Bioavailability of Casein-Bound Vitamin D₃ From Fortified Cheese and its Effects on the Mental Health Status of the Institutionalized Elderly

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
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University of Toronto

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2012

ABSTRACT

All populations risk vitamin D inadequacy. We conducted a randomized double-blind trial of vitamin D₃ fortified cheddar cheese to study bioavailability based on serum 25-hydroxyvitamin D [25(OH)D] concentrations, and its effects on mental health scores in older adults. Once a week, 28 subjects received 200 IU or 28,000 IU of vitamin D₃ per fortified cheese serving. The mean increases in 25(OH)D over 8 weeks were: 4.2±11.4 and 29.4±16.2 for the 200 IU/week and 28,000 IU/week dose groups, respectively (groups differ, P<0.001). Subjects who consumed 28,000 IU/week cheese improved their Mental Component Summary (MCS) scores, based upon the SF-36v2 questionnaire conducted at baseline and at 8 weeks (P<0.05). There was also a positive correlation between the change in MCS score and the change in 25(OH)D (1 tail; P<0.05). These data demonstrate the suitability of fortified cheddar cheese, and provide evidence of neurocognitive benefits with higher 25(OH)D levels.
To my parents, Sadia and Mohamed Taha, a constant source of love and support

I would like to express my sincere gratitude to my supervisor, Dr. Reinhold Vieth, for his guidance and mentorship. I would also like to thank my committee members Dr. David Jenkins, and Dr. Thomas Wolever for their advice and insight.
Table of Contents

Abstract..................................................................................................................ii

List of Tables and Figures.......................................................................................vi

List of Abbreviations...............................................................................................viii

Contributions.............................................................................................................ix

Acknowledgements................................................................................................x

Chapter 1: Literature Review...............................................................................1

1.0: Vitamin D...........................................................................................................2

1.1: Historical Perspective.........................................................................................2

1.2: Formation and Ingestion of vitamin D.............................................................3

1.3: Absorption and Serum Transport of Vitamin D.............................................4

1.4: Metabolism of Vitamin D..................................................................................5

1.5: Mechanism of Action and Health Outcomes.................................................7

1.6: Safety and Toxicity...........................................................................................9

1.7: Serum 25-hydroxyvitamin D concentrations in the population....................10

1.8: Vitamin D and mental health status.................................................................11

1.9: SF-36v2 health survey.......................................................................................13

1.10: Food policy.....................................................................................................16

1.11: Rationale........................................................................................................17

Chapter 2: Bioavailability of vitamin D fortified cheddar cheese.......................26

2.0: Serum 25(OH)D levels and the bioavailability of vitamin D₃ fortified cheddar cheese in older institutionalized adults living in Canada: a pilot study..........27
Chapter 3: Well-being in older adults.................................................................50

3.0: Vitamin D₃ fortified cheese increases serum 25(OH)D response and
improves Mental Health scores of elderly in a Canadian retirement
home..................................................................................................................51

Chapter 4: Implications.....................................................................................67

4.0 Implications..................................................................................................68
List of Tables and Figures

Chapter 1:

Figure 1.2: The chemical structures of vitamin D$_3$ and D$_2$ .................................4
Figure 1.4: Schematic diagram of the metabolism of vitamin D ..............................7
Figure 1.9: SF-36v2 health survey ..............................................................................14
Figure 1.10: Food policy in Canada ...........................................................................16

Chapter 2:

Figure 1: Consort diagram showing the flow of participants throughout the study ...41
Table 1: Baseline characteristics and change in subject’s biochemistry at the end of the intervention ........................................................................................................42
Figure 2: Serum 25(OH)D concentrations in subjects consuming vitamin D Fortified cheese .........................................................................................................................43
Figure 3: Changes in serum 25 (OH)D concentration in subjects consuming vitamin D fortified cheddar cheese ......................................................................................................44
Figure 4: Baseline serum 25 (OH)D concentrations in subjects taking a vitamin D supplement, before consumption of the vitamin D fortified cheese ..........45

Chapter 3:

Figure 2: Box plot shows the change in mental health component summary (MCS) by dose group .................................................................................................................61
Figure 3a: The 95 % confidence bands for the cohort at the end of the study; arrow points to the extreme outlier removed for statistical analysis .................62
Figure 3b: The relationship between the change in Mental Component Summary score (MCS) and the change in serum 25(OH)D through the 8 weeks of this clinical trial........................................................................63

Table 2: Baseline and change (mean ± SD) in components of SF-36v2 health survey per dose group.........................................................................................................................................................64
### List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>25(OH)D</td>
<td>25-hydroxyvitamin D or calcidiol</td>
</tr>
<tr>
<td>1,25 (OH)_2D</td>
<td>1,25-dihydroxyvitamin D or calcitriol</td>
</tr>
<tr>
<td>24-OHase</td>
<td>24-hydroxylase or CYP24A1</td>
</tr>
<tr>
<td>25-OHase</td>
<td>25-hydroxylase or CYP27A1</td>
</tr>
<tr>
<td>1α-OHase</td>
<td>1-α hydroxylase or CYP27B1</td>
</tr>
<tr>
<td>24,25(OH)_2D</td>
<td>24,25-dihydroxyvitamin D</td>
</tr>
<tr>
<td>1,24, 25(OH)_2D</td>
<td>Calcitroic acid</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>BCTX</td>
<td>Bone C-telopeptide</td>
</tr>
<tr>
<td>CV</td>
<td>Coefficient of variation</td>
</tr>
<tr>
<td>DBP</td>
<td>Vitamin D binding protein</td>
</tr>
<tr>
<td>DRI</td>
<td>Dietary reference intake</td>
</tr>
<tr>
<td>FFQ</td>
<td>Food frequency questionnaire</td>
</tr>
<tr>
<td>IOM</td>
<td>Institute of Medicine</td>
</tr>
<tr>
<td>IU</td>
<td>International units (1μ g vitamin D = 40 IU)</td>
</tr>
<tr>
<td>MCS</td>
<td>Mental component summary</td>
</tr>
<tr>
<td>PTH</td>
<td>Parathyroid hormone</td>
</tr>
<tr>
<td>RCT</td>
<td>Randomized controlled trial</td>
</tr>
<tr>
<td>RDA</td>
<td>Recommended daily allowance</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>UL</td>
<td>Tolerable upper intake levels</td>
</tr>
<tr>
<td>UVB</td>
<td>Ultraviolet B radiation</td>
</tr>
<tr>
<td>VDR</td>
<td>Vitamin D receptor</td>
</tr>
<tr>
<td>VDRE</td>
<td>Vitamin D response element</td>
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</table>
Contributions

The work presented here is a result of combined efforts of several individuals. My own contributions to this work are outlined below.

The fortified cheeses were manufactured by Agropur at the Food Research and Development Centre (FRDC) in Saint-Hyacinthe, Quebec. I was present in observing the cheese manufacturing process. I performed the analysis of the vitamin D fortified cheese and the required calculations under the guidance and direction of Dr. Reinhold Vieth. I also prepared and vacuum packaged the cheese to be distributed at each weekly visit.

For the clinical trial, I participated in obtaining the human trial ethics approval from Mount Sinai Hospital and Health Canada. I also participated in in recruiting subjects, obtaining the informed consent, and carrying out the trial. I saw all of the subjects in Aurora at each weekly visit and distributed the cheese. I took their blood and urine samples and collected data (health records, medications, height etc.). I ran the samples for 25(OH)D and BCTX analysis on the Liaison, and Roche, and the rest of the blood and urine biochemistries were done at Mount Sinai Hospital’s clinical chemistry laboratory. To assess their well-being status, I interviewed all of the participants at the beginning and at the end of the trial and entered all of the data for certified analysis by Qualitymetric (the company that issued the health surveys).

I did the statistical analysis, analyzed the data and wrote 2 manuscripts (to be submitted) under the supervision and assistance of Dr. Reinhold Vieth.
Acknowledgements

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We sincerely thank Dr. Pierre Geoffroy for being the principal clinical investigator, and physician for the subjects in this clinical trial. We also thank the Kingsway Arms Aurora Retirement Center staff for being very generous and receptive, and in allowing for the conduct of the project. In particular, we thank Maret Cox, the executive director of the Aurora Retirement Center, and Melissa McDonald, a resident care co-coordinator who provided constant support and assistance throughout the study.

We thank Dr. Azar Azad, Michelle Rodrigues of Mount Sinai Hospital Services, as well as their staff, for their excellent efforts and laboratory work in analyzing all of the blood and urine samples in a timely and efficient manner.

I would like to thank my lab mates, Dennis Wagner and Banaz Al-Khalidi, for their valuable insight and constant support throughout my research studies. Finally, we would like to thank all 28 of the participants and their families who were a part of the clinical trial for their time and commitment to the study.
Chapter 1: Literature review.
1.0 Vitamin D

1.1 Historical Perspective

Rickets was first characterized clinically as a skeletal deformity in children by Glisson, Deboot and Whistler, in 1650 \cite{1}. By the turn of the 20\textsuperscript{th} century this phenomenon was prevalent in industrialized cities of northern Europe and northeastern United States \cite{2}. However, it was in 1822 that Sniadecki noted the role of sunshine in preventing rickets in children \cite{3}. This was reported epidemiologically in 1942 by Palm who documented the relationship between sunlight and the incidence of rickets in children living in the inner cities of Great Britain and those living in underdeveloped countries \cite{4}.

In the realm of food science, in 1924, Steenbock and Black, and Hess and Weinstock, exposed a variety of foods to ultraviolet radiation and found that it imparted anti-rachitic activity to the food substances \cite{5,6}. This led Steenbock to the utility of irradiating foods for the prevention and cure of rickets in children \cite{7}. As a result, rickets was eradicated as a significant medical problem, by the addition of provitamin D to milk and the irradiation of it to impart anti-rachitic activity. This discovery also allowed for the isolation and characterization of vitamin D, and the direct fortification of milk.

In the realm of chemistry, in 1931, Askew et al. determined the structure of vitamin D\textsubscript{2} from the irradiation of plant sterols \cite{8}. Windaus et al. isolated 7-dehydrocholesterol from the skin and subsequently synthesized and converted that to vitamin D\textsubscript{3} \cite{9}. They also provided the chemical syntheses and confirmed the structures of the vitamin D compounds, thus making them available for the treatment of disease \cite{10}. 
1.2 Formation and ingestion of vitamin D

The two forms of vitamin D are vitamin D$_2$ (ergocalciferol) and vitamin D$_3$ (cholecalciferol). Vitamin D$_2$ is different from vitamin D$_3$ by an additional double bond between the 22-23 carbon and an extra 24-methyl group (Figure 1.2; page 4). Vitamin D is not easily found in the food supply. Vitamin D$_2$ is synthesized by the ultraviolet irradiation of yeast and the plant sterol, ergosterol. Vitamin D$_3$ is naturally synthesized in the skin and is found in fatty fish and cod liver oil. Evidence suggests that ingestion of vitamin D$_3$ is more efficient than vitamin D$_2$ in raising serum 25-hydroxyvitamin D [25(OH)D] concentrations in humans [11-13].

Vitamin D$_3$ is produced naturally in the skin by the ultraviolet (UV) irradiation of 7-dehydrocholesterol, a precursor of cholesterol. With sunlight exposure, ultraviolet B photons with wavelengths between 290 and 315 nm penetrate the skin and are absorbed by the epidermal and dermal stores of 7-dehydrocholesterol (provitamin D$_3$) [14]. This causes the cleavage of the 9-10 carbon bond of 7-dehydrocholesterol to form a 9,10-secosterol called previtamin D$_3$, which is biologically inert and must undergo an isomerization via the skin’s temperature to form vitamin D$_3$ in the skin. Once vitamin D$_3$ is formed it enters the dermal capillary bed where it binds to the vitamin D binding protein (DBP) and enters the circulation [14].
1.3 Absorption and serum transport of vitamin D

Vitamin D is absorption in the small intestine is independent of vitamin D status. Ingested vitamin D is emulsified with bile salts and is solubilized within micelles in the duodenum then is passively absorbed with other lipids in the jejunum. It is then incorporated into chylomicrons within the enterocytes in the mesenteric lymph and is transported to the systemic circulation. While it is in the lymph, some of the vitamin D is transferred from the chylomicrons to the serum DBP \(^{15}\).

Vitamin D and its metabolites are transported in the serum via DBP \(^{16}\). It has been reported that humans produce 10mg/kg/day (approximately 172 nmol) of DBP, which exceeds its vitamin D ligands with only 5% of DBP molecules being occupied by a vitamin D sterol ligand \(^{17}\). The total binding capacity of DBP is nearly 4700nmol/L for vitamin D and its metabolites \(^{18}\). Naturally occurring and synthetic vitamin D sterols in the extracellular fluid have a specific, high capacity, and high affinity interaction with DBP \(^{19}\). DBP binds at single binding site, but with varying affinities for the metabolites \(^{20}\). The sequence from greatest affinity is for 25(OH)D and 24,25(OH)\(_2\)D, and then for
1,25 (OH)₂D and the parent vitamin D [18]. The binding of vitamin D and its metabolites to DBP facilitates their distribution into the adipose and tissue compartments of the body and their delivery to sites of metabolism and action [21].

### 1.4 Metabolism of vitamin D

Vitamin D, whether synthesized in the skin or obtained from the diet, is metabolized in the liver by the 25-hydroxylase enzyme (CYP27A1) to form 25-hydroxyvitamin D [25(OH)D] (Figure 1.4; page 7). This is the major circulating vitamin D metabolite, and serves as the clinical indicator of vitamin D status. The production of 25(OH)D is dependent on substrate concentration [22]. About 75% of the circulating vitamin D is hydroxylated to 25(OH)D on a single pass through the liver. This vitamin D metabolite is still biologically inactive since it has minimal capacity to bind to the vitamin D receptor [23].

The DBP-25(OH)D complex is preferentially removed from the plasma by liver and kidney that possess the protein megalin, a DBP binding protein, and catabolizes the DBP and releases the 25(OH)D into the tissues. 25(OH)D can undergo a regulated metabolism in the kidney by the enzyme 1-alpha-hydroxylase (1α-OHase) or CYP27B1 to form 1,25-dihydroxyvitamin D [1,25(OH)₂D] or Calcitriol, the active hormonal form of vitamin D [16] Figure 1.4; page 7). This activation step can also occur in various tissues including the placenta, brain, prostate, keratinocytes, macrophages and osteoblasts [23]. Most tissues are thought to acquire vitamin D metabolites by the penetration of “free” metabolite.
The vitamin D endocrine system is tightly regulated by renal 1α-OHase activity that maintains the 1, 25(OH)₂D within its homeostatic range, regardless of the level of 25(OH)D substrate. The main controlling factors are the serum concentrations of calcium, phosphorus, parathyroid hormone (PTH), and 1, 25(OH)₂D itself through direct feedback control. Hypocalcemia causes an increase in PTH response which then stimulates 1α-OHase to increase 1, 25(OH)₂D synthesis [24]. Also, low serum calcium or phosphorus levels may activate renal 1α-OHase, independently of PTH [25].

Circulating 1, 25(OH)₂D is regulated by the balance of its rates of synthesis and catabolism. This is the balance between 25(OH)D-1-hydroxylase and 1,25(OH)₂D-24-hydroxylase [22]. Additionally, 1, 25(OH)₂D can induce the expression of the 24-hydroxylase enzyme (CYP24A1), which catabolizes each of 25(OH)D and 1, 25(OH)₂D into biologically inactive 24,25-dihydroxyvitamin D [24,25(OH)₂D] and 1,24, 25(OH)₂D respectively, with eventual side chain cleavage and production of water-soluble calcitroic acid [26]. Thus, the circulating levels of 1, 25(OH)₂D are tightly regulated, and the non-calcemic tissues that are able to produce 1, 25(OH)₂D locally do not affect the circulating levels.
Figure 1.4. This image has been reproduced from medical.siemens.com. Permission from publisher was not required since it is publicly available. Schematic diagram of the metabolism of vitamin D. Vitamin D$_3$ is converted in the liver by the enzyme vitamin D-25 hydroxylase to 25(OH)D. This is the major circulating form of vitamin D that is used by clinicians to measure vitamin D status. It is biologically inactive and is converted in the kidney by the enzyme 25-hydroxyvitamin D-1α-hydroxylase to 1,25(OH)$_2$D, which is the biologically active hormone form. 1,25(OH)$_2$D is involved in regulating calcium, phosphorus, and bone metabolism—its “classic” endocrine function. 1,25(OH)$_2$D feedback regulates its own synthesis and decreases the synthesis and secretion of PTH in the parathyroid glands.

1.5 Mechanism of action and health outcomes

The 1, 25(OH)$_2$D hormone has a high affinity for the intracellular vitamin D receptor (VDR). VDR is widely expressed in the vitamin D target organs (intestine, bone, kidney and parathyroid glands) and in various non-calcium regulating organs including the skin, muscle, prostate, breast, colon, pancreas, and immune cells \cite{27}. 1, 25(OH)$_2$D enters the cell by diffusion, facilitated entry (i.e. via megalin), or is locally synthesized
(i.e. autocrine pathway), and then binds to VDR in the cytoplasm. This complex binds to
the retinoic acid receptor (RXR) to form a heterodimer [28-30]. The VDR-RXR
heterodimer can then bind to vitamin D response elements (VDREs) in target genes and
control their expression [30].

The “classic” endocrine function of 1, 25(OH)₂D is to increase calcium
absorption in the intestine and thus to promote bone mineralization. Chronic severe
deficiency of vitamin D in infants and children causes deformities in their bones, known
as rickets. This is portrayed as osteomalacia in adults. Less severe vitamin D inadequacy
in adults can lead to secondary hyperparathyroidism, increased bone turnover and thus an
increased risk of falls, fractures and osteoporosis [31-33].

The autocrine/paracrine role of vitamin D functions beyond calcium homeostasis
and bone development [24]. In the extra-renal system, 1, 25(OH)₂D serves as a signaling
molecule between cells, thus controlling the expression of over 200 genes involved in
cell differentiation, replication and immunity [34]. The paracrine function of vitamin D
and its related health benefits is an active area of research. Evidence, at the levels of basic
science and clinical epidemiology, suggests that vitamin D nutritional status plays a role
in certain types of cancer [35-38], cardiovascular disease and insulin resistance [39-42],
multiple sclerosis [43-45], depression [46-48], and infectious diseases [49,50]. However,
higher vitamin D nutritional status has been, in rare circumstances, associated with higher
rates of prostate and pancreatic cancer in northern latitudes that have minimal sun
exposure during the winter. However, it has been suggested that this is because of the
large seasonal fluctuations of 25(OH)D in the far North. Hence, it is not only higher, but
stable, levels of 25(OH)D levels that are optimal [22].
1.6 Safety and Toxicity

Humans have a high physiological capacity to produce vitamin D₃ in the skin. Exposure to UVB light can generate the equivalent of 10,000 - 20,000 IU of vitamin D in the skin [2]. It is common for people living in sunny areas to have 25(OH)D levels over 100 nmol/L, and up to 225 nmol/L [51,52]. Farmers in Puerto Rico and lifeguards in St. Louis were shown to have 25(OH)D levels above 130 nmol/L. Therefore, serum 25(OH)D concentrations <225 nmol/L are common in sunny environments, even without the use of supplements, and should be regarded as natural and safe [51,53].

Vitamin D toxicity is manifested as hypercalcemia or hypercalciuria. Published literature suggests no adverse effects in vitamin D₃ intakes up to 40,000 IU/day. Clinical trials that administered oral vitamin D₃ intakes of 4000 IU [46,54], 10,000 IU [55] and 40,000 IU [56] did not report any adverse events.

Vitamin D intoxication is very rare but can be caused by the ingestion of excessively high doses. Prolonged excessive intakes can lead to hypercalcemia, dehydration, kidney damage, and soft tissue calcification. Increased calcium intake, reduced renal function, reduced estrogen levels, and granulomatous conditions such as sarcoidosis can all predispose an individual to vitamin D intoxication [57].

The first manifestation of vitamin D intoxication is hypercalciuria (i.e. excess calcium in the urine) followed by raised serum calcium concentrations [51]. The urinary calcium: creatinine ratio was shown to be an effective screening tool to detect hypercalciuria caused by abnormalities in calcium metabolism [58]. Vitamin D toxicity that results from hypercalcemia reflects the role of calcium in many tissues and targets, including bone, the cardiovascular system, nerves and cellular enzymes. Initial symptoms
of hypervitaminosis D include generalized muscle weakness and fatigue, nausea, vomiting, constipation, confusion, drowsiness, difficulty in concentration, and depression [57].

The probable mechanism for the toxicity of vitamin D is that high 25(OH)D concentrations causes excessive production of 1,25(OH)D. Along with the vitamin D and its other metabolites, 25(OH)D causes displacement of the hormone from DBP therefore increasing the amount of free, circulating 1,25(OH)D that is accessible to target cells [59]. Also, at toxic doses, the freely circulating vitamin D and its metabolites accumulate in adipose tissue and muscle. Thus far, the reported cases of vitamin D intoxication have been industrial accidents or were poisonings from an unknown source [60].

1.7 Serum 25-hydroxyvitamin D concentrations in the population

The Institute of Medicine (IOM) report suggests that persons at risk of vitamin D deficiency have serum 25(OH)D levels below 30nmol/L (12ng/mL), inadequacy at serum 25(OH)D levels between 30-50 nmol/L (12 and 20 ng/mL), and sufficiency at serum 25(OH)D levels of at least 50nmol/L (20ng/mL) [61]. However, most experts define 50nmol/L to be the cut-off point of deficiency, and 75 nmol/L or greater to be the optimal level [62-64]. If 25(OH)D levels <40-50 nmol/L and <52-72 nmol/L are the cut-off points for deficiency and insufficiency respectively, then an estimated 1 billion people worldwide have vitamin D deficiency or insufficiency [62].

The prevalence of an inadequate vitamin D nutritional status in many populations remains largely unrecognized worldwide. In Canada, data from the 2007-2009 Canadian Health Measures Survey showed that 16% of the population (ages 6-79) had serum
25(OH)D levels < 40nmol/L in the winter months, during which the cutaneous synthesis is minimal. A total of 31% had 25(OH)D levels <50nmol/L during winter \[^{65}\]. In a sample of healthy university students of diverse ancestries (mean age 21) 85% of the East Asian population had serum 25(OH)D <50nmol/L and 97% had levels < 75nmol/L. 34% of the students of European ancestry had 25(OH)D <50nmol/L and 84% had <75nmol/L. As for students of South Asian ancestry, 94% of them had wintertime serum 25(OH)D levels <50nmol/L and 100% had concentrations <75nmol/L \[^{66}\].

1.8 Vitamin D and mental health status

Vitamin D has been linked to neurocognitive benefits, including improved mood and depression scores. In 1989, Stumpf and Privette were the first to suggest the relationship between vitamin D and depression when they noted the higher prevalence of seasonal affective disorder (SAD) rates at high latitudes \[^{67}\]. Almost ten years later, in a randomized controlled trial (RCT) of 15 patients with SAD, an improvement of serum 25(OH)D levels was significantly associated with an improvement in depression scores \[^{68}\]. Another RCT showed that mood improved in adults after supplementation with 400 and 800 IU of vitamin D3, during winter \[^{69}\]. [Note: RCT’S for vitamin D and mental health are further discussed in Chapter 3 discussion; pages 58-60].

Low serum 25(OH)D levels in the early stages of life, may also contribute to the onset of depression in later stages. A UK-based birth cohort showed an association between low serum 25(OH)D at age 9 years and higher depressive symptoms at age 11 and 14 years \[^{70}\].
Vitamin D deficiency has been associated with lower mood in older adults \[^{71}\]. A 6-year prospective study of older adults aged 65 or older showed that those with 25(OH)D levels that were below 50nmol/L at baseline had significantly higher depression scores at 3 and 6 year follow up, in comparison to those with 25(OH)D levels that were greater than 50 nmol/L \[^{48}\]. A prospective study in members of the Women’s Health Initiative (aged 50 -79 years at baseline) reported that after controlling for other factors, those with the highest intake of dietary vitamin D had lower depressive symptom scores at a 3 year follow up \[^{72}\].

Several cross-sectional surveys explored the relationship between serum 25(OH)D levels and depression. A survey of older adults (aged 65 and older) reported an increased risk of depressive symptoms in those with vitamin D deficiency in northern latitudes \[^{73}\]. Another survey showed that low vitamin D levels were associated with depressive symptoms, especially in individuals with a history of depression \[^{74}\]. It was also reported that lower 25(OH)D levels in older adults (aged 65-95 years) increased the risk of both minor and major depression. Also, there was an association of depression status and severity with decreased serum 25(OH)D levels and increased serum parathyroid hormone (PTH) levels \[^{75}\].

Few cross-sectional studies did not show any associations between 25(OH)D and depression. A population- based cross-sectional study in middle-aged and older Chinese men and women (aged 50-70 years) did not find depressive symptoms to be associated with serum 25(OH)D levels \[^{76}\]. Similarly, a cohort of Japanese municipal employees (21-67 years of age) did not find any associations between higher 25(OH)D levels and lower depressive symptoms when surveyed in November and July. However, they did
show lower depressive symptoms scores in individuals with higher 25(OH)D levels, when surveyed in late autumn [77]. A cross-sectional study in the US (adults ≥ 20 years) also showed no significant association between 25(OH)D levels and depression after adjusting for multiple cofounders [78]. An RCT in older women (age ≥ 70 years) who were given a high dose of vitamin D₃ (500,000IU vitamin D₃ once per year versus a placebo for 3-5 years) showed no association between vitamin D and depression [79].

1.9 SF-36v2 health survey

The SF surveys are a validated tool for assessing well-being from a patient’s point of view and are amenable to a meta-analysis [80]. The SF-36v2 health survey asks 36 questions and provides scores for each of eight health domains that are related to the quality of life. The information from the eight health domains is then aggregated to provide total summary measures of the respondent’s physical and mental health (Figure 1.9; page 14). The scores are standardized to T scores (Mean = 50, Standard deviation = 10), such that a score of 50 represents an “average” North American reference. Standardized scores of 47 or greater are considered to be at least average in relation to the general population, whereas standardized scores that are below 47 indicate impaired functioning or well-being.

An RCT in women (aged 70 or more) that received calcium and vitamin D supplementations for 6 months, showed that 800 IU per day did not lead to an improvement in the mental health scores of the SF-12 questionnaire [81]. This suggests that 800 IU/day of vitamin D may not be enough for mental health benefits in older adults.
3a. Vigorous Activities
3b. Moderate Activities
3c. Lift, Carry Groceries
3d. Climb Several Flights
3e. Climb One Flight
3f. Bend, Kneel
3g. Walk Mile
3h. Walk Several Hundred Yards
3i. Walk One Hundred Yards
3j. Bathe, Dress

4a. Cut Down Time
4b. Accomplished Less
4c. Limited in Kind
4d. Had Difficulty

7. Pain- Magnitude
8. Pain- Interference

1. EVGFP Rating
11a. Sick Easier
11b. As Healthy
11c. Health to Get Worse
11d. Health Excellent

9a. Full of Life
9e. Energy
9g. Worn Out
9i. Tired

6. Social Extent
10. Social-Time

5a. Cut Down Time
5b. Accomplished Less
5c. Less Careful

9b. Nervous
9c. Down in Dumps
9d. Peaceful
9f. Depressed/ Downhearted
9h. Happy

Physical Functioning (PF)
Role-Physical (RP)
Bodily Pain (BP)
General Health (GH)
Vitality (VT)
Social Functioning (SF)
Role-Emotional (RE)
Mental Health (MH)

PHYSICAL HEALTH SUMMARY
MENTAL HEALTH SUMMARY
Figure 1.9. SF-36v2 health survey measurement model. All health domain scales contribute to the scoring of both the Physical and Mental Component Summary measures. Scales contributing most to the scoring of the summary measures are indicated by a connecting solid arrow (➡️). Scales contributing to the scoring of the summary measures to a lesser extent are indicated by a dashed line (..). This figure is reproduced from the User’s manual guide for the SF-36v2 health survey.
1.10 Food Policy

The current median intake of vitamin D from foods, with or without supplementation, in Canada is around 200 IU/day (Figure 1.10). This falls severely below the recommended 600 IU/day and 800 IU/day for adults and older adults respectively. Food policy in Canada allows for the mandatory fortification of milk with 100 IU of vitamin D per 250 ml serving, and 53 IU per 10g of margarine [61]. Thus a potential solution for this is to fortify foods with higher concentrations of vitamin D per serving, or provide more food options that are fortified with vitamin D.

Figure 1.10. Vitamin D intakes from food alone in Canada [82]. Data show the median and the 95% confidence limits for individuals’ intakes across ages and genders. The solid line indicates the RDA of vitamin D for older adults (800 IU/day) and the dashed line indicates the RDA for adults (600 IU/day) [61].
1.11 Rationale for the present investigations

Given that the vitamin D intakes amongst all age groups of Canadians are inadequate (Figure 1.10; page 16), strategies to rectify this are: 1) increase sun exposure 2) increase dietary intake from natural food sources 3) increase the fortification level in milk and margarine 4) increase supplementation via a vitamin D pill or liquid supplement or 5) increase the options of available fortified foods. Each of these options has its advantages and can be important in increasing vitamin D intakes. However, due to the modern day diet and minimal sun exposure the former strategies may be unrealistic or unfeasible. Supplementation or the fortification of more foods may be the most appropriate approaches, especially for individuals that are lactose intolerant.

We previously fortified cheddar cheese with vitamin D$_3$ and showed that it is bioavailable in adults [$^{83}$]. There was a small loss (at least 10%) in the whey protein by-product during the cheese production process. In this study, we developed an improved fortification method that retains the vitamin D by binding it to the casein protein found in milk. Vitamin D binds tightly to casein as was shown by charcoal stripping experiments in our lab, and so the casein bound vitamin D is expected to remain intact during the cheese production.

We also showed a significant improvement in well being with a 4000 IU per day vitamin D$_3$ dose in comparison to a 600 IU per day dose [$^{46}$]. Although there were several cross-sectional surveys (outlined in section 1.8) that assessed 25(OH)D and mental health status, these studies were conducted in community-dwelling older adults that are younger and more active than those residing in nursing homes. Also, there are a few RCT’s that assessed this, and the IOM report concluded that more studies are needed to confirm the
neurocognitive benefits of vitamin D $^{61}$. Therefore, our objectives for the present investigations are:

**Primary objectives:**

1. Measure the bioavailability in older adults by comparing serum 25(OH) D levels before and after consumption of the fortified cheese.

2. Assess the senior’s overall mental health status using a validated questionnaire (SF-36v2).

**Secondary objectives:**

1. Assess the older adults bone C-telopeptide, a biomarker of bone health.

2. Assess the homeostatic model assessment- insulin resistance (HOMA-IR) as an indicator of insulin resistance.

**Hypothesis:**

1. The consumption of fortified cheddar cheese will increase serum 25(OH) D levels in the elderly.

2. We can detect improvements in the mental health component of the SF-36v2 scores in those receiving 4000 IU/day vitamin D$_3$.

3. Biochemical markers of bone health and insulin resistance will improve in those receiving 4000 IU/day vitamin D$_3$. 


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Chapter 2: Bioavailability of vitamin D fortified cheddar cheese.

Note: The following chapters 2 and 3 will be submitted for publication and are in manuscript form. Therefore, there is some repetition of material.
2.0: Serum 25(OH)D levels and the bioavailability of vitamin D₃ fortified cheddar cheese in older institutionalized adults living in Canada: a pilot study

Abstract

The current food choices alone are insufficient to maintain the Institute of Medicine target serum 25-hydroxyvitamin D [25(OH)D] levels of 50nmol/L in many Canadians, especially during winter. We previously fortified cheddar cheese with vitamin D₃ and showed that it is as bioavailable as vitamin D₃ from a liquid supplement. We have developed a different fortification technique, by prebinding vitamin D₃ to the casein protein naturally found in milk. Our aim was to measure the bioavailability of fortified cheddar cheese in older institutionalized adults in Canada, since they are particularly susceptible to low vitamin D levels. Twenty-eight subjects were randomized to a weekly serving of cheddar cheese fortified with either 200IU or 28000IU of vitamin D₃ per 46 and 47g of cheese, respectively. The primary outcome was the comparison of vitamin D bioavailability, measured as serum 25(OH)D response between groups. The mean increase in 25(OH)D over the eight week intervention period was: 4.2±11.4 and 29.4±16.2 for the 200 IU/week and 28,000 IU/ week dose groups, respectively. These changes were different from one another (P<0.001). At baseline, subjects that were not meeting the 800IUper day RDA through the use of a supplement were well below the 50nmol/L target level, in comparison to subjects that did (P<0.001). These data demonstrate the need to increase options of vitamin D fortified foods, and the suitability of cheddar cheese for vitamin D fortification.

Introduction
Vitamin D insufficiency is widely prevalent and remains an unrecognized public health problem \[1,2\]. Cross-sectional or case-control epidemiological data associate the risk and prevalence of several diseases with latitude and UVB exposure \[3\]. These include a greater incident risk of certain types of cancer and increased cancer mortality \[4-7\], cardiovascular diseases \[8,9\], insulin resistance \[10,11\], infectious diseases \[12,13\] and multiple sclerosis\[14-16\]. Overall, the relationship between vitamin D and health point to a desirable serum 25-hydroxyvitamin D \[25(OH)D\] concentration that exceeds 75 nmol/L\[17,18\]. This is higher than the 50 nmol/L that is reported to be sufficient for all of the population groups by the Institutes of Medicine (IOM) Dietary Reference Intakes for vitamin D and calcium \[19\].

Older adults living in long-term care institutions generally have 25(OH)D levels that are below the optimal 75 nmol/L \[20,21\]. This is attributed to a less efficient response to vitamin D than in younger adults \[22-24\], limited sun exposure, inadequate dietary intake \[19\] or the extensive use of medications, which may interfere with vitamin D absorption \[25\]. The major source of vitamin D in the body is the endogenous production through sunlight exposure. However, serum 25(OH)D levels are affected by the endemic environment, culture, latitude, skin pigmentation, age and sunscreen use \[26-30\]. North Americans often rely on dietary supplements and fortified foods in order to meet their vitamin D requirements since it is not naturally present in many foods \[31\]. However, it has been suggested that long-term care residents, both with and without dementia, have a low rate of supplement use \[32\].

Vitamin D fortification is mandatory in Canada for beverage milk (100 IU/250ml) and margarine (53 IU/10g) and is optional in the United States for milk (96 IU/250 ml).
However, cross-sectional studies show that the current North American fortification practices are not effective in preventing vitamin D insufficiency in adults or the elderly. This is likely because there is no variety of available fortified foods and because these foods are often under-fortified, which means that they do not sufficiently increase circulating serum 25(OH)D concentrations \cite{31,33-35}. Data from the 2004 Canadian Community Health Survey Cycle 2.2 shows that the median vitamin D intakes from food alone for both sexes and across all age groups averages at around 200IU/day, which is well below the Recommended Daily Allowance (RDA) of 600IU/day for adults and 800 IU/day for older adults \cite{36}. Thus, there is an obvious need to increase the vitamin D sources of fortified foods in the food supply in order to meet the requirements of the general population, including older adults living in retirement homes.

We have previously shown that vitamin D fortified cheese is an appropriate means to provide vitamin D in adults, and it is as bioavailable as a supplement containing the same amount of vitamin D \cite{37}. Cheese is suitable for vitamin D fortification since it has <0.1 % of lactose, making it a source of vitamin D and calcium for individuals with lactose intolerance and those who do not drink milk.

In our previous work, 10 % of the vitamin D was lost in the whey protein by-product during the cheese manufacturing process. In this phase of our work, we pre-bound the vitamin D to the milk protein casein that is naturally present in milk in order to reduce the loss into the by-product. We investigated in a double-blind, randomized trial whether vitamin D is bioavailable from casein-bound vitamin D\textsubscript{3} fortified cheddar cheese in older adults living in a retirement home in Canada.
We tested the following hypothesis which we set a priori: the change in serum 25(OH)D (from week 0 to week 8) will be significantly higher in the group receiving the higher (28,000IU per week) dose than in the lower (200IU per week) dose. Retirement home residents are an ideal population to target for food fortification with vitamin D. The dietary vitamin D guidelines for older adults are higher than for younger adults [19]. Also, because their diets are well defined and that their exposure to sunlight limited, potential confounders are largely controlled for.

**Materials and Methods**

The study protocol was approved by Health Canada and by the Research Ethics Board at Mount Sinai Hospital (Toronto, Canada). All study subjects signed a form indicating their informed consent. The trial is registered at clinicaltrials.gov (identifier: NCT01555424).

**Subjects and recruitment criteria**

Subjects were recruited at the Aurora Kingsway Arms Retirement Center and were informed about the study through a group information session. Subjects were then approached to sign a consent form. If they were unable to make a decision, family members were approached to discuss the study protocol. Men and women that were deemed unable to participate by their attending physician were excluded from the study. They were not included in the study if any of the following criteria were present: 1) The presence of hypercalcemia/hypercalciuria 2) The use of medications that could interfere with vitamin D metabolism 3) Potential for significant sun exposure (e.g. travel to a
sunny location within the month prior to or during the study. Between February 9-16 2012, we recruited otherwise ‘healthy’ 28 men and women who met the eligibility requirements and resided at the Aurora Kingsway Arms Retirement Center (Latitude 44°N).

**Study Design**

The 28 eligible subjects were randomly assigned to 1 of 2 interventions: 1) Cheddar cheese fortified with 28,000 IU of vitamin D per week (47g/serving); 2) Cheddar cheese fortified with 200 IU of vitamin D per week (46g/serving). Each serving was consumed one day a week at the retirement home for a total of 8 weeks between the months of February and April of 2012. Endogenous synthesis of vitamin D from UV-B sunlight at this time of the year would be negligible \[^{30}\]. The weekly dose consumed is equivalent to a daily dose of 4000 IU and 28.6 IU respectively of cholecalciferol \[^{38}\].

Once a week, subjects received their portion of cheese during their mid-morning snack, in order to ensure compliance. At 0 (baseline), 4 and 8 weeks of the study, fasting venous blood and urine samples were obtained from each subject for biochemical testing. The primary outcome was the comparison of the serum 25(OH)D levels between groups at the week 8 time point. Secondary outcome measures included the homeostasis model assessment model (HOMA) of insulin resistance (IR), serum calcium, creatinine, phosphate, parathyroid hormone (PTH), alkaline phosphatase (ALP), and bone C-telopeptide (BCTX), as well as urine calcium, creatinine, and phosphate.

We expected an increase in the participant’s 25(OH)D levels from baseline. This was based on our previous experience, which showed a mean increase of 65.3 ± 24.1
nmol/L in adults consuming cheese fortified with an equivalent dose to 4000 IU per day of vitamin D₃, between baseline and 8 weeks [37]. We calculated that to study the bioavailability of vitamin D, a sample size of 50 per group will provide 80% power to detect a 0.5 SD difference between treatments at alpha = 0.05 (This is calculated using the Harvard power calculator, found at (http://hedwig.mgh.harvard.edu/sample_size/size.html). However, due to legal reviews we were only able to recruit subjects from one retirement home, which drastically cut down our sample size. Two other nursing homes eventually agreed to participate, but these approvals came too late to include them for this trial.

The randomization sequence was generated by randomly permuted blocks of 6 allocations, on Microsoft excel. This was done by the principal investigator, who was not involved in the implementation of the assignments. Subject names and their corresponding cheese were numerically coded so that both the investigators and subjects did not know which dose of vitamin D fortified cheese they were given. The code was revealed to the investigators once recruitment, data collection, and laboratory analyses were complete.

**Materials**

Both doses of cheese were industrially manufactured by Agropur Cooperative using standard cheese-making methodologies. The preliminary production step involved the addition of vitamin D₃ to the whole milk [4% fat (wt: wt)] destined for cheddar cheese manufacture. We fortified the milk destined for the high dose cheese with 60.55 IU/g and the milk destined for the low dose cheese with 0.39 IU/g of milk, using an in-
house made casein-bound vitamin D premix (186,500 IU/ml). The vitamin D content of
the cheese was measured as previously described [37,39] with the modifications: 1) the
mass of the cheese sample and distilled water was reduced 5-fold; 2) sample preparation,
heated saponification, and lipid extraction steps were all performed in a single test tube.

The mean vitamin D concentration was 593.6 IU/g for the high dose, and 4.3 IU/g
for the low dose. Vitamin D content was confirmed to be homogenous throughout the
cheeses, by sampling random sites from 2-kg cheese blocks and measuring the vitamin
D. After analysis, the cheese was portioned into weekly servings of high dose cheese
(47g/serving) and low dose cheese (46g/serving), corresponding to 28,000 IU vitamin
D$_3$/serving and 200 IU vitamin D$_3$/serving respectively. All cheese servings were
individually vacuum-packaged in plastic food bags, numerically coded so that the
dosage remained undisclosed, and kept refrigerated at 4-8°C. The vitamin D content of
the cheeses did not change over the duration of the study [40]. The tastes and physical
appearances of both cheeses were indistinguishable.

Measurements

We measured the anthropometrics of each subject (i.e. age, weight, and height) at
the baseline visit. At each weekly visit, the empty plastic cheese packages were collected
from each subject in order to ensure compliance. All residents were fed the same 5-week
menu at the retirement home. At the end of each study month, a Food Frequency
Questionnaire (FFQ) was completed and was used to calculate the mean background
daily intake of vitamin D and calcium from food. The food composition database used
for the FFQ was the most recent version of the Canadian Nutrient File. All subjects
maintained their typical dietary habits throughout the study.

Serum 25(OH)D was measured by radioimmunoassay (DiaSorin). The assay has a limit of detection of 3.75 nmol/L, an intra-assay CV of 8%, and an interassay CV of 16%. For serum 25 (OH)D, all samples from a single subject were measured within the same run to minimize assay variation. All samples were analyzed the same day and using the same reagent kit to minimize day-to-day or kit-to-kit variability.

Serum calcium, phosphate, creatinine, PTH, ALP, fasting glucose, fasting insulin and BCTX, we well as urine calcium, phosphate and creatinine were measured on a Modular Analytics Serum Work Area (Roche) within less than 4 hours after obtaining the blood and urine samples. Urinary calcium excretion was calculated as the ratio of millimolar concentrations of urine calcium and urine creatinine.

**Statistical analysis**

The results are expressed as mean ± SEM . All data were analyzed with SPSS software (version 20.0) or Graphpad Prism 5 for Windows. Graphs were created with GraphPad Prism 5. The criterion for statistical significance was set at P<0.05. The difference between groups for the baseline characteristics, the change in the secondary outcome measures and change in 25(OH)D levels were analyzed using an independent samples t-test. Person’s correlation test was used to examine the associations between the secondary outcome measures.
Results

Characteristics of subjects

The flow of participants through the study is illustrated (outlined in Figure 1; page 41). Of the 28 enrolled subjects, 24 completed the entire protocol (3 males and 21 females). One subject dropped out after giving a blood sample and before consuming any of the cheese. Two subjects dropped out, one halfway and one at the end of the study, and there was one death. Compliance, measured by counts of empty cheese packages was 97% (97% for the high dose group; and 98% for the low dose group). The analysis was intention-to-treat and involved all 28 subjects who were randomly assigned to groups. The demographics and baseline characteristics of the participants were very similar for the two intervention groups (Table 1; page 42). Baseline intake of vitamin D and calcium (from food) did not differ significantly between the two groups since they were all fed the same diet.

Baseline characteristics

At baseline, serum creatinine values were correlated to fasting blood glucose ($r=0.45$, $P=0.015$), BCTX ($r=0.62$, $P=0.000$), parathyroid hormone PTH ($r=0.42$, $P=0.027$), and phosphorus ($r=0.39$ $P=0.04$). PTH was also correlated to BCTX ($r=0.442$, $P=0.019$). In the total group (n=28; 4 males and 24 females) the mean baseline serum 25(OH)D concentration was $60.1 \pm 24.5$. Of these subjects 20 (72%) had less than desirable 25(OH)D concentrations below $<75$ nmol/L, 10 (36%) had low concentrations below $<50$ nmol/L, and 5 (18%) had concentrations that could indicate osteomalacia ($\leq 25$nmol/L).
Serum 25(OH)D concentrations

Upon completion of the 8-week intervention, a 25(OH)D concentration of ≥ 75 nmol/L was attained in 5 of 11 subjects (45%) consuming the low dose, and in 10 of the 13 (77%) consuming the high dose (Figure 2; page 43). Changes in serum 25(OH)D concentration [Δ 25(OH)D; nmol/L], calculated as the difference between baseline and week 8, was higher in the group that consumed the higher dose (29.4 ± 16.2) than in the group that consumed the lower dose (4.2 ±11.4) of vitamin D (P<0.001; Figure 3; page 44).

Secondary outcome measures

The changes (from baseline to week 8) in both groups are summarized in Table 1; page 42. Fasting glucose and insulin did not differ from baseline to week 8 between any of the groups, as well as serum creatinine, phosphate, ALP, and BCTX and urine biochemical measures (P>0.05) using an unpaired t-test. The change in serum PTH significantly decreased in the higher dose group in comparison to the lower dose group (P<0.05) and when compared with baseline values (P<0.001). Serum calcium remained unchanged (P>0.5). In all subjects that gave more than one blood sample (n=26), serum calcium concentrations remained within the normal reference range (2.2-2.6 nmol/L) at each time point. No subject developed hypercalcemia (serum calcium > 2.75 nmol/L) or hypercalciuria (millimolar ratio of urine calcium: urine creatinine >1). None of the subjects reported any adverse effects.
Supplements

Several subjects had prior to the start of this study been prescribed a daily multivitamin pill that contained 400IU of vitamin D, or 1000-2000IU daily of a vitamin D supplement. Figure 4 (page 45) shows baseline serum 25(OH)D concentration in subjects consuming below and above 800 IU of supplement per day, which is the current RDA for the elderly. Those who are meeting the RDA of 800 IU or more (by taking 1000 or 2000 IU supplements) had significantly higher serum 25(OH)D levels than those who are taking no supplements or a multivitamin supplement containing 400IU of vitamin D. Remarkably, without the use of a supplement, none of the subjects met the 50nmol/L serum 25(OH)D RDA target level.

Discussion

Our results show that the use of casein-bound vitamin D to fortify cheddar cheese is bioavailable from fortified cheddar cheese in older adults. The change in serum 25(OH)D levels from baseline to 8 weeks was significantly greater in subjects who consumed the high dose (28,000IU/week) of vitamin D than subjects who consumed the low dose (200IU/week). The increase in serum 25(OH)D in the high dose group was significantly higher than baseline after 4 and 8 weeks of the intervention. Our results also show that at baseline, older adults over the age of 70 who do not consume a vitamin D supplement that is at least 800 IU/day do not meet the target RDA of a serum 25(OH)D >50nmol/L. Once they consumed the high dose fortified cheese that provided 28,000 IU per week, their serum 25(OH)D concentrations increased to 91.3 ± 26.3, regardless of the supplementation level they were taking throughout the intervention.
Serum and urine calcium concentrations are the standard safety indices for vitamin D excess and those did not change by the intervention, suggesting the safety and efficacy of vitamin D supplementation at a 4000IU/day dose, in older adults. The PTH concentration decreased over the 8 weeks of the study intervention. Other markers of bone and mineral metabolism, including serum and urine phosphate, serum ALP, and serum BCTX remain unchanged by the consumption of the fortified cheese, as did the HOMA-IR index. These bone variables should be expected to improve with vitamin D concentration. However, our study may have been largely unpowered to detect any clinical significance.

There is limited health information on the vitamin D status of older institutionalized adults above the age of 70 and living in Canada, so it is crucial to understand the prevalence of vitamin D deficiency and sufficiency in this population. This study showed that 36% of older adults had insufficient serum 25(OH)D concentrations, below 50 nmol / L. The Canadian Health Measures Survey showed a U-shaped distribution in which children aged 6–11 y and adults aged 60–79 y had higher 25(OH)D concentrations than did adolescents and younger adults. However, this survey was conducted in community-dwelling older adults of whom 72% gave blood samples during the summer, and 60% took vitamin D supplements [41]. For institutionalized residents, however, exposure to sunlight is limited because they rarely seek out direct exposure of skin to sunlight and their diet generally does not maintain the necessary 25(OH)D levels. Some retirement home residents are prescribed vitamin D, thus adding to their burden of pill taking.
Our dosing protocol delivered 28,000 IU or 200 IU of cholecalciferol per weekly serving, which is equivalent to a daily vitamin D intake of 4000 IU or 29 IU respectively. This intake spans across the 100-400 IU vitamin D/30-50 g serving of cheese fortification level permitted by North American food regulations. One of us had previously shown that cheese fortified with vitamin D, using a fat emulsion premix, was as bioavailable as a supplement in adults [37]. Similarly, Natri et al. [42], demonstrated that bread fortified with cholecalciferol increases serum 25(OH)D as effectively as a supplement, in adults.

Another bioavailability study that used vitamin D fortified bread (5000IU/day) in institutionalized older adults demonstrated substantial benefits to bone density in one year [43]. In a study by Cherniak et al, veterans consuming 2000 IU per day for 6 months showed a similar response in serum 25(OH)D to the 4000IU/day dose group in this study [44]. Johnson et al demonstrated that in adults (≥ 60 years old) the consumption of fortified processed cheese containing 600 IU of vitamin D for 2 months did not produce a detectable increase in serum 25(OH)D [45]. These findings and ours are consistent with previous reports that suggest that 600 IU vitamin D/day is insufficient to increase serum 25(OH)D in the elderly [1,46,47].

The 28,000 IU vitamin D intake given in this study was chosen to produce a rise in serum 25(OH)D that would increase the statistical power to detect possible differences in bioavailability among our treatment groups, and for any potential health benefits. The 200IU/week dose was chosen to serve as an amount of vitamin D in cheese that would be commercially permissible, in accordance with the current milk fortification practices in Canada. Ingestion of 4000 IU/day cholecalciferol from fortified cheese safely increased
the vitamin D status of our subjects, ensuring a serum 25(OH)D concentration ≥ 75 nmol/L in 77% of the high dose vitamin D- treated subjects, and significantly decreased mean PTH levels.

In conclusion, we found that cheese fortified to 28,000IU/week enhances the vitamin D status in older adults living in retirement homes. The availability of vitamin D fortified foods in the food supply is a population- wide and cost effective method to improve vitamin D intakes in the population, including the institutionalized elderly. This may help to bring about the public health benefits that many experts are suggesting result from greater intakes of vitamin D.
Figure 1. Consort diagram shows the flow of participants recruited in the study.
<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Change</th>
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<tr>
<td></td>
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<td>n</td>
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<tr>
<td>Age, y</td>
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<tr>
<td>BMI, kg/m²</td>
<td>23.1 ± 3.6</td>
<td>28.8 ± 7.7</td>
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<tr>
<td>Vitamin D intake</td>
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<td>22.6 ± 29.5</td>
</tr>
<tr>
<td>Calcium intake</td>
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<tr>
<td></td>
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<td>176.3</td>
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<tr>
<td>Serum 25(OH)D, nmol/L</td>
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<td>&lt;75 nmol/L, n (%)</td>
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<td>≤25 nmol/L, n (%)</td>
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<tr>
<td>Fasting glucose (mmol/L)</td>
<td>5.7 ± 1.4</td>
<td>5.2 ± 1.2</td>
</tr>
<tr>
<td>Fasting insulin (pmol/L)</td>
<td>57.4 ± 31.1</td>
<td>53.5 ± 24.6</td>
</tr>
<tr>
<td>Serum calcium, mmol/L</td>
<td>2.3 ± 0.1</td>
<td>2.3 ± 0.1</td>
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<tr>
<td>Urine calcium, mmol/L</td>
<td>2.4 ± 1.9</td>
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<td>Serum creatinine, µmol/L</td>
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<td>97 ± 35.2</td>
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<tr>
<td>Urine creatinine, mmol/L</td>
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<td>Urinary calcium excretion, mmol:mmol creatinine</td>
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<td>0.3 ± 0.2</td>
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<td>Serum phosphate, mmol/L</td>
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<td>1.2 ± 0.1</td>
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<tr>
<td>Urine phosphate, mmol/L</td>
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<td>Serum PTH, pmol/L</td>
<td>5.1 ± 2.7</td>
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<td>Serum Alkaline phosphatase (ALP), U/L</td>
<td>75.1 ± 16.7</td>
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<tr>
<td></td>
<td>604.1 ±</td>
<td>524.5 ±</td>
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<tr>
<td>Serum bone C-telopeptide (BCTX) ng/L</td>
<td>313.1</td>
<td>291.4</td>
</tr>
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</table>

Table 1. Baseline characteristics and change in subject’s biochemistry at the end of the intervention. HOMA-IR was calculated as (fasting glucose x fasting insulin)/22.5. Values are means ±SD. Groups differ, ***P<0.001 and * P< 0.05 using an independent samples t-test.
Figure 2. Serum 25(OH)D concentrations in subjects consuming vitamin D fortified cheese. Values are means ±SEM. ** P<0.01, *** P<0.001 Significantly different from baseline (time 0) by paired t-test. n=14 for week 0 low dose, n=12 for week 4 low dose, n=11 for week 8 low dose; n= 14 for week 0 high dose, n=14 for week 4 high dose, n=13 for week 8 high dose.
Figure 3. Changes in serum 25 (OH)D concentration in subjects consuming vitamin D fortified cheddar cheese. Boxes represent the range of the middle 50% of the sample population, with its mid-line indicating the median value. The whiskers show the highest and lowest values of the summarized data. Means differ significantly with unpaired t-test, *** P<0.001. n= 11 for 200IU/wk dose; n=13 for 28,000IU/wk dose.
Figure 4. Baseline serum 25 (OH)D concentrations before consumption of the vitamin D fortified cheese in subjects taking a vitamin D supplement. This figure is stratified to indicate the vitamin D consumption as the supplement, to permit comparison of 25(OH)D in those 12 subjects consuming less than the current RDA amount versus those 16 subjects who consumed a vitamin D supplement at least equal to the current RDA of 800 IU/day for those older than 70 years. Boxes represent the range of the middle 50% of the sample population, with its mid-line indicating the median value. The whiskers show the highest and lowest values of the summarized data. Means differ significantly with unpaired t-test, *** P<0.001. n=12 for <800IU/d; n=16 for ≥ 800IU/d. The dashed line indicates the 50nmol/L level minimum that indicated adequacy in vitamin D nutrition according to the most recent IOM report on vitamin D.


Chapter 3: Well-being in older adults.
3.0: Vitamin D₃ fortified cheese increases serum 25(OH)D response and improves mental health scores of elderly in a Canadian retirement home

Abstract

Little is known about the vitamin D status and mental health status of older adults living in retirement homes in Canada. The Institute of Medicine increased the Recommended Daily Allowance (RDA) to 800 IU for adults over 70 years of age. However, the median intake of vitamin D from foods and supplements is around 200 IU per day. In the previous chapter, we were able to fortify cheddar cheese with vitamin D and show that it is bioavailable in older adults. Twenty-eight participants were randomized to a weekly serving of cheddar cheese fortified with either 200 IU or 28000 IU of vitamin D₃ per 46 and 47g of cheese, respectively. Our primary outcomes were to assess, in a double-blinded randomized trial, whether the vitamin D was bioavailable and whether higher serum 25-hydroxyvitamin D levels [25(OH)D] resulted in an improvement in mental health scores, based upon the SF-36v2 health survey. A Man-Whitney U test showed that there was a significant difference in the change in Mental Component Summary (MCS) between groups (P=0.045). There was a positive correlation between the change in MCS score and the change in serum 25(OH)D, (1-tail significance; P=0.036), indicating an improvement in MCS with higher 25(OH)D levels. An outlier was excluded for the analysis. There were no significant correlations between the other components of the SF-36v2 questionnaire and 25(OH)D concentrations.
Introduction

In the previous chapter, we were able to show that the consumption of cheddar cheese fortified with 4000 IU per day of vitamin D\textsubscript{3} is bioavailable in older adults, and this increased the serum 25(OH)D levels by 29.4±16.2. This change was significantly higher, at \( P<0.001 \), than in the group that consumed 29 IU per day \( (4.2±11.4) \).

Extra-renal associations have been found between vitamin D and diseases, including multiple types of cancer and cancer mortality \([1-4]\), multiple sclerosis \([5-7]\), insulin resistance and cardiovascular disease \([8-11]\), as well as infectious diseases \([12,13]\). Similar to other tissues, the brain has the nuclear receptors for 1,25(OH)\textsubscript{2}D as well as the necessary enzymes to locally hydroxylate vitamin D to 1,25(OH)\textsubscript{2}D \([14]\). The brain also possesses the degradation enzyme for 1,25(OH)\textsubscript{2}D, vitamin D\textsubscript{3} 24-hydroxylase, suggesting that both its activation and inactivation can occur in the brain \([15]\).

To our knowledge, not many studies have explored the benefits of vitamin D supplementation on mental health status, specifically in older adults residing in retirement homes. In this study, we aimed to characterize the effect of the consumption of cheese fortified with 29 IU/day and 4,000IU/day of vitamin D\textsubscript{3} on the mental health of older institutionalized adults living in Canada, using the SF-36v2 health survey. We expected an improvement in the mental health status of subjects that consumed the higher dose cheese, and therefore showed an increase in their serum 25(OH)D levels. Our a priori hypothesis was based on our previous work, where 4000 IU daily of vitamin D\textsubscript{3} showed an improvement in well-being scores in adults, during the winter months \([16]\).
Materials and Methods

This clinical trial was approved by Health Canada and by the Research Ethics Board at Mount Sinai Hospital (Toronto, Canada). Written informed consent was obtained from all subjects. The trial is registered at clinicaltrials.gov (identifier: NCT01555424).

Participants

Subjects over the age of 70 were recruited at a retirement home in Aurora, Ontario (latitude: 44°N). Participants were first approached through a group information session and signed an informed consent. If they were unable to form a decision, family members were approached to discuss the study protocol. Men and women that were deemed unable to participate by their attending physician were excluded from the study. They were not included in the study if any of the following criteria were present: 1) The presence of hypercalcemia/ hypercalciuria 2) The use of medications that could interfere with vitamin D metabolism 3) Potential for significant sun exposure (e.g. travel to a sunny location within the month prior to or during the study. Between February 9-16 2012, we recruited otherwise ‘healthy’ 28 men and women who met the eligibility requirements.

Study design

The 28 subjects were randomly assigned to 1 of 2 interventions: Cheddar cheese fortified with 28,000 IU of vitamin D per week (47g/serving) or 2) Cheddar cheese fortified with 200 IU of vitamin D per week (46g/serving). Each weekly serving was equivalent to consuming 4000 IU/day and 29 IU/day, respectively [17]. Each serving was
consumed orally once a week at the retirement home for a total of 8 weeks between the months of February and April of 2012, during which the endogenous synthesis of vitamin D would be negligible [18]. At baseline and at week 8 of the study, after the final dose of cheese was consumed, the participants were interviewed using the SF-36v2 script.

The randomization sequence was generated by randomly permutated blocks of 6 allocations, on Microsoft excel. This was done by the principal investigator who did not play a role in the implementation of assignments. Subject names and their corresponding cheese assignment were numerically coded so that both the investigators and subjects did not know which dose of vitamin D fortified cheese they were given. The code was revealed to the investigators once recruitment, data collection, and laboratory analyses were complete.

Materials

Both doses of cheese were manufactured industrially by Agropur Cooperative using standard cheese-making methodologies. The details are described in Chapter 2. Briefly, we added vitamin D bound to the casein protein (which is naturally found in milk), to milk that was destined for cheddar cheese manufacture. The mean vitamin D concentrations were 593.6 IU/g for the high dose, and 4.3 IU/g for the low dose cheese. The cheese was then portioned into weekly servings of high dose cheese (47g/serving) and low dose cheese (46g/serving), such that each serving provided 28000 IU of vitamin D₃ and 200 IU of vitamin D₃ per serving. All of the cheeses were vacuum-packaged in plastic food bags, numerically coded so that the dosage remained undisclosed, and kept
refrigerated at 4-8 °C. The tastes and physical appearances of both cheeses were indistinguishable.

**Measurements**

The anthropometrics of each subject (i.e. age, weight, and height) were measured at the baseline visit. At each weekly visit, the empty cheese packages were collected from each subject in order to confirm compliance. All of the residents were fed the same 5-week menu at the retirement home, and their typical dietary habits were maintained throughout the study. At the end of each study month, a Food Frequency Questionnaire (FFQ) was completed and was used to calculate the mean background daily intake of vitamin D and calcium and food. The food composition database used for the FFQ was the most recent version of the Canadian Nutrient File.

Serum 25(OH)D was measured by radioimmunoassay (DiaSorin). The assay has a limit of detection of 3.75 nmol/L, an intra-assay CV of 8%, and an interassay CV of 16%. For serum 25 (OH)D, all samples from a single subject were measured within the same run to minimize assay variation. All samples were analyzed the same day and the same reagent kit was used to minimize day-to-day or kit-to-kit variability.

Well-being was assessed by the SF-36v2 health survey. This survey consists of 36 questions that measure both physical and mental health from a patient’s point of view. The physical component assesses function and an evaluation of one’s ability to perform a physical activity. The mental health component assesses psychological distress and well being, social functioning, and overall vitality. Responses to the questions were analyzed by Qualitymetric using a certified scoring system, and were sent back to our lab for
Serum calcium, phosphate, creatinine, PTH, ALP, fasting glucose, fasting insulin and BCTX, we well as urine calcium, phosphate and creatinine were measured on a Modular Analytics Serum Work Area (Roche) within less than 4 hours after obtaining the blood and urine samples. Urinary calcium excretion was calculated as the ratio of millimolar concentrations of urine calcium to urine creatinine.

**Statistical Analysis**

The results are expressed as mean ± SD. All data were analyzed with SPSS software (version 20.0). The criterion for statistical significance was set at P<0.05. The difference between groups for the baseline characteristics, the change in the secondary outcome measures, and change in 25(OH)D levels were analyzed using an independent samples t-test. A non-parametric Mann Whitney was used to examine the Mental Component Summary (MCS) score differences between both groups. A Pearson’s correlation test was used to test the a priori 1-tail hypothesis that higher 25(OH)D levels improved MCS scores, as well as to examine the associations between the different components of well-being and the secondary outcome measures.

**Results**

**Subject Characteristics**

The flow of subjects throughout the study is shown in Chapter 2. Figure 1; page 41. A total of 28 enrolled subjects were randomly assigned to 1 of 2 groups; and 24 completed the entire protocol (3 males and 21 females). One subject dropped out after
giving a blood sample and before consuming any of the cheese. Two subjects dropped out by the end of the study, and there was one death. Compliance, defined as percent of dose consumed, was 97% for the high dose group and 98% for the low dose group, as measured by the counts of the returned empty cheese packages. The analysis was intention-to-treat and involved all 28 subjects. The demographics and baseline characteristics of subjects were very similar for the two intervention groups (Chapter 2, Table 1; page 42). Baseline intake of vitamin D and calcium (from food) did not differ significantly between the two groups since they were all fed the same diet at the retirement home.

**Serum 25(OH)D concentrations**

At baseline, the mean baseline serum 25(OH)D concentration was 60.1 ± 24.5 for all of the subjects (n=28; 4 males and 24 females). Changes in serum 25(OH)D was higher in the group that consumed the higher dose (29.4 ± 16.2) than in the group that consumed the lower dose (4.2 ± 11.4) of vitamin D (P<0.001; Chapter 2).

The change in serum PTH significantly decreased in the higher dose group in comparison to the lower dose group (P<0.05) and when compared with baseline values (P<0.001). Serum calcium remained unchanged (P>0.05). None of the subjects reported any adverse effects.

**SF-36v2 Mental Health component outcome**

A boxplot of all of the data shows the difference between the medians of the Mental Component Summary (MCS) of the SF-36v2 questionnaire in the high and low
dose groups (Figure 2; page 61). These data were not significantly different from one another using a non-parametric Mann Whitney test, at a P value that is >0.05 (P=0.119). However, SPSS provides an objective criterion (1.6 times the standard deviation) for determining outliers. Therefore, for further analysis the 2 outliers evident in the figure 2 (points 32 and 33) were excluded. A Man-Whitney test showed that there was a significant difference in the MCS between groups with the removal of the two outliers (P=0.011).

A further scatterplot analysis of the outliers showed that a single datum point (point 33 on figure 2: page 61) lies outside the 95% confidence interval bands for all of the subjects (This is pointed out with an arrow in Figure 3a; page 62). With this one outlier removed, the difference in median values between the groups still remained significant (two-tailed, P=0.045). Also, without the extreme outlier, a regression plot showed the positive correlation between the change in MCS score and the rise in serum 25(OH)D through the 8 weeks of this clinical trial (Figure 3b; page 63). This relationship was significant at a one-tail end, and indicates that the higher the change in serum 25(OH)D levels, the greater the improvement in the MCS score (1-tail; P=0.036). There were no significant correlations between the other components of the SF-36v2 questionnaire and serum 25(OH)D concentrations. The changes in scores between baseline and week 8 are expressed as means± SD in Table 2; page 64.

Discussion

We were able to show that the group that consumed 4000 IU/day of vitamin D₃ fortified cheese had a significantly higher change in the MCS scores, in comparison to
the group that consumed 29 IU/day of vitamin D₃ fortified cheese. Also, the higher the change in serum 25(OH)D concentrations, the greater the improvement in the MCS scores (1-tail significance; \( P=0.036 \) with the removal of one outlier). This indicates that 4000 IU per day of vitamin D, provided for eight weeks, improved the mental health aspect in older adults.

We did not find any significant changes between the various sub-components of the SF-36v2 health survey and serum 25(OH)D levels. This may be because our study was underpowered to detect any significance. To our knowledge, there is no published literature that assessed the relationship between vitamin D and mental health status in older adults using the SF health surveys. What is consistent with the present findings is a cross-sectional epidemiologic report in a subset of the Comprehensive Dialysis Study (CDS) in adults, that showed that lower 25(OH)D levels were independently associated with lower scores on the Mental Component Summary (MCS). The authors also reported that each 25% decrease in 25(OH)D concentration was associated with a 1-point lower score on the MCS[^19].

Vieth et al. showed that both 4000 IU and 600 IU per day of vitamin D₃ improved well-being scores over two consecutive winters in adults, with 4000 IU resulting in a better improvement[^16]. Jorde et al. showed that serum 25(OH)D levels below 40 nmol/L were associated with lower scores on the Beck Depression Inventory (BDI) in overweight and obese adults[^20]. In older adults, serum 25(OH)D levels that were below 50 nmol/L were associated with higher depression scores, using the Center for Epidemiological Studies-Depression Scale (CES-D)[^21].
More recently, Kjaergaad et al. showed that low serum 25(OH)D levels were associated with depressive symptoms (using the BDI, Hospital Anxiety and Depression Scale, Seasonal Pattern Assessment Scale and Montgomery-Asberg Depression Rating Scale). However supplementation with 40,000 IU of vitamin D3 per week for six months did not show an improvement in depressive symptoms \[^{22}\]. These findings may be because the population in the study was young (mean age of 53 years), otherwise healthy adults, who the majority of, were not initially depressed.

The strengths of this study were that it was a double-blinded RCT in a population that is highly affected by low mental health status, showing evidence of benefit with a simple food-based strategy. Limitations were the small sample size and hence a relatively severe effect of outliers.

It has been suggested that individuals that live to an older age, do so with a decreased quality of life and more disease burdens \[^{23}\]. The present RCT findings confirm that higher serum 25(OH)D levels resulted in a positive change in the Mental Component Summary aspect of the SF-36v2 health survey, which translates to a better mood and well-being. We also showed that 4000IU of vitamin D3 per day is safe and tolerable in older adults. Older adults living in retirement homes are particularly susceptible to low 25(OH)D levels, and fortification may be a key way to achieve optimal vitamin D nutritional status, and improve overall well-being.
Figure 2: The boxplot shows the change in the mental component summary (MCS) by dose group. Boxes represent the range of the middle 50% of the sample population, with its mid-line indicating the median value. The whiskers show the highest and lowest values of the summarized data not classified as an outlier by the SPSS software. The group that consumed the higher dose 28000 IU/wk vitamin D fortified cheese showed a higher change in their MCS, which indicates an improvement. With both outliers on the boxplot (points 32 and 33) removed, a Man-Whitney U test showed that there is a significant difference in the Mental Component Summary (MCS) between groups (P=0.011; n= 10 for 200IU/wk dose; n=12 for 28000 IU/wk dose). If only point 33 was excluded as an outlier, the difference between groups still remained significant at (P=0.045; n= 11 for 200IU/wk dose; n=12 for 28000 IU/wk dose)
Figure 3a. This figure includes the 95% confidence bands for the cohort at the end of the study, and the arrow points to the extreme outlier removed for statistical analysis. (n= 11 for 200IU/wk dose; n=13 for 28000 IU/wk dose). White circles indicate subjects consuming the low dose, black circles indicate subjects consuming the high dose.
Figure 3b. The relationship between the change in Mental Component Summary score (MCS) and the change in serum 25(OH)D through the 8 weeks of this clinical trial, with the extreme outlier removed (n= 11 for 200IU/wk dose; n=12 for 28000 IU/wk dose). The 95 % confidence interval bands are for the regression line of the subjects. This relationship was significant at the 1-tail end (P=0.036, Mann Whitney test) and is consistent with the a priori hypothesis. White circles indicate subjects consuming the low dose, black circles indicate subjects consuming the high dose.
<table>
<thead>
<tr>
<th>Components of SF-36v2 survey</th>
<th>Vitamin D$_3$ dose per week</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
</tr>
<tr>
<td></td>
<td>200 IU</td>
</tr>
<tr>
<td>Physical functioning</td>
<td>35.7 ± 9.4</td>
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<tr>
<td>Role- physical</td>
<td>44.6 ± 9.6</td>
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<td>Bodily pain</td>
<td>47.9 ± 7.7</td>
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<tr>
<td>General health</td>
<td>46.0 ± 9.4</td>
</tr>
<tr>
<td>Vitality</td>
<td>48.7 ± 10.9</td>
</tr>
<tr>
<td>Social functioning</td>
<td>44.3 ± 12.1</td>
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<tr>
<td>Role- emotional</td>
<td>47.5 ± 11.8</td>
</tr>
<tr>
<td>Mental health</td>
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<td>Physical component summary (PCS)</td>
<td>40.6 ± 10.0</td>
</tr>
<tr>
<td>Mental component summary (MCS)</td>
<td>51.8 ± 9.0</td>
</tr>
</tbody>
</table>

Table 2. Values are mean ± SD. Baseline and the change in subjects scores of the sub-components of the SF-36v2 health survey per dose group (change is calculated as calculated as Week 8 scores - Week 0 scores). These data points do not include the two outliers. A positive change indicates an improvement. Means differ with a Man Whitney test (*P=0.011; n= 10 for 200IU/wk dose; n=12 for 28000 IU/wk dose).


14. Eyles DW, Burne TH, McGrath JJ. Vitamin D, effects on brain development, adult brain function and the links between low levels of vitamin D and neuropsychiatric disease. *Front Neuroendocrinol.* Jul 11 2012.


Chapter 4: Implications.
The overall purpose of the clinical trial reported here was to test the hypothesis that firstly, vitamin D fortified cheddar cheese is bioavailable in older adults, and secondly, that higher vitamin D nutritional status improves the mental health status in institutionalized older adults, who are particularly vulnerable to vitamin D deficiency. This hypothesis was suggested by work previously done in our lab, where vitamin D₃ fortified cheese (4000 IU per day) was shown to be as bioavailable as a supplement in adults [¹], and that 4000 IU per day of vitamin D₃ showed improvements in the well-being of adults, during the winter months [²].

For our first objective, we succeeded in showing that cheese fortified with vitamin D₃ is bioavailable in older adults. Also, older adults who took a supplement of at least 800 IU daily of vitamin D₃ had what the IOM defines as the target “sufficient” levels of serum 25(OH)D [³]. For our second objective, we showed that there was a significant positive change in the Mental Component Summary (MCS) of the participants that had a mean serum 25(OH)D change of 29.4 ± 16.2 nmol/L by the consumption of the cheese fortified with 4000IU/day of vitamin D₃. Also, the relationship between the change in 25(OH)D and the change in MCS was positive and significant at a 1-tailed end. The mental health status improved in those that had a mean final serum 25(OH)D level of 91.3 ± 26.3 nmol/L. This suggests that it is at levels that are >50nmol/L that mental health benefits are seen in older adults.

This study had some limitations. We had a small sample size, which may have reduced the statistical power of detecting differences in other biochemical or physical health parameters of the SF-36v2 between the two groups. However, we were still able to
show significant differences in the change in serum 25(OH)D levels and the change in mental health status, although there was an outlier that was excluded in our analysis in order to detect significance. We were not able to detect any changes between the groups in their HOMA-IR scores or in bone C-telopeptide results, which were secondary outcome measures. Therefore, it is of interest to replicate the study at a larger scale, and to assess insulin resistance and outcomes of bone turnover with higher 25(OH)D levels. Also, a longer period of supplementation may be necessary to obtain clinically significant benefits in bone health.

Strengths of our study are that it was a small-scale pilot study that consisted of older institutionalized adults in Canada, since they are a group that are susceptible to having low vitamin D nutritional status and are difficult to study. Also, to our knowledge, there are no data on the serum 25(OH)D levels of Canadian institutionalized adults over the age of 79, since the 1997 report by Lui and his colleagues that showed the mean 25(OH)D levels (nmol/L) in institutionalized older adults (> 65 years old) was 44.9 ± 16.9 during fall and 39.9 ± 19.7 during spring [4]. This is a randomized double-blind controlled trial and can be repeated in a larger scale trial to further provide evidence of neurocognitive benefits to increase the RDA requirements of vitamin D.

The present study has important implications in the fields of nutrition and public health. First, the present results show that at baseline a supplement of at least 800 IU/day resulted in serum 25(OH)D concentrations that are >50 nmol/L which confirms the RDA for older adults. Second, cheddar cheese is lactose-free and an enjoyable alternative to consuming supplements and we have shown it to be an effective means of increasing the serum 25(OH)D levels and benefiting this population group. Third, many Canadians,
especially those of non-European ancestry and older adults, have serum 25(OH)D levels that are below 50 nmol/L. Fortified cheese is a public health approach that will target a wider population, and improve vitamin D nutritional status in vulnerable groups. Lastly, we showed that 4000 IU per day of vitamin D₃ is safe and tolerable in older adults.

In summary, the advanced hypothesis that Vitamin D₃ fortified cheddar cheese increases serum 25(OH)D levels and provides mental health benefits in older adults, has been supported by the studies conducted. The findings support the utility and the suitability of vitamin D fortified cheese in Canada, and provide evidence of benefit with a simple solution.

