

**INVESTIGATING THE SAFETY AND THERAPEUTIC POTENTIAL OF VITAMIN D<sub>3</sub>  
WITH CALCIUM SUPPLEMENTATION IN PATIENTS WITH MULTIPLE SCLEROSIS**

by

Samantha Kimball

A thesis submitted in conformity with the requirements for  
the degree of PhD  
Graduate Department of Nutritional Sciences  
University of Toronto

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# INVESTIGATING THE SAFETY AND THERAPEUTIC POTENTIAL OF VITAMIN D<sub>3</sub> WITH CALCIUM SUPPLEMENTATION IN PATIENTS WITH MULTIPLE SCLEROSIS

PhD, 2011  
Samantha Kimball  
Department of Nutritional Sciences  
University of Toronto

*“Desperate diseases must have desperate remedies.”*

English Proverb

## ABSTRACT

Low vitamin D status has been consistently associated with an increased risk of multiple sclerosis (MS). Further, preclinical and *in vitro* data demonstrate immune regulatory properties of 1,25-dihydroxyvitamin D that may be beneficial for patients with MS. To date evidence of beneficial *in vivo* immunomodulation by supplementation with vitamin D<sub>3</sub> in humans is lacking. In a one-year, open-label, phase I/II dose-escalation study of vitamin D<sub>3</sub> (average ~14,000 IU/d over one year) with calcium (1,200mg/d) in patients with MS, we compared the effects of treatment on safety outcomes, clinical outcomes and selected biomarkers of immune system activity, relative to matched MS patients [age, sex, disease duration, disease modifying therapy, and expanded disability status scale (EDSS)] randomized to receive no supplementation. Mean serum 25(OH)D concentrations were 78.1±27.0 nmol/L at baseline and at one-year were 82.7±34.8 and 179.1±76.1 nmol/L in control and treated groups, respectively. Serum and urinary calcium and all other safety outcomes were unchanged throughout the trial. Compared to controls, treated patients tended to have fewer relapses (McNemar, p=0.09) and a greater

proportion had a stable or improved EDSS at study end ( $p=0.018$ ). We observed significantly reduced lymphocyte proliferative responses to antigenic challenge in the treatment group at one year, compared to baseline and control group responses. High serum 25(OH)D concentrations were not associated with short-term adverse effects in patients with MS, but with evidence of clinical improvement and beneficial immunomodulation.

## ACKNOWLEDGEMENTS

Reflecting back on the past four years I am extremely grateful to many people. I have been lucky to have been afforded many opportunities and to have worked within a collaboration of many skilled clinicians and scientists.

I would like to express my deep thanks to my supervisor, Reinhold Vieth. He has fulfilled the roles of teacher, mentor and colleague. I appreciate how he was able to provide intellectual support and guidance while giving me freedom to explore possibilities and learn my own style of writing and research.

I would like to thank Jodie Burton for teaching me about multiple sclerosis. It was a pleasure to work closely with her through all aspects of designing the trial that comprises this work through to its completion.

I am very appreciative to have been able to work with the many amazing people who agreed to participate in this trial, for their interest in vitamin D and for demonstrating a hope and an overall attitude towards life that I admire.

I am grateful to my advisory committee, Wendy Ward, Jennifer Gommerman and Tibor Heim who provided advice, encouragement and practical suggestions. I am appreciative of all the members of the Vieth lab, particularly Heather and Dennis, for all the conversations, support and laughs.

I would like to thank my sisters for never asking if 'my thesis was done yet' and for being my biggest fans. I thank my friends Tammy, Chama and Anne Marie for their support and for listening, even when I wasn't making any sense.

To my parents, I could never articulate how much their continued support and encouragement has meant to me, thank you.

Finally, I truly cannot adequately put into words my appreciation for my best friend and my partner, Peter, for his neverending support and encouragement. Thank you.

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## CONTRIBUTIONS

As with most clinical trials, the study presented in this thesis is the result of the combined efforts of a team of skilled investigators, without whom this work would not have been possible. The VitD4MS study investigators include myself, Dr. Jodie Burton (neurologist, St. Michael's), Dr. Reinhold Vieth (Mt Sinai), Dr. Paul O'Connor (St. Michael's), Dr. Hans Michael Dosch (Sick Kids), Dr. Amit Bar-Or (Montreal Neurological Institute, MNI), Dr. Cheryl D'Souza (University of Toronto), Dr. Melanie Ursell (community neurologist), Roy Cheung (Sick Kids) and Donald Gagne (MNI). The design of the trial was influenced at some stage by all those involved.

My specific roles in the clinical trial included working closely with the lead neurologist and principal investigator for the trial, Dr. Jodie Burton. Together, we wrote ethics submissions, consent forms, study forms and coordinated with various departments (for example ultrasound, ECG, and the lab) at St. Michael's Hospital to facilitate required testing at each study visit. We submitted ethics applications and obtained approval from the Research Ethics Boards at St. Michael's Hospital, Mt Sinai Hospital and the University of Toronto. Dr. Vieth obtained approval from Health Canada. Dr.'s Vieth and O'Connor applied for and obtained funding for the study.

With respect to collection of study data, my role was integral. I solicited patients from the MS Clinic at St. Michael's Hospital and written, informed consent from all study participants was obtained by myself or Dr. Burton. I coordinated patient visits, collected, aliquotted and stored samples. For all visits between baseline and end-of-study I was responsible for patient interviews. I performed many of the sample analyses myself (matrix metalloproteinases,

cytokines, c reactive protein, bone markers and vitamin D metabolites) and was responsible for quality control testing of the vitamin D<sub>3</sub> dose batches prepared by the pharmacy at St. Michael's hospital. The routine biochemistry testing done at each study visit was done by the lab at St. Michael's. Lymphocyte proliferation assays were performed by Roy Cheung at Sick Kids, cytokine analyses were performed by myself and Donald Gagne and quantitation of vitamin D binding protein performed by my lab-mate Banaz Al-Khalidi. Together, Dr. Burton and I performed statistical analyses and wrote the papers presented in chapters 4-6. Dr. Burton took the lead for the paper presented in chapter 4 and the papers presented in chapters 5 and 6 were largely written by myself, but all of the study investigators discussed the results, reviewed and edited all manuscripts. Overall, it has been a successful and rewarding collaboration.

I have presented the data contained within this thesis at several meetings over the last few years, including at the European Congress for Treatment and Research in Multiple Sclerosis (ECTRIMS 2008 and 2009), Experimental Biology (2008), End MS (Multiple Sclerosis Society of Canada, 2007) and the European Charcot Foundation (2010).

## **PUBLICATIONS (Published or Submitted)**

Chapter 1, Section 1.2:

**Samantha Kimball**, Ghada El-Hajj Fuleihan, and Reinhold Vieth. **Vitamin D: A growing perspective.** *Cri Rev Clin Lab Sci* 2008. 45:339-414.

Chapter, Section 1.4:

**Samantha Kimball**, Heather Hanwell, and Brenda Banwell. **Vitamin D and multiple sclerosis.** (To be submitted).

Chapter 4.0:

Jodie Burton, **Samantha Kimball**, Reinhold Vieth, Amit Bar-Or, Hans Michael Dosch, Roy Cheung, Donald Gagne, Cheryl D'Souza, Melanie Ursell, and Paul O'Connor. **A phase I/II dose-escalation trial of vitamin D<sub>3</sub> and calcium in multiple sclerosis.** *Neurology* 2010; 74(23):1853-9.

Chapter 5.0:

**Samantha Kimball**, Reinhold Vieth, Amit Bar-Or, Hans-Michael Dosch, Roy Cheung, Donald Gagne, Paul O'Connor, Cheryl D'Souza, Melanie Ursell, and Jodie Burton. **Evidence of *in vivo* immunomodulation by vitamin D<sub>3</sub> with calcium in multiple sclerosis.** *Journal of Clinical Endocrinology and Metabolism* (submitted).

Chapter 6.0 (majority):

**Samantha Kimball**, Jodie Burton, Banaz Al-Khalidi, Paul O'Connor, and Reinhold Vieth. **Vitamin D metabolite response to high-dose cholecalciferol in patients with multiple sclerosis.** *Journal of Clinical Endocrinology and Metabolism* (submitted).

Chapter 6.0 (urinary calcium data):

**Samantha Kimball**, Jodie Burton, Paul O'Connor, and Reinhold Vieth. **Urinary calcium response to high-dose cholecalciferol in patients with multiple sclerosis.** *Clinical Biochemistry* (submitted).

Chapter 6.0 (cross-reactivity data for LC-MS/MS method):

Dennis Wagner, Heather E. Hanwell, Kareena Schnabl, Mehrdad Yazdanpanah, **Samantha Kimball**, Lei Fu, Gloria Sidhom, D errick Rousseau, David E.C. Cole, Reinhold Vieth. **The ratio of serum 24,25-dihydroxyvitamin D<sub>3</sub> to 25-hydroxyvitamin D<sub>3</sub> predicts 25-hydroxyvitamin D<sub>3</sub> response to vitamin D<sub>3</sub> supplementation.** *Clinical Chemistry* 2010 (In Press).

## LIST OF ABBREVIATIONS

1 $\alpha$ -OHase	1 $\alpha$ -hydroxylase; CYP27B1
24-OHase	24-hydroxylase; CYP24A1
25-OHase	25-hydroxylase; CYP27A1
1,24,25(OH) <sub>3</sub> D <sub>3</sub>	1,24,25-trihydroxyvitamin D <sub>3</sub> ; calcitric acid
1,25(OH) <sub>2</sub> D <sub>3</sub>	1,25-dihydroxyvitamin D <sub>3</sub> ; calcitriol
25(OH) D <sub>3</sub>	25-hydroxyvitamin D <sub>3</sub> ; calcidiol
24,25(OH) <sub>2</sub> D	24,25-dihydroxyvitamin D

Abbos	BSAp147 (an epitope of BSA)	CCPGSMS	Canadian Collaborative
ALT	Alanine Transaminase		Project on Genetic Susceptibility to
ALP	Alkaline Phosphatase		Multiple Sclerosis
APC	Antigen Presenting Cell	CD	Cluster Designation
AST	Aspartate Transaminase	CNS	Central Nervous System
BBB	Blood Brain Barrier	CRP	C Reactive Protein
BAP	Bone Alkaline Phosphatase	CS	Casein
BLG	Beta Lacto-Globulin	CSF	Cerebrospinal Fluid
BSA	Bovine Serum Albumin (BSAp193 is an epitope of BSA)	CTL	Cytotoxic T lymphocyte (CD8+)
		CTx	C Teloptide
BMC	Bone Mineral Content	Cyt-c	Cytochrome c
BMD	Bone Mineral Density	DC	Dendritic Cell

DBP	Vitamin D Binding Protein	Ig	Immune globulin (IgG or IgM)
DMD	Disease Modifying Drug	IL	Interleukin (e.g. IL-2)
DNA	Deoxyribonucleic Acid	IFN	Interferon (IFN- $\gamma$ )
DRIP	Vitamin D Receptor Interacting Protein	KLK	Kallikrein (KLK-6)
DZ	Dizygotic (twin)	LT $\alpha$	Lymphotoxin $\alpha$
EAE	Experimental Allergic Encephalomyelitis	MBP	Myelin Basic Protein
ECG	Electrocardiogram	MHC	Major Histocompatibility Glycoprotein
ECM	Extracellular Matrix	MMP	Matrix Metalloproteinase (MMP-9)
EDSS	Expanded Disability Status Scale	MRI	Magnetic Resonance Imaging
Ex-2	Exon-2 (epitope of MBP)	MZ	Monozygotic (twin)
FFQ	Food Frequency Questionnaire	NHANES	National Health And Nutrition Examination Survey
GAD	Glutamic Acid Decarboxylase (GAD-555 is an epitope GAD)	NO	Nitric Oxide
Gd	Gadolinium	NF $\kappa$ B	Nuclear transcription Factor $\kappa$ B
GFAP	Glial Fibrillary Acidic Protein	OPN	Osteopontin
HLA	Histocompatibility Leukocyte Antigen	PBM	Peak Bone Mass
HPLC	High Performance Liquid Chromatography	PBMC	Peripheral Blood Mononuclear Cell
iNOS	inducible Nitric Oxide Synthase	PHA	Phytohemagglutinin
		PI	Pro Insulin
		PLP	Proteolipid Protein

PPMS	Primary Progressive Multiple Sclerosis	VCAM	Vascular Cell Adhesion Molecule (VCAM-1)
PTH	Parathyroid Hormone	VDR	Vitamin D Receptor
RANKL	Receptor Activator of NFκB Ligand	VDRE	Vitamin D Response Element
RCT	Randomized Controlled Trial	VDDRI	Vitamin D-Dependent Rickets type I
RRMS	Relapsing-Remitting MS	VLA-4	Very Late Antigen 4; α4β1 integrin
RXR	Retinoic acid Receptor		
S-100	Glial antigen		
SPMS	Secondary Progressive MS		
Tep69	Epitope of Pro Insulin		
Th	T helper cell (Th1, Th2, Th17)		
Treg	Regulatory T lymphocyte		
TGF	Tumor Growth Factor		
TIMP	Tissue Inhibitor of Metalloproteinase (TIMP-1)		
TLR	Toll Like Receptor		
TNF	Tumor Necrosis Factor (TNF-α)		
TT	Tetanus Toxin		
UL	Tolerable Upper Intake Level		
USP	United States Pharmacopeia		
UVB	Ultraviolet B		
UVR	Ultraviolet Radiation		

## **1.0 INTRODUCTION & LITERATURE REVIEW**

*“Science is nothing but developed perception, interpreted intent, common sense rounded out and minutely articulated.”*

*Thomas Henry Huxley*

### **1.1.1 INTRODUCTION**

There is growing interest in the potential for nutrition to impact human health. In particular, vitamin D status is a modifiable environmental agent implicated in the susceptibility and treatment of many disorders, from cancer to autoimmune disease (1). Recently, a reappraisal of vitamin D<sub>3</sub> intakes was performed by the Institute of Medicine (IOM) and Health Canada. Where past recommendations consisted of adequate intakes (AI), new recommendations cite estimated average requirements (EAR), which may be considered an improvement as it suggests that the quality of evidence is higher and more abundant studies were available for the assessment. Although there were slight increases made for the intake recommendations and upper levels of intake (UL), it seems the IOM has still failed in this regard. Evidence suggests that not only are a great majority of Canadians vitamin D deficient (defined as serum 25(OH)D levels below 75nmol/L), 70-97% was the most recent estimate (2), but that intakes required to achieve serum 25(OH)D concentrations considered sufficient for optimal bone health are much higher. Whiting et al. reviewed recent studies to examine the requirements for replete vitamin D status (25(OH)D <75nmol/L) and found that adults under the age of 65 would need 2,000 IU/d, whereas adults over the age of 65 require much more at 5,000 IU/d to achieve this (3).

However, there is contention with what is considered “deficient.” The most common definition for vitamin D deficiency is serum 25(OH)D concentrations <75nmol/L because these levels have been shown to be necessary to optimize bone health (4;5). However, Health Canada and the IOM resist and define deficiency as <50nmol/L, resulting in a distribution shift to the left and a lower population prevalence of vitamin D deficiency. There is obviously a need for a consensus on this point to establish recommendations which meet the requirement for achieving the desired serum 25(OH)D concentration. Further, recommendations made by policy makers are intended for healthy individuals. Disturbingly, they have chosen to ignore the thousands of studies which have shown that higher vitamin D status is associated with improved health of various organs: heart, brain, breast, prostate, pancreas, muscle, nerve, eye, colon, liver, mood and immune function. The reason cited for not implementing dietary guidance that might prevent disease is the need of randomized controlled clinical trials that investigate several doses of vitamin D<sub>3</sub> supplementation.

The interest in vitamin D grows with each discovery of new roles it plays in various cell and tissue processes. Historically, vitamin D was known for its roles in calcium homeostasis and bone health. However, the number of tissues and cells that possess the capability to make and utilize the active metabolite of vitamin D, 1,25-dihydroxyvitamin D [1,25(OH)<sub>2</sub>D], extends far beyond those involved in calcitropic functions. The number of genes that contain vitamin D response elements (VDREs), more than 900 (6), continues to increase. *In vitro* evidence demonstrates that 1,25(OH)<sub>2</sub>D impacts cell growth, induces differentiation, stimulates apoptosis, enhances intracellular signaling pathways and, in particular, has various effects on immune cell regulation (7;8). However, no data exist demonstrating an *in vivo*

immunomodulatory effect of vitamin D supplementation in humans, either in patients with multiple sclerosis (MS) or in healthy states. Multiple sclerosis is a neurodegenerative, inflammatory, autoimmune disease for which vitamin D therapy has the potential to beneficially alter immune system responses. This thesis research investigates the therapeutic potential of vitamin D<sub>3</sub> with calcium supplementation in patients with MS.

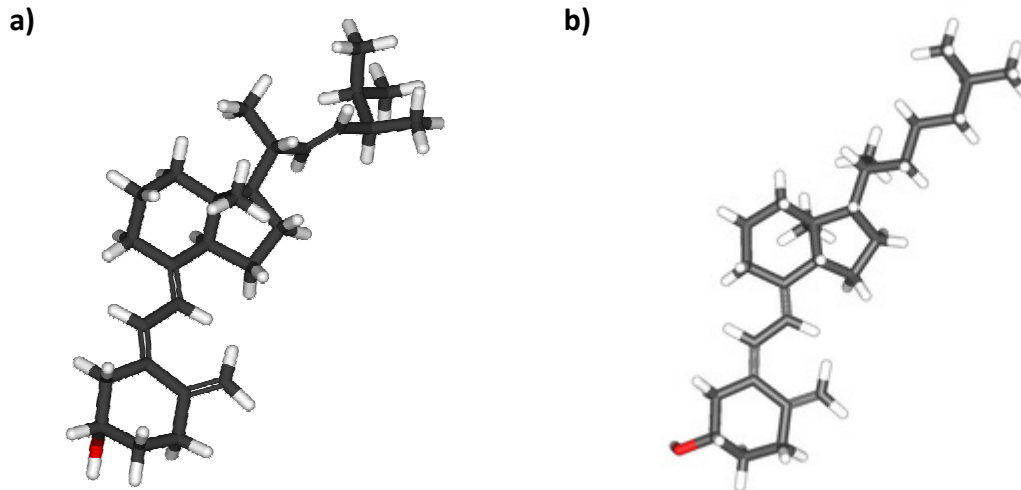
## 1.2 VITAMIN D

*“The most important thing in science is not so much to obtain new facts as to discover new ways of thinking about them.”*

*Sir William Lawrence Bragg*

### 1.2.1 Vitamin D Metabolism

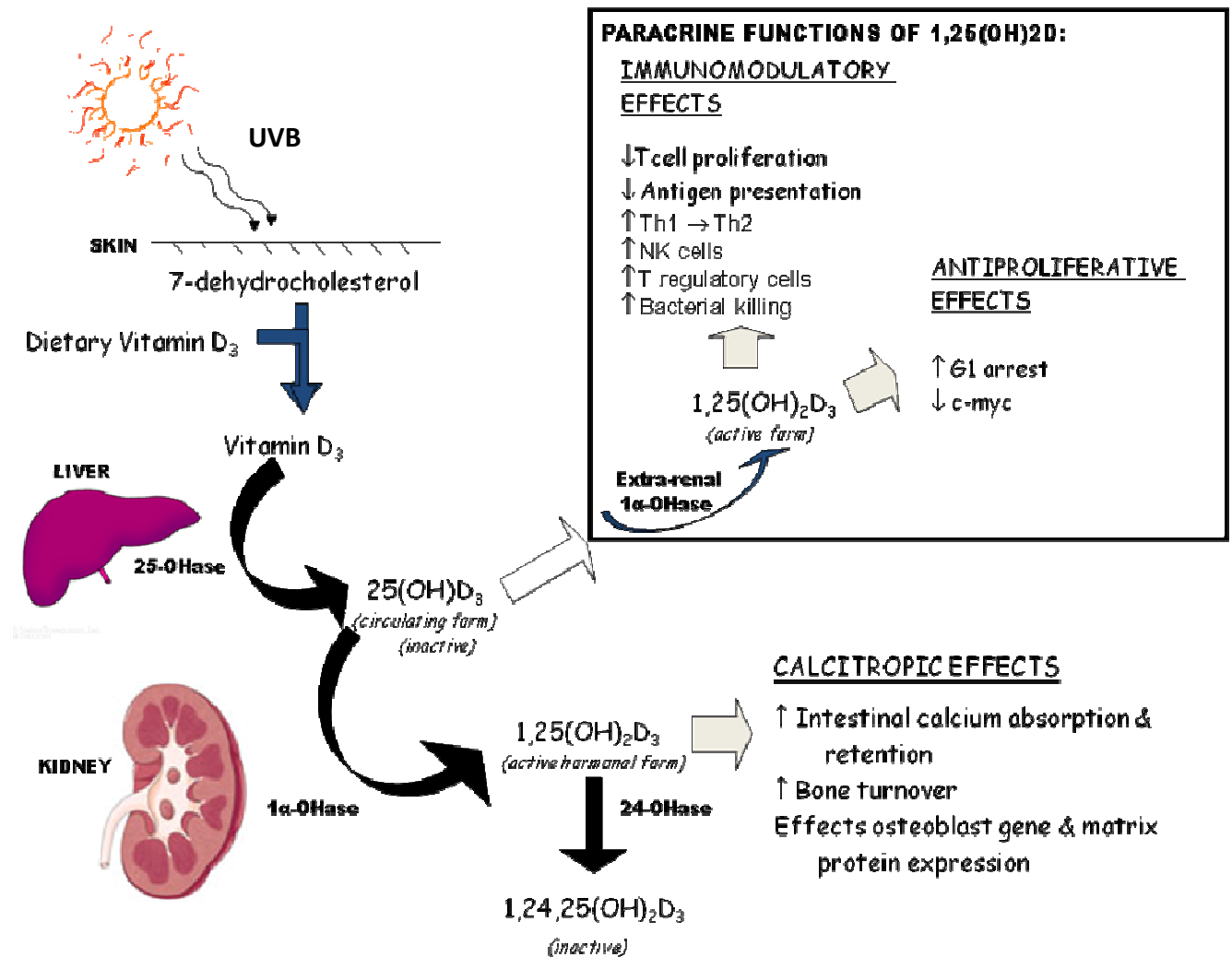
Vitamin D is a unique nutrient; vitamin D can be obtained in the diet or synthesized in the skin of humans and animals in response to ultraviolet B (UVB) radiation. The term ‘vitamin D’ encompasses many metabolites and 2 major isoforms: vitamin D<sub>2</sub> and vitamin D<sub>3</sub> (**Figure 1.1**). Vitamin D<sub>2</sub>, ergocalciferol, is of plant origin and differs from vitamin D<sub>3</sub>, cholecalciferol, by the presence of an additional double bond between the 22-23 carbon groups and an additional methyl group at the 24 carbon. The two metabolites also differ in potency. Vitamin D<sub>3</sub> has been shown to be 2- to 3-fold more effective than vitamin D<sub>2</sub> at raising serum 25-hydroxyvitamin D [25(OH)D] concentrations (9;10), the accepted measure of vitamin D status (11). Vitamin D<sub>3</sub> is a natural metabolite generated in the skin from its precursor, 7-dehydrocholesterol. The capacity to synthesize vitamin D<sub>3</sub> is dependent on a large number of factors, including zenith angle of the sun (influenced by latitude, season, and time of day), clothing coverage, use of sunscreen, age, skin pigmentation, ozone, pollution and cloud cover (12).



**Figure 1.1. Chemical structures of a) vitamin D<sub>2</sub> and b) vitamin D<sub>3</sub>.**

The molecules, derived from the 5-ring cholesterol backbone, differ by the presence of an additional double bond between the 22-23 carbon groups and an additional methyl group at the 24 carbon on the vitamin D<sub>2</sub> form.

The vitamin D metabolic pathway is similar for vitamin D<sub>3</sub> and vitamin D<sub>2</sub> and is illustrated in **Figure 1.2**. Whether synthesized, ingested as a supplement or obtained in the diet, vitamin D<sub>3</sub> is converted in the liver by P450 oxidases, 25-hydroxylase (either mitochondrial CYP27A1 or microsomal CYP2R1; 25-OHase), by the addition of a hydroxyl group at C-25 to produce 25-hydroxyvitamin D<sub>3</sub> [25(OH)D<sub>3</sub>]. Serum 25(OH) D<sub>3</sub> is the major circulating metabolite. Vitamin D<sub>2</sub>, obtained in the diet or as a supplement, undergoes the same hydroxylation reactions by the same enzymes as does vitamin D<sub>3</sub>. For example, vitamin D<sub>2</sub> is metabolized to 25(OH)D<sub>2</sub> by 25-OHase. Subscript numbers, D<sub>2</sub> and D<sub>3</sub>, of different metabolites distinguish the different parent compounds. However, for simplicity and because it is the more biologically relevant form, we will focus on vitamin D<sub>3</sub>.



**Figure 1.2. Vitamin D<sub>3</sub> Metabolism.**

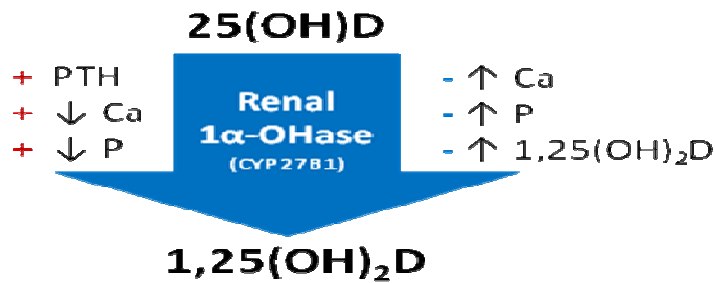
The photoconversion of 7-dehydrocholesterol in the skin of humans and animals is induced by exposure to ultraviolet B (UVB) radiation (295-315nm). Vitamin D enters the systemic circulation where the majority is hydroxylated by 25-hydroxylase [25-OHase] in the liver to produce the major, biologically inactive, circulating form, 25-hydroxyvitamin D [25(OH)D]. Hydroxylation of 25(OH)D occurs, via the action of 1 $\alpha$ -hydroxylase [1 $\alpha$ -OHase], primarily in the kidney to produce circulating 1,25-dihydroxyvitamin D [1,25(OH)<sub>2</sub>D], the biologically active form of vitamin D<sub>3</sub>, whose main function is in the regulation of calcium homeostasis. Many other cells possess 1 $\alpha$ -OHase and the nuclear receptor for 1,25(OH)<sub>2</sub>D, the vitamin D receptor (VDR), allowing for local conversion and use with effects ranging from cell differentiation and proliferation to immune cell regulation. Th1=T helper cell type 1 (pro-inflammatory); Th2= T helper cell type 2 (anti-inflammatory); NK= Natural Killer cells; G1=gap 1 of cell cycle (start of interphase); c-myc=transcription factor (oncogene).

At physiological doses, circulating vitamin D<sub>3</sub> is readily converted to 25(OH)D<sub>3</sub>, with a half-life of 4-6 hr (13). Approximately 75% converted on a single pass through the hepatic circulation (14). The activity of 25-OHase follows first-order kinetics and conversion rates are dependent on substrate availability, or concentrations of vitamin D<sub>3</sub>. Although biologically inactive, 25(OH)D<sub>3</sub> has a half-life of 8 weeks (15;16) and is the accepted measure of vitamin D<sub>3</sub> nutritional status (11).

Activation of 25(OH)D occurs by the addition of another hydroxyl group at the C-1 position by 1 $\alpha$ -hydroxylase (CYP27B1; 1 $\alpha$ -OHase) to produce the active hormonal form of vitamin D, 1,25-dihydroxyvitamin D<sub>3</sub> [1,25(OH)<sub>2</sub>D<sub>3</sub>]. Renal expression of 1 $\alpha$ -OHase controls circulating concentrations of 1,25(OH)<sub>2</sub>D<sub>3</sub> which are tightly regulated by reciprocal changes in rates of synthesis and degradation (14). Unlike 25(OH)D<sub>3</sub>, 1,25(OH)<sub>2</sub>D<sub>3</sub> has a short half-life of approximately 8 hours in the systemic circulation (17). Both 25(OH)D<sub>3</sub> and 1,25(OH)<sub>2</sub>D<sub>3</sub> metabolites are inactivated by a third cytochrome P450 enzyme, 24-hydroxylase (CYP24A1; 24-OHase), which oxidizes the side chain to produce 24,25-dihydroxyvitamin D<sub>3</sub> [24,25(OH)<sub>2</sub>D<sub>3</sub>] and 1,24,25-dihydroxyvitamin D<sub>3</sub> [1,24,25(OH)<sub>3</sub>D<sub>3</sub>], respectively (18). Further degradation of 24,25(OH)<sub>2</sub>D<sub>3</sub> and 1,24,25(OH)<sub>3</sub>D<sub>3</sub> via 24-OHase results in the final catabolic product calcitric acid.

The major physiological function of 1,25(OH)<sub>2</sub>D<sub>3</sub> is maintenance of extracellular calcium concentrations. Circulating concentrations of 1,25(OH)<sub>2</sub>D<sub>3</sub> are tightly controlled primarily by induction of renal 1 $\alpha$ -OHase and 24-OHase expression (**Figure 1.3**). In response to low calcium concentrations, calcium receptors in the parathyroid gland stimulate the release of parathyroid

hormone (PTH). In turn, PTH acts on renal tubular cells to induce expression of  $1\alpha$ -OHase (19) while simultaneously suppressing 24-OHase activity (20). Induction of  $1\alpha$ -OHase is



**Figure 1.3. Regulation of renal  $1\alpha$ -hydroxylase.**

Stimulation (+) or inhibition (-) of  $1\alpha$ -OHase occurs by extracellular hormonal control (PTH, parathyroid hormone;  $1,25(\text{OH})_2\text{D}$ , 1,25-dihydroxyvitamin D) and mineral concentrations (Ca, calcium; P, phosphorus).

independently augmented by hypocalcemic and hypophosphatemic signals (21). To reestablish calcium levels,  $1,25(\text{OH})_2\text{D}_3$  functions to increase intestinal calcium absorption, increase renal calcium reabsorption and, in concert with PTH, mobilizes calcium from bone. Negative feedback inhibition by  $1,25(\text{OH})_2\text{D}_3$  acts to depress  $1\alpha$ -OHase activity and stimulate 25-OHase. In contrast, during states of hypercalcemia  $1\alpha$ -OHase activity is suppressed while 24-OHase activity is induced. Renal expression of  $1\alpha$ -OHase and 24-OHase are thus significant determinants of circulating  $1,25(\text{OH})_2\text{D}_3$  concentrations (22).

Systemic transport of the vitamin D sterols, including vitamin  $\text{D}_2$  and  $\text{D}_3$  metabolites, occurs in complex with vitamin D binding protein (DBP). From the blood stream, DBP-25(OH) $\text{D}^1$

<sup>1</sup> The terms 25(OH)D and  $1,25(\text{OH})_2\text{D}$  encompass both forms of vitamin D: 25(OH) $\text{D}_3$  and 25(OH) $\text{D}_2$ , and  $1,25(\text{OH})_2\text{D}_3$  and  $1,25(\text{OH})_2\text{D}_2$ , respectively.

is removed by a variety of target tissues where 25(OH)D is released into the cell and DBP is degraded. In addition to transport, DBP functions in binding and solubilization. The affinity with which DBP binds the various vitamin D metabolites varies due to different orientations of the ligands within the DBP binding pocket. DBP shows the greatest affinity for 25(OH)D and 24,25(OH)<sub>2</sub>D followed by 1,25(OH)<sub>2</sub>D, vitamin D<sub>3</sub> and vitamin D<sub>2</sub> (23).

Recent evidence suggests that DBP also has roles in inflammation and in the immune system. DBP associates with vitamin D sterols and a number of other molecules including, globular actin, fatty acids and various cell membrane components. DBP binds actin and is believed to act as an actin scavenger during tissue damage and necrosis (24). Further, DBP can associate with the plasma membrane of immune cells, including lymphocytes (25;26), monocytes (25;27), and neutrophils (28;29). DBP can be post-transcriptionally modified to form macrophage-activating factor, a molecule that primes macrophages to become cytotoxic (30;31). Although the function of cell-associated DBP is not fully elucidated, it is thought that neutrophil-DBP plays a role in chemotaxis by enhancing neutrophil response to complement protein C5a (32;33). Overall, DBP plays a role in the turnover and actions of vitamin D sterols and has other roles in inflammatory and immune processes that have not yet been fully clarified.

### **1.2.2 Mechanism of Action & Biological Functions of Vitamin D**

Biological functions of  $1,25(\text{OH})_2\text{D}^2$  are primarily mediated through binding with the Vitamin D Receptor (VDR). Once  $1,25(\text{OH})_2\text{D}$  enters the target cell it binds VDR in the cytoplasm of the cell and translocates to the nucleus where the VDR- $1,25(\text{OH})_2\text{D}$  complex then binds the Retinoic Acid Receptor (RXR) to form a heterodimer (34;35). The heterodimer, VDR- $1,25(\text{OH})_2\text{D}$ -RXR, elicits cellular responses by binding to genomic vitamin D response elements (VDREs). Bound VDREs can induce or inhibit gene transcription. Modulation of gene expression is dependent on the recruitment of coregulator protein complexes, else the VDRE-bound heterodimer is silenced by corepressors (36). To induce positive gene regulation, VDR- $1,25(\text{OH})_2\text{D}$ -RXR recruits coactivators and subsequently basal transcription factors and RNA polymerase II are recruited, and results in target gene transcription (6). As expected, VDR is expressed in target tissues involved in calcium homeostasis: intestine, kidney, bone, and parathyroid gland (37). In bone,  $1,25(\text{OH})_2\text{D}$  regulates the transcription of numerous osteoblast genes and matrix proteins to promote bone mineralization (38). In addition, when  $1,25(\text{OH})_2\text{D}$  concentrations are high,  $1,25(\text{OH})_2\text{D}$  stimulates osteoblasts to activate osteoclasts through the induction of the receptor activator of nuclear factor  $\kappa$ -B ligand (RANKL) (39;40). RANKL induces osteoclast differentiation and results in bone resorption which, during a state of hypocalcemia, helps to reestablish physiological calcium concentrations. These direct effects on calcium absorption and bone mineralization are the reason that vitamin D deficiency during growth can result in undermineralized bone that during infancy causes rickets.

In the past couple of decades, research has begun to demonstrate other functions of  $1,25(\text{OH})_2\text{D}$ , from roles in immune system activity to cellular proliferation, differentiation, and

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<sup>2</sup> The terms  $25(\text{OH})\text{D}$  and  $1,25(\text{OH})_2\text{D}$  encompass both forms of vitamin D:  $25(\text{OH})\text{D}_3$  and  $25(\text{OH})\text{D}_2$ , and  $1,25(\text{OH})_2\text{D}_3$  and  $1,25(\text{OH})_2\text{D}_2$ , respectively.

apoptosis. VDREs have been found in over 900 genes (6;41). Cellular differentiation is induced by  $1,25(\text{OH})_2\text{D}$  (42). Immune cell function is modulated by  $1,25(\text{OH})_2\text{D}$  and is discussed in further detail in **Section 1.4.3**. Briefly, pro-inflammatory cytokine expression is down-regulated in dendritic cells and Th1 lymphocytes by  $1,25(\text{OH})_2\text{D}$ , antigen-presenting capacity of macrophages and dendritic cells is down-regulated by  $1,25(\text{OH})_2\text{D}$  and an anti-inflammatory Th2 lymphocyte phenotype and regulatory T cell (Treg) differentiation are promoted by  $1,25(\text{OH})_2\text{D}$  (43). Many genes in prostate, colon and breast cancer cells are regulated through the VDR (44). The presence of VDR and  $1-\alpha\text{OHase}$  in extra-renal tissues suggests autocrine/paracrine production and utilization of  $1,25(\text{OH})_2\text{D}$  which has diverse physiological functions. Furthermore, extrarenal  $1-\alpha\text{OHase}$  is subject to different regulation than the renal enzyme and appears to augment local production of  $1,25(\text{OH})_2\text{D}$  in target cells (45). Vitamin D nutrition, therefore, has effects beyond bone health, and optimal vitamin D status may serve to improve the function of multiple systems and improve general health. Further, vitamin D may have beneficial effects on several disease states such as cancer and autoimmune disease, specifically multiple sclerosis (1;46).

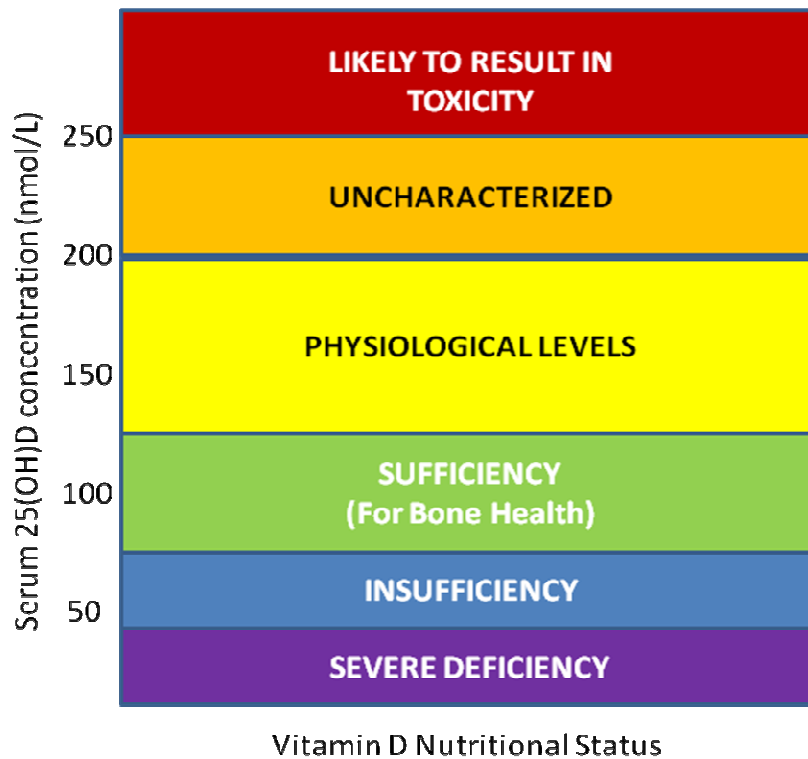
### **1.2.3 Vitamin D Nutritional Status**

Vitamin  $\text{D}_3$  nutritional is unique among vitamins and minerals because it varies with different environmental influences; status is affected by latitude, culture, and food fortification laws. The most important source of vitamin  $\text{D}_3$  is sunshine exposure (47), which is constantly demonstrated by seasonal variations in vitamin  $\text{D}_3$  levels. Seasonal variation is exhibited among

many races, ages and countries (48-58), even at southerly latitudes such as Florida (59;60) and Italy (60).

Vitamin D<sub>3</sub> status is measured by assessing circulating concentrations of 25(OH)D. Vitamin D<sub>3</sub>, obtained by diet, supplements or derived from sun exposure, is readily metabolized to 25(OH)D in the liver within ~3-5 d (61-63). Further, evidence to date suggests that oral supplements are as effective at maintaining vitamin D<sub>3</sub> status as sun-derived vitamin D<sub>3</sub> acquisition. The active metabolite, 1,25(OH)<sub>2</sub>D, has a half-life of 8 hours and its concentrations are ~1000-fold less than 25(OH)D. Further, circulating concentrations of 1,25(OH)<sub>2</sub>D are under tight regulation by parathyroid hormone and compensatory mechanisms which will produce normal levels of 1,25(OH)<sub>2</sub>D even when 25(OH)D concentrations are deficient. Thus, 25(OH)D concentrations are used to determine vitamin D nutritional status and to determine whether an individual is vitamin D deficient or not. **Figure 1.4** is a schematic of broad classifications of vitamin D status based on serum 25(OH)D concentrations and the available evidence.

“Severe deficiency” of vitamin D in infancy or childhood causes a mineralization defect of the collagen matrix that is laid down by osteoblasts. The structural support of the matrix is compromised as a result and increases bone deformity and fracture (64). In childhood, severe vitamin D deficiency results in rickets and is consistently associated with 25(OH)D concentrations <25nmol/L (65). Rickets typically develops during the early months of life and early signs consist of growth failure, lethargy and irritability, followed by more detectable clinical changes such as craniotables, costochondral beading, swelling of the distal ends of long



**Figure 1.4. Vitamin D nutritional status classifications based on serum 25(OH)D levels.**

bones (wrists & ankles), and bowing of the long bones (66). Symptoms of hypocalcemia can cause convulsions, stridor and neuromuscular irritability (spasms) (67) and fractures may occur. In children 1-3 years of age, stunting of growth, bowing of the legs, muscle weakness, walking problems and deformation of the pelvis signal vitamin D deficiency. Healing of skeletal deformities are largely reversible by vitamin D treatment (68).

In adolescence rickets can occur during the pubertal growth spurt (69). A waddling gait, lower limb and back pain, bowing of the legs and muscle weakness are common symptoms (66). Hypocalcemic tetany is also common (69). Skeletal deformation may be permanent after late rickets occurs despite corrective treatment with vitamin D, emphasizing the need to ensure adequate vitamin D intake during adolescence. Heaney et al. (70) stress that rickets reflects

severe vitamin D deficiency and that milder degrees of insufficiency may not produce clinical symptoms but can cause reduced efficiency in utilization of dietary calcium, impeding full acquisition of the genetic potential for bone mass.

According to Osteoporosis Canada ([www.osteoporosis.ca](http://www.osteoporosis.ca)), 2 million Canadians suffer from osteoporosis with a staggering 1.9 billion dollars spent on treatment per year. The causes of osteoporosis are multifactorial and include low peak bone mass (PBM), gender, genetics, and nutrient intake (71). A strategy to prevent osteoporosis, characterized by low bone mass and deterioration of bone, involves maximizing PBM during adolescence to help prevent bone resorption later in life (72).

Bone density and fracture prevention studies are used to determine serum 25(OH)D concentrations that are considered “sufficient.” Data collected from 4,100 Americans (>60y) in The Third National Health and Nutrition Examination Survey (NHANES III) found an association between increased 25(OH)D levels and increased bone mineral density (BMD) within a range of 25(OH)D of 22.5-94 nmol/L (73). In a meta-analysis of 12 randomized controlled trials (RCTs) for non-vertebral fractures (n=42,279) and 8 RCTs for hip fractures (n=40,886), anti-fracture efficacy of vitamin D supplementation was found to be dose-dependent and increased significantly with 25(OH)D concentrations >75nmol/L and doses of vitamin D<sub>3</sub> >400 IU/d (5). Prevention of fractures with vitamin D supplementation was not found if doses were 400 IU/d or less (5). Vitamin D supplementation with 482-770 IU/d was found to reduce non-vertebral fractures by 20% and hip fractures by 18% (5). Further, a meta-analysis of 8 RCTs (n=2,426) found that supplementation with 700-1000 IU/d of vitamin D reduced the risk of falls by 19% (4), which are closely associated with incidence and risk of fracture (74), especially in the

elderly. Risk of falling was reduced only when serum 25(OH)D concentrations above 60 nmol/L were achieved (4). All evidence to date demonstrates that for optimal bone health, and thus for a status of vitamin D “sufficiency,” serum 25(OH)D concentrations >75 nmol/L and intakes of 700-1000 IU/d of vitamin D<sub>3</sub> are required (75). Henceforth, we consider serum 25(OH)D concentrations between 25 and 75 nmol/L as “insufficient”.

If the requirement for vitamin D were based on the serum concentrations of 25(OH)D found in a healthy population of individuals, the target population for such physiological values of 25(OH)D would be acquired by natural means, i.e. we should look to those individuals who are exposed, unblocked, to sunlight on a regular basis. Mean 25(OH)D concentrations of lifeguards, who worked in intense sunlight for at least 8 h/d, were  $133 \pm 84$  nmol/L (76), Puerto Rican farmers were found to have 25(OH)D concentrations of  $133 \pm 50$  nmol/L (77), and Nigerian toddlers (2 mo - 5 y, n=33) were found to have mean serum 25(OH)D concentrations of  $109 \pm 74$  nmol/L (unpublished data). Normal “physiological” values of 25(OH)D are likely in the range of 100-150 nmol/L, although this remains controversial.

Dose-response studies have demonstrated that to increase 25(OH)D concentrations by 10nmol/L requires intakes of vitamin D<sub>3</sub> of 400 IU/d in adults and adolescents (78-80). The recommended intakes of vitamin D<sub>3</sub> published by Health Canada were updated in November of 2010. Although increased to 600 IU/d for people under the age of 70y (infants excepted) and 800 IU/d for people over the age of 70y (previously 200 and 600 IU/d, respectively), the relevance of these intakes are still disputed. On the other end of the spectrum, the tolerable upper intake level (UL) for vitamin D<sub>3</sub> was increased from 2,000 IU/d to 4,000 IU/d for the ages of 9 years and up. This UL is still considered too restrictive by many and it is not based on

current evidence (81). Hathcock *et al.* (82) conducted a risk assessment based on human clinical trials of vitamin D supplementation and found no evidence of toxicity in trials with vitamin D doses as high as 10,000 IU/d. In contrast to Health Canada's guidelines, the Canadian Cancer Society and the Pediatric Association both recommend intakes of 1,000 IU/d. Osteoporosis Canada recently published a guideline statement (83) advocating serum 25(OH)D concentrations >75 nmol/L and increasing recommended intakes of vitamin D of 800-2,000 IU/d for older adults. One must consider that the majority of Canadians live close to the Canadian-US border, latitude of ~44°N, and thus are not able to synthesize vitamin D through the winter months (November through March). Factor in expected dose-response to the recommended 600 IU/d, which only raises serum 25(OH)D concentration by 15 nmol/L, and the increased likelihood of deficiency for at least 6 months of the year is undeniable. Intakes of 4,000 IU/d may be necessary to obtain "physiological" serum 25(OH)D concentrations of ~100 nmol/L (81), and optimal vitamin D<sub>3</sub> intakes for other health outcomes may be even higher. Due to the broad expression of 1 $\alpha$ -OHase and VDR, it has been hypothesized that vitamin D status may have many uncharacterized affects in health and disease, particularly with respect to immune system function. Vitamin D status is therefore of interest in autoimmune diseases such as multiple sclerosis.

## 1.3 MULTIPLE SCLEROSIS

*“Lack of understanding about the cause of MS has generated a remarkable variety of hypotheses over the last century. Few mechanisms have not been proposed to explain the bewildering phenomena associated with this disease.”*

*George Ebers, 1998*

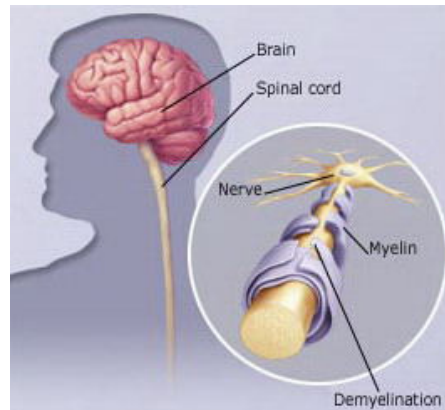
### 1.3.1 Multiple Sclerosis

There are two major types of cells in the CNS: neurons and glial cells. Oligodendrocytes are glial cells that surround nerve cell bodies and axons to serve several functions including: providing supporting elements and structural support, and the formation of myelin (84). Myelin, a lipid-rich protein, is formed in compacted spirals around the axons of neurons in the CNS (Schwann cells are the counterpart of oligodendrocytes in the peripheral nervous system) (85). Myelin functions as an electrical insulator allowing for rapid salutatory propagation of signals along the nerve and protects against axonal damage (86).

Demyelination, or loss of myelin (**Figure 1.5**), characterizes neurodegenerative disease and results in the disruption of nerve signaling. Diverse symptoms are the result of the CNS region affected and the function of the neurons involved.

Multiple Sclerosis (MS) is a demyelinating disease of the central nervous system (CNS) and the most common neurological disease in adults between 20 and 40 years of age. “Multiple” refers to both temporal and spatial changes, and signifies the many areas in the CNS where demyelination occurs and repeated attacks over time, respectively. “Sclerosis” refers to the scarring that accumulates in the CNS when inflammatory attacks are resolved.

MS was first described by Jean-Martin Charcot in 1868 in patients with intermittent episodes of neurological dysfunction as *'la sclerose en plaques.'* Charcot noted the accumulation of inflammatory cells within the brain and spinal cord with a perivascular distribution (87). Lesions of the CNS adversely affect the neural pathways involved in that region of the brain or spinal cord, and translate to clinical deficits. For example, the involvement of long tract neural pathways would predominantly impair the lower extremities. Motor and sensory signs and symptoms are common abnormalities that result from lesions of the CNS and thus represent some of the symptoms at clinical presentation in patients with multiple sclerosis.



**Figure 1.5. Demyelination in multiple sclerosis.**

### **1.3.2 Clinical Presentation and Subtypes of Multiple Sclerosis**

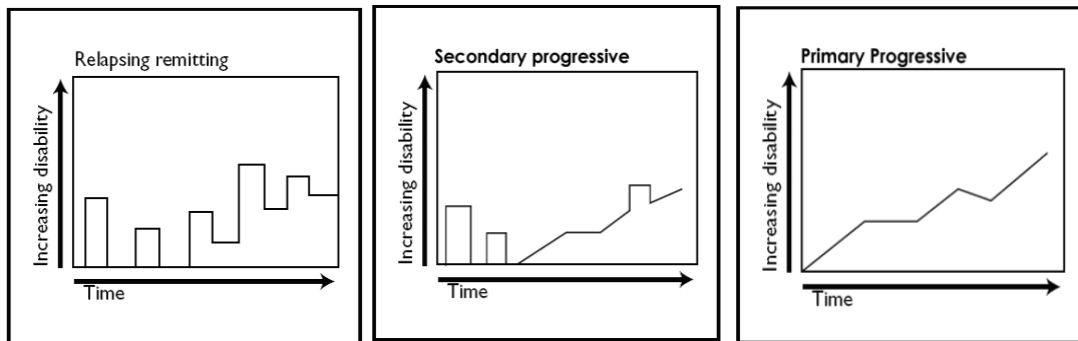
Multiple sclerosis is an extremely complex and heterogeneous disease. MS can lead to substantial disability through deficits of sensation, motor, autonomic and cognitive function in some individuals, whereas others may live with a 'benign' course of disease in which little

disability accumulates. Almost any neurological symptom can appear in patients with MS (**Table 1.1**) and few are disease-specific. Some examples of symptoms include weakness, numbness, tingling sensations, dizziness, extreme fatigue, poor coordination, balance problems, stumbling, paralysis, blurred vision, slurred speech, facial and body spasms, chronic pain, heat sensitivity, bladder and bowel problems, and sexual dysfunction. Cognitive changes, such as difficulties in concentration, and psychological problems, such as depression, can also occur. Heterogeneity exists in all aspects of MS including variability in patient signs and symptoms, pathological and histological findings, presence of immune cells and antibodies, response to therapy, and prognosis.

**Table 1.1 Signs and symptoms of multiple sclerosis.**

<b>Physical</b>	<b>Cognitive</b>	<b>Psychologic</b>
Vision loss	Information processing	Depression
Spasticity	Executive function	Personality and mood changes
Paralysis	Problem solving	
Chronic pain	Attention span	
Fatigue	Learning and memory	
Sensory symptoms		
Paroxysmal symptoms		
Bladder, bowel, and sexual dysfunction		
Heat intolerance		

Multiple sclerosis can be categorized into two main forms (**Figure 1.6**): i) relapsing-remitting, typified by recurrent attacks of symptoms followed by periods of improvement, and ii) progressive MS, either primary or secondary, characterized by worsening of MS symptoms over time without periods of improvement. It is not clear what factors are responsible for the different courses.



**Figure 1.6 Subtypes of multiple sclerosis characterized by patterns of increasing disability over time.**

- i) *Relapsing-remitting multiple sclerosis (RRMS)* is the most frequent type. Approximately 80% of patients present with an acute attack from which they recover completely. Typically, RRMS presents with sensory disturbances, including optic neuritis, Lhermitte’s symptom (an electrical sensation that runs down the spine or limbs evoked by neck flexion), weakness of limbs, clumsiness, gait ataxia, bladder and bowel symptoms, and fatigue that is often worse in the afternoon. Uhthoff’s phenomenon, a worsening of symptoms with increases in core body temperature, may also be seen (88). Attacks of symptoms will recur at variable intervals separated by periods of mostly complete recovery. At some stage (after 5-25 years) patients fail to recover neurological function after relapses and transition to the secondary progressive stage in which, following relapses, disability starts to accrue (88).
  
- ii) *Secondary progressive multiple sclerosis (SPMS)* is generally a second stage of RRMS for most patients and after 10 years approximately 40-45% of disability progresses independently of relapses, in addition to which relapses become less frequent (89).

Progression involves persistent signs of CNS dysfunction that may, or may not, occur after a relapse. Eventually, patient symptoms may evolve to include any of the following: cognitive impairment, depression, speech problems, difficulties swallowing, vertigo, progressive limb paralysis, sensory loss, tremors, spasticity, pain, sexual dysfunction and other manifestations of CNS dysfunction (88).

iii) *Primary progressive multiple sclerosis (PPMS)* is characterized by a gradually progressive clinical course without evidence of relapse or remission periods. About 10-15% of patients present with insidious disease onset and steady progression (89). Often PPMS presents with a slowly evolving deficit in the lower limbs that gradually spreads and worsens.

The etiology of MS remains unclear, despite decades of research, and no single factor has been shown to confer susceptibility, thus MS is likely a multifactorial disease. Indeed, there is likely heterogeneity within the pathological processes in different individuals given the variability in clinical presentation and clinical course of the disease. It is not known what determines the different phases, nor can it be predicted when the transition from one phase of MS will occur. It is also unknown if RRMS and PPMS represent different clinical manifestations of the same disease process or whether there are different pathogenic mechanisms involved. Evidence suggests that MS is an inflammatory demyelinating disease and is likely autoimmune in nature (88). The fundamental concept underlying the autoimmune classification of MS is that a wide array of immune cells and inflammatory mediators interact to cause destruction of myelin and axonal damage which, in turn, results in a variety of neurological deficits.

### **1.3.3 Pathophysiology of Multiple Sclerosis**

The most widely accepted paradigm of multiple sclerosis pathogenesis is that of an autoimmune disease. The major reasons that MS is considered autoimmune are: 1) the animal model of MS, experimental allergic encephalomyelitis, is induced by administration of a self-peptide, myelin; 2) inflammatory cells are prevalent in MS lesions; 3) genetic linkage studies show a strong association with a major histocompatibility allele, presumably because of the role of this encoded protein in antigen presentation and T lymphocyte activation; and 4) current MS therapies target immune cell function and trafficking (90). If inflammation is the driving force of tissue injury in MS, immunological mechanisms are a logical and effective target for altering the disease process and the major reason vitamin D<sub>3</sub> therapy is proposed to be of benefit in MS. However, it remains unknown whether inflammation precedes demyelination, or vice versa, and there are proponents of a neurodegenerative model of MS.

#### ***i) Genes and Environment***

The prevalence of MS in the general population of Canada is 111/100,000 and lifetime incidence of MS is 0.1%. Rates of MS vary across the world from 6 to 20/100,000. The risk of developing MS for the general population in Canada is 1/1000 (91), but for family members the risk increases with degree of shared genes (92). Further, prevalence of MS varies with gender, age, geography and ethnic background, but epidemiological data clearly indicate that there is a genetic predisposition (93).

The Canadian Collaborative Project on Genetic Susceptibility to Multiple Sclerosis (CCPGSMS) is a population-based cohort of patients with MS, and their families from across

Canada, in which researchers examined the genetic contribution of risk of MS. MS patients eligible for the CCPGSMS study were one of the following: a twin [monozygotic (MZ) or dizygotic (DZ)], an adoptee, adopted siblings,  $\geq$  one half-sibling, of non-European descent, of a conjugal MS partnership, or a member of a multiplex family (94). Genetically identical twins provide a unique tool to investigate the non-genetic factors influencing MS development, whereas half-siblings make it possible to examine the genetic contribution from mothers or fathers independently.

Prevalence is substantially increased in family members of MS patients with a cumulative effect of sharing genes. First degree relatives of affected individuals have a 2-5% higher risk of developing MS. Half-siblings of MS patients have a risk of approximately 2%, whereas full siblings have a risk of 3% (94). A parent-of-origin effect has also been demonstrated in which maternal contribution confers a risk of 2.4% versus a paternal contribution of 3% (94;95). The risk of MS in twin-pairs is higher in MZ pairs than in DZ, with a female concordance rate of 34% and 3.8%, respectively (91). Almost all twin studies in MS have demonstrated a higher degree of risk in MZ than DZ pairs (91;96-99), but rates vary between studies. The variation in twin concordance rates is thought to reflect the background prevalence of MS, as prevalence varies between countries. Further, a female preponderance exists, with an increasing sex ratio over the past 100 years, with a female to male ratio of greater than 3:1 (100).

Studies clearly demonstrate a genetic component to MS susceptibility; however, concordance rates in twins suggest a complex interplay between environmental and genetic factors. More than 20 whole genome screens have been performed in different MS populations

and different geographical areas, and, similar to other autoimmune diseases, the strongest association is found within the genes of the Histocompatibility Leukocyte Antigen (HLA) class II region (101;102). In particular, the HLA DRB1 and DQB1 alleles have been identified as predisposing (103), which is thought to account for 10-60% of the genetic risk of MS (104;105). Genotypes DRB1\*1501, DRB5\*0101, DQA1\*0102 and DQB2\*0602 have been identified (92;106) as risk alleles with a strong association in northern Europeans. The encoded proteins, Major Histocompatibility (MHC) molecules, play an important role in how T cells recognize antigen and thus in immune system responsiveness.

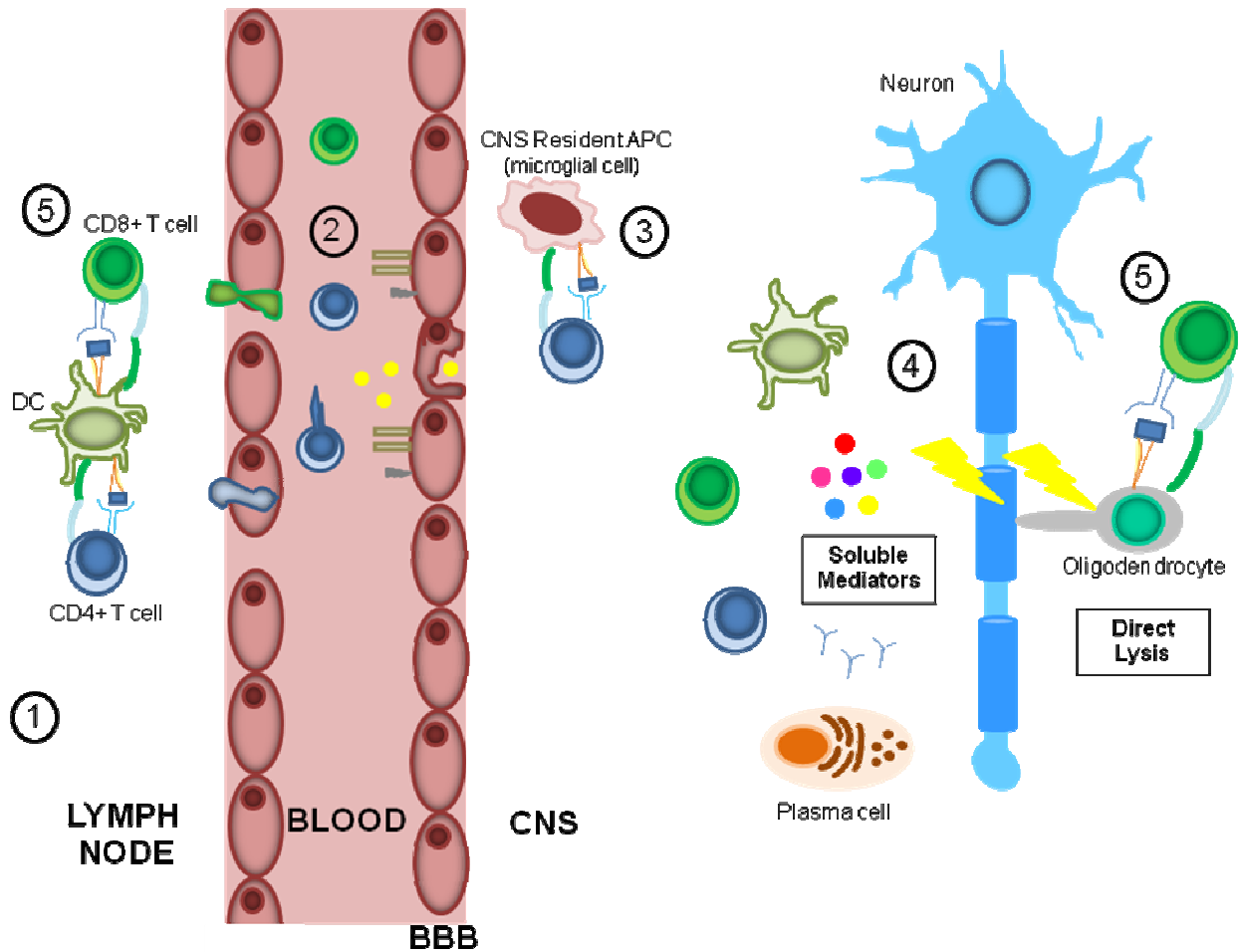
Among the putative environmental factors, infectious agents and lifestyle or behavioural influences have been proposed to contribute to development of MS. Further, because women are more susceptible than men, about 3:1 (100), and because relapses abate during pregnancy and rebound afterward, hormonal environment may also be a risk factor (107).

The hygiene hypothesis posits that individuals not exposed to infections early in life, because of a clean environment, develop aberrant responses to infections when encountering antigenic challenges as young adults and the result is autoimmune, inflammatory disease. Viral and bacterial infections are also candidates as environmental triggers of MS. Molecular mimicry occurs when the receptors of T and B cells share epitopes between infectious antigens and self-antigens; molecular mimicry is a logical explanation for auto-reactivity. Patients with MS are reported to have been infected with measles, mumps, rubella, and Epstein Barr Virus (EBV) at later ages than HLA-DR2 matched controls (108). EBV is the best candidate of these infections and shares four DRB1-restricted T Cell Receptor (TCR)-peptide contacts with myelin basic

protein (MBP) (109), such that an immune response generated against the virus may inadvertently cross-react with myelin and ultimately induce demyelination. A second hypothesis is the 'bystander activation' theory whereby T cell receptor (TCR)-independent bystander activation of autoreactive T cells occurs by means of inflammatory cytokines, superantigens, or molecular pattern recognition. Current data suggest that MS could be induced and/or exacerbated by various microbial infections, and the responsible agents are most likely ubiquitous pathogens in the general population, but hard evidence is scarce (90). Vitamin D status is also postulated as an environmental agent involved in the susceptibility and progression of MS, and is discussed in detail in **Section 1.4**.

## **ii) Pathogenesis**

The steps in the pathogenesis of MS, illustrated in **Figure 1.7**, are thought to begin with activation of autoreactive T cells which can recognize MHC complexes displaying peptides derived from myelin basic protein (MBP), the protein coating of nerves in the CNS. Although MBP-specific T cells can be isolated from MS patients and controls (110-112), in MS patients T cells reactive to MBP have been shown to have a higher activation state and a proinflammatory phenotype (113). T cells recognizing other myelin sheath components, including myelin oligodendrocyte glycoprotein (MOG) and proteolipid protein (PLP) have also been described. T cell migration through the blood brain barrier (BBB) is a central pathologic event in the process of lesion formation in MS. The sequence of cellular events includes adhesion of T cells to endothelial cells, chemoattraction and proteolysis of the basement membrane surrounding the BBB, followed by influx of inflammatory immune cells into the CNS. Activated autoreactive T



**Figure 1.7. Pathogenesis of multiple sclerosis.**

**1)** CD4+ T lymphocytes are primed in the periphery by antigen presenting cells (APCs), such as dendritic cells (DCs), presenting myelin epitopes within the cleft of MHC class II molecules. Co-stimulatory molecules and release of IL-12 by DCs activates the T cell. APCs residing in the central nervous system (CNS) capture myelin antigens and migrate to cervical lymph nodes, or soluble myelin antigens can drain from the CNS to lymph nodes where they are phagocytosed by local APCs. **2)** T cells express  $\alpha 4\beta 1$  integrin to bind endothelial vascular adhesion molecule 1 (VCAM1), or osteopontin (OPN). Release of matrix metalloproteinases (MMPs) by activated T cells and inflamed endothelial cells aids in the degradation of the blood brain barrier (BBB). T cells transmigrate the perivascular space and transverse the ECM to enter the CNS. **3)** T cells are re-activated in the CNS by APCs expressing myelin epitopes. **4)** T cells and APCs release inflammatory cytokines (IL-12, IL-2, IL-1 $\beta$ , TNF- $\alpha$ , IL-17) which recruit and activate other immune cells. The release of soluble mediators contributes to myelin destruction: APCs (IL-1 $\beta$ , TNF, free radicals), Th1 and Th17 lymphocytes (IFN- $\gamma$ , IL-17, IL-23, MMPs), plasma cells (Ig), complement, CTL (granzyme B, perforin, LT $\alpha$ , TNF, MMPs). Myelin protein degradation products then become phagocytosed and the cycle is perpetuated. **5)** CD8+ cytotoxic T cells are primed by cross-presentation by DCs presenting MHC class I-myelin peptide complexes in lymph nodes and follow the same steps 2-5 as CD4+ T cells. CD8+ T cells secrete soluble mediators and can directly lyse oligodendrocytes expressing MHC class I-myelin epitopes.

cells start to express  $\alpha 4\beta 1$  integrin (or very late antigen-4, VLA-4) which binds vascular cell adhesion molecule-1 (VCAM-1) expressed by inflamed endothelial cells of the CNS allowing transmigration through the BBB (114). Osteopontin, expressed by inflamed endothelial cells, acts as a cell adhesion molecule for activated T cells and may also play a role in transmigration of T cells through the BBB (114). T cells also produce matrix metalloproteinases (MMPs), among other mediators, that degrade both the basement membrane and the extracellular matrix (115), allowing T cells direct access into the CNS. Kallikrein 6 (KLK-6) has also been shown to be produced by activated T cells and likewise has protease activity to cleave BBB components (116;117) and may contribute to CNS access.

The major characteristic of MS is focal demyelination. Demyelination occurs within an inflammatory milieu of cells including T cells (CD4+ and CD8+) (118-120), B cells, plasma cells and extensive macrophage and microglial activation occurs (121). A variety of inflammatory cytokines are also found within the area of a lesion, such as interleukin-2 (IL-2), interferon gamma (IFN- $\gamma$ ) and tumor necrosis factor (TNF) (122;123). Osteopontin is also secreted which may increase the survival of activated CD4+ T helper 1 and 17 (Th1 and Th17) cells and may have a role in increasing pathogenic Th cell survival (114).

CD8+ T cells are also involved in lesion formation (124;125) and axonal destruction is associated with the presence of CD8+ T cells and macrophages (126). Autoreactive CD8+ cytotoxic T cells (CTLs) respond to peptide-MHC class I complexes presented by oligodendrocytes and astrocytes. CTLs produce MMPs and cytokines such as lymphotoxin  $\alpha$  (LT  $\alpha$ ) and TNF that can damage the myelin sheath, and may directly kill neurons via perforin/granzyme-mediated mechanisms. There is also evidence that antibodies to myelin

proteins, such as myelin basic protein (MBP) and myelin oligodendrocyte glycoprotein (MOG), participate (127). Oligodendrocytes, responsible for myelin formation, are susceptible to damage via a number of immune mediators including MMPs, OPN, oxygen and nitrogen radicals, antibodies, and complement-, perforin- and granzyme-mediated destruction (128).

Pro-inflammatory chemokines released by T cells, macrophages and microglia recruit neutrophils which cause damage. Among the mediators released, MMPs and OPN can degrade myelin and promote the release of more pro-inflammatory cytokines from the various cell types. In addition, nitric oxide (NO) produced by phagocytes, blocks nerve conduction pathways and contributes to neuronal degradation. Antigen presenting cells in the CNS (microglia and astrocytes) further perpetuate myelin damage and inflammation by presenting myelin antigens due to the destruction already occurring. Myelin is destroyed by macrophages, monocytes, cytokines, oxygen and nitrogen radicals produced during inflammation. But, inflammatory cells are not always present in active areas of demyelination, and leukocytes also play a role in repair by producing growth factors (129). Thus, the exact pathogenic role of the inflammatory response in MS is not clear.

Remission of MS is thought to occur when anti-inflammatory cytokines and growth factors produced by cells in the inflammatory infiltrate offset the autoimmune attack and allow the oligodendrocytes to remyelinate the damaged nerves. However, over time, the buildup of scar tissue around the nerves, coupled with the accumulating assaults on the oligodendrocytes, may prevent remyelination. At this point it is thought that the patient enters the progressive stage in which incomplete recovery from a relapse is characteristic.

In summary, the pathogenesis of MS is believed to be mediated by T cells reactive to CNS auto-antigens (including epitopes of the myelin sheath) which are activated in the peripheral circulation when they encounter antigen presenting cells (APCs) presenting myelin epitopes. These activated T cells then migrate through the blood brain barrier into the CNS where, upon presentation of myelin antigens by resident APCs, they are reactivated. Axonal damage results from the inflammatory milieu of soluble mediators and directly from cytotoxic cell responses. It is unknown what initiates and maintains this autoreactivity, but there is strong evidence to support the concept of T cell-mediated autoimmune disease pathogenesis.

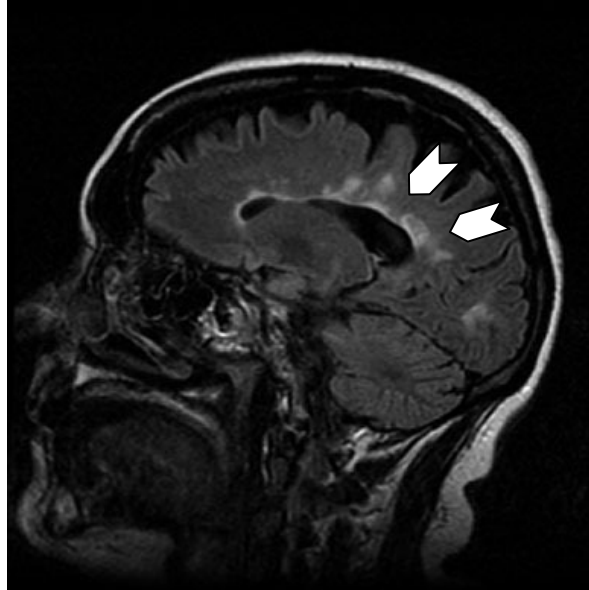
#### **1.3.4 Diagnosis and Measures of Disease Activity and Progression**

The diagnosis of MS is based on the detection of neurological dysfunction “disseminated in space and time” (130). The principle of diagnosis is to establish from clinical and laboratory evidence that demyelination within the CNS has affected more than one part of the CNS and on more than one occasion. MS frequently begins slowly with symptoms that are mild or vague but may be present for years before a patient is referred to a neurologist. Neurological examination consists of testing the following: eye and face movements (eyes following movements of an object), vision (acuity test), reflexes (e.g. reflex to running of a sharp instrument up the toe), limb strength (by resisting pressure on arms and legs), sensation (pin prick awareness on extremities) and coordination (walking a straight line heel-to-toe) (88). In many cases, presence of clinical abnormalities of motor, sensory, visual or autonomic systems is sufficient for

diagnosis; but, given the variability of symptoms, when clinical findings are ambiguous laboratory evidence can aid diagnosis.

***i) Magnetic resonance imaging (MRI)***

MRI imaging is used to visualize soft tissues. When elements with an odd atomic weight, such as water, are exposed to a magnetic field, the nuclei behave as spinning magnets and align in the direction of the applied field (131). Radio frequency applied systematically can alter their alignment to produce a detectable rotating magnetic field that can be used to construct an image (131). MRI is a technique used to image the brain and spinal cord and is the most sensitive technique used to aid in the diagnosis of MS, allowing the visualization of lesions located in white matter. MRI images are based on proton relaxation times which reveal differences in tissue water concentration. The relaxation times for differing compounds vary such that water and fat have different times and can thus be distinguished. Two types of relaxation are used in basic MRI scans: T1 (spin-lattice relaxation) and T2 (spin-spin relaxation). MRI shows focal areas of demyelination, presenting as white (T2 hyperintense) areas (**Figure 1.8**), in white matter in more than 95% of patients (132). The presence of multifocal lesions of various ages supports the clinical picture to aid in diagnosis of MS. However, MRI abnormalities alone are insufficient for the diagnosis of MS because lesions can appear in people without clinical evidence of disease; many elderly people have non-specific white matter lesions (132).



**Figure 1.8. Magnetic resonance image of areas of demyelination typical of multiple sclerosis.** (<http://www.health.com/health/static/hw/media/medical/hw/h9991221.jpg>).

There are several MRI metrics traditionally used in MS for measuring tissue destruction, including T2 hyperintense lesions, T1 hypointense lesions (or black holes), and contrast-enhanced lesions (gadolinium-enhanced T1 lesions). Gadolinium (Gd) is an element whose paramagnetic properties cause a change in local magnetization making it a useful contrast agent. Gd-enhancing lesions are obtained by intravenous injection of gadolinium prior to MRI and typically combined with T1-weighted sequences. Blood-brain barrier (BBB) leakage is a consistent early feature of lesion evolution, linked with entry of immune cells into the CNS and resulting inflammation. Gadolinium leakage reflects BBB disruption and indicates areas of active inflammation on MRI, usually perivascular, and represents new areas of disease activity. An association between the occurrence of Gd-enhancing lesions and exacerbations has been found in several longitudinal studies (133). Gd-enhancing lesions are commonly used in clinical studies to assess therapeutic efficacy. Studies have found that contrast-enhanced lesions predicted

relapses but not future disability, whereas measures of global atrophy have been shown to correlate better with disability (134). The characterization of white matter damage detected using conventional MRI techniques is routinely used in the diagnosis of MS, but is only weakly correlated with clinical symptoms (135). Beneficial clinical effects as a result of treatment in MS have not been seen without effects occurring on MRI and, although it is not a great marker of clinical disease activity, MRI remains the cornerstone of MS clinical trial outcomes. Overall, MRI measures are useful in aiding the diagnosis of MS and are the most widely used technique for monitoring disease activity, particularly for detection of new lesions, but their predictive value of neurological outcome in MS remains limited.

### ***ii) Relapse Activity***

Relapse is generally defined as a new episode of neurological dysfunction or re-emerging impairment that occurs for longer than 24 hours (in the absence of fever or infection). In clinical trials, relapse rates, often converted to an annualized relapse rate, are often measured as a primary endpoint (136). In addition, the proportion of patients who are relapse free at the end of a study is also commonly used as a measure of relapse activity. Natural history data on patients followed from disease onset demonstrate that early relapse rates are predictive of future disability (137).

### ***iii) Cerebrospinal fluid (CSF)***

CSF is the fluid, produced by the choroids plexus, that occupies the subarachnoid space and the ventricular system around and inside the brain. For laboratory studies, CSF is obtained by lumbar puncture. Because MS pathology involves abnormalities in the CNS,

including the influx of immune cells, measurement of various reactivities of cells in CSF has been postulated to reflect disease activity.

- a. Oligoclonal bands are electrophoretically detected immunoglobulins of identical specificity. Oligoclonal bands found in the CSF aid in the diagnosis of MS, with elevations reported in 90% of affected subjects (138-141). However, they are not used in clinical trials because they are not affected by MS therapies. Antigen-specificities of CSF oligoclonal Ig bands remain undefined (138).
- b. Myelin basic protein (MBP) is frequently increased in the CSF of patients with MS. A review of the literature found that CSF MBP correlates with gadolinium-enhancing lesions, severity of relapses, EDSS scores, and that CSF MBP levels remain elevated for weeks after symptom onset and further levels normalize with treatment (142). However, MBP presence in CSF is elevated in a number of neurological diseases and is therefore relatively nonspecific for patients with MS.

#### ***iv) Kurtzke's Expanded Disability Status Scale (EDSS)***

EDSS is the most widely used method to measure neurological impairment in patients with MS. EDSS quantifies disabilities in eight functional systems (pyramidal, cerebellar, brainstem, sensory, bowel and bladder, visual, cerebral and other) on an ordinal scale from 0 (normal health) to 10 (death) (143). For example, an EDSS score of 0-4.5 represents a fully ambulatory patient with mild disability, whereas a score of 5-9.5 represents greater disability and impairment to ambulation. Although EDSS classifies patients with respect to

disease severity, it has been criticized for its emphasis on ambulation status. EDSS scores are commonly used in clinical trials to measure worsening of disease.

Outcome measures for clinical trials are chosen to reflect a treatment's efficacy and the qualities most reflective of disease activity. There are many endpoints that have been used, or investigated for use, in studies investigating potential therapies in patients with MS. The most common clinical outcomes include relapse activity and EDSS scores. Radiological endpoints include lesion volume or number of lesions on T2 or Gd-enhanced MRI. Other endpoints may be included to investigate the effect of a treatment on, for example, immune system behavior. However, MS lacks a practical and reliable immunological marker of disease activity.

### **1.3.5 Other Biomarkers of Interest in the Pathogenesis of MS**

There are a number of molecules that have proposed roles in the pathogenesis of multiple sclerosis, though none have clinical utility at this time. Measurement of these molecules, however, may aid in determining the mechanism of action of an investigative treatment such as vitamin D<sub>3</sub>.

#### ***i) Serine Proteases***

Serine proteases, enzymes involved in coagulation and fibrinolysis, have proposed roles in a number of other biological processes, including cell migration, neurite outgrowth, neurite

pathfinding, synaptic remodeling, cell excitability, and glial and neuronal cell survival (144-151). Serine proteases function in a “trypsin-like” manner to cleave proteins and as such are able to activate proenzymes, degrade extracellular membrane proteins and bind cell surface receptors. Roles for matrix metalloproteinases and kallikreins have been suggested in the pathogenesis of multiple sclerosis.

Matrix metalloproteinases (MMPs) are a family of serine proteases which degrade protein components of Extracellular Matrix (ECM). MMP activity is regulated at a transcriptional level, through enzymatic activation (secreted in latent form) and through the activity of inhibitors. MMPs function in cell migration through connective tissue and into blood and lymph vessels. Normally, the CNS contains low levels of MMPs, but in MS several MMPs become upregulated and are believed to play a role in disease pathogenesis. In MS, the key function of MMPs is thought to be breakdown of the blood-brain barrier (BBB), altering permeability and promoting inflammatory cell entry into the CNS (**Figure 1.7**) (152-154). CD4+ T helper cell type 1 (Th1) migration from the periphery into the CNS is dependent upon MMP-2 and -9 (155). MMPs are secreted by a number of cells in the CNS, including neurons, astrocytes, oligodendrocytes, microglia, endothelial cells and invading leukocytes and macrophages (156;157). Further, MMP-2 and MMP-9 have been shown to cleave MBP *in vitro* and are believed to be involved in the attack on CNS proteins (158). *In vitro* T cells transmigrate through the basement membrane by release of MMPs. MMPs are inhibited by tissue inhibitors of metalloproteinases (TIMPs) (159-162) and it has been suggested that imbalance between levels of MMP-9 and its inhibitor, TIMP-1, may lead to persistent proteolytic activity and degradation of MBP in MS (156;163).

In patients with MS, serum levels of MMP-9 and MMP-9/TIMP-1 ratio have been shown to be elevated in comparison with healthy controls (164-167). Increased serum levels of MMP-9 have been found to correlate with Gadolinium-enhancing lesions on MRI (164) and have been considered predictive of disease activity (168;169). Likewise, CSF concentrations of MMP-9 have been found to be elevated in patients with MS in comparison with healthy controls (165). Elevated levels of MMP-9 and MMP-7 have been found at all stages of lesion formation in MS (170). These results suggest that an excess of MMP-related proteolytic activity occurs in the brain of patients with MS.

Timms et al. found that serum 25(OH)D concentrations were inversely associated with MMP-9 concentrations in patients with MS (171). Vitamin D deficiency was associated with increased MMP-2 and MMP-9 concentrations and importantly, this was corrected with vitamin D supplementation (171). Interferon- $\beta$ , a disease-modifying treatment used in MS, was found to reduce MMP-9 and MMP-9/TIMP-1 after 6 months of treatment (172). Entry of leukocytes into the CNS is dependent on several factors, including MMP secretion. Thus, MMP activity provides a target for therapy in patients with MS and has been associated with vitamin D status.

Kallikreins (KLK) are biomarkers in neoplastic conditions (173) of which KLK-3 (Prostate Specific Antigen, PSA) is widely used in the clinical diagnosis and prognosis of prostate cancer. In patients with MS, KLK-6 has been found to be elevated in demyelinating lesions (174-177) and in the serum in comparison with healthy controls (178). Further, KLK-6 levels have been associated with EDSS score worsening (177). In the mouse model of multiple sclerosis, Experimental Allergic Encephalomyelitis (EAE), MS-like symptoms have been attenuated by

inhibition of KLK-6 (174). A role for KLK-6 is postulated in MS based upon the fact that KLK-6 is up-regulated in activated T cells (116), and its ability to cleave components of the BBB and degrade myelin (117). KLK-6 is abundantly expressed by infiltrating inflammatory cells in the CNS, although its function is not well understood.

Many KLKs are under the regulatory control of steroid hormones (179-181). Further, supplementation with a moderate dose of vitamin D<sub>3</sub> (2,000 IU/d) was found to significantly decrease the rise in PSA in patients with prostate cancer (182). Taken together, it is possible that vitamin D<sub>3</sub> supplementation in patients with MS can alter levels of KLK-6.

### ***i) Osteopontin***

Osteopontin (OPN) is a member of small integrin-binding ligand, N-linked glycoprotein (Sibling) family of proteins. OPN is present in the extracellular matrix (ECM) of vascular endothelial cells, as an immobilized molecule on the ECM of mineralized tissues, and acts as a cytokine in body fluids. Pro-inflammatory cytokines (IL-1 $\beta$  and TNF- $\alpha$ ) stimulate OPN transcription via activation of protein kinase C (183). Further, a cell adhesion molecule expressed by lymphocytes,  $\alpha$ 4 $\beta$ 1 integrin, which binds vascular cell adhesion molecule 1 (VCAM1), also binds OPN through which OPN may assist T cell migration into the CNS. Osteopontin is involved in many biological processes, ranging from cell adhesion to coagulation (114).

Osteopontin may have pleiotropic functions in the demyelination processes of MS and EAE. Glial cells and neurons produce OPN that may attract Th1 cells and is believed to provide protection for autoreactive T cells from death (184) perpetuating the inflammatory immune

response in MS. In EAE, OPN has been shown to trigger recurrent relapses, promote worsening of paralysis and induced neurological deficits (184). EAE mice demonstrated increased expression of OPN in dendritic cells, OPN receptor expression was increased on T cells, and OPN was also shown to induce IL-17 production by CD4+ T cells (185). In addition, anti-OPN treatment reduced the severity of EAE (185).

Osteopontin is widely expressed in lesions of patients with MS and OPN mRNA was detected in active MS lesions but not in controls (186). OPN was found to be expressed by endothelial cells and macrophages in lesions and in adjacent white matter, in addition to expression by astrocytes and microglia (186). In comparison with control subjects, MS patients had higher concentrations of OPN in plasma (187;188) and increased expression of OPN during relapse in comparison with remission (187-189). OPN concentrations have also been found to be elevated in the cerebrospinal fluid of patients with MS (190).

Interestingly, it has been proposed that some biological effects of 1,25(OH)<sub>2</sub>D may be mediated by PKC signaling (191), possibly providing a link between the two modulatory molecules. Further, 1,25(OH)<sub>2</sub>D has been demonstrated to regulate OPN expression in osteosarcoma cells (192), and it is feasible that 1,25(OH)<sub>2</sub>D may also influence OPN expression in other cells/tissues.

## ***ii) Serum Cytokines***

Cytokines are believed to play a central role in the pathogenesis of MS. Certain cytokines trigger the inflammatory response (e.g. IL-6, IL-17, TGF-β and IFN-γ). Other cytokines regulate growth (e.g. IL-2), or contribute to an anti-inflammatory response (e.g. IL-10, TGF-β

and IL-4). Cytokines also represent a common therapeutic intervention strategy in autoimmune diseases.

MS symptoms have been associated with an increased production of inflammatory cytokines IL-2, TNF- $\alpha$ , and IFN- $\gamma$  and a decrease in anti-inflammatory cytokines TGF- $\beta$ 1 and IL-13. Sharif and Hentges (193) demonstrated a correlation between serum TNF- $\alpha$  concentrations and disability, with TNF- $\alpha$  predictive of development of disability over 2 years, but these results have not been reproduced. Gene expression studies have revealed high levels of IL-12 transcripts in MS lesions (194), presumably due to its importance in stimulating inflammatory T cell responses. These studies do not show consistent patterns of gene regulation, but generally genes associated directly or indirectly in cellular and humoral immunity, including cytokines, are upregulated in lesions compared with normal brain tissue (195).

It is postulated that decreased pro-inflammatory and/or increased anti-inflammatory cytokine concentrations would reflect beneficial changes in inflammatory and autoimmune diseases (196). Mahon et al. (197) compared the effects of supplementation with 1,000IU/d vitamin D<sub>3</sub> with calcium (800mg/d) to placebo plus calcium (800mg/d) in patients with MS. After 6 months of treatment, a significant increase in serum TGF- $\beta$ 1 concentrations was observed (197). In patients with congestive heart failure supplemented with 2,000IU/d vitamin D<sub>3</sub> for 9 months, a significant treatment effect was found for pro-inflammatory TNF- $\alpha$  and anti-inflammatory IL-10 (198). Evidence suggests that vitamin D<sub>3</sub> treatment may alter cytokine concentrations as a biomarker of beneficial immunomodulation in patients with MS.

## **1.4 LINKING VITAMIN D & MULTIPLE SCLEROSIS**

*“The most difficult things to explain are those which are not true.”*

*A.S. Wiener, 1956*

### **1.4.1 Vitamin D as an Environmental Factor in MS Development**

There are several lines of evidence that suggest a low vitamin D status may contribute to the development and progression of multiple sclerosis. Although the exact etiology of multiple sclerosis is unknown, both genetic and environmental factors are believed to play a role. A role for vitamin D is suggested by epidemiological and cross-sectional data (summarized in **Table 1.2**), as well as preclinical studies that demonstrate a role for vitamin D in immune regulation (199).

A geographical distribution of MS has been well established in which the prevalence of MS increases with latitude by birthplace for people of European descent (200-209). The link between vitamin D and MS was first postulated in the late 1970s. Goldberg hypothesized that the environmental factor contributing to the latitudinal gradient of MS risk was vitamin D nutritional status (210). The logic is clear: latitude influences the amount of sunshine in a given area and vitamin D levels, specifically serum 25(OH)D concentrations, are reliant on UVB from sunlight which is unavailable at higher latitudes during the winter months. Thus regions with higher incidence of MS have lower vitamin D status in winter months, whereas regions with lower rates of MS have a more stable vitamin D status.

**Table 1.2. Evidence suggesting a link between low vitamin D status and development of MS.**

<b>Link Between Vitamin D and MS Susceptibility</b>	<b>References</b>
<b>Geographical variation: MS prevalence increases with latitude, corresponds to months of low vitamin D synthesis due to reduced sunlight exposure at higher latitudes</b>	(200-209)
<b>UV radiation exposure correlates with risk of MS</b>	(211;212)
<b>Season of birth: MS incidence is higher in people born in spring months when vitamin D levels are lowest; incidence is lower in fall months when vitamin D levels are highest</b>	(213-221)
<b>Higher oral intake of vitamin D (supplements and food) associated with a reduced risk of MS</b>	(222;223)
<b>High serum 25(OH)D concentrations associated with a protective effect against developing MS; Low 25(OH)D associated with increased risk</b>	(224-226)
<b>Serum 25(OH)D concentrations are low when MRI activity is high; serum 25(OH)D concentrations are high when MRI activity is low</b>	(227;228)
<b>Serum 25(OH)D concentrations are higher in remission and lower during relapse</b>	(229;229;230)

Geographical variation in incidence of MS has been demonstrated in which northern regions have higher prevalence rates of MS in comparison with more southerly regions. US veteran data, stratified by sex and ethnic origin, demonstrated that MS risk was halved when men/women born in northern states moved to southern states for active duty (231). Month of birth has been associated with risk of MS also. Specifically, there is an increase in the number of births of patients with MS in spring months, April to June, and decreased number of births in the fall months, October to December (213-221). In a pooled cohort of cases of MS and controls from Canada, Denmark, Great Britain and Sweden, Willer et al. (217) demonstrated that significantly fewer cases of MS were born in November with significantly more born in May when compared to the month of birth in controls. Sadovnick et al. (215) found a decreased number of births in November in patients with RRMS in comparison with unaffected siblings, wherein the May/November birth ratio that was significantly greater in RRMS patients (1.27)

than siblings (1.18) ( $\chi^2=10.70$ ,  $p=0.001$ ) (215). Furthermore, Ramagopalan et al. (214) provided support for an environmental-gene interaction by demonstrating that significantly fewer MS cases who carried the HLA-DRB1\*15 risk allele were born in November while a significantly higher number were born in April in comparison with those not carrying the allele. This supports the notion that the risk factor of month/season of birth interacts with the gene conferring the single strongest genetic effect in MS, the HLA-DRB1\*15 allele (214). This group also demonstrated a mechanistic link between the HLA-DRB1\*15 allele and vitamin D by demonstrating the presence of a Vitamin D Response Element (VDRE) in DNA within the promoter region of *HLA-DRB1* (232). In addition, Tremlett et al. (233) have associated month of birth with an effect on disease progression. They found that patients born in January had a 40% increased chance of requiring a cane than those born in other months of the year (233).

Gene-environment effects via allelic variation in vitamin D metabolic genes have been investigated in MS with conflicting results. An association between polymorphisms in the CYP27B1 (the  $1\alpha$ -hydroxylase enzyme) has been found in Australian, New Zealand and Swedish MS cohorts (234), but not in a Canadian population of patients with MS (235). Studies investigating an effect of polymorphisms in the VDR gene on MS risk have not yielded a connection in Caucasian populations (235-237), but in a Japanese cohort an association was demonstrated (238). In a study of Canadian twins in which at least one twin had MS, a genetic influence on the regulation of circulating 25(OH)D was found (239). Interestingly, in a case report of 3 patients with vitamin D-dependent rickets type I (VDDR1), resulting from a CYP27B1 loss-of-function mutation, all 3 patients were later diagnosed MS (240). Taken together these

findings suggest that variation within the vitamin D metabolic genes, particularly those involving the production and use of 1,25(OH)<sub>2</sub>D, may influence risk of MS.

Ecological data demonstrate a strong negative correlation between UVR exposure and prevalence of MS in Australia ( $r=-0.91$ ,  $p=0.01$ ) (211). Similarly, Vukusic et al. examined prevalence rates of MS in a stable population of French farmers to find that prevalence rates of MS were significantly higher in north-eastern regions (~100/100,000) compared with south-western regions (~50/100,000) (241). In a population-based dietary assessment from NHANES II, Munger et al. demonstrated an inverse association between total vitamin D intake and relative risk of MS in women, such that those in the highest quintile had the lowest risk of MS (RR=0.67,  $p<0.03$  for trend), as was the case for women taking vitamin D supplements  $\geq 400$  IU/d (222). In a case-control study in Tasmania, van der Mei et al. demonstrated that higher sun exposure during childhood and adolescence (6-15y) was associated with a decreased risk of MS (OR 0.31, 95% CI 0.16, 0.59). When serum 25(OH)D concentrations were compared between cases and controls, MS patients were more likely to have insufficient vitamin D status (defined as serum 25(OH)D concentrations of 25-40nmol/L) and an association between low 25(OH)D concentrations and increased disability was also found (242). Cross-sectional data demonstrate that low vitamin D status, as measured by serum 25(OH)D concentrations, is associated with increased risk of MS. Overall, increasing latitude is associated with an increased risk of MS and UVR seems to be a good candidate for the contributing environmental factor.

Serum 25(OH)D concentrations, the accepted measure of vitamin D nutritional status, directly link vitamin D status with MS prevalence and incidence. In a nested-case control study, Munger et al. ascertained MS cases and matched controls, with at least 2 serum samples stored

in the US Department of Defense Serum Repository, from over 7 million US military personnel over 20 years. Individuals with 25(OH)D concentrations above 99.2nmol/L had a 62% lower odds risk of MS than those with serum 25(OH)D less than 63.3nmol/L (225). Further, a 41% decrease in risk of MS with every 50nmol/L increase in 25(OH)D concentrations was found (225). This suggests that serum 25(OH)D concentrations are an important predictor of MS risk. Overall, a causal effect of low vitamin D status on risk of developing MS is supported by temporal data of moderate strength, biological gradient, and biological plausibility (**section 1.4.3**) of the observed association.

#### **1.4.2 Vitamin D and MS Activity and Progression**

Links between vitamin D status and disease activity are provided by some studies. Cross-sectional, case-control and longitudinal data demonstrate that serum 25(OH)D concentrations are lower in patients with MS during relapse than in remission (226;229;230). Cross sectional studies also show that MS disease activity is inversely correlated with serum 25(OH)D concentrations (242-245). Although this may be explained by less sun exposure in patients with more severe MS, a systemic effect of higher 25(OH)D concentrations is suggested. In support, a clear biphasic seasonal fluctuation in MRI activity has been demonstrated in patients with MS wherein the highest activity was seen in spring/early summer and the lowest in autumn (227). Embry et al. using a similar population of German men, correlated serum 25(OH)D concentrations, which were lowest in spring months and highest in the autumn, with the frequency of lesions by month and found a significant inverse relation between the curves

(228). CSF concentrations of 25(OH)D, which would better reflect CNS concentrations, have been examined in one study but no association was found between CSF or serum 25(OH)D and presence of relapse or gadolinium-enhancing lesions (246).

Several small clinical studies (**Table 1.3**) have examined the potential of a protective role in patients with MS, although safety has been the main outcome measure (197;210;247;248). All of these trials (discussed in **Section 1.4.4**) have been small and have used varying doses and differing forms of vitamin D<sub>3</sub> treatment. Collectively these studies suggest that a dose of vitamin D<sub>3</sub> of 4,000-10,000IU/d can maintain 25(OH)D concentrations in the range of 150nmol/L without adversely affecting calcium homeostasis in patients with MS.

**Table 1.3. Clinical trials of vitamin D in patients with multiple sclerosis.**

Reference	Study Design	N	Main Outcome	Results
<b>Goldberg (210)</b>	1-yr open label Active MS Mg(10mg/d), Ca(16mg/d), VitD(5,000IU/d)	N=16  (6M, 10F)	Annualized Relapse Rates	↓ Relapses during trial; ↓ 60% from predicted
<b>Mahon (197)</b>	6-mo blinded RCT Control: Ca (800mg/d) + Placebo Treated: Ca (800mg/d) + Vitamin D <sub>3</sub> (1,000IU/d)	N=39  Control: 22 Treated: 17	Serum: TGF-β  PBMC: IL-2, TNF-α, IFN-γ	Treated: ↑ TGF-β
<b>Kimball (248)</b>	28-wk open label Active MS pts Ca (1,200mg/d) + Vitamin D <sub>3</sub> (4,000-40,000IU/d)	N=12  (5M, 7F)	Serum/Urine Ca, New MRI (Gd+) lesions, EDSS & Relapse Rates	No change in serum/urine calcium  ↓Gd+lesions
<b>Wingerchuk (247)</b>	48-wk open label Active MS pts Ca (800mg/d) + 1,25(OH) <sub>2</sub> D (0.5-2.5µg/d)	N=15  (3M, 12F)	New MRI (Gd+) lesions, EDSS & Relapse Rates	No change clinically *hypercalcemia in 5/15 patients

### 1.4.3 Biological Plausibility of a Role for Vitamin D in Multiple Sclerosis

Biological plausibility is provided by the observed *in vitro* effects exerted by 1,25(OH)<sub>2</sub>D on cells of the immune and central nervous systems. Upon binding of 1,25(OH)<sub>2</sub>D to the Vitamin D Receptor (VDR), heterodimerization with a Retinoid X Receptor (RXR) occurs, and these heterodimers recruit co-regulatory protein complexes and vitamin D receptor interacting protein (DRIP). These complexes bind vitamin D response elements (VDREs) within the regulatory regions of target genes to promote or inhibit target gene transcription. *In vitro* and animal data demonstrate that 1,25(OH)<sub>2</sub>D effects cell proliferation and apoptosis, differentiation of immune cells and modulates immune responses.

A variety of cells from the innate and adaptive arms of the immune system express VDR as well as 1 $\alpha$ -hydroxylase, the enzyme required to produce 1,25(OH)<sub>2</sub>D. As such, immune cells possess the capability to convert circulating 25(OH)D to 1,25(OH)<sub>2</sub>D and use it locally. Antigen Presenting Cells (APC), including macrophages and some dendritic cells, and activated T cells and B cells express VDR and synthesize 1,25(OH)<sub>2</sub>D (249;250). Interestingly, these cells may all play roles in the pathogenesis of MS. The responses of these cells to 1,25(OH)<sub>2</sub>D-treatment *in vitro* are summarized in **Table 1.4**.

Antigen Presenting Cells (APC) are integral to the innate immune response and directly influence the adaptive immune response. Toll-like receptors (TLR) are membrane-bound pattern recognition receptors which recognize bacterial components such as lipopolysaccharide and peptidoglycan. TLR engagement triggers DC maturation and the production of defensins by epithelial cells. Upon activation of TLR-2/1, monocytes/macrophages up-regulate VDR and 1 $\alpha$ -

hydroxylase and, provided there is sufficient 25(OH)D available, local production of 1,25(OH)<sub>2</sub>D stimulates up-regulation of cathelicidin, an antimicrobial protein, which promotes bacterial killing (251) and the generation of autophagosomes (252). In addition, the promoter region of the  $\beta$ -defensin 4 gene contains a VDRE (253;254) which can be induced with 1,25(OH)<sub>2</sub>D with activation of NF- $\kappa$ B and IL-1 signaling (255). Together, these findings suggest that 1,25(OH)<sub>2</sub>D production by innate immune cells targets optimization of bacterial killing. APCs participate during inflammation by perpetuating self-destruction through the secretion of inflammatory factors and activation of autoreactive T cells (256). Thus, altering the phenotype of APCs may alleviate some of the inflammatory milieu that contributes to the autoimmune environment.

Monocytes exposed to 1,25(OH)<sub>2</sub>D have reduced MHC class II and co-stimulatory molecule (CD40, CD80 and CD86) expression (257) limiting their ability to activate T cells. Monocyte differentiation into DCs is inhibited by 1,25(OH)<sub>2</sub>D with suppression of IL-12 (258;259). DCs are likewise profoundly affected by 1,25(OH)<sub>2</sub>D treatment, and since DCs directly induce and regulate T cell responses, these effects extend to T cells. For example, depending on the type of pathogen and the profile of co-stimulatory molecules, DCs can drive the development of proinflammatory Th1, Th17 or Th2 or protective Treg cells. Exposure of DCs to 1,25(OH)<sub>2</sub>D *in vitro* inhibits maturation and differentiation, reduces expression of MHC class II molecules and co-stimulatory receptors; decreases production of pro-inflammatory cytokines; and inhibits maturation and differentiation (257;258;260-265). Dendritic cells respond to 1,25(OH)<sub>2</sub>D by arresting in a semi-mature state and attenuating antigen presentation (258;266). The resulting tolerogenic DCs can induce Treg differentiation with an increase in IL-10 secretion (260;265;267;268). Further, the production of IL-12 by DCs, the major cytokine

**Table 1.4. Effects of 1,25-dihydroxyvitamin D on Immune cells *In Vitro***

	Immune cell type				
	Monocytes/ macrophages	Dendritic cells	CD4+ T lymphocytes	CD8+ T lymphocytes	B lymphocytes
<b>CYP27B1 Expression</b>	Upregulated with activation (TLR 2/1 or TLR4 triggering)	Upregulated with maturation	Inducible with activation	Inducible with activation	Inducible with activation
<b>VDR Expression</b>	Constitutive	Constitutive	Low in resting cells Upregulated with activation	Inducible with 1,25(OH) <sub>2</sub> D	Inducible with 1,25(OH) <sub>2</sub> D
<b>Effects Produced by 1,25(OH)<sub>2</sub>D</b>	↑ Proliferation ↑ CYP27B1 ↑ VDR ↓ MHC class I & II ↓ CD40, CD80, CD86 ↓ TLR2, TLR4 ↓ CD1a ↓ IL-12 ↑ TNF-α ↑ IL-1β ↑ Cathelicidin ↑ Defensin β4 ↑ iNOS	↓ Maturation ↓ MHC class II ↓ CD40, CD80, CD86 ↓ TLR2, TLR4 ↓ IL-12 ↓ IL-10 ↓ TNF-α ↓ IL-2 ↓ CCR7 ↑ PDL-1	↓ Differentiation ↓ Proliferation ↓ Activation ↓ IL-2 ↓ IFN-γ ↓ IL-17 ↓ IL-16 ↓ IL-23 ↑ IL-4 ↑ IL-5 ↑ IL-10 ↑ FoxP3	↓ Proliferation ↓ Cytotoxicity	↓ Proliferation ↓ IgG production ↓ IgM production ↓ Plasma cell differentiation ↑ CYP24A1 ↑ VDR
<b>Overall Effects of 1,25(OH)<sub>2</sub>D</b>	↑ Antigen processing ↑ Bacterial killing ↑ Phagocytosis ↑ Superoxide synthesis	↑ Tolerogenic DCs ↑ Treg response ↓ Th1 response	↑ Treg response ↑ Th2 response ↓ Th1 response ↓ Th17 response ↓ Th1 IL-2 driven B cell IgG synthesis	↓ CTL response	↓ Humoral response

driving Th1 responses, is inhibited by 1,25(OH)<sub>2</sub>D (259) and thus indirectly inhibits Th1 cell development. Not surprisingly, 1,25(OH)<sub>2</sub>D –treated dendritic cells are able to suppress Th1 proliferation and IFN-γ production by Th1 cells (265;269). Recently, Bartels et al. have demonstrated intrinsic production of 1,25(OH)<sub>2</sub>D and with profound effects of 25(OH)D treatment on cultured DCs. Treated DCs demonstrated reduced maturation, reduced production of inflammatory IL-12 and TNF, as well as reduced IL-10 production, reduced antigen presentation and reduced chemotaxis (270). In sum, by modulating APC function, 25(OH)D and 1,25(OH)<sub>2</sub>D may act to promote tolerogenic adaptive immunity.

Dysregulation of the Th1 response to self-antigen has been largely regarded as responsible for the autoimmune attack directed against myelin in MS and, in support, CD4+ T cells are widely found in lesions of MS patients (271;272). T cells have been shown to metabolize 25(OH)D to 1,25(OH)<sub>2</sub>D and 25(OH)D treatment resulted in decreased proliferation of CD4+ T cells (229). Treatment with 1,25(OH)<sub>2</sub>D inhibits Th1 production of IL-2, IFN- $\gamma$ , and TNF (273) by VDR-mediated down-regulation of gene transcription (274;275). The production of pro-inflammatory IL-2, IL-6, IFN- $\gamma$  and GM-CSF by Th1 cells is inhibited by 1,25(OH)<sub>2</sub>D (262;276-279). Th1 cell differentiation and proliferation are also inhibited by 1,25(OH)<sub>2</sub>D (280). It has recently been recognized that pathogenic CD4+ Th17 cells play a role in the pathogenesis of MS. 1,25(OH)<sub>2</sub>D inhibits the production of IL-6 and IL-17 from pathogenic Th17 cells while promoting IL-10-producing T cells (Tregs) (229). Thus, the effects of 1,25(OH)<sub>2</sub>D are mediated through induction of Tregs and through elimination of proinflammatory effector T cells.

Treatment of CD4+ T cells with 1,25(OH)<sub>2</sub>D *in vitro* inhibits differentiation and proliferation and inhibits the production of Th1 phenotypic cytokines (IL-2, IFN- $\gamma$  and TNF) while simultaneously increasing Th2 phenotypic cytokine production (263;279;281;282). The result is a shift towards an “anti-inflammatory” Th2 response. Further, 1,25(OH)<sub>2</sub>D induces naïve CD4+ T cells to differentiate into IL-10 producing Treg cells (283). Ultimately, vitamin D<sub>3</sub> treatment may be able to beneficially alter the T cell compartment in patients with MS, such that pathogenic Th1 and Th17 cells are inhibited and anti-inflammatory Th2 and protective Treg cells are promoted.

In addition to effects on APCs and T cells, 1,25(OH)<sub>2</sub>D influences B cell responses. Activated B cells exposed to 1,25(OH)<sub>2</sub>D have reduced proliferation, immunoglobulin production and

undergo apoptosis (284). Although the role of B cells in MS is unclear, the presence of plasma cells and immunoglobulin within MS lesions suggests that some damage is caused by activation of B cells. Local production of  $1,25(\text{OH})_2\text{D}$  may prove to be important in the maintenance of homeostasis of B cells.

Experimental Allergic Encephalomyelitis (EAE) is a mouse model of MS, in which MS-like disease is induced by immunization with myelin peptides (MBP, MOG or PLP) with Complete Freund's Adjuvant. Vitamin D-deficient mice have accelerated onset of EAE (285). However, mice born to vitamin D-deficient mothers have been found to have milder symptoms and delayed onset of EAE (286). Studies in which EAE has been treated with vitamin D metabolites are summarized in **Table 1.5**. Treatment with  $1,25(\text{OH})_2\text{D}$  reduces incidence of EAE and has been shown to prevent clinical and pathological signs of EAE (285;287) in a VDR-dependent manner (288). As well,  $1,25(\text{OH})_2\text{D}$  treatment ameliorates symptoms in already active EAE mice (285). Animals treated with  $1,25(\text{OH})_2\text{D}$  that developed EAE experienced a milder disease with prolonged survival (287;289-295), and  $1,25(\text{OH})_2\text{D}$ -treatment resulted in a reduction of autoreactive T cells and reduction of peptide-specific proliferation and Th1 cell development (287). *In vivo* animal studies suggest that modulation of immune activity in EAE by  $1,25(\text{OH})_2\text{D}$  results in a shift from Th1 to Th2 response, i.e. a shift from a pro-inflammatory to anti-inflammatory response, with the induction of Treg cells. Treatment with the parent compound vitamin  $\text{D}_3$ , or cholecalciferol, has also been demonstrated to inhibit EAE in female mice but not in males (296). In summary, vitamin D metabolites have a protective role against EAE development and progression. Importantly, as shown *in vitro*, self-regulation of T cells and APCs may be induced by local conversion and use of  $1,25(\text{OH})_2\text{D}$  from circulating  $25(\text{OH})\text{D}$ .

**Table 1.5. Treatment of Experimental Allergic Encephalomyelitis (EAE) with vitamin D.**

	<b>Prevents EAE</b>	<b>Ameliorates Symptoms</b>
<b>Ultraviolet Radiation</b>	√ (297)	No data
<b>Vitamin D<sub>3</sub></b>	√ (296;298-300)	No data
<b>25(OH)D</b>	No effect (297)	No effect (297)
<b>1,25(OH)<sub>2</sub>D</b>	√ (285;287;288;291;301-303)	√ (285;304)

No effect= no difference between 25(OH)D-treated and control mice.

#### **1.4.4 Vitamin D in the Treatment of MS**

The effect of vitamin D<sub>3</sub> supplementation on immune responses in humans has not been systematically investigated and there are only a handful of studies that have investigated the effects of supplementation with vitamin D<sub>3</sub> in patients with multiple sclerosis (summarized in **Table 1.3**), and only one of these has been a randomized controlled trial. When the therapeutic benefit of a drug is tested there is a sequence of trials that are conducted to establish the safety and efficacy of the compound of interest. Phase I trials are designed to test the safety and tolerability of the compound in a small group of patients or healthy volunteers. Phase II trials usually involve a control group and are used to assess dosing requirements and to determine short-term side effects. Phase III are randomized placebo-controlled trials, usually multi-centered, and are designed to test efficacy. Phase IV are post-marketing surveillance studies that monitor ongoing safety and efficacy after the drug has been approved for sale by Health Canada or the FDA in the United States. Vitamin D<sub>3</sub> is a nutrient and clinical trials have bypassed these phases, but because of the intention of its use as a therapeutic agent, designing and performing these phases of study is necessary and essential for ensuring that efficacy is not missed due to suboptimal dosing regimens.

In a randomized controlled trial, Mahon et al. (197) randomized patients with MS to receive 800mg/d of calcium with placebo (n=22) or calcium (800mg/d) with 1,000IU/d of vitamin D<sub>3</sub> for 6 months (n=17). Anti-inflammatory cytokine transforming growth factor β1 (TGF-β1) concentrations in the serum were significantly increased in the treatment group, but no changes were reported in other measured cytokines (TNF, IFN-γ, IL-13) (197). Clinical outcomes were not addressed in this study.

Two un-blinded prospective studies have investigated the disease modifying effects of vitamin D<sub>3</sub> in MS; however, both were small, and without a control group. Goldberg et al. (210) treated 16 patients with MS (10 completed the trial) with 5,000IU/d vitamin D (as cod-liver oil). Disease stabilization was reported, with a 50% reduction in the number of relapses during the 2 years of treatment. In a phase I trial, we assessed the safety of escalating doses of vitamin D<sub>3</sub> (4,000-40,000IU/d) D with 1,200mg/d of calcium in 12 patients with MS. After 28 weeks the treatment was found to be safe, with no evidence of altered calcium homeostasis, despite serum 25(OH)D concentrations of nearly 400nmol/L. In addition, mean gadolinium-enhancing lesions on MRI were significantly reduced (248). A fourth trial investigated the effects of 48 weeks of treatment with the active hormonal metabolite, 1,25(OH)<sub>2</sub>D (0.5-2.5μgd), in 15 patients with MS (247). Exacerbation rates were reduced by 27% at end-of-study, but hypercalcemia resulted in the withdrawal of 2 patients and dose adjustments for 2 others.

These studies vary in dose, ranging from 1,000 to 40,000IU/d of vitamin D<sub>3</sub>, and form of vitamin D<sub>3</sub>, cholecalciferol vs. 1,25(OH)<sub>2</sub>D. Nevertheless, taking into account the considerable physiological data suggesting a plausible biological context, the results are encouraging and provide justification for more extensive phase II and III trials. It cannot be ruled out, and

evidence certainly suggests the opposite, that supplementation with vitamin D<sub>3</sub> can provide neurological and immunological benefit in patients with MS.

## 2.0 RATIONALE AND OBJECTIVES

*“The best way to have a good idea is to have lots of ideas.”*

*Sir Francis Bacon*

### 2.1 Rationale

Multiple sclerosis (MS) is an inflammatory, neurodegenerative disease of the central nervous system (CNS) of unknown etiology. Although the pathophysiology of MS is not fully understood, it is thought to occur when peripheral T helper lymphocytes (pathogenic Th1 and Th17 cells) reactive against CNS antigens (including epitopes of the myelin sheath) migrate through the blood-brain barrier (BBB) to initiate and propagate localized areas of inflammation (305). Regulatory T cells (Treg), that would normally contribute by suppressing pathogenic responses in a healthy T cell compartment, have been found to have compromised activity in patients with MS (306;306;307). Further, a number of partially effective immune therapies are utilized in autoimmune disease treatment, including MS, and T cells are thought to function as one of the major targets (196).

Vitamin D<sub>3</sub> may have therapeutic potential in MS, either alone or in combination with available immune therapies. Experimentally, the active metabolite of vitamin D, 1,25(OH)<sub>2</sub>D, is able to skew the T cell compartment away from a pro-inflammatory state into a more anti-inflammatory and regulated profile. In the mouse model of MS, Experimental Allergic Encephalomyelitis (EAE), treatment with 1,25(OH)<sub>2</sub>D can prevent EAE and ameliorates symptoms with treatment after disease onset (285). These findings suggest a therapeutic role for vitamin D<sub>3</sub> in MS, but only a few small, clinical trials have examined this potential in an

unstructured manner (197;210;247;248). Further, no data exist demonstrating an *in vivo* immunomodulatory effect of vitamin D supplementation in humans, either in patients with MS or in general.

The current study attempts to characterize responses to high serum concentrations of 25-hydroxyvitamin D [25(OH)D], the accepted measure of vitamin D status, in patients with MS. The primary outcomes, presented in chapter 4.0, examined the safety of high serum 25(OH)D concentrations and included several related measurements including, renal ultrasounds, electrocardiograms, liver function enzymes, and serum creatinine (renal function). Vitamin D toxicity, which manifests as hypercalcemia, was regarded as an important adverse event. As such, serum and urinary calcium measures were monitored every 6 weeks in treated patients. Serum creatinine, a measure of kidney function, and liver function enzymes were also measured at 6-week intervals. At baseline, mid-study and at one year (end-of-study) renal ultrasounds were performed to ensure absence of soft tissue calcification. Likewise, at baseline and end-of-study electrocardiograms were performed to ensure normal cardiac function.

Clinical outcomes included measurement of disease progression by the Expanded Disability Status Scale (EDSS) score and relapse rates. Clinical evaluations were performed for all study participants at baseline and end-of-study. As relapse events occurred they were evaluated and treated by the attending neurologist (Dr. Jodie Burton or Dr. Paul O'Connor) as per standard practice in the MS Clinic at St. Michael's Hospital. EDSS scores were evaluated at baseline and end-of-study. These measures were utilized to assess clinical adverse outcomes, as well as to indicate any clinical efficacy of vitamin D treatment in patients with MS. Because of the small sample size we did not expect significant differences between treatment groups.

In addition to safety and clinical outcomes, we measured several markers of immune system function, presented in chapter 5.0, including lymphocyte proliferative response to antigenic stimulation and cytokine concentrations. Modest vitamin D<sub>3</sub> supplementation has been shown to affect cytokine profiles (197;198). We sought to determine if high serum 25(OH)D concentrations would have detectable effects on markers of the immune system activity, specifically if high serum 25(OH)D levels could alter proliferative responses of T lymphocytes to antigens known to elicit responses in patients with MS (308). We further examined cytokine concentrations to determine if changes in these chemical signals may provide a mechanistic link with any changes found.

Because of the unique opportunity to examine the effects of high serum 25(OH)D concentrations on inflammatory markers, particularly molecules that have been found to be skewed in patients with MS, we measured a number of other markers at baseline, mid-study and end-of-study in treatment and control participants (**Figure 5.1**). Normal blood-brain barrier (BBB) function establishes and maintains CNS homeostasis. Increased BBB permeability is a characteristic of many CNS diseases, including MS (309;310). Cytokines, chemokines and other inflammatory molecules can influence the endothelial cell barrier to increase permeability (311). Matrix metalloproteinases are proteolytic enzymes that attack the extracellular matrix (ECM) and elevated levels of MMP-9 have been found in patients with MS. Kallikrein-6, another serine protease, and osteopontin (OPN), a pro-inflammatory molecule, have likewise been implicated in transmigration of T cells across the BBB and have been demonstrated to be elevated in MS patients (186). Putative regulatory roles for 1,25(OH)<sub>2</sub>D have been described for

all of the above molecules (171;192;312), which led us to measure concentrations of MMP-9, TIMP-1, KLK-6, and OPN in treated patients and controls (**Figure 2.6**).

In healthy individuals supplemented with vitamin D serum 25(OH)D concentrations have been found to have an inverse relationship with CRP (171). Although this relationship has not been demonstrated in patients with MS (197);(171), the ranges of 25(OH)D concentrations studied have been limited. CRP was measured in the present study to assess the affect of a much broader range of 25(OH)D than previously examined.

Vitamin D<sub>3</sub> is necessary for optimal bone health and skeletal muscle function (73). We sought to determine if, in a population known to be at risk of osteoporosis, serum 25(OH)D concentrations >75nmol/L would alter bone turnover responses as measured by a marker of bone formation, bone alkaline phosphatase (BAP), and a marker of degradation, C telopeptide (CTX).

As presented in chapter 6.0, we measured the response of a number of vitamin D metabolites including, 25(OH)D, 1,25(OH)<sub>2</sub>D and 24,25-dihydroxyvitamin D [24,25(OH)<sub>2</sub>D]. Further, concentrations of vitamin D binding protein (DBP) were measured at baseline and end-of-study. Due to the high range of serum 25(OH)D responses expected, this study provided a unique opportunity to characterize the response of vitamin D metabolites in response to high-dose vitamin D<sub>3</sub> supplementation, especially considering that DBP and 24,25(OH)<sub>2</sub>D have not been previously addressed. Further, urinary calcium in response to a wide range of 25(OH)D concentrations was also investigated.

## **2.2 Objectives:**

1. To characterize the safety profile of high-dose vitamin D<sub>3</sub> supplementation with calcium in patients with multiple sclerosis. Specifically, we sought to establish the effects of high serum 25(OH)D concentrations on urinary and serum measures of calcium homeostasis and other safety endpoints, including biochemical (serum creatinine, PTH, and liver enzymes), renal ultrasound and electrocardiograms.

2. To assess the effects of high serum 25(OH)D concentrations on measures of disease activity and progression in patients with MS. Comparisons were made between treated patients and matched controls, both within groups between baseline and end-of-study, as well as between groups (changes over time).

3. To investigate high serum 25(OH)D concentrations in relation to immune system activity. We compared lymphocyte proliferation responses to disease-specific antigens and cytokine concentrations (inflammatory and anti-inflammatory) between treatment groups and responses were compared within groups between baseline and end-of-study (one year later). Associations between serum 25(OH)D concentrations and other inflammatory markers were also examined including, matrix metalloproteinase-9, tissue inhibitor of MMP-9 (TIMP-1), kallikrein 6, osteopontin and C reactive protein.

4. To investigate the affects of high serum 25(OH)D on markers of bone turnover we measured a marker of bone formation, bone specific alkaline phosphate, and bone resorption, C-telopeptide. Concentrations of bone markers were compared within (baseline vs. end of study) and between groups.

5. To characterize the response of vitamin D metabolites in response to high dose vitamin D<sub>3</sub> supplementation we measured 25(OH)D, 24,25(OH)<sub>2</sub>D, 1,25(OH)<sub>2</sub>D, and vitamin D binding protein (DBP). The concentrations of metabolites were compared within groups (baseline vs. end of study) and between groups.

### **2.3 Hypotheses:**

1. Based on the findings of our small pilot study (248), we hypothesized that high-dose vitamin D<sub>3</sub> supplementation with calcium in patients with multiple sclerosis, would not result in hypercalcemia nor did we expect any adverse outcomes.
2. We expected serum 25(OH)D to increase dramatically in the treatment group and remain unchanged or decreased in the control group. We expected serum 1,25(OH)<sub>2</sub>D concentrations to remain within the reference range (39-193 pmol/L) because we did not expect to see any increase in serum and urinary calcium concentrations.

3. We hypothesized that by elevating serum 25(OH)D concentrations, via high-dose vitamin D<sub>3</sub> supplementation, an ample supply of substrate to immune cells, would alter immune cell responses via the ability to locally produce (via 1- $\alpha$ -hydroxylase expression) and utilize 1,25(OH)<sub>2</sub>D (via expression of the vitamin D receptor, VDR). As such, we hypothesized that T cell responsiveness would be reduced post-treatment and that some of the pro-inflammatory markers measured (cytokines, MMP-9, TIMP-1, KLK-6, OPN, CRP) may be decreased in the vitamin D<sub>3</sub>-treated group in comparison with the control group.

### **3.0 VITD4MS STUDY DESIGN**

*“It is a wise mans part, rather to avoid sickness, than to wish for medicines.”*

*Thomas More*

#### **3.1 Inclusion & Exclusion Criteria**

##### **3.1.1 Inclusion Criteria:**

We planned to enroll patients with any of the three major clinical subtypes of multiple sclerosis (relapsing-remitting, secondary progressive, or primary progressive) in the age range of 18 to 55 years. Patients with a Expanded Disability Status Scale (EDSS) score of 0-7 were to be enrolled (i.e. a range of disability from no disability, EDSS of 0, to patients who were wheelchair restricted but still able to carry out their own transfers and perform activities of daily living including self-care functions, EDSS of 4-7). Patients to be enrolled included patients treated with currently available disease modifying drugs (DMDs) including interferon- $\beta$  (Avonex<sup>®</sup>, Rebif<sup>®</sup>, Betaseron<sup>®</sup>) and glatiramer acetate (Copaxone<sup>®</sup>) as well as patients not taking DMDs. In addition, patients were not restricted in taking other medications used for treatment of MS-related symptoms, or medications prescribed for other medical diagnoses unless indicated in the exclusionary criteria. The purpose of this wide range of disability, treatment and age was to be inclusive and to provide a safety profile of high-dose vitamin D<sub>3</sub> with calcium supplementation in a sample representative of the MS population in our clinic.

### 3.1.2 Exclusion Criteria:

Patients participating in other research trials were excluded from study enrollment. Patients were excluded if they were taking medications that could potentially interact with vitamin D<sub>3</sub>, including magnesium-containing antacids, digoxin, phenytoin, and thiazide-containing diuretics. Patients with a history of renal disease were excluded due to the risk of kidney stone development with vitamin D<sub>3</sub> and calcium supplementation (313). Patients with co-morbid disease were not offered enrollment in this study. Because of the possibility of ectopic vitamin D<sub>3</sub> synthesis by 1 $\alpha$ -hydroxylase localized to granulomatous tissue, participants who had ever received a diagnosis of granulomatous disease (including sarcoidosis, tuberculosis, silicosis, chronic or active fungal infections or lymphoma) were excluded from participation in this study (314). Patients with cardiac disease history, including cardiac arrhythmia, were not offered study enrollment due to a small potential for cardiac arrhythmia as a result of hypercalcemia (315). Further, at screening, any participant with abnormal results were not offered enrollment, including abnormal serum or urinary calcium, serum albumin, liver function enzymes (alanine transaminase, aspartate transferase, or alkaline phosphatase), or an abnormal electrocardiogram or renal ultrasound. Patients with recent disease activity were not offered study enrollment. This included relapse within 60 days, steroid use within 30 days, or treatment with chemotherapy within 12 months. Female patients were not offered enrollment if they were pregnant, planning to become pregnant, or did not use adequate contraception. Participants were excluded if they had been taking >4,000 IU/d of vitamin D<sub>3</sub> supplementation or who had a serum 25(OH)D concentration >150nmol/L at screening.

### **3.2 Study Design:**

This was an open-label, stratified study design in which treatment participants underwent a dose-escalation of vitamin D<sub>3</sub> with calcium supplementation. Patients were recruited from the Multiple Sclerosis Clinic at St. Michael's Hospital in Toronto between June 2006 and March 2007. Magnetic resonance imaging (MRI) scans of the central nervous system (CNS) were not performed during this study. MRI studies were completed in the pilot phase (248) and no adverse effects were detected on MRI following treatment with up to 40,000IU/d of vitamin D<sub>3</sub> with calcium.

Ethics & Protocol Approval: The trial was approved by the institutional review boards at St. Michael's Hospital, University of Toronto, The Hospital for Sick Children, and McGill University. The trial was registered with clinicaltrials.gov through the National Institute of Health (ID NCT00644904).

Consent: All patients were asked if they were interested in participating in any of the trials at the St. Michael's hospital for which they were eligible by their clinic physician. If patients were interested in the present trial, Samantha Kimball or Jodie Burton would speak with the patient at their request. All participants provided written informed consent.

Pairing & Randomization:

Patients who met the inclusion/exclusion criteria were matched with other enrollees as follows:

- a) Age: 18-30y, 31-40y, 41-55y
- b) Disease duration: 0-9y, 10-19y,  $\geq 20$ y
- c) EDSS Score:  $\leq 2.5$ , 3.0-5.5, 6.0-6.5
- d) Disease modifying drug: interferon, glatiramer acetate, none
- e) Sex

Once a matching pair was available, the 2 members were randomized to treatment or control groups by blinded drawing from a hat. The primary role of control group participants was to determine if any changes seen in secondary endpoints were related to vitamin D<sub>3</sub> and calcium supplementation. The rationale was that, although indicated in MS pathology, most of these markers have not been characterized longitudinally in patients with MS, thus changes seen over time may not be related to treatment and the control group was included for this purpose.

Study Length: The duration of this study was one year.

Sample Size: Sample size was based on a power of 0.90 at a 2-sided 0.05 significance level. If a difference between treatment and control groups of 1 standard deviation was considered a clinically meaningful increase in calcium-related parameters most likely to be adversely affected by vitamin D<sub>3</sub> supplementation, a sample of 50 patients provided  $>0.90$  power with an alpha of 0.05 for primary meaningful effect size of 0.30 mmol/L difference in serum calcium.

The calculated sample size was also considered large enough to detect any meaningful differences in secondary outcome measures, although specific calculations for each measurement were not performed.

Vitamin D<sub>3</sub> Preparation: Vitamin D<sub>3</sub> was supplied as an ethanol-based solution that could be added to any beverage. Hyperconcentrated solutions of vitamin D<sub>3</sub> were supplied in amber glass vials at every dose-changing visit. Patients were instructed to take anywhere between one to four mL to obtain the appropriate weekly dose of vitamin D<sub>3</sub> depending on that week's dose. Vitamin D<sub>3</sub> could be mixed with any beverage.

US Pharmacopeia (USP)-grade vitamin D<sub>3</sub> (cholecalciferol) was purchased in crystalline form from Sigma (Sigma, St. Louis, USA) and dissolved in USP-grade ethanol. Ultraviolet absorption spectra obtained on a diode array spectrophotometer (Hewlett-Packard, Palo Alto, California, USA) and high performance liquid chromatography (HPLC) were used for measurement of the molar concentration of vitamin D<sub>3</sub>. For spectrophotometric calculations an extinction coefficient of  $18,300 \text{ Au} \cdot \text{mol}^{-1} \cdot \text{L}^{-1}$  was used. Vitamin D<sub>3</sub> was quantified using an Agilent 1,100 series isocratic HPLC, equipped with detectors at 266 and 228nm. Absorbance data were recorded to a computer, and ChemStations software (Agilent, Mississauga, Canada) was used to integrate the peak areas. The retention time of vitamin D<sub>3</sub> was established using known concentrations of crystalline vitamin D<sub>3</sub> in mobile phase ( $1\text{-}35 \mu\text{g} \cdot \text{mL}^{-1}$ ). A C<sub>18</sub> HPLC column (5 $\mu\text{m}$  particles, 4.6mm i.d. 20cm length) (Grace Vydac, Toronto, Canada) was utilized throughout the HPLC analysis.

Quality control testing was performed for each batch and dose of vitamin D<sub>3</sub> prepared and again at study completion. An arbitrary expiration date of 6 months was used for each batch. The preparation and quality testing of ethanol solution of vitamin D<sub>3</sub> has been previously described (248).

Calcium: Powdered tricalcium phosphate (Rhodia, Cranbury, NJ) was supplied to treatment participants at each visit. Calcium could be mixed with food or beverage. Participants were given measuring cups and instructed to take a measured quantity (7.5mL) equivalent to 1,200 mg/d elemental calcium.

### **3.3 Intervention**

The current study was an open-label design, meaning that participants were not blinded to which trial arm they were in. As such, control participants did not have to attend as many clinic visits as treated participants. Treated participants were required to visit the clinic at least every 6 weeks in order to obtain subsequent doses of vitamin D<sub>3</sub> and for the assessment of serum and urinary calcium measurements.

#### **3.3.1 Control Group**

Participants randomized to the control group did not receive vitamin D<sub>3</sub> or calcium supplementation. If control group participants were taking calcium and/or vitamin D<sub>3</sub> supplementation prior to study enrollment, they are able to continue doing so provided vitamin D<sub>3</sub> supplementation was at a dose less than 4,000 IU/d.

#### **3.3.2 Treatment Group**

Participants randomized to the treatment group followed a dose-escalation schedule of vitamin D<sub>3</sub> supplementation as outlined in **Table 3.1** accompanied by a dose of calcium at 1,200mg/d. All treatment group patients received the same treatment with calcium and vitamin D<sub>3</sub> supplements free of charge.

Calcium: Treatment group participants began calcium supplementation (1,200mg/d) at the first study visit and continued taking this dose until week 48 of the study. Calcium supplementation was incorporated into the study design because studies using the animal model of MS have shown that calcium supplementation is necessary for optimal suppression of disease activity with the active form of vitamin D<sub>3</sub> (1,25(OH)<sub>2</sub>D), thus calcium supplementation at the current recommended adequate intake of 1,200mg/d for women and men over the age of 50, was supplied in addition to vitamin D<sub>3</sub> (303).

**Table 3.1. Treatment group supplementation schedule.**

Study Visit	Week of Study	Supplementation		Calcium (mg/d)
		Vitamin D3 IU/week	Vitamin D3 IU/day (Equivalent to)	
1	1	0	0	1,200
2	3	28,000	4,000	1,200
3	5	70,000	10,000	1,200
4	11	112,000	16,000	1,200
5	17	224,000	32,000	1,200
6	23	280,000	40,000	1,200
7	29	70,000	10,000	1,200
8	35	70,000	10,000	1,200
9	41	28,000	4,000	1,200
10	49	0	0	0
11	52	0	0	0

Vitamin D<sub>3</sub>: The doses of vitamin D<sub>3</sub> ranged from 4,000 to 40,000 IU/d, taken as a weekly dose (28,000 to 280,000 IU/week, respectively). Vitamin D<sub>3</sub> supplementation was initiated at week 2 of the study and was taken for 2 weeks to rule out immediate hypersensitivity to vitamin D<sub>3</sub>. In

healthy, non-pregnant subjects,  $1\alpha$ -hydroxylase, found primarily in the kidney, mediates conversion of  $25(\text{OH})\text{D}$  to  $1,25(\text{OH})_2\text{D}$ . In a few disease states, the  $1\alpha$ -hydroxylase found in other tissues functions in an unregulated manner (e.g. macrophage  $1\alpha$ -hydroxylase in sarcoidosis). Hypercalcemia or hypercalciuria can result indicating hypersensitivity to vitamin  $\text{D}_3$  therapy (316). Diseases in which ectopic conversion of vitamin  $\text{D}_3$  to the active hormone,  $1,25(\text{OH})_2\text{D}$ , may occur include sarcoidosis, tuberculosis, certain fungal infections and silicosis, and more rarely Hodgkin's and non-Hodgkin's lymphoma (314). Because these diseases are rare, and every attempt was made to exclude patients with co-morbid disease, the risk of hypersensitivity to vitamin  $\text{D}_3$  occurring in our patient population was expected to be close to, or equal to zero. Nonetheless, patients were evaluated after only 2 weeks of the lowest dose of vitamin  $\text{D}_3$  (4,000 IU/d) to detect hypersensitivity.

Subsequent doses of vitamin  $\text{D}_3$  were approximately doubled every 6 weeks up to 40,000 IU/d (week 28). The purpose of the dose-escalation design was to rapidly increase serum  $25(\text{OH})\text{D}$  concentrations in a short period, not to assess the safety of each dose. The half-life of serum  $25(\text{OH})\text{D}$  is approximately 4-9 weeks (16;317), the dose escalation schedule was intended to result in an increase in  $25(\text{OH})\text{D}$  corresponding to each half-life, i.e. the dose of vitamin  $\text{D}_3$  would be approximately doubled every 6 weeks. Our objective was to increase serum  $25(\text{OH})\text{D}$  concentrations above a the 'normal' value of 75 nmol/L, to a physiological range ( $\sim 150$  nmol/L), for the majority of the study in order to characterize safety and determine if these values affected secondary outcomes.

Vitamin  $\text{D}_3$  toxicity manifests as hypercalcemia (81;318). Because any elevations in calcium first appear in the urine (319), urinary calcium:creatinine ratios were calculated at each

visit to provide an early detection for elevated calcium concentrations. For each visit, safety was determined by assessing serum calcium and urinary calcium:creatinine ratios. The reference ranges at St. Michael's Hospital, where measurements were performed, are 2.1-2.6mmol/L for serum calcium and <1.0 for urinary calcium:creatinine. If serum calcium concentrations were within the reference range and urinary calcium:creatinine did not exceed 1.0, the participant was able to proceed with the next dose of vitamin D<sub>3</sub>. If abnormal serum or urinary calcium concentrations were detected, they were handled as outlined below.

The maintenance phase involved supplementation with vitamin D<sub>3</sub> at 10,000 IU/d was maintained for 12 weeks. This is a physiological dose of vitamin D<sub>3</sub> that can be obtained naturally with sufficient sun-exposure (320), and believed to be the optimal dose. An 8-week down-titration to 4,000 IU/d vitamin D<sub>3</sub> supplementation was followed by a 4-week wash-out period.

*Non-compliance:* Failure of 25(OH)D to increase at 2 consecutive study visits with increasing dose of vitamin D<sub>3</sub> and was taken as evidence of possible non-compliance. Non-compliant patients would have been followed in the same manner as those taking the supplements, but may have been excluded from analyses. No patient in the treatment group was suspected of non-compliance during the trial.

### **3.4 Measurements & Monitoring:**

#### **3.4.1 Measurements for All participants:**

All patients underwent a general medical examination at baseline and end-of-study. The patient's medical chart was reviewed and a health history was taken with careful reference to any history of cardiac disease, granulomatous disease (history of tuberculosis, sarcoidosis, lymphoma, chronic fungal infection or silicosis) and nephrolithiasis or renal disease. Participants with positive health histories for these conditions, or any other significant co-morbid disease (excluding diabetes), were not offered participation.

A complete physical examination and neurological examination was conducted by either Dr. Jodie Burton or Dr. Paul O'Connor. This examination included an assessment of Expanded Disability Status Scale (EDSS) score. Fresh blood samples were collected for lymphocyte proliferation assays at baseline and end-of-study. Serum was collected at each study visit and aliquots were cryopreserved (-70°C) for all other biomarkers. In addition to scheduled study visits, patients were seen between visits for relapse events. Relapse assessments were performed by Dr. Jodie Burton. Patients experiencing relapse (defined as an objective new/re-emerging neurologic abnormality present for at least 24 hours in the absence of fever/infection) were treated as deemed appropriate by the attending neurologist (intravenous methylprednisolone or oral prednisone). All events were documented on adverse event reporting forms.

The methodologies for respective measurements are described in subsequent chapters.

### 3.4.2 Measurements in the Control Group

Control group participants were seen 4 times throughout the study as outlined in **Table 3.2**, unless a relapse occurred for which an additional visit may have occurred. Serum was collected at baseline (week 1), mid-study (weeks 11 and 29), and end-of-study (week 52). These mid-study visits corresponded to the treatment group visits following the first 8 weeks of vitamin D<sub>3</sub> supplementation (following the first 8 weeks of supplementation) and following 6 weeks at the highest vitamin D<sub>3</sub> dose (40,000 IU/d) when serum 25(OH)D concentrations would be at their highest in the treatment group.

**Table 3.2. Monitoring and measurement schedule for control group participants.**

Monitoring or Measurement	Study Week			
	1	11	29	52
Physical and Neurological Exam	X			X
Serum calcium, albumin, 25(OH)D	X	X	X	X
Urinary calcium:creatinine	X		X	X
Lymphocyte proliferation assays	X			X
Serum cytokines	X			X
C reactive protein	X		X	X
Serine proteases: MMP-9, TIMP-1, KLK-6	X	X	X	X
Vitamin D metabolites: 1,25(OH) <sub>2</sub> D, 24,25(OH) <sub>2</sub> D	X		X	X
D Binding Protein (DBP)	X			X
Bone markers: BALP, C-Tx, OPN	X		X	X

### 3.4.3 Measurements in the Treatment Group

Treatment patients were seen every 6 weeks as outlined in **Table 3.3** for measurements/monitoring and to obtain subsequent doses of vitamin D<sub>3</sub> and calcium supplements. Serum calcium, albumin, 25(OH)D, PTH, liver function enzymes (ALT, AST, ALP), creatinine, urea and urinary calcium:creatinine ratios were measured in treated patients at all study visits. After the screening visit, participants were provided with 2 labeled sterile urine containers and a biohazard sample bag. Participants were instructed to collect a urine sample the evening prior to the scheduled clinic visit and the morning of the visit to account for daily variance in calcium excretion. The average urinary calcium:creatinine measurement was used for assessment of safety. Participants did not begin taking the next dose of vitamin D until serum and urinary calcium concentrations were found to be within reference ranges (2.2-2.6mmol/L and <1.0, respectively).

Renal ultrasounds were performed for treatment group participants at baseline (week 1), mid-study (week 29), and end-of-study (week 52). These studies were performed to ensure that deposition of calcium in renal tissue did not occur. This is not expected to occur, given that urine calcium did not change in the pilot study (248). However, this radiologic intervention provided an added objective measurement to evaluate the safety of high doses of vitamin D<sub>3</sub> with calcium. Renal ultrasound provided the advantage of detecting soft-tissue calcification, which might indicate an increased risk of renal stone formation if found. Further, electrocardiograms (ECG) were performed at baseline and end-of-study to ensure normal cardiac function and that calcification of cardiac tissue did not occur either.

Abnormal Calcium Measurements (Applicable to all subsequent visits):

In the event abnormal calcium values were encountered:

1. If urinary calcium:creatinine exceeded 1.0 but serum calcium was within the reference range: the participant was instructed to stop taking the calcium supplement and asked to return to the clinic within 3 days for repeat testing. If calcium:creatinine returned to normal subsequent to stopping the calcium supplementation, the participant discontinued calcium supplementation and continued with the schedule for vitamin D<sub>3</sub> supplementation.
2. If serum calcium exceeded the reference range (>2.6mmol/L) and urinary calcium:creatinine were <1.0: the participant was instructed to return for repeat testing. If the second measurement was within the reference range, the patient continued with supplementation as before. If, on repeat, serum calcium still exceeded 2.6mmol/L the patient discontinued all supplementation and was followed for the duration of the study as if they were still taking supplementation.
3. If both serum calcium and urinary calcium:creatinine were found to be above their respective reference ranges: the patient was asked to discontinue taking the calcium supplement and to return to the clinic for repeat testing. If both measurements were found to be normal the participant continued with the vitamin D<sub>3</sub> supplementation without further calcium supplements. In the event that either measurement still exceeded the reference range from the second sample, the patient discontinued all supplementation and was followed for the duration of the study as if they were still taking supplementation.

4. If the participant had already stopped calcium at a previous visit due to a high urinary calcium:creatinine and again had a ratio that exceeded 1.0: the test was repeated as before to rule out laboratory error or simple diurnal variation. If the abnormality persisted, vitamin D<sub>3</sub> supplementation was discontinued as well and the participant was followed for the duration of the study as if they were still taking supplementation

**Table 3.3. Monitoring and measurement schedule for treatment group participants.**

Monitoring/Measurements	Week of Study											
	Sc	1	3	5	11	17	23	29	35	41	49	52
Physical & Neurological Exam	X											X
Serum calcium, albumin, 25(OH)D, ALT, AST, ALP, creatinine, PTH	X		X	X	X	X	X	X	X	X	X	X
Urinary Calcium:creatinine	X		X	X	X	X	X	X	X	X	X	X
Lymphocyte proliferation assays	X											X
Serum cytokines	X											X
C reactive protein	X							X				X
Serine proteases: MMP-9, TIMP-1, KLK-6	X				X			X				X
Vitamin D metabolites: 1,25(OH) <sub>2</sub> D, 24,25(OH) <sub>2</sub> D	X											X
Vitamin D binding protein (DBP)	X											X
Bone markers: BALP, C-Tx, OPN	X							X				X
Renal Ultrasound	X							X				X
Electrocardiogram	X											X

### **3.5 Adverse Events**

The risk of venipuncture included temporary discomfort, local pain, bruising, swelling and rarely phlebitis. Some participants may have demonstrated a vasovagal reaction to venipuncture and develop dizziness or fainting; however, this did not occur in the present trial.

With vitamin D<sub>3</sub> and calcium supplementation there may be a very small increased risk of developing kidney stones (313). Participants with a history of kidney disease, including kidney stones, were excluded from the study.

The most important risk to participants taking vitamin D<sub>3</sub> with calcium supplementation is vitamin D<sub>3</sub> toxicity and the development of hypercalcemia. Symptoms of hypercalcemia include malaise, tiredness, weakness, confusion, anorexia, pain, increased thirst and urination, constipation, nausea and vomiting (316). The most serious adverse events are hypercalcemia-related neurological, cardiac and renal effects, including muscle weakness, decreased level of consciousness, irregular heart rhythms (arrhythmia), low blood pressure and renal impairment (315). Of note, vitamin D<sub>3</sub> supplementation capable of resulting in the most serious of these adverse events involves the long-term intake of doses >40,000 IU/d (81).

Vitamin D<sub>3</sub> toxicity is not expected to occur in this study based on the results of the pilot study (248). Further, sun-exposure can produce physiologic amounts of vitamin D<sub>3</sub> equivalent to 10,000 IU in a mere 15 minutes with total body exposure, nor has toxicity ever been observed at the levels targeted in the present trial (81). A trial in healthy men from Nebraska (n=67) has demonstrated the safety of 10,000 IU/d over 28 weeks, with no reported side effects or incidence of hypercalcemia (78).

## 4.0 A PHASE I/II DOSE-ESCALATION TRIAL OF VITAMIN D<sub>3</sub> AND CALCIUM IN MULTIPLE SCLEROSIS

*“No great discovery was ever made without a bold guess.”*

*Sir Isaac Newton*

### 4.1 Abstract

**Background:** Low vitamin D status has been associated with multiple sclerosis (MS) prevalence and risk. However, it remains unclear if vitamin D<sub>3</sub> is *per se* beneficial in established MS, and in particular, at what dose. **Objectives:** Here we characterize the safety profile of high-dose oral vitamin D<sub>3</sub> in MS. **Methods:** In this prospective open-label 52-week trial, patients with MS were matched (sex, age, subtype, EDSS, disease duration and disease modifying drug) and pairs were randomized to treatment or control groups. Treatment patients received escalating vitamin D<sub>3</sub> doses over 28 weeks (4,000-40,000 IU/d) followed by 10,000 IU/d (12 weeks), and down-titrated. Calcium (1,200 mg/d) was given throughout the trial. Primary endpoints were to detect toxicity, most importantly any change in serum or urinary calcium. Secondary endpoints included other biochemical measures, clinical (EDSS and relapses) and investigations of immunological biomarkers. **Results:** Forty-nine patients (25 treatment, 24 control) with MS were enrolled. At baseline 25(OH)D concentrations were  $78.1 \pm 27.0$  nmol/L and by end-of-study were  $82.7 \pm 34.8$  and  $179.1 \pm 76.1$  nmol/L in control and treatment groups, respectively. All calcium measures were normal throughout the trial. Despite a mean peak 25(OH)D of  $414.7 \pm 146.4$  nmol/L, no significant adverse events were observed. Compared to controls, treated patients tended to have fewer relapses, greater relapse rate reduction, and a greater

proportion completed the trial with stable/improved EDSS. Treated patients had persistent reduction in T-cell proliferation compared to controls. **Discussion:** High-dose vitamin D<sub>3</sub> (~10,000 IU/d) in MS is safe, with evidence of clinical improvement and immunomodulation.

## 4.2 Introduction

Multiple sclerosis (MS) risk has a well-documented geographical distribution, with prevalence and risk rising with increased distance from the equator in either direction (211;212;321;322). Sunlight or UVB radiation exposure, the primary source of vitamin D in humans, is inversely correlated with MS risk and prevalence, and likely represents a major intermediary between latitude and MS (212;323). In support, serum 25-hydroxyvitamin D [25(OH)D] concentrations, a measure of vitamin D status, are also inversely correlated with MS risk and prevalence (212;225;323).

Consistently, MS patients have lower serum 25(OH)D levels than healthy controls (226;230). Although sun-avoidant behaviors and MS therapies might explain low serum 25(OH)D values, MS disease activity, clinically and radiologically, increases during periods of low UVR exposure and decreases in periods of high exposure (226;228;230), suggesting a role for vitamin D in active disease.

Vitamin D in its active hormonal form, 1,25(OH)<sub>2</sub>D, with calcium pre-treatment in the EAE mouse has been shown to prevent clinical or pathological disease development and treatment after induction ameliorated disease activity (303). Vitamin D has “anti-inflammatory” actions *in vitro*, with affects on dendritic cell differentiation, macrophage phagocytic activity, and promotes a generally less pro-inflammatory profile (7).

Despite some positive evidence (197;210;247;248), it remains unclear whether or not vitamin D<sub>3</sub> supplementation can be beneficial to MS patients. One challenge in the field is the fear of vitamin D toxicity, and another is the uncertainty of what constitutes a ‘normal’ value in

the general population. Here we established the clinical acceptability of high serum 25(OH)D concentrations in the absence of altered calcium homeostasis (i.e. toxicity) and a lack of negative impact on disease course in established MS patients. Despite the low statistical power, rising serum 25(OH)D concentrations (over 500%) for less than a year tended to positively affect disease outcomes on measured parameters.

## **4.3 Methods**

### **4.3.1 Participants**

Patients were enrolled at St. Michael's Hospital MS Clinic in Toronto, Canada between June 2006 and March 2008 for a 52-week treatment trial. All patients provided written informed consent. Inclusion criteria included patients with clinically definite MS according to McDonald criteria (130) between 18-55 years of age with Expanded Disability Status Scale (EDSS) scores of 0 to 6.5. Patients were excluded if any of the following characteristics were present: relapse event within 60 days, steroid use within 30 days, chemotherapy within 12 months, pregnancy or in adequate contraception, vitamin D<sub>3</sub> intake >4,000 IU/d or serum 25(OH)D level of >150nmol/L, lymphoma, granulomatous disease, cardiac arrhythmia, nephrolithiasis, kidney dysfunction or disordered calcium metabolism.

### **4.3.2 Trial Design**

Fifty-one patients were screened and 49 enrolled. Patients were grouped according to age (18-30, 31-40, 41-55), disease duration (0-9 years, 10-19 years, ≥20 years), EDSS (≤2.5, 3.0-5.5, 6.0-6.5) and disease modifying drug (interferon, glatiramer acetate, or none). Pairs were enrolled and members were randomized to treatment or control groups by blindly drawing a letter out of a hat.

Patients in the treatment group began calcium supplementation (1,200mg/d) at visit 1, initiated vitamin D<sub>3</sub> two weeks later and followed the escalating supplementation schedule in

**Table 3.1.** Patients in the control group were permitted to take  $\leq 4,000$  IU/d of vitamin D<sub>3</sub> daily and supplemental calcium as per the MS Clinic’s standard of care at St. Michael’s Hospital.

All patients underwent a general medical examination at baseline and end-of-study, including assessment of EDSS. Serum calcium, albumin, and 25(OH)D concentrations were measured for all patients at baseline, mid-study, and end-of-study. All assessments, biochemical and immunological measurements were performed according to the schedule in

**Tables 4.1.** Treatment patients were assessed every six weeks for safety markers and were instructed to start taking the next dose of vitamin D<sub>3</sub> only after calcium and urinary calcium/creatinine were found to be within normal limits.

Patients experiencing possible relapse or adverse events were seen within 72 hours by the treating physician. Patients requiring relapse treatment received steroid therapy (intravenous methylprednisolone or oral prednisone) as deemed appropriate by the treating physician. All events were documented on adverse event reporting forms.

**Table 4.1. Biomarker measurement schedule for all study participants.**

Measurement	Week of Study			
	1	11	29	52
<b>Immune markers: Lymphocyte proliferative responses, serum cytokines</b>	X			X
<b>Inflammatory markers: MMP-9, TIMP-1, OPN, KLK-6</b>	X	X	X	X
<b>C reactive protein</b>	X		X	X
<b>Bone turnover markers: BAP, CTx</b>	X		X	X

### **4.3.3 Vitamin D<sub>3</sub> Preparation and Administration**

The preparation and quality testing of vitamin D<sub>3</sub> solution has been previously described (248). Hyperconcentrated solutions were supplied in light-resistant amber glass vials at every visit. Patients were instructed to take anywhere between one to four mL per week (equivalent to between 4,000 and 40,000 IU/d) depending on the scheduled dose. Patients were also supplied with tricalcium phosphate and instructed to take a measured amount equivalent to 1,200mg/d of elemental calcium.

### **4.3.4 Immunological Investigations**

Peripheral blood T-cell proliferative responses to a panel of 10 MS-related dietary, self and control antigens were measured as previously described (324). MMP-9 and TIMP-1 were measured with the Quantikine ELISA system (R&D Systems Inc., Minneapolis, MN) according to manufacturer's directions. Cytokine samples were drawn from serum and the supernatant from T-cell proliferation assays. Cytokine concentrations (IL-1 $\beta$ , -2, -4, -5, -6, -10, -12p40, -13, IFN- $\gamma$ , and TNF- $\alpha$ ) were measured simultaneously by multiplex bead immunoassays and read on the Luminex LX100 as previously described (325).

### **4.3.5 Statistical analysis**

Sample size was based on a power of 0.90 at a 2-sided 0.05 significance level. If a difference between treatment and control groups of 1 standard deviation was considered a clinically meaningful increase in calcium related parameters most likely to be adversely affected

by vitamin D<sub>3</sub> supplementation, a sample of 50 patients provided >0.90 power with an alpha of 0.05 for primary meaningful effect size of 0.30 mmol/L difference in serum calcium. Sign and sign rank testing were used to evaluate continuous outcomes. Wilcoxon sign rank tests, McNemar testing, and Chi-square and Fisher's exact tests were used to analyze categorical and binary outcomes while repeated measures were calculated using mixed modeling procedures and time series analysis. Logistic regression was used to analyze the role of covariates on primary outcome modeling. All statistical tests were performed using SAS 9.1.3™.

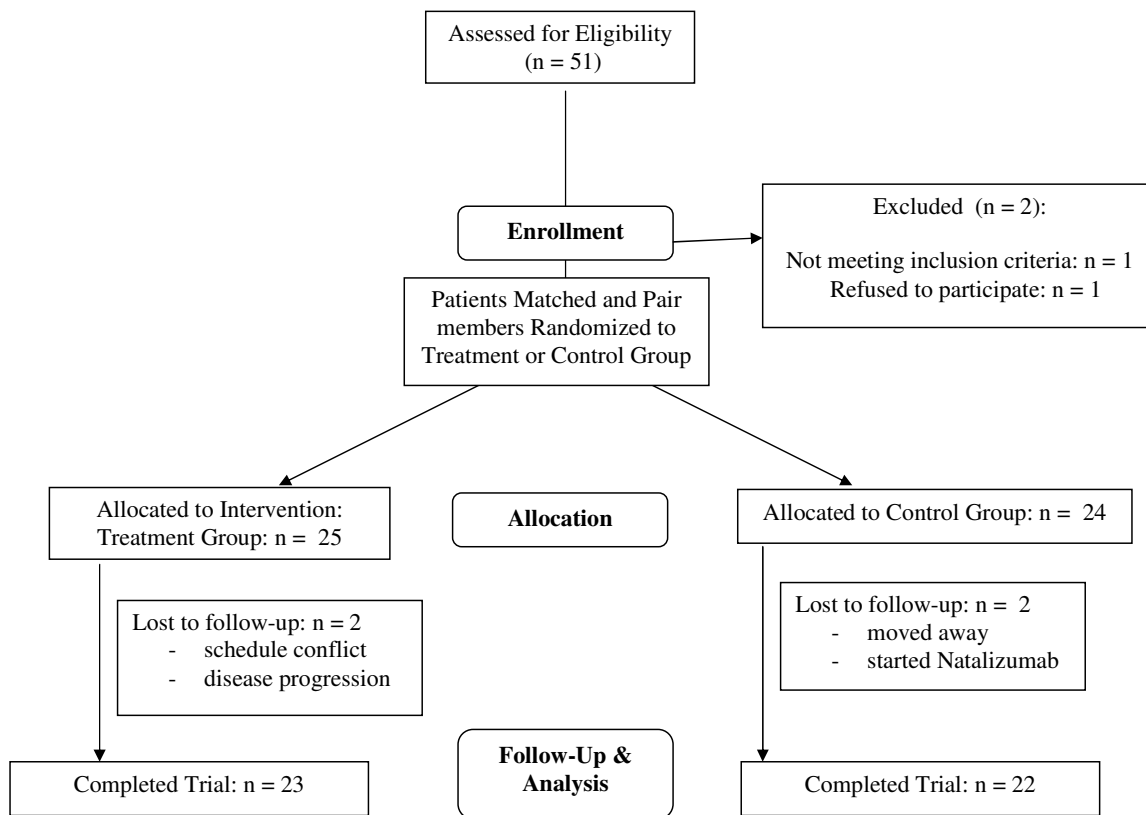
## 4.4 Results

Between June 2006 and March 2007, 51 patients were screened and the eligible 49 enrolled. Patients were matched and pair members were randomized to either treatment or control groups. Forty-five patients had relapsing-remitting MS while the remaining four had secondary progressive MS. Forty women and nine men participated, with mean age 40.5 years (range 21-54y), mean EDSS 1.34 (range 0-6.0) and disease duration of 7.8y (range 1-25y). At baseline there were no significant differences between groups as presented in **Table 4.2**, including pre-trial vitamin D<sub>3</sub> intake and relapse rate. Two patients in each group withdrew during the trial; none related to adverse events (**Figure 4.1**).

**Table 4.2. Patient Demographics at baseline**

Variable	Treatment Group	Control Group	p-value
Age (years)	41.1 (22-54)	39.9 (21-53)	NS
Gender	21 F: 4 M	19 F: 5M	NS
MS Subtype	23 RR: 2 SP	22 RR: 2 SP	NS
Disease Duration (years)	8.2 (1-25)	7.4 (1-21)	NS
Baseline EDSS	1.46 (0-6.0)	1.23 (0-6.0)	NS
Season Enrolled	Sp/Su 16: F/W 9	Sp/Su 11: F/W 13	NS
Annualized Relapse Rate	0.44 (0-3)	0.54 (0-2)	NS
DMD Use	IFN 12: GA 2: NO 11	IFN 12 :GA 2: NO 10	NS
Vitamin D <sub>3</sub> dose (IU/d)	1,160 (0-4,000)	991 (0-4,000)	NS
Serum 25(OH)D (nmol/L)	73 (38-146)	83 (38-154)	NS
Serum calcium (mmol/L)	2.32 (2.08-2.57)	2.32 (2.16-2.47)	NS

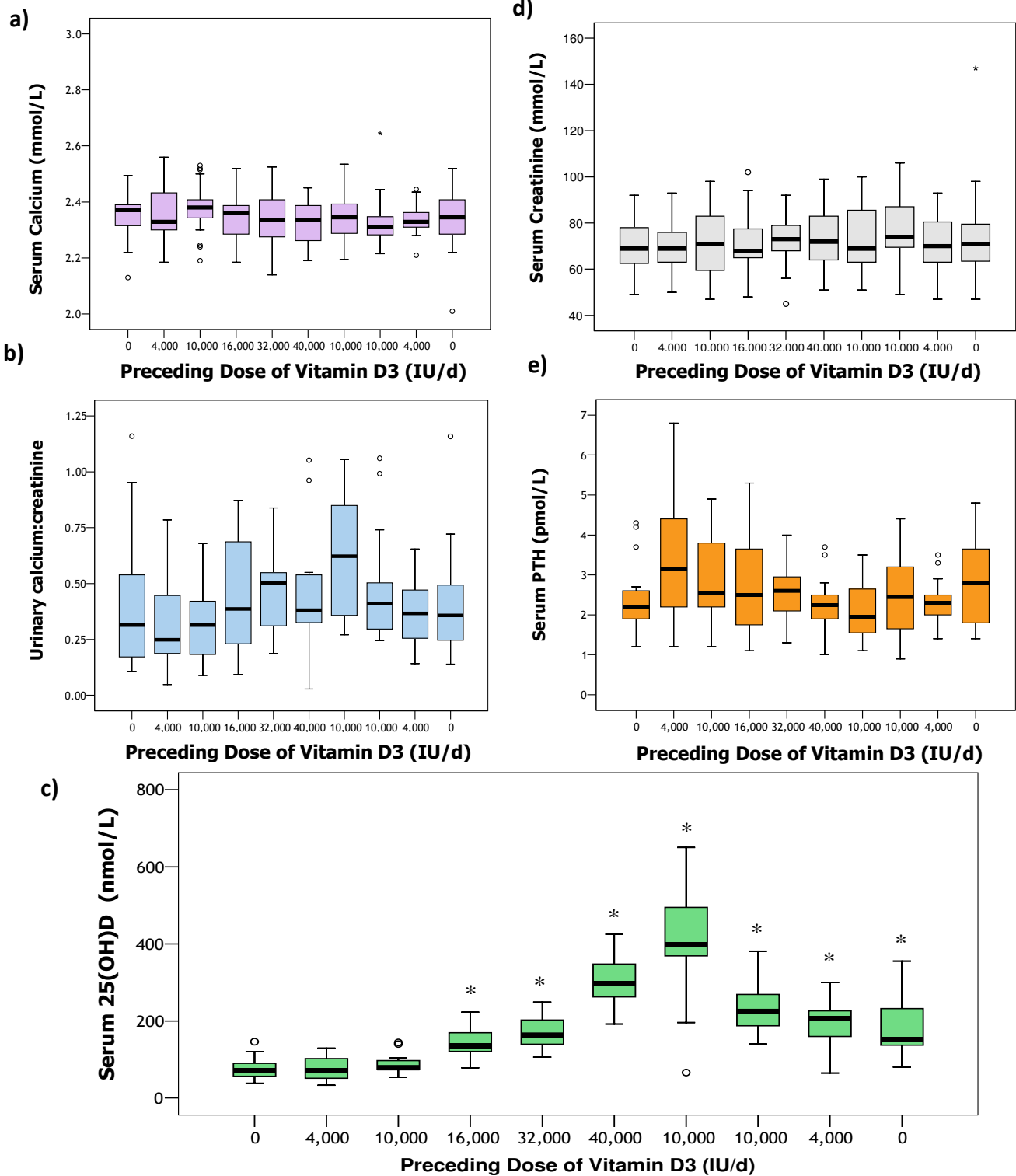
DMD (disease modifying medication), IFN (interferon beta), GA (glatiramer acetate), NO (none), Sp/Su (spring/summer), F/W



**Figure 4.1. Flowchart of patient enrollment and follow-up.**

#### **4.4.1 Biochemical Outcomes**

Serum calcium and urinary calcium:creatinine ratios were used to detect any sign of vitamin D<sub>3</sub> toxicity in the treated group. Serum calcium, evaluated by repeated measures, did not differ significantly with increased vitamin D<sub>3</sub> doses, nor were any values above the reference range (2.1-2.6 mmol/L) detected (**Figure 4.2a**). Urinary calcium:creatinine ratios did not exceed the upper limit (<1.0). During the weeks patients were taking the highest doses urinary calcium/creatinine ratios were increased (maximum mean ratio 0.61), but remained



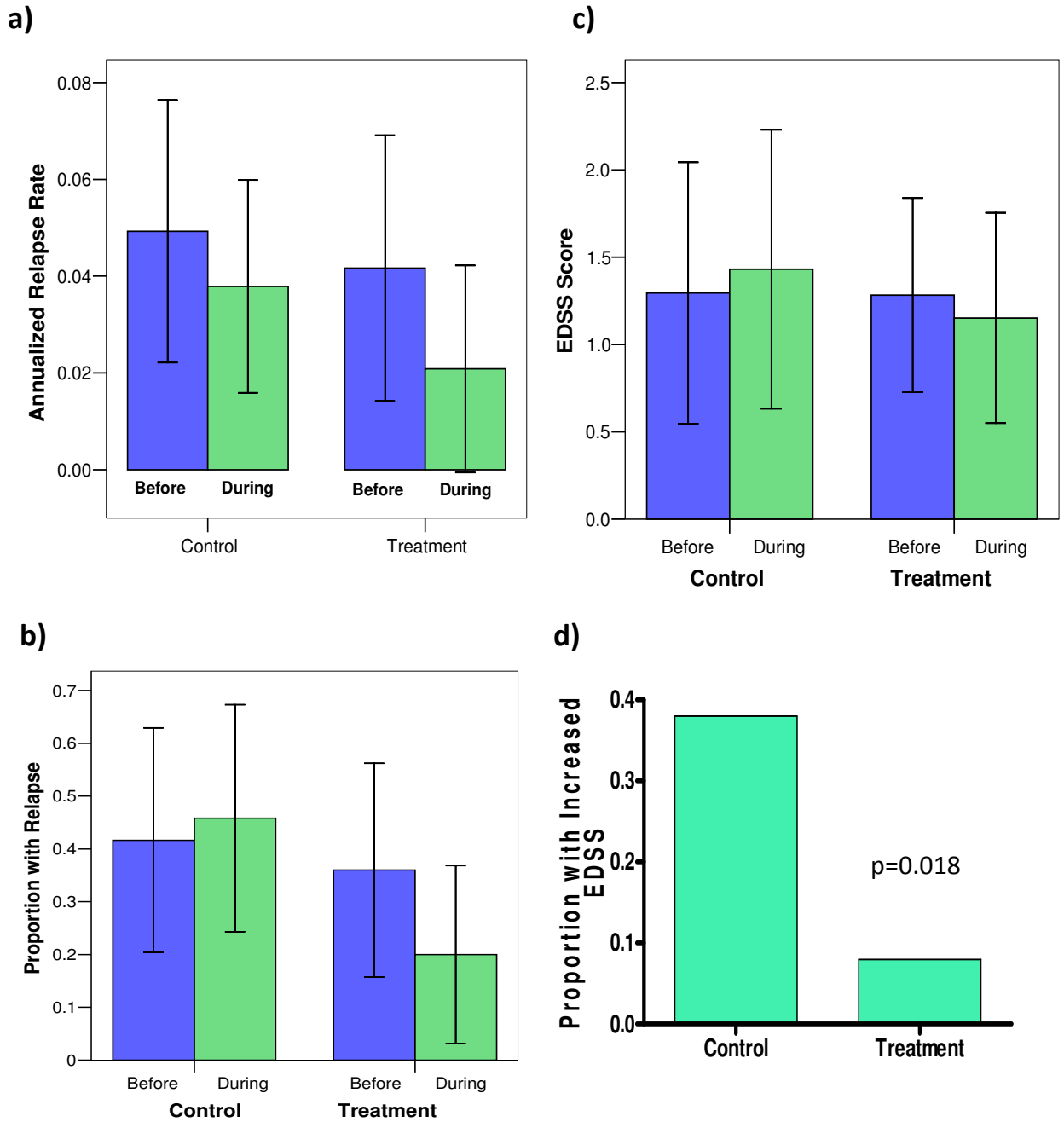
**Figure 4.2. Biochemical responses to high-dose vitamin D<sub>3</sub> with calcium supplementation in the treatment group.** a) Serum calcium, reference range 2.1-2.6 mmol/L; b) Urinary calcium:creatinine ratios, reference range <1.0; c) Serum 25(OH)D; d) Serum creatinine, a measure of kidney function; e) Serum PTH, reference range 1.6-6.9 pmol/L. Boxes represent the central 50%, whiskers show the highest and lowest values, and the line indicates the median value. \*Denotes significant difference from baseline, p-value <0.005.

within the normal range, indicating the appropriateness of utilizing urinary calcium as an indicator of increased calcium (**Figure 4.2b**). As expected, serum 25(OH)D concentrations increased steadily and significantly from a mean of  $73.5 \pm 26.4$  nmol/L, reaching a mean of  $414.7 \pm 146.4$  nmol/L ( $p < 0.001$ ) after the highest dose of vitamin D<sub>3</sub>, and was  $179.1 \pm 76.1$  nmol/L at end-of-study ( $p < 0.001$ ). This peak value is well above the often cited “toxic” value 250 nmol/L. In fact, patient mean serum 25(OH)D values were above this “threshold” for over 18 weeks with no measureable biochemical or clinical adverse consequences (**Figure 4.2c**), including abnormalities of serum creatinine (**Figure 4.2e**), urea, ALT, AST, or ALP values. PTH did not differ across doses (**Figure 4.2f**). The influence of season was ruled out using time series analysis. In the control group, serum 25(OH)D concentrations remained unchanged (baseline  $82.9 \pm 27.4$  and end-of-study  $82.7 \pm 34.8$  nmol/L).

No patient had evidence of calcification in renal structures on ultrasound during the trial. Incidental chronic pelviectasis was found in one patient without clinical symptoms or evidence of nephrolithiasis. Known chronic hepato-hemangiomas in one patient were unaffected by the trial, and known, transient hepatosteatorosis attributed to previous steroid use in another patient resolved spontaneously. No patient developed disturbance of cardiac rhythm detected by electrocardiogram. Thus, we conclude, that high serum 25(OH)D concentrations, above 150nmol/L, can be safely achieved and sustained in adults with MS for longer than 4 months.

#### **4.4.2 Clinical Outcomes**

This toxicity trial was not designed to have the power to detect any but dramatic clinical impact. However, the mean annualized relapse rate in treatment patients fell from 0.44 to 0.26 (41% reduction) while in control patients, it also fell, but only from 0.54 to 0.45 (17% reduction) ( $p=0.13$ ) (**Figure 4.3a**). The proportion of patients who experienced relapses during the trial was 0.16 in the treatment group compared to 0.37 in the control group (Fisher's exact,  $p=0.11$ ). The proportions in the year preceding the trial were 0.36 and 0.40, respectively (**Figure 4.3b**). As well, mean disability status as measured by EDSS dropped from 1.46 to 1.23 in the treatment group while it rose from 1.15 to 1.45 in controls, but due to small cohort sizes, this trend did not reach significance (**Figure 4.3c**). The proportion of patients whose EDSS increased over the 52-week trial was almost five times greater in the control group than in the treatment group (0.08 versus 0.38,  $p=0.018$ ) (**Figure 4.3d**).



**Figure 4.3. Clinical measures from the year prior to study entry in comparison with the year of the study.** a) Annualized relapse rates were not significantly different between groups for the year of study; b) The proportion of patients experiencing a relapse showed a trend to be less in the treatment group for the year of study (McNemar,  $p=0.09$ ); c) EDSS changes did not differ significantly between groups; d) The proportion of patients who had an increase in EDSS score at end-of-study vs. baseline was significantly smaller in the treatment group (Fisher's exact,  $p=0.018$ ). Error bars represent 95% CI.

#### **4.4.3 Immunological Outcomes**

T-cell proliferative responses were compared at baseline and one year later at end-of-study. The total T cell proliferation, (a score was generated based on proliferation in response to antigenic stimulation), decreased significantly in the treated group in comparison with baseline (Wilcoxon Sign Ranks,  $p=0.002$ ). Differences in post-trial T cell scores between treated and control arms were also detected ( $p=0.003$ ) (data not shown).

Matrix metalloproteinase 9 (MMP-9) and its inhibitor, tissue inhibitor of metalloproteinase 1 (TIMP-1), are among a group of tissue-active enzymes thought to enhance access of inflammatory immune cells into the CNS (167;326). We measured serum levels of both proteins and when treated and control groups were individually analyzed as repeated measures over the course of the trial small reductions in the MMP-9/TIMP-1 ratio were observed in both trial arms (i.e. not treatment-related) between visits, but comparing changes across the year between treated and control patients did not reveal any significant difference between groups (data not shown).

Cytokine measurements for IL-1 $\beta$ , -2, -4, -5, -6, -10, -12p40, -13, IFN- $\gamma$ , and TNF- $\alpha$  were made at baseline and end-of-study in both serum samples and supernatants from the T cell proliferation assays. There were no consistent patterns of change in cytokine titers within or between groups over the 52-week period (data not shown). A more detailed analysis of T cell proliferation assays and cytokine profiles is provided in **Chapter 5.0**.

#### **4.4.4 Adverse Events**

No adverse events associated with the use of vitamin D<sub>3</sub> were reported. Four treatment patients experienced mild constipation early in the trial attributed to calcium supplementation in powdered form. With switch to calcium tablets in 3 patients and discontinuation of calcium in one, these symptoms resolved.

## **4.5 Discussion**

The present trial has demonstrated the safety of high serum 25(OH)D concentrations, well above the assumed toxic 250 nmol/L threshold, in patients with MS. Specifically, no episodes of hypercalcemia nor persistent hypercalciuria occurred despite peak 25(OH)D concentrations of >400 nmol/L. Treated patients spent 36/52 weeks on doses of vitamin D<sub>3</sub> ≥ 10,000 IU/d. The mean daily dose for the year was roughly 14,000 IU/d.

Although the study was not powered to measure even short term clinical impact on MS course, emerging clinical trends hint at treatment-related improvements in disease course. Treated patients had a greater reduction in relapse rate and a lower proportion of these patients had relapse events compared to control patients although this did not reach significance. While most patients in the trial had relatively mild MS, those in the treatment group were significantly less likely to complete the trial with a higher EDSS than at baseline, while a significant proportion of control patients did ( $p=0.018$ ).

Measurements of a number of other pro- and anti-inflammatory markers in serum and T cell culture supernatants did not reveal any differences within or between groups. However, parallel measurement of multiple, abnormal and MS-associated T cell reactivities enhanced the power of T cell proliferative responses in detecting treatment-associated changes over the trial course. Thus, T cell proliferation scores, i.e. the number of positive responses, declined significantly in the treated patients, and overall, the decline of T cell reactivity was most pronounced in patients who maintained a serum 25(OH)D concentration of >100 nmol/L at end-of-study. While longer term treatment in larger, randomized, blinded studies will be

required to seek relationships between clinical course improvements and T cell reactivities, the latter observation may provide a first, tentative target range for therapeutic serum 25(OH)D levels in (mild) MS.

Unlike earlier trials of vitamin D supplementation in MS, the present trial employed a control group with the disease, and used high-dose vitamin D<sub>3</sub> (the immediate product of UVB exposure of human skin), as opposed to the active metabolite, 1,25-dihydroxyvitamin D<sub>3</sub> [1,25(OH)<sub>2</sub> D<sub>3</sub>] (247). Our effort was the first controlled trial in any population to test the safety of such high doses of vitamin D<sub>3</sub> with high serum 25(OH)D levels sustained over several months. It appears that our dose titration schedule worked well to avoid possible toxicity at higher doses of vitamin D<sub>3</sub>, in fact, none were observed throughout the duration of the trial. Whether such toxicities emerge upon longer exposures remains to be established, but the trial identified achievable, non-toxic target levels of serum 25(OH)D that may be able to improve measures of autoimmunity.

The addition of calcium supplementation allowed our safety data to be generalized to those already on calcium supplementation, and this combination was effective in both animal experiments and human cancer prevention trials (303;327).

The present trial had sufficient power to interpret a primary safety endpoint, serum calcium levels (210;247). The use of vitamin D<sub>3</sub> provided additional safety from hypercalcemia, as this form has low calcemic potential. In fact, a previous trial of supplementation with 1,25(OH)<sub>2</sub>D did encounter symptomatic and asymptomatic hypercalcemia at serum 25(OH)D levels much lower than those observed here (81).

The main limitations of the present study were its focus on clinical safety, lack of MRI data, and its associated lack of power to measure clinical impact of vitamin D<sub>3</sub>. The relatively small size of the trial cohort made exact matching in the trial arms difficult. Randomization with stratification provided more balanced trial arms, but without blinded placebo control some inadvertent selection bias is likely, such that some patients perhaps were more active in their MS care and nutritional choices. While the primary outcome (biochemical measures of calcium status), would not be expected to be vulnerable to a lack of blinding, clinical outcomes are subject to bias.

The results of this trial clearly demonstrate that doses of vitamin D<sub>3</sub> well above current recommendations, and 25(OH)D concentrations well beyond a still poorly supported “normal” range, do not expose patients with MS to adverse biochemical or clinical outcomes. In comparison to a control group whose serum 25(OH)D concentrations were considered sufficient, i.e mean serum 25(OH)D was >75nmol/L, our treatment group demonstrated improvement in MS clinical outcomes and abnormal T-cell reactivity.

Vitamin D<sub>3</sub> has a robust and consistent impact on MS risk, and a therapeutic effect in established MS would be intuitively appealing. Previous studies and the present trial point to a reduction of disease progressivity that deserves further study. Vitamin D<sub>3</sub> now appears to play a role in a growing spectrum of medical conditions (328). Without a factual consensus regarding “normal” levels, and with uncertainty of whether normal levels are sufficient in some or all conditions impacted by vitamin D status, development of a multi-center randomized, blinded and control phase trial is necessary, desirable and must have a primary focus on MRI lesions, clinical response to vitamin D status at the time of clinically isolated syndrome presentation.

## 5.0 EVIDENCE OF *IN VIVO* IMMUNOMODULATION BY VITAMIN D<sub>3</sub> WITH CALCIUM SUPPLEMENTATION IN PATIENTS WITH MULTIPLE SCLEROSIS

*“Hormones, vitamins, steroids and depressives are oils upon the creaky machinery of life. Principal item, however, is the machinery.”*

*Martin H. Fischer*

### 5.1 Abstract

**Background:** In vitro and animal studies show that vitamin D, specifically 1,25(OH)<sub>2</sub>D, has immunomodulatory properties. **Objectives:** We conducted a clinical trial to determine if the sun-dependent nutrient, cholecalciferol, can alter disease-associated cellular immunity in patients with multiple sclerosis (MS). **Methods:** 48 patients with MS were matched (for age, sex, disease duration, disease modifying drug, and disability) and randomized either to 12 mo of no treatment, or to a 6-month protocol of increasing doses of cholecalciferol (4,000-40,000IU/d) and calcium (1,200mg/d), followed by equilibration to a moderate, physiological intake (4000IU/d) when tested at 12 mo. At enrollment and at 12 mo, peripheral blood T cell proliferative responses to disease-associated, MS-relevant and control antigens were measured, along with serum biochemical markers. **Results:** At 12 mo, mean serum 25-hydroxyvitamin D [25(OH)D] concentrations were 83±35nmol/L in the control group, and 179±76nmol/L in the treated group (paired t, p<0.001). Serum 1,25-dihydroxyvitamin D [1,25(OH)<sub>2</sub>D] values remained unchanged from baseline in both groups. In the treated group, 12-mo values for T cell proliferative responses to neuronal antigens Ex-2 and MBP were suppressed (p=0.002). In the control group there were no significant changes in disease-associated T cell reactivities of control patients. There were no significant differences between groups in levels of selected cytokines, matrix metalloproteinase-9 and its tissue inhibitor-1,

kallikrein-6, C reactive protein, osteopontin, bone specific alkaline phosphatase or c  
telopeptide. **Conclusions:** Our results demonstrate that MS-associated, abnormal T cell  
reactivities were suppressed in vivo by cholecalciferol at serum 25(OH)D concentrations higher  
than the current therapy target of 75nmol/L.

## 5.2 Introduction

Multiple sclerosis (MS) is a demyelinating, neuroinflammatory and neurodegenerative disease of the central nervous system (CNS). While its etiology remains enigmatic, there is longstanding consensus that abnormal pools of disease-associated T lineage effector cells fundamentally contribute to the CNS tissue lesions that characterize MS pathology (324;329;330). Most of the current, partially effective, immune therapies for MS are thought to target these abnormal T cell pools (196).

Vitamin D<sub>3</sub> is a seco-steroid, readily metabolized by the liver to 25-hydroxyvitamin D [25(OH)D], an inactive metabolite which reflects vitamin D<sub>3</sub> nutritional status. Ultraviolet light exposure can produce physiological levels of serum 25(OH)D as high as 225nmol/L (81). Although known for its effects on calcium homeostasis and bone mineral density, various cells metabolize 25(OH)D into 1,25-dihydroxyvitamin D [1,25(OH)<sub>2</sub>D], the signaling molecule that interacts with the vitamin D receptor (VDR) in target tissues. Vitamin D<sub>3</sub> may have therapeutic potential in MS because preclinical *in vitro* and *in vivo* animal experiments show that 1,25(OH)<sub>2</sub>D affects cell proliferation and apoptosis, differentiation of immune cells and modulates immune responses. T cells and antigen presenting cells express the VDR (249;250) and possess the capability to produce 1,25(OH)<sub>2</sub>D from 25(OH)D via the expression of 1- $\alpha$ -hydroxylase (229;331). Dendritic cells (DCs) respond to 1,25(OH)<sub>2</sub>D with differentiation arrest, attenuated antigen presentation (258) and such tolerogenic DCs can induce regulatory T (Treg) cells (268), promoting a shift towards Th2 predominance in proinflammatory tissue lesions (46). In the mouse, 1,25(OH)<sub>2</sub>D pre-treatment prevents the development of experimental allergic

encephalomyelitis (EAE), and after onset of active disease 1,25(OH)<sub>2</sub>D treatment ameliorates symptoms (285).

Much of the current data on the anti-inflammatory effects, signaling through VDR, are focused on the final metabolite, 1,25(OH)<sub>2</sub>D, with little evidence that the nutrient compound, cholecalciferol (vitamin D<sub>3</sub>), has effects in animals or humans. However, indirect human data imply desirable effects of cholecalciferol in the context of MS. The number of gadolinium enhanced MRI lesions are higher in winter than in summer (227;228), and higher serum 25(OH)D concentrations predict lower relapse rates in both adults and children (226;245;332;333). Small clinical trials report reduced development of new gadolinium-enhancing lesions (248) and reduction of circulating TGF-β1 after cholecalciferol supplementation in patients with MS (197).

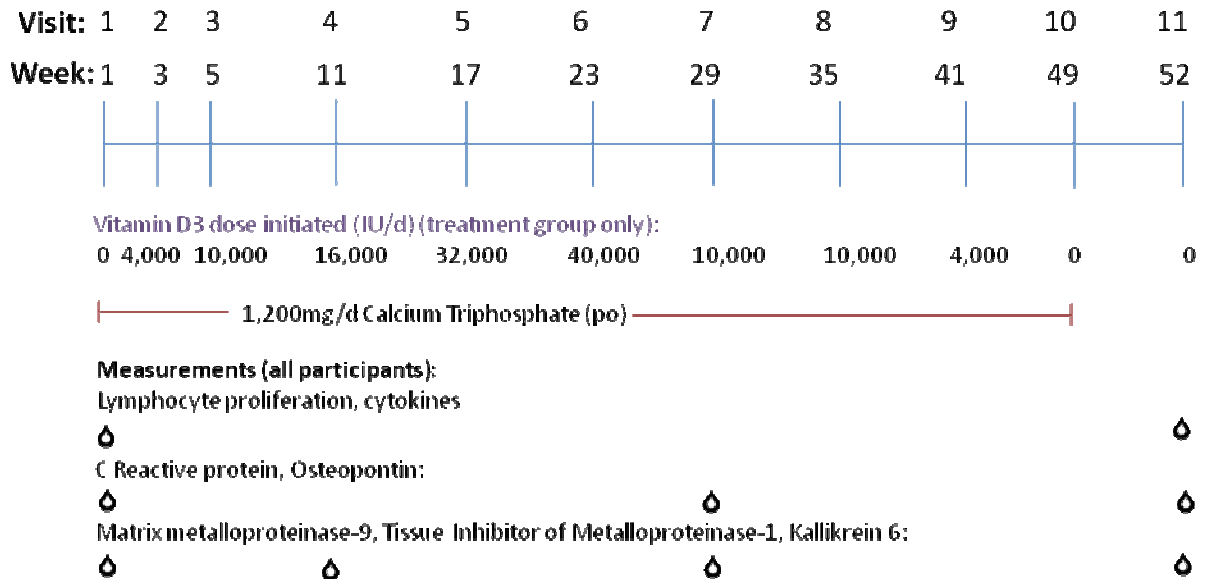
MS patients, both adults and children, show abnormal T cell proliferative responses to islet and neuronal autoantigens (324;329). *In vitro* 1,25(OH)<sub>2</sub>D-treatment of T cells from MS patients reduces their proliferative responses to MS-relevant autoantigens (229;334). To our knowledge no clinical intervention with a nutrient, such vitamin D<sub>3</sub>, has been shown to moderate these responses.


In the present clinical trial of high-dose cholecalciferol in MS patients, we have previously shown successful increase in serum 25(OH)D concentrations and observed positive impact on clinical MS parameters (335). Here we report that our trial protocol significantly reduced responses of disease-associated T cell pools, providing a mechanistic correlate for the clinical impact of increased serum 25(OH)D concentrations within the physiological range in patients with MS.

## 5.3 Methods

### 5.3.1 Vitamin D Study Design

Details of the study design have been discussed in Chapter 3 and elsewhere (335). A timeline of study visits is presented in **Figure 5.1**.



**Figure 5.1. Schedule of supplementation (treated only) and biomarker measurements (all participants).** The supplements outlined were received by the treatment group only; control group participants received no intervention. Measurements were conducted as indicated at time points marked by .

### 5.3.2 Proliferation assays

Heparinized blood samples were collected from all patients at 2 time points, baseline and one year later at end of study. Peripheral blood mononuclear cells (PBMC) were purified on Ficoll-Hypaque gradients, washed and cultured ( $10^5$  PBMC plus 10U IL-2/well) for one week in protein-free Hybrimax 2997 medium (Sigma, St. Louis, MS), when proliferative responses to an array of test- and control antigens (0.01-10  $\mu$ g/well) were measured by  $^3$ H-thymidine incorporation. Replicate responses (cpm) were averaged and normalized to generate stimulation indices (SI = cpm test antigen/cpm cells alone). Positive responses (SI  $\geq$ 1.5 (324)) to test antigens were added for antigen subset scores (dietary, islet, or neuron test antigens) and the total proliferation score (308;336).

Our T cell activation array has been described (308;329) and validated for studies of disease-associated T cell pools in patients with MS (324) or type 1 Diabetes (336;337). The array included positive controls [*tetanus toxin (TT)* and *phytohemagglutinin (PHA)*], negative controls [*cytochrome c (Cyt-c)* and *actin*], dietary (milk) antigens [*casein (CS)*,  *$\beta$ -lactoglobulin (BLG)*, *bovine serum albumin (BSA)*, and *BSA epitopes BSAp193 and BSAp147(ABBOS)*], human islet antigens [*Tep69*, *glutamic acid decarboxylase (GAD)*, *GADp555*, *proinsulin (PI)*], and neuron antigens [*myelin basic protein (MBP)*, *exon-2 of MBP (Ex-2)*, *glial fibrillary acidic protein (GFAP)* and *a glial antigen, S100B*].

Culture supernatants were cryopreserved. Supernatant pools were prepared for each subset of antigens based on proliferation responses, including: positive (PHA + TT), negative (cyt-c +, actin), dietary (BSA + BSAp193), islet (PI), and neuronal (MBP + Ex-2) antigens. Cytokine

concentrations were measured in stored supernatant pools for all treatment samples (n=23) and a random selection of control samples (n=13). Concentrations of Interleukin-1 $\beta$ , -2, -4, -5, -6, -10, -12p40, -13, interferon (IFN)- $\gamma$ , and TNF- $\alpha$  were measured simultaneously in serum samples (below) and culture supernatants, using multiplex Luminex X100 bead immunoassays (Luminex, Austin, TX) and calibrated reagents (Bio-Plex Precision Pro, Bio-Rad Lab, Hercules, CA) (325).

### ***5.3.3 Serum samples***

Sera were cryopreserved (-70°C) from all participants at baseline, twice at mid-study (at week 7, corresponding to 2 weeks of 4,000 IU/d cholecalciferol, and at week 29 corresponding to 6 weeks of 40,000 IU/d cholecalciferol in the treatment group), and at one-year after protocol completion. Samples were analyzed together in the same run. **Figure 5.1** depicts measurements with respect to the treatment group dosing schedule.

### ***5.3.4 Cytokine concentrations***

Concentrations of Interleukin-1 $\beta$ , -2, -4, -5, -6, -10, -12p40, -13, interferon (IFN)- $\gamma$ , and TNF- $\alpha$  were measured simultaneously in serum samples (below) and culture supernatants, using multiplex Luminex X100 bead immunoassays (Luminex, Austin, TX) and calibrated reagents (Bio-Plex Precision Pro, Bio-Rad Lab, Hercules, CA) (325).

### **5.3.5 Other biomarker measurements**

Replicate measurements of serum 1,25(OH)<sub>2</sub>D concentrations employed an immunoassay-based kit (IDS Ltd, Tyne and Wear, UK) and discussed in further detail in Chapter 6. High sensitivity C reactive protein (CRP) and C telopeptide (CTx) were measured by an electrochemiluminescent immunoassay and an immunoturbidimetric assay, respectively, on a Roche Modular analyzer (Roche Diagnostic, Mannheim, Germany). Matrix metalloproteinase 9 (MMP-9), tissue inhibitor of metalloproteinase 1 (TIMP-1), osteopontin (OPN) and bone specific alkaline phosphatase (BAP) were measured by commercial ELISA kits (R & D Systems Inc, Minneapolis, MN, USA) and kallikrein 6 (KLK-6) was measured by an in-house ELISA developed in Dr. E.P. Diamandis's lab at Mount Sinai Hospital, Toronto, Canada (181).

### **5.3.6 Statistical Analyses**

Statistical analyses were performed with SPSS v16.0 (SPSS Inc., Chicago, IL). Graphs were produced using GraphPad Prism 4.0 (GraphPad Software Inc., La Jolla, CA). Because results of proliferation assays and cytokine concentrations did not follow a normal distribution, the Wilcoxon signed ranks tests were used for within-group comparisons (baseline vs. end-of-study) and between-group comparisons (treatment vs. control). Bonferroni corrections were made for multiple comparisons. Mixed modeling procedures were used to compare markers that were measured at various time points throughout the study. ANOVA testing was used to distinguish trends. Chi-square testing and Fisher's exact testing were used to compare proportions. Values are given in the text as mean ± standard deviation.

## 5.4 Results

At baseline, the mean serum 25(OH)D concentration was  $78 \pm 27$  nmol/L, with no difference between groups randomized to receive no supplements or cholecalciferol plus calcium. One year later, at study end, 25(OH)D concentrations were  $179 \pm 76$  nmol/L and  $83 \pm 27$  nmol/L for treated and control groups, respectively (paired t,  $p < 0.001$ ). Significant differences in 25(OH)D concentrations between groups were detected starting at week 11 (following 6 weeks at 10,000 IU/d of cholecalciferol in the treated group) ( $p = 0.02$ ) and at each time point throughout the study ( $p < 0.001$ ). There were no significant changes in serum 25(OH)D concentrations in the treated group from week 35 to week 52, suggesting a steady-state vitamin D status when proliferation was evaluated. Serum 1,25(OH)<sub>2</sub>D concentrations were also measured at baseline and at 12 mo. In controls, end-of-study 1,25(OH)<sub>2</sub>D levels did not change with respect to baseline ( $162.9 \pm 44.7$  and  $164.5 \pm 39.1$  pmol/L, respectively). In treated participants 1,25(OH)<sub>2</sub>D levels were  $155.3 \pm 58.6$  pmol/L at baseline and  $184.4 \pm 46.7$  pmol/L at 12 mo (ns, paired t). No significant clinical or biochemical adverse events occurred.

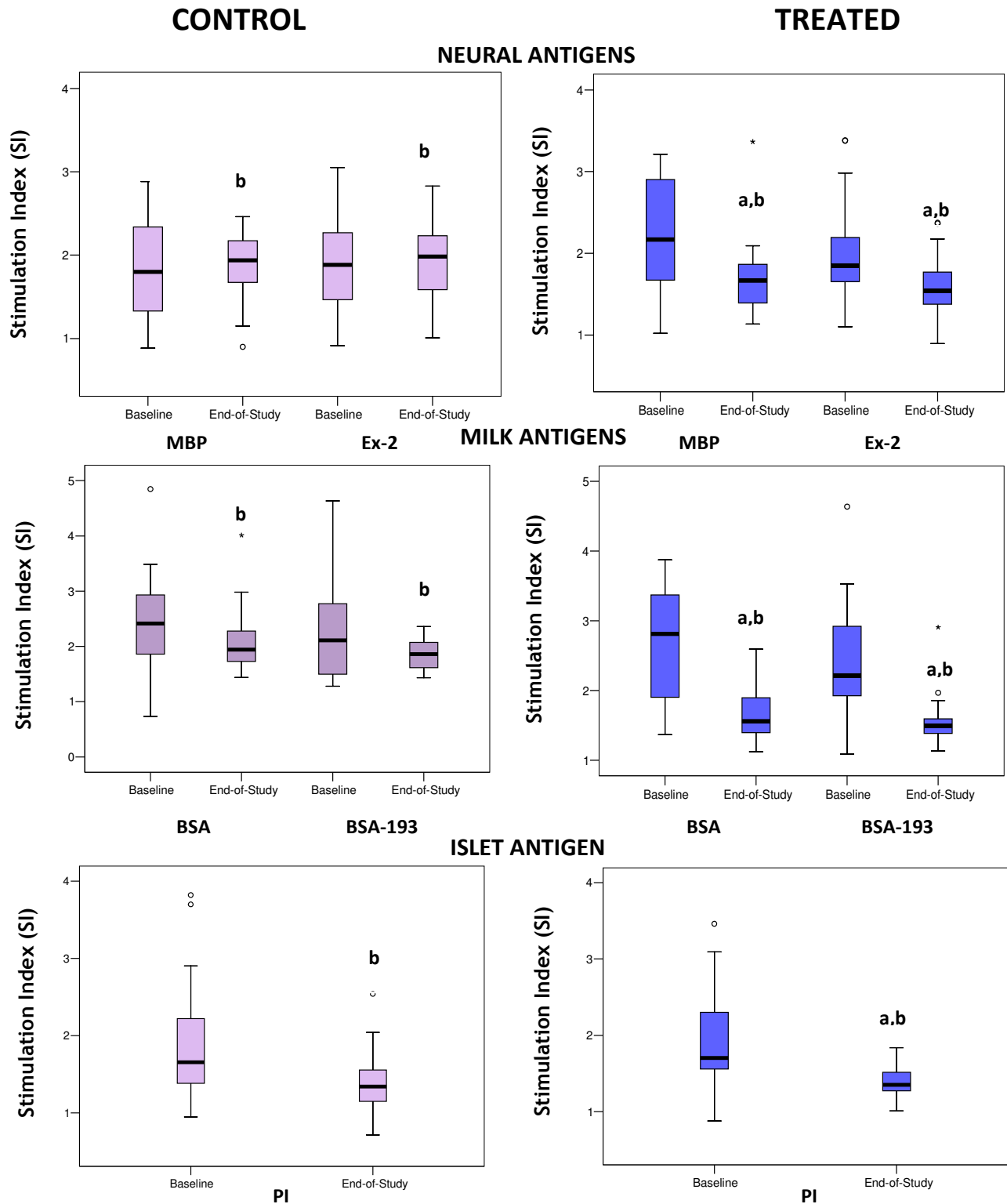
We investigated the *in vivo* effects of cholecalciferol supplementation on abnormal, disease-associated T cell reactivities (324;338) at baseline and one year later, at the conclusion of the study, using a validated array of test antigens including neuron, dietary and islet antigens (308;324;329;336;337). At baseline, proliferative responses of bulk T cell cultures did not differ between groups for any analyte measured, including the most MS-implicated responses to the BSAp193 and MBP epitopes, which are also peptides that cause EAE in mice (308;324). During the ensuing year, no patient developed additional or enhanced responses to these antigens in either group, and T cell responses to positive and negative controls remained similar across the

trial. At the end of the trial, in the treatment group, several specific, previously MS disease-associated responses were significantly reduced. These desirable changes were significant both from comparison of final values with baseline, as well as from comparison of final data between treated and control groups (Table 5.1). As shown in Figure 5.2, proliferative responses declined for 2 out of 4 neuronal antigens (Ex-2 and MBP,  $p=0.001$ ), 2 out of 5 milk antigens (BSA and BSAp193,  $p<0.001$ ), and 1 out of 4 islet antigens (pro-insulin,  $p<0.001$ ). In contrast, polyclonal

**Table 5.1. Comparison of MS-associated antigen-stimulated lymphocyte proliferation between treated and control groups.**

Antigenic Stimulus	Baseline		End-of-Study		Within-Group (pre vs. post) <sup>a</sup>		Control vs. Treated <sup>a</sup>
	Control	Treated	Control	Treated	Control	Treated	End-of-Study
<b>MILK:</b>							
CS	1.19±0.40	1.11±0.30	1.12±0.18	1.05±0.22	ns	Ns	ns
BLG	1.45±0.87	1.18±0.22	1.21±0.27	1.11±0.29	ns	Ns	ns
BSA	2.36±0.87	2.75±0.97	2.09±0.59	1.65±0.36	ns	<0.001	<0.001
BSA-193	2.21±0.88	2.44±0.78	1.84±0.27	1.54±0.36	ns	<0.001	<0.001
Abbos	1.10±0.17	1.13±0.17	1.03±0.14	1.06±0.09	ns	Ns	ns
<b>ISLET:</b>							
Tep	1.06±0.15	1.07±0.14	1.03±0.14	1.07±0.08	ns	Ns	ns
Gad	1.08±0.16	1.12±0.27	1.04±0.16	1.02±0.11	ns	Ns	ns
Gad-555	1.05±0.16	1.04±0.12	1.04±0.08	1.35±1.81	ns	Ns	ns
PI	1.86±0.76	1.95±0.60	1.41±0.40	1.36±0.23	ns	<0.001	0.005
<b>NEURAL:</b>							
Ex-2	1.82±0.68	1.95±0.62	1.94±0.44	1.61±0.36	ns	0.001	0.001
MBP	1.79±0.57	2.22±0.91	1.89±0.42	1.67±0.46	ns	<0.001	0.001
GFAP	1.11±0.17	1.14±0.18	1.32±1.13	1.03±0.10	ns	Ns	<0.001
S100	1.12±0.15	1.10±0.18	0.99±0.16	1.00±0.12	ns	Ns	ns
<b>POS CTL:</b>							
PHA	30.10±16.05	27.22±6.57	26.9±9.42	26.9±6.06	ns	Ns	ns
TT	16.05±6.47	15.23±4.39	14.29±4.55	14.82±3.07	ns	Ns	ns
<b>NEG CTL:</b>							
Cyt-c	1.05±0.15	1.05±1.15	1.02±0.09	1.01±0.11	ns	Ns	ns
Actin	1.04±0.18	1.07±0.09	1.00±0.10	0.95±0.10	ns	Ns	ns
N	24	25	22	23	22	23	22

\*ns=not significant; <sup>a</sup>Wilcoxon signed-ranks test; Bonferonni corrected p-value for multiple comparisons,  $p=0.0038$



**Figure 5.2. Lymphocyte proliferative responses to MS disease-associated antigenic challenge.** Thymidine incorporation was measured in response to antigen stimulation. MBP= myelin basic protein; ; Ex-2=exon-2; BSA=bovine serum albumin; PI=pro-insulin. Boxes represent central 50%, whiskers show highest/lowest values, and lines indicate median values. <sup>a</sup>Denotes significant difference within group (baseline vs. end-of-study) (Wilcoxon,  $p < 0.01$ ); <sup>b</sup>Denotes a difference between groups (control vs. treated) in change from baseline (Wilcoxon,  $p < 0.01$ ).

PHA and recall responses to tetanus toxoid did not change. Therefore, *in vivo* treatment with cholecalciferol and the associated increased serum 25(OH)D concentrations selectively affected disease-associated T cell pools in the circulation of treated MS patients.

As reported previously, there was evidence of clinical improvement with vitamin D<sub>3</sub> treatment. Compared to controls, treated patients tended to have fewer relapses (p=0.09) and a greater proportion had a stable or improved EDSS at end of study (p=0.018) (335).

Cytokines can attenuate or enhance a pro-inflammatory tissue lesion, and we considered whether the vitamin D<sub>3</sub> treatment affected cytokine profiles. However, levels of IL-1 $\beta$ , -2, -4, -5, -6, -10, -12p70, -13, IFN- $\gamma$ , and TNF- $\alpha$  in pooled culture supernatants (data not shown) and in sera were mostly near or below detection sensitivities, and coefficient of variation for each cytokine was as follows: 27%, 37%, 34%, 46%, 27%, 25%, 26%, 40%, 45%, and 31%, respectively. No differences were found within groups between baseline and one year, nor were any differences detected between groups for any of the measured cytokines (**Table 5.2**; Bonferroni correction significant p-value=0.005).

**Table 5.2. Serum cytokine concentrations compared within- and between-groups.**

	Control			Treatment			Between groups* p-value
	Baseline (BL)	End-of-Study (ES)	p-value	Baseline (BL)	End-of-Study (ES)	p-value	
<b>IL-1<math>\beta</math></b>	4.0 $\pm$ 6.8	5.2 $\pm$ 8.1	0.139	2.5 $\pm$ 4.5	2.7 $\pm$ 4.2	0.374	<b>0.906</b>
<b>IL-4</b>	1.1 $\pm$ 2.6	1.8 $\pm$ 4.7	0.325	0.8 $\pm$ 0.9	1.8 $\pm$ 5.8	0.344	<b>0.202</b>
<b>IL-5</b>	57.6 $\pm$ 178.1	59.7 $\pm$ 140.4	0.374	74.3 $\pm$ 103.5	41.7 $\pm$ 75.6	0.091	<b>0.026</b>
<b>IL-6</b>	51.0 $\pm$ 87.9	66.5 $\pm$ 90.0	0.256	41.9 $\pm$ 34.0	67.2 $\pm$ 74.1	0.576	<b>0.841</b>
<b>IL-10</b>	38.7 $\pm$ 63.7	42.6 $\pm$ 55.7	0.328	30.7 $\pm$ 22.2	41.2 $\pm$ 55.1	0.965	<b>0.546</b>
<b>IL-12</b>	8.3 $\pm$ 12.7	8.7 $\pm$ 8.7	0.569	8.8 $\pm$ 7.8	8.9 $\pm$ 6.6	0.842	<b>0.681</b>
<b>IL-13</b>	10.0 $\pm$ 7.0	12.4 $\pm$ 9.7	0.046	10.5 $\pm$ 6.2	10.8 $\pm$ 5.2	0.889	<b>0.266</b>
<b>IFN-<math>\gamma</math></b>	3.9 $\pm$ 4.3	4.8 $\pm$ 3.5	0.381	4.0 $\pm$ 3.3	6.5 $\pm$ 13.5	0.610	<b>0.296</b>
<b>TNF</b>	5.1 $\pm$ 4.9	10.1 $\pm$ 12.8	0.038	4.5 $\pm$ 4.7	17.6 $\pm$ 26.8	0.015	<b>0.407</b>

Values are mean concentration  $\pm$  standard deviation (pg/mL). IL=Interleukin; IFN=Interferon; TNF=Tumor Necrosis Factor. \*Between groups comparison = Control (ES-BL) vs. Treatment (ES-BL)

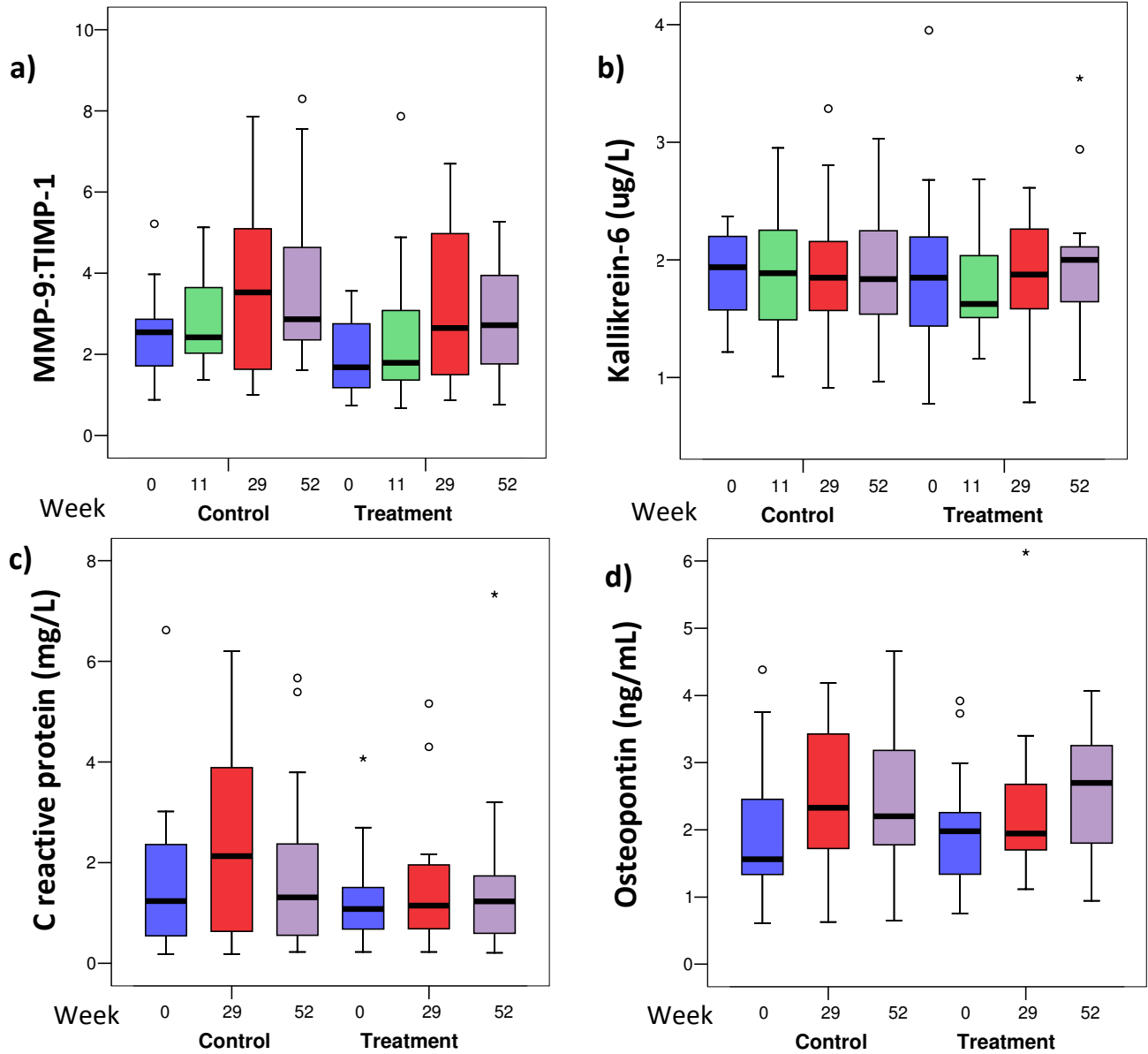
We investigated several candidate markers associated with CNS inflammation. We measured markers involved in the disruption of the blood-brain barrier and trafficking of immune cells into the CNS of patients with MS including, matrix metalloproteinase-9 (MMP-9), tissue inhibitor of metalloproteinase (TIMP-1), kallikrein-6 (KLK-6), C reactive protein (CRP) and osteopontin (OPN). Although changes over time were detected for MMP-9, MMP-9:TIMP-1 ratio, and OPN (mixed modeling,  $p < 0.001$ ,  $p = 0.001$ ,  $p = 0.001$ , respectively), these were consistent between groups (**Figure 5.3**). Likewise, these analyses revealed no differences between control and treated groups (**Table 5.3**).

**Table 5.3. Mean ( $\pm$  standard deviation) sera concentrations of biomarkers associated with MS disease activity.** These molecules are believed to be involved in the disruption of the blood-brain barrier, influx of inflammatory cells and the degradation of myelin, thus playing roles in the pathogenesis of MS. Vitamin D<sub>3</sub> treatment was not associated with change in any of these markers.

Metabolite	Group	Week of Study			
		1	11	29	52
MMP-9 (ng/mL)	Control	4.04 $\pm$ 2.18	4.73 $\pm$ 2.12	6.49 $\pm$ 3.85	6.15 $\pm$ 4.42
	Treatment	2.76 $\pm$ 1.43	3.71 $\pm$ 2.37	4.69 $\pm$ 3.07	4.77 $\pm$ 2.62
TIMP-1 (ng/mL)	Control	1.56 $\pm$ 0.38	1.69 $\pm$ 0.33	1.67 $\pm$ 0.35	1.69 $\pm$ 0.32
	Treatment	1.48 $\pm$ 0.38	1.57 $\pm$ 0.35	1.56 $\pm$ 0.29	1.68 $\pm$ 0.32
MMP-9:TIMP-1 <sup>a</sup>	Control	2.55 $\pm$ 1.04	2.84 $\pm$ 1.34	3.92 $\pm$ 2.26	3.67 $\pm$ 2.58
	Treatment	1.93 $\pm$ 0.92	2.46 $\pm$ 1.69	3.11 $\pm$ 1.97	2.86 $\pm$ 1.43
KLK-6 ( $\mu$ g/L)	Control	1.91 $\pm$ 0.40	1.88 $\pm$ 0.51	1.94 $\pm$ 0.52	1.92 $\pm$ 0.52
	Treatment	1.89 $\pm$ 0.67	1.77 $\pm$ 0.41	1.88 $\pm$ 0.51	1.98 $\pm$ 0.55
CRP (mg/L)	Control	2.0 $\pm$ 2.5		2.7 $\pm$ 2.9	2.5 $\pm$ 3.3
	Treatment	2.1 $\pm$ 3.5		1.6 $\pm$ 1.5	1.9 $\pm$ 0.8
OPN (ng/mL) <sup>a</sup>	Control	1.9 $\pm$ 0.9		2.3 $\pm$ 1.0	2.5 $\pm$ 1.1
	Treatment	1.9 $\pm$ 0.8		2.2 $\pm$ 1.1	2.5 $\pm$ 0.9
BAP ( $\mu$ g/L) <sup>b</sup>	Control	22.4 $\pm$ 7.2		20.5 $\pm$ 6.8	20.4 $\pm$ 5.1
	Treatment	18.9 $\pm$ 5.6		20.5 $\pm$ 6.7	21.2 $\pm$ 6.0
CTx (pg/mL)	Control	234.9 $\pm$ 158.8		244.2 $\pm$ 222.6	224.3 $\pm$ 139.0
	Treatment	274.0 $\pm$ 184.5		234.7 $\pm$ 143.2	228.0 $\pm$ 130.1

<sup>a</sup>Differences over time detected in control and treatment groups, mixed modeling,  $p = 0.05$ .

<sup>b</sup>Difference between groups at baseline, mixed modeling,  $p = 0.007$ .



**Figure 5.3. Serum biomarkers associated with MS disease activity were not affected by increased serum 25(OH)D concentrations.** a) Ratio of serum matrix metalloproteinase 9 (MMP-9) to its inhibitor (TIMP-1 ); b) Kallikrein 6 concentrations; c) C reactive protein; d) Osteopontin. Boxes represent central 50%, whiskers represent highest/lowest values, and lines indicates median values. There were no significant differences detected between groups.

Bone turnover is a continuous process involving bone degradation and formation which can be estimated by measurement of Type I collagen C-terminal telopeptide (CTx) and bone alkaline phosphatase (BAP), respectively. Although a difference was found between groups at baseline for BAP, there were no differences found over time or between groups for either bone formation (BAP) or resorption (CTx) (**Table 5.3**). As expected, BAP and CTx were highly correlated ( $r=0.77$ ,  $p<0.001$ ). Correlations were also found between OPN and both bone markers, BAP ( $r=0.51$ ,  $p=0.003$ ) and CTx ( $r=0.48$ ,  $p=0.009$ ).

We conclude that cholecalciferol treatment selectively attenuated MS-associated T cell pools thought to drive progression of pro-inflammatory lesions in the CNS. The treatment did not measurably affect polyclonal nor cognate recall T cell reactivities in these patients.

## 5.5 Discussion

Elevated steady-state 25(OH)D levels following oral administration of cholecalciferol had desirable clinical effects in the present cohort, as we previously reported (335). Here we analyzed MS disease-associated T cell responsiveness to an array of test antigens previously validated in blinded MS and Diabetes studies. The rise of mean serum 25(OH)D concentrations from 78 nmol/L at baseline to 179nmol/L at study conclusion, achieved in the treated group, significantly reduced abnormal T cell responsiveness by one year, while polyclonal and recall T cell responses were unaffected. In the untreated control group, T cell responsiveness to these same antigens remained unchanged over this period. To our knowledge, this is the first indication that a non-toxic intervention can selectively reduce disease-associated T cell pools in humans with tissue-selective autoimmune disease. Moreover, although it was a small trial, this is the first study to link these pools with clinical course.

Serum 25(OH)D concentrations correlate inversely with the percentage of Treg cells in patients with MS (339) and cholecalciferol supplementation in healthy individuals resulted in an increase in percentage of Treg cells (340). Addition of 1,25(OH)<sub>2</sub>D *in vitro* improves Treg-mediated suppression of CD4+ T cell proliferation (229;229). However, it remains unclear if provision of more 25(OH)D via supplementation with cholecalciferol will promote local production and use of the signaling metabolite, 1,25(OH)<sub>2</sub>D. In fact, the ultimate vitamin D effector mechanisms remain unknown. In support, circulating 1,25(OH)<sub>2</sub>D concentrations were not affected by increased 25(OH)D levels and suggest local utilization by immune cells.

Although we measured several serum markers related to compromised blood-brain barrier integrity in patients with MS and a general marker of inflammation, C-reactive protein,

we did not detect any differences between treated and control groups. MMP-9 and KLK-6 may have roles in the degradation of the blood-brain barrier in patients with MS. Elevated MMP-9 concentrations have been demonstrated in the CSF and serum of patients with MS (168) and vitamin D status correlated inversely with MMP-9 levels (171). KLK-6 has been found to be elevated in inflammatory MS lesions (177) and in the serum of patients with MS (178;341). We found no changes in serum concentrations of MMP-9, TIMP-1 or KLK-6 in treated patients. Osteopontin (OPN), also referred to as T cell activation gene-1, is a pleiotropic molecule that has roles in bone turnover, inflammation and immune response to infectious disease (342). OPN has also been found to be elevated in plasma (188) and CSF levels were elevated during relapse in patients with MS (189). C reactive protein is a general marker of inflammation. Serum 25(OH)D concentrations in this group of patients were already at what is widely regarded as “optimal” (>75nmol/L). We attribute the lack of changes in these markers to high baseline 25(OH)D values. The inverse relationship previously shown between 25(OH)D with C-reactive protein and cytokines occurs at lower 25(OH)D baseline concentrations (171;197;343;344). Similarly, correlation between serum 25(OH)D and MMP-9 concentrations occurred in patients with MS, but baseline serum 25(OH)D concentrations were less than 50nmol/L, and post-vitamin D<sub>3</sub> supplementation MMP-9 values decreased in patients with MS (171). However, normal or optimal levels of vitamin D remain a moving target and ultimately will require formal (and larger) trials such as ours in relevant target populations of healthy as well as affected subjects from different environments.

Markers of bone turnover were not affected by increased serum 25(OH)D concentrations. Bone density studies demonstrate an optimal 25(OH)D concentration of

75nmol/L, meaning that above this value the influence of 25(OH)D on bone turnover is negligible (345). Not surprisingly, we did not detect any change in bone specific alkaline phosphatase or C telopeptide concentrations. We attribute this lack of change to high baseline serum 25(OH)D concentrations as well. Correlations were found between OPN and both bone markers, BAP and CTx. These results support those of Vogt *et al.* who have recently found a similar correlation between OPN and CTx (346).

This was a randomized interventional clinical trial design with objective outcomes that cannot be attributed to a placebo effect. The range in serum 25(OH)D concentrations achieved in treated patients is well within the natural physiological range achievable through environmental exposure and, in fact, no adverse events occurred. Because of the long half-life of 25(OH)D (8-12 weeks) (81), serum 25(OH)D concentrations were stable during the three months leading up to the time of final sampling for the proliferation assays (335). The number of subjects in this RCT was limited because of its exploratory character, yet we were surprised by the extent and the strength of statistical confidence obtained for the changes elicited for key, disease-associated-T cell pools.

Our observations of 25(OH)D-induced amelioration of MS-associated T cell reactivities provide a mechanistic explanation of recent evidence that higher serum 25(OH)D concentrations or vitamin D supplementation are associated with lower rates of relapse (210;226;229;332) and to fewer gadolinium-enhancing lesions (248). Considering our data and the short half lives of T lineage cells (~3d), it appears likely that the peripheral blood T lymphocytes analyzed here contain recently activated T cells recirculating from inflamed tissue sites, in this case the CNS. T cell activation substantially raises vitamin D receptor expression

(347) which would be the simplest explanation of the selectivity of immune impact observed in the cholecalciferol-treated subjects in our trial.

## 6.0 VITAMIN D METABOLITE RESPONSE TO HIGH-DOSE CHOLECALCIFEROL IN PATIENTS WITH MULTIPLE SCLEROSIS

*“All truths are easy to understand once they are discovered; the point is to discover them.”*

*Galileo Galilei*

### 6.1 Abstract

#### Abstract

**Background:** Vitamin D metabolism involves three activating enzymes. Final production and circulating concentrations of 1,25(OH)<sub>2</sub>D, via CYP27B1, is under complex regulatory control to serve its physiological role in calcium homeostasis. **Objectives:** To characterize the response of vitamin D metabolites and D binding protein (DBP) to high-dose cholecalciferol supplementation. **Method:** In an open-label, phase I/II dose-escalation study of vitamin D<sub>3</sub> (4,000-40,000IU/d) with calcium (1,200mg/d) in patients with MS, we compared the effects over one year on safety measures and vitamin D metabolites, DBP and C reactive protein (CRP), versus results for matched MS patients (age, sex, disease duration, disease modifying drug, and disability) randomized to receive no additional vitamin D<sub>3</sub> nor calcium. **Results:** Increasing doses of vitamin D<sub>3</sub> supplementation resulted in significant increases in 25(OH)D concentrations. Interestingly, the variability between individual 25(OH)D response (i.e. standard deviation) increased as vitamin D<sub>3</sub> supplementation dose increased ( $r^2 = 0.93$ ,  $p < 0.001$ ). Seasonality of serum 25(OH)D was not present in control group participants. As serum 25(OH)D increased, its catabolite, 24,25(OH)<sub>2</sub>D, increased correspondingly with a ratio of 4:1. There were no differences detected in concentrations of DBP. At mid-year, serum 1,25(OH)<sub>2</sub>D concentrations

increased above the reference range; however, calcium measures remained within reference ranges. DBP concentrations correlated positively with CRP ( $r=0.451$ ,  $p=0.003$ ). **Discussion:** Serum 25(OH)D concentrations well above those considered “sufficient” were attained without evidence of altered calcium homeostasis. Production of 24,25(OH)<sub>2</sub>D increased corresponding increased serum 25(OH)D, suggesting that 25(OH)D may induce its own catabolism. Furthermore, a relationship between DBP and CRP was identified.

## 6.2 Introduction

The vitamin D metabolic pathway is well defined and production of the active metabolite, 1,25-dihydroxyvitamin D [ $1,25(\text{OH})_2\text{D}$ ], involves two hydroxylation steps. Vitamin  $\text{D}_3$  is first hydroxylated in the liver (via CYP27A1 or CYP2R1) and the resulting 25-hydroxyvitamin D [ $25(\text{OH})\text{D}$ ] is further hydroxylated to form  $1,25(\text{OH})_2\text{D}$  in the kidney or extra-renal tissues (via CYP27B1). C-1 hydroxylation is the rate-limiting step in this pathway, whereas 25-hydroxylation follows first-order kinetics. While renal synthesis of  $1,25(\text{OH})_2\text{D}$  serves to maintain serum calcium within narrow physiological concentrations, other tissues are capable of producing and utilizing  $1,25(\text{OH})_2\text{D}$ . Vitamin D metabolites circulate in the blood in complex with vitamin D binding protein (DBP). DBP predominantly functions in the binding, solubilization and serum transport of the vitamin D sterols (317), though recent evidence suggests more diverse activities for DBP including roles in inflammation and immune cell function.

Further hydroxylation, via 24-hydroxylation (CYP24A1), of  $25(\text{OH})\text{D}$  or  $1,25(\text{OH})_2\text{D}$  results in the catabolic products 24,25-dihydroxyvitamin D [ $24,25(\text{OH})_2\text{D}$ ] and 1,24,25-trihydroxyvitamin D [ $1,25(\text{OH})_3\text{D}$ ]. Circulating concentrations of  $1,25(\text{OH})_2\text{D}$  are regulated by reciprocal control of the activating and deactivating enzymes by  $1,25(\text{OH})_2\text{D}_3$  itself (348).

Vitamin D toxicity manifests as hypercalcemia, with symptoms of dehydration, calcification of soft tissues and ultimately renal failure (349). Establishing the safety of high-dose supplementation with vitamin  $\text{D}_3$  is established in practice by monitoring urinary calcium:creatinine concentrations and serum calcium concentrations (350). The administration of doses of vitamin  $\text{D}_3$  as high as 10,000IU/d have been shown to be safe (81;82). A

mathematical relationship between vitamin D<sub>3</sub> input and 25(OH)D response has been defined (78). However, the effect of high-dose vitamin D<sub>3</sub> supplementation on other vitamin D metabolites, including 24,25(OH)<sub>2</sub>D and DBP, has not been characterized.

In a phase I/II dose-escalation trial of vitamin D<sub>3</sub> with calcium in patients with multiple sclerosis (MS) we examined the response of serum and urinary calcium and parathyroid hormone (PTH) concentrations. Further we characterized the response of vitamin D metabolites to high-dose vitamin D<sub>3</sub> supplementation, including 25(OH)D, 1,25(OH)<sub>2</sub>D and 24,25(OH)<sub>2</sub>D, as well as concentrations of DBP.

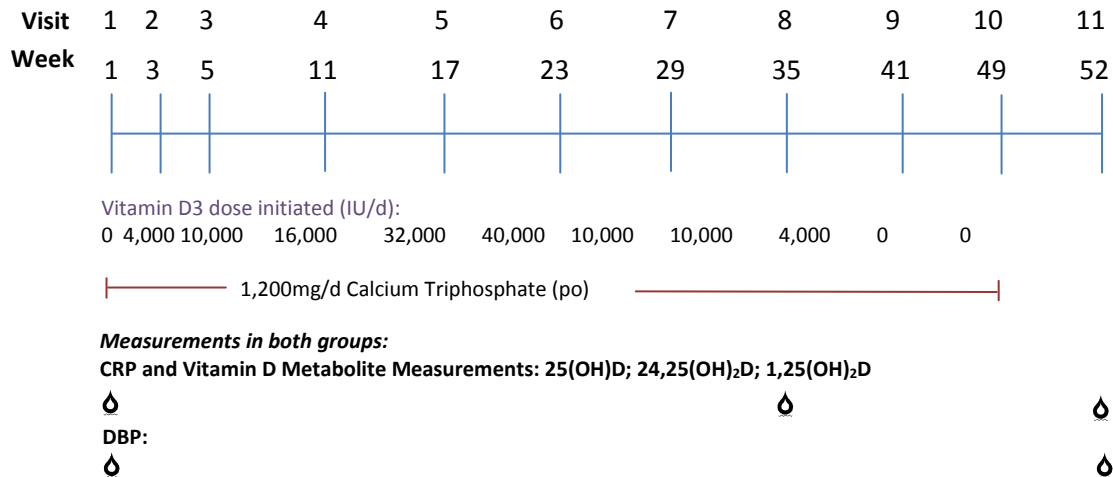
## **6.3 Methods**

### **6.3.1 Vitamin D Study Design**

Details of the study design have been discussed in **Chapter 3** and elsewhere (335). A timeline of treatment group study visits and supplementation is outlined in **Figure 6.1**. Measurements made in both treated and control group participants are also depicted. The safety protocol involved monitoring urinary and serum calcium concentrations, serum creatinine, PTH and 25(OH)D at each visit in treated participants. For all study participants, vitamin D metabolites [25(OH)D, 24,25(OH)<sub>2</sub>D, and 1,25(OH)<sub>2</sub>D] and CRP measurements were characterized at baseline, mid-study and end-of-study. DBP was measured at baseline and study conclusion.

### **6.3.2 Biochemical Measurements**

Urinary calcium:creatinine, serum calcium, creatinine, 25(OH)D and PTH concentrations were analyzed in the clinical laboratory at St. Michael's hospital, Toronto, ON, as previously described (248).



**Figure 6.1. Timeline of supplementation (treated only) and measurements in control and treated participants.** At each study visit and for safety reasons, treatment group participants underwent testing for the following: urinary calcium:creatinine, serum calcium, creatinine, 25(OH)D and PTH.

### 6.3.3 Vitamin D metabolite measurements

Serum 25(OH)D and 24,25(OH)<sub>2</sub>D, were quantitated by a mass spectrophotometric (LC-MS/MS) method. Briefly, samples spiked with d<sub>6</sub>-25-hydroxyvitamin D<sub>3</sub> internal standard were extracted with methyl-t-butyl ether (MTBE). Residues obtained by evaporation of the ether phase were resuspended in methanol and analysed by HPLC (Agilent Technologies 1200 series) employing methanol-water mobile phases in increasing gradients to 100% methanol at a flow rate of 0.8mL/min on an Eclipse C8 column at 50°C.

The API 5000 (Applied Biosystems/Sciex, Concord, ON, Canada) mass spectrometer equipped with an atmospheric pressure chemical ionization (APCI) source and operated in the positive mode was used with ion-transitions of m/z 401.4 → 383.4 was monitored for 25-hydroxyvitamin D<sub>3</sub>, m/z 417.4 → 399.4 was monitored for 24,25-dihydroxyvitamin D<sub>3</sub>, m/z

413.4 → 395.4 was monitored for 25-hydroxyvitamin D<sub>2</sub> and m/z 407.5 → 389.4 was monitored for d<sub>6</sub>-25-hydroxyvitamin D<sub>3</sub>. Concentrations of metabolites were derived using Analyst software (version 1.4.2).

Concentrations of 1,25(OH)<sub>2</sub>D were measured by immunoextraction followed by EIA quantitation according to the manufacturer's directions (Immunodiagnosics Ltd. (IDS), Tyne and Wear, UK).

To measure DBP concentrations, serum was spiked with 5,000nmol/L of 25(OH)D and incubated at 37°C for 1 hour. Bound and free fractions were separated with dextran-coated charcoal and centrifugation. Supernates were decanted and 25(OH)D was extracted with 3:2 mixture of hexane:isopropanol. Sodium sulphate was added to remove non-lipid components. After centrifugation, the hexane-rich layer was extracted and analyzed by HPLC on a normal-phase silica column eluted with 9:1 of hexane:isopropanol. This is a modification of a previously published method (351). A method validation between the DBP binding assay and a conventional DBP immunoassay was performed. The coefficient of variation for the binding assay was 16% and 19% for the immunoassay. Regression analysis found a significant correlation between the two methods (<0.0001, r=0.687, y-int=3.04, slope=0.03; data not shown).

High sensitivity C reactive protein (CRP) was measured by chemiluminescent immunoassay on a Roche Modular analyzer (Roche Diagnostic, Mannheim, Germany).

### **6.3.4 Cross-reactivity experiments**

Pooled serum from 5 healthy donors was utilized in which the concentration of 1,25(OH)<sub>2</sub>D was measured in samples of neat serum (n=5) and serum spiked (n=5) with all of the following: 100nM vitamin D<sub>3</sub>, 400nM 25(OH)D, and 40nM of 24,25(OH)<sub>2</sub>D. Based on the results from these initial analyses and to elucidate which metabolite the method was cross-reacting with, 1,25(OH)<sub>2</sub>D concentrations were measured in duplicate in the serum from a healthy donor that was spiked with each metabolite individually in the same concentrations as above. All 1,25(OH)<sub>2</sub>D samples were analyzed with routine samples by a technologist unaware of the investigation or the possible cross-reactants contained within the samples.

The specificity of LC-MS/MS to detect 24,25(OH)<sub>2</sub>D was questioned due to the highly significant relationship between 25(OH)D and 24,25(OH)<sub>2</sub>D that was found. A charcoal-stripped serum sample with a baseline concentration of 6pmol/L 1,25(OH)<sub>2</sub>D and 58nmol/L 25(OH)D, was used to perform detection studies for 24,25(OH)<sub>2</sub>D. The serum was spiked with either 400nM 25(OH)D, 40nM 24,25(OH)<sub>2</sub>D, or both and analysed by LC-MS/MS as described above. Blinded samples were run in duplicate in at least 3 different batches.

### **6.3.5 Statistical analyses**

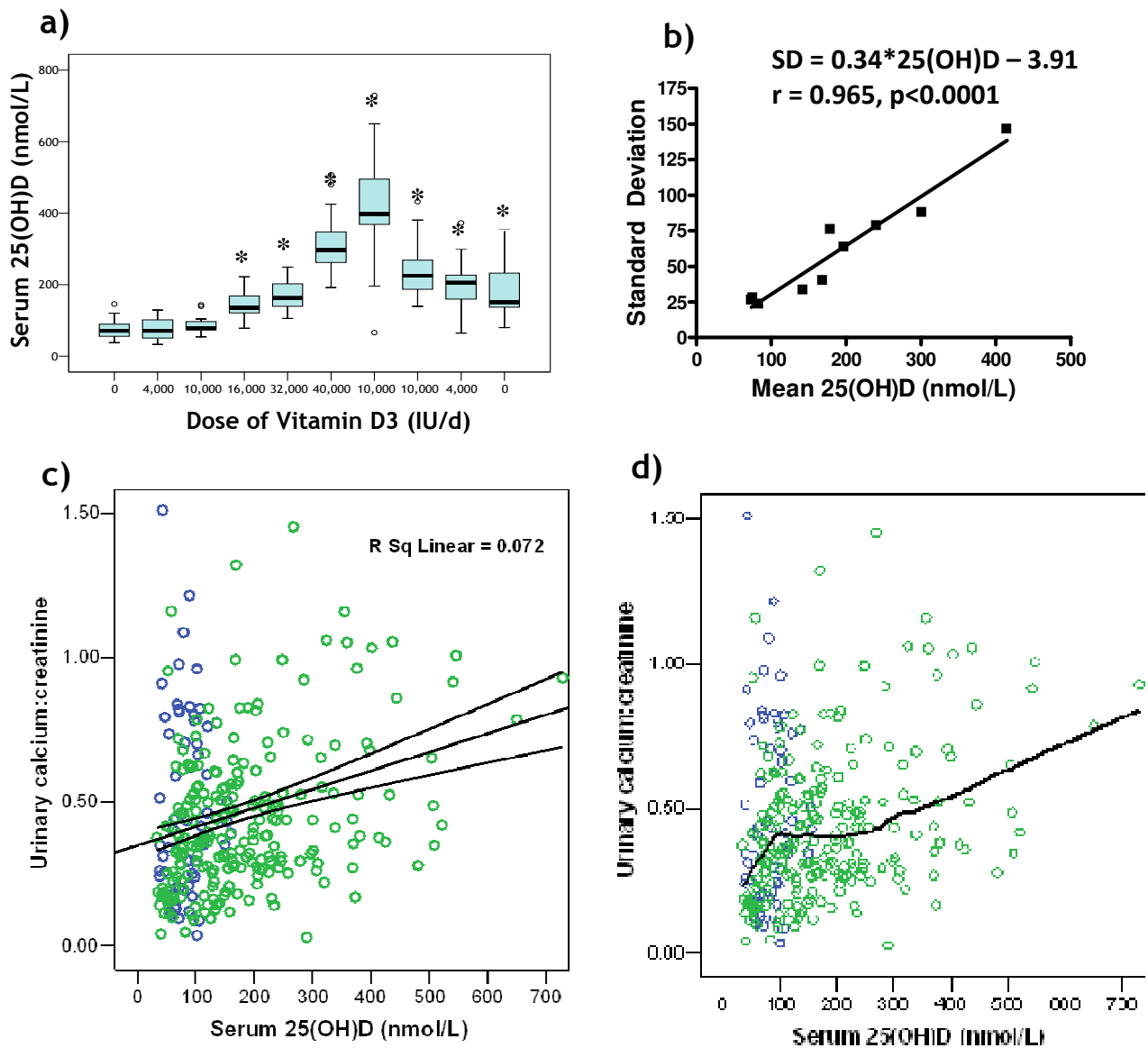
Results are presented as means ± SD. SPSS v16.0 (SPSS Inc., Chicago, IL) software was utilized for statistical analyses. Biochemical associations were assessed with Pearson correlation and regression analyses. Within-group changes over time were analyzed by paired 2-tailed *t* tests with Bonferroni correction. Variability between individuals was tested by linear

regression of mean vs. standard deviation. Seasonality of 25(OH)D in the control group was assessed by spearman ranks analysis.

## 6.4 Results

As expected, serum 25(OH)D concentrations rose significantly in the treatment group in a dose-dependent manner (**Figure 6.2a**), such that by week 11, following 6 weeks of 10,000IU/d of vitamin D<sub>3</sub> supplementation, a significant increase in 25(OH)D concentrations was observed (with respect to baseline, paired t,  $p=0.02$ ) and maintained significantly increased from baseline until study completion. Interestingly, as serum 25(OH)D concentrations increased, the variation between treatment subjects (i.e. standard deviation) increased proportionately (**Figure 6.2b**);  $r=0.965$ ,  $p<0.001$ . Further, 25(OH)D concentrations tracked by patient (Spearman ranks,  $p=0.02$ ), such that patients who responded to supplementation with higher serum 25(OH)D concentrations tended to have consistently higher concentrations at each measurement, and vice versa for those patients who responded with lower concentrations (data not shown).

Safety outcomes included serum calcium and urinary calcium:creatinine ratios which were monitored at each study visit as outlined in **Figure 6.1**. Serum calcium remained unaffected and within reference ranges (2.1-2.6 mmol/L) and urinary calcium:creatinine also remained within the reference range ( $<1.0$ ) at all study visits over one year of treatment. We analyzed the relationship between urinary calcium:creatinine and 25(OH)D concentrations by linear regression and found a significant correlation ( $r=0.261$ ,  $p<0.001$ , **Figure 6.2c**). In adults, calcium excretion is essentially a measure of calcium absorption. Heaney et al. demonstrated a positive relationship between increasing 25(OH)D and calcium absorption efficiency that plateaued at 25(OH)D concentrations above 80nmol/L (352). Using 25(OH)D concentrations defined a priori and based upon calcium absorption ( $<75$ nmol/L), a “physiological” range of



**Figure 6.2. Serum 25(OH)D and urinary calcium:creatinine responses to high-dose vitamin D<sub>3</sub>.** a) Serum 25(OH)D concentrations in the treatment group; b) Increasing variability of individual responses to increasing doses of vitamin D<sub>3</sub> supplementation in the treatment group; c) Linear regression analysis with 95%CI of relationship between serum 25(OH)D and urinary calcium:creatinine; d) Non-parametric analysis (Loess plot) of relationship from c). Green circles represent treated patients, blue circles represent control patients. \*denotes difference with respect to baseline.

**Table 6.1. Regression analyses of the relationship between serum 25(OH)D concentrations and urinary calcium: creatinine ratios.**

25(OH)D (nmol/L)	R	y-intercept	Slope	p-value
< 80	0.288	14.1	0.435	0.017
80-225	0.013	42.5	0.008	ns
> 225	0.332	22.2	0.100	0.007
All data points	0.268	34.8	0.065	<0.001

25(OH)D (75-225nmol/L) and “supraphysiological” values (>225nmol/L), we dissected the response of calcium excretion in relation to serum 25(OH)D concentrations. When 25(OH)D values were <75nmol/L or >225nmol/L, serum 25(OH)D and urinary calcium:creatinine correlated positively, validating the use of this marker as an early detection for vitamin D toxicity. However, at 25(OH)D concentrations between 75 and 225nmol/L, no relationship existed (**Table 6.1**). A non-parametric Loess plot reflects this concentration-dependent relationship (**Figure 6.2d**). PTH remained within the reference range (1.6-6.9 pmol/L) throughout the study with no difference between time points. PTH concentrations correlated strongly with serum calcium and serum creatinine concentrations ( $p<0.001$  and  $p=0.042$ , respectively).

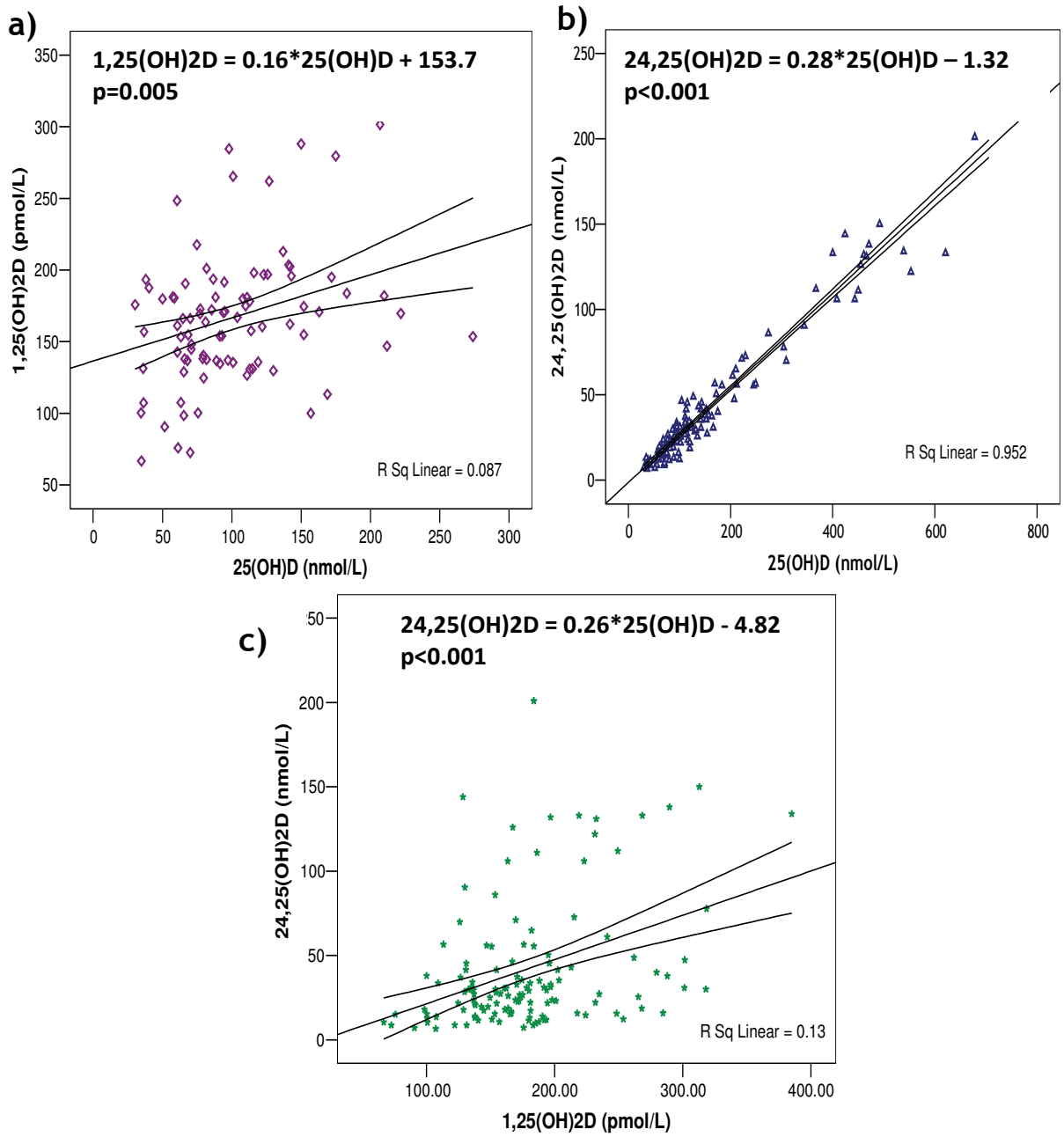
To characterize the response of vitamin D metabolites to high-dose vitamin D<sub>3</sub> supplementation, we measured serum 25(OH)D, 1,25(OH)<sub>2</sub>D and 24,25(OH)<sub>2</sub>D (**Table 6.2**). In treated patients, serum 1,25(OH)<sub>2</sub>D concentrations increased significantly from baseline values of  $155.3\pm 58.6$  to  $221.3\pm 66.6$  pmol/L at mid-study, after the highest dose of vitamin D<sub>3</sub> supplementation (40,000 IU/d for 6 weeks) (paired t,  $p=0.014$ ), but by study conclusion the difference from baseline was no longer significant. As expected, no difference in serum 1,25(OH)<sub>2</sub>D was seen over time in control group participants. A weak positive linear relationship between serum 25(OH)D and 1,25(OH)<sub>2</sub>D concentrations ( $r=0.295$ ,  $p=0.005$ ) was found (**Figure 6.3a**). Serum 24,25(OH)<sub>2</sub>D concentrations were highly correlated with 25(OH)D ( $r=0.975$ ,  $p<0.001$ ) (**Figure 6.3b**). The relationship between 1,25(OH)<sub>2</sub>D and 24,25(OH)<sub>2</sub>D was found to be much weaker ( $r=0.361$ ,  $p<0.001$ ) (**Figure 6.3c**) than for 25(OH)D and 24,25(OH)<sub>2</sub>D.

**Table 6.2. Vitamin D metabolite levels in treated and control groups at baseline and one year.**

Vitamin D Metabolite	Group	Baseline	Mid-Study	End-of-Study
<b>25(OH)D</b> (nmol/L)	Control	89.7±30.7	89.1±30.6	77.5±27.3
	Treatment	80.7±33.8	416.0±144.0	155.2±46.7
<b>1,25(OH)2D</b> (pmol/L)	Control	162.9±44.7	178.0±47.1	164.5±39.1
	Treatment	155.3±58.6	221.3±66.6*	184.4±46.7
<b>24,25(OH)2D</b> (nmol/L)	Control	23.1±8.0	19.6±8.4	20.1±8.5
	Treatment	21.6±10.0	113.0±44.4	46.1±14.3
<b>N</b>	Control	24	22	21
	Treatment	25	24	23

\*Denotes difference with respect to baseline, paired t p<0.05

We questioned the specificity of the assays employed to quantitate 24,25(OH)<sub>2</sub>D (LC-MS/MS) and 24,25(OH)<sub>2</sub>D (immunoassay) and investigated whether high serum 25(OH)D and vitamin D<sub>3</sub> concentrations interfered with the respective methods of measurement. The distinction between 24,25(OH)<sub>2</sub>D and 25(OH)D by mass spectrophotometry was found to be complete (**Table 6.3**). However, the immunoassay employed to measure 1,25(OH)<sub>2</sub>D was found to have a small but significant amount of cross-reactivity with 25(OH)D (**Table 6.4a**), but at a level that does not account for the significant increase found in patient samples. Analyses of serum spiked individually with the different metabolites (**Table 6.4b**) revealed that 25(OH)D and 24,25(OH)<sub>2</sub>D both interfered with 1,25(OH)<sub>2</sub>D quantitation to a small degree. According to the manufacturer cross-reactivity for vitamin D metabolites is as follows: 24,25(OH)<sub>2</sub>D at 100%; 24,25(OH)<sub>2</sub>D at <0.1%; 25(OH)D at <0.01% and vitamin D<sub>3</sub> at <0.01%. Our investigation suggests that the cross-reactivity of 25(OH)D and 24,25(OH)<sub>2</sub>D at serum concentrations of 25(OH)D above 250nmol/L is much higher, in the range of 1%.



**Figure 6.3. Correlations between 25-hydroxyvitamin D, 24,25-dihydroxyvitamin D and 1,25-dihydroxyvitamin D.** Correlation between a) 25(OH)D and 1,25(OH)2D; b) 25(OH)D and 24,25(OH)2D; c) 1,25(OH)2D and 24,25(OH)2D.

**Table 6.3. Cross-reactivity analysis in the quantitation of 24,25-dihydroxyvitamin D by mass spectrophotometry.**

Sample	Added metabolite conc. (nM)		Detected metabolite conc. (nM)	
	25(OH)D	24,25(OH) <sub>2</sub> D	25(OH)D	24,25(OH) <sub>2</sub> D
S1	0 (58)*	0	31 ± 0.2	5 ± 0.1
S2	400 (458)	0	534 ± 10	4 ± 1
S3	0 (58)	40	30 ± 1.0	66 ± 9
S4	400 (458)	40	491 ± 23	69 ± 2

\*including baseline serum 25(OH)D concentration of 58nM as measured on Liaison

**Table 6.4a. Cross-reactivity of anti-1,25(OH)<sub>2</sub>D<sub>3</sub> antibody employed in Immunodiagnosics (IDS) immunoassay with other metabolites of vitamin D.**

Sample	1,25(OH) <sub>2</sub> D <sub>3</sub> (pmol/L) [Mean ± SD]	Range (pmol/L)
Neat serum (n=5)	124.8 ± 14.7	105-138
*Spiked serum (n=5)	139.4 ± 5.7	130-144

\*pooled serum from a healthy volunteers was spiked with 100nM vitamin D<sub>3</sub>, 400nM 25(OH) D<sub>3</sub> and 40nM 24,25(OH)<sub>2</sub>D<sub>3</sub> to determine cross-reactivity of other vitamin D metabolites with the IDS 1,25(OH)<sub>2</sub>D<sub>3</sub> immunoassay.

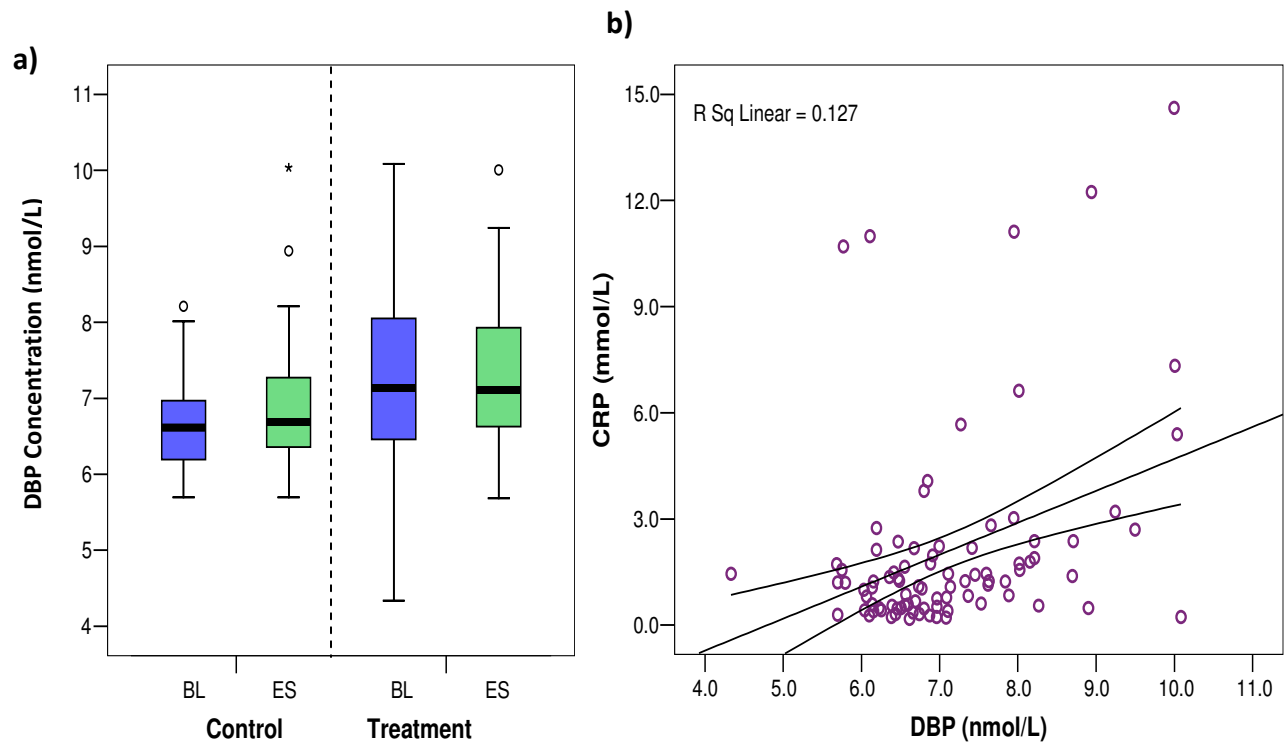
**Table 6.4b. Cross-reactivity of anti-1,25(OH)<sub>2</sub>D<sub>3</sub> antibody employed in Immunodiagnosics (IDS) immunoassay with other metabolites of vitamin D.**

Sample	Conc. of metabolites added (nmol/L)			Measured 1,25(OH) <sub>2</sub> D (pmol/L)
	25(OH)D <sub>3</sub>	24,25(OH) <sub>2</sub> D	vitamin D <sub>3</sub>	
1	0 (118)	0	0	72
2	300 (418)	0	0	79
3	0 (118)	40	0	82
4	0 (118)	0	10	69
5	300 (418)	40	10	79

(Concentration) including baseline as measurement

DBP was measured at baseline and end-of-study to determine whether the dose-escalation design affected DBP concentrations. We hypothesized that an increased concentration of DBP may help to buffer high 25(OH)D concentrations by reducing the unbound portion of 25(OH)D and 1,25(OH)<sub>2</sub>D. No changes were detected between baseline and end-of-study concentrations of DBP in either group (**Figure 6.4a**). However, DBP concentrations correlated with C reactive protein (CRP) in the whole sample ( $r=0.451$ ,  $y\text{-int}=-5.414$ ,

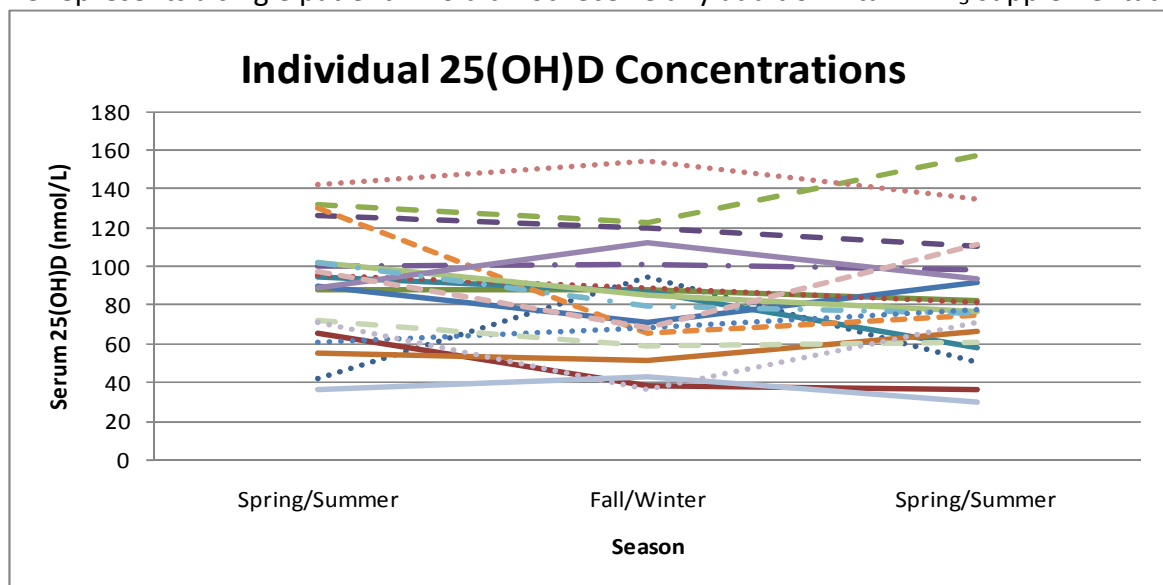
slope=0.001,  $p=0.002$ ; **Figure 6.4b**) and separately at baseline ( $r=0.404$ ,  $p=0.05$ ) and end-of-study ( $r=0.681$ ,  $p<0.001$ ). To determine if this relationship was a reflection of serum protein concentrations, we characterized the relationship between serum albumin and DBP, as well as albumin and CRP. Albumin correlated negatively with DBP ( $r=0.254$ ,  $y\text{-int}=44.78$ , slope=  $-0.702$ ,  $p=0.018$ ) and CRP ( $r=0.347$ ,  $y\text{-int}= 40.54$ , slope=  $-0.383$ ,  $p=0.001$ ). The relationship between DBP and CRP was positive, whereas both compounds correlated negatively with albumin, suggesting the relationship is true.



**Figure 6.4. Vitamin D binding protein (DBP) and a relationship with C reactive protein (CRP).** a) Serum DBP concentrations at baseline (BL) and end-of-study (ES) in control and treated patients; b) a correlation between DBP and CRP concentrations in both groups ( $r=0.637$ ,  $y\text{-int}=40.9$ , slope= $0.616$ ;  $p=0.002$ ). Boxes represent the central 50%, whiskers the highest/lowest values, and lines indicate the median values.

We sought to examine the effect of season on serial measurements of 25(OH)D in the control group. Measurements of 25(OH)D were made at baseline and at weeks 29 and 52, but due to staggered enrolment, sample collection occurred during all months of the year. **Figure 6.5** depicts serum 25(OH)D concentrations in control patients (i.e. no additional vitamin D<sub>3</sub> supplied) by month of sample draw; no seasonality was observed. We examined the correlation of serum 25(OH)D concentrations within an individual to determine if those individuals with comparatively low summer values were likely to have low winter 25(OH)D values, and vice versa for higher summer and winter values. Correlation analysis revealed a significant relationship between 25(OH)D concentrations within individuals ( $r=0.637$ ,  $y\text{-int}=40.9$ ,  $\text{slope}=0.616$ ;  $p=0.002$ ). Supplemental intake data, gathered at baseline, did not predict serum 25(OH)D concentration at any time point (data not shown).

**Figure 6.5. Seasonality of serum 25(OH)D concentrations in control group participants.** Each line represents a single patient who did not receive any addition vitamin D<sub>3</sub> supplementation.



## 6.5 Discussion

In the present study we found no indication of vitamin D<sub>3</sub> toxicity in patients with MS despite attainment of a mean serum 25(OH)D concentration of 416nmol/L after the highest dose of vitamin D<sub>3</sub>. Surprisingly, concentrations of 1,25(OH)<sub>2</sub>D were found to increase by mid-year with increasing 25(OH)D concentration. The reference range for 1,25(OH)<sub>2</sub>D (39-193 pmol/L) was exceeded by 62.5% (15/24) of treated patients after 6 weeks of the highest dose of vitamin D<sub>3</sub> (40,000IU/d) when the mean 25(OH)D was 416nmol/L, and was exceeded by 47.8% (11/23) by end-of-study when the mean 25(OH)D concentration was 155nmol/L. However, serum and urinary calcium concentrations remained within reference ranges for the duration of the study. Further there was no indication of clinical adverse events. Together this suggests that elevated 1,25(OH)<sub>2</sub>D concentrations *per se* are not toxic. It has been reported that toxicity results from an increase in unbound 1,25(OH)<sub>2</sub>D (349), suggesting that DBP concentrations in this population were adequate to bind both 1,25(OH)<sub>2</sub>D and 25(OH)D. It is unknown whether a sustained increase in 1,25(OH)<sub>2</sub>D levels in these patients would have remained safe, but more likely, sustained concentrations of 25(OH)D > 400nmol/L may have caused hypercalcemia if there were an excessive intake of calcium (81).

It has been estimated that 10 mg/kg/day of DBP is produced in humans (353) with a half-life of 2.5 days (354;355) and the total binding capacity of vitamin D metabolites is approximately 4,700 nmol/L (351). DBP concentrations are known to increase during pregnancy (356) and decrease in states of malnutrition (357).

High-sensitivity CRP, as a general marker of inflammation, was measured to investigate any association between CRP and 25(OH)D concentrations, but no association was found. DBP has been associated with the inflammatory process. DBP acts as an actin scavenger, binding actin to prevent thromboembolic events (358). DBP also stimulates chemotaxis by phagocytic neutrophils (32) and activates the phagocytic function of macrophages (30). However, the breadth of involvement of DBP response to inflammation and the mechanism that triggers it have not been elucidated. DBP correlated positively with CRP concentrations in all participants, suggesting a common stimulus between the two compounds. Although a teleological rationale for connecting CRP and DBP is evident, we are not aware of any publication examining the relationship between DBP and CRP.

We observed that the response to vitamin D<sub>3</sub> supplementation varied greatly between individuals, such that as 25(OH)D concentrations increased, a proportionate increase in variation between patients was seen. Genetic influence has been found to play a role in the regulation of seasonal 25(OH)D concentrations in twins with MS from the Canadian Collaborative Project on Genetic Susceptibility of MS (CCPGSMS) study (239). Associations were found between *CYP27B1* SNPs and 25(OH)D concentrations, but *CYP27A1* genotype polymorphisms were not investigated. Further, we examined serial measurements of 25(OH)D in the control group for seasonal influence, however, no seasonality was observed. As demonstrated in control group participants (Figure 6.5), those individuals who had high summer 25(OH)D concentrations were also likely to have high winter 25(OH)D concentrations, and those with lower summer 25(OH)D concentrations were more likely to have lower winter values. Supplemental intake was not found to predict 25(OH)D concentration, but one may

speculate that the lack of seasonality observed may be due to an awareness of a potential benefit for vitamin D<sub>3</sub> supplementation in this population of patients with MS as seen by the relatively high proportion who reported taking vitamin D supplements (13/24).

The relationship between 24(OH)D and 24,25(OH)<sub>2</sub>D was much stronger than the correlation found between 1,25(OH)<sub>2</sub>D and 24,25(OH)<sub>2</sub>D, a relationship that supports recent findings by Wagner et al. (359). It is known that 1,25(OH)<sub>2</sub>D induces its catabolism by activating the CYP24A1 enzyme; it would appear the 25(OH)D can also induce its own catabolism as seen by the strong correlation between 24,25(OH)<sub>2</sub>D and 25(OH)D, even in control group participants.

In summary, high dose vitamin D<sub>3</sub> supplementation with calcium in patients with multiple sclerosis produced significant effects on systemic vitamin D metabolite concentrations. Inherent differences were observed between individuals, both in response to high-dose vitamin D<sub>3</sub> supplementation and with respect to seasonal concentrations in participants not receiving additional vitamin D<sub>3</sub>.

## 7.0 CONCLUSIONS & DISCUSSION

*“Not everything that counts can be counted, and not everything that can be counted counts.”*

*Albert Einstein*

### 7.1 KEY FINDINGS

This work provides several important findings:

1. **Vitamin D<sub>3</sub> is safe at high intakes:** Peak mean serum 25(OH)D concentrations of 415 nmol/L were attained in patients with multiple sclerosis without evidence of adverse effects. An average vitamin D<sub>3</sub> intake of 14,000 IU/d with 1,200 mg/d of calcium over one year resulted in physiologically achievable serum 25(OH)D concentrations of approximately 180 nmol/L.
2. **Evidence of Clinical Efficacy:** Clinical efficacy was suggested with vitamin D<sub>3</sub> treatment. Although unblinded, treated patients tended to have fewer relapses compared to controls (p=0.09). Further, a greater proportion of treated patients had a stable or improved Expanded Disability Status Scale scores at end of study (p=0.018).
3. **Vitamin D<sub>3</sub> desirably diminished autoreactivity:** Lymphocyte reactivity to MS-associated antigenic challenge was significantly reduced in treated patients, in comparison with baseline responses and in comparison with controls (p<0.05). At

physiological concentrations of 25(OH)D (180 nmol/L) a significant *in vivo* immunomodulatory effect of high serum 25(OH)D concentrations was observed.

4. **New data on serum 24,25(OH)<sub>2</sub>D:** In vitamin D<sub>3</sub>-treated and control MS patients, metabolites of vitamin D<sub>3</sub> were found to be highly correlated, particularly 25(OH)D and 24,25(OH)<sub>2</sub>D, consistent with the notion that 25(OH)D induces its own catabolism.
5. **Significant increases in 1,25(OH)<sub>2</sub>D at mid-study** in the treatment group were observed, but without excessive changes in serum or urinary calcium. By end-of-study, serum 1,25(OH)<sub>2</sub>D concentrations were within the reference range (39-193 pmol/L) for both treated and control patients.
6. **Elucidated the relationship between 25(OH)D and urinary calcium excretion** by demonstrating no changes at concentrations of 25(OH)D between 80nmol/L to 225nmol/L. Below this range, higher serum 25(OH)D increased urinary excretion of calcium (which closely parallels the absorption of calcium from the gut). Below this range (<80nmol/L) higher serum 25(OH)D improved calcium regulation. Beyond the physiologic range (>225nmol/L) urinary calcium excretion began to increase again, suggesting the start of the aberrant and excessive absorption of calcium from the gut that eventually reveals vitamin D toxicity. These results indicate that the “safe,” physiological range of 25(OH)D is between 80 and 225nmol/L.

7. **Vitamin D treatment did not adversely affect markers of bone deposition** (bone specific alkaline phosphatase) or resorption (C telopeptide). This indicates that high serum 25(OH)D did not cause excessive bone resorption, which some fear.
  
8. **Vitamin D binding protein correlated with C reactive protein:** DBP, which had been suggested to have a role in inflammation, was found to correlate with CRP, an acute phase protein released in response to inflammation. These results suggest a common inflammatory stimulus for both proteins.

## **7.2 CONCLUSIONS & IMPLICATIONS**

Overall, the aim of this thesis was to investigate the safety and therapeutic potential of vitamin D<sub>3</sub> supplementation in patients with multiple sclerosis. The present study provides conclusive evidence that physiologically attainable serum 25(OH)D concentrations of 180nmol/L are safe in patients with multiple sclerosis. Clinical outcomes suggested efficacy and demonstrated a trend for improvement in the treated group, with stability or improvement in disease progression in treated patients and a trend for fewer relapses in vitamin D-treated patients. This provides ample impetus for larger randomized controlled trials. Importantly, T cell proliferative responses to MS-associated antigen stimulation were significantly reduced in treated patients at one-year, both in comparison to baseline and in comparison with control group responses. The 25(OH)D-induced amelioration of abnormal T cell reactivities provide a mechanistic explanation of recent evidence that higher serum 25(OH)D concentrations or vitamin D supplementation are associated with lower rates of relapse (210;226;229;332) and to fewer gadolinium-enhancing lesions (248). Our data indicate that T cell pools responsive to MS-associated antigens were reduced in cholecalciferol-treated patients, which may indicate that these T cell pools that may have responded were rendered more tolerant or anergic. We could find no evidence of alterations in T cell trafficking and since T cell activation substantially raises vitamin D receptor expression (347), this would be the simplest explanation for the reduction in T cell proliferation. Alternatively, reduced T cell proliferation may suggest an epigenetic effect of T cell lines in which anti-inflammatory and regulatory T cells are promoted. Of particular interest, a recent Cochrane review of vitamin D supplementation in multiple sclerosis included

the clinical trial presented in this thesis as the only study pertinent to the field (360). Despite the low power of the present study, it is the strongest work in this area to date. This research should serve to motivate more rigorous clinical trials of vitamin D in the treatment and prevention of multiple sclerosis. This research also opens the way for further studies of vitamin D supplementation for treatment and prevention of other disorders. These studies are required, warranted and may eventually impact recommendations to the Canadian public.

### **7.3 LIMITATIONS**

The main limitation of the present study was its focus on clinical safety and the associated lack of power to measure clinical impact of vitamin D<sub>3</sub> in patients with multiple sclerosis. Randomization with stratification provided balanced trial arms, but without blinded placebo control, some inadvertent selection bias is likely. Further, bias may have been introduced because of greater clinical attention in the treated group wherein clinic visits were greater in number (10 vs. 4 in the control) and frequency than the attention given to control group participants. It has been suggested that being part of a clinical trial alone may help to improve clinical outcomes. Primary biochemical outcome measures were objective, but lack of blinding may have led to vulnerability with clinical outcome measures. The size of the study population, although sufficiently powered for safety outcomes, was relatively small and makes the subsequent immune marker investigations exploratory and hypotheses-generating and the *in vivo* anti-proliferative effects will need to be confirmed in larger RCTs. However, the extent and the strength of statistical confidence obtained in treated patients for the changes elicited for key, disease-associated-T cell pools was surprising. Cytokine concentrations, transient in nature, were found to be below detectable levels in the majority of samples assayed, limiting the interpretation of these results. Further, several cytokines characteristic of other immune cell phenotypes (for example, Th17 and regulatory T cells) were not investigated and may have contributed more meaningful results. Finally, the clinical trial design, in which the recommended daily intake of calcium was included in the treatment regimen, does not

preclude a possible contribution of calcium to the observed immunomodulatory effects in treated patients.

## **7.4 FUTURE RESEARCH DIRECTIONS**

Randomized controlled trials investigating of the therapeutic potential of vitamin D<sub>3</sub> supplementation in multiple sclerosis are seriously lacking. This study is one of only four found in the literature, and the only one considered acceptable for the Cochrane Review completed by Jagannath et al. (360). The limitations of previous studies include varying doses and forms of vitamin D<sub>3</sub>, small sample size, lack of blinding and placebo groups, and these studies have been conducted without a consensus on an optimal dose of vitamin D<sub>3</sub>. There have not been any studies adequately powered to detect clinical changes induced by vitamin D<sub>3</sub> supplementation in patients with MS. However, despite these limitations the studies performed to date have demonstrated that patients with multiple sclerosis receiving vitamin D<sub>3</sub> supplementation have reduced relapse rates and fewer new lesions on MRI, suggesting that vitamin D<sub>3</sub> supplementation may have benefit in these patients. Large randomized blinded placebo-controlled studies are needed. An ideal trial of this nature would involve a larger, multi-centered population (n>250) with primary outcome measures of new Gadolinium-enhancing lesions on MRI, relapse events, annualized relapse rates and disease progression (EDSS). A trial to adequately ensure efficacy would need to be at least two years in duration. The immunomodulatory properties of vitamin D<sub>3</sub> supplementation in multiple sclerosis should also be confirmed. In addition, mechanistic studies are needed to determine the mechanism through which high serum 25(OH)D concentrations exert this effect. Further, the relationship between vitamin D binding protein and c reactive protein has not been confirmed nor explored, but may be better examined in a healthy population.

As a separate issue from treatment of MS, vitamin D<sub>3</sub> prevention studies are also required to determine if adequate supplementation in utero, during childhood and/or during adolescence can reduce the risk of MS. An example of how a trial of this nature may be conducted would involve a stable, homogenous, nation-wide population in which half that population was supplemented with at least 4,000 IU/d of vitamin D and the other half would receive the recommended intake of 600 IU/d. A good example would be the population of French farmers observed in the Vukusic study, in which prevalence rates displayed an astounding south-north gradient with significantly higher rates (~100/100,000) in the north versus those in the south (~50/100,000) (241). Randomization of vitamin D<sub>3</sub> doses by province and stratified by latitude, and the capture of incidence of multiple sclerosis tracked over at least 50 years would provide a good assessment. However, the population-based nature, funding issues and practicality of these trials combined with the late onset of MS are likely to be prohibitive.

## 8.0 REFERENCES

*“The devil can cite Scripture for his purpose.”*

*English Proverb*

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