OH)D refers to the summed total of the two vitamers.

4.3.3   Anthropometric Measurements

Study staff measured weight and height for each participant, and body mass index (BMI=kg/m^2) was calculated.

4.3.4   Ancestry

The participants completed a personal questionnaire that assessed ancestry. The questionnaire asked detailed questions regarding the places of birth, ethnicity, native language, migration history, and present residence of each participant, and we also included questions about parental and grandparental places of birth, ethnicity and native language.

4.3.5   Dietary Assessment

Daily intake of vitamin D from dietary and supplemental sources was estimated using a Food Frequency Questionnaire (FFQ). We have previously validated this FFQ for assessment of vitamin D and calcium intake in young adults of diverse ancestry (Wu et al., 2009). Subjects were provided with portion size aids and recorded their food, beverage and supplement intake. The FFQs were analyzed with the computer program Food Processor (version 8.0 and its revisions, ESHA Research Inc., Salem OR, which included the 2007 Canadian Nutrient File from Health Canada); Canadian foods were always chosen where Canadian fortification was different from elsewhere (e.g., margarine and breakfast cereals).

4.3.6   DNA Collection and Genetic Analysis

A sample of each participant's blood was collected in a 4-mL EDTA tube. Collected blood was stored at -20˚C until DNA extraction took place. Genomic DNA extraction was carried out using the E.Z.N.A. Blood DNA Midi Kit (Omega Bio-Tek, Georgia, United States).

The p.D432E and p.T436K SNPs were genotyped with phase assignment based on allele-specific amplification of the p.T436K site followed by restriction endonuclease digestion of the p.D432E site. Allele-specific amplification was carried out in a 20μL reaction mixture containing 1X PCR buffer (Qiagen), 0.2 mM each of dNTPs, 50 ng genomic DNA, 0.5U HotStarTaq™ (Qiagen) and 0.3-1 μM of the following primers: 5'-GGCATGTTTCACTTTCTGATCTC-3' (forward), 5'-ACCAGCTTTGCCAGT<u>A</u>CCG-3' (wild-type reverse) and 5'-GCAAAGTCTGAGTGCTTGTTA<u>TG</u>CAGCTTTGCCAGTT<u>G</u>CT-3' (mutant reverse). The underlined bases in the primer sequences are mismatched nucleotides introduced to avoid cross priming. After the initial DNA denaturation and HotStarTaq™ activation at 95°C for 15min, the amplification went through 35 cycles of denaturation at 94°C for 20 sec, annealing at 58°C for 20 sec and extension at 72°C for 20 sec with an increment of 1 sec after each subsequent cycle and a final extension at 72°C for 5 min. Eight microliters of the amplified products were run in a 2% NuSieve gel containing ethidium bromide and then visualized using UV illumination. The p.T436K wild-type allele produced a 246bp band and the mutant allele a 270bp band. Another 8μl of amplicon was digested with *HaeIII* (New England BioLabs) at 37°C overnight and the digested products were analyzed by repeat electrophoresis in 2% NuSieve gel. The DNA bands containing the wild-type p.D432E allele remained unchanged while those with p.D432E mutant allele were cut, producing 221bp fragments.

4.3.7   Sun/UVR Exposure

Each participant completed a UVR Exposure questionnaire that assessed their sun exposure habits, use of sunscreen and extent of body exposure. For the present study we used the dichotomous variable of "average time spent outside between 9 a.m. to 5 p.m. during the summer months" as an estimate of summertime sun exposure (< or > 30 minutes per day).

4.3.8   Skin Pigmentation

Constitutive skin pigmentation (pigmentation in unexposed areas of the skin) was measured in the inner upper arm using a narrow-band reflectometer (Dermaspectrometer, Cortex Technology, Hadsund, Denmark) (Shriver and Parra, 2000). Melanin Index values calculated using the Dermaspectrometer typically range from the low 20s to almost 100, with individuals with the

lightest skin pigmentation having the lowest values and those with the darkest pigmentation having the highest (Shriver and Parra, 2000)

4.3.9   Statistical Analyses

Deviations from Hardy-Weinberg proportions were evaluated using a chi-square test for each SNP (Rodriguez et al., 2009). Multiple regression was used to test the association of each genetic marker with serum 25(OH)D concentrations in both the fall and the winter. In addition to the relevant genetic markers, other predictors were also added to the multiple regression model because of their potential effect on 25(OH)D concentrations (sex, BMI, total vitamin D intake, UV exposure and skin pigmentation). Outliers and influential points were assessed using normality tests (Shapiro-Wilk and Anderson-Darling) and Cook's distance, respectively. When outliers and/or influential points were identified, the test was run with and without the outliers, and the results compared. Results are presented with outliers and influential points removed.

Multiple linear regression was used to assess the contribution of each of the genetic markers (the two SNPS, D432E and T436K and the GC diplotypes) on serum 25(OH)D concentration in both the fall and the winter visit. We also considered several other predictor variables that may influence serum 25(OH)D. In the fall regression models, log serum 25(OH)D was the dependent variable and the predictors were BMI, sex, skin pigmentation, reported sun exposure, vitamin D intake, year and genotype or diplotype. For the winter visit, the dependent variable was log winter serum 25(OH)D concentration, while predictors were BMI, sex, skin pigmentation, reported vitamin D intake, calendar year and genotype/diplotype. The goal of the regression was the calculation of the partial r^2 values for each predictor variable. The partial r^2 statistic reflects the amount of variation captured by each predictor in the model, while controlling for the effect of all other predictors.

In the Multiple Regression, the dependent variable 25(OH)D had a significantly skewed distribution and was log transformed before the analysis. All statistical tests were performed with SPSS (Version 15.0, SPSS Inc., 2006) and Minitab (Version 15.1.1, Minitab Inc., 2007).

A power analysis using the software G*Power (Version 3) (Erdfelder et al., 1996) indicated that with a significance level of $\alpha=0.05$, the multiple regression analysis (sample size = 351 and up to

13 predictors) has >99% power to detect a medium effect (f^2=0.15) for the fall 25(OH)D concentration and, and for the winter values (with a sample size of 321 individuals and up to 13 predictors), the analysis has >99% power to detect a medium effect (f^2=0.15). The power was reduced for the analyses in the individual population groups (with a sample size of ~100 individuals, and up to 13 predictors), yielding 64-78% power for detecting a medium effect (f^2=0.15), at a significance level of α=0.05, but the power to detect a large effect (f^2=0.35) was >98%.

4.4 Results

4.4.1 Sample Characteristics, 25(OH)D Concentrations and Associated Variables

Genetic data and corresponding serum 25(OH)D measurements were available for 351 subjects (225 females, 126 males) in the fall and 322 (202 females, 120 males) in the winter. Participants were grouped into broadly defined ancestral subsets based on the geographic origin of the subjects, their parents and grandparents. For example, individuals who stated that their geographic origin was from China, Japan or Korea were classified as East Asian, while those who reported origin from India or Pakistan were grouped as South Asian. The majority of the participants were of African, East Asian, European, Middle Eastern or South Asian ancestry. Individuals who reported being of multiple ancestries were placed into a subgroup designated as "Other". Because of the small sample size, the African (N=12), Middle Eastern (N=16) and "Other" (N=13) subgroups were not included in the subgroup analyses.

Table 4-1 summarizes the relevant characteristics for the total sample and the three population subgroups [East Asian (N=104), European (N=110) and South Asian (N=95)]. Mean age at fall visit for the total sample was 21.1 ± 3.0 years and mean BMI was 23.2 ± 4.2 kg/m^2. Mean serum 25(OH)D for the entire fall sample was 54.4 nmol/L (SD= 24.7 nmol/L; Range= 12.8-150.1 nmol/L). Analyzed by ancestral subgroup, mean 25(OH)D concentrations were 48.2 ± 15.3 nmol/L, 76.9 ± 24.3 nmol/L, and 37.5 ± 15.1 nmol/L for East Asian, European and South Asian subgroups, respectively. In the winter visit, mean age was 21.4 ± 3.0 years and mean BMI was 23.5 ± 4.4 kg/m^2. The mean winter 25(OH)D concentration for the full sample was 38.4 nmol/L (SD= 19.4 nmol/L; Range: 8.51-127.0 nmol/L), and ancestral subgroup means were 33.3 ± 15.4

nmol/L, 52.4 ± 19.7 nmol/L and 29.4 ± 14.7 nmol/L for the East Asians, Europeans and South Asians, respectively. Mean fall vitamin D intake for the full sample was 304 ± 298 IU/day (7.60 ± 7.45 µg/day), and mean winter vitamin D intake 290 ± 318 IU/day (7.25 ± 7.95 µg/day).

Significant sex differences were found for: BMI, 25(OH)D and vitamin D intake. Significant calendar year differences were found for: 25(OH)D, skin pigmentation and vitamin D intake (one way ANOVAs, data not shown). To control for these effects, year and sex were included as predictors in the multiple regression analyses.

4.4.2 Vitamin D Binding Protein (VDBP) Polymorphisms, Ancestry and 25(OH)D Levels

Table 4-2 shows the allele and genotype frequencies for both the D432E (rs7041) and the T436K (rs4588) SNPs in the total sample, and in each of the three ancestral subgroups. Table 4-2 also includes the frequencies of the GC-1f, GC-1s and GC-2 isoforms, as well as the resulting diplotype combinations. There was no significant deviation from Hardy Weinberg equilibrium for either SNP, whether looking at the full sample or ancestral subgroups (results not shown). Table 4-3 shows mean 25(OH)D concentrations for each of the genetic markers considered in the study, stratifying the sample by ancestral subgroup and season, and also in the total sample.

Table 4-4 reports the contribution of each of the predictors to 25(OH)D levels during the fall, measured as the partial r^2. This statistic reflects the amount of variation captured by each predictor in the model, while controlling for the effect of all other predictors. For each ancestry group, three regression analyses were performed, one with each SNP (D432E and T435K) in addition to the other factors, and finally one with the GC diplotypes in addition to the other factors. Additionally, a regression was performed including the total sample so that relative contributions of each of the predictors to 25(OH)D concentrations in the models could be better understood. Table 4-5 summarizes the same results as Table 4-4 but for the winter visit.

4.4.2.1 Effect of the T436K Polymorphism on 25(OH)D Levels

The T436K polymorphism had a significant effect on 25(OH)D concentrations in the total sample, and after controlling for other relevant predictors, explained 2.3% of the variation in 25(OH)D levels in the fall and 1.9% in the winter (Tables 4-4 and 4-5). However, there were

differences in the strength of the association depending on ancestral subgroup and season. The effect of T436K was significant in the fall in both the East Asian and European samples, explaining 20.5% (p<0.001) and 7% (p<0.01) of the variation in 25(OH)D concentrations, respectively (Table 4-4). T436K also had a significant effect on wintertime 25(OH)D concentrations in the East Asian sample (r^2=0.14, p<0.01), but the effect was not significant in the European sample (Table 4-5). In the South Asian sample, neither the fall or winter 25(OH)D concentrations were significantly associated with T436K.

4.4.2.2   Effect of the D432E Polymorphism on 25(OH)D Levels

Similarly to what was observed for T436K, the D432E polymorphism was a significant predictor of 25(OH)D concentration in the total sample, and explained 1.8% and 1.6% of the variation observed in 25(OH)D levels in the fall and the winter, respectively (Tables 4-4 and 4-5). There was also evidence of heterogeneity in the strength of association of D432E with 25(OH)D concentrations, depending on ancestral subgroup and season. During the fall, D432E was significantly associated with 25(OH)D concentrations in East Asians (r^2=0.11, p<0.01) and there was evidence of a similar trend in Europeans, as indicated by the borderline p-value (r^2=0.035, p=0.061). During the winter, D432E was a significant predictor of 25(OH)D concentrations only the East Asian sample. D432E was not a significant predictor of 25(OH)D concentrations in either the fall or winter visit in South Asians.

4.4.2.3   Effect of GC Diplotypes on 25(OH)D Levels

The GC diplotypes were not significant predictors of 25(OH)D concentrations in the total sample, or any of the three subgroups examined for either the fall or winter visit (summed partial r^2 in Tables 4-4 and 4-5, individual diplotype p-values not shown). However, similar trends were observed in terms of the direction of the effects already described for the individual markers. In particular, the lowest concentrations observed in the individual population groups, as well as the global sample, correspond to the individuals with diplotype GC2-2 (Table 4-3).

4.4.3   Relative Role of Vitamin D Intake, Skin Pigmentation, Sun Exposure and GC Polymorphisms on 25(OH)D Concentrations

One of the major goals of our study was to elucidate the relative role of major factors known to influence 25(OH)D concentrations in a sample of young adults of diverse ancestry. The results

for the total sample in the fall visit (Table 4-4) indicate that skin pigmentation, total vitamin D intake, sun exposure, recruitment year and both GC polymorphisms (T436K and D432E) are significant predictors of 25(OH)D concentrations. Overall, skin pigmentation is the best predictor of fall 25(OH)D concentrations, alone accounting for between 13-14% of the variation in 25(OH)D, depending on the regression model. Total vitamin D intake is the second best predictor of fall 25(OH)D concentrations, and it alone explains approximately 9% of the variation in 25(OH)D, while sun exposure is third, accounting for approximately 3% of the variation in 25(OH)D. Comparatively, T436K accounts for 2.3% of the variation in 25(OH)D, while D432E explains about 1.8%.

In the winter regression model for the total sample (see Table 4-5), skin pigmentation, total vitamin D intake and both *GC* polymorphisms are significant predictors of 25(OH)D concentrations. Total vitamin D intake is the best predictor of wintertime 25(OH)D concentrations, and it accounts for approximately 22% of the variation in 25(OH)D levels. Skin pigmentation is also a significant predictor of 25(OH)D concentrations in the fall, accounting for approximately 10-11% of the variation. In comparison, T436K explains 1.9% of the variation in wintertime 25(OH)D concentrations, while D432E accounts for 1.6%.

4.4.4   Tables

Table 4-1: Description of the raw variables collected in the total sample, and stratified by ancestral subgroup.

Mean and standard deviation (in parenthesis) values are reported.

	East Asian	*European*	*South Asian*	*Total Sample*
Fall	(n = 104)	(n=110)	(n=95)	(n=351)
Age (yrs)	20.4 (±2.31)	22.1 (±3.97)	20.7 (±1.89)	21.1 (±2.99)
BMI (kg/m^2)	22.3 (±3.69)	23.6 (±4.97)	23.3 (±3.74)	23.2 (±4.22)
Skin Pigmentation (Melanin Index)	32.4 (±2.75)	30.4 (±2.91)	39.2 (±6.00)	34.6 (±7.30)
25(OH)D (nmol/L)	48.2 (±15.3)	76.9 (±24.5)	37.5 (±15.1)	54.4 (±24.7)
Total Vitamin D Intake (µg/day)	6.33 (5.55)	8.45 (±7.78)	7.78 (±6.35)	7.60 (±7.45)
Winter	(n = 92)	(n=97)	(n=93)	(n=321)
Age (yrs)	20.7 (±2.28)	22.6 (±4.08)	21.0 (±1.89)	21.4 (±3.00)
BMI (kg/m^2)	22.6 (±3.88)	23.8 (±5.09)	23.6 (±3.93)	23.5 (±4.38)
Skin Pigmentation (Melanin Index)	30.9 (±2.94)	28.1 (±2.63)	37.8 (±5.50)	33.1 (±7.35)
25(OH)D (nmol/L)	33.3 (±15.4)	52.4 (±19.7)	29.4 (±14.7)	38.4 (±19.4)
Total Vitamin D Intake (µg/day)	5.63 (±4.88)	7.48 (±7.53)	7.60 (±7.33)	7.25 (±7.95)

Table 4-2: Proportion of individuals with specific genotypes for the two vitamin D binding protein SNPs and the GC haplotypes for the full sample, and stratified by ancestry.

Genetic Polymorphism		East Asian N	East Asian %	European N	European %	South Asian N	South Asian %	Total Sample N	Total Sample %
D432E (rs7041)									
Alleles	G		32		59		58		49
	T		68		41		42		51
Genotypes	GG	12	12	39	35	31	33	93	27
	TG	43	41	52	47	48	50	159	45
	TT	49	47	20	18	16	17	99	28
T436K									
Alleles	A		27		28		26		26
	C		73		72		74		74
Genotypes	AA	7	67	8	7	7	7	24	7
	CA	43	41	46	41	36	38	136	39
	CC	54	52	57	52	52	55	191	54
GC Diplotypes									
Alleles	1f		44		19		24		30
	1s		30		55		52		46
	2		26		26		24		24
Diplotypes	1f-1f	20	19	9	8	10	11	52	15
	1f-1s	25	24	15	4	19	20	65	19
	1f-2	26	25	9	8	7	7	44	13
	1s-1s	11	11	37	33	27	28	86	24
	1s-2	14	14	34	31	25	26	81	23
	2-2	7	7	7	6	7	7	22	6

Table 4-3: **Serum 25(OH)D concentrations by *GC* genotype and diplotype.**

Genetic Polymorphism			East Asian		European		South Asian		Total Sample	
			Fall mean 25(OH)D (nmol/L)	Winter mean 25(OH)D (nmol/L)	Fall mean 25(OH)D (nmol/L)	Winter mean 25(OH)D (nmol/L)	Fall mean 25(OH)D (nmol/L)	Winter mean 25(OH)D (nmol/L)	Fall mean 25(OH)D (nmol/L)	Winter mean 25(OH)D (nmol/L)
Single Nucleotide Polymorphism	Rs No.	Genotype								
D432E	rs7041									
		GG	62.7	43.7	79.1	50.6	37.8	29.4	61.4	41.5
		TG	46.6	32.6	77.2	54.9	37	30.4	53.5	38.4
		TT	46.1	31.6	69.5	50.3	38.5	26.5	49.3	35.2
T436K	rs4588									
		AA	34.2	24.3	59.5	53.5	41.5	23.4	45.8	33.8
		CA	42.7	29.6	73.4	52.2	39	30.7	52	37.7
		CC	54.3	37.3	81.4	52.5	36	29.3	57.2	39.3
GC Diplotypes										
1f-1f			53.5	39.6	72.8	53.7	48.5	40.6	54	41.1
1f-1s			49.9	32.1	87.3	59.2	33.3	29.1	53.6	36.3
1f-2			43.4	26.6	74.5	48.6	37	28.6	48.6	32.8
1s-1s			63.7	44.8	79.6	50.4	35.1	27.2	61.5	41.2
1s-2			40.9	33.9	73.8	52.8	37.9	29.4	53.7	39.8
2-2			34.2	24.3	57.9	52.2	41.5	23.4	43.9	32

Table 4-4: **Fall Visit: Relative contribution (partial correlations, r^2 values) for predictors in regression models for 25(OH)D concentrations, based on genetic marker included in model [T436K, D432E or GC Diplotypes (GC Dip.)]**

Genetic marker in models	East Asian			European			South Asians			Total Sample		
	T436K	D432E	GC Dip.	T436K	D432E	GC Dip.	T436K	D432E	GC Dip.	T436K	D432E	GC Dip.
BMI (kg/m^2)	0.007	0.005	0.008	0.037	**0.044**[a]	**0.058**[a]	0.016	0.019	0.018	0.004	0.006	0.004
Sex (M or F)	**0.043**[a]	0.029	0.033	0.000	0.000	0.001	**0.063**[a]	**0.070**[a]	**0.057**[a]	0.002	0.001	0.002
Skin Pigmentation (Melanin Index)	0.034	**0.079**[b]	**0.073**[b]	0.011	0.011	0.013	0.000	0.000	0.005	**0.144**[c]	**0.128**[c]	**0.138**[c]
Total Vitamin D Intake (IU/day)	**0.132**[c]	**0.154**[c]	0.149	0.027	0.028	0.032	**0.159**[c]	**0.153**[c]	**0.148**[c]	**0.091**[c]	**0.091**[c]	**0.090**[c]
UVR Exposure (<30> min/day)	0.006	0.001	0.011	0.004	0.001	0.009	0.001	0.000	0.000	**0.028**[a]	**0.028**[a]	**0.033**[b]
Year (1 or 2)	0.019	0.029	0.024	0.020	0.022	0.014	0.031	0.026	0.018	**0.014**[a]	**0.016**[a]	**0.013**[a]
T436K	**0.205**[c]	-	-	**0.07**[b]	-	-	0.028	-	-	**0.023**[b]	-	-
D432E	-	**0.110**[b]	-	-	0.035	-	-	0.003	-	-	**0.018**[a]	-
GC Diplotypes	-	-	0.053	-	-	0.050	-	-	0.119	-	-	0.007

a=<0.05
b=<0.01
c=<0.001

Table 4-5: **Winter Visit: Relative contribution (partial correlations, r^2 values) for predictors in regression models for 25(OH)D concentrations, based on genetic marker included in model [T436K, D432E or GC Diplotypes (GC Dip.)]**

Genetic marker in models	East Asian			European			South Asians			Total Sample		
	T436K	D432E	GC Dip.	T436K	D432E	GC Dip.	T436K	D432E	GC Dip.	T436K	D432E	GC Dip.
BMI (kg/m^2)	0.004	0.003	0.001	0.006	0.007	0.014	**0.076**[b]	**0.076**[b]	**0.082**[b]	0.005	0.006	0.004
Sex (M or F)	0.007	0.005	0.008	0.029	0.030	0.033	0.025	0.021	0.029	0.001	0.002	0.001
Skin Pigmentation (Melanin Index)	0.028	**0.052**[a]	**0.056**[a]	0.027	0.028	**0.034**[a]	0.008	0.006	0.005	**0.114**[c]	**0.099**[c]	**0.106**[c]
Total Vitamin D Intake (IU/day)	**0.329**[c]	**0.298**[c]	**0.305**[c]	**0.072**[a]	**0.072**[a]	**0.083**[b]	**0.480**[c]	**0.483**[c]	**0.456**[c]	**0.224**[c]	**0.222**[c]	**0.216**[c]
Year (1 or 2)	0.001	0.000	0.004	0.000	0.000	0.000	0.000	0.000	0.001	0.003	0.003	0.004
T436K	**0.138**[b]	-	-	0.004	-	-	0.004	-	-	**0.019**[a]	-	-
D432E	-	**0.055**[a]	-	-	0.006	-	-	0.007	-	-	**0.016**[a]	-
GC Diplotypes	-	-	0.130	-	-	0.053	-	-	0.034	-	-	0.004

a=<0.05
b=<0.01
c=<0.001

4.5 Discussion

We found that *GC* polymorphisms were significant predictors of 25(OH)D concentrations in a sample of young Canadian adults (Tables 4-4 and 4-5). However, the strength of the associations was dependent on ancestry subgroup and season. We observed that the T436K SNP was significantly associated with serum 25(OH)D concentrations in both East Asians and Europeans at the fall visit and in the East Asians at the winter visit. As shown in Table 4-3, the presence of the variant 436K (rs4588 A allele) is associated with a reduction in 25(OH)D concentrations in both the East Asian and European samples, and in the total sample, particularly at the fall visit. Likewise, the D432E SNP was found be a significant predictor of fall 25(OH)D concentrations in the East Asian sample at both the fall and winter visits, and also showed a borderline p-value in the European sample for the fall visit (p=0.06). The variant 432E (rs7041 T allele) is associated with lower 25(OH)D concentrations in the East Asian, European and total Sample. Based on the partial r^2 values observed for both polymorphisms, it appears that T436K has a stronger effect on 25(OH)D levels than D432E (Tables 4-4 and 4-5). Although the *GC* diplotypes were not found to be significantly associated with 25(OH)D concentrations at either visit, this may be due to the reduction in power resulting from the increase number of predictors in the regression model. In fact, as expected from the trends described for the individual markers, we observed that individuals with the GC2-2 diplotype had the lowest 25(OH)D concentrations in the fall in the East Asian, European and Total Sample (see Table 4-3). We did not observe any significant associations between any of the *GC* polymorphisms and 25(OH)D concentrations in the South Asian sample (see Tables 4-4 and 4-5). One potential explanation for these discordant results is the presence of gene-environment interactions. It is possible that the effect of the *GC* polymorphisms is mediated by environmental factors, and these factors show variation between population groups. We further discuss the issue of gene-environment interactions below. Another interpretation is that there may be some heterogeneity in the effects of the *GC* variants on 25(OH)D levels in different population groups.

 Interestingly, the relative contribution of the *GC* genetic markers to 25(OH)D concentrations appears to differ not only by ancestry but also by season. The partial r^2 values corresponding to the genetic markers are substantially higher in the fall than in the winter. Conversely, the proportion of variation in 25(OH)D levels explained by vitamin D intake is substantially higher

in the winter than in the fall (Tables 4-4 and 4-5). In our study, the fall and winter 25(OH)D measurements correspond to the same subjects, so this seasonal effect points again to the presence of gene-environment interaction. The main difference between the two seasonal measurements is that in Canada during the summer and the fall there is endogenous vitamin D synthesis in the skin, but this is not the case in the winter. This is reflected in substantially higher 25(OH)D concentrations during the fall than during the winter (see Table 4-1), something that is typically observed in high-latitude countries. Our data indicate that the genetic effects of the *GC* polymorphisms are easier to identify during the fall, when there is endogenous synthesis of vitamin D in the skin and higher circulating levels of 25(OH)D. It is important to note that in a recent study of older Canadian women, Sinotte et al. also observed that the relationship between the *GC* SNPs and 25(OH)D concentrations was more obvious when the concentrations of 25(OH)D are higher, particularly in the months when cutaneous vitamin D synthesis is possible (Sinotte et al., 2009). Given these findings, one potential explanation for the lack of significant effects observed for the *GC* polymorphisms in our South Asian sample is that 25(OH)D concentrations were very low in this sample both in the fall and the winter (Table 4-1).

Significant associations between *GC* polymorphisms and serum 25(OH)D concentrations have been observed in other recent studies. In a cross sectional study of postmenopausal women, Lauridsen et al. (2005) characterized the major GC isoforms by isoelectric focusing and reported that the highest 25(OH)D concentrations were observed in subjects with the GC1-1 phenotype and the lowest in subjects with the GC2-2 phenotype, while individuals with the GC1-2 phenotype had intermediate concentrations (Lauridsen et al., 2005). Kurylowicz et al. (2006) observed that a greater proportion of Graves disease patients that were either heterozygotes (TK) or homozygotes (KK) at T436K (rs4588) had 25(OH)D concentrations below 50 nmol/L compared to homozygotes for the wildtype variant (TT) (Kurylowicz et al., 2006). Engelman et al. (2008) reported that T436K (rs4588) and D432E (rs7941) both showed associations with 25(OH)D in their sample of Hispanics and African Americans. In particular, the genotypes associated with the GC-2 isoform were associated with lower levels of 25(OH)D in their study, but concentrations were not specified (Engelman et al., 2008). In a sample of postmenopausal women in Germany, Abbas et al. (2008) observed that mean 25(OH)D concentrations were lowest in women with the GC2-2 diplotype and the highest concentrations were observed in women with the GC1f-1s diplotype (Abbas et al., 2008). Fang et al. (2008) examined serum

25(OH)D concentrations and *GC* haplotypes in the Rotterdamn study sample (n=1,317) and found a significant association with 25(OH)D concentrations the GC isoforms: GC-1S was associated with a 7 nmol/L increase in 25(OH)D concentrations, GC-2 was associated with a 16 nmol/L decrease in 25(OH)D and no significant differences were observed in 25(OH)D concentrations for the GC-1F isoform (Fang et al., 2009). In a small sample of healthy subjects from Toronto, Fu et al. (2009) also observed that KK homozygotes at T436K had lower mean 25(OH)D concentrations (46 nmol/L), than TK heterozygotes (65 nmol/L) and TT homozygotes (66 nmol/L) genotypes (Fu et al., 2009). Sinotte et al. (2009) examined T436K and D432E in a sample of premenopausal white women living in Quebec, Canada and reported a significant association of both SNPS with 25(OH)D concentrations. In their study, the presence of the variant allele in either SNP was associated with lower 25(OH)D concentrations (Sinotte et al., 2009). Ahn et al. (2009) also found an association between 25(OH)D concentrations and the D432E polymorphism, with homozygotes (EE) having the lowest 25(OH)D concentrations, in comparison to heterozygotes (DE) and homozygotes (DD) (Ahn et al., 2009). Finally, a recent genome wide study of 4,501 persons of European ancestry has also found highly significant association between 25(OH)D concentrations and polymorphisms in the *GC* gene, including D432E (rs7041) (Ahn et al., 2010), an association which has confirmed by an even larger consortium of ~ 30,000 individuals of European ancestry (Wang et al., 2010).

Although a large number of studies have shown that *GC* polymorphisms, in particular T436K (rs4588) and D432E (rs7041), are significantly associated with 25(OH)D concentrations, the mechanisms underlying these associations remain unclear. The associations may be driven by the differences in affinity for 25(OH)D between the GC isoforms, or to differences in metabolism related to the differential patterns of glycosylation. An important goal of future research will be, not only to elucidate the exact mechanism by which *GC* polymorphisms affect serum 25(OH)D concentrations, but also to explore the potential role of these variants in other important aspects of vitamin D metabolism, such as the vitamin D autocrine pathway. Recent research indicates that VDBP may be involved in the active transport of vitamin D metabolites into the cell in local tissue (Lin and Scanlan, 2005). In the kidney, the epithelial cells of the proximal tubule express the proteins megalin and cubulin, which bind to and then internalize the circulating 25(OH)D that is bound to the VDBP as a 25(OH)D-VDBP complex (Rowling et al., 2006). Rowling et al (2006) used fluorescent microscopy to observe that breast cancer cells can quickly internalize

VDBP via megalin-mediated endocytosis, which was also associated with the activation of the vitamin D receptor (VDR) by 25(OH)D, further suggesting that mammary cells in vivo are also able to transport VDBP and to activate the VDR pathway (Atkins et al., 2007; Rowling et al., 2006). Studies of human osteoblasts show that these cells can express CYP25B1 mRNA, which led to a detectable secretion of 1,25(OH)$_2$D (Atkins et al., 2007). Furthermore, osteoblasts were also shown to express the receptors for the VDBP, cubulin and megalin, suggesting they are also able to internalize vitamin D binding protein-25(OH)D complexes via receptor-mediated endocytosis and to respond to 25(OH)D *in vivo* (Atkins et al., 2007). In addition to these tissues, megalin is expressed in several other body tissues, including both male and female reproductive organs (Lin and Scanlan, 2005). Unfortunately, to our knowledge, no research has been carried out to explore the potential effect of *GC* polymorphisms on receptor-mediated endocytosis.

One of the issues that we wanted to explore in this study was the relative role of different factors that can potentially influence 25(OH)D levels (vitamin D intake, skin pigmentation, UV exposure, BMI, sex and *GC* polymorphisms). In order to do this, it is important to consider the full sample because it increases statistical power, while also providing a much better representation of some of the critical factors involved. For example, skin pigmentation variation is quite limited within each ancestral subgroup, but the full sample encompasses a wide range of pigmentary phenotypes, making it possible to explore in more detail the role of skin pigmentation on 25(OH)D concentrations. The results of this analysis indicate that, although both *GC* polymorphisms have a significant effect on serum 25(OH)D concentrations, their effect is relatively small compared to some of the other predictors that we included in our models. In the fall, the major predictors of 25(OH)D concentrations are skin pigmentation (13-14%), total vitamin D intake (9%), sun exposure (3%), and finally the *GC* markers, T436K (2.3%) and D432E (1.8%). The prominent role of skin pigmentation in determining 25(OH)D concentrations in the fall is not surprising, given the important role that skin pigmentation plays in the endogenous synthesis of vitamin D when enough UV radiation is available. During the winter, our analysis found that the majority of the variation in 25(OH)D was explained by vitamin D intake (22%) and skin pigmentation (10-11%), with only small amounts of variation accounted for by T436K (1.9%) and D432E (1.6%). Again, it is not surprising that vitamin D intake becomes the major factor affecting 25(OH)D in the winter, when no endogenous vitamin D synthesis is possible in Canada because of insufficient UVR (Webb et al. 1997). The observation

that skin pigmentation has a large effect on wintertime 25(OH)D concentrations can be accounted for by the differential cutaneous vitamin D production during the summer months, which in turn influences 25(OH)D levels during the wintertime. This is supported by the observation that when incorporating fall 25(OH)D concentrations in the winter model, the effect of skin pigmentation is no longer significant (Gozdzik et al., 2010).

The main limitation of this study is the relatively small sample size (particularly when independently considering each ancestral subgroup). However, the effect of some of the factors included in the analysis (including the two *GC* SNPs) on 25(OH)D concentrations was strong enough to identify statistically significant associations. Our research has several strengths in comparison with previous studies. Our sample included subjects from three different population groups, East Asian, European and South Asian. We were able to measure the most important factors that affect vitamin D levels, including skin pigmentation, vitamin D intake and UV exposure, and this makes it possible to explore the relative effects of the different predictors on 25(OH)D concentrations. Another strength of our study is that we were able to measure 25(OH)D concentrations during the late summer/early fall and again in the winter, capturing the seasonality that is observed in 25(OH)D concentrations at our latitude.

In conclusion, we report that two non-synonymous SNPs located within the *GC* gene (T436 and D432E) are significantly associated with 25(OH)D concentrations in a sample of young Canadian adults of diverse ancestry. Individuals carrying the T436K (rs4588) K allele and D432E (rs7041) D allele appear on average to have lower 25(OH)D concentrations. The relationship between these SNPs and 25(OH)D concentrations appears to be more apparent when serum 25(OH)D concentrations are high (during times of cutaneous synthesis) than during the wintertime, when 25(OH)D concentrations are much lower, and this points to gene-environment interaction effects. Our study, as well as many other previous studies, indicate that genetic markers within the *GC* gene influence vitamin D status, and therefore may be relevant for vitamin D associated diseases.

4.6 Acknowledgements

4.7 References

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5 Concluding Remarks

5.1 Introduction

This thesis aims to improve our knowledge of the vitamin D status of Canadian young adults, and also to expand our understanding regarding the main factors influencing vitamin D levels. A sample of young adults of diverse ancestry living in the Greater Toronto Area was studied, and my research evaluated: 1) vitamin D status (measured as serum 25(OH)D concentrations) of the participants during the fall and the winter; 2) vitamin D intake patterns; 3) seasonal trends in 25(OH)D levels; 4) impact of Body Mass Index (BMI), sun exposure, skin pigmentation and vitamin D intake on 25(OH)D concentrations; and 5) effect of vitamin D binding protein (VDBP) polymorphisms on 25(OH)D levels. Sections 5.1.1 to 5.1.5 summarize the major findings and contributions of my research.

5.2 Summary of Findings

5.2.1 Vitamin D Levels in Canadian Young Adults of Diverse Ancestry

Living at high latitudes increases the risk of vitamin D insufficiency, due to the lack of available UVB for cutaneous vitamin D synthesis during the winter. Therefore, it is not surprising that previous studies in Canada and in other high latitude countries have shown that vitamin D levels are low in many otherwise healthy adults, particularly during the winter months (Rucker et al., 2002; Vieth et al., 2001). However, there is a lack of knowledge regarding the vitamin D status of individuals of non-European ancestry living in Canada, who are at a relatively higher risk of vitamin D insufficiency due to the higher melanin content in the skin, which interferes with vitamin D synthesis during times of adequate UVB incidence. One of the major goals of my thesis was to explore vitamin D status in a sample with an adequate representation of individuals from different ancestral groups.

In agreement with previous studies, vitamin D levels were low in my sample, particularly during the wintertime. The proportion of healthy young adults of European ancestry that had low vitamin D levels was quite consistent with previous research in Canada. A novel finding of this thesis is that the prevalence of low vitamin D levels is much higher in East Asian and South Asian young adults than in those of European ancestry. During the wintertime, half of the South Asians and one-third of the East Asians had 25(OH)D concentrations below 25 nmol/L, a widely used cutpoint to define vitamin D deficiency. This contrasts with only 7% of Europeans. Nearly

all the South Asian and East Asian participants (93% and 90%, respectively) had 25(OH)D levels below 50 nmol/L, which many experts consider the minimum concentration to maintain healthy bone and mineral metabolism (Henry et al., 2010). Again, these values stand in sharp contrast to the percentage observed in Europeans (48%). These findings have been described in Chapters 2 and 3 of the thesis. Given the potential health consequences of vitamin D insufficiency, these findings have important public health implications and also demonstrate the need for further research into the vitamin D status of these and other populations groups living in Canada.

5.2.2 Vitamin D Intake in Young Adults of Diverse Ancestry

In spite of recent efforts, such as the Canadian Health Measures Survey (CHMS), information about vitamin D intake among Canadians is still incomplete. This is particularly the case for Canadians of non-European ancestry. For example, in the recent CHMS survey, all the non-European participants were included in a group called "Other" because of limitations with the sample size (Langlois et al., 2010b). My thesis is a significant contribution to our knowledge of vitamin D intakes in young adults of non-European ancestry, particularly those of East Asian and South Asian ancestry.

Using information collected during my first study (Chapter 2), a Food Frequency Questionnaire (FFQ) designed to estimate vitamin D and calcium intake was validated using a 7-day food diary. This FFQ was used in the follow-up studies and allowed me to evaluate the proportion of the total vitamin D intake coming from the diet and from supplements, and also the extent to which vitamin D intake influences 25(OH)D concentrations (Chapter 3). I found that the majority of vitamin D intake came from dietary sources, during both the fall and winter visits, in all three ancestral groups (Europeans, East Asians and South Asians). The relative proportion of vitamin D coming from supplements increased slightly during the winter, but this was primarily driven by smaller dietary intakes in the winter than in the fall (Chapter 3). However, vitamin D intakes were not able to compensate for the lack of cutaneous synthesis during the winter: I observed a strong decrease in 25(OH)D concentrations from the fall to the winter (see below).

My research indicates that current Health Canada recommendations are insufficient to ensure adequate vitamin D concentrations. Many participants who reported vitamin D intakes in excess

of the 200 IU/d recommended by Health Canada had 25(OH)D concentrations lower than 50 nmol/L, and even 25 nmol/L. My study lends support for a need to increase vitamin D intakes in those at higher risk for vitamin D insufficiency (living at high latitudes, lack of UVR exposure and darker skin pigmentation).

5.2.3   Seasonal Trends in Vitamin D Levels

In Chapter 3, I examined seasonal fluctuations in vitamin D levels by measuring 25(OH)D concentrations at two key points in each participant: when vitamin D is being synthesized in the skin (early fall), and when cutaneous synthesis is not possible because of insufficient solar radiation (winter). I found that there were large seasonal changes in 25(OH)D concentrations. The largest decrease in 25(OH)D concentrations was observed in Europeans (24.5 nmol/L), followed by East Asians (14.9 nmol/L) and South Asians (8.1 nmol/L). I also observed that the absolute decrease in 25(OH)D was correlated to baseline 25(OH)D concentrations; that is, individuals with higher baseline 25(OH)D concentrations showed the largest absolute drop in 25(OH)D levels. This is not surprising, given the absence of endogenous vitamin D synthesis during the winter, the seasonal patterns of vitamin D intake, and the half-life characteristics of 25(OH)D. Very few studies in Canada have analyzed in detail seasonal trends in vitamin D levels, and this is an important contribution of my research. Vieth (2009) recently hypothesized that to minimize disease risk, it is not only important to ensure that 25(OH)D concentrations reach optimal levels but also that they remain stable throughout the year. In light of this hypothesis, the strong seasonal fluctuations of vitamin D observed at high latitudes in this and other studies may be of concern and the potential health effects of these fluctuations merit further research.

5.2.4   Impact of Various Factors Affecting Vitamin D Levels

Another goal of this thesis was to evaluate the major factors that affect vitamin D levels in young adults living in Canada. Among the variables evaluated were vitamin D intake, sun exposure, skin pigmentation and BMI.

Not surprisingly, vitamin D intake was a significant predictor of 25(OH)D levels in both the winter and in the fall. During the fall, sun exposure and skin pigmentation were also important predictors of 25(OH)D concentrations. Interestingly, skin pigmentation was also a predictor of

wintertime 25(OH)D concentrations. It is known that, at the latitude of Toronto (43°N), there is inadequate UVB to synthesize vitamin D in the skin from mid-October to mid-March (Webb et al., 1988). The significant association between skin pigmentation and wintertime serum 25(OH)D concentrations is probably due to the increased vitamin D synthesis in individuals with lower melanin levels during times of adequate UVB incidence (late spring, summer and early fall). This hypothesis is supported by the observation that, when baseline fall 25(OH)D concentrations were included in the winter linear regression model, fall serum concentration was the best predictor for the winter variance, and skin pigmentation was no longer significant (Chapter 3).

5.2.5 Effect of Genetic Variants of Vitamin D Binding Protein on Vitamin D levels

Several studies have reported associations between Vitamin D Binding Protein (*VDBP)* polymorphisms and serum 25(OH)D concentrations. The goal of Chapter 4 was to assess the role of two VDBP genetic variants (T436K and D432E) on 25(OH)D concentrations, while also taking into account the variables that have already been found to have an effect on 25(OH)D: skin pigmentation, sun exposure and vitamin D intake. In agreement with previous research, I found that both VDBP polymorphisms were significant predictors of 25(OH)D concentrations. However, the T436K polymorphism had a stronger effect on 25(OH)D levels than the D432E variant. Interestingly, the strength of the association was dependent on ancestry subgroup and season. As described in Chapter 4, this is probably due to gene-environment interaction effects. Overall, evidence is accumulating that genetic markers within the *VDBP* gene influence vitamin D status, and therefore knowledge of these polymorphisms may be relevant for vitamin D associated diseases.

5.3 Conclusions

Based on results of the data collected for this thesis, I conclude that:

1) vitamin D levels (measured as 25(OH)D concentrations) are low in Canadian young adults, particularly in those of non-European ancestry;

2) vitamin D intake was on average in excess of 200 IU/day (Adequate Intake recommended by Health Canada for this age group) in each of the ancestral groups studied. However, this intake was inadequate to maintain sufficient vitamin D levels;

3) vitamin D levels undergo large seasonal changes. Winter 25(OH)D concentrations are substantially lower than those observed during the fall. Additionally, the largest decreases in 25(OH)D concentrations correspond to the individuals with the highest fall concentrations.

4) vitamin D intake is an important year-round predictor of 25(OH)D concentrations, but skin pigmentation and sun exposure are also important predictors during the times when UVB is adequate for cutaneous synthesis; and

5) vitamin D binding protein (VDBP) polymorphisms (T436K and D432E) are significant predictors of 25(OH)D concentrations, but their effects vary by ancestry and season, indicating gene-environment interaction effects.

One of the major conclusions derived from my research is that higher vitamin D intakes are needed to offset the seasonal drop in vitamin D levels and to ensure adequate vitamin D levels year-round for those at higher risk of insufficiency. In this sense, my study lends support to the recent recommendations of the Canadian Cancer Society, which take into account risk factors for vitamin D insufficiency (seasonality, UVR exposure and skin pigmentation). The Canadian Cancer Society recommends an intake of 1000 IU per day during the fall and winter, or year-round for those at higher risk of having low vitamin D levels (those with darker skin, who stay indoors or always cover up) (Society, 2007). While an increase in vitamin D intake, particularly during the winter, is clearly needed, further work is required to elucidate the vitamin D intakes that are necessary to achieve and maintain optimal vitamin D levels in individuals of different ancestry.

5.4 References

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