RANDOMIZED TRIAL OF THE EFFECTS OF VITAMIN D ON TISSUE VITAMIN D METABOLITES AND ON PROSTATE CANCER PATHOLOGY

by

Dennis Wagner

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy (PhD)
Graduate Department of Nutritional Sciences
Faculty of Medicine
University of Toronto

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Randomized Trial of the Effects of Vitamin D on Tissue Vitamin D Metabolites and on Prostate Cancer Pathology

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2013

Abstract

Epidemiologic, laboratory and clinical data suggest that vitamin D plays a favourable role in the prevention and prognosis of prostate cancer (PCa) and other malignancies. However, the metabolism and function of vitamin D in tissues beyond those involved in bone metabolism are poorly understood. The objective of this thesis was to investigate whether higher\(^1\) levels of vitamin D consumed orally or achieved in the circulation result in increased concentrations of vitamin D metabolites in human tissue, and how this affects cellular biology. The hallmark of this work is a randomized clinical trial of oral vitamin D\(_3\) (400-, 10,000-, or 40,000 IU/d) in PCa patients to evaluate the effects of supplementation on prostatic vitamin D metabolism and on PCa pathology. Various methods to measure vitamin D metabolites in serum were evaluated and modified to allow for measurement of these metabolites in tissue. Ultimately, I developed a robust tissue extraction method coupled to enzyme immunoassay and liquid chromatography-tandem mass spectrometry for measurement of calcitriol hormone, as well as 25-hydroxyvitamin D (25(OH)D) and 24,25-dihydroxyvitamin D (24,25(OH)\(_2\)D), respectively. Human colon tissue

\(^1\) In this context, “higher” denotes intake or circulating concentrations of vitamin D that are near or beyond the upper end of exposure attainable through normal physiology
was analyzed first and found to contain calcitriol at physiologically relevant concentrations partly determined by serum calcitriol, with some evidence of local colonic synthesis. In the clinical trial, prostate tissue and serum levels of calcitriol, 25(OH)D and 24,25(OH)₂D increased dose-dependently (p<0.02) without adverse side effects. The level of calcitriol attained in prostate tissue was inversely associated with the expression of Ki67 protein, a proliferation marker (p<0.05). Serum parathyroid hormone (PTH) and prostate specific antigen (PSA) declined from baseline in the combined higher-dose groups (10,000-40,000 IU/d) (p<0.02). We provide clinical trial evidence that prostatic vitamin D metabolism can be modulated \textit{in vivo} by oral consumption of vitamin D₃. Higher prostate calcitriol and vitamin D doses also showed suggestion of clinical benefit, including lowered Ki67 expression and modest reductions in serum PSA and PTH. Further studies are needed to validate the potential utility of dietary vitamin D₃ supplemenation in cancer prevention and therapy.
This PhD thesis is dedicated to my parents, Adriana and Carlos Wagner. Mom and Dad, your unwavering support and unconditional love are what have made this possible.
Acknowledgments

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<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,24,25(OH)(_3)D</td>
<td>1,24,25-trihydroxyvitamin D</td>
</tr>
<tr>
<td>1,25(OH)(_2)D</td>
<td>1,25-dihydroxyvitamin D or calcitriol</td>
</tr>
<tr>
<td>1α-OHase</td>
<td>1α-hydroxylase</td>
</tr>
<tr>
<td>24,25(OH)(_2)D</td>
<td>24,25-dihydroxyvitamin D</td>
</tr>
<tr>
<td>24,25:25(OH)D to 25(OH)D ratio</td>
<td>24,25(OH)(_2)D to 25(OH)D ratio</td>
</tr>
<tr>
<td>25(OH)D: 25-hydroxyvitamin D or calcidiol</td>
<td>25(OH)D: 25-hydroxyvitamin D or calcidiol</td>
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<tr>
<td>ADT:</td>
<td>androgen deprivation therapy</td>
</tr>
<tr>
<td>AI:</td>
<td>adequate intake</td>
</tr>
<tr>
<td>ALP:</td>
<td>alkaline phosphatase</td>
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<tr>
<td>ALT:</td>
<td>alanine transaminase</td>
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<tr>
<td>ANOVA:</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>AR:</td>
<td>androgen receptor</td>
</tr>
<tr>
<td>AU:</td>
<td>absorbance unit</td>
</tr>
<tr>
<td>BMD:</td>
<td>bone mineral density</td>
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<tr>
<td>BMI:</td>
<td>body mass index</td>
</tr>
<tr>
<td>Ca:</td>
<td>calcium</td>
</tr>
<tr>
<td>CI:</td>
<td>confidence interval</td>
</tr>
<tr>
<td>CLIA:</td>
<td>chemiluminescent immunoassay</td>
</tr>
<tr>
<td>CV:</td>
<td>coefficient of variation</td>
</tr>
<tr>
<td>CYP24A1:</td>
<td>24-hydroxylase</td>
</tr>
<tr>
<td>CYP27B1:</td>
<td>1α-hydroxylase</td>
</tr>
<tr>
<td>CYP27A1:</td>
<td>mitochondrial 25-hydroxylase</td>
</tr>
<tr>
<td>CYP27B1:</td>
<td>mitochondrial 25-hydroxylase</td>
</tr>
<tr>
<td>DBP:</td>
<td>vitamin D binding protein</td>
</tr>
<tr>
<td>DEQAS:</td>
<td>Vitamin D External Quality Assessment Scheme</td>
</tr>
<tr>
<td>DHT:</td>
<td>dihydrotestosterone</td>
</tr>
<tr>
<td>DRE:</td>
<td>digital rectal exam</td>
</tr>
<tr>
<td>DRI:</td>
<td>dietary reference intake</td>
</tr>
<tr>
<td>EBRT:</td>
<td>external beam radiation therapy</td>
</tr>
<tr>
<td>ECLIA:</td>
<td>electrochemiluminescence immunoassay</td>
</tr>
<tr>
<td>EIA:</td>
<td>enzyme immunoassay</td>
</tr>
<tr>
<td>FFPE:</td>
<td>formalin-fixed paraffin-embedded</td>
</tr>
<tr>
<td>GFR:</td>
<td>glomerular filtration rate</td>
</tr>
<tr>
<td>HPLC:</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>IOM:</td>
<td>Institute of Medicine</td>
</tr>
<tr>
<td>IU:</td>
<td>international units</td>
</tr>
<tr>
<td>IU (1 μg vitamin D = 40 IU)</td>
<td>(1 μg vitamin D = 40 IU)</td>
</tr>
<tr>
<td>LCM:</td>
<td>laser capture microdissection</td>
</tr>
</tbody>
</table>
LC-MS/MS: liquid chromatography-tandem mass spectrometry
LHRH: luteinizing hormone-releasing hormone
LIA: LIAISON
LOD: limit of detection
LOQ: limit of quantification
LOWESS: locally weighted regression and smoothing scatterplot
LRP: laparoscopic radical prostatectomy
miRNA: micro-RNA
OR: odds ratio
P: phosphorus
PCa: prostate cancer
PIN: prostatic intraepithelial neoplasia
PSA: prostate specific antigen

PTH: parathyroid hormone
PZ: peripheral zone (of prostate)
RALRP: robotic-assisted laparoscopic prostatectomy
RDA: recommended dietary allowance
RIA: radioimmunoassay
RR: relative risk
RXR: retinoic acid receptor
SD: standard deviation
TMA: tissue microarrays
TZ: transition zone (of prostate)
USP: United States Pharmacopoeia
UV: ultraviolet (light)
VDR: vitamin D receptor
VDRE: vitamin D response element
CHAPTER 1: Introduction
1.1 Overview

Vitamin D has received considerable attention over the last decade. In 2007, Time magazine recognized “the benefits of vitamin D” in its list of “Top 10 Medical Breakthroughs” [1]. Much of this interest can be attributed to the growing number of scientific studies reporting widespread vitamin D deficiency worldwide and its adverse impacts on human health [2-6]. Indeed, low vitamin D status has been associated with increased risk and progression of several diseases, including osteoporosis [7, 8], cancers [9-11], multiple sclerosis [12, 13], cardiovascular disease [14, 15], diabetes [16, 17], microbial infections [18, 19], and even total mortality [20]. As such, the prevention of vitamin D deficiency remains an important issue in health care. Section 2.1 provides an overview of the vitamin D system, including its metabolism, physiologic functions, safety, toxicity, and vitamin D inadequacy.

Prostate cancer (PCa) is the most common human internal malignancy, and the second most common cause of cancer death in men. The impact of PCa on public health is likely to increase as the population ages. To date, no universally accepted prevention strategy capable of reducing the burden of this disease has been developed. Of the various chemo-preventive approaches being explored, substantial preclinical data supports the clinical evaluation of vitamin D compounds in PCa treatment and prevention. Such evidence includes epidemiologic associations of lower PCa risk with higher exposure to sunlight and vitamin D [11, 21-23], growth inhibitory effects of vitamin D compounds in vitro and in vivo [24-27], and preliminary evidence of clinical benefit of vitamin D repletion in cancer patients [28, 29]. Sections 2.2 and 2.3 provide a summary of PCa and its relationship with vitamin D.
Our understanding of vitamin D biology has evolved considerably. The wide expression of vitamin D receptor (VDR) and vitamin D metabolic enzymes (in particular, 1-α-hydroxylase) across a wide variety of non-calcemic cell types support the concept of local synthesis and accumulation of vitamin D metabolites in tissues to mediate several critical cellular functions, including cell growth regulation and immunomodulation [2]. This, however, remains a theory because direct measurement of vitamin D metabolites in human tissue has not been reported and extra-renal vitamin D metabolism remains poorly understood. Thus, the overall objective of this thesis is to shed some light on this new paradigm of extra-renal vitamin D metabolism by investigating whether higher\(^1\) levels of vitamin D consumed orally or achieved in the circulation result in increased concentrations of vitamin D metabolites, particularly active calcitriol, in human tissue. The hallmark of this work is a randomized clinical trial conducted in PCa patients to evaluate the effects of oral vitamin D\(_3\) supplementation on prostatic vitamin D metabolism and on PCa pathology.

The work leading up to the PCa clinical trial involved a progressive evaluation of analytical methods to measure vitamin D metabolites in serum and in tissue. In Chapter 4, we evaluated novel automated platforms for quantification of 25(OH)D concentrations in human serum [30]. In Chapter 5, we tested a novel liquid chromatography-tandem mass spectrometry (LC-MS/MS) assay for 25(OH)D that offered the advantages of measuring an additional vitamin D metabolite (i.e. 24,25(OH)\(_2\)D) and being less susceptible to the matrix interferences often encountered with immunoassays [31]. In Chapter 6, we performed a comprehensive evaluation of our newly developed method to measure 1,25(OH)\(_2\)D in human tissue [32]. The end result of

---

\(^1\) In this context, “higher” denotes intake or circulating concentrations of vitamin D that are near or beyond the upper end of exposure attainable through normal physiology
our method development work was the establishment of a robust methodology to measure various vitamin D metabolites in serum and human tissue that could be employed in our PCa clinical trial. We also discovered that the hormone $1,25(OH)_2D$ can be successfully detected in human colon at physiologically relevant concentrations determined by both tissue uptake and local synthesis.

The culmination of this PhD thesis is a randomized clinical trial of oral vitamin D$_3$ in PCa patients undergoing radical prostatectomy (Chapter 7). The objectives were to determine whether higher$^1$ oral vitamin D doses taken prior to surgery can modulate vitamin D metabolism and PCa pathology within prostate tissue in vivo. Our results show that higher oral vitamin D$_3$ supplementation produces higher levels of calcitriol within prostate tissue, and that higher prostate calcitriol relates to diminished expression of the proliferation marker Ki67 and increased expression of tumour suppressive micro-RNAs (miRNAs) in PCa tissue. We also found modest declines in serum prostate specific antigen (PSA) and parathyroid hormone (PTH) with the higher vitamin D doses.

Overall, the results of this PhD research shed light on extra-renal vitamin D metabolism in human tissues and how it can be modulated by oral vitamin D intake. It also provides justification for larger randomized clinical trials of vitamin D supplementation in PCa and other cancers to determine whether this vitamin can truly reduce mortality and morbidity from disease.

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$^1$ In this context, “higher” denotes intake or circulating concentrations of vitamin D that are near or beyond the upper end of exposure attainable through normal physiology.
2 CHAPTER 2: Literature Review

Sections of this chapter contain the following published works:

1. Dennis Wagner.
   Fortification of hard cheese with vitamin D$_3$: Stability and bioavailability of vitamin D$_3$

   Vitamin D, nutritional imprinting and prostate cancer. In: Vitamin D Deficiency.
2.1 Vitamin D

2.1.1 Historical Perspective

The discovery of vitamin D and the elimination of rickets as a widespread medical problem represent major achievements in medicine. The work leading to the identification of vitamin D and its role in bone physiology included several important contributions. In 1650, Glisson, DeBoot, and Whistler provided the first scientific description of rickets, a bone disease identified by deformities of the skeleton [33]. The incidence of rickets increased rapidly during the industrial revolution and by the turn of the 20th century this crippling bone disease was epidemic in industrialized cities of northern Europe and the northeastern United States [33].

In 1822, Sniadecki documented the first insight into the role of sunlight in preventing and curing rickets. He observed that children living in the narrow urban streets and poorly lit dwellings of Warsaw, Poland, had a very high incidence of rickets whereas children living in rural areas did not. He correctly concluded that exposure to sunlight could cure rickets [34].

The concept that rickets was caused by a nutritional deficiency was first suggested by the common folklore practice of feeding children cod liver oil to prevent and cure this disease [33]. In 1827, Bretonneau treated a 15-mo-old child with acute rickets with cod liver oil and noted the incredible speed at which the patient was cured [33]. His student, Trouseau, used liver oils from a variety of fish and aquatic mammals for the treatment of rickets and osteomalacia [35]. However, it was not until 1918 when Mellanby reported that he could experimentally induce rickets in dogs and reverse the bone disease with cod liver oil that the scientific community began to consider rickets as a nutritional deficiency disease [36]. Originally, the anti-rachitic factor in cod liver oil was thought to be vitamin A. However, McCollum et al. clearly
demonstrated that the anti-rachitic activity, which he called vitamin D, was separate from vitamin A when he exposed the cod liver oil to heat and oxygen that destroyed the vitamin A activity while maintaining the anti-rachitic activity [37]. At the same time, Huldshinsky [38] and Chick et al. [39] independently showed that rachitic children could be cured with exposure to sunlight or artificially produced ultraviolet (UV) light.

In 1924, Steenbock and Black [40] and Hess and Weinstock [41] discovered that UV irradiation could impart anti-rachitic activity to food. This concept led to the first fortification of milk with vitamin D and the eventual elimination of rickets as a major medical problem. This discovery also led to the development of an available source of vitamin D for isolation and identification. The structure of vitamin D$_2$ was determined in 1931 by Askew et al. [42] from irradiation of plant sterols. Windaus et al. [43] isolated the precursor of vitamin D$_3$ from the skin, namely, 7-dehydrocholesterol. Subsequently, 7-dehydrocholesterol was synthesized and converted to vitamin D$_3$ by irradiation. Windaus’ group provided chemical syntheses of the vitamin D compounds, confirming their structures and thus ending the era of the isolation and identification of nutritional forms of vitamin D and making them available for the treatment of disease [44].
2.1.2 Formation and Ingestion of Vitamin D

There are 2 forms of vitamin D. Vitamin D$_2$ (ergocalciferol) is produced by UV irradiation of the yeast and plant sterol, ergosterol. Vitamin D$_3$ (cholecalciferol) is the natural form of vitamin D produced in the skin and also found in oily fish and cod liver oil. Vitamin D$_2$ differs from Vitamin D$_3$ by an extra double bond between the 22-23 carbon and an additional 24-methyl group (Figure 2.1). Most evidence indicates that vitamin D$_3$ is more effective than vitamin D$_2$ in improving and maintaining vitamin D status [45, 46].

![Vitamin D$_3$ and Vitamin D$_2$](image)

**Figure 2.1.** Forms of Vitamin D. Chemical structures of vitamin D$_3$ and vitamin D$_2$.

A summary of the vitamin D metabolic pathway is illustrated in Figure 2.2. Vitamin D$_3$ is produced naturally in the skin by UV irradiation of 7-dehydrocholesterol, a precursor of cholesterol. During exposure to sunlight, ultraviolet B photons with wavelengths between 290 and 315 nm penetrate into the skin, where they are absorbed by epidermal and dermal stores of 7-dehydrocholesterol (provitamin D$_3$) [47]. This results in a cleavage of the 9-10 carbon bond of 7-dehydrocholesterol to form a 9,10-secosterol called previtamin D$_3$. Previtamin D$_3$ is biologically inert and must undergo isomerization (at the skin’s temperature) to form vitamin D$_3$. Excess previtamin D$_3$ or vitamin D$_3$ is degraded by sunlight into biologically inactive...
photoproducts (e.g. lumisterol, tachysterol) [47]. Once formed in the skin, vitamin D$_3$ enters the dermal capillary bed where it is bound to vitamin D binding protein (DBP) and enters the circulation [47].

Sunlight exposure is the major contributor to vitamin D status. However, the dermal production of vitamin D$_3$ is limited by latitude, season, sunscreen, clothing, glass shielding, age and skin pigmentation [33]. As a result, there is a need to consume vitamin D from the diet, especially during times of insufficient sunlight when endogenous synthesis is absent (e.g. winter). North American populations often rely on fortified foods (primarily milk) and supplements because natural dietary sources of vitamin D are limited to a small number of foods that are eaten infrequently (Table 2.1). Of note, Health Canada recommends that in addition to following Canada’s Food Guide, all adults over 50 years of age should take a daily vitamin D supplement of 400 IU because meeting the recommended intakes for vitamin D through food alone is almost impossible without recommending unrealistic daily consumption of some foods.
Figure 2.2. The Vitamin D system. The vitamin D endocrine pathway, responsible for the “classical” calcitropic actions of vitamin D, is represented by black arrows. The autocrine or paracrine pathway, which mediates the more recently discovered “non-classical” effects of vitamin D, is depicted in red. Lastly, the catabolic pathway, which functions in the degradation of vitamin D metabolites, is shown in blue. Detailed explanations can be found in the text.

*In the circulation, vitamin D metabolites are bound and transported by DBP.
Table 2.1. Sources of Vitamin D\textsuperscript{a}

<table>
<thead>
<tr>
<th>Source</th>
<th>Serving</th>
<th>Vitamin D content</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fortified foods</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Milk (cow, goat, soy)</td>
<td>1 cup</td>
<td>100 IU vitamin D\textsubscript{3}</td>
</tr>
<tr>
<td>Margarine</td>
<td>1 tsp</td>
<td>30 IU vitamin D\textsubscript{3}</td>
</tr>
<tr>
<td>Infant formulas</td>
<td>1 cup</td>
<td>100 IU vitamin D\textsubscript{3}</td>
</tr>
<tr>
<td>Yogurt (select brands)</td>
<td>½ cup</td>
<td>30 IU vitamin D\textsubscript{3}</td>
</tr>
<tr>
<td>Orange juice (calcium-fortified)</td>
<td>1 cup</td>
<td>100 IU vitamin D\textsubscript{3}</td>
</tr>
<tr>
<td><strong>Natural foods</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Salmon</td>
<td>3 oz</td>
<td>500-800 vitamin D\textsubscript{3}</td>
</tr>
<tr>
<td>Sardines, Mackerel, Tuna</td>
<td>3 oz</td>
<td>200-250 vitamin D\textsubscript{3}</td>
</tr>
<tr>
<td>Shiitake mushrooms</td>
<td>3.5 oz</td>
<td>100 IU vitamin D\textsubscript{2}\textsuperscript{b}</td>
</tr>
<tr>
<td>Egg yolk</td>
<td>1 large</td>
<td>20 IU vitamin D\textsubscript{3} or D\textsubscript{2}</td>
</tr>
<tr>
<td><strong>Supplements</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prescription (US only)</td>
<td>1 capsule</td>
<td>50,000 IU vitamin D\textsubscript{2}</td>
</tr>
<tr>
<td>Multivitamins</td>
<td>1 capsule</td>
<td>400 IU vitamin D\textsubscript{3}</td>
</tr>
<tr>
<td>Vitamin D</td>
<td>1 capsule</td>
<td>400-1000 IU vitamin D\textsubscript{3}</td>
</tr>
<tr>
<td>Cod liver oil</td>
<td>1 tsp</td>
<td>400-1000 IU vitamin D\textsubscript{3}</td>
</tr>
<tr>
<td>Sunlight exposure (arms + legs)</td>
<td>5-10 min</td>
<td>3000 IU vitamin D\textsubscript{3}</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Canadian sources, unless otherwise indicated.
\textsuperscript{b} Vitamin D\textsubscript{2} (ergocalciferol) is a less potent form of the vitamin found in certain plants and fungi.

In 2009, the Institute of Medicine (IOM) conducted a review of research studies to assess the current relevant data and update, as appropriate, the Dietary Reference Intakes (DRIs) for vitamin D. The result was a small upward revision of recommended intakes and establishment of a new Recommended Dietary Allowance (RDA) (Table 2.2). However, the IOM report stipulated that 25(OH)D need only be maintained at or above 50 nmol/L (to the disagreement of many experts) to sustain bone density, calcium absorption, and to minimize risk of osteomalacia and rickets. However, Vieth [48] and other investigators point to a large body of evidence that provides compelling reasons to why 25(OH)D should preferably exceed 75 nmol/L, including: (a) scrutiny of actual data specified by the IOM relating 25(OH)D to bone density and osteomalacia shows the desirable minimum 25(OH)D to be 75 nmol/L; (b) humans are primates, optimized through evolution to inhabit tropical latitudes, with serum 25(OH)D exceeding 100 nmol/L; (c) epidemiologic relationships show health benefits if 25(OH)D levels surpass 70 nmol/L, including fewer falls, better tooth attachment, less colorectal cancer, improved depression and wellbeing. Taken together, the evidence makes it very unlikely that further
research will change the conclusion that risk of disease with serum 25(OH)D higher than 75 nmol/L is lower than that if the serum 25(OH)D is approximately 53 nmol/L [48].

**Table 2.2.** Dietary Reference Intakes (DRIs) for Vitamin D

<table>
<thead>
<tr>
<th>Age group</th>
<th>Recommended Dietary Allowance (RDA) per day</th>
<th>Tolerable Upper Intake Level (UL) per day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infants 0-6 months</td>
<td>400 IU (10 mcg)*</td>
<td>1000 IU (25 mcg)</td>
</tr>
<tr>
<td>Infants 7-12 months</td>
<td>400 IU (10 mcg)*</td>
<td>1500 IU (38 mcg)</td>
</tr>
<tr>
<td>Children 1-3 years</td>
<td>600 IU (15 mcg)</td>
<td>2500 IU (63 mcg)</td>
</tr>
<tr>
<td>Children 4-8 years</td>
<td>600 IU (15 mcg)</td>
<td>3000 IU (75 mcg)</td>
</tr>
<tr>
<td>Children and Adults 9-70 years</td>
<td>600 IU (15 mcg)</td>
<td>4000 IU (100 mcg)</td>
</tr>
<tr>
<td>Adults &gt; 70 years</td>
<td>800 IU (20 mcg)</td>
<td>4000 IU (100 mcg)</td>
</tr>
<tr>
<td>Pregnancy &amp; Lactation</td>
<td>600 IU (15 mcg)</td>
<td>4000 IU (100 mcg)</td>
</tr>
</tbody>
</table>

*Adequate Intake rather than Recommended Dietary Allowance.*
2.1.3 Absorption and Transport of Vitamin D

Absorption of dietary vitamin D occurs in the small intestine and is dependent on the presence of intestinal bile and fats. Vitamin D absorption is independent of vitamin D status. Ingested vitamin D is emulsified with bile salts and solubilized within micelles in the duodenum before being passively absorbed in the jejunum along with other lipids [49]. After absorption, vitamin D is incorporated into chylomicrons within the enterocytes and the released chylomicrons convey the vitamin in the mesenteric lymph to the systemic circulation [49]. During the journey in the lymph, an appreciable amount of the vitamin D in the chylomicrons is transferred to the serum vitamin D binding protein (DBP) [49].

The serum transport of vitamin D and its metabolites occurs via DBP. In the circulation, DBP is present in large excess relative to its vitamin D ligands (plasma DBP concentration ~ 4-8 M) [50]. It has been suggested that 10 mg/kg/day of DBP is produced in humans by the liver [51] and that the total binding capacity of vitamin D metabolites is approximately 4700 nmol/L [52]. As well as having weak, nonspecific associations with lipoproteins and albumin, vitamin D sterols in the extracellular fluid form a specific, high-capacity, and high affinity association with DBP [53]. All naturally-occurring and synthetic vitamin D sterols are bound by a single binding site, but with variable affinity [54]. DBP shows the greatest affinity for 25(OH)D and 24,25(OH)₂D, followed by 1,25(OH)₂D and parent vitamin D [52]. The association of vitamin D ligands with DBP is thought to facilitate their dispersal throughout the body, and their delivery to sites of metabolism and action [50]. Specifically, the DBP (bound to vitamin D metabolite) binds to megalin and its co-receptor cubilin on the cell membrane of the renal proximal tubule epithelium, which permits the internalization of vitamin D metabolites into the cell. The mechanism of vitamin D uptake in extra-renal sites is poorly understood.
2.1.4 Metabolism of Vitamin D

Whether it is produced in the skin or ingested from the diet, vitamin D is hydroxylated in the liver by the 25-hydroxylase enzyme (mitochondrial: CYP27A1, microsomal: CYP2R1) to form 25-hydroxyvitamin D [25(OH)D], the major circulating metabolite of vitamin D. The production of 25(OH)D is not significantly regulated, being primarily dependent on substrate concentration. Approximately 75% of circulating vitamin D is 25-hydroxylated in a single pass through the liver [55].

Once in the circulation, the DBP-25(OH)D complex is removed from the plasma by a variety of target tissues. This process releases most of the 25(OH)D into the tissues, whereas DBP undergoes proteolysis. At physiological doses, vitamin D₃ distributes widely into tissues, not just to adipose, but to skeletal muscle and other organs as well [56]. Based on human deprivation studies in which serum 25(OH)D declines upon sudden elimination of sunlight, 25(OH)D has a half-life of approximately 2 months [57, 58]. Like its parent molecule, 25(OH)D is biologically inert at physiologic concentrations, and it requires an additional hydroxylation in the kidney by the 1α-hydroxylase enzyme (CYP27B1) to produce 1,25-dihydroxyvitamin D [1,25(OH)₂D; calcitriol], the most active hormonal metabolite of vitamin D. Both 25(OH)D and 1,25(OH)₂D can be further metabolized by the kidney CYP24A1 enzyme into 24,25-dihydroxyvitamin D [24,25(OH)₂D] and 1,24,25-trihydroxyvitamin D, respectively. Figure 2.3 provides an overview of the various vitamin D metabolites and their production enzymes. The enzyme immunoassay has been the most commonly used analytical method to measure vitamin D metabolite concentrations in serum or plasma (see Section 4.3 for serum 25(OH)D; Section 6.3 for serum 1,25(OH)₂D), although use of LC-MS/MS assays is increasing (see Section 5.3 for combined serum 25(OH)D and 24,25(OH)₂D).
Figure 2.3. Overview of vitamin D₃ metabolism, chemical structures, and production enzymes. Key carbon atoms differentiating the vitamin D metabolites are shown in red. 
N.B. Vitamin D₂ metabolism occurs through a similar pathway, but involves a different backbone vitamin D structure (see Figure 2.1).
Regulation of the vitamin D endocrine system occurs through the stringent control of renal 1α-hydroxylase activity, keeping 1,25(OH)\(_2\)D in strict homeostatic range despite varying amounts of 25(OH)D substrate (Figure 2.4). The main regulatory factors are serum concentrations of calcium and phosphorus, parathyroid hormone (PTH), and 1,25(OH)\(_2\)D itself. The increase in PTH in response to hypocalcemia stimulates 1α-hydroxylase to increase the synthesis of 1,25(OH)\(_2\)D [59]. Furthermore, low serum calcium or phosphorus may activate renal 1α-hydroxylase, independent of PTH [60].

Circulating levels of 1,25(OH)\(_2\)D are regulated and involve reciprocal adjustments to its rates of synthesis and catabolism. To prevent excess synthesis, 1,25(OH)\(_2\)D suppresses its own production in the kidney and the synthesis of PTH by the parathyroid glands [61]. Furthermore, 1,25(OH)\(_2\)D induces the expression of the 24-hydroxylase enzyme (CYP24A1), which metabolizes both 25(OH)D and 1,25(OH)\(_2\)D into 24,25(OH)\(_2\)D and 1,24,25(OH)\(_2\)D, respectively, and into downstream biologically inactive, water-soluble catabolites [62].

24-hydroxylase is expressed ubiquitously in vitamin D target tissues and is highly inducible by 1,25(OH)\(_2\)D. 24-OHase activity can also be simultaneously inhibited by PTH and augmented by hypercalcemia [63]. Therefore, circulating concentrations of 1,25(OH)\(_2\)D are tightly modulated and the non-calcemic tissues that are capable of generating 1,25(OH)\(_2\)D locally do not contribute to circulating concentrations.
Figure 2.4. Regulation of renal 1α-hydroxylase. The activity of 1α-OHase is either upregulated (+) or downregulated (−) by the hormones (PTH, parathyroid hormone; 1,25(OH)₂D, 1,25-dihydroxyvitamin D), minerals (Ca, calcium; P, phosphorus), and other metabolites (24,24(OH)₂D, 24,25-dihydroxyvitamin D) indicated.
2.1.5 Mechanism of Action and Biological Effects

2.1.5.1 The Vitamin D Receptor (VDR)

The biological effects of 1,25(OH)\(_2\)D are primarily mediated by its interaction with the vitamin D receptor (VDR). The VDR belongs to the nuclear receptor superfamily of steroid/thyroid hormone receptors that regulate gene expression in a ligand-dependent manner. A nuclear VDR has been well-characterized and shown to facilitate the genomic effects of 1,25(OH)\(_2\)D. More recently, a second class of VDR has been described as being present in the plasma membrane of several cell types, which is capable of producing the rapid responses observed with 1,25(OH)\(_2\)D (e.g. rapid intestinal absorption of Ca\(^{2+}\), secretion of insulin by pancreatic β cells) [64].

VDR is expressed widely and found in the classic vitamin D target organs (intestine, bone, kidney and parathyroid glands) [65] as well as several non-calcium-regulating organs including the skin, muscle, prostate, breast, colon, pancreas, and immune cells (Table 2.3). The 1,25(OH)\(_2\)D hormone enters the cell by diffusion, facilitated entry with DBP (i.e., via megalin), or is locally synthesized (i.e., autocrine pathway). 1,25(OH)\(_2\)D then binds with high affinity to VDR in the cytoplasm, and this complex then binds the retinoic acid receptor (RXR) to form a heterodimer [66, 67]. The VDR-RXR heterodimer translocates to the nucleus where it can then bind to vitamin D response elements (VDREs) in target genes to regulate their expression [68]. The rapid response, membrane-initiated pathway is less clearly understood, but thought to stimulate a variety of signaling pathways, presumably through direct interactions with G-proteins or non-receptor tyrosine kinases [69].
Table 2.3. Tissue expression of VDR and 1-α-hydroxylase *.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>VDR</th>
<th>1-α-hydroxylase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intestine</td>
<td>+ [70, 71]</td>
<td>+ [72]</td>
</tr>
<tr>
<td>Liver</td>
<td>+ [73]</td>
<td>+ [74] pig</td>
</tr>
<tr>
<td>Kidney</td>
<td>+ [70, 73]</td>
<td>+ [65, 75]</td>
</tr>
<tr>
<td>Parathyroid gland</td>
<td>+ [70]</td>
<td>+ [76]</td>
</tr>
<tr>
<td>Thyroid</td>
<td>+ [77]</td>
<td>+ [78]</td>
</tr>
<tr>
<td>Adrenal gland</td>
<td>+ [79]</td>
<td></td>
</tr>
<tr>
<td>Pituitary</td>
<td>+ [70, 80]</td>
<td></td>
</tr>
<tr>
<td>Brain/neurons</td>
<td>+ [81]</td>
<td>+ [81]</td>
</tr>
<tr>
<td>Skin</td>
<td>+ [70]</td>
<td>+ [82]</td>
</tr>
<tr>
<td>Skeletal muscle</td>
<td>+ [83, 84]</td>
<td>+ [85]</td>
</tr>
<tr>
<td>Colon</td>
<td>+ [82, 86, 87]</td>
<td>+ [86-88]</td>
</tr>
<tr>
<td>Prostate</td>
<td>+ [89, 90]</td>
<td>+ [27, 91]</td>
</tr>
<tr>
<td>Testes</td>
<td>+ [92]</td>
<td></td>
</tr>
<tr>
<td>Breast</td>
<td>+ [93, 94]</td>
<td>+ [94]</td>
</tr>
<tr>
<td>Ovary</td>
<td>+ [95]</td>
<td>+ [95]</td>
</tr>
<tr>
<td>Uterus</td>
<td>+ [96]</td>
<td>+ [96]</td>
</tr>
<tr>
<td>Placenta</td>
<td>+ [97, 98]</td>
<td>+ [98]</td>
</tr>
<tr>
<td>Lung</td>
<td>+ [99]</td>
<td>+ [100]</td>
</tr>
<tr>
<td>Pancreas</td>
<td>+ [101]</td>
<td>+ [82, 101]</td>
</tr>
<tr>
<td>Dendritic cells</td>
<td>+ [102, 103]</td>
<td>+ [104]</td>
</tr>
<tr>
<td>Macrophages</td>
<td>+ [105]</td>
<td>+ [106-108]</td>
</tr>
<tr>
<td>Monocytes</td>
<td>+ [18, 109]</td>
<td>+ [18, 110, 111]</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>+ [112, 113]</td>
<td>+ [114, 115]</td>
</tr>
<tr>
<td>Heart</td>
<td>+ [116]</td>
<td></td>
</tr>
<tr>
<td>Vascular endothelium</td>
<td>+ [117]</td>
<td>+ [118]</td>
</tr>
<tr>
<td>Stomach</td>
<td>+ [70]</td>
<td></td>
</tr>
</tbody>
</table>

*(+) indicates that the expression and/or activity of the enzyme has been reported in the references indicated in parentheses. Absence of (+) indicates that enzyme not present or not assessed. N.B. This list is not exhaustive.

2.1.5.2 Classical endocrine functions of vitamin D

The “classical” function of 1,25(OH)₂D is to maintain blood calcium levels within a narrow range to sustain a wide variety of metabolic and physiologic functions and to optimize bone health. The 1,25(OH)₂D hormone acts on the small intestine to increase the efficiency of calcium absorption. Its role in phosphorus absorption is often stated, but there are no reports demonstrating that 1,25(OH)₂D directly affects phosphorus absorption or transport in the human gut. To a lesser extent, 1,25(OH)₂D can stimulate the renal retention of calcium. Under conditions of inadequate dietary calcium, 1,25(OH)₂D works in conjunction with PTH to mobilize calcium from bone by activating osteoclasts. Therefore, the actions of 1,25(OH)₂D
help maintain a (calcium x phosphate) concentration high enough to allow for proper mineralization of the collagen matrix (osteoid) in bone [119].

Chronic severe vitamin D deficiency in infants and children causes bone deformation due to poor mineralization, commonly known as rickets. In adults, muscle weakness, bone pain, osteomalacia, and pseudofractures may develop. Less severe vitamin D inadequacy prevents children and adolescents from attaining their optimal genetically programmed peak bone mass and in adults leads to secondary hyperparathyroidism, increased bone turnover, and progressive loss of bone, increasing the risk of fractures and osteoporosis [7, 83, 120]. A large body of evidence supports the beneficial effects of vitamin D intake (≥ 800 IU/d) on skeletal health, which include enhanced calcium absorption, suppression of PTH, improved muscle function (i.e. less falls), and reduced risk of osteoporotic hip fractures [120].

2.1.5.3 Non-classical autocrine/paracrine functions of vitamin D

The biological effects of vitamin D are not limited to mineral homeostasis and the maintenance of skeletal health. The VDR has been isolated from cells in most organs, including those not typically associated with calcium regulation or bone metabolism (Table 2.3). In addition, some of these cells and tissues express the 1α-hydroxylase enzyme, allowing them to synthesize 1,25(OH)₂D locally from circulating 25(OH)D. It has been estimated that over 900 genes are regulated by 1,25(OH)₂D [121]. Some of these target genes are involved in cellular growth, differentiation, and apoptosis, as well as immunomodulation. Evidence is growing that the extra-renal synthesis of 1,25(OH)₂D may thus be important for regulating cell growth, the immune system, and other physiological processes that affect health, via paracrine or autocrine mechanisms. Vitamin D has been shown to play a favourable role in various chronic and infectious diseases including cancer [10, 29, 122], multiple sclerosis [12, 13], diabetes [16, 123,
124], cardiovascular disease [15, 125], rheumatoid arthritis [126], osteoarthritis [127], psoriasis [128], depression [129-131] and predisposition to infectious diseases [18, 19, 132]. The investigation into the “non-classical” paracrine actions of vitamin D and their relation to human health and disease is currently an active area of research.
2.1.6 Vitamin D Safety and Toxicity

Vitamin D is increasingly regarded as a beneficial compound with a wide margin of safety. Vitamin D₃ intakes of 4,000 IU/d [133, 134], 10,000 IU/d [135], and 40,000 IU/d [12] have been shown to be safe in humans. The published literature contains no cases of vitamin D intoxication at long-term doses up to 40,000 IU/d [136]. In contrast, 1,25(OH)₂D (calcitriol) has a far more narrow margin of safety and more readily induces hypercalcemia, necessitating careful clinical monitoring of calcium levels during therapy. Many cases of so-called vitamin D intoxication were in fact the result of calcitriol administration, which was erroneously interpreted as vitamin D. Indeed, vitamin D is two metabolic steps away from the active product, is not a hormone, and is not a synonym for 1,25(OH)₂D (calcitriol), its analogs or deltanoids [137].

To provide some perspective, the human body has a high physiologic capacity to produce vitamin D₃ in the skin. Exposure of a healthy adult in a bathing suit to one minimal erythemal dose (a light pinkness to the skin) of sunlight or tanning bed UV radiation is equivalent to taking between 10,000 IU and 20,000 IU of vitamin D₃ orally [33]. Healthy farmers in Puerto Rico and lifeguards in St. Louis had mean serum 25(OH)D concentrations well in excess of 130 nmol/L [136]. Although not strictly within the “normal” range for a clothed, sun-avoiding population, serum 25(OH)D concentrations ≤20 nmol/L are consistent with certain environments, are not unusual in the absence of vitamin D supplements, and should be regarded as being within the physiologic range for humans [136].

Vitamin D intoxication is very rare but can be caused by inadvertent or intentional ingestion of excessively high doses. Prolonged excessive intakes of vitamin D can lead to hypercalcemia, dehydration, kidney damage, and soft tissue calcification. Factors that might predispose an individual to vitamin D intoxication include increased calcium intake, reduced
renal function, reduced estrogen levels, and granulomatous conditions such as sarcoidosis [138]. The first signs of vitamin D intoxication are hypercalciuria (\textit{i.e.}, excess calcium in the urine) followed by elevated serum calcium concentrations (hypercalcemia) [136]. Wills et al. [139] demonstrated that the urinary calcium:creatinine ratio can be an effective screening tool to detect hypercalciuria caused by abnormalities in calcium metabolism. The clinical manifestations of vitamin D toxicity resulting from hypercalcemia reflect the essential role of calcium in many tissues and targets, including bone, the cardiovascular system, nerves, and cellular enzymes [138]. Initial signs and symptoms of hypervitaminosis D include generalized weakness and fatigue, which can be followed by nausea, vomiting, abdominal cramps, constipation, confusion, drowsiness, heart arrhythmias, difficulty concentrating, and depression [138].

The mechanisms of vitamin D intoxication can be explained by three features of the vitamin D system: the binding capacity of DBP, the level of 1α-hydroxylase activity, and the capacity to clear vitamin D metabolites from the body [140]. Because free, not bound, 1,25(OH)\textsubscript{2}D is functional \textit{in vivo}, a probable mechanism for the toxicity of vitamin D is that high 25(OH)D concentrations will cause excessive synthesis of 1,25(OH)\textsubscript{2}D and, together with vitamin D and its other metabolites, cause displacement of the hormone from DBP thereby increasing the amount of free 1,25(OH)\textsubscript{2}D that is accessible to target cells [140]. Furthermore, at toxic doses, the freely circulating vitamin D and its metabolites can accumulate in adipose tissue and muscle [56].

The vast majority of cases of vitamin D intoxication have involved vitamin D\textsubscript{2}. The situations involving vitamin D\textsubscript{3}, to date, have been industrial accidents or poisonings from an unknown source [56]. One explanation for the toxicity of vitamin D\textsubscript{2} is the poorer stability and greater impurity of vitamin D\textsubscript{2} compared to vitamin D\textsubscript{3} preparations. There are newer reasons
why vitamin D₂ has a greater potential for harm. First, DBP has a weaker affinity for the vitamin D₂ metabolites than for 25(OH)D₃ and 1,25(OH)₂D₃ [134]. This means that the proportions of free 25(OH)D₂ and 1,25(OH)₂D₂ are higher and more biologically available. Second, unique biologically active metabolites are produced from vitamin D₂ in humans and there are no such metabolites derived from vitamin D₃ [134]. Vitamin D₂ should be regarded as a synthetic analogue of vitamin D that is not naturally-occurring, with different chemical, biological, and toxicological properties.
2.1.7 Vitamin D Inadequacy

The objective measure of vitamin D status is the 25(OH)D concentration in blood serum or plasma, since this concentration reflects vitamin D inputs from cutaneous synthesis and dietary intake. The most widely used method for measuring serum 25(OH)D in clinical samples has been the immunoassay, although liquid chromatography-tandem mass spectrometry (LC-MS/MS) have been gaining in performance and clinical use. Despite the lack of an official consensus on optimal levels of serum 25(OH)D, vitamin D deficiency is now defined by most experts as a 25(OH)D level of less than 50 nmol/L [2]. 25(OH)D levels are inversely associated with PTH levels until the former reach 75 to 100 nmol/L, at which point PTH levels are claimed to level off [141]. However, more rigorous analysis argues against such a PTH plateau [131, 142].

Intestinal calcium transport was increased by 45 to 65% in women when 25(OH)D levels were increased from 50 to 80 nmol/L [143]. Given such data, a 25(OH)D concentration of 50 to 75 nmol/L can be considered to indicate a relative insufficiency of vitamin D, and a level of 75 nmol/L or greater can be considered to indicate sufficient or optimal vitamin D status [2]. Based on a review by Bischoff-Ferrari [144], shifting the population to >75 nmol/L would result in an expected increase in bone mineral density (BMD) by up to 4–5% in younger and older adults, an increase in lower extremity function by up to 4–6% among older adults, reduce hip or any non-vertebral fractures by about one fourth, and reduce cancer incidence by about 17%. Desirable vitamin D status for prevention of diseases unrelated to bone have not been clearly defined and warrant further clinical intervention studies.
Mounting evidence suggests that vitamin D inadequacy constitutes a largely unrecognized public health issue in many populations worldwide. Vitamin D insufficiency, defined as serum 25(OH)D < 40-50 nmol/L (i.e., the older cut-off), has been reported to occur in 26% of young adult females in Toronto [6], 32% of young adults in Boston [145], 39% of healthy western Canadians [146], 46% of Iranian young adults [4], 57% of general medicine inpatients in the United States [147], and particularly in the elderly and dark-skinned people, including 76% of elderly Italian women [3] and 42% of African American women [5]. According to several studies, 40 to 100% of U.S. and European elderly men and women still living in the community (not in nursing homes) are deficient in vitamin D [2]. Children and young adults can also be at high risk for vitamin D deficiency. For example, 52% of Hispanic and black adolescents in Boston [148] and 48% of white preadolescent girls in Maine [149] had 25(OH)D levels below 50 nmol/L. At a Boston hospital, 32% of healthy students, physicians and residents were vitamin D–deficient, despite drinking a glass of milk, taking a multivitamin daily and eating salmon at least once a week [145]. Recent data from the Canadian Community Health Survey indicated that the average 25(OH)D levels of Canadian adults, in year-round sampling, was 67 nmol/L.

Vitamin D deficiency and insufficiency is particularly common among non-white individuals and populations with low dietary/supplemental vitamin D intake or minimal exposure to sunlight [150]. A study of Asian adults in the United Kingdom showed that 82% had 25(OH)D levels less than 30 nmol/L during the summer, with the proportion increasing to 94% during the winter [151]. Lastly, we systematically examined the vitamin D status of young Canadian adults of diverse ancestry during the winter months and found that the prevalence of vitamin D insufficiency was very high (~ 75%), particularly among those of non-European
ancestry (85-100%) [152]. In summary, it has been estimated that one billion people worldwide have vitamin D deficiency or insufficiency [2].
2.2 Prostate Cancer

2.2.1 Prostate Cancer Incidence and Mortality

Prostate cancer (PCa) is the most common cancer among North American men (excluding non-melanoma skin cancer). In 2011, approximately 25,500 Canadian men and 240,890 American men were diagnosed with PCa [153]. That same year, about 4,100 Canadians and 33,720 Americans died from this disease [153]. It has been estimated that, on average, 70 Canadian men will be diagnosed with PCa and 11 Canadian men will die from it every day [153]. During their lifetime, 1 in 7 men will develop PCa (the risk is highest after age 60) and 1 in 28 will die of it [153]. Canadian PCa statistics are summarized in Table 2.4 and Figure 2.5.

Prostate cancer incidence varies across continents and countries (Figure 2.6 and 2.7). Incidence is high in the United States, Canada, Australia/New Zealand and Northern/Western Europe. The lowest incidence rates are found in China and other parts of Asia. Mortality resulting from PCa also differs considerably around the world, but the differences are substantially smaller than for incidence. Survival for PCa is greater in high-risk countries: 87% in the US compared to 45% in developing countries [154]. Following breast (17.9%) and colorectal cancer (11.5%), PCa is the third most prevalent cancer worldwide, with 9.6% of cases [155].

In general, there has been an overall upward trend in the incidence rate of PCa since 1980, which is likely due to increased early detection (i.e. through higher use of prostate specific antigen (PSA) testing and transurethral resection of the prostate (TURP) procedures) or possible changes in risk factors. Mortality rates for PCa rose much more slowly during the same period, averaging 2-8% every 5 years [156]. Since the 1990s, PCa mortality declined in several
developed countries as a result of decreased diagnosis of distant-stage disease and improved treatment [154].

**Table 2.4.** Prostate cancer statistics in Canada

<table>
<thead>
<tr>
<th>Year: 2011</th>
<th>Males</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cases</td>
<td>25,500</td>
</tr>
<tr>
<td>Incidence rate</td>
<td>122</td>
</tr>
<tr>
<td>Incidence rank</td>
<td>1st</td>
</tr>
<tr>
<td>Deaths</td>
<td>4100</td>
</tr>
<tr>
<td>Death rate</td>
<td>21</td>
</tr>
<tr>
<td>5-year survival (2004-2006)</td>
<td>96%</td>
</tr>
</tbody>
</table>

*age-standardized to the 1991 Canadian Standard Population (per 100,000)

Source: ref [153]
Figure 2.5. Percentage distribution of estimated new cases and deaths for selected cancers (males, Canada, 2011). Source: ref [153].
Figure 2.6. Prostate cancer incidence worldwide. Source: Globocan 2008 (IARC).
Figure 2.7. Prostate cancer incidence and mortality across geographic regions.  
Source: Globocan 2002 (IARC).
Prostate Cancer Pathology

The prostate is the walnut shaped gland in males surrounding the urethra just below the urinary bladder and in front of the rectum, that is involved in the liquefaction of the male ejaculate. Typically, the mean weight of a “normal” prostate in adult males ranges between 7 and 16 grams [157]. The gland is made up of secretory cells with a surrounding connective tissue stroma. Histologically, the secretory epithelium is mainly pseudostratified, comprising tall columnar cells and basal cells which are supported by a fibroelastic stroma containing randomly orientated smooth muscle bundles.

Prostate cancer is the transformation of the secretory cells into malignant cells that have the potential to grow more rapidly and spread outside of the prostate. Almost all cancers of the prostate are adenocarcinomas. They tend to arise from the peripheral (outer) zone of the prostate in 85% of cases [158]. Adenocarcinoma of the prostate occurs in more than one site (multifocal) in more than 85% of cases [158]. Prostate adenocarcinomas are graded by the pathologist according to their degree of differentiation and overall aggressiveness (see Section 2.2.4: Prostate Cancer Diagnosis). Typically, PCa will start out localized in the prostate, but can then spread into adjacent tissues and lymph nodes if not detected early. The most common site of metastatic disease is the bone, but it can also spread to the lung, liver and other organs.

Prostatic intraepithelial neoplasia (PIN) is an abnormality of prostatic glands and believed to precede the development of prostate adenocarcinoma. PIN consists of architecturally benign prostatic ducts lined with cytologically atypical cells [158]. When high-grade PIN is found on needle biopsy, there is a 30 to 50% risk of detecting carcinoma on subsequent biopsies [159]. Although high grade PIN appears to be a precursor lesion to many peripheral intermediate grade
and high grade adenocarcinomas, PIN is not a required precursor for carcinomas to arise within the prostate [158].

Benign prostatic hyperplasia (BPH) (also referred to as enlarged prostate) is the nonmalignant enlargement of the prostate gland. BPH is more common in older men, and occurs when the prostate gland enlarges to the point where it presses on the urethra thereby constricting urine flow and leading to urinary difficulties. BPH is not cancer, does not progress into cancer, and in many cases does not cause significant symptoms. However, BPH can be a real medical problem for some men. If it requires treatment, medications can often be used to shrink the size of the prostate (e.g. 5α-reductase inhibitors) or to relax prostatic muscles (alpha blockers), which usually helps with urine flow. If medications are not successful, a surgery known as a transurethral resection of the prostate (TURP) may be needed. This procedure involves the surgical removal of a part of the prostate through the urethra and usually relieves BPH symptoms.
2.2.2 Prostate Cancer Risk Factors

Prostate cancer (PCa) is likely the result of a combination of factors, although its specific causes remain relatively unknown. The primary risk factors for PCa are age and genetics (family history). Environmental risk factors include cigarette smoking, alcohol consumption, cadmium exposure, occupation, infectious agents, ionizing radiation, ultraviolet light, physical inactivity, body mass index and diet.

2.2.2.1 Age

Prostate cancer is very rare in men younger than 45, but becomes more common with advancing age (Table 2.5). More than 80% of all PCa’s are diagnosed in men over 65 years old and 90% of men who die from the disease are in this age group. The average age at the time of diagnosis is 70. However, many men never know they have PCa. Autopsy studies of men who died of other causes have found PCa in 15-30% of men older than 50 yrs and 60-70% of men older than 80 yrs [154]. Although the incidence of clinical PCa varies greatly worldwide, the age-specific incidence of histological PCa does not, suggesting that the initiation of PCa is similar globally, and related to age.

Table 2.5. Risk for developing PCa by age group

<table>
<thead>
<tr>
<th>Age group (yrs)</th>
<th>Risk of PCa $^*$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;50</td>
<td>0.22</td>
</tr>
<tr>
<td>50-59</td>
<td>2.12</td>
</tr>
<tr>
<td>60-69</td>
<td>6.71</td>
</tr>
<tr>
<td>70-75</td>
<td>5.25</td>
</tr>
<tr>
<td>&gt;75</td>
<td>11.6</td>
</tr>
</tbody>
</table>

$^*$Average annual risk. Source: ref [160].
2.2.2.2 Genetics

Genetic background may contribute to PCa risk, as suggested by associations with family history, race, and specific gene variants. Ten to 15% of men with PCa have a family history of the disease, and those with a family history of PCa tend to develop PCa at an early age (e.g. under 55 y) [160]. Men who have a first-degree relative (father or brother) with PCa have a 2-fold increased risk of developing PCa during their lifetime [161]. With 2 affected first-degree relatives, PCa risk increases 5-fold, and with 3 or more first-degree relatives the risk is very high – almost 100% [160]. Prostate cancer occurs more often in African-American men than in men of other races. African-American men are also more likely to be diagnosed at an advanced stage, and are more than twice as likely to die of PCa as white men [162]. Prostate cancer occurs less often in Asian-American and Hispanic/Latino men than in non-Hispanic whites, although the reasons for these racial and ethnic differences are not clear. Studies of twins in Scandinavia suggest that over 40% of PCa risk can be attributed to inherited factors; PCa heritability was highest amongst the cancer types studied (e.g. breast cancer, 27%) [163].

Several different genes have been associated with PCa, although these probably account for only a small number of cases overall. Inherited mutations of the BRCA1 and BRCA2 genes, important risk factors for breast and ovarian cancers in women, have also been shown to increase PCa risk in some men [164]. Other genes that have been implicated in PCa include the Hereditary Prostate Cancer gene 1 (HPC1), the androgen receptor and the vitamin D receptor [165]. TMPRSS2-ETS gene family fusion, particularly TMPRSS2-ERG, has been shown to promote PCa cell growth and present in 40-80% of prostate cancers in humans [166]. Loss of tumour suppressor genes, including p53, PTEN, KAI1, E-cadherin, CD44, and several others, has been reported in early prostate carcinogenesis and metastasis [167].
2.2.2.3 **Environmental Factors**

The role of diet in PCa is still unclear. Epidemiological studies suggest little or no association between total fruit and vegetable consumption and risk of PCa [168]. Consumption of red and processed meat has been shown to increase PCa risk in some studies [169], although a recent meta-analysis found no association between meat intake and PCa [170]. Dietary fat, as well as obesity, have been linked to higher PCa risk by increasing levels of PCa growth-promoting androgens [171-173]. Despite considerable preclinical evidence for a chemopreventive role of vitamin E and selenium in PCa [174], a large clinical trial of these nutrients alone or in combination (SELECT) showed no evidence of clinical benefit in PCa patients [175]. Lycopene (found in tomatoes), crucifers (found in broccoli, cabbage, sprouts) and green tea polyphenols have demonstrated anticancer properties *in vitro* and *in vivo* [174], although large randomized clinical trials are warranted to confirm their efficacy in PCa. Excessive use of multivitamin or folic acid supplements have been related to increased PCa risk [176, 177], although these associations are typically not found when the nutrients are derived from the diet. The results of some [178-181] but not all [182, 183] studies suggest that a high intake of calcium may increase the risk of PCa. Lower exposure to sunlight and/or vitamin D has been linked to increased risk for PCa. The relationship between vitamin D and PCa is discussed in Section 2.3.

In 2007, the World Cancer Research Fund and the American Institute for Cancer Research published a systematic review of preventive approaches to preventing cancer worldwide [184]. A panel of distinguished expert judges concluded that: “Foods containing lycopene, as well as selenium or foods containing it, probably protect against prostate cancer. Foods containing calcium are a probable cause of this cancer. It is unlikely that beta-carotene (where from food or supplements) has a substantial effect on the risk of this cancer. There is limited evidence that
pulses (legumes) including soya and soya products, foods containing vitamin E, and alphatocopherol supplements are protective; and that processed meat, and milk and dairy products are a cause of this cancer [184].” However, this review preceded the publication of the SELECT study findings, which may have changed the Panel judgment on the chemopreventive utility of selenium and vitamin E.

Other environmental factors can also predispose an individual to develop PCa. Infection or inflammation of the prostate (prostatitis) may increase PCa risk while other studies indicate that infection may help prevent PCa by increasing blood to the area. In particular, infection with the sexually transmitted infections chlamydia, gonorrhea, or syphilis seems to increase risk [185]. Use of cholesterol-lowering drugs (i.e. statins) may also decrease risk of PCa, particularly of aggressive cases [186].
2.2.3 Prostate Cancer Diagnosis

Most PCa’s are first detected during screening with a prostate specific antigen (PSA) blood test and/or a digital rectal exam (DRE).

Prostate specific antigen (PSA) is a glycoprotein produced for the ejaculate, where it liquefies semen in the seminal coagulum and allows sperm to swim freely [187]. It is also believed to be instrumental in dissolving cervical mucus, allowing the entry of sperm into the uterus [187]. Elevated levels of PSA in serum (>4 ng/mL) may suggest the presence of PCa. However, PSA levels can be also increased by prostatitis, BPH and other factors. Furthermore, PCa can also be present in the complete absence of an elevated PSA level. Despite some limitations, the PSA blood test has had a relatively long history of use as a standard diagnostic tool to detect PCa in men aged 50 and older. However, the value of PSA testing has recently come under scrutiny. The United States Preventive Services Task Force (USPSTF, 2012) does not recommend PSA screening, noting that the test may result in “over-diagnosis” and “overtreatment” because most PCa is asymptomatic, and treatments involve risks of complications. The USPSTF concluded the potential benefit of PSA screening does not outweigh the expected harms. Despite the ongoing debate on the value of the PSA test, this blood test is still considered very useful by several medical bodies in diagnosing PCa and Prostate Cancer Canada recommends that all men should begin annual or semiannual PSA monitoring at age 50 [160].

The digital rectal exam is another part of the early detection and diagnosis of PCa. During a DRE, a doctor inserts a lubricated gloved finger into the rectum and feels the prostate gland
through the rectal wall to check for bumps or abnormal areas. Typically, the results of the DRE are used in conjunction with those of the PSA test to determine if further follow-up is necessary.

Early prostate cancers usually do not cause symptoms, but more advanced cancers are sometimes first found because of symptoms they cause, including urination problems, blood in the urine/semen, erectile difficulties and bone pain (metastatic cases). Whether PCa is suspected based on screening tests or symptoms, the actual diagnosis can only be made with a prostate biopsy [162].

If certain symptoms or the results of early detection tests suggest possible PCa (i.e. elevated PSA and/or abnormal DRE), a prostate biopsy is offered expeditiously. During a biopsy, a urologist obtains tissue samples from the prostate via the rectum. Guided by transrectal ultrasound, a biopsy gun inserts and removes special hollow-core needles (usually three to six on each side of the prostate) in less than a second. Prostate biopsies are routinely done on an outpatient basis and rarely require hospitalization. The biopsy tissue samples are then examined under a microscope by a trained pathologist to determine whether cancer cells are present, and to evaluate the microscopic features (or Gleason score) of any cancer found [162].

Pathologists grade PCa according to the Gleason system. This system assigns a Gleason grade based on how much the cells in the cancerous tissue look like normal prostate tissue, using numbers from 1 (normal-looking) to 5 (very abnormal). Since prostate cancers often have areas with different grades, a grade is assigned to the two areas that make up most of the cancer. These two grades are added together to yield the Gleason score (also called the Gleason sum) between 2 and 10. PCa with Gleason score of 6, 7 and 8-10 are termed well-differentiated/low-grade, moderately-differentiated/intermediate-grade and poorly-differentiated/high-grade,
respectively. The higher the Gleason score, the more likely it is that the PCa will grow and spread quickly [162].
2.2.4 Prostate Cancer Treatment

The first decision to be made in managing PCa is whether any treatment at all is needed. The most common, low-grade forms of PCa found in the typical elderly patient often grow so slowly that no treatment is required at all. Treatment may also be inappropriate if the patient has other serious health problems, co-morbidities or is not expected to live long enough for symptoms to appear. The most appropriate option depends on the stage of the disease, the Gleason score, and the PSA level. Other important factors are age, general health, and patient views about potential treatments and their possible side effects. Because all treatments can have significant side effects, such as erectile dysfunction and urinary incontinence, treatment discussions often focus on balancing the goals of therapy with the risks of lifestyle alterations [188].

2.2.4.1 Active surveillance

Many patients diagnosed with low-risk PCa are eligible for active surveillance. This approach involves careful observation of the tumour over time, with the intention of curative treatment if there are signs of cancer progression. In active surveillance, the tumour is monitored for signs of growth or the appearance of symptoms. The monitoring process may involve serial PSA blood tests, physical examination of the prostate, and/or repeated biopsies. The goal of surveillance is to avoid overtreatment and the sometimes serious, permanent side effects of treatment for a slow-growing or self-limited tumour that would never cause any problems for the patient [162]. Active surveillance is not used for aggressive cancers, but it may cause anxiety for patients who wrongly believe that all cancer is fatal or life-threatening. For the 50-75% of patients with PCa that will cause no harm before the man dies of something unrelated, active surveillance may be the best option [162].
One question that arises is whether anything might be done during active surveillance to prevent the progression of the cancer. That question is also a fundamental basis for the theme of this thesis, whether greater intakes of a dietary supplement might not be added to an active surveillance protocol. The main findings of this thesis support the plausibility of using vitamin D to prevent the progression of PCa. This work helps to justify clinical trials looking into the use of vitamin D supplementation during active surveillance, to improve patients’ outcomes.

2.2.4.2 Surgery for Prostate Cancer

Surgery is a common approach to attempt to cure PCa if it is not thought to have spread outside the gland. The main type of surgery for PCa is known as radical prostatectomy. In this operation, the surgeon removes the entire prostate gland with some of the tissue around it, including the seminal vesicles. A radical prostatectomy can be performed in an open or laparoscopic manner. In general, the overall 10-year cure rate ranges from 70-80% for both the open and laparoscopic method [189, 190]. The cure rate with radical prostatectomy is the same regardless of whether it is open, laparoscopic or robotic. (N.B. I do realize that the word “cure” is controversial, since one can never be certain that residual cancer exists somewhere. I use the word here in its common intent, to indicate that there is no further evidence that the cancer has arisen during the person’s lifetime).

In the traditional, open approach to conducting a prostatectomy, the surgeon operates through a single long incision to remove the prostate and nearby tissues. In radical retropubic prostatectomy, the surgeon makes a skin incision in the lower abdomen, from the belly button down to the pubic bone. If there is a reasonable chance the cancer may have spread to the lymph nodes (based on the PSA level, DRE, and biopsy results), the surgeon may first remove lymph
nodes from around the prostate, which are then inspected by a pathology lab to determine if they contain cancer cells [162].

When removing the prostate, the surgeon will pay close attention to 2 tiny bundles of nerves that run on either side of the prostate and are responsible for controlling erections. For those patients that were able to have erections before surgery, the surgeon will try not to injure these nerves (known as a nerve-sparing approach). However, if the cancer is growing into or very close to the nerves the surgeon will need to remove them, increasing the likelihood that the patient will be unable to have spontaneous erections [162]. Typically, it takes at least a few months to a year after surgery to regain the ability to have an erection because the nerves have been handled during the operation, which temporarily impairs their function. Other possible side effects of prostatectomy are described below.

Another type of prostate surgery is called radical perineal prostatectomy, in which the surgeon makes the incision in the skin between the anus and scrotum (the perineum) [162]. This approach is used less often because the nerves cannot easily be spared and lymph nodes cannot be excised. However, it is often a shorter operation with a quicker and less painful recovery phase.

Laparoscopic approaches to prostatectomy use several smaller incisions and special surgical tools to remove the prostate. This can be done with the surgeon either holding the tools directly, or using a control panel to precisely move robotic arms that hold the tools. In a laparoscopic radical prostatectomy (LRP), the surgeon makes several small incisions, through which special long instruments are inserted to remove the prostate [162]. One of the instruments has a small video camera on the end, which lets the surgeon view inside the abdomen. LRP has
some advantages over the usual open radical prostatectomy, including reduced blood loss and pain, shorter hospital stays (usually no more than 1 day), and faster recovery times. A newer approach is to perform the laparoscopic surgery remotely using a robotic interface (called the da Vinci system), which is known as robotic-assisted laparoscopic prostatectomy (RALRP). In this operation, the surgeon sits at a panel near the operating table and controls robotic arms to perform the surgery through several small incisions in the patient's abdomen [162]. Like direct LRP, RALRP has advantages over the open approach in terms of pain, blood loss, and recovery time. Overall, there seems to be little difference between robotic and direct LRP for the patient.

There are possible risks and side effects with any type of surgery for PCa. The risks with any type of radical prostatectomy are similar to those of any major surgery, including risks from anesthesia. Among the most serious, there is a small risk of heart attack, stroke, blood clots, and infection at the incision site. If lymph nodes are removed, a collection of lymph fluid (i.e. lymphocele) can form and may need to be drained. Another risk is excessive bleeding during and after the operation. Rarely, part of the intestine might be cut during surgery, which could lead to infections in the abdomen and might require corrective surgery. In extremely rare cases, people die because of complications related to this operation. Risk of death depends partly on the patient’s overall health and age, as well as the skill of the surgical team [162].

The major possible side effects of radical prostatectomy are urinary incontinence (being unable to control urine) and impotence (being unable to have erections). It should be noted that these side effects can also occur with other forms of treatment for PCa. Other side effects of radical prostatectomy may include: changes in orgasm, loss of fertility, lymphedema (rare), change in penis length, and inguinal (groin) hernia [162].
2.2.4.3 Radiation Therapy for Prostate Cancer

Radiation therapy uses high-energy rays or particles to kill cancer cells. The cure rate for radiation therapy is similar to that of radical prostatectomy. Radiation may be used:

- As the initial treatment for low-grade cancer that is still confined within the prostate gland.
- As part of the first treatment (along with hormone therapy) for cancers that have grown outside of the prostate gland and into nearby tissues.
- If the cancer is not completely resected or recurs in the area of the prostate after surgery.
- If the cancer is advanced, to reduce the size of the tumour and to provide relief from symptoms [162].

Two main types of radiation therapy can be used: external beam radiation and brachytherapy (internal radiation). Both appear to be effective methods of treating PCa.

In external beam radiation therapy (EBRT), beams of radiation are focused on the prostate gland from a machine outside the body [162]. This type of radiation can be used to try to cure earlier stage cancers, or to help relieve symptoms such as bone pain if the cancer has metastasized to the bone. Typically, EBRT involves treating the patient 5 days a week (few minutes per treatment) in an outpatient center for 7 to 9 weeks. Each treatment is similar to getting an x-ray. Standard (conventional) EBRT is used much less often than in the past. Newer techniques provide higher doses of radiation to the prostate gland while reducing the radiation exposure to nearby healthy tissues [162]. These techniques have fewer side effects than standard EBRT and may also stand a better chance of curing the cancer. These newer techniques include: three-dimensional conformal radiation therapy, intensity modulated radiation therapy (now used routinely in many major hospitals), image guided radiation therapy and conformal proton beam radiation therapy. Possible side effects of standard EBRT include: bowel problems, bladder
problems, urinary incontinence, erectile difficulties (including impotence), fatigue, lymphedema and urethral stricture [162]. The risks of the newer EBRT techniques described above are likely to be lower.

Brachytherapy (also called seed implantation or interstitial radiation therapy) uses small radioactive pellets, or “seeds”, each about the size of a grain of rice, which are inserted directly into the prostate [162]. Imaging tests such as trans-rectal ultrasound, CT scans, or MRI are used to help guide the placement of the radioactive pellets. Brachytherapy is generally used only in men with early stage PCa that is relatively slow growing (i.e. low-grade tumors) [162]. There are 2 types of prostate brachytherapy.

In permanent (low dose rate, or LDR) brachytherapy, pellets (seeds) of radioactive material (e.g. iodine-125 or palladium-103) are placed inside thin needles, which are inserted through the skin in the perineum and into the prostate [162]. The pellets are left in place as the needles are removed and emit low doses of radiation for weeks or months. Radiation from the seeds travels a very short distance, so the seeds can emit a very large amount of radiation to a very small area. This reduces damage to the healthy tissues that are close to the prostate. Typically, anywhere from 40 to 100 seeds are inserted. Because they are so small, the seeds cause little discomfort and are simply left in place after their radioactivity is expended [162].

Temporary (high dose rate, or HDR) brachytherapy is a newer technique. In this procedure, hollow needles are inserted through the skin in the perineum and into the prostate [162]. Soft nylon tubes (catheters) are placed in these needles. The needles are then removed but the catheters remain in place. Radioactive iridium-192 or cesium-137 is then placed in the
catheters, usually for 5 to 15 minutes. Generally, about 3 brief treatments are given over 2 days, and the radioactive substance is removed each time [162].

Brachytherapy is often combined with EBRT given at a lower dose than if used alone. The total dose of radiation is computed so that it is high enough to kill all the cancer cells. The advantage of this approach is that most of the radiation is concentrated in the prostate gland itself, sparing the urethra and the tissues around the prostate (e.g. nerves, bladder, and rectum) [162]. Like EBRT, brachytherapy can also cause bowel problems, urinary problems, and problems with erections.

2.2.4.4 Other Therapies for Prostate Cancer

Cryosurgery

Cryosurgery (also called cryotherapy or cryoablation) is sometimes used to treat early stage PCa by freezing it. In this approach, several hollow probes are placed through the skin in the perineum. Very cold gases are then passed through the needles, creating ice balls that destroy the prostate gland. Warm saltwater is circulated through a catheter in the urethra during the procedure to keep it from freezing. Cryosurgery is less invasive than radical prostatectomy, so there is usually less blood loss, a shorter hospital stay, shorter recovery period, and less pain than with surgery [162]. However, doctors know much less about the long-term effectiveness of cryosurgery compared with surgery or radiation therapy. For this reason, cryosurgery is not often used as first-line treatment for PCa, but is sometimes recommended if the cancer has recurred after other treatments. Typical side effects of cryosurgery include swelling of the penis or scrotum, pain or burning sensations in the bladder and intestine, and erectile dysfunction [162].
**Hormone Therapy**

The goal of hormone therapy (also called androgen deprivation therapy (ADT) or androgen suppression therapy) is to reduce levels of male hormones, called androgens, in the body, or to prevent them from reaching PCa cells [162]. The main androgens are testosterone and dihydrotestosterone (DHT). Androgens, which are produced mainly in the testicles, stimulate PCa cells to grow. Lowering androgen levels or stopping them from entering PCa cells often makes tumours shrink or grow more slowly for a time. However, hormone therapy alone does not cure PCa.

Hormone therapy may be used in the following circumstances [162]:

- If the patient is not able to have surgery or radiation or cannot be cured by these treatments because the cancer has already spread beyond the prostate gland
- If the cancer remains or recurs after treatment with surgery or radiation therapy
- In combination with radiation therapy as initial treatment if the patient is at higher risk of recurrence after treatment (based on a high Gleason score, high PSA level, and/or growth of the cancer outside the prostate)
- Before radiation to try to shrink the cancer to make treatment more effective

Several types of hormone therapy can be used to treat PCa and include the following [162]:

- Orchiectomy (surgical castration): this surgery involves the permanent removal of the testicles, where most of the androgens are generated. With this source removed, most prostate cancers stop growing or shrink for a time.
- Luteinizing hormone-releasing hormone (LHRH) analogs: these drugs lower the amount of testosterone produced by the testicles. Treatment with these drugs is sometimes called chemical castration because they reduce androgen levels just as well as orchiectomy.
- **LHRH antagonists**: LHRH antagonists work similarly to LHRH agonists, but they decrease testosterone levels more quickly and do not cause tumor flare like the LHRH agonists can do.

- **Anti-androgens**: these compounds impede the body’s ability to use any androgens by blocking androgen receptors or affecting androgen production. Even after orchiectomy or during treatment with LHRH analogs, the adrenal glands still produce small amounts of androgens. Anti-androgen treatment may be combined with orchiectomy or LHRH analogs as first-line hormone therapy (i.e. combined androgen blockade).

- **Other androgen-suppressing drugs**: Estrogens were once the main alternative to orchiectomy for men with advanced PCa. Because of their possible side effects (including blood clots and breast enlargement), estrogens have been largely replaced by LHRH analogs and anti-androgens. Ketoconazole (Nizoral®), first used for treating fungal infections, inhibits androgen production. It is most often used to treat patients recently diagnosed with advanced PCa, as it offers a quick way to lower testosterone levels [162].

**Chemotherapy**

Chemotherapy (chemo) uses anti-cancer drugs injected into a vein or given by mouth. These drugs enter the bloodstream and circulate throughout the body, making this treatment potentially useful for cancers that have metastasized to distant organs. Chemotherapy is sometimes used if PCa has spread outside the prostate and hormone therapy is not working. Chemo is not a standard treatment for early PCa. Chemotherapy is administered in cycles (each lasting a few weeks), with each period of treatment followed by a rest period to allow the body time to recover [162]. For PCa, chemo drugs are typically used one at a time and include:
Docetaxel (Taxotere®), Cabazitaxel (Jevtana®), Mitoxantrone (Novantrone®), Estramustine (Emcyt®), Doxorubicin (Adriamycin®), Etoposide (VP-16), Vinblastine (Velban®), Paclitaxel (Taxol®), Carboplatin (Paraplatin®), Vinorelbine (Navelbine®) [162].

In most cases, the first chemo drug given is docetaxel, combined with the steroid drug prednisone. If this drug does not work (or stops working), a newer drug called cabazitaxel is often the next chemo drug attempted (although there may be other treatment options as well). Both of these drugs have been shown to help patients live several months longer than older chemotherapy drugs [162]. They may slow the growth of the cancer and also reduce symptoms, resulting in a better quality of life. Still, chemotherapy for PCa is very unlikely to result in a cure.
2.3 Relationship between Vitamin D and Prostate Cancer

2.3.1 Epidemiological Evidence

Known major risk factors for PCa include age, race, and geography (discussed in Section 2.2.2). The risk of developing PCa increases with age, as 80-90% of all PCa diagnoses and mortalities occur in men over 65 years of age [160]. PCa is considerably more common in Black men compared to other races. Moreover, Black men are more likely to be diagnosed at an advanced stage, and are more than twice as likely to die of PCa than White men [162]. Finally, several studies have demonstrated geographic patterns in PCa incidence and mortality such that these rates are higher in northern latitudes.

In 1990, Schwartz hypothesized that several major risk factors for PCa (i.e. age, race, geography) are all associated with decreased synthesis of vitamin D [191]. The elderly are frequently vitamin D deficient for several reasons including reduced exposure to sunlight [192] and the decreased capacity of older skin to generate vitamin D [193]. In terms of race, the higher melanin content in darker skin blocks the UV-B light required for vitamin D synthesis [194], which often results in lower serum 25(OH)D levels among pigmented races [152]. Inadequate sunlight/UV-B exposure has been consistently implicated as a risk factor for PCa incidence and mortality in several studies. Table 2.6 summarizes the epidemiological studies on the association between sunlight exposure and PCa risk. Hanchette et al [22] demonstrated that age-adjusted PCa mortality rates in the US are inversely proportional to UV radiation, the principle source of vitamin D. These findings were supported by data from the NHANES I cohort, which demonstrated that lower risk of PCa was significantly associated with residence in the South US at baseline (RR = 0.68, CI = 0.41-1.13), state of longest residence in the South US (RR = 0.62, CI = 0.40-0.95), and high solar radiation in the state of birth (RR = 0.49, CI = 0.30-0.79) [195].
A similar epidemiological study reported that, in addition to sun exposure in adulthood, early-life sun exposure was associated with a significant 51% reduced risk of PCa [196]. Of note, this study suggests that vitamin D exposure during early life could influence subsequent PCa risk later in life. Lastly, PCa patients were found to have better prognosis (15-50%) if they were diagnosed with PCa during the summer or autumn compared to the winter months [23]. Similar geographic patterns in cancer risk and mortality have been demonstrated for other cancers, including those of the colon and breast [197-200].

The association between dietary intake of vitamin D and PCa is less consistent as shown in Table 2.6. A prospective study of 3612 men followed for 8-10 years found no association between vitamin D intake and PCa risk [201]. A meta-analysis of 45 observational studies indicated that dietary vitamin D intake (<400 IU/d) was not related to PCa risk [202]. However, Ahn et al [21] reported a 40% reduction in PCa risk with >600 IU of supplemental vitamin D compared to no supplements. These findings suggest that vitamin D may be protective against PCa only when dietary consumption levels exceed recommended intakes.

Several studies have evaluated PCa risk in relation to serum levels of vitamin D metabolites, but the results have been mixed (Table 2.6). Corder et al [203] showed that PCa risk decreased with higher levels of 1,25(OH)2D, especially in those men with low 25(OH)D (OR = 0.15, CI = 0.03-0.85). A nested case-control study from Finland found that PCa risk was more than 3 times higher among young men (<52 years) with the lowest serum 25(OH)D (≤40 nmol/L) concentrations compared to older men (>51 years) [204]. Tretli et al [11] reported that PCa patients with medium (50-80 nmol/L) and high (>80 nmol/L) 25(OH)D concentrations had a 67% and 84% lower risk of death from PCa, respectively, compared to those with low
### Table 2.6. Summary of selected epidemiological studies linking sunlight exposure, vitamin D intake, and serum vitamin D metabolites to prostate cancer risk

<table>
<thead>
<tr>
<th>First author, year, study place</th>
<th>Study design</th>
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<td><strong>Sunlight exposure and PCa risk</strong></td>
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<td></td>
</tr>
<tr>
<td>Hanchette, 1992, US [22]</td>
<td>Ecological</td>
<td>N/A (Epidemiological index: n = 220, UV count: n = 3069, Latitude: n = 3073)</td>
<td>Epidemiological index: r = -0.25, P &lt; 0.0002, UV count: r = -0.15, P &lt; 0.0001, Latitude: r = 0.19, P &lt; 0.0001</td>
<td>PCa mortality exhibits a north-south trend, with lower rates in the South. UVB radiation may inhibit the development of clinical PCa.</td>
</tr>
<tr>
<td>John, 2004, US [195]</td>
<td>Cohort</td>
<td>153/3414</td>
<td>High solar radiation at longest residence: 0.62 (0.40-0.95), High solar radiation at place of birth: 0.49 (0.30-0.79)</td>
<td>Sunlight exposure reduces risk of PCa and has important implications for PCa prevention.</td>
</tr>
<tr>
<td>John, 2007, US [196]</td>
<td>Cohort</td>
<td>161/3367</td>
<td>High solar radiation in state of birth: 0.49 (0.27-0.90), Frequent recreational sun exposure in adulthood: 0.47 (0.23-0.99)</td>
<td>Sun exposure in both early life and adulthood protects against PCa.</td>
</tr>
<tr>
<td>Robsahm, 2004, Norway [23]</td>
<td>Cohort</td>
<td>16,457 PCa deaths, 39,583 PCa cases</td>
<td>Summer diagnosis: 0.80 (0.75-0.84), Fall diagnosis: 0.70 (0.66-0.74)</td>
<td>Higher UV radiation at the time of diagnosis (e.g. summer, fall) may improve prognosis of PCa.</td>
</tr>
<tr>
<td><strong>Vitamin D intake and PCa risk</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tseng, 2005, US [201]</td>
<td>Prospective cohort</td>
<td>131/3612</td>
<td>0.6 (0.3-1.2), P = 0.16</td>
<td>After adjustment for calcium intake, vitamin D was not associated with PCa risk.</td>
</tr>
<tr>
<td>Huncharek, 2008, US [202]</td>
<td>Review meta-analysis</td>
<td>26769</td>
<td>1.16 (0.98-1.38)</td>
<td>Dietary intake of vitamin D was not related to PCa risk.</td>
</tr>
<tr>
<td>Ahn, 2007, US [21]</td>
<td>Cohort</td>
<td>1910/29509</td>
<td>0.61 (0.41-0.89), P = 0.05</td>
<td>PCa risk tended to decrease with greater vitamin D from supplemental sources, with a 40% reduction in men who used &gt;600 IU of supplemental vitamin D compared with men not using vitamin D supplements.</td>
</tr>
<tr>
<td><strong>Serum vitamin D metabolites and PCa risk</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corder, 1993, US [203]</td>
<td>Case-control</td>
<td>181/181</td>
<td>0.15 (0.03-0.85)</td>
<td>PCa risk decreased with higher levels of</td>
</tr>
<tr>
<td>Study (Year, Location)</td>
<td>Study Design</td>
<td>Sample Size</td>
<td>25(OH)D Level</td>
<td>1,25(OH)2D Level</td>
</tr>
<tr>
<td>-----------------------</td>
<td>--------------</td>
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</tr>
<tr>
<td>Ahonen, 2000, Finland [204]</td>
<td>Nested case-control</td>
<td>149/566</td>
<td>1.7 (1.0-3.0), P = 0.01</td>
<td></td>
</tr>
<tr>
<td>Tretli, 2009, Norway [11]</td>
<td>Cohort</td>
<td>19/52</td>
<td>25(OH)D (50-80 nmol/L): 0.33 (0.14-0.77) 25(OH)D (&gt;80 nmol/L): 0.16 (0.05-0.43)</td>
<td></td>
</tr>
<tr>
<td>Fang, 2011, US [205]</td>
<td>Cohort</td>
<td>166/429</td>
<td>For lethal PCa (low vs high 25(OH)D): 1.59 (1.06-2.39), P = 0.006</td>
<td></td>
</tr>
<tr>
<td>Shui, 2012, US [206]</td>
<td>Nested case-control</td>
<td>1260/1331</td>
<td>For lethal PCa (low vs. high 25(OH)D): 0.43 (0.24-0.76), P = 0.001</td>
<td></td>
</tr>
<tr>
<td>Ahn, 2008, US [207]</td>
<td>Nested case-control</td>
<td>749/781</td>
<td>1.08 (0.77-1.53), P = 0.15</td>
<td></td>
</tr>
<tr>
<td>Gann, 1996, US [208]</td>
<td>Nested case-control</td>
<td>232/414</td>
<td>25(OH)D: 0.92 (0.56-1.50), P = 0.82 1,25(OH)2D: 0.88 (0.53-1.45), P = 0.50</td>
<td></td>
</tr>
<tr>
<td>Platz, 2004, US [209]</td>
<td>Case-control</td>
<td>460/460</td>
<td>25(OH)D: 1.19 (0.79-1.79), P = 0.59 1,25(OH)2D: 1.25 (0.82-1.90), P = 0.16</td>
<td></td>
</tr>
<tr>
<td>Albanes, 2011, Finland [210, 210]</td>
<td>Nested case-control</td>
<td>1000/1000</td>
<td>Highest (&gt;75 nmol/L) vs Lowest (&lt;25 nmol/L): 1.56 (1.15–2.12), P = 0.01</td>
<td></td>
</tr>
<tr>
<td>Tuohimaa, 2004, Norway, Finland, Sweden, [211]</td>
<td>Longitudinal nested case-control</td>
<td>622/1451</td>
<td>Lowest 25(OH)D: 1.5 (0.8-2.7) Highest 25(OH)D: 1.7 (1.1-2.4)</td>
<td></td>
</tr>
</tbody>
</table>
(<50 nmol/L) 25(OH)D concentrations. In a cohort from the Health Professionals Follow-up Study and Physicians’ Health Study, patients with the lowest 25(OH)D quartile were more likely to die of their cancer compared to those in the highest quartile [205]. Recently, Shui et al [206] demonstrated that higher 25(OH)D levels were associated with a 57% reduction in the risk of lethal PCa, and that SNPs among several vitamin D-related genes were related to lethal PCa risk. Vieth et al [212] showed that among PCa patients undergoing a watchful waiting protocol, the rise in prostate specific antigen (PSA), the most widely-used clinical biomarker of PCa, was slower in the spring and summer when serum 25(OH)D levels were rising. However, other studies failed to demonstrate a correlation between PCa risk and vitamin D status [207-209].

Recently, Albanes et al [210] indicated that Finnish men with the highest 25(OH)D quintile (75 nmol/L) had a higher risk for developing PCa than those with lower (<25 nmol/L) levels. Tuohimaa et al [211] reported a U-shaped risk curve, whereby both low (<19 nmol/L) and high (>80 nmol/L) serum 25(OH)D concentrations were associated with higher PCa risk. In a Danish study, all-cause mortality was shown to be significantly higher at very low (10 nmol/L) and high (140 nmol/L) serum 25(OH)D levels [213], although several other studies reported reduced mortality with higher 25(OH)D throughout the reference range. Vieth [214, 215] pointed out that this rare “U” or “J” risk phenomenon is specific to high latitudes, and hypothesized that it is attributable to the potentially detrimental consequences of dynamically fluctuating 25(OH)D supplies on vitamin D metabolic enzymes that occur with seasons at high latitudes. Interestingly, this phenomenon has only been reported in studies of Scandinavian populations. Scandinavians are unique because they are among the few populations that still commonly consume cod liver oil as a major source of vitamin D (e.g. 44% use in Norwegian women [216]). A potential issue with cod liver oil is that it can contain excessive doses of
vitamin A that inhibit vitamin D at the receptor level [217] and impair its function [218]. Thus, the antagonism between vitamin D and vitamin A that may occur with excessive cod liver oil use may explain the rare but reported U-shaped risk phenomenon between vitamin D and disease.
2.3.2 Experimental Evidence

Considerable evidence implicates the prostate as a target organ for vitamin D. Skowronski et al [219] reported VDR expression in LNCaP, PC-3, and DU 145 PCa cell lines, as well as growth inhibition by 1,25(OH)\textsubscript{2}D (1-100 nmol/L) treatment amounting to ~60%, ~40-50%, and ~10% of controls, respectively. Similar 1,25(OH)\textsubscript{2}D-induced growth arrest (ED\textsubscript{50} = 0.25-1 nmol/L) was demonstrated in primary epithelial cells derived from normal, benign prostate hyperplasia and cancerous regions of prostate obtained during prostatectomy [220].

Numerous studies have reported 1α-OHase expression in the prostate, supporting the chemotherapeutic utility of 25(OH)D through local prostatic conversion to 1,25(OH)\textsubscript{2}D [24, 221-223]. Growth of primary cultures of normal [24] and malignant [221] prostate cells was inhibited by physiological concentrations of 25(OH)D (i.e. 100 nmol/L) at an equal potency to pharmacological (and less safe) doses of 1,25(OH)\textsubscript{2}D. However, this therapeutic approach may be limited because some PCa cell lines exhibit lower 1α-hydroxylase [222] and higher 24-hydroxylase [224] expression, which reduces the availability of prostatic 1,25(OH)\textsubscript{2}D. Recently, Tokar and Webber [27, 225] reported that normal and malignant prostate epithelial cell lines constitutively express 25-OHase (CYP27A1), an enzyme capable of directly converting vitamin D into 25(OH)D and 1,25(OH)\textsubscript{2}D. Treatment of these cells with physiological levels of vitamin D\textsubscript{3} inhibited growth, induced differentiation, and decreased invasion by upregulating VDR, retinoid X receptor, and AR [27, 225]. The unique ability of prostate to locally produce 1,25(OH)\textsubscript{2}D from circulating vitamin D as well as paracrine 1,25(OH)\textsubscript{2}D synthesis by neighbouring normal PCa cells could circumvent the lower 1α-OHase and higher 24-OHase expression observed in prostate carcinoma. Taken together, these findings suggest that high-dose vitamin D can be an effective agent for PCa treatment and possibly prevention.
The \textit{in vivo} effects of vitamin D in PCa animal models have been mostly studied using 1,25(OH)$_2$D or synthetic vitamin D analogs. Blutt et al [226] showed that intraperitoneal injections of the vitamin D analog EB1089 substantially reduced the growth of LNCaP tumour xenografts in nude mice, without inducing hypercalcemia. Studies of the highly metastatic MAT LyLu Dunning tumour model in rats showed that 1,25(OH)$_2$D, as well as the Ro23-6760 and EB1089 vitamin D analogs, inhibited tumour growth and reduced lung metastases in these animals [227, 228]. Xue et al [229] demonstrated that a high fat, low calcium, and low vitamin D diet fed to rats resulted in hyperproliferation of the prostate epithelium, a process which was subsequently inhibited by increasing levels of calcium and vitamin D in the rat diet. Recently, 2 mouse studies provided further evidence supporting the potential utility of dietary vitamin D supplementation in PCa prevention and therapy. Kovalenko et al [25] showed that a diet low in vitamin D$_3$ (25 IU/kg diet) increased proliferation (measured by Ki67 staining), suppressed apoptosis, and increased the severity of prostatic intraepithelial neoplasia (PIN) lesions (see Section 2.2.2) in wild-type and PCa transgenic mice. In mouse xenograft models of breast (i.e. MCF-7) and prostate (i.e. PC-3) cancer, dietary vitamin D$_3$ resulted in inhibition of tumour growth (>50\%) and gene expression changes (e.g. ER decrease, p21 increase) that were equivalent to calcitriol treatment [26]. Overall, preclinical data provide strong support for the clinical evaluation of dietary vitamin D$_3$ as a chemopreventive or chemotherapeutic agent.

There appear to be multiple genomic and non-genomic mechanisms by which 1,25(OH)$_2$D induces growth inhibition, including the previously mentioned effects on cell cycle arrest, apoptosis, metastasis, and angiogenesis. In one study, treatment of LNCaP cells with 1,25(OH)$_2$D increased the expression of CDK inhibitor p21 and decreased CDK2 activity, leading to a reduction in phosphorylation of the retinoblastoma protein and repression of E2F
transcriptional activity, resulting in G1 arrest of the cells [230]. The elevations in p21 may be mediated indirectly by 1,25(OH)₂D induced upregulation of insulin-like growth factor binding protein-3 expression [231]. Blutt et al [232] demonstrated evidence of apoptosis in LNCaP cells exposed to 1,25(OH)₂D, including the downregulation of the pro-apoptotic proteins Bcl-2 and Bcl-XLa. In prostate epithelial cell lines, 1,25(OH)₂D or its precursors increased expression of PSA [225, 233], a differentiation marker, and decreased expression of vimentin, a dedifferentiation indicator [27], in part due to upregulation of AR. Furthermore, 1,25(OH)₂D, along with its analogs and precursors, have also been shown to reduce markers of tumour invasion and metastasis, including matrix metalloproteinases [27, 234] and cell-surface integrins [235]. Studies have indicated that 1,25(OH)₂D can inhibit tumour cell-induced angiogenesis in mice [236] and reduce the angiogenic signaling molecule angiopoietin-2 in tumour-derived endothelial cells [237]. Lastly, cDNA microarray analyses have revealed several novel putative vitamin D target genes in prostate cells including MAP kinase phosphatase 5 and superoxide dismutase 2, which are involved in protection from oxidative stress, metallothionein cell survival factors, tumour necrosis factor, and several others [238, 239].
2.3.3 Clinical Trial Evidence

A small number of human intervention studies have examined the safety and efficacy of vitamin D treatment in PCa. However, most of these investigations used 1,25(OH)\(_2\)D or its analogs, rather than dietary vitamin D or vitamin D supplements, and have yielded mixed results. A small phase II dose-escalation trial of oral 1,25(OH)\(_2\)D (0.5-1.5 μg/d) in 14 patients with advanced metastatic androgen-independent PCa (AIPCa) reported no responses (i.e. response was defined as >50% reduction in PSA or >30% reduction in tumour mass) with treatment [240]. Gross et al [241] conducted a pilot trial of increasing doses of 1,25(OH)\(_2\)D (0.5-2.5 g/d) in 7 patients with early recurrent PCa following radiation or prostatectomy. This study found a substantial reduction in the rate of PSA rise in all patients, suggesting that 1,25(OH)\(_2\)D therapy could be effective in slowing PCa progression. However, in both of these early trials, hypercalciuria or hypercalcemia limited the clinical utility of 1,25(OH)\(_2\)D in PCa therapy.

More recent clinical trials have attempted to circumvent the calcemic side effects of 1,25(OH)\(_2\)D administration by altering its dosing regimen or employing less calcemic analogs. Beer et al [242] showed that high-dose weekly oral 1,25(OH)\(_2\)D (0.5 μg/kg) for a median of 10 months was safe (no hypercalcemia or renal calculi was detected) in PCa patients with rising PSA after therapy, although it did not achieve the endpoint of 50% reduction in PSA. DN-101 (Novacea Inc., San Francisco, CA), a proprietary formulation of high-dose 1,25(OH)\(_2\)D (45 μg weekly), was shown to be safe and able to attain the high systemic exposures (>1 nmol/L of 1,25(OH)\(_2\)D) required for anti-tumour activity [243, 244]. In the AIPCa Study of Calcitriol Enhancing Taxotere (ASCENT) phase II trial, the addition of DN-101 to weekly docetaxel was associated with a significant 33% reduction in the risk of death, although this combination did not produce a significant improvement in PSA response [245]. The improved survival led to a
phase III trial of this regimen (ASCENT-2), in which survival was a primary endpoint. Unfortunately, ASCENT-2 was terminated abruptly after enrolment of 900 men due to excess number of deaths in the DN-101 arm. It is unclear whether the negative findings of ASCENT-2 were related to 1,25(OH)_{2}D *per se* or flawed study design [246]. Of note, ASCENT-2 had serious methodological issues, including: 1) the docetaxel dosing regimen in the DN-101 arm (weekly, 36 mg/sqm) was different and shown by other trials to be inferior to that of the control group (every 3 weeks, 75 mg/sqm); as such, this study design violates one of the primary tenets of RCT design; and 2) the DN-101 dose was selected based more on convenience than RCT substantiation [i.e. no maximum-tolerated dose (MTD) or optimal dose of DN-101 for PCa therapy has been defined to date] [246].

Analogs of 1,25(OH)_{2}D exhibit an improved safety profile over 1,25(OH)_{2}D, however, their clinical efficacy in PCa has not been confirmed. A phase I/II trial of paricalcitol (Abbot Pharmaceuticals, Abbott Park, IL) in AIPCa patients did not show a response to treatment (i.e. PSA decline), although paricalcitol did reduce PTH, which was inversely associated with survival [247]. Similarly, phase II trials of doxercalciferol alone [248] or with docetaxel [249] failed to show objective responses in PSA or survival in AIPCa. Other vitamin D analogs, including seocalcitol (EB-1089; LEO Pharma, Ballerup, Denmark), inecalcitol (Hybrigenics, Paris, France), 22-oxa-calcitriol, calciptotriol (LEO Pharma), KH1060 (LEO Pharma), and RO24-5531 are also being investigated as potential anticancer drugs.

The limited success of clinical trials of vitamin D-based compounds in PCa may relate to the choice of the therapeutic agent itself. To date, most studies have focused on the anticancer capacity of 1,25(OH)_{2}D or its analogs, but these require pharmacological doses and careful monitoring of calcium levels. However, preclinical evidence suggests that physiological levels
of nutrient vitamin D₃ can exert similar antiproliferative effects as supraphysiological 1,25(OH)₂D concentrations without inducing calcemic side effects (see section on experimental evidence). In 2005, Woo et al [29] described the first clinical trial evaluating the efficacy of nutrient vitamin D₃ (2000 IU/d) in 15 patients with recurrent PCa. Vitamin D₃ treatment decreased or stabilized PSA values in 9 patients for as long as 21 months of follow-up, delaying the implementation of androgen ablation. Furthermore, there was a statistically significant reduction in the rate of PSA rise after vitamin D₃ administration. Overall, 14 out of 15 patients had a prolonged PSA doubling time, which increased significantly from 14.3 to 25 months after commencing vitamin D₃ supplementation. The positive findings from this pilot trial led to a phase II randomized trial of higher doses of oral vitamin D₃ [40 000, 10 000, or 400 (control) IU/d] administered preoperatively to early-stage PCa patients undergoing radical prostatectomy (ClinicalTrials.gov Identifier: NCT00741364). The major outcomes of this study were vitamin D metabolite concentrations in prostate tissue and immunohistochemical markers of prostate cell proliferation (described in Section 7). Since the commencement of this clinical trial, two additional clinical studies of dietary vitamin D provided further support for the vitamin D hypothesis of PCa therapy. In a small, open-label study, patients with advanced PCa were advised to take a vitamin D supplement, of which most consumed 1000 IU vitamin D₂/d. Of the 26 patients that took vitamin D, five (20%) responded to the supplementation with a mean (range) reduction in PSA level of 45.3 (15.9–95.1)%, and a mean duration of response of 4–5 months [250]. In a more recent open-label clinical trial, low-risk PCa patients under active surveillance (n=48) were treated with vitamin D₃ (4000 IU/d for 1 yr) and followed up until repeat biopsy. The vitamin D₃ supplementation led to a significant reduction in positive cores at repeat biopsy; specifically, 55% of patients exhibited a decrease in the number of positive cores.
or Gleason score, 11\% of patients showed no change, and 34\% showed an increase in positive cores or Gleason score [28].
2.3.4 Vitamin D and Prostate Cancer: Research Frontiers

Despite its relatively ancient roots in the Victorian era of medicine, vitamin D remains an exciting area of research in disease epidemiology, prevention, and therapy. Definitive results from clinical studies are needed to support the persuasive epidemiological and experimental data reported to date, and to allay the fears caused by “U-shaped risk curves”. In particular, the optimal agent (i.e. whether it be the nutrient vitamin D₃, its metabolites, or analogs), dose, and route of administration that provide maximal benefit specific to PCa prevention and therapy require further investigation. Given the substantial molecular heterogeneity in PCa, design of future studies should also consider the genetic variability in metabolic and functional responses to vitamin D dosing. The combination of vitamin D with other agents, including chemotherapeutic drugs, catabolic (i.e. 24-hydroxylase) inhibitors, soy isoflavones (e.g. genistein), retinoids, glucocorticoids, and other VDR ligands [246, 251] is a compelling strategy being tested that could yield synergistic efficacy in cancer prevention and therapy. Moreover, identification of critical windows during which vitamin D can prevent and/or treat disease is imperative for minimizing the risk of later disease and for implementing public health programs.
3 CHAPTER 3: Hypotheses and Objectives
3.1 **Hypothesis**

Much of this thesis is based upon a series of my published manuscripts that progressively reported on evaluation of the analytical validity and clinical utility of various methods for measuring the concentration of various vitamin D metabolites in serum (Chapter 4, 5) and in human tissue (Chapter 6, 7). These methodologies, which include a novel method to measure vitamin D metabolites in human tissues, were ultimately used in our randomized clinical trial of vitamin D₃ in order to investigate the clinical effects of vitamin D₃ supplementation on prostatic vitamin D metabolism and on PCa pathology (Chapter 7).

**Overall Hypotheses:** Higher¹ levels of vitamin D consumed orally or achieved in the circulation will result in increased concentrations of vitamin D metabolites, particularly active calcitriol, in human tissue. Higher oral vitamin D doses and/or tissue levels of vitamin D metabolites will relate to measurable changes in cell biology, such as the reduction of cell proliferation markers in clinical prostate tissue samples.

**Specific Hypotheses:**

1. Existing analytical platforms for quantification of vitamin D metabolites can be modified to reliably measure serum and tissue vitamin D metabolites in our PCa clinical trial samples:
   
   i. The DiaSorin LIAISON 25(OH)D chemiluminescent immunoassay will demonstrate strong correlation and comparable precision with the reference RIA 25(OH)D method (Chapter 4).

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¹ In this context, “higher” denotes intake or circulating concentrations of vitamin D that are near or beyond the upper end of exposure attainable through normal physiology.
ii. A novel liquid chromatography tandem mass spectrometry (LC-MS/MS) assay for simultaneous determination of 25(OH)D and 24,25(OH)_{2}D can provide clinically useful information on vitamin D supplementation and may be a more appropriate method for our PCa clinical trial samples (Chapter 5).

iii. An enzyme immunoassay can be adapted to measure calcitriol concentrations in clinical colon samples, and show that tissue calcitriol levels correlate with those in matched serum samples (Chapter 6).

2. Higher oral doses of dietary vitamin D\textsubscript{3} given to PCa patients prior to radical prostatectomy will induce changes in prostatic vitamin D metabolism and in PCa pathology.

i. Patients taking the higher doses of oral vitamin D\textsubscript{3} will show higher levels of vitamin D metabolites in their prostate tissue collected at surgery (Chapter 7).

ii. Higher levels of vitamin D\textsubscript{3} consumed orally or achieved in prostate tissue will relate to reduced prostate cell proliferation, as measured by Ki67 immunohistochemistry (Chapter 7).
3.2 Objectives

Overall Objectives: We sought to determine whether higher\textsuperscript{1} levels of vitamin D consumed orally or achieved in the circulation will result in increased concentrations of vitamin D metabolites, particularly active calcitriol, in human tissue. Furthermore, we aimed to investigate whether higher oral vitamin D doses and/or tissue levels of vitamin D metabolites will relate to measurable changes in cell biology, such as reduced markers of cell proliferation in clinical prostate tissue samples.

Specific Objectives:

1. To conduct method development and validation to evaluate whether existing analytical platforms for quantification of vitamin D metabolites can be modified to reliably measure serum and tissue vitamin D metabolites in our PCa clinical trial samples:
   
i. To compare two new automated assays with the well-established reference method, DiaSorin radioimmunoassay (RIA), for quantitation of serum total 25(OH)D (Chapter 4).

   ii. To assess the analytical validity and clinical utility of a novel liquid chromatography tandem mass spectrometry (LC-MS/MS) assay for simultaneous determination of 25(OH)D and 24,25(OH)\textsubscript{2}D (Chapter 5).

   iii. To determine whether calcitriol is present in human colon tissue and to characterize the relationship between human colon tissue and serum calcitriol concentrations (Chapter 6).

\textsuperscript{1} In this context, “higher” denotes intake or circulating concentrations of vitamin D that are near or beyond the upper end of exposure attainable through normal physiology.
2. To conduct a randomized clinical trial to evaluate whether higher oral doses of dietary vitamin D$_3$ given to PCa patients prior to radical prostatectomy can induce changes in prostatic vitamin D metabolism and in PCa pathology.
   i. To determine whether oral vitamin D$_3$ supplementation results in higher vitamin D metabolite levels in human prostate tissue (Chapter 7).
   ii. To determine whether higher levels of vitamin D$_3$ consumed orally or achieved in prostate tissue can reduce Ki67, an immunohistochemical marker of prostate cell proliferation (Chapter 7).
CHAPTER 4: Study 1 – An evaluation of automated methods for measurement of serum 25-hydroxyvitamin D

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4.1 Abstract

Objectives: To compare two new automated assays with the well-established reference method, DiaSorin radioimmunoassay (RIA), for quantitation of serum total 25-hydroxyvitamin D [25(OH)D].

Methods: 25(OH)D from human sera (n = 158) was measured using DiaSorin RIA and two automated platforms, DiaSorin “LIAISON 25 OH Vitamin D TOTAL”, and Roche Modular “Vitamin D₃ (25-OH)”. Methods were compared by regression and Bland-Altman analyses.

Results: DiaSorin LIAISON demonstrated a stronger correlation (r = 0.918) and better agreement (bias = -0.88 nmol/L) with DiaSorin RIA than the Roche Modular assay (r = 0.871, bias = -2.55 nmol/L). Precision ranges (CV%) for the RIA, LIAISON, and Roche Modular assays, respectively, were: within run (6.8-12.9%, 2.8-8.1%, and 1.9-5.5%), and total precision (7.4-14.5%, 7.3-17.5%, and 7.6-14.5%).

Conclusion: DiaSorin LIAISON displayed the best correlation and agreement with DiaSorin RIA. The DiaSorin LIAISON 25 OH Vitamin D TOTAL assay is an accurate and precise automated tool for serum total 25(OH)D determination.
4.2 Introduction

The most reliable indicator of vitamin D status is measurement of 25-hydroxyvitamin D [25(OH)D] in serum or plasma. 25(OH)D, the major circulating metabolite of vitamin D, is produced in the liver by a hydroxylation of vitamin D at carbon 25. Two distinct forms of 25(OH)D exist: 25(OH)D$_3$, formed from vitamin D$_3$ (cholecalciferol), and 25(OH)D$_2$, produced from vitamin D$_2$ (ergocalciferol). Vitamin D$_3$ is synthesized naturally in skin exposed to UV radiation and also found in fatty fish. Vitamin D$_2$ is generated by UV irradiation of the plant sterol, ergosterol, and is less potent than vitamin D$_3$ [45, 46, 252]. Low circulating 25(OH)D concentrations have been associated with increased risk and progression of several diseases, including osteoporosis [7, 8], cancers [9-11], multiple sclerosis [12, 13], and cardiovascular disease [14, 15]. Such research into the role of vitamin D beyond calcium homeostasis has substantially increased clinical interest in vitamin D.

The measurement of 25(OH)D is challenging because circulating 25(OH)D is highly lipophilic, bound strongly to protein, present in low (nanomolar) concentrations, and exists in two structurally similar forms, 25(OH)D$_3$ and 25(OH)D$_2$ [253]. Several published methods exist for determining 25(OH)D concentrations, including competitive protein-binding assays, radioimmunoassay (RIA), High Performance Liquid Chromatography (HPLC), liquid chromatography-mass spectrometry (LC-MS), and the more recent automated immunoassays. In 1989, the International External Quality Assessment Scheme for Vitamin D metabolites (DEQAS, Northwest Thames, United Kingdom) was established to monitor the analytical reliability of 25(OH)D assays [254]. However, several reports have demonstrated large inconsistency and variability in 25(OH)D measurements between methods and laboratories [255-257]. As a result, some groups have emphasized a need for appropriate reference materials and
standardization of 25(OH)D assays [255, 256].

The rising clinical demand for assessment of vitamin D status has increased the need for simple, high through-put methods for measuring 25(OH)D in patient samples. Protein binding assays, HPLC, and LC-MS are manual methods that can be time and labour intensive, technique and operator dependent, and require costly equipment and large sample volumes. The DiaSorin RIA was the first vitamin D test approved for clinical diagnosis by the US Food and Drug Administration (FDA) and has been the most widely used method since. However, being a manual method, the RIA has been challenged by the rapidly increasing demand for 25(OH)D testing. Recently, automated chemiluminescence-based immunoassays have become available which offer higher through-put capacity, lower sample volume requirement, and reduced operator error. In 2007, DiaSorin received FDA approval for clinical use of its second-generation automated “LIAISON 25 OH Vitamin D TOTAL” chemiluminescent immunoassay (CLIA). More recently, Roche Diagnostics released an automated electrochemiluminescence immunoassay (ECLIA) called “Vitamin D₃ (25-OH)” that can be performed on their Elecsys, Modular Analytics, and Cobas analyzers. The objective of the present study was to compare the analytical performance of these two new automated assays (LIAISON and Roche) with the reference method (DiaSorin RIA) for the determination of serum 25(OH)D.
4.3 Materials and Methods

Samples

Human serum samples (n = 400) were obtained from a clinical trial in Toronto, Canada (latitude 43°N) in which healthy adults received either 28,000 IU vitamin D$_3$/wk or a placebo for 8 weeks [258]. Serum aliquots were stored at -80°C until analysis. Under these storage conditions, 25(OH)D is very stable in serum or plasma over a prolonged time and repeated freeze-thaw cycles [253, 259, 260]. Quantitative determination of serum 25(OH)D was performed in singleton by: DiaSorin “25-hydroxyvitamin D $^{125}$I RIA” in April 2007 (n = 390), DiaSorin “LIAISON 25 OH Vitamin D TOTAL” CLIA in September 2007 (n = 390), and Roche Modular “Vitamin D$_3$ (25-OH)” ECLIA in October 2007 (n = 158). The DiaSorin 25(OH)D RIA served as the reference method. Out of the 400 serum samples acquired, 390 samples were analyzed by DiaSorin RIA and DiaSorin LIAISON TOTAL (10 samples had insufficient volume) and 158 samples were analyzed by the Roche Modular assay because there were not enough reagent kits to analyze the full 400 samples. Therefore, direct method comparisons were limited to those samples that were measured by all three assays (n = 158). The 158 samples pertained to baseline and end-of-study (wk 8) measurements of 20 subjects taking placebo and 59 subjects taking vitamin D$_3$ [258].

25(OH)D assays

DiaSorin 25(OH)D $^{125}$I RIA

The DiaSorin 25(OH)D RIA method is based on a competitive principle with a goat antibody against 25(OH)D, an iodinated ($^{125}$I) 25(OH)D$_3$ tracer, and donkey anti-goat
precipitating complex as secondary antibody. The first part of the assay involves a rapid extraction of 25(OH)D and other hydroxylated metabolites from serum or plasma with acetonitrile. Following extraction, the sample, antibody, and tracer are incubated for 90 minutes at 20-25°C. Phase separation is accomplished after a 20 minute incubation at 20-25°C with the secondary antibody. A buffer is then added prior to centrifugation to reduce non-specific binding. Radioactivity is measured by a gamma counter and is inversely proportional to the concentration of 25(OH)D in the sample.

**DiaSorin LIAISON 25(OH)D TOTAL CLIA**

The LIAISON 25 OH Vitamin D TOTAL Assay is a direct competitive chemiluminescence immunoassay for human serum or plasma intended for use on the DiaSorin LIAISON automated analyzer. The assay uses magnetic particles (solid phase) coated with antibody against 25(OH)D and 25(OH)D conjugated to an isoluminol derivative (tracer). During the first incubation phase (10 minutes), 25(OH)D is dissociated from binding protein by buffer containing 10% ethanol and then binds to the anti-25(OH)D antibody on the solid phase. After a second 10 minute incubation with the tracer, the unbound material is washed off and starter reagents are added to generate a flash chemiluminescent signal which is measured by a photomultiplier and is inversely related to 25(OH)D concentration.

This assay differs from its older version, “LIAISON 25 OH Vitamin D”, due to alterations in the on-board extraction procedure, the addition of a second incubation step, and the use of human serum-based calibrators instead of horse serum.
**Roche Modular 25(OH)D ECLIA**

The Roche Vitamin D₃ (25-OH) assay is a direct competitive electrochemiluminescence immunoassay for human serum or plasma intended for use on Roche automated immunoassay analyzers. In this study, the Modular Analytics analyzer was used. The assay employs microparticles coated with streptavidin and a polyclonal sheep antibody against 25(OH)D, which is labeled with ruthenium. In the first incubation, 25(OH)D₃ in the sample competes with biotin labelled 25(OH)D for binding with the anti-25(OH)D antibody. In the second incubation, the biotin-25(OH)D/anti-25(OH)D antibody immunocomplex becomes bound to the microparticles via interaction of biotin and streptavidin. The microparticles are then magnetically captured onto the surface of an electrode. A voltage is applied to the electrode to produce a chemiluminescent emission, which is measured by a photomultiplier and is inversely proportional to 25(OH)D concentration.

Specifications for the three assays, as stated by the manufacturer, are listed in Table 4.1. According to the product inserts, none of the analytical methods are significantly affected by levels of hemolysis or lipemia typically encountered in conventionally collected and prepared samples.

*Quality assessment*

All assays were performed in accordance with the manufacturer’s instructions and complied with our standard operating procedures for good laboratory practice. DiaSorin RIA and LIAISON 25(OH)D results from our laboratory consistently fall within one standard deviation of the group mean in the international DEQAS proficiency surveys. In the January 2009 DEQAS results, the “all methods” mean ± SD (CV%) for a test sample was 47.2 ± 6.1
nmol/L (12.9%), compared to 46.7 ± 7.7 nmol (16.5%) for DiaSorin RIA, 46.6 ± 6.0 nmol/L (12.9%) for DiaSorin LIAISON TOTAL, and 52.1 ± 5.9 nmol/L (11.3%) for the Roche assay.
Table 4.1. Assay specifications, as stated in the manufacturer’s product insert.

<table>
<thead>
<tr>
<th></th>
<th>DiaSorin 25-Hydroxyvitamin D$_{25}$ I RIA</th>
<th>DiaSorin LIAISON 25 OH Vitamin D TOTAL</th>
<th>Roche Vitamin D$_3$ (25-OH)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Assay Format</td>
<td>extraction, equilibrium RIA</td>
<td>direct, competitive, CLIA</td>
<td>direct, competitive, ECLIA</td>
</tr>
<tr>
<td>Platform</td>
<td>manual</td>
<td>automated</td>
<td>automated</td>
</tr>
<tr>
<td>Analyzer(s)</td>
<td>N/A</td>
<td>LIAISON</td>
<td>Elecsys, Modular Analytics, or Cobas</td>
</tr>
<tr>
<td>Sample volume</td>
<td>50 μL</td>
<td>25 μL</td>
<td>35 μL</td>
</tr>
<tr>
<td>Sample type</td>
<td>serum or plasma (EDTA, Hep)</td>
<td>serum or plasma (EDTA, Hep)</td>
<td>serum or plasma (EDTA, Hep)</td>
</tr>
<tr>
<td>Assay time</td>
<td>110 minutes</td>
<td>20 minutes</td>
<td>18 minutes</td>
</tr>
<tr>
<td>Analytical sensitivity</td>
<td>3.75-NR</td>
<td>10-375 nmol/L</td>
<td>10-250 nmol/L</td>
</tr>
<tr>
<td>Analytical specificity</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vitamin D$_3$</td>
<td>% Cross-reactivity 0.8</td>
<td>% Cross-reactivity &lt;1</td>
<td>% Cross-reactivity &lt;1</td>
</tr>
<tr>
<td>Vitamin D$_2$</td>
<td>0.8</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>25(OH)$_2$D$_3$</td>
<td>100</td>
<td>100</td>
<td>&lt;10</td>
</tr>
<tr>
<td>25(OH)$_2$D$_2$</td>
<td>11</td>
<td>17</td>
<td>Up to 100</td>
</tr>
<tr>
<td>1,25(OH)$_2$D$_3$</td>
<td>11</td>
<td>40</td>
<td>NR</td>
</tr>
<tr>
<td>1,25(OH)$_2$D$_2$</td>
<td>11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Precision</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>within-run</td>
<td>% CV 8.6-12.5</td>
<td>% CV 2.9-5.5</td>
<td>% CV 3.5-4.9</td>
</tr>
<tr>
<td>total</td>
<td>8.2-11.0</td>
<td>6.3-12.9</td>
<td>4.2-7.8</td>
</tr>
<tr>
<td>Method Comparison</td>
<td>NR</td>
<td>1. n = 155, against RIA LIAISON = 0.99 (RIA) + 2.4 r = 0.97</td>
<td>1. n = 291, against automated assay Roche = 1.272 (other) – 0.045 r = 0.912</td>
</tr>
<tr>
<td></td>
<td>DiaSorin RIA is usually the reference method</td>
<td></td>
<td>2. n = 771 against LC-MS-MS Roche = 1.008 (LC-MS-MS)+ 0.045 r = 0.902</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>NR = not reported</td>
</tr>
</tbody>
</table>
Statistical Analyses

Concentrations of 25(OH)D are given in nmol/L units. All data were analyzed with SPSS software (version 13.0) and Analyse-it for Microsoft Excel. The criterion for significance was set at P < 0.05.
4.4 Results

Samples

Overall, 158 complete cases were evaluated. None of the samples tested showed visible signs of hemolysis or lipemia. Descriptive statistics of the 25(OH)D concentrations measured by the DiaSorin RIA, DiaSorin LIAISON, and Roche Modular assays, respectively, were: mean ± SD (76.4 ± 39.5, 75.5 ± 39.3, and 73.8 ± 31.2 nmol/L; P > 0.05), median (66.0, 67.0, and 68.4 nmol/L), 95% CI (70.2-82.6, 69.3-81.7, and 68.9-78.7 nmol/L), and range (16.0-183.0, 17.1-176.0, and 16.7-189.6 nmol/L). Table 4.2 compares the assays based on the proportion of samples fulfilling commonly-used decision criteria for 25(OH)D status.

Precision

The precision of the RIA, LIAISON, and Roche Modular assays was determined by using 5 human serum-based quality controls (kit, in-house pooled serum, and patient samples), spanning a 25(OH)D range of 35-180 nmol/L. Each control sample was assayed in 2-6 replicates per run for 3-5 runs. Precision values are shown in Table 4.3. Precision ranges (CV%) for the RIA, LIAISON, and Roche Modular assays, respectively, were: within run (6.8-12.9%, 2.8-8.1%, and 1.9-5.5%), and total precision (7.4-14.5%, 7.3-17.5%, and 7.6-14.5%). These precision values fall within the CV ranges typically encountered with 25(OH)D methods in DEQAS (10-20%).
Table 4.2. Concordance of assays to 25(OH)D decision criteria.

<table>
<thead>
<tr>
<th>Serum 25(OH)D</th>
<th>RIA</th>
<th>LIAISON</th>
<th>Roche</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>158</td>
<td>158</td>
<td>158</td>
</tr>
<tr>
<td>&lt; 40 nmol/L, % (n)</td>
<td>21 (33)</td>
<td>24 (38)</td>
<td>11 (18)</td>
</tr>
<tr>
<td>&lt; 75 nmol/L, % (n)</td>
<td>54 (85)</td>
<td>55 (87)</td>
<td>58 (91)</td>
</tr>
</tbody>
</table>
### Table 4.3. Within-run and total precision of the 25(OH)D assays evaluated

<table>
<thead>
<tr>
<th>Assay/Sample</th>
<th>n</th>
<th>Mean (nmol/L)</th>
<th>Within-run precision (%CV)</th>
<th>Total precision (%CV)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>RIA</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kit control 1</td>
<td>6</td>
<td>36.8</td>
<td>10.6</td>
<td>12.2</td>
</tr>
<tr>
<td>Kit control 2</td>
<td>5</td>
<td>164.6</td>
<td>6.8</td>
<td>7.4</td>
</tr>
<tr>
<td>In-house Level 1</td>
<td>15</td>
<td>41.2</td>
<td>9.9</td>
<td>10.2</td>
</tr>
<tr>
<td>In-house Level 2</td>
<td>15</td>
<td>88.3</td>
<td>9.2</td>
<td>10.9</td>
</tr>
<tr>
<td>In-house Level 3</td>
<td>15</td>
<td>176.9</td>
<td>12.9</td>
<td>14.5</td>
</tr>
<tr>
<td><strong>LIAISON</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kit control 1</td>
<td>16</td>
<td>40.5</td>
<td>7.3</td>
<td>11.3</td>
</tr>
<tr>
<td>Kit control 2</td>
<td>18</td>
<td>125.5</td>
<td>5.7</td>
<td>12.8</td>
</tr>
<tr>
<td>In-house Level 1</td>
<td>6</td>
<td>57.5</td>
<td>8.1</td>
<td>15.9</td>
</tr>
<tr>
<td>In-house Level 2</td>
<td>7</td>
<td>93.0</td>
<td>7.8</td>
<td>17.5</td>
</tr>
<tr>
<td>In-house Level 3</td>
<td>7</td>
<td>152.9</td>
<td>2.8</td>
<td>7.3</td>
</tr>
<tr>
<td><strong>Roche</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kit control 1</td>
<td>7</td>
<td>71.9</td>
<td>5.5</td>
<td>10.0</td>
</tr>
<tr>
<td>Kit control 2</td>
<td>7</td>
<td>93.0</td>
<td>5.0</td>
<td>9.5</td>
</tr>
<tr>
<td>Kit control 3</td>
<td>7</td>
<td>147.2</td>
<td>2.8</td>
<td>8.5</td>
</tr>
<tr>
<td>Sample A</td>
<td>4</td>
<td>37.3</td>
<td>1.9</td>
<td>14.5</td>
</tr>
<tr>
<td>Sample B</td>
<td>4</td>
<td>106.9</td>
<td>2.2</td>
<td>7.6</td>
</tr>
</tbody>
</table>
Method correlations

The three methods were compared by both linear and Deming regression. Regression parameters are shown in Table 4.4. Deming regression plots of the 25(OH)D assays are presented in Figure 4.1. Based on the regression analysis, the DiaSorin LIAISON platform correlated best with DiaSorin RIA ($r = 0.918$, $n = 158$). Furthermore, this correlation, based on samples measured by all 3 assays ($n = 158$), was equivalent to the correlation between DiaSorin LIAISON and RIA 25(OH)D assays in the larger trial cohort ($r = 0.917$, $n = 390$). The Roche Modular method correlated reasonably well with DiaSorin RIA ($r = 0.871$, $n = 158$) and LIAISON ($r = 0.862$, $n = 158$).

Method agreement

The agreement among 25(OH)D assays was analyzed by the mean, difference method of Bland and Altman [261]. Bland-Altman analyses of the 25(OH)D methods are presented in Figure 4.2. LIAISON showed little bias when compared to DiaSorin RIA [$\text{bias} +/\text{- SD (95\% CI)} = -0.88 +/\text{- 15.95 (-3.38 to 1.63) nmol/L}$]. Roche Modular demonstrated higher bias compared to DiaSorin RIA [-2.55 +/\text{- 19.67 (-5.64 to 0.54) nmol/L}] than to LIAISON [-1.67 +/\text{- 20.14 (-4.83 to 1.50) nmol/L}].
**Table 4.4.** Regression (linear, Deming) and correlation parameters of the methods being compared

<table>
<thead>
<tr>
<th>Comparison</th>
<th>n</th>
<th>Linear regression</th>
<th>Deming regression</th>
<th>Correlation</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIAISON vs. RIA</td>
<td>158</td>
<td>LIAISON = 0.91 (RIA) + 5.75</td>
<td>LIAISON = 0.99 (RIA) - 0.42</td>
<td>r = 0.918</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sy</td>
<td>x = 15.625</td>
<td>Sy</td>
</tr>
<tr>
<td></td>
<td>390</td>
<td>LIAISON = 0.91 (RIA) + 5.80</td>
<td>LIAISON = 0.99 (RIA) - 0.90</td>
<td>r = 0.917</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sy</td>
<td>x = 14.02</td>
<td>Sy</td>
</tr>
<tr>
<td>Roche vs. RIA</td>
<td>158</td>
<td>Roche = 0.69 (RIA) + 21.30</td>
<td>Roche = 0.76 (RIA) + 15.57</td>
<td>r = 0.871</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sy</td>
<td>x = 15.365</td>
<td>Sy</td>
</tr>
<tr>
<td>Roche vs.</td>
<td>158</td>
<td>Roche = 0.68 (LIAISON) + 22.20</td>
<td>Roche = 0.77 (LIAISON) + 16.04</td>
<td>r = 0.862</td>
</tr>
<tr>
<td>LIAISON</td>
<td></td>
<td></td>
<td>Sy</td>
<td>x = 15.894</td>
</tr>
</tbody>
</table>
Figure 4.1. Deming regression of 25(OH)D quantitation comparisons: DiaSorin RIA reference method with DiaSorin LIAISON Total and Roche Modular automated platforms. (A) DiaSorin RIA vs. DiaSorin LIAISON Total (left: n = 158; right: n = 390, full cohort). (B) DiaSorin RIA vs. Roche Modular (n = 158). (C) DiaSorin LIAISON Total vs. Roche Modular (n = 158). Both the Pearson and the non-parametric Spearman correlation coefficients are indicated. Dotted lines indicate 95% confidence intervals.
Figure 4.2. Bland/Altman plots, showing means of paired differences between 25(OH)D quantitation comparisons: DiaSorin RIA reference method with DiaSorin LIAISON Total and Roche Modular automated platforms. (A) DiaSorin RIA vs. DiaSorin LIAISON Total (left: n = 158; right: n = 390, full cohort). (B) DiaSorin RIA vs. Roche Modular (n = 158). (C) DiaSorin LIAISON Total vs. Roche Modular (n = 158). Thick solid lines show bias (means of paired differences), denoted numerically as bias ± SD. Thin solid lines represent lines of identity. Dashed lines show 95% limits of agreement (bias ± 1.96*SD). Dotted lines indicate 95% confidence intervals.
4.5 Discussion

The assessment of vitamin D status, through the measurement of 25(OH)D in serum or plasma, has received considerable attention in the last decade. A growing number of studies have reported widespread vitamin D deficiency in apparently healthy populations worldwide [2-6]. Low vitamin D status, defined by low circulating 25(OH)D, has been associated with several diseases, including osteoporosis [7, 8], cancers [9-11], multiple sclerosis [12, 13], cardiovascular disease [14, 15], diabetes [16, 17], and microbial infections [18, 19]. A recent meta-analysis demonstrated that vitamin D supplementation was associated with a 7% reduction in total mortality [20]. The latest consensus indicated that a serum 25(OH)D concentration of 75 nmol/L or greater is sufficient or optimal for health [262], putting the majority of the North American and European population at varying levels of deficiency. Therefore, the measurement of circulating 25(OH)D is becoming increasingly important and the clinical demand for 25(OH)D assays has risen substantially.

The DiaSorin 125I-based RIA has been the method of choice for measuring 25(OH)D concentrations. However, the RIA is time-consuming, labour intensive, and employs radioactive compounds, which pose a health hazard and limit automation. The increasing demand for non-radioactive, high-throughput 25(OH)D measurement has led to the development of several automated platforms. DiaSorin and Roche Diagnostics have recently introduced fully automated immunoassay systems employing chemiluminescent technology for 25(OH)D determination in serum or plasma. Here, we have compared these automated methods with the reference method, DiaSorin RIA. To our knowledge, the comparison of DiaSorin LIAISON Total, Roche Modular, and DiaSorin RIA methods for 25(OH)D has never been reported in the literature. We found that DiaSorin LIAISON demonstrated a stronger correlation (r = 0.918) and better agreement (bias =
-0.88 nmol/L) with the DiaSorin RIA reference method than the Roche Modular assay (r = 0.871, bias = -2.55 nmol/L).

This study employed the second-generation and most recent version of the LIAISON assay, “LIAISON 25 OH Vitamin D TOTAL”. Our results indicate that this modified version showed a higher correlation with DiaSorin RIA than reported previously with the older version of the assay, “LIAISON 25 OH Vitamin D” [257, 263]. Recently, Roth et al [264] evaluated the accuracy of several 25(OH)D methods, including the LIAISON and Roche assays presented here. However, they chose LC-MS/MS as their reference method and made no direct comparisons between the automated methods and RIA, making it difficult to compare data. DiaSorin RIA is the more appropriate reference method because its use is the basis of virtually all the research linking circulating 25(OH)D to health and disease outcomes and reference values [120, 262]. In contrast, there have been no large clinical trials demonstrating decision-based reference values for 25(OH)D based upon clinical data using LC-MS methods. Nonetheless, the LIAISON and Roche assays demonstrated good correlation and agreement with LC-MS/MS. Leino et al [265] recently showed that the Roche 25(OH)D assay performed similarly against DiaSorin RIA (r = 0.836, n = 163) to what we report here. Our investigation had the advantage of having compared two new automated assays with a method (DiaSorin RIA) that has set the standard for clinical diagnosis of vitamin D deficiency [253]. Furthermore, we analyzed a large number of samples with a wider range of 25(OH)D concentrations than previously reported because we used the baseline and final serum samples from a placebo-controlled, vitamin D dosing clinical trial [258]. An appropriate methods evaluation should test a broad range of analyte concentrations. This is particularly relevant for vitamin D because there is a wide distribution of 25(OH)D in the general population.
Clinical diagnosis and treatment decisions related to vitamin D are based on assessment of total 25(OH)D concentration. Therefore, the analytical method of choice should detect 25(OH)D₃ and 25(OH)D₂ equally to report an accurate total 25(OH)D value. The major limitation of the Roche assay is its inability to detect 25(OH)D₂. As stated in the product insert (Table 1), the version of the Roche assay which we evaluated here had <10% cross-reactivity with 25(OH)D₂ (Roche has since redesigned their assay method to overcome this shortcoming). In contrast, the DiaSorin RIA and LIAISON assays claim 100% cross-reactivity with 25(OH)D₂ and 25(OH)D₃ on an equimolar basis. Consumption of supplements or foods containing vitamin D₂ (e.g. mushrooms) will contribute to total 25(OH)D concentrations; however, this contribution would be underestimated by the Roche assay. It is unlikely that vitamin D₂ significantly affected the total 25(OH)D concentrations measured in our Canadian subjects because vitamin D₃ is more commonly used in Canada. However, this problem would be more pronounced in the US, where vitamin D₂ is commonly used, and also in patients receiving pharmaceutical preparations of high-dose vitamin D, which only exist as vitamin D₂ (e.g. Calciferol or Drisdol). It is more likely that the lower correlation and agreement of the Roche assay with the reference method is related to the assay itself. As shown in Table 2 and Figure 2B, the Roche Modular assay tended to overestimate 25(OH)D at low concentrations (< 40-50 nmol/L) and underestimate 25(OH)D at high concentrations (> 75-100 nmol/L). For example, when DiaSorin RIA reference values of 25 nmol/L and 150 nmol/L are applied to the Deming regression equations, the corresponding DiaSorin LIAISON concentrations are 24.3 nmol/L and 148.1 nmol/L, and the Roche Modular values are 34.6 nmol/L and 129.6 nmol/L, respectively. Furthermore, following our analysis, we noticed the same discrepancy in the January 2009 DEQAS results. For example, the DiaSorin RIA (n = 16 laboratories) mean ± SD for a “low” DEQAS test sample was 26.5 ± 4.2 nmol, compared to 22.1 ± 3.5 nmol/L for DiaSorin LIAISON TOTAL (n = 99), and 44.8 ± 9.9 nmol/L.
for the Roche assay (n = 15). In contrast, the DiaSorin RIA (n = 16) mean ± SD for a “high” DEQAS test sample was 79.3 ± 13.8 nmol, compared to 73.3 ± 8.8 nmol/L for DiaSorin LIAISON TOTAL (n = 100), and 53.0 ± 6.1 nmol/L for the Roche assay (n = 15). The discordant sensitivity at the lower and upper end of the measuring range may be related to the extraction procedure, the antibody used, or matrix effects that lead to variability in individual patient samples (36). Furthermore, this analytical issue with the Roche assay could have negative implications in the clinical assessment of vitamin D status. However, we believe that the performance and validity of the Roche assay can be improved by modifying the method to correct the problems outlined.

Within-run precision was generally higher in the automated LIAISON and Roche Modular assays compared to the manual RIA. The Roche method displayed the best within-run precision, however, its quality controls cover a relatively high 25(OH)D range (~ 60 – 180 nmol/L), compared to the ranges encompassed by the DiaSorin RIA and LIAISON quality controls (~ 30 – 180 nmol/L) which are more representative of values observed in the general population. Total precision was not substantially different among the three assays. Of note, our precision values were slightly lower than those reported in the product inserts (Table 1). A more comprehensive evaluation of precision performance would have used a greater number of replicates and runs than those used in the present study.

The variation among 25(OH)D methods observed in the present study was smaller than previously reported, but still illustrates the need for standardization of 25(OH)D assays. We conclude that the DiaSorin LIAISON 25 OH Vitamin D TOTAL assay is an accurate and precise tool for the determination of 25(OH)D. The LIAISON assay exhibited better correlation and agreement with the reference RIA method than the recently introduced Roche assay. Automated,
accurate 25(OH)D methods provide greater speed and convenience, and improve work flow and efficiency in the high-throughput clinical laboratory as it continues to meet the increasing demand for 25(OH)D testing. The diagnostic field awaits an automated assay for determination of 1,25-dihydroxyvitamin D [1,25(OH)₂D], the active hormonal metabolite of vitamin D.

ACKNOWLEDGEMENTS

We thank DiaSorin for donating the LIAISON 25 OH Vitamin D TOTAL kits and Roche Diagnostics for donating the Vitamin D₃ (25-OH) kits.
CHAPTER 5: Study 2 – The ratio of serum 24,25-dihydroxyvitamin D₃ to 25-hydroxyvitamin D₃ is predictive of 25-hydroxyvitamin D₃ response to vitamin D₃ supplementation

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5.1 Abstract

24,25-dihydroxyvitamin D (24,25(OH)$_2$D) is the major catabolite of 25-hydroxyvitamin D (25(OH)D) metabolism, and may be physiologically active. Our objectives were to: 1) characterize the response of serum 24,25(OH)$_2$D$_3$ to vitamin D$_3$ supplementation; 2) test the hypothesis that a higher 24,25(OH)$_2$D$_3$ to 25(OH)D$_3$ ratio (24,25:25(OH)D$_3$) predicts 25(OH)D$_3$ response.

Serum samples (n = 160) from wk 2 and wk 6 of a placebo-controlled, randomized clinical trial of vitamin D$_3$ (28,000 IU/wk) were analyzed for serum 24,25(OH)$_2$D$_3$ and 25(OH)D$_3$ by mass spectrometry.

Serum 24,25(OH)$_2$D$_3$ was highly correlated with 25(OH)D$_3$ in placebo- and vitamin D$_3$-treated subjects at each time point (p < 0.0001). At wk 2, the 24,25:25(OH)D$_3$ ratio was lower with vitamin D$_3$ than with placebo (p = 0.035). From wk 2 to wk 6, the 24,25:25(OH)D$_3$ ratio increased with the vitamin D$_3$ supplement (p < 0.001) but not with placebo, such that at wk 6 this ratio did not significantly differ between groups. After correcting for potential confounders, we found that 24,25:25(OH)D$_3$ at wk 2 was inversely correlated to the 25(OH)D$_3$ increment by wk 6 in the supplemented group (r = -0.32, p = 0.02) but not the controls.

There is a strong correlation between 24,25(OH)$_2$D$_3$ and 25(OH)D$_3$ that is only modestly affected by vitamin D$_3$ supplementation. This indicates that the catabolism of 25(OH)D$_3$ to 24,25(OH)$_2$D$_3$ rises with increasing 25(OH)D$_3$. Furthermore, the initial ratio of serum 24,25(OH)$_2$D$_3$ to 25(OH)D$_3$ predicted the increase in 25(OH)D$_3$. The 24,25:25(OH)D$_3$ ratio may therefore have clinical utility as a marker for vitamin D$_3$ catabolism and a predictor of serum 25(OH)D$_3$ response to vitamin D$_3$ supplementation.
5.2 Introduction

Vitamin D has received considerable attention because of associations between low vitamin D status and increased risk for several diseases, including osteoporosis, cancers, multiple sclerosis, diabetes, cardiovascular disease, and microbial infections [7, 10, 13, 14, 18, 266, 267]. The determinants of serum 25-hydroxyvitamin D (25(OH)D), the classic measure of vitamin D status, include environmental (e.g. season, latitude, sunlight, diet) [268, 269], demographic [e.g. ethnicity, body mass index (BMI)] [270], and genetic factors (e.g. polymorphisms in metabolism and transport genes) [271-273]. However, the factors that modify response to vitamin D supplementation warrant further study, especially in view of the large inter-individual variation that has been reported in serum 25(OH)D response to supplementation with identical doses of vitamin D [136, 272, 274]. An analysis of 24,25-dihydroxyvitamin D (24,25(OH)₂D), the major metabolite of 25(OH)D, could provide clinically relevant information that may shed light on these inter-individual differences.

24,25(OH)₂D is produced via 24-hydroxylation of 25(OH)D by the cytochrome P450 24-hydroxylase enzyme (CYP24A1; \( V_{\text{max}} = 0.088 \ \text{mol/min/mol} \ \text{P450} \), \( K_m = 160 \ \text{nM} \)) [275]. In addition, CYP24A1 catalyzes the side-chain metabolism of 1,25-dihydroxyvitamin D (1,25(OH)₂D), considered to be the primary active metabolite. CYP24A1 is expressed in many tissues [276-279] but the biological activity of 24,25(OH)₂D remains controversial. The general view is that 24,25(OH)₂D production is the first step to inactivate 25-hydroxylated metabolites of vitamin D, thus regulating synthesis of 1,25(OH)₂D [280, 281]. However, there is considerable evidence demonstrating that 24,25(OH)₂D has unique biological properties, including physiological roles in embryogenesis, cartilage development, and fracture repair [64, 282-287]. Recently, Larsson et al demonstrated that 24,25(OH)₂D binds to catalase, suggesting that
24,25(OH)₂D-mediated signal transduction may occur through modulating hydrogen peroxide production [288].

Few clinical studies have reported circulating 24,25(OH)₂D concentrations [289-295], likely because its measurement is technically challenging and its physiological role is unclear. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) has received increased attention because it is capable of measuring 25(OH)D₂ and 25(OH)D₃ separately, but the capability for measuring 24,25(OH)₂D has not been widely exploited. Furthermore, the effects of vitamin D₃ supplementation on serum 24,25(OH)₂D₃ concentrations in humans are unknown. Here, we characterize the biochemical response of serum 24,25(OH)₂D₃ to vitamin D₃ supplementation in healthy adults using a highly sensitive and specific LC-MS/MS assay for simultaneous determination of serum 25(OH)D₃ and 24,25(OH)₂D₃ concentrations. We hypothesized that a higher 24,25(OH)₂D₃ to 25(OH)D₃ ratio (24,25:25(OH)D₃) would predict a smaller serum 25(OH)D₃ response to an increased vitamin D₃ intake because a relatively higher 24,25(OH)₂D₃ would indicate higher catabolism.
5.3 Materials and Methods

Study samples

Human serum samples (n = 160) were obtained from a randomized, double-blind, placebo-controlled clinical trial carried out in Toronto, Canada (latitude 43ºN). Healthy young adults, half of whom were female, received either 28,000 IU vitamin D₃/wk as a supplement or fortified cheese of equivalent bioavailability (n = 60), or a placebo (n = 20), for 8 weeks during the winter months [258]. Serum aliquots were stored at -80°C until analysis. Under these storage conditions, vitamin D metabolites are stable in serum or plasma over a prolonged time and repeated freeze-thaw cycles [259, 260]. Samples were available from subjects at wk 2 (n = 80) and wk 6 (n = 80) of the dosing protocol. The study protocol was approved by the Research Ethics Boards of the University of Toronto and of Mount Sinai Hospital (Toronto, Canada).

25(OH)D and 24,25(OH)₂D assays

Aliquots of 200 µL serum were spiked with 50 µL of d₆-25-hydroxyvitamin D₃ [d₆-25(OH)D₃] (Medical Isotopes Inc., Pelham, NH, USA) 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 4:1 methanol:water 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 1:1 methanol:water and transferred into an HPLC autosampler vial. A 20 µL aliquot was analyzed by LC-MS/MS.
The chromatographic separation of 25(OH)D₃, 25(OH)D₂, and 24,25(OH)₂D₃ was carried out using an Agilent Technologies 1200 series HPLC system in linear gradient mode at a flow rate of 0.80 ml/min on an Eclipse C8 column (50 x 3.0 mm, 1.8 μm) employing a mobile phase consisting of methanol-water (37:63) increasing to 100% methanol over 4 min and maintained at 100% methanol for 1 min. The column was re-equilibrated with methanol-water (37:63) for 1 min. The column temperature was maintained at 50°C. The total chromatographic run time for each sample was 6.5 min and typical retention times for 24,25(OH)₂D₃, d6-25(OH)D₃, 25(OH)D₃, and 25(OH)D₂ were 2.92, 3.65, 3.66, and 3.72 min, respectively.

An API 5000 mass spectrometer (Applied Biosystems/Sciex, Concord, ON, Canada) was equipped with an atmospheric pressure chemical ionization (APCI) source and operated in the positive mode. The ion source temperature was maintained at 400°C, the corona current adjusted to 3.0 A, and collision gas, nebulizer gas and curtain gas pressures set to 5, 40, and 30, respectively, the collision energy set to 24V and the declustering potential set to 100V. The ion-transitions of m/z 417.4 → 399.4, 407.5 → 389.4, 401.4 → 383.4, and 413.4 → 395.4 were monitored to detect and quantify 24,25(OH)₂D₃, d6-25(OH)D₃, 25(OH)D₃, and 25(OH)D₂, respectively. The dwell time per transition was set to 50 ms.

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 of 25(OH)D₃, 25(OH)D₂, and 24,25(OH)₂D₃ (Sigma Aldrich) prepared in 100% ethanol that were analyzed within the same analytical run. The absolute concentrations of the calibrators were assigned using the Agilent 8453 E ultraviolet/visible spectrophotometer and calculated using the Merck Index molar absorptivity of 18 300 AU·mol⁻¹·L⁻¹ at 265 nm.
Serum 25(OH)D was also determined by DiaSorin “25-hydroxyvitamin D \(^{125}\)I Radioimmunoassay (RIA)” and DiaSorin “LIAISON 25 OH Vitamin D TOTAL” chemiluminescent immunoassay (LIA), as reported previously [30], and used for confirmatory analyses. Serum 24,25(OH)\(_2\)D\(_3\) and 25(OH)D\(_3\) concentrations measured by LC-MS/MS are presented, unless otherwise indicated.

**LC-MS/MS method evaluation**

Between-day imprecision was assessed by measuring vitamin D metabolites in low (L1), medium (L2), and high (L3) plasma control pools in duplicate over 20 working days. Within-run imprecision was evaluated by measuring vitamin D metabolites in 20 different aliquots of L1, L2, and L3. Linearity of the analytical measurement range was evaluated by measuring vitamin D metabolite calibrators in triplicate. The measurement response was classified as linear if a straight line was drawn within an allowable systemic error of 10% of each calibrator point.

The limit of detection (LOD) and limit of quantification (LOQ) are defined as the peaks that give signal to noise ratios of 3:1 and 10:1, respectively, and were determined by running the calibration curve in triplicate with the following calculations: 

\[
\text{LOD} = \frac{3 \times SD_0 \text{ calibrator}}{\text{slope}_{\text{curve}}},
\]

\[
\text{LOQ} = \frac{10 \times SD_0 \text{ calibrator}}{\text{slope}_{\text{curve}}},
\]

Functional sensitivity was evaluated by diluting L1 and measuring it 5 times to determine the concentration that gives a coefficient of variation (CV) near 20%.

The specificity of the LC-MS/MS assay to measure 24,25(OH)\(_2\)D\(_3\) and 25(OH)D\(_3\) separately was evaluated by spiking pooled serum with either 25(OH)D\(_3\) (~500 nmol/L), 24,25(OH)\(_2\)D\(_3\) (~50 nmol/L), or both, and assaying as described above. Samples were run in triplicate on 2 separate days. The method was evaluated for potential interference of high
bilirubin, hemoglobin, and lipemic conditions by spiking separate control plasma pools with bilirubin (800 μmol/L), haemoglobin (3 g/L), and lipids (100 mmol/L), and assaying as described above.

LC-MS/MS assay 25(OH)D measurements were compared to Diasorin RIA (n = 160) and Diasorin LIA (n = 160) values. Method comparisons were not performed for 24,25(OH)2D3 measurements because there is no published reference method for this metabolite.

*Other biochemical measurements*

Calcium, phosphate, and creatinine in serum and urine, as well as serum parathyroid hormone (PTH), were measured on the Modular Analytics Serum Work Area (Roche) as previously described [258]. Glomerular filtration rate (GFR) was estimated from serum creatinine using the Modification of Diet in Renal Disease (MDRD) Study equation [296].

*Statistical analyses*

The study was powered for a probability of 80% to detect a difference of 1 SD in 25(OH)D; this required a sample size of at least 34. Results are presented as means ± SD. All data were analyzed with SPSS software (version 18.0). Associations between biochemical measures were assessed using Pearson correlation coefficients (r). For regression lines plotted non-parametrically, we used the locally weighted regression and smoothing scatterplot (LOWESS) approach. Within-group changes in biochemical variables over time were analyzed with paired 2-tailed t tests. Between-group differences in biochemical measures at each time point were analyzed with independent sample 2-tailed t tests. The cut-off for statistical significance was set at p < 0.05.
5.4 Results

*LC-MS/MS method evaluation*

All data were normally distributed, as indicated by the Kolmogorov-Smirnov test. LC-MS/MS assay performance characteristics are shown in Table 5.1. Total imprecision for all vitamin D metabolites (CV = 7.3-14%) was comparable to immunoassays (5-15%) [260]. Linearity was confirmed across the analytical measurement range for all vitamin D metabolites. The functional sensitivity for all vitamin D metabolites (≤1 nmol/L) was lower (i.e. higher sensitivity) than immunoassays (≤10 nmol/L). 25(OH)D2 was not detected in any sample. Specificity experiments indicated no cross-reactivity (i.e. complete resolution) between 24,25(OH)2D3 and 25(OH)D3. Bilirubin, hemolysis, and triglycerides did not interfere with measurement of vitamin D metabolites. Lastly, serum 25(OH)D3 concentrations determined by LC-MS/MS correlated well with those measured by RIA (r = 0.915, p < 0.0001) and LIA (r = 0.907, p < 0.0001). However, both the RIA and LIA 25(OH)D methods demonstrated significant positive bias compared to LC-MS/MS (15.0 and 13.6 nmol/L, respectively, p < 0.0001), likely because these immunoassays have 100% cross-reactivity with 24,25(OH)2D [294].

*Biochemical responses*

Linear regression analysis indicated that serum 24,25(OH)2D3 and 25(OH)D3 were highly correlated in the total sample (Figure 5.1), and separately in the placebo- and vitamin D3-treated sub-groups at wk 2 (r = 0.81, r = 0.86, respectively; p < 0.0001) and wk 6 (r = 0.92, r = 0.81, respectively; p < 0.0001). LOESS (LOcally Estimated Scatterplot Smoothing) fitting supported these findings but suggested slight deviation from linearity at 25(OH)D3 concentrations >100
Table 5.1. LC-MS/MS assay performance characteristics

<table>
<thead>
<tr>
<th>Parameter</th>
<th>24,25(OH)₂D₃</th>
<th>25(OH)D₃</th>
<th>25(OH)D₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imprecision (%CV)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Between-day</td>
<td>7.3-9.6</td>
<td>5.3-6.5</td>
<td>7.5-13</td>
</tr>
<tr>
<td>Within-run</td>
<td>5.2-7.4</td>
<td>4.7-6.6</td>
<td>5.3-7.0</td>
</tr>
<tr>
<td>Total</td>
<td>9.1-12</td>
<td>7.3-8.5</td>
<td>10.0-14.0</td>
</tr>
<tr>
<td>Linearity</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Analytical measurement range (nmol/L)</td>
<td>0-60</td>
<td>0-437</td>
<td>0-403</td>
</tr>
<tr>
<td>Linear calibration curve</td>
<td>y₁ = 0.024x - 0.002</td>
<td>y = 0.034x + 0.003</td>
<td>y = 0.025x - 0.079</td>
</tr>
<tr>
<td></td>
<td>r = 0.9992</td>
<td>r = 1.0000</td>
<td>r = 0.9997</td>
</tr>
<tr>
<td>Sensitivity (nmol/L)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Limit of detection²</td>
<td>0.24</td>
<td>0.25</td>
<td>0.17</td>
</tr>
<tr>
<td>Limit of quantification³</td>
<td>0.8</td>
<td>0.83</td>
<td>0.57</td>
</tr>
<tr>
<td>Functional sensitivity⁴</td>
<td>1.13</td>
<td>0.59</td>
<td>1.14</td>
</tr>
<tr>
<td>Specificity (nmol/L)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- cross-reactant</td>
<td>5 ± 0.1</td>
<td>31 ± 0.2</td>
<td>ND⁵</td>
</tr>
<tr>
<td>+ 25(OH)D₃ (~500 nmol/L)</td>
<td>4 ± 1.0</td>
<td>534 ± 10</td>
<td>ND</td>
</tr>
<tr>
<td>+ 24,25(OH)₂D₃ (~50 nmol/L)</td>
<td>66 ± 9.0</td>
<td>30 ± 1.0</td>
<td>ND</td>
</tr>
<tr>
<td>+ both</td>
<td>69 ± 2.0</td>
<td>491 ± 23.0</td>
<td>ND</td>
</tr>
<tr>
<td>Interference (nmol/L, % change)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- interferant</td>
<td>10.6</td>
<td>75.3</td>
<td>10.6</td>
</tr>
<tr>
<td>+ bilirubin (800 μmol/L)</td>
<td>12.1, + 14%</td>
<td>77.5, + 2.9%</td>
<td>12.1, + 14%</td>
</tr>
<tr>
<td>+ hemoglobin (3 g/L)</td>
<td>11.3, + 6.6%</td>
<td>79.6, + 5.7%</td>
<td>11.3, + 6.6%</td>
</tr>
<tr>
<td>+ triglyceride (100 mmol/L)</td>
<td>10.9, +2.8%</td>
<td>71.7, - 4.8%</td>
<td>10.9, + 2.8%</td>
</tr>
<tr>
<td>Method comparison</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Regression/correlation</td>
<td>not reported,</td>
<td>LC-MS/MS = 0.82 (RIA) + 0.32</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>no reference method for 24,25(OH)₂D₃</td>
<td>r = 0.92, n = 160</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>LC-MS/MS = 0.82 (LIA) + 1.24</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>r = 0.91, n = 160</td>
<td></td>
</tr>
<tr>
<td>Agreement</td>
<td>not reported,</td>
<td>RIA bias = 15.0 ± 13.4 nmol/L</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>no reference method for 24,25(OH)₂D₃</td>
<td>LIA bias = 13.6 ± 13.8 nmol/L</td>
<td></td>
</tr>
</tbody>
</table>

¹y = peak area vitamin D/peak area IS, x = concentration (nmol/L)
²calculated as (3*SD)/slope
³calculated as (10*SD)/slope
⁴CV = 20%
⁵Not detected
Figure 5.1. Strong correlation between serum 24,25(OH)$_2$D$_3$ and 25(OH)D$_3$ concentrations in the total sample (n = 160), with slight deviation from linearity at serum 25(OH)D concentrations >100 nmol/L. Linear regression line, with 95% confidence intervals (curved lines), shown in black. LOESS fit line depicted in red. The linear regression equation and Pearson correlation coefficient (r) are also indicated.

Equation: $24,25$(OH)$_2$D$_3 = 0.14(25$(OH)D$_3) - 0.27$

$n = 160$

$r = 0.91$

$p < 0.0001$
nmol/L. All correlations persisted when serum 25(OH)D values previously measured by RIA and LIA were used (p < 0.0001). On average, serum 24,25(OH)₂D₃ values were 14% of 25(OH)D₃ concentrations.

In the vitamin D₃ group, serum 24,25(OH)₂D₃ and 24,25:25(OH)D₃ ratio also correlated with serum creatinine at wk 2 (r = -0.46, r = -0.39, respectively; p < 0.005) and wk 6 (r = -0.39, r = -0.39, respectively; p < 0.005). However, neither serum 24,25(OH)₂D₃ nor 24,25:25(OH)D₃ ratio correlated significantly with estimated GFR, nor with PTH, calcium, or phosphate in serum or urine.

Table 5.2 shows the absolute 25(OH)D₃ and 24,25(OH)₂D₃ concentrations at wk 2 and wk 6. After 2 wk of treatment, both serum 25(OH)D₃ and 24,25(OH)₂D₃ concentrations were significantly greater in the vitamin D₃ group (69.6 ± 17.5 and 8.9 ± 3.1 nmol/L, respectively) compared to placebo (40.2 ± 17.2 nmol/L and 5.9 ± 2.5, respectively) (p < 0.0001). By wk 6, serum 25(OH)D₃ and 24,25(OH)₂D₃ had increased to 90.5 ± 19.7 and 12.8 ± 3.6 nmol/L with vitamin D₃ supplementation, respectively (p < 0.0001), but remained unchanged in the placebo group (p = 0.26). The increases in serum 25(OH)D₃ and 24,25(OH)₂D₃ during this time period was significantly greater in the supplemented group (21.2 ± 9.1 and 4.1 ± 2.0 nmol/L, respectively) than in controls (-1.1 ± 4.1 and -0.3 ± 1.3 nmol/L, respectively) (p < 0.0001).

At 2 wk of treatment, the ratio of 24,25(OH)₂D₃ to 25(OH)D₃ was lower in the vitamin D₃ group (0.127 ± 0.02) than in the placebo group (0.155 ± 0.05) (p = 0.03). From wk 2 to wk 6, the 24,25:25(OH)D₃ ratio increased with the vitamin D₃ supplement to 0.142 ± 0.02 (p < 0.001) but remained unchanged in controls (wk 6: 0.146 ± 0.04, p = 0.34). At wk 6, the 24,25(OH)₂D₃ to 25(OH)D₃ ratio did not differ significantly between vitamin D₃- and placebo-treated subjects.
Table 5.2. Serum 25(OH)D₃ and 24,25(OH)₂D₃ concentrations over time in subjects consuming placebo (n = 20) or vitamin D₃ (n = 60)₁

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Placebo-treated</th>
<th>Vitamin D-treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>25(OH)D₃ (nmol/L)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>wk 2</td>
<td>40.2 ± 17.2</td>
<td>69.6 ± 17.5**</td>
</tr>
<tr>
<td>wk 6</td>
<td>39.2 ± 17.1</td>
<td>90.5 ± 19.7**⁺,†</td>
</tr>
<tr>
<td>Change</td>
<td>-1.1 ± 4.1</td>
<td>21.2 ± 9.1**</td>
</tr>
<tr>
<td>24,25(OH)₂D₃ (nmol/L)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>wk 2</td>
<td>5.9 ± 2.5</td>
<td>8.9 ± 3.1**</td>
</tr>
<tr>
<td>wk 6</td>
<td>5.6 ± 2.6</td>
<td>12.8 ± 3.6**⁺,†</td>
</tr>
<tr>
<td>Change</td>
<td>-0.3 ± 1.3</td>
<td>4.1 ± 2.0**</td>
</tr>
<tr>
<td>24,25:25(OH)D₃ ratio</td>
<td></td>
<td></td>
</tr>
<tr>
<td>wk 2</td>
<td>0.155 ± 0.05</td>
<td>0.127 ± 0.02⁺</td>
</tr>
<tr>
<td>wk 6</td>
<td>0.146 ± 0.04</td>
<td>0.142 ± 0.02⁺</td>
</tr>
<tr>
<td>Change</td>
<td>-0.009 ± 0.04</td>
<td>0.016 ± 0.02⁺</td>
</tr>
</tbody>
</table>

₁Values are means ± SD.
**p < 0.0001,  *p < 0.05 (compared to placebo)
⁺p < 0.001 (compared to wk 2)
(p = 0.61). These results were confirmed when using RIA and LIA serum 25(OH)D values. When stratifying by gender in the vitamin D_3 group, the 24,25:25(OH)D_3 ratio was not significantly different between genders at wk 2 (females: 0.131 ± 0.02, males: 0.122 ± 0.02; p = 0.20) but at wk 6 it was significantly higher in females (0.149 ± 0.02) compared to males (0.134 ± 0.02) (p = 0.01).

Linear regression indicated that the 24,25:25(OH)D_3 ratio at wk 2 and wk 6 was significantly inversely correlated with the change in serum 25(OH)D_3 (i.e., wk 6 – wk 2) in the vitamin D_3 group (r = -0.38, p = 0.004; r = -0.30, p = 0.03; respectively) but not in the placebo group (p > 0.40) (Figure 5.2). These correlations were essentially the same, irrespective of 25(OH)D measurement method (p < 0.05, data not shown). Significant correlation between 24,25:25(OH)D_3 and 25(OH)D_3 response to vitamin D_3 persisted at wk 2 and wk 6 after controlling for serum 25(OH)D_3 (wk 2 and 6), 24,25(OH)_2D_3 (wk 6), BMI, age, gender, PTH, calcium, phosphate, and creatinine (p < 0.05). After controlling for baseline serum 25(OH)D (as measured by RIA and LIA) and 24,25(OH)_2D_3 at wk 2, the association between 24,25:25(OH)D_3 and 25(OH)D_3 response was attenuated at wk 6 (r = -0.21, p = 0.14) but not at wk 2 (r = -0.35, p = 0.01). The 24,25:25(OH)D_3 ratio at wk 2 also correlated significantly with the overall change in serum 25(OH)D_3 (i.e., wk 8 – wk 0) (r = -0.40, p = 0.003) in the vitamin D_3 group but not in the placebo group (p = 0.22).
Figure 5.2. Inverse correlation between the serum 24,25(OH)₂D₃ to 25(OH)D₃ ratio at wk 2 and change (wk 6 – wk 2) in 25(OH)D₃ concentrations in subjects consuming vitamin D₃ (n = 60). Linear regression line, with 95% confidence intervals (curved lines), is shown. The linear regression equation and Pearson correlation coefficient (r) are also indicated.
5.5 Discussion

Our data suggest a new clinical indication utility for measuring serum 24,25(OH)\(_2\)D\(_3\), the major metabolite of 25(OH)D\(_3\), by a novel LC-MS/MS assay for simultaneous determination of 25(OH)D\(_3\) and 24,25(OH)\(_2\)D\(_3\). The developed LC-MS/MS method was highly sensitive, specific, and the first to quantify 24,25(OH)\(_2\)D\(_3\) in serum. Investigators should therefore exploit the capability of LC-MS/MS methods to measure both serum 24,25(OH)\(_2\)D and 25(OH)D simultaneously. Indeed, 24,25(OH)\(_2\)D is the most abundant 25(OH)D metabolite and its roles in fracture healing and cartilage growth [64, 284-287] support its physiological relevance beyond vitamin D catabolism.

We found that serum 24,25(OH)\(_2\)D\(_3\) concentrations were highly correlated with serum 25(OH)D\(_3\), indicating that the catabolism of 25(OH)D\(_3\) into 24,25(OH)\(_2\)D\(_3\) rises with increasing 25(OH)D\(_3\) concentrations. This is consistent with the findings of other investigators [289, 294, 295]. In our study, the correlation between these variables was remarkably strong; indeed, 82% of the variation in serum 24,25(OH)\(_2\)D\(_3\) could be explained by 25(OH)D\(_3\) concentrations. Furthermore, serum 24,25(OH)\(_2\)D\(_3\) increased in parallel with 25(OH)D\(_3\) levels during the 4 weeks of 28,000 IU/wk vitamin D\(_3\) supplementation. In fact, the two variables are so closely related that one might argue that serum 24,25(OH)\(_2\)D\(_3\) could serve as an alternative marker of vitamin D status. Taken together, the strong correlation and similar response of serum 24,25(OH)\(_2\)D\(_3\) with 25(OH)D\(_3\) indicate that 24,25(OH)\(_2\)D\(_3\) measurement provides clinically useful information pertaining to vitamin D status and supplementation.

Since 24,25(OH)\(_2\)D\(_3\) concentration changed in proportion to that of 25(OH)D\(_3\), we normalized serum 24,25(OH)\(_2\)D\(_3\) response by calculating the ratio of 24,25(OH)\(_2\)D\(_3\) to
25(OH)D₃. This ratio served as an index of vitamin D₃ clearance since 24,25(OH)₂D₃ is the major initial catabolite of 25(OH)D₃ metabolism. Interestingly, the 24,25:25(OH)D₃ ratio at wk 2 was significantly lower in the vitamin D₃ group than placebo, indicating a possible lag in 24-hydroxylation during the early phase of supplementation. We speculate that this lag effect is the result of: 1) the large incremental increase in 25(OH)D₃ observed during the first 2 wk of dosing, which was greater than that observed at any other time interval, and 2) the slower reaction kinetics of CYP24A1 [turnover number (TN) = 2-20 min⁻¹] compared to CYP27A1 (25-hydroxylase; TN = 40-50 min⁻¹) [275, 278]. By wk 6, however, the 24,25:25(OH)D₃ ratio had increased significantly with supplementation, as a response to the vitamin D₃ loading. Overall, these results suggest that catabolism is induced with vitamin D₃ supplementation but these adaptations may occur over weeks not days. Indeed, in vitro studies indicate that a variety of molecular mechanisms may be involved, including gene expression up-regulation and enzyme trafficking [297]. Future studies should investigate the genetic influences of CYP24A1 genotypes on vitamin D catalytic activity and biochemical response.

Our data provide insight on the in vivo effects of this altered expression and kinetic behaviour of the CYP24A1 enzyme. Firstly, the correlation in the vitamin D₃-treated group of serum 24,25(OH)₂D₃ and 24,25:25(OH)D₃ ratio with serum creatinine, a measure of renal function, is supportive of the idea of variable renal CYP24A1 action in 25(OH)D₃ metabolism. Accordingly, the 24,25:25(OH)D₃ ratio may be useful in monitoring kidney function during vitamin D₃ supplementation but this needs to be studied directly. The increase in 24,25:25(OH)D₃ over time is consistent with the induction of renal CYP24A1 catabolic capacity with increasing vitamin D₃ loading. The concept of induction proportional to load is also supported by the LOWESS fit line (Figure 5.1), which appears to become more curvilinear at
serum 25(OH)D₃ concentrations exceeding 100 nmol/L. Lastly, we found that the 24,25:25(OH)D₃ ratio was significantly higher in supplemented women compared to men at wk 6. This suggests that females were catabolising 25(OH)D₃ at a slightly faster rate than males during the later parts of vitamin D₃ supplementation, an effect that may be related to estrogen. Further research is needed to elucidate the regulation of CYP24A1 activity by gender and varying 25(OH)D₃ concentrations.

A major finding of this study was that the 24,25:25(OH)D₃ ratio alone predicted the magnitude of the serum 25(OH)D₃ change resulting from vitamin D₃ supplementation. This inverse correlation remained significant at wk 2 after controlling for other variables that may affect serum 25(OH)D₃ response, including baseline 25(OH)D, BMI, gender, serum PTH, and serum calcium. Although moderate (r = -0.38), this correlation was similar to those commonly reported with more conventional correlates of vitamin D response and status, including BMI (r = -0.41) [270] and PTH (r = -0.34) [258]. Taken together, these results suggest that relative 24,25(OH)₂D₃ concentration, as assessed by a ratio of circulating 24,25(OH)₂D₃ to 25(OH)D₃ early after dosing commences, is a potentially important determinant of serum 25(OH)D₃ response to supplementation. Consequently, this ratio may assist in identifying individuals who are more likely to experience a lower serum 25(OH)D₃ response and thereby require more vitamin D₃ due to a higher 24,25:25(OH)D₃ ratio (i.e. higher 25(OH)D₃ catabolism) during the early loading stage (i.e. wk 2) of the supplementation protocol.

Data on 24,25(OH)₂D can also be evaluated from the perspective of the biological activities of the vitamin D metabolites. Differential 24,25(OH)₂D production and 25(OH)D response may impact bioactive 1,25(OH)₂D levels, particularly in extra-renal 1,25(OH)₂D synthesis, which may well depend on 25(OH)D substrate supply, and in the renal failure
population, which exhibit abnormalities in renal vitamin D metabolism. Also, 25(OH)D itself has been reported to be a functional ligand of VDR and to exert genomic actions independent of 1,25(OH)₂D [298]. Therefore, differences in serum 25(OH)D responses due to increased vitamin D catabolism or other factors might directly affect 25(OH)D-mediated responses such as cell growth regulation. Lastly, there is substantial evidence supporting unique biological properties for 24,25(OH)₂D, particularly with respect to bone and cartilage [64, 282, 284-286]. In fact, preliminary evidence for the presence of a unique, non-nuclear membrane receptor for 24,25(OH)₂D has been reported [299].

The availability of robust LC-MS/MS methods for simultaneous determination of 25(OH)D₃ and 24,25(OH)₂D₃, like the one presented here, will also help elucidate the functional role of 24,25(OH)₂D in human physiology. Furthermore, the 24,25:25(OH)D₃ ratio may indicate not only metabolic differences in serum 25(OH)D response but also differential functioning of 24,25(OH)₂D between individuals and/or target tissues. For instance, the 24,25:25(OH)D₃ ratio (i.e. local or systemic), may be important in investigating the rate of putative 24,25(OH)₂D-dependent processes, such as fracture healing, whereby a higher ratio could hypothetically indicate faster healing.

Several limitations bear mention. Serum 24,25(OH)₂D₃ concentrations at baseline and end-of-study were not available. However, baseline 24,25(OH)₂D₃ levels in the vitamin D₃ group would, in all probability, be similar to those at wk 2 in the placebo group, particularly since baseline 25(OH)D concentrations did not differ significantly between groups. However, end-of-study (wk 8) 24,25(OH)₂D₃ determination may have provided additional meaningful data. The relatively small increment in 25(OH)D in the vitamin D₃ group from 6 to 8 wk is certainly compatible with the notion of proportional catabolism, but a direct test of this supposition is
warranted. Nonetheless, our evidence indicates that metabolic clearance rate at wk 2, as reflected in the 24,25:25(OH)D₃ ratio, appears to be the key determinant of 25(OH)D₃ response, such that the inclusion of baseline or end-of-study 24,25(OH)₂D₃ measurements would not have substantially changed our findings.

In conclusion, the measurement of serum 24,25(OH)₂D₃ in conjunction with 25(OH)D₃ shows promise as a novel marker of vitamin D₃ catabolism and predictor of serum 25(OH)D₃ response to vitamin D₃ supplementation. It should be emphasized that LC-MS/MS assay methods can be modified to measure both serum 25(OH)D₃ and 24,25(OH)₂D₃ simultaneously, thus providing more comprehensive data regarding vitamin D status and repletion. Moreover, further in vivo evidence may confirm the biological activity of 24,25(OH)₂D in physiological processes such as fracture repair, making its measurement ever more important. Future research should continue to explore the clinical utility of 24,25(OH)₂D measurement in vitamin D testing. Ultimately, this information may aid clinicians in adjusting vitamin D₃ dose for optimum individual benefit, thus contributing to the goal of personalized medicine and nutrition.

Acknowledgments

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CHAPTER 6: Study 3 – Determination of 1,25-dihydroxyvitamin D concentrations in human colon tissues and matched serum samples

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Dennis Wagner, Andre G. Dias, Kareena Schnabl, Theodorus van der Kwast, Reinhold Vieth


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6.1 Abstract

Background: A novel method to measure 1,25-dihydroxyvitamin D (1,25(OH)₂D) in human tissue was developed and validated. The objectives of this study were to determine whether 1,25(OH)₂D is present in human colon tissue and to characterize the relationship between human colon tissue and serum 1,25(OH)₂D concentrations.

Materials and Methods: Normal colon tissue specimens and matched serum samples were obtained from 30 patients who had undergone colectomy. Colon 1,25(OH)₂D was measured by lipid extraction followed by enzyme immunoassay (EIA). Serum 1,25(OH)₂D and 25-hydroxyvitamin D (25(OH)D) were measured by EIA. Vitamin D binding protein (DBP) was measured in a subset of serum and tissue samples.

Results: Regression analysis indicated a significant positive correlation between serum and colon 1,25(OH)₂D concentrations (r = 0.58, p = 0.0008). The corresponding intercept at zero serum 1,25(OH)₂D was 21.5 pmol/kg (95% CI = 16.95-25.98; p<0.001). Colon 1,25(OH)₂D did not correlate significantly with serum 25(OH)D. DBP levels in tissue samples were negligible.

Conclusion: The hormone 1,25(OH)₂D can be successfully detected in human colon at physiologically relevant concentrations partly determined by serum 1,25(OH)₂D. The results support the notion of in vivo synthesis of 1,25(OH)₂D within colon tissues.


6.2 Introduction

Epidemiological data have demonstrated that the risk of colon cancer and other malignancies decreases with increasing exposure to UV-B light and vitamin D [9]. A recent meta-analysis indicated that higher circulating 25-hydroxyvitamin D (25(OH)D), the clinical indicator of vitamin D status, was associated with a 33% reduced risk of colorectal cancer [300]. Gorham et al have shown that higher serum 25(OH)D (>= 82 nmol/L) or vitamin D intake (>= 1000 IU/d) reduced colorectal cancer incidence by 50% [301, 302]. In a prospective study of colorectal patients, higher plasma 25(OH)D levels were associated with a 48% reduction in overall mortality [303]. A 4-year randomized controlled trial of vitamin D₃ (1100 IU/d) and calcium (1400-1500 mg/d) revealed that vitamin D alone decreased all-cancer risk by up to 77% in post-menopausal women [10].

Several tissues, including the colon, express the vitamin D receptor (VDR) and the vitamin D-activating enzyme CYP27B1 [24, 76, 224, 304, 305], which mediate the anti-proliferative effects of 1,25-dihydroxyvitamin D (1,25(OH)₂D), the active vitamin D metabolite. Accordingly, it has been postulated that 1,25(OH)₂D accumulates in various tissues to locally induce growth inhibitory, pro-differentiating and pro-apoptotic actions on cells [2, 215, 306-308]. Although tissue 1,25(OH)₂D measurement has been reported in mice [309], no clinical observations in human tissues are available because of the technical difficulty in measuring 1,25(OH)₂D in human tissue. Furthermore, the relationship between tissue and serum levels of vitamin D metabolites has not been evaluated.

We have developed a novel, robust method to measure 1,25(OH)₂D concentration in human tissue. The objectives of this study were to validate the tissue 1,25(OH)₂D extraction
method, determine whether 1,25(OH)₂D is present in human colon tissue and characterize the relationship between human colon and serum 1,25(OH)₂D concentrations. Our findings support the analytical validity of the tissue extraction method and we demonstrate that 1,25(OH)₂D is present in human colon tissue at physiologically relevant concentrations partly determined by serum 1,25(OH)₂D, with some evidence of local colonic synthesis.
6.3 Materials and Methods

*Extraction and measurement of $1,25(\text{OH})_2\text{D}$ in human colon tissue*

Serum and normal colon specimens were obtained from 30 patients who had undergone colectomy surgery. For each patient, resected colonic mucosa and muscularis regions were sampled and pooled for $1,25(\text{OH})_2\text{D}$ analysis. The pooled colon tissue was washed in PBS to remove blood and feces, and 200 mg samples were minced with a scalpel to disrupt connective tissue prior to transfer into borosilicate glass tubes. The vitamin D-containing fraction was extracted using a modification of a previously described method [310]. Briefly, tissue samples were reconstituted in distilled water (1 mL) and homogenized in 2:1 methanol:methylene chloride (3.75 mL) at high speed for 5 min using a hand-held homogenizer (PRO 200, PRO Scientific Inc., CT, USA). Next, methylene chloride was added to the mixture (1.25 mL), followed by distilled water (1.25 mL). All samples were vortexed thoroughly after the addition of each reagent. After centrifugation (3500 rpm, 20 min), the lower lipid-containing layer was collected and the remaining protein residue was re-extracted with methylene chloride (1.25 mL). The collected methylene chloride extracts were dried under nitrogen gas and reconstituted in $1,25(\text{OH})_2\text{D}$-stripped human sera. This sera was prepared by treating pooled human serum with a charcoal-dextran suspension (Sigma Chemicals, Saint Louis, MO, USA), followed by mixing (2 h) and centrifuging (3500 rpm, 20 min) to collect the $1,25(\text{OH})_2\text{D}$-free supernatant. Absence of artifactual $1,25(\text{OH})_2\text{D}$ in the stripped serum was confirmed by enzyme immunoassay (EIA). Reconstituted tissue extracts were filtered using micro-filtration tubes (Nanosep MF, Pall Corporation, Port Washington, NY, USA) prior to $1,25(\text{OH})_2\text{D}$ assay.
The 1,25(OH)\(_2\)D levels in tissue extracts and serum samples were measured by a commonly-used EIA (Immunodiagnostic Systems, Scottsdale, AZ, USA). Serum 25(OH)D was measured by a chemiluminescent immunoassay (DiaSorin LIAISON, Stillwater, MN, USA). Vitamin D binding protein (DBP) was measured in a subset of patient serum (n=13) and colon homogenates (mucosa, n=5; muscularis, n=8) by ELISA (Immunodiagnostik, Bensheim, Germany).

Validation of human colon 1,25(OH)\(_2\)D assay

As there is no reference method or material for 1,25(OH)\(_2\)D measurement in human tissue, validation experiments were conducted to evaluate the ability of the extraction method to accurately and reproducibly extract 1,25(OH)\(_2\)D from a tissue matrix. Bovine muscle tissue (200 mg) was homogenized (PRO 200), spiked and left to incubate (1 h) with either radiolabelled \([\textsuperscript{3}H]1\alpha,25\text{(OH)}\textsubscript{2}D_3\) (17,940 cpm/mL; Amersham Biosciences, UK) dissolved in ethanol, n=10; crystalline 1α,25(OH)\(_2\)D\(_3\) dissolved in ethanol (5 µg/mL; Sigma Chemicals), n=10; or pooled control human serum containing known, physiological levels of 1,25(OH)\(_2\)D (≈ 163 nmol/L), n=10. All samples were extracted as described above and 1,25(OH)\(_2\)D was quantified, respectively, by liquid scintillation radioactivity analysis (TRI-CARB 2900TR Liquid Scintillation Analyzer; PerkinElmer, Shelton, CT, USA); HPLC (column: Zorbax SIL [5 µm particles, 4.6 mm i.d., 25 cm length; Agilent, Mississauga, ON, Canada], mobile phase: hexane:isopropanol (9:1)) and EIA (Immunodiagnostic Systems). The extraction methodology was also tested on unspiked neat human serum (200 µL) in place of tissue with EIA quantification.
EIA measurement confirmed the absence of artifactual 1,25(OH)₂D in the bovine muscle tissue and stripped serum used for method development. Results of the various quantification platforms were used to calculate the % recovery of added 1,25(OH)₂D achieved by our extraction method. A subset of colon and serum samples, as well as the stripped serum, was assayed multiple times to determine method precision.

Statistical analyses

Colon 1,25(OH)₂D and DBP values are expressed as pmol/kg and nmol/kg (wet tissue weight), respectively. Serum 1,25(OH)₂D and DBP values are expressed as pmol/L and nmol/L, respectively. The concentrations of free 1,25(OH)₂D were calculated as the ratio between the molar concentrations of 1,25(OH)₂D and DBP (“free 1,25(OH)₂D index”) as described previously [311]. All data were analyzed by SPSS software (version 13.0) and graphs were created using GraphPad Prism.
6.4 Results

Validation of human colon 1,25(OH)\(_2\)D assay

Table 6.1 shows the 1,25(OH)\(_2\)D extraction recoveries across the various quantification methods used to confirm recovery and analytical validity of the tissue extraction procedure. The results indicated that the extraction method consistently yielded excellent recovery of 1,25(OH)\(_2\)D (mean: 100.1 ± 6.5 %). The tissue 1,25(OH)\(_2\)D assay was reproducible as the within-assay variation (mean: 6.7 ± 2.4%) was comparable to those reported by the kit manufacturer (3-5%) and other immunoassays (Table 6.2). Taken together, these results demonstrated that the developed tissue extraction method was suitable for assay of 1,25(OH)\(_2\)D in human colon.

Human colon and serum vitamin D metabolite levels

Figure 6.1 shows the relationship between human colon and serum levels of 1,25(OH)\(_2\)D. Colon tissue 1,25(OH)\(_2\)D concentrations correlated significantly with serum 1,25(OH)\(_2\)D levels (r = 0.58, p<0.001). Regression analysis indicated a significantly positive slope (0.12, 95% CI = 0.05-0.18; p<0.001) of colon tissue 1,25(OH)\(_2\)D (pmol/kg) with serum 1,25(OH)\(_2\)D (pmol/L). The corresponding intercept at zero serum 1,25(OH)\(_2\)D was also significantly positive, 21.5 pmol/kg (95% CI = 16.95-25.98; p<0.001), suggesting that the colon tissue 1,25(OH)\(_2\)D response could not be attributable to serum 1,25(OH)\(_2\)D alone.

Serum 1,25(OH)\(_2\)D levels did not exceed the upper limit of the normal reference range (200 pmol/L) in any patient. On average, colon 1,25(OH)\(_2\)D levels were 50% lower than serum 1,25(OH)\(_2\)D concentrations (p<0.001) (Table 6.3). DBP levels in colon were deemed negligible.
Table 6.1. Recovery of $1,25(\text{OH})_2\text{D}$ achieved with tissue extraction procedure followed by confirmatory quantification methods.

<table>
<thead>
<tr>
<th>Matrix</th>
<th>n</th>
<th>Added $1,25(\text{OH})_2\text{D}$</th>
<th>$1,25(\text{OH})_2\text{D}$ quantification method</th>
<th>$1,25(\text{OH})_2\text{D}$ recovery (% ± CV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine muscle tissue</td>
<td>10</td>
<td>None (ethanol vehicle)</td>
<td>EIA</td>
<td>Not detected</td>
</tr>
<tr>
<td>Bovine muscle tissue</td>
<td>10</td>
<td>$[^3]H1\alpha,25(\text{OH})_2\text{D}_3$</td>
<td>Liquid scintillation radioactivity analysis</td>
<td>90.5 ± 3.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(17,940 cpm/mL)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bovine muscle tissue</td>
<td>10</td>
<td>$1\alpha,25(\text{OH})_2\text{D}_3$</td>
<td>HPLC</td>
<td>104.9 ± 4.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(5 g/mL)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bovine muscle tissue</td>
<td>10</td>
<td>Pooled human serum $[1,25(\text{OH})_2\text{D}: \sim 163 \text{ pmol/L}]$</td>
<td>EIA</td>
<td>101.9 ± 3.4</td>
</tr>
<tr>
<td>Serum*</td>
<td>10</td>
<td>None</td>
<td>EIA</td>
<td>103.1 ± 10.3</td>
</tr>
</tbody>
</table>

*Serum samples were extracted with described tissue extraction method, reconstituted in stripped serum, and quantified by enzyme immunoassay (EIA). Results were compared to those obtained using the same serum sample assayed neat (i.e. no tissue extraction step) by EIA. CV: coefficient of variation.
Table 6.2. Precision of 1,25(OH)₂D assay.

<table>
<thead>
<tr>
<th>Matrix</th>
<th>n</th>
<th>Mean 1,25(OH)₂D (pmol/L)</th>
<th>Within-assay</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>SD</td>
</tr>
<tr>
<td>Bovine muscle tissue</td>
<td>10</td>
<td>Not detected</td>
<td>N/A</td>
</tr>
<tr>
<td>Stripped serum</td>
<td>10</td>
<td>Not detected</td>
<td>N/A</td>
</tr>
<tr>
<td>Colon 1</td>
<td>2</td>
<td>24.5</td>
<td>2.1</td>
</tr>
<tr>
<td>Colon 2</td>
<td>2</td>
<td>28.5</td>
<td>2.1</td>
</tr>
<tr>
<td>Colon 3</td>
<td>2</td>
<td>43.8</td>
<td>1.8</td>
</tr>
<tr>
<td>Serum 1 (neat)</td>
<td>3</td>
<td>44.0</td>
<td>3.6</td>
</tr>
<tr>
<td>Serum 2 (neat)</td>
<td>3</td>
<td>104.3</td>
<td>2.1</td>
</tr>
<tr>
<td>Serum 3 (neat)</td>
<td>3</td>
<td>162.3</td>
<td>9.3</td>
</tr>
</tbody>
</table>

SD: standard deviation; CV: coefficient of variation; N/A: not applicable.
Figure 6.1. Correlation of human colon tissue and serum concentrations of 1,25(OH)\(_2\)D.
Table 6.3. Vitamin D metabolite and DBP measurements in human colon tissue and serum.

<table>
<thead>
<tr>
<th>Matrix</th>
<th>1,25(OH)$_2$D pmol/kg</th>
<th>DBP nmol/kg</th>
<th>Free 1,25(OH)$_2$D index (x 10$^5$)</th>
<th>25(OH)D nmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colon</td>
<td>28 ± 8</td>
<td>1194 ± 507</td>
<td>2.4 ± 1.0</td>
<td>Not measured</td>
</tr>
<tr>
<td>Serum</td>
<td>63 ± 41</td>
<td>4929 ± 821</td>
<td>1.2 ± 0.4</td>
<td>62 ± 26</td>
</tr>
<tr>
<td>P-value (colon vs. serum)</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.006</td>
<td>N/A</td>
</tr>
</tbody>
</table>

*Mean of 5 colon mucosa and 8 colon muscularis samples. DBP: vitamin D binding protein.
as measured values were below or near the concentration of the lowest calibrator (1717 nmol/L), substantially below the normal reference range of serum DBP (3903-10733 nmol/L), and significantly lower than serum DBP concentrations ($p<0.001$) (Table 3). Furthermore, colon 1,25(OH)$_2$D levels did not correlate significantly with DBP concentrations separately in colon mucosa ($r=0.63$, $p=0.37$), colon muscularis ($r=-0.50$, $p=0.39$) or both ($r=0.51$, $p=0.16$). Colon 1,25(OH)$_2$D did not correlate significantly with serum 25(OH)D ($r=0.09$, $p=0.66$) or serum DBP ($r=-0.14$, $p=0.71$). The free 1,25(OH)$_2$D index was significantly higher in colon tissue (2.4 ± 1.0) compared to serum (1.2 ± 0.4) ($p=0.006$).
6.5 Discussion

Evaluations of vitamin D with various health outcomes including cancer have been investigated predominantly in the context of serum levels of vitamin D metabolites. However, these outcomes have not been associated with tissue levels of vitamin D metabolites. Intracellular concentrations of vitamin D may indeed be more important than serum levels, particularly given the multitude of cells that differentially express the genes that activate (i.e. CYP27B1) and respond to (i.e. VDR) vitamin D [24, 76, 224, 304, 305]. Thus, it has been hypothesized that it is the local accumulation of 1,25(OH)\(_2\)D in tissues, not serum per se, that drives the biological effects of vitamin D in cell growth regulation and immunomodulation [2, 215, 306-308]. However, the direct measurement of 1,25(OH)\(_2\)D in human tissue has not been reported. We have developed a novel and robust method to measure 1,25(OH)\(_2\)D concentration in human colon tissue.

Our results describe for the first time that the 1,25(OH)\(_2\)D hormone can be detected in human colon tissue at physiologically relevant concentrations partly determined by serum 1,25(OH)\(_2\)D. Colon tissue 1,25(OH)\(_2\)D levels were ~50% of those in serum, and correlated significantly with serum 1,25(OH)\(_2\)D as would be expected for a hormone and its target tissue. Although the precise origin of the colonic 1,25(OH)\(_2\)D (i.e. local synthesis vs. tissue uptake) is difficult to ascertain without the administration of radio-labeled agent, the data also provided evidence of some degree of local 1,25(OH)\(_2\)D production within colon tissue in vivo. Had the 1,25(OH)\(_2\)D detected in colon been simply the result of serum deposition, then one would also expect to detect physiological levels of DBP, the carrier protein of circulating vitamin D metabolites, in the colon. However, colon DBP levels (1194 ± 507 nmol/kg) were negligible as these values were below or near the lower limit of assay sensitivity and far below the normal
reference range of serum DBP (3903-10733 nmol/L). Specifically, DBP levels were four-fold lower in colon than serum, and could not account for the two-fold difference in 1,25(OH)\(_2\)D concentrations observed between colon and serum. Furthermore, the free 1,25(OH)\(_2\)D index was twice as high in colon (2.4 ± 1.0) compared to serum (1.2 ± 0.4), suggesting increased accessibility and physiological activity of 1,25(OH)\(_2\)D hormone at the tissue level. This was an important finding as it shows a higher accumulation of active agent at the target organ compared to the circulation. Another indication of local, paracrine production of 1,25(OH)\(_2\)D in tissue was the significantly positive intercept of colon 1,25(OH)\(_2\)D (21.5 pmol/kg) at zero serum 1,25(OH)\(_2\)D, implying a basal level of 1,25(OH)\(_2\)D production in the colon independent of serum 1,25(OH)\(_2\)D. Although serum 25(OH)D was not associated with colon 1,25(OH)\(_2\)D, the low serum 25(OH)D levels (62 nmol/L) in this patient population may have been insufficient to produce detectable amounts of 1,25(OH)\(_2\)D in colon tissue. Only three patients had serum 25(OH)D concentrations greater than 100 nmol/L, the level which is believed to be optimal for extra-renal metabolism and cancer prevention [120, 302]. Of note, we have recently completed a Phase II clinical trial of high-dose vitamin D\(_3\) administration to prostate cancer patients (NCT00741364), which will allow us to address such issues in the context of intra-prostate vitamin D metabolism.

The data also demonstrated that our method for measuring 1,25(OH)\(_2\)D in tissue is accurate and precise. Despite having developed our protocol independently from Nittke et al [309], both methods are similar in principle in that they employ a double extraction procedure coupled to EIA. However, the recovery of 1,25(OH)\(_2\)D achieved by the present method (100.1 ± 6.5 %) was higher than that of Nittke et al (85 %) and was confirmed by several quantification platforms. The higher recovery could be attributed to differences in the lipid extraction step and
the present use of 1,25(OH)$_2$D-stripped serum to reconstitute the tissue extract prior to EIA. Future work should optimize the tissue 1,25(OH)$_2$D method to apply to other types of tissues and vitamin D metabolites.

This study had some limitations. The small quantity of available colon tissue (~300 mg) necessitated the pooling of colonic tissue regions and restricted the analyses to 1,25(OH)$_2$D and DBP. Therefore, the 1,25(OH)$_2$D or other vitamin D metabolites (e.g. 25(OH)D) could not be measured separately in colon mucosa and muscularis regions. Regional differences in vitamin D metabolism across various tissue types should be explored in future studies, and will be investigated in our clinical trial of vitamin D$_3$ in prostate cancer (NCT00741364).

Higher risk of colon cancer has been consistently associated with lower levels of serum 25(OH)D [9, 300-303]. A plausible explanation for this relationship is the inadequate local colonic conversion of inactive circulating 25(OH)D into active 1,25(OH)$_2$D as the colon expresses the vitamin D-activating enzyme CYP27B1. However, direct evidence for this hypothesis has been lacking because there has been no reported method to quantify 1,25(OH)$_2$D concentration in human colon. The present measurement of tissue 1,25(OH)$_2$D shows that this hormone can be found in human colon at physiologically relevant concentrations partly determined by serum 1,25(OH)$_2$D, and also provides preliminary evidence of local synthesis of 1,25(OH)$_2$D within colon tissue in vivo. The colon 1,25(OH)$_2$D method described here will enable researchers to quantify vitamin D metabolites in accessible human tissue (e.g. surgical specimens) and facilitate an enhanced understanding of vitamin D metabolism at the tissue level. Such a tool should stimulate research examining the relationship between tissue vitamin D metabolites and the various health outcomes that are believed to be linked with vitamin D intakes.
CHAPTER 7: Study 4 – Randomized clinical trial of cholecalciferol doses on tissue vitamin D metabolite levels and Ki67 immunohistochemistry at radical prostatectomy in prostate cancer patients

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Dennis Wagner, Dominique Trudel, Theodorus Van der Kwast, Larisa Nonn, Angeline Antonio Giangreco, Doris Li, Andre Dias, Monique Cardoza, Sanda Laszlo, Karen Hersey, Laurence Klotz, Antonio Finelli, Neil Fleshner, Reinhold Vieth.

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7.1 Abstract

A chemopreventive strategy based on higher cholecalciferol (vitamin D₃) doses is supported by preclinical evidence that prostate cells can locally synthesize the active hormone calcitriol. To determine the effects of oral cholecalciferol on calcitriol levels in human prostate and on PCa pathology, PCa patients (n=66) were randomized to daily cholecalciferol (400, 10,000, or 40,000 IU/d) for 3-8 wk prior to radical prostatectomy. Prostate and serum were assayed for vitamin D metabolites. Ki67 protein expression in prostate tissue was assessed by quantitative digital immunohistochemistry. Secondary outcomes included vitamin D metabolism gene expression, serum parathyroid hormone (PTH) and prostate specific antigen (PSA). Prostate tissue calcitriol and the free calcitriol index increased dose-dependently (p<0.02). Both measures of prostate calcitriol differed between the 400 and 40,000 IU dose groups (p<0.04). Prostate vitamin D metabolites correlated positively with serum levels (p<0.0001) and with mRNA expression of their production enzymes in cancer tissue (p<0.04). Ki67 measures did not differ significantly among vitamin D dose groups. However, the level of calcitriol attained in prostate tissue was inversely associated with Ki67 intensity, Ki67 histoscore and Ki67 (3+) % positive nuclei in PCa and benign tissue (p<0.05). Safety measures, including plasma and urine calcium, did not change adversely with dosing. Compared to the 400 IU/d group, serum PTH and PSA were lower in the combined higher-dose groups at end-of-study (p<0.02). We provide clinical trial evidence that prostatic vitamin D metabolism can be modulated in vivo by oral consumption of the nutrient, cholecalciferol. Higher prostate calcitriol and vitamin D doses also showed suggestion of clinical benefit, including lowered Ki67 expression and modest reductions in serum PSA and PTH.
7.2 Introduction

The circulating concentration of the hormone precursor, 25-hydroxyvitamin D (25(OH)D, calcidiol), is the direct result of both oral intake of vitamin D₃ (cholecalciferol) and skin exposure to ultraviolet light. Risk of prostate cancer (PCa) is lower in men with a life history of greater sun exposure [312]. Furthermore, survival rates of breast, colorectal, and PCa are 25% higher if they are diagnosed and treated in summer compared to winter [23]. There is also seasonality in the rate of rise in prostate specific antigen (PSA) in patients monitored for untreated low-grade PCa, with the slowest rate of increase during spring-summer [212]. Consistent with that, the rate of rise in PSA in men being evaluated for failed PCa surgery or radiation appears to slow down with cholecalciferol supplementation [29].

Virtually all previous clinical trials addressing the relevance of "vitamin D" in the context of PCa have used the active metabolite calcitriol (1,25-dihydroxyvitamin D; 1,25(OH)₂D₃) or its analogs [241, 242, 246, 313-315]. The disadvantage of the systemic administration of calcitriol or its analogs as a chemotherapeutic agent is that supraphysiological doses are required that rely on a spill-over of the active agent from blood into prostate tissue [241, 242]. Calcitriol and its analogs have narrow margins of safety, with high risk of hypercalcemia, and since these agents have short half-lives, they are present at target tissues only intermittently if given at the commonly used weekly dosing interval [56]. In contrast, the inactive precursor cholecalciferol has a long half-life, a wide margin of safety [274], and will generate a steady, sustained therapeutic action.

A clinically more effective strategy designed to achieve higher serum 25(OH)D concentrations is supported by reports that physiologic concentrations of 25(OH)D exhibit anti-
proliferative effects on prostate cells to a degree equivalent to a 100-fold superphysiologic excess of calcitriol [24, 27, 316]. Furthermore, the traditional, liver-and-kidney model for calcitriol synthesis does not apply to the prostate, because prostate cells possess both of the enzymes (i.e. 25-hydroxylases: CYP27A1 (mitochondrial) and CYP2R1 (microsomal), 1α-hydroxylase: CYP27B1) needed to convert cholecalciferol to the active paracrine hormone, calcitriol [225]. Moreover, physiological levels of cholecalciferol have been reported to inhibit growth, induce differentiation of prostate epithelial cells, and to up-regulate among others, the androgen receptor [26, 225]. Therefore, we hypothesized that cholecalciferol will increase intraprostate calcitriol concentration and affect prostate biology.

Direct measurement of vitamin D metabolites in human prostate tissue has not been reported and vitamin D metabolism within the prostate remains poorly understood. Furthermore, the effects of oral cholecalciferol dosing on prostate cell proliferation in clinical samples have not been evaluated in a clinical trial. Recently, we developed and validated a novel method to measure calcitriol concentration in human tissues [32]. Here, we describe the results from our randomized clinical trial on the tissue effects of various doses of cholecalciferol given orally to PCA patients scheduled to undergo radical prostatectomy. The primary objectives were to characterize the clinical effects of vitamin D dosing on prostate tissue levels of vitamin D metabolites and on prostate cell proliferation assessed by Ki67 immunochemistry. Secondary outcomes included mRNA expression of vitamin D-metabolism genes in prostate tissue, safety-related measures and serum PSA and parathyroid hormone (PTH) responses.
7.3 Materials and Methods

The study protocol was approved by the Research Ethics Boards of the University of Toronto, Mount Sinai Hospital, University Health Network (UHN), and Sunnybrook Health Sciences Centre. All patients signed a form indicating their informed consent. The trial was registered with [www.clinicaltrials.gov](http://www.clinicaltrials.gov) (Trial Registration ID: NCT00741364).

Patients

Patients with localized PCa scheduled to undergo radical prostatectomy were recruited from the urologic oncology clinics of Princess Margaret Hospital (at UHN) and Sunnybrook Health Sciences Centre. Patients were considered eligible for the study if they met the following criteria: diagnosis of Gleason score 6-7 adenocarcinoma of the prostate biopsy, plasma calcium < 2.62 mmol/L, urine calcium (mmol/L) to creatinine (mmol/L) ratio < 1.0, plasma phosphate < 1.40 mmol/L, serum PTH < 6.9 pmol/L, plasma creatinine < 200 mol/L, plasma ALT < 40 U/L, and plasma ALP < 150 U/L. Patients were excluded for: previous treatment of prostate carcinoma or benign prostatic hyperplasia (e.g. 5 alpha reductase inhibitors), regular use of vitamin D supplementation in excess of 2,000 IU/d, significant sunlight exposure during study (e.g. travel to a sunny/tropical destination without regularly using sunscreen), duration for intervention < 3 wk, and history of hypercalcemia, sarcoidosis, or urolithiasis. Consented patients that could not participate in the study due to insufficient duration for intervention (<3 wk) but otherwise met all other eligibility criteria, were enrolled (without randomization) into the control (untreated) arm of the study.
Study design

Our study was a multicenter, double-blind, randomized controlled trial of vitamin D in PCa patients. Eligible patients were randomly allocated to 1 of 3 vitamin D₃ (cholecalciferol) doses: 1) 400 IU (10 μg), 2) 10,000 IU (250 μg), and 3) 40,000 IU (1000 μg). Patients in the control (non-randomized) arm of the study did not receive any supplemental vitamin D (0 IU). Treated patients consumed the vitamin D orally, once per day, for a 3-8 week period ending the day prior to radical prostatectomy. Up to an additional 2 weeks of treatment were permitted if surgery was delayed for unrelated reasons. Overall, the protocol was ‘incidental’ and did not affect or delay primary treatment in any way. Analysis was intention-to-treat and involved all patients randomized to groups.

Materials

Liquid vitamin D doses were prepared by dissolving crystalline US Pharmacopoeia (USP)-grade cholecalciferol (Sigma, St Louis, USA) into USP-grade ethanol (Commercial Alcohols Inc., Brampton, Canada) as described previously [258]. Quality control was performed by spectroscopy and also independently by a licensed pharmaceutical testing laboratory using USP method 31. Vitamin D concentrations were tested bimonthly and remained unchanged. Vitamin D doses were physically identical, unidentifiable, and consumed daily by each patient by mixing 1 mL of the ethanolic solution into juice or water prior to drinking it.

Assessments and sampling

Treated patients were evaluated during a screening (baseline) clinic visit, biweekly follow-up assessments, and on the day of surgery prior to entering the operating room (i.e. final visit).
At baseline, the following information was obtained: eligibility criteria, use of concomitant therapies (medications, supplements, or herbal preparations), and anthropometrics (age, height, weight, ethnicity, and Gleason score). During intervention, patients were interviewed biweekly via telephone to record any adverse events or changes in concomitant therapies since the previous assessment. Venous blood and urine samples were collected from each treated patient at the baseline and final visits for biochemical testing. Serum aliquots were stored at -80°C until analyses. A urine sample was provided 2 weeks after the start of intervention for additional safety monitoring. Compliance was monitored by measuring the remaining volume of liquid in the vials returned at the final visit.

Untreated control patients were all evaluated on the day of surgery and some had also attended a previous screening visit. Venous blood and urine samples were obtained from at least one of these visits for biochemical testing. Day of surgery (i.e. final visit) assessments are presented for untreated control patients.

Upon radical prostatectomy, the resected prostate specimen was weighed, measured, and inked to indicate margins. Fresh tissue (100-500 mg) from the peripheral zone (PZ) and the transition zone (TZ) of prostate was then excised by a pathologist during gross examination and stored at -80°C until analyses. The remaining prostate specimen was then fixed in formalin for > 48 hours and submitted in toto for paraffin embedding and sectioning as per routine pathology practice. The formalin-fixed paraffin-embedded (FFPE) prostate blocks were stored at ambient temperature to be used for gene expression and immunohistochemical analyses.
**Outcome measures**

One primary outcome was the serum and prostate tissue levels of vitamin D metabolites attained in the patients after oral vitamin D₃ administration. These metabolites included: 25-hydroxyvitamin D₃ [25(OH)D₃], calcitriol [1,25-dihydroxyvitamin D, 1,25(OH)₂D], and 24,25-dihydroxyvitamin D₃ [24,25(OH)₂D₃]. The other primary outcome was the expression of the proliferation marker Ki67 (MIB-1) in normal and malignant prostate cells, as measured by immunohistochemical staining. The key safety indicator was urinary calcium excretion (ratio of millimolar concentrations of urine calcium and urine creatinine), 2 weeks into vitamin D treatment. Other safety-related measures were plasma levels of calcium, phosphate, creatinine, alanine transaminase (ALT), and alkaline phosphatase (ALP), measured at the baseline and final visits. Secondary outcomes included the mRNA expression of genes involved in vitamin D metabolism (i.e. CYP27A1: mitochondrial 25-hydroxylase, CYP2R1: microsomal 25-hydroxylase, CYP27B1: 1α-hydroxylase and CYP24A1: 24-hydroxylase) and the VDR, measured from laser-capture microdissected (LCM) FFPE prostate tissue. The effects of vitamin D supplementation on serum PSA and PTH were also evaluated.

**Analytical methods**

Serum 25(OH)D₃ and 24,25(OH)₂D₃ were measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS) [317]. Serum calcitriol was determined by enzyme immunoassay (EIA) (Immunodiagnostic Systems, AZ, USA). Prostate vitamin D concentrations were measured separately in prostate PZ, the region surrounding the tumour, and in prostate TZ, which envelops the PZ. Measurement of vitamin D metabolites in prostate involved an initial tissue pre-extraction procedure reported elsewhere by our group [32]. In the tissue extracts,
calcitriol was measured by EIA, while 25(OH)D₃ and 24,25(OH)₂D₃ were quantified by LC-MS/MS.

Vitamin D binding protein (DBP) was measured in a subset of patient serum (n=41) and prostate TZ homogenates (n=37, 100 mg) by ELISA (Immunodiagnostik, Bensheim, Germany). Measures of “free” 25(OH)D₃, calcitriol, and 24,25(OH)₂D₃ were calculated as the ratio between the molar concentrations of the respective vitamin D metabolite and DBP [32]. Calcium, creatinine, phosphate, ALT, ALP, PSA and PTH were measured by routine hospital laboratory methods.

Validation of prostate tissue vitamin D metabolite assay

The prostate tissue vitamin D metabolite assays were evaluated as previously reported for colon calcitriol [32]. In bovine tissue free of endogenous vitamin D, mean recoveries (% ± CV) of exogenously-added 25(OH)D₃, calcitriol and 24,25(OH)₂D₃ were 94.4 ± 6.3, 95.8 ± 8.3, and 91.5 ± 14.9, respectively. A subset of prostate (n=8) and serum (n=14) samples was assayed in duplicate to determine method precision. Total within-run precision (%CV) of 25(OH)D₃, calcitriol and 24,25(OH)₂D₃ measurement were similar in prostate tissue (13.0%, 11.1%, and 14.3%, respectively) and serum (7.0%, 10.2%, and 9.4%, respectively).

Ki67 immunochemistry and digital immunoscopying

Tissue microarrays (TMAs) were created from pathologist-annotated FFPE blocks from each of the study cases. Three normal and 3 PCa cores (1 mm diameter) were extracted from each case. TMAs were then sectioned and immunostained for a polyclonal antibody (1:1000 dilution) specific for Ki67 protein (Novus Biologicals, Littleton, CO). TMA slides were scanned
onto a computer using Aperio scanner and segmented using TMAlab. Nine random zones were delimitated and analyzed by a trained pathologist. Parameters of nuclear algorithm v9 were adjusted until most results were within 35% of variation. Genie tissue pattern recognition software (Aperio, Vista, CA) was then trained to recognize glands. The classifier was accepted when sensibility was over 90% and was merged with the adjusted nuclear algorithm. Genie analysis was conducted on 32 whole cores (24 malignant cores) and correlated with results of the adjusted nuclear algorithm on manually annotated cores. Further analysis was conducted by Genie software, either on whole core or on selected zones of the core if tissues other than tissue of interest were recognized by Genie; cores with 500 or less nuclei as recognized by Genie analysis were rejected. Each core was analyzed individually by a trained pathologist.

The correlation between the evaluation of Ki67 staining in PCa by a pathologist compared to the adjusted nuclear algorithm v9 was high ($r^2 = 0.88$). Genie classifier sensitivity and specificity for glands were 94.8% and 63.5%, respectively. Using a threshold of more than 500 nuclei recognized by Genie, analysis on whole core or area of cores containing the tissue of interest correlated well with analysis using adjusted nuclear algorithm ($r^2 = 0.85$). Atrophy and high grade prostatic intraepithelial neoplasia were rejected. After evaluation of each core, 356 cores out of 438 (81.3%) were available for evaluation.

The quantitative digital immunoscopy platform provided the following Ki67 measures, which were used in the analysis: mean % positive nuclei = positive nuclei (1+, 2+, 3+) / total nuclei, intensity score = (1+): weak, (2+): moderate, (3+): intense, histoscore = % positive nuclei * intensity score, mean (3+) % positive nuclei = positive nuclei (3+ only) / total nuclei.
**Vitamin D metabolism gene mRNA expression**

Normal and PCa prostate tissue was extracted from FFPE blocks (pathologist-annotated) by laser-capture microdissection, as described by Nonn et al [318]. Samples were stored overnight at -80°C until RNA was extracted, quantified and reverse-transcribed as previously reported [318]. For the mRNA pre-amplification, specific mRNA TaqMan-designed probes were diluted to 0.2X and pooled for the reaction. A diluted pre-amplified product was used as a template for qRT-PCR with TaqMan assays. Fold-changes were calculated using 2^{-\Delta\Delta CT} method and normalized to the expression of mRNA housekeeping genes (B2M, HPRT, Actin, GAPDH, and/or CK18). Information on the TaqMan gene expression assays we used is displayed in **Supplementary Table S7.1.**

**Statistical analyses**

The randomization sequence was generated using computer software to produce randomly permutated blocks of 6 (i.e. 2 of each dose in each sequence of 6 study ID numbers), stratified by center [Random Allocation Software 1.0 (2004), by M. Saghaei]. All data were analyzed with SPSS software (version 20). Graphs were created with GraphPad Prism 4 for Windows. Within-group changes in biochemical variables over time were analyzed with paired 2-tailed t tests. Between-group differences in biochemical variables were analyzed with 1-way ANOVA followed by Bonferroni post hoc testing. Associations between biochemical measures were examined by means of the Spearman rho or Pearson correlation coefficient. The criterion for significance was set at P < 0.05.
**Supplementary Table S7.1.** TaqMan gene expression assays employed in our study.

<table>
<thead>
<tr>
<th>Assay</th>
<th>Gene</th>
<th>Part number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hs0018742_m1</td>
<td>B2M</td>
<td>4331182</td>
</tr>
<tr>
<td>Hs03929097_g1*</td>
<td>GAPDH</td>
<td>4331182</td>
</tr>
<tr>
<td>Hs00357333_g1</td>
<td>ACTIN</td>
<td>4331182</td>
</tr>
<tr>
<td>Hs00989011_g1</td>
<td>CYP24A1</td>
<td>4331182</td>
</tr>
<tr>
<td>Hs01017992_g1</td>
<td>CYP27A1</td>
<td>4331182</td>
</tr>
<tr>
<td>Hs00168017_m1*</td>
<td>CYP27B1</td>
<td>4331182</td>
</tr>
<tr>
<td>Hs01379776_m1*</td>
<td>CYP2R1</td>
<td>4331182</td>
</tr>
<tr>
<td>Hs01045844_m1</td>
<td>VDR</td>
<td>4448892</td>
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<tr>
<td>Hs01003267_m1</td>
<td>HPRT1</td>
<td>4448892</td>
</tr>
</tbody>
</table>
7.4 Results

Patients

Between October 2008 and July 2011, 94 patients were screened for eligibility and 66 patients were randomized to intervention, while 10 additional patients were enrolled as untreated control subjects, to provide blood samples and prostate tissue (Figure 7.1). Overall, the mean duration of vitamin D₃ treatment was 33.6 ± 9.5 d, without differences among treatment groups (P = 0.53). Compliance to vitamin D treatment was 97%. Four patients withdrew prematurely from the study for reasons unrelated to intervention. Demographics and baseline characteristics of study patients were similar among treatment groups (Table 7.1).

Primary outcomes:

Vitamin D metabolite analyses

Serum 25(OH)D₃ increased significantly from baseline to final in all 3 vitamin D₃-treated groups (P < 0.01) in a dose-dependent manner such that the highest levels were attained in the 40,000 IU/d group (P < 0.05) (Figure 7.2A). Serum calcitriol levels rose significantly with dosing in the 10,000- and 40,000 IU/d groups (P < 0.0001) and the highest serum calcitriol concentration was achieved in the highest dose group (P < 0.0001) (Figure 7.2B). Serum 24,25(OH)₂D₃ increased significantly over time (P < 0.001) in a dose-dependent fashion (P < 0.05) (Figure 7.2C).

Prostate tissue 25(OH)D₃ concentrations (in PZ and TZ) trended upwards with dose [P (linear trend) < 0.0001] and were significantly highest in the 40,000 IU/d group (P < 0.0001) (Figure 7.2D). The calcitriol level in prostate tissue was higher in the 40,000 IU/d group than in
Figure 7.1. CONSORT diagram. Enrollment, randomization, and follow-up of the study patients.
<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Total</th>
<th>400 IU/d</th>
<th>10,000 IU/d</th>
<th>40,000 IU/d</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>66</td>
<td>21</td>
<td>22</td>
<td>23</td>
</tr>
<tr>
<td>Age – y</td>
<td>57.4 ± 6.8</td>
<td>58.9 ± 6.2</td>
<td>55.9 ± 7.3</td>
<td>57.6 ± 6.7</td>
</tr>
<tr>
<td>Ethnicity – no. (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>White</td>
<td>54 (83)</td>
<td>18 (90)</td>
<td>17 (77)</td>
<td>19 (83)</td>
</tr>
<tr>
<td>Black</td>
<td>4 (6)</td>
<td>1 (5)</td>
<td>3 (14)</td>
<td>0</td>
</tr>
<tr>
<td>Other</td>
<td>7 (11)</td>
<td>1 (5)</td>
<td>2 (9)</td>
<td>4 (17)</td>
</tr>
<tr>
<td>BMI – kg·m²</td>
<td>27.8 ± 4.0</td>
<td>27.9 ± 4.5</td>
<td>27.8 ± 3.5</td>
<td>27.8 ± 4.2</td>
</tr>
<tr>
<td>Gleason score – no. (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>33 (52)</td>
<td>10 (50)</td>
<td>9 (42.9)</td>
<td>14 (60.9)</td>
</tr>
<tr>
<td>7</td>
<td>31 (48)</td>
<td>10 (50)</td>
<td>12 (57.1)</td>
<td>9 (39.1)</td>
</tr>
<tr>
<td>Serum PSA – μg/L</td>
<td>6.99 ± 4.56</td>
<td>7.08 ± 4.55</td>
<td>7.02 ± 4.75</td>
<td>6.87 ± 4.59</td>
</tr>
<tr>
<td>Serum 25(OH)D₃ – nmol/L</td>
<td>65.4 ± 23.9</td>
<td>59.4 ± 13.9</td>
<td>65.2 ± 26.5</td>
<td>71.4 ± 27.7</td>
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<tr>
<td>&lt;100 nmol/L – no. (%)</td>
<td>50 (91)</td>
<td>17 (100)</td>
<td>18 (90)</td>
<td>15 (83)</td>
</tr>
<tr>
<td>&lt;75 nmol/L – no. (%)</td>
<td>39 (71)</td>
<td>15 (88)</td>
<td>14 (70)</td>
<td>10 (56)</td>
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<tr>
<td>&lt;50 nmol/L – no. (%)</td>
<td>13 (24)</td>
<td>4 (23)</td>
<td>5 (25)</td>
<td>4 (22)</td>
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<td>&lt;25 nmol/L – no. (%)</td>
<td>2 (4)</td>
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<td>2 (10)</td>
<td>0</td>
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<tr>
<td>Serum calcitriol – pmol/L</td>
<td>103.3 ± 33.7</td>
<td>108.3 ± 38.6</td>
<td>108.4 ± 33.9</td>
<td>94.2 ± 28.3</td>
</tr>
<tr>
<td>Serum 24,25(OH)₂D₃ – nmol/L</td>
<td>14.7 ± 6.6</td>
<td>11.4 ± 3.5</td>
<td>14.8 ± 7.1</td>
<td>17.6 ± 7.2</td>
</tr>
<tr>
<td>Serum DBP – nmol/L</td>
<td>7561.7 ± 1328.2</td>
<td>7332.9 ± 1506.5</td>
<td>7664.5 ± 1355.2</td>
<td>7657.6 ± 1227.8</td>
</tr>
<tr>
<td>Serum free 25(OH)D₃ index³ x 10⁵</td>
<td>8.59 ± 3.73</td>
<td>8.13 ± 2.78</td>
<td>8.87 ± 4.93</td>
<td>8.71 ± 3.24</td>
</tr>
<tr>
<td>Serum free calcitriol index³ x 10⁵</td>
<td>1.34 ± 0.42</td>
<td>1.45 ± 0.32</td>
<td>1.51 ± 0.42</td>
<td>1.06 ± 0.38</td>
</tr>
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<td>Serum free 24,25(OH)₂D₃ index³ – x 10⁵</td>
<td>1.88 ± 0.98</td>
<td>1.50 ± 0.61</td>
<td>2.09 ± 1.39</td>
<td>2.01 ± 0.63</td>
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<tr>
<td>Plasma Calcium – mmol/L</td>
<td>2.37 ± 0.08</td>
<td>2.38 ± 0.09</td>
<td>2.39 ± 0.07</td>
<td>2.36 ± 0.08</td>
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<tr>
<td>Urine Calcium – mmol/L</td>
<td>2.91 ± 2.29</td>
<td>2.83 ± 2.41</td>
<td>3.09 ± 2.64</td>
<td>2.81 ± 1.89</td>
</tr>
<tr>
<td>Plasma Creatinine – mol/L</td>
<td>83.4 ± 13.0</td>
<td>88.2 ± 14.6</td>
<td>82.4 ± 13.9</td>
<td>79.8 ± 9.3</td>
</tr>
<tr>
<td>Urine Creatinine – mol/L</td>
<td>12258.1 ± 6287.5</td>
<td>13590.6 ± 8133.7</td>
<td>11982.4 ± 6266.3</td>
<td>11261.8 ± 3913.2</td>
</tr>
<tr>
<td>Urine Calcium:Creatinine – ratio</td>
<td>0.25 ± 0.16</td>
<td>0.24 ± 0.16</td>
<td>0.25 ± 0.17</td>
<td>0.26 ± 0.16</td>
</tr>
<tr>
<td>Plasma Phosphate – mmol/L</td>
<td>1.03 ± 0.16</td>
<td>1.03 ± 0.14</td>
<td>1.06 ± 0.18</td>
<td>1.01 ± 0.17</td>
</tr>
<tr>
<td>Serum PTH – pmol/L</td>
<td>4.65 ± 1.33</td>
<td>5.11 ± 1.37</td>
<td>4.66 ± 1.64</td>
<td>4.23 ± 0.80</td>
</tr>
<tr>
<td>Plasma ALT – U/L</td>
<td>28.7 ± 10.8</td>
<td>25.0 ± 8.8</td>
<td>30.7 ± 12.5</td>
<td>30.1 ± 10.1</td>
</tr>
<tr>
<td>Plasma ALP – U/L</td>
<td>74.9 ± 18.3</td>
<td>73.4 ± 12.6</td>
<td>70.4 ± 13.2</td>
<td>80.6 ± 25.0</td>
</tr>
</tbody>
</table>

a does not include untreated control patients (N = 10) for which only vitamin D metabolites and DBP were measured at the final visit; these patients had similar demographic characteristics as treated patients.

bBaseline values did not differ among treatment groups, P > 0.05.

cmean ± SD, where applicable.

dcalculated as the ratio between the molar concentrations of the vitamin D metabolite and DBP.
Figure 7.2. Serum (left) and prostate (right) responses of 25(OH)D$_3$ (A, D), calcitriol (B, E), and 24,25(OH)$_2$D$_3$ (C, F) metabolites to vitamin D$_3$ dosing. Values are means ± SE. Means without a common letter differ significantly between groups (P < 0.05). *indicates a significant difference from baseline to final within groups (P < 0.05). PZ: peripheral zone, TZ: transition zone.
every other dose group (TZ: P = 0.01, PZ: P = 0.08) (Figure 7.2E).  Likewise, in both prostate TZ and PZ, 24,25(OH)_{2}D_{3} concentrations trended upwards with dose (P < 0.001) (Figure 7.2F) and were higher in the 40,000 IU/d group than in any other group (P < 0.001).  Of note, vitamin D metabolite measures in serum and prostate tissue did not differ significantly (P > 0.30) between the 400 IU/d group and the untreated controls (0 IU/d).  Furthermore, prostate tissue 25(OH)D_{3}, calcitriol and 24,25(OH)_{2}D_{3} concentrations did not differ significantly between PZ and TZ regions (P > 0.05) in any dose group.  Therefore, to improve precision for subsequent analyses, we used the average of the PZ and TZ metabolite concentration values.

Serum levels of 25(OH)D_{3}, calcitriol, and 24,25(OH)_{2}D_{3} were strongly correlated with the average prostate PZ and TZ readings (r = 0.83, P < 0.0001; r = 0.73, P < 0.0001; r = 0.51, P < 0.0001, respectively) (Figure 7.3).  The relationships between serum and prostate metabolite levels were best characterized by non-linear (quadratic) regression, all of them showing significantly positive intercepts at zero values of serum 25(OH)D_{3} (95% CI, 56.9-99.1 nmol/kg), calcitriol (7.9-51.0 pmol/kg), and 24,25(OH)_{2}D_{3} (12.4-19.9 nmol/kg) (P < 0.05).  Based on multiple regression analysis, 43% of the variability in prostate calcitriol was attributable to the serum calcitriol concentration (standardized beta coefficient: 0.55, P = 0.001) and to CYP27B1 mRNA (see below) within the PCa regions (standardized beta coefficient: 0.30, P = 0.04).  Furthermore, 25(OH)D_{3} levels in serum and in prostate were positively correlated with prostate calcitriol (r = 0.44, P = 0.001; r = 0.33, P = 0.017; respectively) (Figure 7.3D).

Since it could be argued that vitamin D metabolites detected within the prostate were an artifact of residual blood within the tissue, we measured the extracellular transport protein for vitamin D in the plasma, vitamin D-binding protein (DBP), in homogenates of prostate tissue
Figure 7.3. Relationships between serum and mean prostate levels of 25(OH)D$_3$ (A), calcitriol (B), and 24,25(OH)$_2$D$_3$ (C), as well as relationship between serum 25(OH)D$_3$ and prostate calcitriol (D). Line of best fit (A-C: quadratic function, D: linear) with 95% confidence intervals shown in red (n=52).
Supplementary Figure S7.1. Serum and prostate responses of DBP (A) and free 25(OH)D₃ (B), free calcitriol (C), and free 24,25(OH)₂D₃ (D) indices to vitamin D₃ dosing. Values are means ± SE. Means without a common letter differ significantly between groups (P < 0.05). *indicates a significant difference from baseline to final within groups (P < 0.05).
used for the metabolite assays. Serum DBP did not change significantly as a result of the intervention in any of the dose groups (P > 0.05), nor did DBP differ among study groups (serum: P = 0.92; prostate: P = 0.71) (Supplementary Figure S7.1A). In the prostate tissue, average DBP concentrations were 890.9 ± 207.7 nmol/kg, which was much lower than serum levels, 7029.4 ± 1108.3 nmol/L (P < 0.001). The free 25(OH)D₃ index [ratio of 25(OH)D₃ to DBP] increased dose-dependently in both serum (P < 0.0001) and prostate tissue to levels that were highest in the 40,000 IU/d group (P < 0.0001) (Supplementary Figure S7.1B). Similarly, the prostate tissue free calcitriol index increased dose-dependently (P = 0.013 for linear trend).

Although both prostatic free and total calcitriol of the 10,000 IU group were not significantly different from the other groups, both of these variables differed significantly between the 400 and 40,000 IU dose groups (free calcitriol, P = 0.024; total calcitriol, P = 0.036) (Supplementary Figure S7.1C). The free index of every vitamin D metabolite was substantially higher in prostate than in serum (P < 0.0001), showing that the metabolites measured in the prostate were indeed distinct to prostate tissue.

**Ki67 proliferation measures**

To assess the validity of the digital immunohistochemistry data, we compared the various Ki67 proliferation measures between tissue regions (PCa vs. benign) and Gleason scores. As expected, Ki67 % positive nuclei (PCa: 0.67%, N: 0.26%, P < 0.0001), Ki67 histoscore (PCa: 1.36, N: 0.56, P < 0.0001) and Ki67 (3+) % positive nuclei (PCa: 0.20%, N: 0.10%, P = 0.002) were significantly higher in PCa compared to benign tissue. Comparing Gleason Scores, Ki67 % positive nuclei (Gleason 6: 0.58%, Gleason 7: 0.88%, P = 0.027), Ki67 histoscore (Gleason 6: 1.13, Gleason 7: 1.89, P = 0.045) and Ki67 (3+) % positive nuclei (Gleason 6: 0.16%, Gleason 7:
0.29%, \( P = 0.047 \)) in PCa were significantly higher among patients with Gleason score 7 than those with Gleason score 6. This indicates that measures of proliferation were higher in tissue and patients with more advanced disease, as would be expected. Lastly, all Ki67 proliferation measures correlated with each other within PCa and benign tissue (\( P < 0.02 \), for each comparison between Ki67 % positive nuclei, Ki67 intensity, Ki67 histoscore and Ki67 (3+) % positive nuclei).

In PCa and benign tissue, Ki67 % positive nuclei, Ki67 intensity, Ki67 histoscore and Ki67 (3+) % positive nuclei did not differ significantly among vitamin D dose groups. However, linear regression analyses by vitamin D metabolite level demonstrated inverse correlations between the prostate level of calcitriol and Ki67 intensity, Ki67 histoscore and Ki67 (3+) % positive nuclei in PCa and benign tissue (\( P < 0.05 \)) (Table 7.2). Apart from calcitriol, no other vitamin D metabolite, in serum or prostate, was significantly related to any of the Ki67 proliferation measures. The relationships between prostate calcitriol and Ki67 intensity, Ki67 histoscore and Ki67 (3+) % positive nuclei exhibited an asymptotic-like relationship, suggesting a threshold effect. Upon stratifying by the attained prostate calcitriol level, patients at the highest quartile of prostate calcitriol (\( \geq 37 \) pmol/kg) showed significantly lower Ki67 intensity (PCa: \( P = 0.048 \), Figure 7.4A) and Ki67 (3+) % positive nuclei (PCa: \( P = 0.01 \), B: \( P = 0.049 \)) (Figure 7.4B, C) compared to those with lower levels.

Secondary outcomes:

Expression of the vitamin D-metabolism genes and VDR

The mRNA expression of vitamin D metabolism genes and VDR, measured in laser capture-microdissected PCa and normal prostate epithelium, were not significantly different
### Table 7.2. Correlations between vitamin D metabolite levels and Ki67 measures in prostate cancer (PCa) and benign normal (N) tissue

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Tissue Type</th>
<th>Ki67 % positive nuclei</th>
<th>Ki67 intensity</th>
<th>Ki67 histoscore</th>
<th>Ki67 % (3+) positive nuclei</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prostate calcitriol (pmol/kg)</td>
<td>PCa:</td>
<td>rho = -0.13, p = 0.44</td>
<td>rho = -0.38, p = 0.02</td>
<td>rho = -0.21, p = 0.18</td>
<td>rho = -0.41, p = 0.01</td>
</tr>
<tr>
<td></td>
<td>N:</td>
<td>rho = -0.26, p = 0.08</td>
<td>rho = -0.35, p = 0.02</td>
<td>rho = -0.29, p = 0.048</td>
<td>rho = -0.30, p = 0.04</td>
</tr>
<tr>
<td>Serum calcitriol (pmol/L)</td>
<td>PCa:</td>
<td>rho = -0.02, p = 0.89</td>
<td>rho = -0.30, p = 0.02</td>
<td>rho = -0.10, p = 0.45</td>
<td>rho = -0.26, p = 0.06</td>
</tr>
<tr>
<td></td>
<td>N:</td>
<td>rho = -0.03, p = 0.82</td>
<td>rho = -0.24, p = 0.06</td>
<td>rho = -0.10, p = 0.47</td>
<td>rho = -0.11, p = 0.42</td>
</tr>
<tr>
<td>Prostate 25(OH)D (nmol/kg)</td>
<td>PCa:</td>
<td>rho = -0.06, p = 0.70</td>
<td>rho = -0.27, p = 0.10</td>
<td>rho = -0.12, p = 0.45</td>
<td>rho = -0.21, p = 0.19</td>
</tr>
<tr>
<td></td>
<td>N:</td>
<td>rho = -0.04, p = 0.79</td>
<td>rho = 0.09, p = 0.56</td>
<td>rho = -0.01, p = 0.99</td>
<td>rho = 0.06, p = 0.70</td>
</tr>
<tr>
<td>Serum 25(OH)D (nmol/L)</td>
<td>PCa:</td>
<td>rho = -0.08, p = 0.59</td>
<td>rho = -0.15, p = 0.30</td>
<td>rho = -0.08, p = 0.58</td>
<td>rho = -0.20, p = 0.16</td>
</tr>
<tr>
<td></td>
<td>N:</td>
<td>rho = -0.06, p = 0.68</td>
<td>rho = -0.04, p = 0.79</td>
<td>rho = -0.05, p = 0.71</td>
<td>rho = -0.02, p = 0.91</td>
</tr>
<tr>
<td>Prostate 24,25(OH)2D (nmol/kg)</td>
<td>PCa:</td>
<td>rho = -0.07, p = 0.68</td>
<td>rho = -0.05, p = 0.78</td>
<td>rho = -0.07, p = 0.69</td>
<td>rho = -0.23, p = 0.15</td>
</tr>
<tr>
<td></td>
<td>N:</td>
<td>rho = 0.04, p = 0.79</td>
<td>rho = 0.14, p = 0.36</td>
<td>rho = 0.08, p = 0.61</td>
<td>rho = 0.09, p = 0.55</td>
</tr>
<tr>
<td>Serum 24,25(OH)2D (nmol/L)</td>
<td>PCa:</td>
<td>rho = -0.06, p = 0.69</td>
<td>rho = -0.13, p = 0.39</td>
<td>rho = -0.06, p = 0.69</td>
<td>rho = -0.17, p = 0.26</td>
</tr>
<tr>
<td></td>
<td>N:</td>
<td>rho = -0.16, p = 0.24</td>
<td>rho = -0.01, p = 0.97</td>
<td>rho = -0.14, p = 0.32</td>
<td>rho = -0.09, p = 0.52</td>
</tr>
</tbody>
</table>
Figure 7.4. Responses of Ki67 intensity in PCa tissue (A), and Ki67 (3+) % positive nuclei in PCa (B) and N (C) tissue according to prostate calcitriol level attained after vitamin D₃ dosing. Values are means ± SE. Groups defined by prostate calcitriol upper quartile (37 pmol/kg). *indicates a significant difference between groups (P < 0.05).
Supplementary Figure S7.2. Responses of CYP27A1 (A), CYP2R1 (B), CYP27B1 (C), CYP24A1 (D), and VDR (E) mRNA expression levels to vitamin D₃ dosing and across all patients, in laser capture-microdissected PCa and normal prostate epithelium.
among dose groups (P < 0.12) (Supplementary Figure S7.2). Among all 76 patients, CYP27B1 mRNA was higher, and CYP24A1 mRNA was lower, in PCa compared to normal prostate tissue (P = 0.04) (Supplementary Figure S7.2C, D).

Prostate 25(OH)D₃ levels were positively correlated with mRNA expression of CYP27A1 (r = 0.54, P = 0.001) (Figure 7.5A) and CYP2R1 (r = 0.49, P = 0.003) in PCa tissue. After controlling for serum 25(OH)D₃, significant correlations persisted between prostate 25(OH)D₃ and PCa mRNA expression levels of CYP27A1 (r = 0.44, P = 0.008) but not of CYP2R1 (r = 0.32, P = 0.06). Prostate tissue calcitriol concentration, but not serum calcitriol (data not shown for non-significant serum measures), was positively correlated with CYP27B1 mRNA (r = 0.36, P = 0.036) in PCa tissue (Figure 7.5B). This correlation remained unchanged after controlling for serum levels of calcitriol (r = 0.36, P = 0.035). Prostate 24,25(OH)₂D₃ concentrations were inversely associated with CYP24A1 mRNA expression in PCa tissue (r = -0.35, P = 0.03) (Figure 7.5C). Prostate and serum levels of 25(OH)D₃ were positively correlated with CYP27B1 mRNA levels (r = 0.41, P = 0.01; r = 0.32, P = 0.04; respectively) in PCa tissue (Figure 7.5D). After controlling for 25(OH)D₃ (serum or prostate) and calcitriol (serum and prostate) concentrations, the relationship between CYP27B1 mRNA expression in PCa tissue and 25(OH)D₃ remained significant in prostate tissue (r = 0.40, P = 0.02) but not in serum (r = -0.22, P = 0.22). Lastly, prostate levels of calcitriol, but not serum calcitriol, were positively correlated with VDR mRNA expression in PCa tissue (r = 0.42, P = 0.01) (Figure 7.5E).

Safety measures and adverse events

Plasma calcium concentrations did not change with dosing and remained well within the normal reference range (2.20-2.60 mmol/L) throughout the study (Table 7.3). In all 3 treatment
Figure 7.5. Relationships between CYP27A1 (A), CYP27B1 (B), and CYP24A1 (C) mRNA expression levels and their vitamin D metabolite products in PCa tissue. Relationship between CYP27B1 mRNA (PCa) and its substrate (prostate calcitriol) (D), and between prostate calcitriol and VDR mRNA (E) also shown. Linear regression line with 95% confidence intervals in red.
### Table 7.3. All safety-related biochemical measures and adverse events in vitamin D-treated study patients

<table>
<thead>
<tr>
<th>Measure</th>
<th>400 IU/d</th>
<th>Change (from baseline)</th>
<th>10,000 IU/d</th>
<th>Change (from baseline)</th>
<th>40,000 IU/d</th>
<th>Change (from baseline)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Final</td>
<td></td>
<td>Final</td>
<td></td>
<td>Final</td>
<td></td>
</tr>
<tr>
<td>Plasma Calcium – mmol/L</td>
<td>2.37 ± 0.08a</td>
<td>-0.01 ± 0.09</td>
<td>2.37 ± 0.11a</td>
<td>-0.01 ± 0.08</td>
<td>2.36 ± 0.08a</td>
<td>-0.001 ± 0.11</td>
</tr>
<tr>
<td>Hypercalcemia (&gt; 2.60 mmol/L) – n (%)</td>
<td>0</td>
<td></td>
<td>0</td>
<td></td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Urine Calcium (mmol/L)/Creatinine (mmol/L) – ratio</td>
<td>0.11 ± 0.07a</td>
<td>-0.10 ± 0.13*</td>
<td>0.13 ± 0.11a</td>
<td>-0.12 ± 0.14*</td>
<td>0.15 ± 0.10a</td>
<td>-0.09 ± 0.12*</td>
</tr>
<tr>
<td>wk 2</td>
<td>0.36 ± 0.23a</td>
<td>0.16 ± 0.23*</td>
<td>0.25 ± 0.14a</td>
<td>0.01 ± 0.15</td>
<td>0.36 ± 0.22a</td>
<td>0.10 ± 0.25</td>
</tr>
<tr>
<td>Hypercalciuria (&gt; 1.0) – n (%)</td>
<td>0</td>
<td></td>
<td>0</td>
<td></td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Plasma Creatinine – μmol/L</td>
<td>83.7 ± 13.9a</td>
<td>-3.7 ± 12.4</td>
<td>80.2 ± 14.2a</td>
<td>-2.3 ± 7.3</td>
<td>81.7 ± 10.8a</td>
<td>1.8 ± 7.8</td>
</tr>
<tr>
<td>Creatinine elevation (&gt; 110 μmol/L) – n (%)</td>
<td>0</td>
<td></td>
<td>0</td>
<td></td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Plasma Phosphate – mmol/L</td>
<td>0.92 ± 0.15a</td>
<td>-0.15 ± 0.16*</td>
<td>0.99 ± 0.19a</td>
<td>-0.07 ± 0.21</td>
<td>0.96 ± 0.16a</td>
<td>-0.05 ± 0.20</td>
</tr>
<tr>
<td>Hypophosphatemia (&lt; 0.80 mmol/L) – n (%)</td>
<td>5 (27.8)</td>
<td>4 (19.0)</td>
<td>3 (14.3)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma ALT – U/L</td>
<td>24.1 ± 7.4a</td>
<td>-0.6 ± 6.4</td>
<td>30.7 ± 15.0a</td>
<td>0.05 ± 11.4</td>
<td>29.3 ± 10.9a</td>
<td>-0.95 ± 6.7</td>
</tr>
<tr>
<td>ALT elevation (&gt; 40 U/L) – n (%)</td>
<td>1 (5.6)</td>
<td>2 (9.5)</td>
<td>1 (4.5)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma ALP – U/L</td>
<td>69.7 ± 12.3a</td>
<td>-2.1 ± 10.6</td>
<td>70.2 ± 15.1a</td>
<td>-0.8 ± 7.0</td>
<td>74.1 ± 21.0a</td>
<td>-6.4 ± 13.9</td>
</tr>
<tr>
<td>ALP elevation (&gt; 150 U/L) – n (%)</td>
<td>0</td>
<td></td>
<td>0</td>
<td></td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Diarrhea – n (%)</td>
<td>0</td>
<td></td>
<td>0</td>
<td></td>
<td>1 (4.3)</td>
<td></td>
</tr>
<tr>
<td>Constipation – n (%)</td>
<td>0</td>
<td></td>
<td>1 (4.5)</td>
<td></td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Muscle weakness – n (%)</td>
<td>1 (4.8)</td>
<td>1 (4.5)</td>
<td>2 (8.7)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Abdominal pain – n (%)</td>
<td>1 (4.8)</td>
<td>0</td>
<td>2 (8.7)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Itchy skin – n (%)</td>
<td>2 (9.5)</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Values are means ± SD, where applicable. Final values in a row without a common superscript letter differ significantly (P < 0.05, between-group).

*Final and baseline values differ significantly (P < 0.05, within-group).

n = number of patients experiencing adverse event. All adverse events were grade 1 (mild) and judged to be unrelated to intervention by attending oncologist.

Abbreviations: ALT = aspartate aminotransferase, ALP = alkaline phosphatase
groups, the urine calcium:creatinine ratio decreased significantly from baseline (P < 0.01) to a level that was similar among groups at the final visit (P = 0.40). At wk 2, the urine calcium:creatinine ratio was equivalent among dosing groups (P = 0.16). Urinary calcium excretion remained well within normal values (urine calcium:creatinine ratio < 1.0) in all patients throughout the study. There were no cases of hypercalcemia or hypercalciuria.

Plasma creatinine, ALT, and ALP levels did not change with dosing (P > 0.16). Plasma phosphate decreased slightly over time with 400 IU/d only (P = 0.001). A small number of patients experienced marginal and asymptomatic hypophosphatemia and ALT elevation. These cases were distributed evenly among dose groups and were likely attributed to differences in blood sampling conditions (i.e. baseline: fed, final: fasting).

No deaths have occurred. All reported adverse events were classified as grade 1 (mild), evenly balanced among treatment groups and judged to be unrelated to intervention by the attending uro-oncologist.

**Serum PTH and PSA**

As anticipated, serum PTH decreased over time with in the highest dose (P < 0.0001), but this was not statistically significant in the other dose groups (P > 0.32) (Figure 7.6A). Final serum PTH was also lower in the 40,000 IU/d group than in the other groups (P < 0.0001). Combining the higher treatment doses (10,000- and 40,000 IU/d) indicated a statistical decline in serum PTH from baseline to final (P = 0.0001).

Serum PSA decreased over time in the groups receiving 10,000 IU/d (P = 0.04) or 40,000 IU/d (P = 0.19), while the level remained unchanged in the 400 IU/d group (P = 0.81) (Figure
Figure 7.6. Serum PTH (A) and PSA (B) responses to vitamin D₃ dosing. Values are means ± SE. Means without a common letter differ significantly between groups (P < 0.05). *indicates a significant difference from baseline to final within groups (P < 0.05).
7.6B). Reductions in serum PSA (final vs. baseline) were observed in 61%, 70%, and 81% of patients treated with 400-, 10,000-, and 40,000 IU/d of vitamin D₃, respectively. Grouping the higher vitamin D doses (10,000- and 40,000 IU/d) also showed a significant decrease in serum PSA (P = 0.017). Serum PSA and PTH levels were not correlated (r = 0.03, P = 0.83).
7.5 Discussion

Skepticism that cholecalciferol taken orally could produce a significant increase in prostatic calcitriol is why previous clinical trials focused on oral calcitriol and its analogs [241, 242, 246, 313-315]. To our knowledge, the present study describes the first comprehensive analysis of vitamin D metabolism in human tissue. This work provides direct clinical trial evidence that vitamin D metabolism occurs in vivo within prostate, can be modulated by oral consumption of the nutrient cholecalciferol and that higher levels of prostate tissue calcitriol can affect prostate biology. We found that supplementation with 40,000 IU/d of vitamin D₃, leading to mean serum 25(OH)D₃ concentrations of 296 ± 69 nmol/L, resulted in the highest accumulation of 25(OH)D₃, calcitriol, and 24,25(OH)₂D₃ metabolites within prostate tissue. Furthermore, higher levels of calcitriol attained within prostate tissue related to diminished expression of Ki67, a cell-cycle protein that is associated with prostate cell proliferation.

Our vitamin D metabolite measurements provide important information on the mechanism of prostatic vitamin D metabolism. Prostate levels of all 3 vitamin D metabolites were positively correlated with their respective serum concentrations, suggesting that the vitamin D metabolites detected in prostate tissue may be derived, at least in part, from the circulation. However, our data also provide considerable evidence of local vitamin D metabolism within prostate in vivo. Regression analyses indicated that prostate 25(OH)D₃, calcitriol, and 24,25(OH)₂D₃ concentrations remained significantly positive when their respective serum levels were extrapolated to zero, implying a basal level of vitamin D metabolism within prostate that is independent of serum. Supporting this, the prostate tissue expressed the full set of enzymes required for vitamin D metabolism (CYP27A1, CYP2R1, CYP27B1 and CYP24A1), which is consistent with previous reports [27, 225, 319] that prostate cells can locally convert vitamin D
into 25(OH)D, calcitriol, and 24,25(OH)2D. Importantly, prostate tissue concentrations of 25(OH)D3 and calcitriol correlated positively with mRNA expression of the enzymes that produce those metabolites (CYP27A1 and CYP27B1, respectively), independently of serum. Together, serum calcitriol concentration and prostate tissue CYP27B1 mRNA accounted for 43% of the variability in prostate calcitriol. Furthermore, the carrier protein of vitamin D metabolites in the circulation, DBP, was virtually absent in the prostate tissue, indicating that the vitamin D metabolites we measured in prostate were not artifacts from the blood. Finally, the free indices of vitamin D metabolites were much higher in prostate compared to serum, suggesting increased accessibility and biological activity of these metabolites at the tissue level [320]. Taken together, these data demonstrate that vitamin D supplementation can lead to an accumulation of vitamin D metabolites within target tissues through a combination of local synthesis and tissue uptake from the circulation.

It has been hypothesized that the accretion of vitamin D metabolites within prostate tissue has important implications for PCa because the metabolites can exhibit varying degrees of anti-proliferative activity. The growth inhibitory effects of calcitriol have been well-characterized in preclinical PCa models [219, 220]. In addition, 25(OH)D at physiological concentrations (i.e. 100 nmol/L) can also suppress the growth of normal [24] and malignant [221] prostate cells at an equal potency to pharmacological doses of calcitriol, likely through intracellular conversion of 25(OH)D to calcitriol. Recently, Swami et al [26] demonstrated that dietary vitamin D3 and calcitriol exhibited equivalent reductions in tumour volume in mouse xenograft models of breast (MCF-7) and prostate (PC-3) cancer. The present study extends these preclinical findings into the clinical setting, by providing some evidence of the biological effects of increased levels of calcitriol in prostate. Through quantitative digital immunohistochemistry, we measured Ki67 protein, a cellular marker of proliferation that is present during all active phases of the cell cycle.
(G1, S, G2, and mitosis), but is absent from resting cells (G0) [321]. Although the vitamin D doses did not significantly affect Ki67 measures *per se*, the level of calcitriol attained in prostate tissue correlated negatively with Ki67 intensity and histoscore. In fact, those patients that achieved higher prostate calcitriol levels (in particular, >37 pmol/kg) showed a substantially lower proportion of nuclei stained strongly positive (3+) for Ki67. As Ki67 staining intensity, reflective of protein expression [322], has been shown to generally increase during growth phases of the cell cycle [321, 323-325], these findings suggest a possible cell-cycle effect of calcitriol on Ki67, whereby higher prostatic levels of calcitriol reduce Ki67 expression to ultimately delay cell cycle progression. Indeed, a reduction in Ki67 expression with higher calcitriol may help explain *in vitro* findings of calcitriol-induced growth arrest of PCa cells at G1 [219, 220], since Ki67 is associated with progression from G1 to S. Our results are also supported by a recent study by Kovalenko et al [25], which demonstrated that mice fed a low vitamin D diet, or had their VDR deleted, exhibited higher proliferation (i.e. increased Ki67 labeling index) and lower apoptosis in their prostate. Lastly, the effects of prostate calcitriol on Ki67 may be mediated by VDR as we found that higher prostate tissue levels of calcitriol were also associated with higher mRNA levels of VDR, the primary mediator of calcitriol biological action, in PCa tissue. This finding is consistent with preclinical studies [326-328] and could indicate enhanced responsiveness of PCa cells to calcitriol hormone in the short term. Overall, the relationship between calcitriol and Ki67 requires further study and validation because despite the well-characterized role of the Ki67 labeling index as a proliferation marker, the functional significance of the Ki67 protein *per se* remains unclear.

Our secondary outcomes provide further insight on the mechanism of prostatic vitamin D metabolism, the safety of high-dose vitamin D₃ supplementation and the therapeutic potential of enhanced vitamin D status. In our gene expression studies, we discovered that prostate vitamin
D metabolites measured in benign tissue related exclusively to expression of vitamin D metabolism genes measured in PCa tissue, not in benign prostate. This implies that tumoural vitamin D metabolites transfer to surrounding prostatic regions in a paracrine fashion. However, we were not able to obtain sufficient quantities of fresh surgical cancer tissue to measure the metabolites. Nonetheless, our findings suggest a potential protective mechanism in PCa cells to increase local concentrations of active vitamin D metabolites via paracrine or autocrine pathways.

Interestingly, when pooling the gene expression data, we found in our clinical samples that mRNA levels of CYP27B1 were higher, and of CYP24A1 were lower, in PCa compared to normal prostate tissue. Down-regulation of CYP24A1 expression in PCa compared to adjacent benign lesions is supported by a recent investigation of paired human prostate samples [329]. In contrast to our findings, Hsu et al [222] demonstrated that CYP27B1 activity is decreased in PCa compared to normal prostatic epithelial cells. The reason for these discrepant findings are unclear but may reflect differences between the in vivo and in vitro environments of our respective studies.

Another implication of the present finding, that prostate calcitriol is determined both by circulating calcitriol and by local synthesis, is that other physiological determinants of serum calcitriol may be relevant to PCa. This is particularly important given the inter-individual variability in prostate calcitriol production observed in the present study. Calcium might be one such determinant of tissue calcitriol levels. Circulating calcitriol normally increases in response to a low dietary intake of calcium, to stimulate the calcium-absorptive efficiency of the gut [330]. A high dietary intake of calcium suppresses calcitriol, and this suppression may account for the epidemiological association between high calcium intakes and PCa risk [178]. Indeed, low
serum calcitriol levels are related to increased risk of PCa [203]. Therefore, dietary strategies for preventing PCa need to take into account the relationships between calcium and vitamin D [331].

Safety of short-term high-dose oral vitamin D supplementation in PCa patients was an important outcome that was monitored in our trial. Despite the high 25(OH)D₃ and calcitriol levels attained in our patients, both of which had increased from baseline with the higher oral doses, the classic safety indices for vitamin D excess, namely plasma and urine calcium concentrations, were unaffected. Furthermore, kidney and liver function were not impaired by vitamin D dosing, and all reported side effects were minor and unrelated to study intervention. Similarly, an excellent safety profile for high-dose vitamin D supplementation has been demonstrated in multiple sclerosis patients [12, 274], tuberculosis patients [332] and healthy adults [134, 135, 258]. Overall, these findings indicate that, at least in the short term, high doses of vitamin D₃ (up to 40,000 IU/d) can be safely tested in PCa patients without adverse outcomes. The doses used here were high because the timeframe for intervention was relatively short. The present trial does not serve as a basis to advocate higher doses of cholecalciferol for the prevention or treatment of PCa, but it does justify further research.

Our findings also provide evidence that high-dose vitamin D₃ supplementation (≥10,000 IU/d) should be studied further as a way to moderate serum PSA and PTH responses in PCa patients. Although the declines we observed in serum PSA were small, they occurred over a very brief course of vitamin D₃ treatment (~1 month), and are consistent with previous reports showing that oral vitamin D₃ (2000 IU/d) [29] or calcitriol [241] can slow the rate of PSA rise in PCa patients. Furthermore, a recent clinical trial demonstrated that vitamin D₃ supplementation can benefit PCa patients without significant changes in PSA. In that study, vitamin D₃ treatment (4000 IU/d) for one year led to a decrease in the number of positive cores (or decrease in
Gleason score) in PCa patients under active surveillance [28]. The declines in serum PTH are also of interest. An oncogenic role for PTH in the prostate is supported by preclinical studies [333, 334] and clinical evidence showing that elevated PTH levels correlated with reduced survival in patients with androgen-independent PCa [247]. Therefore, the suppression of PTH with cholecalciferol may not only reduce PCa mortality but could also decrease skeletal morbidity in PCa (e.g. fracture risk, bone pain), which is often exacerbated by androgen deprivation therapy [335].

The strengths of this study are that this was a double-blinded randomized clinical trial involving a meaningfully large range of vitamin D doses, and the primary outcome of a dose-dependent increase in prostate calcitriol was appropriately demonstrated. A weakness was that there was no clinically meaningful outcome, but since this was a Phase II randomized clinical trial, there is now reason to study clinical outcomes in a larger sample size with a longer duration of treatment.

In conclusion, our clinical trial data support the hypothesis that vitamin D metabolism occurs within prostate in vivo and can be modulated by high oral vitamin D dosing. Furthermore, higher prostate calcitriol and vitamin D doses (10,000- and 40,000 IU/d) showed suggestion of clinical benefit, with relations to lowered Ki67 expression, and modest declines in serum PSA and PTH, respectively. The vitamin D doses (400-40,000 IU/d) were well-tolerated by PCa patients without signs of toxicity. Further studies are needed to validate the potential utility of dietary vitamin D₃ supplementation in PCa prevention and therapy.
8 CHAPTER 8: General Discussion
8.1 General Discussion

Vitamin D continues to attract considerable attention in the medical and lay community because of epidemiologic associations linking higher vitamin D status with lower risk for a host of diseases ranging from cancer to diabetes [2]. The biological plausibility of such claims is supported by preclinical data demonstrating that the vitamin D modulates the expression of over 900 genes [121], has been subject to strong evolutionary pressures [336] and exerts its biologic effects across a wide range of tissues [2]. Such evidence points to an autocrine or paracrine role for vitamin D, adding to its well-characterized endocrine action in bone metabolism. This extra-renal function becomes particularly plausible given the wide expression of VDR and vitamin D metabolic enzymes (in particular, 1-α-hydroxylase) across a multitude of non-calcemic cell types (see Table 2.3). As such, it is thought that vitamin D metabolites are synthesized locally and accumulate in various tissues to mediate several critical cellular functions, including cell growth regulation and immunomodulation. This however remains a theory because direct measurement of vitamin D metabolites in human tissue has not been reported and extra-renal vitamin D metabolism remains poorly understood. Thus, the overall objective of this thesis was to shed some light on this new paradigm of extra-renal vitamin D metabolism by investigating whether higher levels of vitamin D consumed orally or achieved in the circulation result in increased concentrations of vitamin D metabolites, particularly active calcitriol, in human tissue. The hallmark of this work was a randomized clinical trial conducted in PCa patients to evaluate the effects of oral vitamin D₃ supplementation on prostatic vitamin D metabolism and on PCa pathology.

The measurement of vitamin D metabolites in biological specimens is a technical challenge because these metabolites can be highly lipophilic, bound strongly to protein, present in low
concentrations and exist in structurally similar forms. As such, several different methods exist for measuring vitamin D metabolite concentrations in blood serum or plasma, all of which vary in analytical performance. Therefore, the objectives of my first two studies were to assess the analytical validity of promising novel methods (Chapter 4: automated immunoassays, Chapter 5: LC-MS/MS) for measuring serum 25(OH)D and/or 24,25(OH)₂D concentrations in clinical samples. One of these methods would ultimately be used in determining serum 25(OH)D and 24,25(OH)₂D levels in our PCa clinical trial samples.

In Study 1 (Chapter 4), we compared the analytical reliability of two new automated immunoassays with the well-established reference method, DiaSorin radioimmunoassay (RIA), for quantitation of 25(OH)D concentration in human serum. Of the two automated platforms evaluated, the DiaSorin LIAISON assay demonstrated a stronger correlation and better agreement with the reference method than the Roche Modular assay [30]. We concluded that the DiaSorin LIAISON assay was accurate, precise and the best available automated tool for 25(OH)D determination in serum/plasma samples [30]. Consequently, this work contributed to the adoption of the DiaSorin LIAISON assay by Mount Sinai Hospital reference laboratory, which helped increase throughput capacity for vitamin D testing and meet the ~2400% increase in testing volumes Ontario had experienced from 2004-2010 [337]. The DiaSorin LIAISON platform would have been a perfectly acceptable method to measure serum 25(OH)D concentrations in our PCa clinical trial samples, however, we decided against its use for two major reasons. First, the LIAISON 25(OH)D assay did not perform well with extracted tissue samples. Recovery of 25(OH)D with LIAISON quantification (following tissue extraction) was unsatisfactory (62.5%) and significantly lower than with LC-MS/MS (Appendix A, unpublished data). The low 25(OH) recovery with LIAISON quantification may have been the result of interfering compounds in the extracted tissue matrix, an issue that can be encountered with
immunoassays [338]. Furthermore, the automated nature of the LIAISON immunoassay makes it difficult to adequately refine components of the method (e.g. reagents, volumes) to suit a different matrix. In theory, these matrix issues would be resolved with a more robust extraction and quantification procedure, as offered with LC-MS/MS assays.

In Study 2 (Chapter 5), we assessed the analytical validity and clinical utility of a novel LC-MS/MS assay for simultaneous determination of 25(OH)D and 24,25(OH)_2D in serum/plasma. This novel method exhibited high sensitivity (1 nmol/L), excellent specificity (i.e. no cross-reactivity between vitamin D metabolites), negligible interference (i.e. with elevated levels of bilirubin, hemoglobin and triglyceride), and strong correlation with serum 25(OH)D levels as measured by DiaSorin RIA (r = 0.92) or LIAISON (r = 0.91) [317]. Furthermore, the LC-MS/MS assay can provide more comprehensive data concerning vitamin D status and repletion, as we discovered that the ratio of serum 24,25(OH)_2D to 25(OH)D was predictive of 25(OH)D response in subjects randomized to vitamin D₃ supplementation (28,000 IU/wk). Specifically, a higher ratio, reflective of increased catabolic clearance of vitamin D, related to a smaller serum 25(OH)D response to vitamin D dosing. Taken together, these methodological and clinical features provided strong basis to use the LC-MS/MS assay for our PCa clinical trial samples. Indeed, subsequent method development efforts with bovine muscle tissue demonstrated that the LC-MS/MS quantification platform consistently produced vitamin D metabolite recoveries >90% (compared to 62% with LIAISON) and was thus suitable for the newly developed tissue extraction method (Appendix A). Thus, LC-MS/MS was the method of choice to measure 25(OH)D and 24,25(OH)_2D concentrations in the serum and prostate tissue samples from our PCa clinical trial.
In Study 3 (Chapter 6), we performed a comprehensive evaluation of our newly developed method to measure 1,25(OH)₂D in human tissue. As there is no reference method or material for 1,25(OH)₂D measurement in human tissue, validation experiments were conducted to evaluate the ability of the extraction method to accurately and reproducibly extract 1,25(OH)₂D from a tissue matrix. Using our tissue extraction method, assay of bovine muscle tissue (free of endogenous vitamin D) spiked with exogenous 1,25(OH)₂D yielded excellent 1,25(OH)₂D recovery (mean: 100.1 ± 6.5%), as confirmed by multiple quantification methods [32]. This extraction method performed similarly well for tissue 25(OH)D and 24,25(OH)₂D determination employing LC-MS/MS quantification (see Section 7.4). Taken together, these results demonstrated that the developed tissue extraction method was suitable for assay of 1,25(OH)₂D in human tissue. Therefore, we used this method to measure 1,25(OH)₂D levels in human colon tissue and matched serum samples obtained from patients who had undergone colectomy. The objectives were to determine whether 1,25(OH)₂D is present in human colon tissue and to characterize the relationship between human colon tissue and serum 1,25(OH)₂D concentrations. Our results described for the first time that the 1,25(OH)₂D hormone can indeed be detected in human colon tissue at physiologically relevant concentrations partly determined by serum 1,25(OH)₂D. There was also evidence of local 1,25(OH)₂D production within colon tissue in vivo as the levels of DBP, the carrier protein of circulating vitamin D metabolites, were negligible in the tissue and linear regression showed a significant positive intercept of colon 1,25(OH)₂D at zero serum 1,25(OH)₂D. Thus, having shown that physiological levels of 1,25(OH)₂D can be detected in human tissue, the next step was to investigate whether these tissue levels can be modulated by oral vitamin D supplementation.
Study 4 (Chapter 7) was the culmination of my PhD thesis work. The goal was to evaluate in a randomized clinical trial whether higher\(^1\) oral doses of dietary vitamin D\(_3\) given to PCa patients prior to radical prostatectomy can induce changes in prostatic vitamin D metabolism and in PCa pathology. Thus, we randomized early-stage PCa patients to daily vitamin D\(_3\) (400, 10,000, or 40,000 IU/d) for 3-8 wk and obtained blood and prostate tissue specimens at surgery. The first objective was to determine whether the oral vitamin D\(_3\) supplementation resulted in higher vitamin D metabolite levels in human prostate tissue. We found a linear upward trend (P < 0.05) in prostate tissue concentrations of 25(OH)D, calcitriol, and 24,25(OH)\(_2\)D\(_3\) among the dose groups, with the 40,000 IU/d group exhibiting the highest accumulation of these metabolites. Similar to our colon data, prostatic levels of vitamin D metabolites were reflective of those in serum, but there was also considerable evidence of local vitamin D metabolism within prostate \textit{in vivo}. The free indices of vitamin D metabolites were much higher in prostate compared to serum and increased dose-dependently, suggesting increased accessibility and biological activity of these metabolites at the tissue level that can be modulated by higher oral vitamin D intake. Furthermore, prostate tissue concentrations of 25(OH)D\(_3\) and calcitriol correlated positively with mRNA expression of the enzymes that produce those metabolites (CYP27A1 and CYP27B1, respectively), independently of serum. Taken together, these data confirmed our hypothesis of higher levels of calcitriol in prostate tissue after higher oral administration of vitamin D, through a combination of local synthesis and tissue uptake from the circulation.

\(^1\) In this context, “higher” denotes intake or circulating concentrations of vitamin D that are near or beyond the upper end of exposure attainable through normal physiology
The second major objective of the PCa clinical trial (Chapter 7) was to determine whether higher levels of vitamin D3 consumed orally or achieved in prostate tissue can affect PCa pathology. Through quantitative digital immunohistochemistry, we measured Ki67 protein, a cellular marker of proliferation that is present during all active phases of the cell cycle (G1, S, G2, and mitosis), but is absent from resting cells (G0) [321]. Analysis by dose group revealed no significant differences in Ki67 proliferation measures among the doses, likely because Ki67 is a lowly-expressed antigen with considerable tissue heterogeneity, thereby requiring higher statistical power than in the present study to detect significant differences. However, we found that the level of calcitriol attained in prostate tissue correlated negatively with Ki67 intensity and histoscore, two measures that were assessed objectively by quantitative digital immunohistochemistry. In fact, those patients that achieved higher prostate calcitriol levels showed a substantially lower proportion of nuclei stained strongly positive (3+) for Ki67. A clinical interpretation of these findings remains unclear because the functional significance of the Ki67 protein per se is largely unknown. However, as Ki67 staining intensity, reflective of protein expression [322], has been shown to generally increase during growth phases of the cell cycle [321, 323-325], these data might suggest a possible cell-cycle effect of calcitriol on Ki67, whereby higher prostatic levels of calcitriol reduce Ki67 expression to ultimately delay cell cycle progression. Indeed, a reduction in Ki67 expression with higher calcitriol may help explain in vitro findings of calcitriol-induced growth arrest of PCa cells at G1 [219, 220], since Ki67 is associated with progression from G1 to S.

In a collaborative study conducted with the University of Illinois at Chicago (PI: Larisa Nonn), our PCa clinical trial FFPE tissue was also used to investigate the effects of oral vitamin D supplementation and tissue vitamin D metabolite levels on the expression of mRNA and micro-RNA (miRNA) related to PCa or vitamin D metabolism (see Appendix B). Similar to our
Ki67 data, there were no significant differences in mRNA or miRNA expression in laser-capture-microdissected benign and PCa tissue among vitamin D₃ dose groups. However, we found that the level of calcitriol attained in patient prostate tissue correlated positively with three tumour suppressive miRNAs, namely miR-100, miR-125b and Let-7a. Upregulation of these miRNAs by 1,25(OH)₂D and their antiproliferative function were confirmed *in vitro* in prostate epithelial cells treated with 1,25(OH)₂D. Taken together with the Ki67 findings, these data suggest that higher levels of 1,25(OH)₂D are beneficial to PCa pathology as this seems to induce changes in the prostatic expression of a cell cycle protein and tumour-suppressive miRNAs. As such, strategies that may maximize calcitriol within prostate for anti-cancer activity warrant further study.

Other important contributions of the PCa clinical trial work bear mention. The higher doses of vitamin D₃, which led to large increases in serum 25(OH)D and calcitriol, exhibited an excellent safety profile without adverse effects on calcium levels, kidney function or liver enzymes. These data support other studies demonstrating the safety of high-dose vitamin D supplementation in multiple sclerosis patients [12, 274], TB patients [332] and healthy adults [134, 135, 258]. Our findings also provide evidence that high-dose vitamin D₃ supplementation (≥10,000 IU/d) should be studied further as a way to moderate serum prostate specific antigen (PSA) and parathyroid hormone (PTH) responses in PCa patients. Although the declines we observed in serum PSA were small, they occurred over a very brief course of vitamin D₃ treatment (~1 month), and are consistent with previous reports showing that oral vitamin D₃ (2000 IU/d) [29] or calcitriol [241] can slow the rate of PSA rise in PCa patients. The declines in serum PTH are also of clinical interest because elevated PTH has been associated with reduced survival in PCa patients [247] and bone loss [339]. Therefore, the suppression of PTH with
vitamin D₃ may not only reduce PCa mortality but could also decrease skeletal morbidity in PCa (e.g. fracture risk, bone pain), which is often exacerbated by androgen deprivation therapy [335].

In conclusion, our data shed light on the new paradigm of extra-renal vitamin D metabolism and action. We demonstrated that higher levels of vitamin D consumed orally (prostate study) or achieved in the circulation (colon study) result in increased concentrations of vitamin D metabolites, particularly active calcitriol, in human tissue. Specifically, our clinical trial data support the hypothesis that vitamin D metabolism occurs within prostate in vivo and can be modulated by high oral vitamin D dosing. Furthermore, there was suggestion of clinical benefit, as higher prostate calcitriol and vitamin D doses (10,000- and 40,000 IU/d) related to lowered Ki67 expression, increased levels of tumour suppressive miRNAs, and modest declines in serum PSA and PTH, respectively. The vitamin D doses (400-40,000 IU/d) were well-tolerated by PCa patients without signs of toxicity. Further studies are needed to validate the potential utility of dietary vitamin D₃ supplementation in PCa prevention and therapy.
8.2 Strengths and Limitations

Tissue Vitamin D Metabolism

We developed a novel and robust method to measure vitamin D metabolite levels in human tissues, and used it to provide the first comprehensive analysis of vitamin D metabolism in tissue. The association between measured prostate calcitriol levels and the expression of both Ki67 (Chapter 7) and micro-RNAs (Appendix B) further support the analytical and clinical utility of our vitamin D extraction method. To our knowledge, we described the first evaluation of vitamin D metabolites in human tissue and its linkage with a biological measure. Another important strength of our tissue method is that it is fully compatible with the most widely assays to quantify serum vitamin D metabolites. Ultimately, our developed method could enable researchers to quantify vitamin D metabolites in accessible human tissue (e.g. surgical specimens) and facilitate an enhanced understanding of vitamin D metabolism at the tissue level. Such a tool should stimulate research examining the relationship between tissue vitamin D metabolites and the various health outcomes that are believed to be linked with vitamin D intakes. Our PCa clinical trial also had the advantage of testing a wide spectrum of doses, ranging from low physiological levels to pharmacological doses, ensuring the likelihood that our study protocol would detect any changes in prostatic vitamin D metabolism elicited by oral intake.

Despite the excellent accuracy and precision of the method, it was not possible to assess the full gamut of vitamin D metabolism, as certain vitamin D metabolites were not measured (i.e. vitamin D; 1,24,25(OH)\(_3\)D) due to restraints in the sensitivity of the analytical method and in specimen quantity (i.e. >200 mg). As such, a complete pharmacokinetic or pharmacodynamic analysis could not be accomplished in our studies. The determination of 1,24,25(OH)\(_3\)D in tissue
is of particular interest because its precursor 24,25(OH)$_2$D was found in high concentration in prostate tissue and 1,24,25(OH)$_3$D has been shown to exhibit similar anti-proliferative activity to 1,25(OH)$_2$D in cancer cells [340]. However, our quantification technologies were not sufficiently sensitive to detect 1,24,25(OH)$_3$D and other such metabolites that are present at low levels, although the three major vitamin D metabolites (i.e. 25(OH)D; 1,25(OH)$_2$D; 24,25(OH)$_2$D) were indeed measured. Furthermore, an ideal method to measure vitamin D metabolites in tissue and serum would allow for the simultaneous determination of a panel of metabolites (e.g. vitamin D; 25(OH)D; 1,25(OH)$_2$D; 24,25(OH)$_2$D; 1,24,25(OH)$_3$D; others). A panel platform would minimize analytical variability and the specimen quantity required.

The tissue vitamin D metabolite method itself had certain limitations. The tissue assay is technically challenging and time-consuming, and thus its performance largely depends on the technical skill of the laboratorian. These aspects also limit the throughput of the method, unless it can be reasonably automated. The assay requires a minimum of 200 mg of tissue, an amount that can impractical to obtain with certain biopsy or animal samples. Lastly, we showed that the tissue method is compatible with colon, prostate and likely other epithelial tissues, although its analytical performance with non-epithelial tissues would need to be validated.

*Prostate cancer pathology*

We describe the first double-blinded randomized clinical trial of vitamin D in PCa involving a meaningfully large range of doses. Another strength is that we enrolled a considerable number of patients and essentially created a virtual tissue bank of study cases (prostate tissue, serum) that could be used in future analyses. The expression of Ki67 was assessed quantitatively (as opposed to the traditional semi-quantitative method) using the latest technologies in immunohistochemistry (i.e. tissue microarrays, digital immunoscopy using
Aperio® software, digital annotations using Genie® tissue pattern recognition algorithm). In particular, these digital automated methods were optimized and validated by experienced pathologists and showed excellent concordance with the traditional manual scoring method. Despite the strength of our randomized design and wide range of vitamin D doses tested, we did not see differences in the Ki67 labeling index (i.e. Ki67 % positive nuclei) among dose groups at radical prostatectomy, likely due to lack of statistical power. Given that the SD of the response variable (i.e. Ki67 % positive nuclei) observed in our study was 0.6%, a total of 128 patients would have to be enrolled in the trial to detect the difference in Ki67 % positive nuclei observed between the lowest and highest vitamin D dose groups (i.e. 0.3%) as significant (at a two-sided 0.05 significance level with 80% power). Indeed, a baseline Ki67 measurement on the prostate biopsy may have provided additional statistical power to detect changes in the Ki67 labeling index within dose groups. However, the small quantity of biopsy material available was inadequate for accurate immunohistochemical and biochemical assessment. Nonetheless, our pathology findings suggest a possible cell-cycle effect of tissue calcitriol on Ki67, whereby higher prostatic levels of calcitriol reduce Ki67 expression to ultimately delay cell cycle progression. However, a clinical interpretation of these findings remains unclear because the functional significance of the Ki67 protein per se is largely unknown. Furthermore, it is difficult to draw mechanistic insight on the effects of calcitriol on the cell cycle without having analyzed additional cell cycle biomarkers. Nonetheless, the similar pattern of prostate calcitriol also being positively correlated with tumour suppressive miRNAs support the concept of changes in prostate biology being mediated by local concentrations of calcitriol. Further analyses of additional cell cycle or molecular markers can be investigated in future studies using the tissue banked from our PCa clinical trial.
8.3 Future Directions

Serum vitamin D metabolites

Automated methods to measure serum 25(OH)D continue to evolve. Notably, there has been an increase in the number of commercial automated 25(OH)D immunoassays in the market (e.g. IDS iSYS, Roche Elecsys, Siemens ADVIA Centaur, Abbott ARCHITECT) owing to the increased demand for clinical vitamin D testing. Such assays claim to offer higher sample throughput capacity and increased analytical sensitivity compared to older methods. Furthermore, LC-MS/MS methods have been gaining in prominence, although these remain largely manual. Considering the proliferation of 25(OH)D assays, it is important that the analytical performance of these new platforms be evaluated against commonly used reference methods. Recently, Farrell et al [341] compared the performance of 5 automated immunoassays, an RIA and 2 LC-MS/MS methods to measure circulating 25(OH)D. They found that the automated immunoassays demonstrated variable performance and not all tests met the minimum performance goals, particularly at 25(OH)D concentrations <20 nmol/L [341]. As noted by the authors, a potential confounder in 25(OH)D measurement is the presence of 25(OH)D 3-epimer forms. Indeed, recent studies have shown that these 3-epi forms are present not only in infants, but also in adults in variable concentrations representing up to 17% of total 25(OH)D [342, 343]. Thus, further research is needed to better understand the influence of 25(OH)D epimer forms and other analytical limitations on the performance of 25(OH)D assays.

Another area that is gaining research momentum relates to the 24,25(OH)₂D metabolite. Considerable evidence now indicates that 24,25(OH)₃D may not simply be a vitamin D catabolite because it possesses unique biological properties [64, 282, 284-286] and possibly a non-nuclear membrane receptor [299]. Furthermore, genetic polymorphisms in CYP24A1, the
enzyme that generates 24,25(OH)\(_2\)D, have been associated with vitamin D status. Therefore, as demonstrated by our evaluation of the serum 24,25(OH)\(_2\)D to 25(OH)D ratio (Study 2), differential 24,25(OH)\(_2\)D production may contribute to the large inter-individual variation that has been reported in serum 25(OH)D response to vitamin D supplementation. Taken together, future studies should continue to measure 24,25(OH)\(_2\)D to confirm its functional role in human physiology and its potential clinical utility as a predictor of serum 25(OH)D response to vitamin D supplementation.

*Tissue vitamin D metabolites*

Up to now, evaluations of vitamin D with various health outcomes have been limited to serum levels of vitamin D metabolites, although tissue concentrations of vitamin D may be more important in reducing disease risk. Our novel method to assay vitamin D metabolites in tissue should enable researchers to shed some light on the new paradigm of extra-renal vitamin D metabolism. Such a tool should stimulate research examining the relationship between tissue vitamin D metabolites and the various health outcomes that are believed to be linked with vitamin D intakes. Ultimately, this sort of work will help determine the tissue vitamin D metabolite concentrations (and thereby vitamin D doses) that should be targeted to maximize the health benefits of vitamin D.

Future research should also consider optimizing the method to measure additional vitamin D metabolites (e.g. parent vitamin D; 1,24,25(OH)\(_3\)D) to gain a more comprehensive understanding of the extra-renal pharmacokinetics of vitamin D. Such work would also be facilitated by the development of a quicker, less technically challenging (but equally reliable) method that permits the simultaneous determination of a panel of vitamin D metabolites in tissue and serum. LC-MS/MS platforms show promise here, as some of these methods have reported
the quantification of multiple vitamin D metabolites including serum 1,25(OH)\textsubscript{2}D \cite{344,345}. Future work could also attempt to minimize the quantity of tissue required for assay and validate the reliability of the tissue vitamin D method on various types of epithelial and non-epithelial tissues that are putative target sites for vitamin D.

An implication of our finding that prostate calcitriol is determined both by circulating calcitriol and by local synthesis, is that other physiological determinants of calcitriol may be relevant to PCa. This is particularly important given the inter-individual variability in prostate calcitriol production observed in the PCa clinical trial. Calcium might be one such determinant of tissue calcitriol levels. A high dietary intake of calcium suppresses circulating calcitriol, and this suppression may account for the epidemiological association between high calcium intakes and PCa risk \cite{178}. Indeed, low serum calcitriol levels are related to increased risk of PCa \cite{203}. Furthermore, genetic polymorphism in vitamin D metabolic enzymes could contribute to inter-individual variability in prostate calcitriol production and in biological responses to vitamin D therapy. Indeed, polymorphisms in genes involved in the vitamin D metabolic pathway (e.g. cholesterol synthesis, DBP, CYP2R1, CYP24A1, VDR) have been associated with vitamin D status, as well as risk of oral and prostate cancers \cite{346,347} Taken together, future studies should identify the physiological regulators of tissue calcitriol, including calcium, calciotropic hormones, vitamin D metabolic enzymes, and other potential determinants.

Prostate cancer pathology

Although not feasible at the time of the study, prostate biopsy material is available for baseline assessments (e.g. baseline Ki67 measures), provided there is a reliable immunohistochemical method for small biopsy samples that can be compared to radical prostatectomy specimens. Given the lack of a dose effect on Ki67 measures at radical
prostatectomy, a baseline Ki67 measurement on the prostate biopsy could increase sensitivity to detect within-patient changes in prostate pathology.

The biological relevance of differences in Ki67 staining intensity (i.e. protein expression) requires further study, particularly since we found this measure to be negatively associated with prostate tissue calcitriol levels in our PCa clinical trial. The biological function of Ki67 needs to be known to interpret findings showing changes in Ki67 expression. Such investigations would be strengthened by analyses of carefully selected cell cycle or molecular markers on the stored clinical prostate tissue to assess whether local concentrations of calcitriol do indeed modulate cell cycle progression.

In light of the suggestive clinical benefits to PCa pathology and modest declines in PSA observed in our study, a larger randomized clinical trial is warranted that evaluates a greater number of PCa patients and a longer duration of vitamin D3 treatment. Such a trial should examine harder endpoints following conventional PCa treatment, such as PCa recurrence or mortality, or more closely investigate the effects of longer-term supplementation on PCa and cell-cycle biomarkers. It is also worth noting that our PCa trial design can be duplicated in another human cancer, such as breast cancer, which has also been shown to respond to vitamin D [26, 348]. In fact, based on the work in this thesis, the high-dose-vitamin D protocol has been funded (Canadian Breast Cancer Foundation) for women scheduled for breast cancer surgery (Dr Angel Arnaout, Ottawa Hospital; Dr. Vieth is a co-investigator of that trial).

Ultimately, a large primary chemoprevention trial of vitamin D would confirm whether vitamin D supplementation can indeed prevent PCa or other cancers at the population level. It should also be important to confirm with a proper, randomized, double-blinded, placebo-
controlled clinical trial the preliminary findings that 4000 IU/d of vitamin D is of benefit to men undergoing active surveillance [28].

In conclusion, vitamin D remains a promising area of research in PCa prevention and treatment. Our findings contribute to the growing body of evidence suggesting that vitamin D can be an effective anticancer agent. Further studies are needed to validate the potential utility of dietary vitamin D₃ supplementation in PCa prevention and therapy. Recently, there has been great concern raised about whether radical prostatectomy is objectively beneficial for the men of the sort (low-moderate grade PCa) who were enrolled for our clinical trial [349]. Furthermore, the recent single-arm clinical trial showing that 4000 IU/d vitamin D₃ may benefit patients undergoing active surveillance [28] is supported by the results of our own trial. What is now needed is a proper clinical trial, involving PCa patients undergoing active surveillance, randomized to low and high intakes of vitamin D. Undoubtedly, the future of this “sunshine vitamin” looks “bright” as we continue to learn more about its vital role in health and disease.
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Table A1. Recovery of exogenously-added vitamin D metabolites achieved with tissue extraction procedure followed by EIA or LC-MS/MS quantification

<table>
<thead>
<tr>
<th>Vitamin D metabolite</th>
<th>Quantification method</th>
<th>Vitamin D metabolite recovery (% ± CV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25(OH)D</td>
<td>EIA (DiaSorin LIAISON)</td>
<td>62.5 ± 5.7*</td>
</tr>
<tr>
<td>25(OH)D</td>
<td>LC-MS/MS</td>
<td>94.4 ± 6.3</td>
</tr>
<tr>
<td>24,25(OH)2D</td>
<td>LC-MS/MS</td>
<td>91.5 ± 14.9</td>
</tr>
<tr>
<td>1,25(OH)2D</td>
<td>EIA (Immunodiagnostic Systems)</td>
<td>95.8 ± 8.3</td>
</tr>
</tbody>
</table>

*Bovine muscle tissue (n=10) free of endogenous vitamin D was spiked with pooled human serum containing known concentrations of vitamin D (25(OH)D: 65 nmol/L, 24,25(OH)2D: 10 nmol/L, and 1,25(OH)2D: 163 pmol/L), extracted with described tissue extraction method, and quantified for each of the vitamin D metabolites. Recovery was calculated based on values obtained for tissue spiked with pooled serum and those for the same serum sample assayed neat (i.e. no tissue extraction step). CV: coefficient of variation.

Recovery was significantly different compared to the other vitamin D metabolites (P < 0.05).
Appendix B

1,25-Dihydroxyvitamin D₃ and vitamin D₃ increase the levels of tumor suppressor microRNAs, miR-100 and -125b, in primary prostate cells and in patient tissue

Running Title: miR-100 and miR-125b are up-regulated by vitamin D₃

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Angeline A Giangreco¹, Avani Vaishnav¹, Dennis Wagner³, Neil Fleshner⁴, Theodorus Van der Kwast⁵, Reinhold Vieth³, Larisa Nonn¹.², *

¹Department of Pathology, University of Illinois at Chicago, IL, USA
²University of Illinois Cancer Center, Chicago, IL, USA
³Department of Pathology, Mt. Sinai Hospital, University of Toronto, ON, Canada
⁴Surgical Oncology, University Health Network, Toronto, ON, Canada
⁵Department of Pathology, University Health Network, University of Toronto, ON, Canada

*Corresponding Author: Department of Pathology, Inonn@uic.edu

840 S. Wood St, Room 130 CSN, Chicago, IL 60612.

Phone: 1(312) 996-0194, Fax: 1(312) 996-7586

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MiR-100 and miR-125b are lost in many cancers and have potential function as tumor suppressors. Using both primary prostatic epithelial cultures and laser-capture-microdissected prostate epithelium from 45 patients enrolled in a vitamin D₃ randomized trial, we identified miR-100 and -125b as targets of 1,25-dihydroxyvitamin D₃ (1,25D). In patients, miR-100 and -125b levels were significantly lower in tumor tissue than in benign prostate. Similarly, miR-100 and -125b were lower in primary PCa cells than in cells derived from benign prostate. Prostatic concentrations of 1,25D positively correlated with these miRNA levels in both PCa and benign epithelium, demonstrating that PCa patients may still benefit from vitamin D₃. In cell assays, upregulation of these miRNAs by 1,25D was vitamin D receptor-dependent. Transfection of miR-100 decreased proliferation in primary cells while miR-125b suppressed migration and clonal growth of PCa cells, supporting a tumor suppressor function. 1,25D suppressed expression of previously bona fide mRNA targets of these miRNAs, E2F3 and Plk1, in a miRNA-dependent manner. Together, these findings demonstrate that vitamin D₃ supplementation augments tumor suppressive miRNAs in patient prostate tissue, providing evidence that miRNAs could be key physiologic mediators of vitamin D₃ activity in prevention and early treatment of PCa.
INTRODUCTION

In the past decade new research has revealed health benefits of vitamin D that extend beyond its role in calcium homeostasis. Preclinical, epidemiological and clinical studies show that maintaining vitamin D status has potential benefits for several conditions including cancer, diabetes, multiple sclerosis, infection, depression, pain, and cardiovascular diseases (1). Recent reports suggest a widespread deficiency of vitamin D in adults (2). In prostate cancer (PCa), the same factors that associate with decreased vitamin D levels (age, African American ethnicity, and residence at northern latitudes) also associate with increased PCa risk (3); suggesting vitamin D status alters PCa risk. The slow growing nature of PCa provides a long window of opportunity for chemopreventive agents, such as vitamin D (4). Please note that we use “vitamin D” in general discussion, whereas the specific form of vitamin D₃/metabolites will be used when describing specific results or experiments.

Several decades of studies support a chemopreventive role for vitamin D in PCa. A recent report showed that men supplemented with 4000 IU/day vitamin D₃ for one year had a decrease in positive cores at repeat biopsy compared to a control population (5). In PCa cell culture and in vivo studies 1,25-dihydroxyvitamin D₃ (1,25D) regulates proliferation(6), apoptosis (7), inflammation (8) and differentiation (8) through binding to the vitamin D receptor (VDR), a transcription factor mediating gene expression (9, 10). The Giovannucci group recently reported that men in the highest quartile of serum 25-hydroxyvitamin D₃ (25D) had a 57% decrease in risk of lethal PCa (11) which is consistent with other studies showing that high serum 25D levels were associated with decreased PCa incidence and mortality (3, 12). However, epidemiological studies do not consistently find association between vitamin D status and PCa risk (13-16). 25D is the major circulating metabolite of vitamin D and precursor to the active 1,25D. The prostate
expresses the VDR protein (17) and CYP27B1, the enzyme which converts 25D into 1,25D (18), demonstrating that local production of 1,25D occurs in the prostate. Therefore, local prostatic levels of 1,25D may be an important factor in determination of vitamin D status and PCa risk.

Given the genomic activity of vitamin D, via VDR binding to DNA and regulating gene transcription, it is likely that both coding genes and non-coding RNAs are regulated by vitamin D. MicroRNAs (miRNAs) are small (~22 nucleotide) non-coding RNAs that canonically function via binding to the 3’ untranslated region of target mRNA resulting in mRNA degradation and/or translational repression (19, 20). Aberrant expression of miRNAs is observed in human cancer tissues/cells and may promote carcinogenesis and progression (21-25). MiRNAs signatures unique to PCa have been identified (21-24) and various oncomiR and tumor suppressive miRNAs characterized. 1,25D and testosterone regulated several miRNAs in LNCaP cells (26). However, to date no studies have investigated miRNAs regulated by vitamin D or vitamin D metabolites in human primary prostatic epithelial cells or in prostate cancer patients.

In the current study, we identified and characterized miRNAs that are regulated by vitamin D. MiRNA expression was profiled in normal human prostatic epithelial cells that were treated with a non-growth inhibitory dose of 1,25D. Candidate miRNAs were validated in laser-capture microdissected epithelium from patient prostate tissue from a clinical trial in which the men were given various doses of vitamin D3 prior to radical prostatectomy. Targets of validated miRNAs and their effects on cell phenotype were further characterized in vitro. This translational study found that the chemopreventive activity of vitamin D in the prostate involves up-regulation of tumor suppressor miRNAs.
MATERIALS AND METHODS

Cell cultures

As described previously (27), primary prostatic epithelial cells (PrE) were established from radical prostatectomy tissue from the University of Illinois at Chicago Medical Center. Fresh tissue from the peripheral zone was selected by the pathologist according to an IRB-approved protocol. Briefly, the tissue is digested in collagenase, and plated on collagen-coated dishes in PrEGM media (Lonza, Walkersville, MD) for epithelial cell outgrowth. Epithelial cells are abbreviated PrE cells (from benign prostate peripheral zone) or PrE-Ca (from an area of >80% cancer). PrE cells do not express cancer markers and PrE-Ca cells express AMACR. All cells were used at secondary passage and 70% confluency. 1,25D was given at 50 nM, a non-growth inhibitory dose for these cells. LNCaP cells were grown in RPMI Media/10% fetal bovine serum.

Clinical Samples

Paraffin blocks of prostatectomy specimens from forty-five patient in the clinical trial were used. In this trial 66 patients (age 42-67) were randomized into three dose groups of vitamin D₃ (cholecalciferol); 400, 10,000 or 40,000 IU/day given orally in the time interval (3-8 weeks) between randomization at diagnosis and prostatectomy. The trial was registered with www.clinicaltrials.gov (NCT00741364). The specimens were obtained from Dr. T. van der Kwast (University Health Network, Toronto, Canada) who, blinded by treatment group, demarcated benign and PCa areas for laser-capture microdissection. Benign: glands in the peripheral zone without signs of atrophy or inflammation and without high grade PIN. PCa: highest density of Gleason grade 3 glands.
**Laser Capture Microdissection (LCM)**

Two 8 micron FFPE sections of each patient prostatectomy specimen were mounted on RNase-free PEN slides (Leica, Buffalo Grove, IL). As previously described by our group (28), specimens were deparaffinized, fixed, and stained with 0.5% toluidine blue. Using the Leica LMD-6000 (Leica) 100-150 acini of benign and PCa epithelium were collected into Eppendorf caps containing digestion buffer (Life Technologies, Grand Island, NY).

**RNA isolation**

For cell cultures, total RNA was isolated with Trizol (Life Technologies). For patient samples, RNA was extracted from the prostate tissue with the RecoverAll kit using a modified protocol as previously described (28). RNA quality and quantity were evaluated by measuring absorbance at 260 and 280 nm using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE).

**qRT-PCR of prostate cells and clinical samples**

Cells: For miRNA analysis, stem-loop cDNAs were generated on 10 ng of RNA using a pool of 5X RT primers and the TaqMan® MicroRNA RT Kit (Applied Biosystems, Foster City, CA). For mRNA analysis, cDNA was generated from 500 ng of RNA with the High Capacity Kit (Applied Biosystems). qRT-PCR was run with either SYBR green (mRNA) or TaqMan Gene Expression master mix (miRNA) (Applied Biosystems) using the StepOne Plus machine (Applied Biosystems). Ct values were normalized to housekeeping genes TBP and/or B2M for mRNA and RNU44 and/or RNU48 for miRNA.
Tissue: For miRNA analysis 45 ng of RNA was used with Megaplex Pool A RT Primers and the TaqMan miRNA RT kit. For mRNAs, RNA (50ng) was reverse-transcribed using the Vilo cDNA Kit (Invitrogen, Carlsbad, CA). cDNAs were pre-amplified according to the manufacturer’s protocol using TaqMan PreAmp master mix and specific TaqMan-designed assays (Table S1). For miRNAs, the Megaplex Pool A Pre-Amp primers were used in the preamplification reaction. Fold-changes were calculated using $2^{-\Delta\Delta CT}$ method and normalized to the expression of RNU44 and RNU48 or mRNA housekeeping genes (B2M, HPRT, Actin, GAPDH, and/or CK18).

**Vitamin D metabolite measurement in serum and prostate tissue**

Serum and tissue 25D levels were measured by liquid chromatography-tandem mass spectrometry as described previously (29). Serum and tissue 1,25D levels were determined by enzyme immunoassay (EIA) (Immunodiagnostic Systems, Scottsdale, AZ). In tissues, vitamin D was pre-extracted prior measurement as previously described (29). Tissue vitamin D metabolites were reported as the mean of two samples (transitional and peripheral zone) of fresh frozen tissue. Full statistical analysis of serum and tissue levels of vitamin D metabolites are reported elsewhere (Wagner et al manuscript currently in review).

**miRNA profiling by TaqMan Low Density Array (TLDA)**

PrE cells (70% confluency) were treated with 1,25D or ethanol for 24H and RNA was extracted. Megaplex pool of RT primers (Applied Biosystems) specific to either TLDA plate A/B and 350 ng RNA was used for the cDNA reaction and PCR was run according to the manufacturer’s protocol. Results were analyzed with RQ Manager, Data Assist Software
and manual 2$^{-\Delta\Delta C_T}$ method. Cts> 33 were omitted and RNU44 and RNU48 were analyzed as endogenous controls.

**Pre-miR transfection**

PrE or LNCaP cells were transfected using NeoFx reverse transfection kit (Ambion, Austin, TX) with a hsa-pre-miR or hsa-anti-miR to miR-100 or miR-125b (Applied Biosystems) at a final concentration of 5-50 nM.

**Cell proliferation Assay**

PrE cells were transfected with a hsa-pre-miR or with hsa-anti-miR at a final concentration 10 nM and plated in triplicate in a 24-well plate at a density of $2.5 \times 10^4$ cells per well for 72H. Cell number was counted with the Cellometer Auto T4 (Nexcelom Bioscience, Lawrence, MA).

**Migration (scratch) assay**

PrE cells ($3.5 \times 10^5$) were scratched 24H after transfection with 50 nM hsa-pre-miR. Pictures were taken at times 0, 2, 8, and 24H using the EVOS Digital Microscope (Advanced Microscopy Group, Bothell, WA). Migration was analyzed by percent closure of the area of the scratch with Image J software.

**Colonogenic assay**

LNCaP cells were transfected in triplicate with 50 nM hsa-pre-miR at a density of 500 cells/60mm dish. After 10 days, the colonies were stained with 0.5% crystal violet and counted.
Immunoblot

PrE cells were either treated with 1,25D and/or transfected (NeoFx) with a VDR or negative siRNA (Santa Cruz Biotechnology, Santa Cruz, CA) for 24H. Proteins were isolated in Cell Lysis buffer (Cell Signaling, Danvers, MA) followed by centrifugation. Protein (10µg) was loaded onto a 10% Bis-Tris NuPAGE gel (Invitrogen) and transferred to PVDF membrane. The membranes were probed with anti-VDR 1:500 (Santa Cruz Biotechnology), anti-β-tubulin 1:1000, anti-actin 1:1000, anti-PLK1 1:500 (Cell Signaling), or anti-E2F3 1:1000 (AbCam, Cambridge, MA). Bands were visualized using 20X LumiGLO reagent (Cell Signaling).

Statistical analysis

Unsupervised hierarchical clustering was done with DataAssist 3.0 (Applied Biosystems). For in vitro experiments the difference between two groups was analyzed with student’s t-test. In patient samples the correlation between miRNA and vitamin D metabolites was determined by Spearman correlation. A paired samples t-test was used to analyze differences in paired benign and PCa tissues. ANOVA analyzed the difference between vitamin D₃ treatment groups. Differences were considered significant at p<0.01 and p<0.05.
RESULTS

1,25D alters miRNA expression profiles in primary human epithelial cells

MiRNA expression was analyzed in PrE cells after 24H of 50 nM 1,25D or vehicle (0.01% ethanol) treatment. To control for cell density-induced changes in miRNAs (30), PrE cells were treated at 70% confluency with a non-growth inhibitory dose of 1,25D. Expression of 667 miRNAs was profiled by MicroRNA TLDA in five different patient-derived primary PrE cells. Of the miRNAs profiled, approximately 30% of miRNAs on Array A and 15% on Array B were detected (CT <33) in PrE cells (Table S2). Up- or down-regulated miRNAs were identified by a >1.5-fold increase or <0.5 decreased and a significance of p< 0.05 across three patients. Paired t-test identified miR-100, miR-125b and 29 other miRNAs that were increased by 1,25D and only one down-regulated miRNA, miR-196b (Table S3).

MiRNAs were selected for validation by magnitude of change and relevance to cancer/PCa from published studies (21-23, 25). miR-100, miR-125b, miR-200c, miR-197, miR-196b, miR-106b, miR-141, miR-103, miR-146a, miR-301a, miR-331-3p, and let-7b were analyzed by individual PCR in three of the original PrE cells and three additional PrE cells. Unsupervised hierarchical clustering of the three patients showed that intra-patient miRNA expression for these miRNAs was more similar than 1,25D-induced expression changes (Figure 1A). However, all 12 miRNAs were significantly regulated by 1,25D in the three original PrE cell lines (Figure 1A-B). Of the 12 miRNAs analyzed, individual qRT-PCR validation confirmed that miR-100 and miR-125b were the most consistently and significantly up-regulated by 1,25D (1.5-2.5 fold) across the six total PrE cells (Figure 1C).
Suppression of miR-100 and miR-125b targets by 1,25D

MiR-100 and miR-125b were further characterized, as they have known tumor suppressor activity (31, 32) and are located on the same chromosomal region 11q13 (33). We examined functional activity of miR-100 and miR-125b by measuring previously bona fide mRNA targets of the miRNAs; Plk1 for miR-100 and E2F3 for miR-125b (34, 35). MiR-125b was inversely correlated with its target E2F3 (r= -0.53, p= 0.01) and miR-100 to its target PLK1 (r= -0.49, p=0.02) in 9 patient-derived PrE cells, supporting regulation of these genes by the miRs. In PrE cells increased levels of miR-125b or miR-100 by 1,25D, decreased levels of E2F3 and PLK1 (Figure 1D). 1,25D dose dependently decreased E2F3 and PLK1 protein expression in PrE cells (Figure 1E).

miR-100, miR-125b, E2F3 and PLK1 are differentially expressed in PCa cells

We examined miR-100 and miR-125b expression in a matched pair of PrE and PrE-Ca cells that were derived from the same patient. The PrE-Ca cells which were AMACR positive (Figure 2A), had lower miR-125b in basal conditions compared to the normal PrE cells. 1,25D treatment increased miR-100 and miR-125b in both the normal PrE cells and PrE-Ca cells compared to their control further suggesting that 1,25D augments these miRNAs in normal and PCa cells (Figure 2B). We also measured expression of PLK1 and E2F3, the targets of the miRNAs, in our PrE cells. Both PLK1 and E2F3 levels were significantly higher in the PrE-Ca cells compared to normal PrE cells and 1,25D treatment decreased expression of both genes in PrE and PrE-Ca cells relative to their untreated control (Figure 2C). Overall these data suggest that miR-100 and miR-125b levels are lower in PCa and their suppression inversely correlates with PLK1 and E2F3.
miR-100 and -125b suppress cancer-associated phenotypes

In vitro phenotypes consistent with tumor suppressive functions of miR-100 and miR-125b (31, 36) were investigated by analysis of cell growth, migration, and clonogenicity, following modulation of miR-100 and miR-125b levels in PrE and LNCaP cells. Cell proliferation was quantified in both PrE and LNCaP cells 72H after transfection with pre-miRs or anti-miRs [negative, -125b, or -100]. MiR-100 overexpression (3-fold, data not shown) significantly decreased proliferation of PrE cells compared to control while neither miR-100 nor miR-125b altered cell proliferation of LNCaP cells (Figure 3A). Transfection of PrE and LNCaP cells with 10 nM anti-miRs only demonstrated a minimal increase in proliferation of LNCaP cells by miR-125b (Figure 3B). When we analyzed the targets, transfection with anti-miR-100 or -125b abrogated regulation of E2F3 and PLK1 by 1,25D, demonstrating that the miRNAs are required for their target regulation by 1,25D (Figure 3C). In addition, miR-125b overexpression in LNCaP cells decreased colony formation compared to the control (Figure 3D) which is suggestive of a malignant phenotype as clonogenicity correlates with tumorigenicity in animals (37). Cell migration by scratch assay showed that miR-125b decreased migration (Figure 3E). These data indicate that miR-100 and miR-125b have anti-migratory, anti-proliferative, and/or anti-colonogenic properties in prostate cells, which is consistent with tumor suppressor actions.

Regulation of miR-100 and miR-125b and their targets by 1,25D is VDR-dependent

Knockdown of VDR by siRNA reduced VDR protein levels ~50% which was sufficient to blunt VDR activity as demonstrated by reduced VDR-responsive CYP24A1 expression (Figure 4A-B). The reduction in VDR abrogated up-regulation of miR-100 and miR-125b by 1,25D (Figure 4C). VDR knockdown with siRNA also confirmed that regulation of E2F3 and PLK1 expression were VDR-dependent (Figure 4D). These results demonstrate that up-regulation of
miR-100 and miR-125 and down-regulation of their targets E2F3 and PLK1 by 1,25D occurs in a VDR-dependent manner.

**miR-100, miR-125b, E2F3 and PLK1 are differentially expressed in tissue**

The *in vitro* findings were validated in prostate tissue from a phase II clinical trial of vitamin D₃ in PCa patients. This trial was run at the University Health Network and Sunnybrook Hospital (Toronto, Ontario, Canada). Patients (N=66) were randomized to three treatment groups; 1=400 IU/day, 2=10,000 IU/day, 3=40,000 IU/day of oral vitamin D₃ (cholecalciferol) for 3-8 weeks prior to surgery (PI: Reinhold Vieth). Tumor and benign prostate epithelium from 15 patients per treatment group was collected by LCM (*Figure 5A*) and the expression of 12 miRNAs (miR-100, miR-125b, miR-103, miR-331-3p, miR-146a, miR-155, miR-197, miR-106b, miR-141, miR-301a, let-7a, and let-7b) was quantified by qRT-PCR. Areas of PCa were AMACR positive (*Figure 5B*) confirming that the tumor was sampled. Both miR-100 and miR-125b were decreased in tumor compared to benign epithelium from PCa patients, p< 0.001 (*Figure 5B*). In addition, the other 9 miRNAs that we analyzed in patient tissue (miR-103, miR-331-3p, miR-146a, miR-155, miR-197, miR-106b, miR-141, miR-301a, let-7a and let-7b) were significantly lower in PCa tissue (*Table S4*). These results are consistent with previous findings that miR-100 and miR-125b are down-regulated in PCa (26). E2F3 and PLK1 expression were measured in a subset of the vitamin D₃-treated clinical trial samples. E2F3 was slightly increased in PCa versus benign epithelium (p= 0.09) and PLK1 was unchanged (*Figure 5C*). Overall these data suggest that miR-100 and miR-125b levels are lower in PCa and their suppression may upregulate PLK1 and E2F3.
miR-100 and miR-125b are upregulated by dietary vitamin D₃ in clinical trial specimens

Wagner and colleagues found that in serum from the high dose of vitamin D₃, 25D levels increased 300% over baseline and 1,25D 80% over baseline. In prostate tissue from the high group, 25D levels increased 30% and 1,25D 10% over the 400IU group (Wagner et al, under review). However, due to high intra-group heterogeneity, no significant difference in miRNA expression was observed when analyzed by treatment group. Irrespective of treatment groups, prostatic 1,25D concentrations positively correlated with miR-100 and miR-125b in both benign and PCa epithelium (Figure 5D, Table 1). miR-100 and miR-125b levels correlated with serum levels of 1,25D or 25D, but the results varied between benign and PCa tissue (Table 1). Six of the other 10 miRNAs analyzed (miR-106b, miR-141, miR-103, miR-331-3p, let-7a and let-7b) also positively correlated with prostatic 1,25D in either benign or PCa prostate epithelium (Table S5). These results fully substantiate the in vitro data and demonstrate that miR-100 and miR-125b and other miRNAs are regulated by vitamin D₃ in the prostate. Importantly, not only does vitamin D₃ augment expression of miRNAs in benign tissue, it also upregulates the miRNA levels in the PCa cells, bringing up the levels closer to normal.

In addition, the correlation between miRNA and target levels were measured in a subset of tissue (N=15) from the clinical trial, where we saw a trend toward PLK1 and E2F3 being correlated to their targets in normal and/or PCa [miR-125b (normal; r= -0.36, p= 0.09) (cancer; r= -0.30, p= 0.15); miR-100 (normal; r= -0.08, p= 0.40) (cancer; r= -0.35, p= 0.09)].
DISCUSSION

In this translational study we report miRNA regulation as a novel mechanism of vitamin D₃ in the prostate. 1,25D \textit{in vitro} and oral administration of vitamin D₃ in patients upregulated miR-100 and miR-125b, known tumor suppressor miRNAs and two of the oldest known animal miRNAs (38).

The miRNAs in our study do not overlap with the few published reports have examined regulation of miRNAs by vitamin D. In human myeloid leukemia, 1,25D increased miR-32 (39) and miR-181 (40) levels and altered differentiation and cell cycle respectively. Other studies examined the dual effects of 1,25D and stress (41) or testosterone (26) on miRNAs and identified miR-182, miR-22, 29ab, -134, 17, 20a as 1,25D-regulated miRNAs (26). miR-22 expression was induced by 1,25D in colon cancer cells (42). 1,25D can have powerful growth inhibitory effects \textit{in vitro} that are dependent upon dose and cell type. Growth inhibition \textit{in vitro} may confound miRNAs studies because cell-cell contact at higher cell density causes a robust upregulation of miRNA expression globally (30). Cell density was carefully controlled in our study and we utilized non-growth inhibitory dose of 1,25D in PrE cells.

By using both \textit{in vitro} and clinical approaches we were able to identify, validate and characterize miR-100 and miR-125b as targets of 1,25D in both cells and patient prostate tissue. Levels of miR-100, miR-125b and 9 other miRNAs were suppressed in PCa tissue compared to benign tissue, which support previous studies that found widespread downregulation of miRNAs in PCa (25). The fact that dietary vitamin D₃ and 1,25D were able to upregulate miRNAs in PCa tissue and PrE-Ca cells, where there was a significant suppression of miRNA expression, implicates an overall benefit of vitamin D in benign and PCa tissue. We observed that serum
25D correlated with the miRNAs only in the PCa areas (Table S5), suggests that cancer may respond to vitamin D₃ supplementation faster than the benign areas.

miR-100 and miR-125b have been shown to have both tumor suppressive and oncogenic properties depending on the cell type (21, 23, 31-33, 43, 44). In PCa, miR-100 levels are lower in tumor compared to normal tissue (31) and in the early stages of hepatocarcinoma suggesting involvement in carcinogenesis (44). miR-125b is hypermethylated in breast cancer (32), has decreased expression in hepatocellular cancer and suppresses malignant phenotypes in vitro and in xenografts (45). However, overexpression of miR-125b decreased anti-apoptotic proteins in PCa xenografts (46), high levels of miR-100 increased the risk of PCa reoccurrence (47) and miR-100 was oncogenic in acute myeloblastic leukemia (43). The seemingly discrepant roles for these miRNAs in cancer may be a result of specific model systems or overexpression methodologies. Our findings were consistent with tumor suppressor activities of miR-100 and 125b as that both miRNAs were significantly lower in PCa compared to benign epithelium and in vitro overexpression of the miRNAs decreased cancer-associated phenotypes.

There are several characterized targets for miR-100 and miR-125b, two of which we examined. Previously, others have validated the interaction between miR-100 and Plk1 and miR-125b and E2F3 with luciferase assays (34, 35). Consistent with these reports, we observed a negative correlation between miR-100 and Plk1 and between miR-125b and E2F3 in vitro. We speculate that due the small N, the association between the miRNAs and their targets was weaker in patient samples. Plk1 and E2F3 are cell cycle proteins that are overexpressed in a variety of cancers including PCa (21, 34, 35, 44). In PCa, E2F3 is positivity correlated with the Enhancer of Zeste Homolog gene 2 (EZH2), an well studied PCa marker and oncogene (48). E2F3-positivity positively correlate with overall PCa survival, indicating that E2F3-EZH2 contribute to
PCa aggressiveness (49). EZH2 expression, measured in a small subset of our patients (N=20), was increased in PCa compared to benign tissue (p=0.05) (Figure S1), but was not correlated with 1,25D or 25D.

Let-7a is located on chromosome 11q13 (33) adjacent to miR-100 and miR-125b-1. A recent report identified let-7, miR-100 and miR-125 as three of the four most frequently selected miRNA classifiers of cancer patient outcome across 46 publications (50). In our study, let-7a was not regulated by 1,25D in our array therefore not included in the original validation. Because of its chromosomal location, we later quantified let-7a in a subset of tissues and found that let-7a also positively correlated with prostatic 1,25D in both benign (p=0.047) and PCa epithelium (p=0.033) (Figure S2). The let-7 family is well studied as tumor suppressive and let-7a suppressed PCa cell proliferation and tumor growth in vivo (51).

In the clinical trial samples, there was heterogeneity in prostatic 1,25D levels within each treatment group, because of which there were no significant differences in miRNA levels when analyzed by treatment group. The variation in prostatic 1,25D may be a result of inter-patient variability in calcium levels and/or vitamin D metabolism. Importantly, when we analyzed prostatic 1,25D concentration there was a strong correlation between prostatic 1,25D and miRNAs, which fully validated our in vitro findings.

There is a controversial epidemiological relationship between vitamin D status and PCa risk that has been based upon serum levels of 25D or 1,25D. While serum 25D measures overall vitamin D status, the results of our clinical trial suggest that serum levels do not directly correlate to tissue levels. In our data, miR-100 and miR-125b correlate better with prostatic 1,25D than to serum 1,25D or 25D and there was no correlation to tissue 25D. The positive correlation between the miRNAs and tissue 1,25D supports the hypothesis that local tissue levels of 1,25D
are important in PCa pathogenesis, as prostate cells express CYP27B1, (18) locally producing 1,25D. Therefore, not only is there an effect of local prostatic 1,25D, but also measurement of prostatic 1,25D may be superior to serum in epidemiological studies on PCa risk.

We show that vitamin D regulates known tumor suppressive miRNAs, miR-100 and miR-125b in both PCa and benign prostate tissue cells and tissue. The tumor suppressive activities of these miRNAs support a chemopreventive role for vitamin D. Our data also indicate that vitamin D$_3$ and 1,25D may upregulate miRNAs globally to counteract the downregulation of miRNAs observed in cancer. In addition, the fact that a seemingly short duration of treatment with vitamin D$_3$ in PCa patients significantly upregulate these miRNAs suggests that further long-term studies with vitamin D$_3$ could be usefully in globally regulating miRNAs in the prostate to alter cancer progression and/or outcome.
Acknowledgements

This research was supported by the NIH grant K22 CA133105 (Nonn), American Institute for Cancer Research Postdoctoral Scholar Grant (Giangreco), the Canadian Cancer Society (Vieth), and by a studentship from Canadian Institutes for Health Research (Wagner). We thank the PCa patients who donated their tissue for primary cell cultures and who participated in the clinical trial, Lindsay Gallagher for sectioning of the clinical trial specimens, Dr. Margaret Wright and Ryan Deaton for statistical assistance and Dr. Alan Diamond for proofreading of the manuscript.
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† Spearman r
‡ p value two-tailed, Gaussian approximation
*p < 0.05
Figure 1. miRNAs regulated by 1,25D in PrE cells. A, unsupervised hierarchical clustering of the top 12 miRNAs altered by 1,25D in three PrE cells. Mean levels of miR-100 and miR-125b expression, B, by TLDA analysis in three PrE cells and, C, by individual qRT-PCR in six PrE cells treated with 50 nM 1,25D for 24H. Error bars = SEM; * p< 0.5. Expression normalized to RNU44/RNU48. D, mean expression of miR-125b, miR-100, E2F3 and PLK1 by qRT-PCR in nine PrE cells treated with 1,25D. miRNA and the target expression are relative to their own control treatment. ** p<0.01, mean ± SEM. E, E2F3 and PLK1 protein measurement by immunoblot in PrE cells treated for 24H with 10-100 nM 1,25D.
Figure 2. Differential expression of miR-100, miR-125b and their targets in 1,25D-treated PrE cells. qRT-PCR shows the expression of AMACR, A, miR-100 and miR-125b, B, and PLK1 and E2F3, C, in 1,25D-treated paired PrE and PrE-Ca cells derived from one patient. Error bars=SD of experimental replicates and experiments were repeated twice.
Figure 3. Functional effects of miR-100 and miR-125b in PrE and LNCaP cells. 

A, Cell proliferation in PrE and LNCaP cells 72H following transfection with pre-miR-neg (open), -100 (horizontal), or -125b (vertical). Mean fold change of cell count 4 separate experiments. Error bars = SEM * p< 0.05. 

B, Cell proliferation in PrE and LNCaP cells 72H following transfection with anti-miRs. Mean fold change of cell count from 3 separate experiments. Error bars = SEM. 

C, E2F3 and PLK1 mRNA expression by qRT-PCR in PrE cells 24H after transfection with anti-miRs (25nM) and 24 h treatment with ethanol or 1,25D. Error bars=SD of experiments with two different PrE cells. 

D, colony formation assay of LNCaP cells transfected with pre-miRNAs (50 nM). Mean of experimental replicates, error bars = SD. Repeated 3 times. 

E, migration of PrE cells 0-48H after scratch. PrE cells were transfected with pre-miRs 24H prior to scratch. Graph is representative of duplicate experiments. Error bars = SD.
**Figure 4.** Up-regulation of miR-100 and miR-125b and their targets by 1,25D is VDR-dependent. 

**A,** VDR protein levels examined by immunoblot 48H after transfection with 20 nM Neg or VDR siRNA and 24H treatment with 1,25D in PrE cells. 

**B,** CYP24A1, miR-100 and miR-125b, PLK1 and E2F3, expression by qRT-PCR. Error bars = SD of experimental replicates and were repeated three times.
Figure 5. MiR-100 and miR-125b expression in prostate epithelium from men treated with vitamin D₃. A, experimental flow chart for analysis. B, miR-100, miR-125b and AMACR expression by qRT-PCR in benign and PCa epithelium (N=42 patients). C, E2F3 and PLK1 expression by qRT-PCR in benign and PCa (Normal N=14, PCa N=16). Error bars = SEM, ## p<0.001 or * p<0.05. D, Spearman correlation between prostatic 1,25D concentration and miR-125b or miR-100 level in benign and PCa epithelium.
11 CONTRIBUTIONS

As with most studies, the work presented in this thesis are a result of the combined efforts of several individuals and here I outline my own contributions to each study.

Study 1: An evaluation of automated methods for measurement of serum 25-hydroxyvitamin D

In this study, I assayed the 390 serum samples for 25(OH)D on both the LIAISON and Roche Modular platforms and compared these values to those obtained from RIA 25(OH)D measurements I had previously conducted during my MSC. Once I had collected these data, I performed the statistical analyses, interpreted the results and wrote the paper presented in this thesis. Heather Hanwell assisted with the biochemical measurements, statistical analyses and review of the paper.

Study 2: The ratio of serum 24,25-dihydroxyvitamin D₃ to 25-hydroxyvitamin D₃ is predictive of 25-hydroxyvitamin D₃ response to vitamin D₃ supplementation

In this study, I conceived (along with Dr. Vieth) the 24,25(OH)₂D to 25(OH)D ratio as a potential indicator of vitamin D catabolism, performed the statistical analyses of data, interpreted the results, presented this work at the “14th Workshop on Vitamin D” (Brugge, Belgium), and wrote the paper described in this thesis. Mehrdad Yazdanpanah and Kareena Schnabl from UHN were primarily responsible for developing the LC-MS/MS assay.

Study 3: Determination of 1,25-dihydroxyvitamin D concentrations in human colon tissues and matched serum samples

In this study, I developed and validated the method to measure vitamin D metabolites from human tissue, conducted all the biochemical measurements, performed the statistical analyses of
data, interpreted the results, presented this work at the “Vitamin D and Analogs in Cancer Prevention and Therapy” conference (Homburg, Germany), and wrote the paper described in this thesis. Andre Dias (undergraduate student) helped during the initial stages of method development.

Study 4: Randomized clinical trial of cholecalciferol doses on tissue vitamin D metabolite levels and Ki67 immunohistochemistry at radical prostatectomy in prostate cancer patients

This clinical trial was the major component of my PhD work. As the study director, I managed the day-to-day operations of the trial, wrote the study protocol and case report forms, trained clinical research staff, addressed study-related enquiries, and liaised with study investigators. Patients were seen by study physicians (Drs. Fleshner, Finelli, Klotz) and their teams of clinical research associates. Prior to study commencement, I was directly involved in study design and regulatory submissions (Health Canada, Research Ethics Boards). Upon study completion, I conducted all the biochemical analyses in tissue and serum, and facilitated the immunohistochemical and gene expression analyses. Tissue microarrays of FFPE prostate tissue were created by Doris Li (undergraduate student). Immunohistochemical staining was performed by a service lab at UHN (PRP lab). Immunohistochemical scoring was conducted by Dr. Dominique Trudel (pathologist, fellow), who optimized the digital scoring algorithm and trained the tissue pattern recognition software (Genie). I performed the statistical analyses of all data, interpreted the results, presented this work at the “FASEB Experimental Biology Conference” (San Diego, California), and wrote the manuscript presented in this thesis.