Vitamin D status and its contribution to multiple sclerosis risk: Insights gained through the
study of children with central nervous system demyelination

by

Heather Elaine Courtney Hanwell

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

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2011

Abstract

Acute demyelination in children may be a monophasic illness or the sentinel attack of multiple sclerosis (MS) – a chronic inflammatory neurodegenerative demyelinating disease. MS risk is largely determined during childhood and vitamin D may protect against MS. The primary objective of this thesis was to evaluate vitamin D status in children presenting with acute demyelinating syndromes (ADS) as a potential contributor to MS outcome. The LIAISON “25 OH Vitamin D TOTAL” assay was validated to assess the biomarker of vitamin D status – serum 25-hydroxyvitamin D (25(OH)D) concentrations. Consecutive patients (<16 y) were enrolled at presentation with ADS and prospectively evaluated at 23 Canadian centres. MS was defined by a second clinical demyelinating event or by MRI evidence of new lesions over time.
Cox proportional hazards regression models assessed risk of MS outcome as a function of serum 25(OH)D tertiles, accounting for factors associated with either MS risk or vitamin D status – age, sex, season, and HLA-DRB1*15 status. Of 211 children with 25(OH)D measured in sera obtained a median of 9 days from onset (interquartile range, 5 – 17 d; maximum 36 days), 20% (n = 41) were diagnosed with MS after 3.7 mos. (3.1 – 7.3 mos.). Risk of MS was lower in children with 25(OH)D levels in the highest tertile (≥ 74 nmol/L) at ADS versus those in the lowest tertile (< 50 nmol/L) (HR 0.41; 95% CI 0.18 to 0.97, adjusted model). Children with higher circulating 25(OH)D concentrations at ADS have a lower risk of MS. Further evidence for a role of vitamin D insufficiency during childhood and adolescence contributing to MS risk comes from three MS patients with suboptimally managed pseudo-vitamin D deficiency rickets. Finally, a sun exposure questionnaire was validated in the latter part of this thesis for use in future research into determinants of vitamin D status and their association with risk of MS.
C:\Users\name\Documents\thesis\acknowledgements\Acknowledgements\Acknowledgements\in\iv\iv

The completion of this thesis would not have been possible without the contributions and support of so many others.

First, I would like to thank my supervisors, Drs. Reinhold Vieth and Brenda Banwell. I feel incredibly privileged to have benefited from the mentorship of such accomplished and wonderfully complementary supervisors.

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I am also very grateful to Dr. Banwell for her unceasing encouragement and enthusiasm and for the many wonderful experiences that she has enabled since we began our collaboration. She is a generous, caring, visionary mentor who sets a high standard for achievement. I thank her for inspiring me to strive to maintain a healthy work/life balance while also settling for nothing less than excellence in my endeavours.

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List of Abbreviations

1,25(OH)₂D 1-alpha,25-dihydroxyvitamin D or calcitriol
1,25D₃-MARRS 1,25(OH)₂D₃-membrane associated, rapid-response steroid-binding
24,25(OH)₂D 24,25-dihydroxyvitamin D
25(OH)D 25-hydroxyvitamin D or calcidiol
ADEM acute disseminated encephalomyelitis
ADS acquired demyelinating syndrome
AI adequate intake
ANCOVA analysis of covariance
ARR annualized relapse rate
B[a]P benzo[a]pyrene
BBB blood brain barrier
BMI body mass index
CCPGSMS Canadian Collaborative Project on the Genetic Susceptibility to Multiple Sclerosis
CI confidence interval
CIS clinically isolated syndrome
CDC Centers for Disease Control
CLIA chemiluminescent immunoassay
CNS central nervous system
CSF cerebrospinal fluid
CV coefficient of variation
CYP cytochrome P450 mixed-function oxidase
DEQAS Vitamin D Quality Assessment Scheme
DIS dissemination in space
DIT dissemination in time
DMT disease modifying therapy
DNA deoxyribonucleic acid
DRI dietary reference intake
EAE experimental autoimmune encephalomyelitis
EAR estimated average intake
EBV Epstein-Barr virus
EDSS Expanded Disability Severity Scale
EDTA ethylenediaminetetraacetic acid
ERp57 endoplasmic reticulum protein of 57 kDa
Gd gadolinium
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
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<tbody>
<tr>
<td>GWAS</td>
<td>genome wide association studies</td>
</tr>
<tr>
<td>Hep</td>
<td>heparin</td>
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<tr>
<td>HLA</td>
<td>human leukocyte antigen</td>
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<tr>
<td>HR</td>
<td>hazard ratio</td>
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<tr>
<td>IFN</td>
<td>interferon</td>
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<tr>
<td>IgG</td>
<td>immunoglobulin G</td>
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<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>IM</td>
<td>infectious mononucleosis</td>
</tr>
<tr>
<td>IOM</td>
<td>Institute of Medicine</td>
</tr>
<tr>
<td>IQR</td>
<td>inter-quartile range</td>
</tr>
<tr>
<td>IU</td>
<td>international units</td>
</tr>
<tr>
<td>LC-MS/MS</td>
<td>liquid chromatography tandem mass spectrometry</td>
</tr>
<tr>
<td>MBP</td>
<td>myelin basic protein</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>MMP</td>
<td>matrix metalloproteinases</td>
</tr>
<tr>
<td>MOG</td>
<td>myelin oligodendrocyte glycoprotein</td>
</tr>
<tr>
<td>MRI</td>
<td>magnetic resonance imaging</td>
</tr>
<tr>
<td>MS</td>
<td>multiple sclerosis</td>
</tr>
<tr>
<td>N/A</td>
<td>not applicable</td>
</tr>
<tr>
<td>NIST</td>
<td>National Institute of Standards and Technology</td>
</tr>
<tr>
<td>NMO</td>
<td>neuromyelitis optica</td>
</tr>
<tr>
<td>NR</td>
<td>not reported</td>
</tr>
<tr>
<td>ON</td>
<td>optic neuritis</td>
</tr>
<tr>
<td>OR</td>
<td>odds ratio</td>
</tr>
<tr>
<td>PAF</td>
<td>population attributable fraction</td>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PDDR</td>
<td>pseudovitamin D deficiency rickets</td>
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<tr>
<td>PLP</td>
<td>proteolipid protein</td>
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<tr>
<td>PPMS</td>
<td>primary progressive multiple sclerosis</td>
</tr>
<tr>
<td>PTH</td>
<td>parathyroid hormone</td>
</tr>
<tr>
<td>RDA</td>
<td>recommended dietary allowance</td>
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<tr>
<td>RIA</td>
<td>radioimmunoassay</td>
</tr>
<tr>
<td>RRMS</td>
<td>relapsing-remitting multiple sclerosis</td>
</tr>
<tr>
<td>RXR</td>
<td>retinoid X receptor</td>
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<tr>
<td>SNP</td>
<td>single nucleotide polymorphism</td>
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<tr>
<td>SPMS</td>
<td>secondary progressive multiple sclerosis</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>STAT3</td>
<td>signal transducer and activator of transcription-3</td>
</tr>
<tr>
<td>SZA</td>
<td>solar zenith angle</td>
</tr>
<tr>
<td>T1D</td>
<td>type 1 diabetes mellitus</td>
</tr>
<tr>
<td>TGF</td>
<td>transforming growth factor</td>
</tr>
<tr>
<td>Th</td>
<td>T helper</td>
</tr>
<tr>
<td>TM</td>
<td>transverse myelitis</td>
</tr>
<tr>
<td>TNF</td>
<td>tumour necrosis factor</td>
</tr>
<tr>
<td>UK</td>
<td>United Kingdom</td>
</tr>
<tr>
<td>UL</td>
<td>Tolerable Upper Limit</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>UVB</td>
<td>ultraviolet B radiation</td>
</tr>
<tr>
<td>UVR</td>
<td>ultraviolet radiation</td>
</tr>
<tr>
<td>VDBP</td>
<td>vitamin D binding protein</td>
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<tr>
<td>VDDRII</td>
<td>vitamin D-dependent rickets type II</td>
</tr>
<tr>
<td>VDR</td>
<td>vitamin D receptor</td>
</tr>
<tr>
<td>VDRE</td>
<td>vitamin D response element</td>
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</table>
1 Introduction

Vitamin D is an essential nutrient unique in that humans are able to acquire it from the diet and through solar ultraviolet B (UVB) radiation-catalyzed synthesis in the skin. While it is best known for its role in calcium homeostasis and bone mineralization, vitamin D is also involved in numerous other functions such as modulating immune function, and regulating cellular proliferation, differentiation, and apoptosis. Given the immune modulating functions of vitamin D and the striking increase in prevalence of Multiple Sclerosis (MS) and other immune-mediated diseases as a function of increased latitude or reduced ambient UVB, vitamin D has been implicated as a putative determinant of MS risk.

MS is a chronic inflammatory disease characterized by demyelinating plaques and neurodegeneration within the central nervous system. Although typically diagnosed in early to mid-adulthood, MS also affects children and adolescents; collaborative research in the area of pediatric MS has served to increase the recognition and awareness of MS in children and also to increase interest in studying these young patients. While the cause of MS remains to be determined, epidemiologic evidence implicates (i) the period of life spanning from gestation through adolescence as the time of risk determination, and (ii) the contribution of both genetic and environmental risk factors.

In this doctoral thesis, a programmatic approach to the evaluation of vitamin D status as a determinant of MS risk is employed. The thesis commences with a critical appraisal of the current level of evidence – as measured against the Bradford-Hill criteria for assessing causality\(^1\) – for vitamin D insufficiency as an environmental risk determinant for MS and as a potential modifying factor for MS disease activity. The second stage of the doctoral work
focuses on methodological approaches to the accurate measurement of vitamin D serum concentrations, yielding evidence-based rationale for the vitamin D assays employed for the core analyses. The core analyses then interrogate vitamin D status as measured at the time of an acute first demyelinating attack of the central nervous system in children to define the relative association of vitamin D status with subsequent MS outcome. Such studies were uniquely enabled through the national pediatric demyelinating disease study involving over 200 children from whom serum was obtained at the time of acute demyelination and MS outcome defined through rigorous prospective observation. The complex interplay between vitamin D and host genetic risk alleles for MS were then explored both in the inception cohort of children, but also in three MS patients with a rare inherited form of disturbed vitamin D metabolism. Finally, in order to evaluate behavioral contributions to individual vitamin D status, a sun-exposure questionnaire was developed and evaluated in a collaborative study involving adults living in Italy. Through a series of published or submitted manuscripts, this programmatic approach provides novel insights into the putative contribution of vitamin D to MS risk in Canadian children.
2 Background and Literature Review

“If I have seen further, it is only by standing on the shoulders of giants.”

– Sir Isaac Newton, 1676

2.1 Multiple Sclerosis

2.1.1 Epidemiology

Multiple Sclerosis (MS) is a chronic disease characterized by inflammation, demyelination, and neurodegeneration within the central nervous system (CNS). Typically diagnosed between the ages of 18 – 40 years, MS is the most common non-traumatic disabling neurological disease of adulthood. MS frequently results in motor, sensory, and cognitive dysfunctions that may be temporary, progressive or permanent. Thus, MS can adversely affect employment, relationships, and overall sense of well-being and quality of life. According to the 2008 MS Atlas, there were at least 1.32 million people living with MS worldwide; given that this estimate does not include a number of large countries, this estimate of global prevalence of MS is almost certainly an underestimate. The estimated global prevalence of MS is approximately 30 people per 100,000 and, in general, prevalence increases with latitude and with lower available ultraviolet radiation (UVR). Canada has the fifth highest prevalence of MS in the world with estimates ranging from 132.5 to 260 people with MS per 100,000.

Accurate prevalence estimates may be challenged by the apparent increases in prevalence over time which may, in part, be due to improved diagnostic tools – that is, improved ascertainment – and also improved survival of those diagnosed with MS. However, these explanations do not
account for the disproportionate increase in the prevalence of females with MS observed by Orton et al.; the female-to-male ratio among Canadians born in the 1930’s was approximately 1.9:1 and has risen to more than 3:1 among those born after the late 1960’s.

The relatively high prevalence of MS in Canada, the observed trend of increasing prevalence and the longer survival of patients with MS who often experience a high level of disability – which limits productivity and often necessitates supportive care – mean that MS also presents a formidable economic burden in Canada. In addition to the aforementioned disability-related costs of MS is the expense of diagnosing, treating and monitoring patients.

2.1.2 Presentation & Diagnosis

In the majority of patients, MS begins with a relapsing-remitting disease course generally accompanied by little or no residual disability during remission (Figure 2-1). However, over time relapses may occur over a backdrop of accruing disability; this is characteristic of a secondary-progressive disease course. In the minority of instances, MS will follow a primary progressive disease course wherein the patient experiences irreversible progression of disability in the absence of relapses.
MS displays considerable variability both intra- and inter-individually. MS attacks or relapses may manifest as a wide variety of signs and symptoms such as ataxia – which is essentially reduced coordination of movement – (reported in up to 80% of patients), spasticity (more than 60% of patients), visual disturbances (60% of patients), fatigue (at least 40% of patients), sensory deficits (approximately 40% of patients), pain (at least 15% of patients), depression (between 15 – 40%) and cognitive impairment (between 40-65% of patients).
Generally speaking, the diagnosis of MS is based upon evidence of disease activity separated in both time and space, i.e. “multiple” deficits affecting more than one region of the CNS and occurring at different times. More specifically, diagnoses of MS are made using carefully constructed criteria, the most commonly used set of criteria now being the McDonald Criteria or the recently-revised, “simplified” McDonald criteria. These criteria for diagnosis of MS incorporate clinical, paraclinical and magnetic resonance imaging (MRI) findings (Table 2-1). MRI not only plays a key role in the diagnosis of MS but also provides another means to monitor disease progression and response to treatment. MRI technology allows clinicians to localize inflammatory lesions within the CNS and – when accompanied by the use of gadolinium – can distinguish between active and quiescent lesions.

To appreciate the varied and often progressive clinical manifestations of MS, it is helpful to review what is known about the underlying pathobiology.
Table 2-1: 2010 Updated McDonald Criteria for MS Diagnosis

<table>
<thead>
<tr>
<th>Criteria</th>
<th>Additional Data Needed for MS Diagnosis</th>
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<tbody>
<tr>
<td>≥ 2 attacks (^a); objective clinical evidence of ≥ 2 lesions or objective clinical evidence of 1 lesion with reasonable historical evidence of a prior attack</td>
<td>None. However, it is desirable that any diagnosis of MS be made with access to imaging based on these Criteria. If imaging or other tests are undertaken (e.g. cerebrospinal fluid, CSF) and are negative, extreme caution needs to be taken before making a diagnosis of MS, and alternative diagnoses must be considered. There must be no better explanation for the clinical presentation.</td>
</tr>
<tr>
<td>≥ 2 attacks (^a); objective clinical evidence of 1 lesion</td>
<td>Dissemination in space (DIS)(^b)</td>
</tr>
<tr>
<td>1 attack (^a); objective clinical evidence of ≥ 2 lesions</td>
<td>Dissemination in time (DIT)(^c)</td>
</tr>
<tr>
<td>1 attack (^a); objective clinical evidence of 1 lesion (ADS)</td>
<td>Both DIS and DIT</td>
</tr>
<tr>
<td>Insidious neurological progression suggestive of MS (primary progressive, PPMS)</td>
<td>1 year of disease progression (retrospectively or prospectively determined) plus 2 of 3 of the following:</td>
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<tr>
<td></td>
<td>1. Evidence for DIS within the brain based upon ≥ 1 T2 lesion in periventricular, juxtacortical, or infratentorial regions</td>
</tr>
<tr>
<td></td>
<td>2. Evidence for DIS in the spinal cord based on ≥ 2 T2 lesions.</td>
</tr>
<tr>
<td></td>
<td>3. Positive CSF (isoelectric focusing evidence of oligoclonal bands or elevated IgG index)</td>
</tr>
</tbody>
</table>

\(^a\)Attack: Patient-reported or objectively observed events typical of an acute CNS inflammatory demyelinating event with duration of ≥ 24 hours, in the absence of fever or infection.

\(^b\)Criteria for Demonstration of Dissemination in Space (DIS): ≥ 1 T2 lesion\(^*\) in at least 2 of 4 areas: Periventricular, Juxtacortical, Infratentorial, Spinal cord (*Note: Symptomatic lesions within spinal cord or brainstem do not contribute to lesion count); or await a further clinical attack implicating a different CNS site.

\(^c\)Criteria for Demonstration of Dissemination in Time (DIT): Simultaneous presence of gadolinium (Gd)-enhancing and non-enhancing lesions at any time or ≥ 1 new T2 or Gd-enhancing lesion(s) on follow-up MRI, with reference to a baseline scan, irrespective of the timing of the baseline MRI.
2.1.3 Pathophysiology

Within the CNS, oligodendrocytes produce myelin – a lipid-rich structure consisting of coiled layers that form a protective axonal sheath. The myelin sheath is periodically punctuated by unmyelinated segments called the nodes of Ranvier which, in concert with the myelinated sections, serve to increase the efficiency and speed at which nervous impulses – called action potentials – are propagated along axons. In MS, lesions form within the brain, spinal cord and optic nerves, primarily affecting the myelin-dense white matter regions, although not exclusively. The lesions are often characterized by a combination of inflammation, edema, demyelination, and, in some cases, permanent axonal injury and scarring. Damage to or destruction of the myelin or axon disrupts nervous signaling; this disruption may manifest as signs and symptoms of MS.

But why does MS produce these CNS demyelinating lesions? The etiology of MS remains unknown but the most widely accepted view of the pathobiology underlying MS is that it is an immune-mediated disease, specifically an autoimmune disease of the CNS. In autoimmune disease, host immune cells inappropriately recognize and respond to host antigens, leading to immune-mediated targeting and injury to host tissue expressing the antigens. Several lines of evidence support the view of MS as an autoimmune disease: the animal model of MS, genetics of MS, characteristics of MS lesions, and efficacy of MS therapies.

First, there are similarities between MS and an induced experimental model in animals, termed experimental autoimmune encephalomyelitis (EAE) – which is induced by immunizing the animals with myelin-derived proteins or peptides such as myelin basic protein (MBP), hydrophobic proteolipid proteins (PLPs), and myelin oligodendrocyte glycoprotein (MOG) or by transfer of immune cells T-helper that have been sensitized to such myelin peptides. Overall, the lesions and clinical signs induced in EAE by these myelin-associated antigens bear
resemblance to the inflammatory demyelinating processes observed in MS. Next, the genes most strongly and consistently associated with risk of MS encode products involved in immune function, such as those of the human leukocyte antigen (HLA) system, thereby implicating dysregulated immune function in the pathobiology of the MS (discussed further in 2.2.1). Furthermore, higher circulating levels of immune cells and immunoglobulins specific to the CNS are found in the cerebrospinal fluid (CSF) and immune cells are found in MS lesions.

The prevailing view of MS immunopathogenesis is that auto-reactive immune cells are activated peripherally and gain access to the CNS via several mechanisms: (i) Interactions between complementary adhesion molecules expressed by activated immune cells and endothelial cells of the blood brain barrier (BBB) allow for adhesion of immune cells to the BBB luminal endothelium; (ii) chemokines expressed on the endothelial lumen attract specific activated immune cells; and (iii) immune cell secretion of matrix metalloproteinase (MMPs) disrupt the integrity of the BBB, allowing for immune cell extravasation from circulation into the CNS. Finally, the disease modifying therapies (DMTs) for MS target aspects of immune signaling; these DMTs reduce relapses and CNS lesion burden seen by MRI once again, indicating the importance of immune function in the pathobiology of MS.

In addition to the inflammation described above, there is also a neurodegenerative component to MS that results in axonal damage and brain atrophy. While the accrual of new demyelinating lesions can be associated with acute clinical relapse, it is the progressive accrual of both clinical and subclinical pathological disease (focal areas of inflammation as well as widespread neuronal cell loss and axonal transaction) that is felt to underlie progression to irreversible disability. The risk of irreversible disability increases with disease duration; however, the pathological features of tissue injury are detectable from clinical disease onset. There is not a clear divide between the inflammation – and its accompanying lesional inflammatory milieu of cytokines, nitric
oxide, oxidative stress, and macrophage-mediated autophagy – and neurodegeneration observed in MS. Effective strategies for neuroprotection against MS have not yet been established.

### 2.1.4 Features of Pediatric Onset MS

Although MS is most commonly diagnosed in early to mid-adulthood, approximately 2 – 5% of all MS patients are diagnosed in childhood or in adolescence before the age of 16 years. Very little study of pediatric MS occurred until the past decade. This was partly due to the relative paucity of MS patients with a childhood-onset but was compounded by a general lack of recognition of MS as a disease entity in children and accompanying under-diagnosis. An increasing number of studies now detail the clinical, neuroimaging, immunological and epidemiological features of pediatric-onset MS- highlighting similarities and distinctions from adult-onset disease. The diagnosis of MS in children, and in adults, is predicated on the exclusion of other disorders, such as non-demyelinating inflammation of the central nervous system, infectious agents, neoplasms, inherited leukodystrophies and metabolic disorders.

The clinical onset of MS, or first demyelination event, may be termed acquired demyelinating syndrome (ADS) or clinically isolated syndrome (CIS). In adults, an ADS/ CIS event represents the sentinel attack of MS in approximately 80% of patients, whereas somewhere between 25-60% of children or adolescents presenting with CIS or ADS will subsequently be diagnosed with MS. The likelihood of MS diagnosis following a sentinel event is influenced by the presenting clinical, laboratory, and MRI features and by genetic and environmental factors. In over 97% of children with MS, the disease manifests with a relapsing-remitting MS (RRMS) course. In contrast, RRMS accounts for only 80% of adult-onset disease and 20% of adults experience a primary progressive form of MS (PPMS). The relapse rate in pediatric MS also
exceeds that of adult-onset disease early in the disease course, as does MRI evidence of lesion accrual.\textsuperscript{43,44} In spite of the higher rate of relapses noted in pediatric MS vs. adult-onset MS, the time from diagnosis to disability, as measured by the Kurtzke Expanded Disability Severity Scale (EDSS) score, is typically longer for pediatric-onset MS.\textsuperscript{45} However, despite the longer time to disability from diagnosis, patients with pediatric-onset MS are still generally younger than adult-onset MS patients at the time when they reach EDSS scores indicating significant physical disability.\textsuperscript{46} It is of interest to note that the EDSS is relatively insensitive to the type of disability that might be more pertinent to children and adolescents with MS; namely, cognitive dysfunction. Current research indicates a significant burden of cognitive dysfunction among pediatric-onset MS patients even early in the course of the disease.\textsuperscript{47-49}

While the relapses may be the clinical hallmarks of the activity of the underlying CNS inflammation, cognitive dysfunction may be an indicator of the neurodegenerative process; in adults with MS, cognitive dysfunction strongly correlates with MRI correlates of neurodegeneration or atrophy, such as brain volume loss.\textsuperscript{50}

Most studies in pediatric-onset MS have found an overall female-to-male sex ratio similar if not higher than that observed in adult-onset MS.\textsuperscript{46,51,52} However, in children experiencing prepubescent onset – before the age of about 10 to 12 years – the female-to-male ratio is generally either approximately 1 or slightly lower.\textsuperscript{43,53} Why sex ratios differ when pediatric cohorts are stratified around the peri-pubescent time is currently unknown. However, sex hormones or other non-hormonal sex-based differences may play a role in modifying susceptibility to MS.\textsuperscript{54} Recent work suggests that the effect of sex on risk of MS may be contingent upon environmental factors. For instance, female to male MS ratio increases as available regional UVB declines\textsuperscript{55} and the ratio of female to male MS patients also increases
with increasing latitude, suggesting a possible interaction between some female-specific feature, low UVB and risk of MS.\textsuperscript{56,57}

2.2 Determinants of MS Risk

Much research has been undertaken to better understand MS but our understanding of the factors involved in the etiology and progression of MS remains incomplete.\textsuperscript{29} Epidemiological studies have shed light on ethnic, seasonal and geographical variations in MS incidence, leading to the various hypotheses regarding genetic, hormonal and environmental risk factors, and their potential interactions.\textsuperscript{11,58-61}

2.2.1 Genetics

Years of intensive study by genetic epidemiologists and molecular geneticists – for instance, the Canadian Collaborative Project on the Genetic Susceptibility to Multiple Sclerosis (CCPGSMS) and the International Multiple Sclerosis Consortium – have yielded many candidate regions of DNA, and specific genes and alleles that relate to MS risk. With the exception of genes encoding the major histocompatibility complex (MHC), the overall contribution of genetic factors to MS risk appears to be relatively small: candidate genes identified in genome-wide association studies (GWAS) may contribute only approximately only 5\% of the variance in risk of developing MS. \textsuperscript{62-65}
The genes with the strongest and most consistent association with MS risk are found on chromosome 6 and cluster within regions of DNA that encode the MHC, also known as the human leukocyte antigen (HLA) system.\textsuperscript{66-69} Given that the pathobiology observed in MS is likely driven by an autoimmune process, it follows that candidate loci would preferentially fall within regions with genes encoding products involved with immune function. The MHC is highly polymorphic, meaning that this region is highly variable, containing many single nucleotide polymorphisms (SNPs) that contribute to the diversity of interpersonal response to infections and the likelihood of aberrant, self-targeting of the immune response, known as autoimmunity. One gene of particular interest in the context of MS is \textit{HLA-DRB1}. This gene encodes the beta chain of a class II antigen-presenting molecule – HLA-DR – which is a heterodimer that consists of both an alpha and beta chain. Class II antigen-presenting molecules such as HLA-DR are found on the surface of the professional antigen-presenting cells of the immune system, i.e. B cells, dendritic cells, and macrophages.\textsuperscript{70} HLA-DR presents peptide antigens to T cells which results in production of antibodies that are specific to the particular antigen presented.\textsuperscript{70} HLA-DRB1 is composed of multiple subtypes with allelic variation being represented by a two digit number following the gene name (e.g. HLA-DRB1*15). Differences in the two digits correspond to the type of serological antigen that is presented by the HLA-DRB1 molecule. Each allelotype can be further resolved to subgroups that differ due to non-synonymous nucleotide substitutions; in other words, the resultant HLA-DRB1 molecule will present the same antigen but differs in its structure by one amino acid. Non-synonymous nucleotide substitutions are represented by the addition of a third and fourth digit (e.g. HLA-DRB1*1501).\textsuperscript{71} Finally, differences in the nucleotide sequence of the \textit{HLA-DRB1} DNA that do not actually result in amino acid differences within the gene product itself – i.e. synonymous substitutions – are represented by an additional two digits (e.g. HLA-DRB1*150101).\textsuperscript{71} Many of the HLA-DRB1 allelotypes are associated with either a higher or lower risk of MS. For instance,
HLA-DRB1*15 is associated with a higher risk of MS. Many studies examining HLA-DRB1 will only resolve to the first two digits; however, the relationship between HLA-DRB1*15 may be more specifically driven by HLA-DRB1*1501 and there is evidence of complex epistatic interactions in terms of the association with resultant MS risk.

Why risk of MS varies between certain allelotypes remains to be determined. One promising area of research pertains to differential regulation of the expression of the allelotypes. For instance, Ramogopalan et al. recently reported that the HLA-DRB1*1501 allelotype possessed a highly conserved, functional vitamin D response element (VDRE) within the gene’s promoter region. Furthermore, the authors demonstrated that addition of the active metabolite of vitamin D (calcitriol) to antigen-presenting cells in vitro resulted in increased expression of HLA-DRB1 on the cellular surface. Therefore, it is plausible that the association between the HLA-DRB1 allelotype and risk of MS may be modified by vitamin D status, which determines the availability of the activated metabolite of vitamin D.

MS risk increases with increasing degree of relatedness or similarity of DNA. In Canada, concordance rates of MS among monozygotic twins – who share virtually 100% of their DNA – are generally between 25-31%. In contrast, concordance rate for MS in dizygotic twins and full siblings – who share approximately 50% of their DNA – is only 4-5%. Even though MS risk is clearly higher in twins or in family members of affected individuals, other factors are clearly required to both explain MS discordance in 70% of identical twins and to understand MS incidence in non-familial cases. Several of the most compelling environmental risk factors studied to date are presented below.
2.2.2 Environmental Contributions to MS risk

MS prevalence varies across different world regions, across different populations, and appears influenced by socioeconomic status and urbanization. Several environmental factors have been studied as putative contributors to regional disparity in MS risk. While the majority of this thesis focuses on vitamin D, other candidate risk factors for MS, such as sex hormones, viral infection (particularly Epstein-Barr virus, EBV) and tobacco smoke exposure will be discussed in the context of interactions with vitamin D status.

Arguably the most compelling environmental contribution to MS risk relates to world region of residence. The prevalence of MS is higher in areas with temperate rather than tropical climates, generally increases with distance from the equator, and is inversely associated with average ambient UVB. The striking difference in prevalence of MS and some other autoimmune diseases as a function of latitude has pointed to UVB – and resultant vitamin D status – as a determinant of autoimmune risk. Several comprehensive reviews on this topic are available. Vitamin D is involved in modulating immune function and cell proliferation, differentiation, and apoptosis, providing biological plausibility for a role of vitamin D in disorders relating to immune dysregulation. In vitro and animal models of immune cell behaviour and central nervous system inflammation have demonstrated a pro-inflammatory impact of vitamin D insufficiency and an anti-inflammatory role for vitamin D supplementation.

The next sections of the text will review vitamin D biology, sources of vitamin D and will outline current views on optimal vitamin D intake and 25(OH)D concentrations in humans. The evidence for a role of vitamin D status in MS will then be elaborated upon and evaluated using the Bradford Hill criteria. Finally, a conceptual framework for future research into the potential for vitamin D
supplementation as a primary risk reduction strategy at the population level and as a therapeutic agent to reduce relapse rate in individuals with MS will be discussed.

2.3 Vitamin D

2.3.1 Metabolism

In humans, vitamin D is obtained either through its synthesis in the skin or through dietary ingestion. Cholecalciferol (vitamin D₃) can be obtained from the diet or produced in the skin following exposure of 7-dehydrocholesterol to UVB radiation, while ergocalciferol (vitamin D₂) can only be obtained via oral consumption. The term “vitamin D” without subscripts encompasses both forms.

Following either cutaneous synthesis or ingestion, vitamin D is transported to the liver bound to the vitamin D binding protein (VDBP, also known as group-specific component of serum or Gc-globulin) which increases solubilization of vitamin D metabolites in circulation. Data from animal studies suggest that – per pass across the liver – approximately 40-60% of circulating vitamin D is taken up by the liver whereby the vitamin D may be oxidatively metabolized to a number of products, the most relevant of which is 25-hydroxyvitamin D₃ (25(OH)D). The 25(OH)D is predominately catalyzed by the hepatic cytochrome P450 mixed-function oxidase (CYP) microsomal CYP2R1 and, possibly to a lesser extent, also by the mitochondrial CYP27A1.

Following the hepatic 25-hydroxylation, 25(OH)D is exported to systemic circulation where it circulates in the nanomolar ranges (typically ~25 – 225 nmol/L) bound to VDBP. VDBP is present in the systemic circulation at micromolar concentrations (~6µmol/L), thus, only 5% of
circulating VDBP is typically occupied by 25(OH)D.\textsuperscript{101} The 25-hydroxylation step operates under first-order kinetics – that is, is dependent upon substrate concentrations only. The circulating concentration of 25(OH)D represents 25-hydroxylated vitamin D obtained from both UVB-catalyzed synthesis and diet and is the accepted biomarker for vitamin D nutritional status.\textsuperscript{97,102} The VDBP-25(OH)D complex binds to megalin, a transmembrane receptor expressed in proximal tubular cells of the kidney, which acts to endocytose the VDBP-25(OH)D complex, or binds to cubulin, a surface receptor that can act in concert with megalin to take up VDBP-25(OH)D. The 25(OH)D metabolite is then trafficked within the cell by intra-cellular vitamin D binding proteins\textsuperscript{103} to be further hydroxylated by the mitochondrial one-alpha hydroxylase (CYP27B1) to form 1,25-dihydroxyvitamin D [1,25(OH)\textsubscript{2}D; calcitriol].

Calcitriol is the most bioactive of the naturally-derived vitamin D metabolites (Figure 2-2). Calcitriol also circulates bound to VDBP; and acts as a potent hormone targeting bone, kidneys and the intestines to modulate calcium and phosphate homeostasis. When circulating calcium concentrations are low, the parathyroid calcium-sensing receptor stimulates secretion of parathyroid hormone (PTH) which increases renal production of calcitriol from 25(OH)D. Calcitriol increases absorption of calcium from the intestines, increases urinary calcium reabsorption, and stimulates bone resorption, resulting in calcium liberation from bone.\textsuperscript{97,104,105} Calcitriol production is regulated, in part, through a biofeedback loop in which the calcitriol-induced gene, $CYP24A1$, encodes an enzyme that initiates the catabolism and clearance of calcitriol and other vitamin D-related metabolites via hydroxylation of carbon 24. Although the main metabolite, 24,25-dihydroxyvitamin D (24,25(OH)\textsubscript{2}D) is primarily regarded simply as the first metabolite in the catabolic pathway, 24,25(OH)\textsubscript{2}D has biological activity which, for the most part, opposes the functions of 1,25(OH)\textsubscript{2}D.\textsuperscript{106}
Mutations in the \textit{CYP27B1} gene that impair or abolish the expression or function of the CYP27B1 enzyme – thus, inhibiting production of calcitriol – produce an autosomal recessive inherited condition known as vitamin D dependent rickets type I or pseudovitamin D deficiency rickets (PDDR). Prompt provision of medicinal calcitriol resolves most of the short- and long-term sequelae of PDDR.\textsuperscript{107}

Although the kidney is the endocrine organ for the 1α-hydroxylation step, the CYP27B1 enzyme is expressed elsewhere in the body, including various immune cells, endothelial cells, and neuronal and glial cells in many regions of both the developing and mature CNS.\textsuperscript{108-110}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{Vitamin_D_Metabolism.png}
\caption{Vitamin D Metabolism (adapted from \textsuperscript{111} with permission)}
\end{figure}
2.3.2 Signaling

Most classical vitamin D-related actions are mediated by calcitriol via the vitamin D receptor (VDR).\textsuperscript{112} Calcitriol binds VDR in the cytoplasm. This complex translocates to the nucleus where they form a heterodimeric complex with the retinoid X receptor (RXR) which, along with other cofactors, is capable of binding to genomic VDREs, modulating expression of a large number of genes.\textsuperscript{113,114} Similar to the CYP27B1 enzyme, numerous cells and tissues throughout the body also express VDR such as the intestines, kidneys, and bones – all sites of vitamin D endocrine action. Also, all of the aforementioned cells that express CYP27B1 also express VDR, including dendritic cells, monocytes, macrophages, and B- and T-lymphocytes cells.\textsuperscript{115-118} These extra-renal tissues can activate vitamin D to calcitriol and locally control vitamin D-influenced biological processes such as apoptosis, proliferation, and immune cell behaviour.\textsuperscript{119,120}

Mutations to the gene encoding VDR that impair or ablate VDR expression or function result in a disease called vitamin D dependent rickets type II (VDDRII). Therapy for VDDRII depends upon the severity of the genetic defect – some forms of VDDRII may be resolved with daily doses of vitamin D of 4,000 international units (IU) or more while more severe presentations may be ameliorated by relatively high doses of calcitriol and/or high doses of elemental calcium. However, while PDDR can be ameliorated with calcitriol, many VDDRII patients remain refractory to treatment.\textsuperscript{121}

In addition to the nuclear VDR, another receptor for calcitriol has been recently described: 1,25(OH)\textsubscript{2}D\textsubscript{3}-membrane associated, rapid-response steroid-binding (1,25D\textsubscript{3}-MARRS) protein, also referred to as ERp57 (endoplasmic reticulum protein of 57 kDa).\textsuperscript{122-124} The 1,25D\textsubscript{3}-MARRS
receptor may be responsible for regulating intestinal calcium absorption and also mediating other relatively rapid or pre-genomic effects attributable to calcitriol.\textsuperscript{112,122,125-127}

### 2.3.3 Endogenous Synthesis and Exogenous Sources

Solar-stimulated cutaneous synthesis of vitamin D by UVB (280-320 nm) is a major driver of vitamin D status. Evidence for the substantial contribution of endogenous vitamin D to overall vitamin D status is the presence of seasonal variation in circulating 25(OH)D concentrations that overshadows the contribution of dietary sources, in spite of mandatory fortification of beverage milk, butter and margarine in Canada.\textsuperscript{128-133} Furthermore, studies investigating determinants of vitamin D status frequently report that sun exposure is the strongest or among the strongest predictor of 25(OH)D concentrations.\textsuperscript{128,134-139}

Many factors contribute to inter- and intra-personal variability in synthesis, and so it is a challenging problem to accurately estimate the amount of vitamin D each person synthesizes in a given amount of time. Factors affecting UVB-stimulated endogenous synthesis can be broadly classified as geoclimatic or personal. A key geoclimatic factor that determines UVB strength and therefore vitamin D synthesis is solar zenith angle (SZA), which is the angle between local vertical (90° perpendicular to the horizon) and the position of the sun in the sky.\textsuperscript{140} A low SZA occurs when the sun appears high in the sky and the solar radiation is traversing through the minimal amount of atmosphere to reach the Earth’s surface. As SZA increases, so does the amount of Earth’s atmosphere that solar UVB must traverse before reaching Earth’s surface. Thus, as SZA increases, available UVB for cutaneous synthesis of vitamin D decreases. SZA varies with several factors such as time of day, latitude, and season. The SZA is smallest at solar noon when the sun is highest in the sky and is higher in the morning and evening when the sun
appears closer to the horizon. SZA also increases with distance from the equator and varies with the seasons due to the tilt of the Earth’s axis; with a solar noon SZA nadir in summer and peak noon SZA in winter. In regions that are far from the equator, such as Canada and the northern USA, the hours of sunlight per day are lower and the large SZA during November through February results in so little UVB reaching the Earth’s surface that the sun cannot stimulate the endogenous production of meaningful amount of vitamin D.

Other geoclimatic factors that affect available UVB are air pollution and airborne particulates (aerosols), ozone, clouds, the amount of sky that is visible in a given location, altitude, and surface albedo. UVB is impeded by aerosols and ozone, while the effect of cloud cover is more variable; for the most part, cloud coverage attenuates UVB reaching the surface but on partly cloudy days where the sky is visible between clouds, UVB may either not be affected or, in some cases, may actually be marginally higher due to reflection from the cloud sides. UVB also tends to be stronger at higher altitudes due in part to fewer aerosols and also because the UVB traverses less atmosphere to get to the surface. Surfaces with high reflectivity (e.g. snow) – known as albedo – also increases available UVB while objects like tall buildings and mountains that reduce the amount of visible sky reduce UVB. It is interesting to note, however, that because of its short wavelength, UV radiation is susceptible to more scattering from atmospheric particles than visible light is. The consequence of this is that the visible shadow cast by such objects is larger than the UV shadow because some UV that has been scattered by atmospheric particles does enter the visible shadow from other parts of the sky.

Even in areas of high ambient UVB, vitamin D synthesis can vary markedly between individuals. Clothing or application of sunscreen can impair endogenous vitamin D synthesis, the extent of which is dependent upon how much skin is covered, and the sun protection factor rating of the fabric or sunscreen. Clothing and time in the sun also contribute to the
seasonal variation in circulating 25(OH)D concentrations – not only does ambient UVB decrease in the late fall through early spring, but the temperatures also decrease, leading people to expose very little skin when outdoors and generally spending less time outdoors. Furthermore, a person’s position also will affect their vitamin D synthesis such that lying horizontally will maximize the amount and intensity of UVB for vitamin D synthesis while standing vertically minimizes the amount of UVB. The importance of the angle of body surfaces in determining the intensity of incident UV radiation is demonstrated by the relatively common occurrence of sunburn on the regions of the body that typically remain horizontal, including the dorsal aspect of the feet, the shoulders, and tops of ears.

Limited evidence suggests that the amount of 7-dehydrocholesterol in the skin decreases with age such that the elderly may have reduced capacity to synthesize vitamin D. Also, people who have more melanin pigment in their skin also typically require longer sun exposure to achieve maximal vitamin D synthesis.

Although endogenous synthesis can account for a substantial proportion of circulating 25(OH)D – with sun exposure alone producing circulating 25(OH)D levels in excess of 200 nmol/L in some cases – there are no documented cases of vitamin D intoxication due to excessive exposure to sunlight. Conversely, although the amount of vitamin D obtained orally from diet and supplements is typically much lower than that which can reasonably be obtained through UVB-catalyzed endogenous synthesis, accidental or intentional oral ingestion can produce vitamin D toxicity. However, large doses producing toxicity are almost invariably in excess of 40,000 IU per day. The first indicator of vitamin D toxicity is usually hypercalciuria, followed by hypercalcemia. Depending on the severity of intoxication, vitamin D toxicity may also produce clinical symptoms and signs associated with hypercalcemia, such as decreased appetite, nausea, vomiting, constipation, lethargy, abdominal pain, altered mental status, and –
in infants or toddlers – persistent irritability, failure to thrive, and hypotonia. Serious intoxication may also produce more serious complications such as renal failure and even death.

The estimation of dietary intake of vitamin D is challenging for several reasons: International differences in mandatory fortification rules means that, between countries, different types of foods are fortified with varying amounts of vitamin D. In some countries, discretionary fortification results in only certain brands or types of those foods containing vitamin D; and the amount of vitamin D naturally present in some foods may vary dramatically. For instance, vitamin D naturally present in animal-derived food products may vary with the season or other aspects of the animals’ environment such as whether the animal was wild or farm-raised. For example, a serving of salmon or tuna can provide anywhere from 100 to 1,000 IU vitamin D per serving depending upon such aforementioned factors.

The government of Canada mandates fortification of cow’s milk and infant formula – presently fortified with 100 IU per 250mL, and margarine – fortified with 25 IU per teaspoon. Canadian vitamin supplements generally contain vitamin D₃ – although some vegan supplements may contain ergocalciferol (vitamin D₂). Most over-the-counter multivitamin supplements contain 400 IU of vitamin D, although the amount can vary from 50 IU to 1,000 IU. It remains to be seen whether the most recent revisions to the Institute of Medicine’s (IOM) Dietary Reference Intakes (DRI) for vitamin D (Table 2-2; Food and Nutrition Board of the IOM) will affect the typical levels of vitamin D found in supplements or fortified foods in Canada or the United States.

2.3.4 Recommended Dietary Allowances for Vitamin D Intake and Status

As highlighted in Table 2-2, considerable variation exists between groups or organizations in terms of recommendations for vitamin D intakes across the lifespan. Intake recommendations...
are intended to reduce adverse events in the population of interest – thus, avoiding both vitamin D deficiency and vitamin D toxicity.

Part of the variability in the recommendations for vitamin D intake arises from the lack of consensus regarding (i) the strength of evidence implicating vitamin D status as having a role in various health outcomes such as falls, fractures, cancers, and immune-mediated diseases; and (ii) the range of serum 25(OH)D concentrations that are considered optimal. The recommendations set forth by the IOM are intended only to optimize bone-related outcomes, asserting that a vitamin D serum 25(OH)D concentration $\geq 50$nmol/L constitutes vitamin D sufficiency. However, many other experts assert that recommendations should be based upon evidence for vitamin D improving not only bone-related outcomes but also for reducing risk of cancers, autoimmune diseases and several other diseases. With these indications in mind, a target of 75 nmol/L is currently regarded by many as the minimum concentration needed to optimize vitamin D status. It is possible that consensus on optimal vitamin D status could be more readily formed if there first was consensus regarding the methods of compiling and evaluating the evidence.
Table 2-2: The Vitamin D Intake and Supplementation Recommendations of Various Organizations, Committees, and Societies.\textsuperscript{160,167-178}

<table>
<thead>
<tr>
<th>Group</th>
<th>Location</th>
<th>Target Group</th>
<th>Daily Vitamin D Recommendation</th>
<th>Date</th>
</tr>
</thead>
<tbody>
<tr>
<td>American Academy of Pediatrics</td>
<td>USA</td>
<td>Infants, children, and adolescents</td>
<td>400 IU intake</td>
<td>2008</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Exclusively and partially breastfed infants, and non-breastfed infants ingesting &lt;1,000 mL/day of vitamin D-fortified formula or milk</td>
<td>400 IU supplement</td>
<td></td>
</tr>
<tr>
<td>Canada’s Food Guide</td>
<td>Canada</td>
<td>Adults &gt;50 years of age</td>
<td>400 IU supplement</td>
<td>2007</td>
</tr>
<tr>
<td>Canadian Cancer Society</td>
<td>Canada</td>
<td>Adults (Fall through spring)</td>
<td>1,000 IU supplement</td>
<td>2007</td>
</tr>
<tr>
<td>Canadian Pediatric Society</td>
<td>Canada</td>
<td>Breastfed Infants</td>
<td>400 IU supplement</td>
<td>2007</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Breastfed Infants ≥ 55° N latitude</td>
<td>800 IU supplement</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pregnant and lactating women</td>
<td>2000 IU supplement</td>
<td></td>
</tr>
<tr>
<td>DirectMS</td>
<td>Canada</td>
<td>≤10 years of age</td>
<td>800 IU supplement</td>
<td>2003</td>
</tr>
<tr>
<td>DirectMS</td>
<td>Canada</td>
<td>&gt;10 years of age</td>
<td>2,000 IU supplement</td>
<td></td>
</tr>
<tr>
<td>Food and Nutrition Board, Institute of Medicine of The National Academies</td>
<td>USA / Canada</td>
<td>Infants under 12 mos</td>
<td>400 IU intake</td>
<td>2010</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Children ≥12 mos to adults ≤70 years of age</td>
<td>600 IU intake</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>≥71 years of age</td>
<td>800 IU intake</td>
<td></td>
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<tr>
<td>Food Standards Agency</td>
<td>United Kingdom</td>
<td>Pregnant and lactating women</td>
<td>400 IU supplement</td>
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<td>Adults of Asian origin</td>
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<td>Adults who are rarely outdoors</td>
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<td></td>
</tr>
<tr>
<td>Group</td>
<td>Location</td>
<td>Target Group</td>
<td>Daily Vitamin D Recommendation</td>
<td>Date</td>
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<td>400 IU supplement</td>
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<td>400 IU intake</td>
<td>2009</td>
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<tr>
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<td>Europe</td>
<td>Infants &lt; 12 mos</td>
<td>1,000 IU intake</td>
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<td></td>
<td>Adults</td>
<td>5,000 IU supplement</td>
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2.4 Assessment of Evidence for a Relationship Between Vitamin D Status and MS: The Bradford Hill Criteria

2.4.1 Overview of Criteria

In 1965, Sir Austin Bradford Hill proposed a set of viewpoints to aid in assessing the evidence for a causal relationship (Figure 2-3). Hill’s criteria are arguably most appropriate for assessing evidence of causality under simplistic models of cause and effect whereby a specific outcome is attributed to a single causal agent. The criteria do not sufficiently capture the complexity of the relationship between causal complexes comprised of environmental and genetic risk factors that may be variably necessary or sufficient to induce a heterogeneous disease such as MS. Nevertheless, the criteria do provide a generally well-rounded structure for a critical evaluation of evidence for causality.

Sir Austin Bradford Hill's Criteria for Assessing Evidence of Causality

1. Strength
2. Consistency
3. Specificity
4. Temporality
5. Biological Gradient
6. Plausibility
7. Coherence
8. Experiment
9. Analogy

Figure 2-3: Hill's Criteria\textsuperscript{1,180}
2.4.1 Strength

The strength of an association can be defined as the magnitude of difference in the risk, odds, or severity of a disease outcome based on variations in exposure to the factor of interest. A strong association supports a causal relationship between two entities. However, a weak association certainly does not negate the possibility of a causal relationship, particularly if the association occurs only in certain contexts. How strong are the links between MS and vitamin D status – as defined by circulating 25(OH)D – or determinants of vitamin D status such as dietary intake of vitamin D, or sun exposure? To explore this, one must consider vitamin D exposure across the lifespan, from in utero to adulthood.

2.4.1.1 Vitamin D status in utero:

Several studies have demonstrated a month of birth effect in MS risk. In Northern Sardinia – an island with very high MS incidence – a case-control study observed an excess of spring births in MS patients (29.4%) relative to both their unaffected siblings (22.1%, P = 0.008) or to the background population (24.6%, P = 0.036).\textsuperscript{181} Pooled month-of-birth data from a case-control study of MS patients in Canada, Denmark, Great Britain and Sweden (n = 42,045) demonstrated an excess of MS patients born in May (odds ratio (OR) 1.10, 95% confidence interval (CI) 1.07 to 1.13) and fewer than expected births in November (OR 0.91, 95% CI 0.87 to 0.95).\textsuperscript{182} Overall, the risk of MS in those born in May was 13% higher than for those born in November (95% CI 5 to 22%). Given the low ambient sunlight in winter months in the countries studied, these results could be interpreted to suggest that low serum 25(OH)D during pregnancy or low
vitamin D in the breast milk during first few months post-birth influence subsequent MS risk.\textsuperscript{129,183}

### 2.4.1.2 Childhood Sun Exposure and MS Risk:

Four case-control studies have demonstrated that increased sun exposure in childhood is related to lower odds of MS. In a study (n = 126 MS and 272 controls) from Tasmania, high sun exposure between the ages of 6-15 years was associated with a decreased risk of MS (OR 0.31, 95% CI 0.16 to 0.59) even after adjustment for skin pigmentation and smoking status prior to MS diagnosis.\textsuperscript{184} Furthermore, the study also found that moderate-to-high grade actinic damage to the skin of the hand, a marker for lifetime sun exposure, was independently associated with a decreased risk of multiple sclerosis (OR 0.32, 95% CI 0.11 to 0.88, adjusted for the same variables and sun exposure post-MS diagnosis). Similar findings were reported in Norway where higher outdoor activities in early life – particularly at 16-20 years of age – were associated with decreased odds of MS (OR 0.55, 95% CI 0.39 to 0.78).\textsuperscript{185}

A North American study of 79 pairs of identical twins discordant for MS found that the unaffected twin reported more sun exposure during childhood than did the twin with MS: A 5% rise in the sun exposure index score was associated with an MS OR of 0.75 (95% CI 0.62 to 0.90).\textsuperscript{186}

Finally, a case-control study consisting of participants from Cuba, Martinique and Sicily – regions of varying latitudes, ambient UVR, and MS prevalence – also observed a consistently reduced risk of MS related to measures of sun exposure before age 15, and increased risk of MS
related to sun protection practices before age 15 years of age. For instance, in multivariate analyses, weekday sun exposure of ≥1 hour per day was associated with decreased MS risk (OR 0.90, 95% CI 0.85 to 0.98) while wearing pants when exposed to sunlight was associated with increased risk (OR 1.90, 95% CI 1.10 to 3.20). These four case-control studies provide evidence supporting the hypothesis that sun exposure in childhood conveys protection against MS.

Further support for the importance of sun exposure in childhood in determining MS risk also comes from studies investigating place of childhood residence, migration patterns, and ethnicity of MS populations. Migration between areas of disparate MS prevalence before or during adolescence results in the individual adopting the risk of the new region. Migration in adulthood, however, does not influence MS risk. In a study of Toronto MS patients, patients with pediatric-onset MS were more likely than adult-onset patients to be first generation Canadians, and were also to have parents born in world regions of low MS prevalence.

### 2.4.1.3 Vitamin D status prior to MS diagnosis:

In a case-control study nested within a prospective cohort of over 7 million US military personnel, a decreased risk of MS (OR 0.38, 95% CI 0.19 to 0.75) was observed among white participants (148 MS patients, 296 controls) with serum 25(OH)D concentrations in the highest quintile (99.1-152.9 nmol/L) compared with the lowest quintile (<63.3 nmol/L). (This paper is discussed further in the section on dose-response)
2.4.1.4 Vitamin D status at the clinical onset of MS:

As noted earlier, an initial demyelinating attack can represent a monophasic illness or the clinical onset of MS. Determination of vitamin D status at the time of this first attack could provide insight into whether vitamin D status predicts individuals destined for relapse and confirmation of MS.

A recent Australian study evaluated serum 25(OH)D status of 216 patients within 6 months of a first demyelinating event in the CNS (ADS) and 395 healthy age-, sex- and region-matched adults. Higher serum 25(OH)D levels were inversely related to the odds of ADS. Cross-sectional comparison of vitamin D status in individuals experiencing first attacks with matched healthy participants reveals that low 25(OH)D is associated with a first attack of demyelination. However, because samples were collected up to 6 months post ADS – a window of time within which substantial changes can occur in 25(OH)D concentrations – these 25(OH)D levels are unlikely to consistently reflect pre-ADS vitamin D status. Furthermore, the participants in this study have not been monitored prospectively to determine whether the observed inverse relationship between vitamin D status and odds of ADS is specific to and driven by low levels among those who are subsequently identified as having MS.

While lower 25(OH)D concentrations in individuals with ADS provide support for vitamin D insufficiency in a cohort at exceptionally high risk of MS, it is also possible that low 25(OH)D concentrations occur as an epiphenomenon of acute illness. Prospective observation of these participants would be needed to determine whether the subgroup of individuals subsequently diagnosed with MS had particularly low 25(OH)D levels at ADS. Also, serial evaluation of
vitamin D status in individuals following a first attack are required to determine whether serum 25(OH)D concentrations remain low in individuals destined to experience further relapses.

2.4.1.5 Vitamin D status in individuals with established MS:

In adults, low serum 25(OH)D concentrations have been recorded at the time of clinical relapses in adults with established MS. Two Finnish studies and one Argentinian study all suggest that mean serum 25(OH)D concentrations were lower during relapses than remission. Similarly, researchers working in Tasmania reported an inverse relationship between relapses and both estimated serum 25(OH)D (r = –0.31, p = 0.057) and erythemal UV exposure (r = –0.32, p = 0.046). An inverse relationship was also observed between serum 25(OH)D levels in Tasmanian RRMS patients and risk of relapse, with each 10nmol/L increase in 25(OH)D resulting in a 12% decrease in relapse risk. Also, amongst patients in the USA with pediatric-onset MS or clinically isolated syndromes (CIS), vitamin D status predicted subsequent rate of relapse: each 25nmol/L increase in seasonally-adjusted 25(OH)D concentrations predicted a 34% decrease in subsequent relapse rate (incidence rate ratio 0.66, 95% CI 0.46 to 0.95).

Serum 25(OH)D concentrations also correlate with some types of MRI evidence of MS disease activity. In one study, low serum 25(OH)D levels predicted an increased likelihood of gadolinium (Gd)-enhancing lesions in MRI scans performed in the subsequent two month period. Although, as mentioned above, lower serum 25(OH)D was observed in relapses, serum 25(OH)D did not correlate with MRI burden of disease (defined as the summed cross-sectional area (in mm²) of lesions in T2-weighted scans) but, importantly, Gd-enhanced images were not included in this study. Taken together, these results provide support for
relationship between vitamin D status and active MS disease as measured by relapses and Gd-enhancing lesions on MRI.

It could be argued that the absolute 25(OH)D concentrations in serum – or more importantly adequate circulating 25(OH)D to produce 1,25(OH)2D at the cell level – are the key issue in considering vitamin D contributions to MS biology. However, it is also relevant to appreciate the behaviors that determine vitamin D status in an individual- both with respect to sun exposure and dietary ingestion of vitamin-D containing foods or supplements. In individuals with established MS, the confounding influence of disease-related limitations in physical and outdoor activity may result in decreased sun exposure and thus, limited skin synthesis of vitamin D. Furthermore, Uhthoff’s phenomenon, a transient heat-induced re-emergence of symptoms in previously demyelinated pathways, can also result in avoidance of sun or warm environments. It is also important to obtain a careful dietary history that includes information on the use of vitamin supplements. Intake of vitamin D as supplements may be increasing due to heightened awareness of the association of vitamin D insufficiency and MS – an awareness made increasingly possible through the internet and other social media. Neurologists may already recommend vitamin D to those with MS. Motivation to improve vitamin D status could be disproportionately higher in individuals with more active disease; therefore, a careful dietary and sun exposure history is required.

### 2.4.2 Consistency

The underlying principles of consistency are that the cause of the disease should be constant across variable settings, times, and populations, and that the relationship remains consistent even if other factors vary. While the relationship should remain constant, it is important to note
that the relative risk conveyed may vary due to interactions with other factors. For example, even if vitamin D insufficiency is consistently associated with MS risk across diverse world regions, the relative contribution may differ due to interaction with variants in vitamin-D responsive genes such as HLA-DRB1*15.\textsuperscript{74,205} Furthermore, consistency of association must be considered and evaluated to determine whether the association alone is sufficient for disease. In other words, vitamin D insufficiency is common in temperate climates, yet not all individuals with low serum 25(OH)D concentrations develop MS. The absence of MS in these individuals does not, however, negate the potential importance of vitamin D insufficiency as a risk factor for MS.

Discussed further in other sections, low sun or UVR exposure – a measure often predictive of lower circulating 25(OH)D – in varying world regions has been consistently associated with increased risk of MS,\textsuperscript{184-187} increased prevalence of MS,\textsuperscript{58,61,80,206} and increased risk of MS-related mortality.\textsuperscript{207}

Consistency of data relating to low 25(OH)D levels and MS is evidenced by studies of both adults and children with MS in Australia,\textsuperscript{208} the United States,\textsuperscript{193,201,209-211} and Europe.\textsuperscript{197,212-214} While low 25(OH)D concentrations in MS patients have been documented across multiple studies, there are some that fail to demonstrate an association.\textsuperscript{215-217} One study found low 25(OH)D in the male MS patients but not in females.\textsuperscript{218} Lacking to date are studies of vitamin D status in world regions where MS is rare, such as peri-equatorial countries, Africa, and certain regions of Asia. Evidence of vitamin D insufficiency at the time of first attack in the rare individuals diagnosed with MS in such regions would strongly support the notion of consistency of association between vitamin D and MS.
Torkildsen et al.\textsuperscript{219} reported a case series of three adult females with MS who were diagnosed with PDDR. The co-existence of this extremely rare genetic form of rickets and MS due to chance alone is highly improbable. All patients were diagnosed prior to the commercial availability of calcitriol and thus, received vitamin D\textsubscript{3} therapy following the diagnosis of PDDR even though they are unable to convert vitamin D to calcitriol. The patients were reported to have “normalized” serum 25(OH)D following treatment but serum concentrations of 25(OH)D were not actually reported. Although this patient series initially appears to suggest that increased risk of MS was conferred before PDDR diagnosis, the use of vitamin D as a therapeutic agent following diagnosis allows for the possibility that cellular availability of calcitriol remained compromised. Thus, risk may also have been conferred post-diagnostically.

### 2.4.3 Specificity

According to the Hill criteria, the likelihood of a causal relationship increases with the specificity of the relationship between a factor and an outcome. However, in describing the utility of this criterion, Hill himself noted that it was the least important of the criteria and did not always apply.\textsuperscript{1} Furthermore, it is important to define “specificity”. Specificity could be interpreted as a disease-specific association or more generally as specificity at the level of biological mechanisms. Given that calcitriol has the potential to modulate expression of hundreds if not thousands of genes involved in a variety of processes,\textsuperscript{114} the manifestations of suboptimal vitamin D status could be relevant to many diseases and could (i) operate either acutely or chronically, (ii) be dependent upon stage of life or status of other nutrients,\textsuperscript{220} and (iii) differ due to variants in genes encoding products involved in vitamin D metabolism\textsuperscript{99,205,217} or response.\textsuperscript{74} Vitamin D
insufficiency has been associated with systemic lupus erythematosus, \textsuperscript{221} inflammatory bowel disease, \textsuperscript{222} asthma and allergy, \textsuperscript{223} type 1 diabetes mellitus (T1D), \textsuperscript{224} rheumatoid arthritis, and other inflammatory disorders. \textsuperscript{225,226} Thus, if one considers specificity as more broadly referring to inflammation or misdirected immunological recognition of self tissue, then an argument for specificity between vitamin D status and MS (as a representative disease) can be made.

2.4.4 Temporality

An important determination of causality is evidence that the exposure precedes outcome. If impaired vitamin D status increases risk of MS, then it can reasonably be expected that vitamin D deficiency or suboptimal vitamin D status would precede MS onset.

Serum 25(OH)D levels are rarely evaluated in apparently healthy individuals prior to the onset of disease; however, one study did demonstrate that vitamin D status in early adulthood was inversely related to subsequent MS risk. \textsuperscript{193}

As discussed in other sections, other studies have used season, latitude, and questionnaire-based data regarding diet and sun exposure as proxies for vitamin D status prior to disease onset. Although these studies are limited in that they do not directly measure circulating 25(OH)D concentrations, they do lend support to the criterion of temporality in that they all implicate low vitamin D-related exposures with higher risk of MS.
2.4.5 Biological Gradient (Dose-Response)

Further evidence for vitamin D as an important determinant in MS can be considered in terms of (i) the degree of vitamin D insufficiency and relative risk of MS; and (ii) the extent of vitamin D supplementation and disease risk or clinical disease response.

Evidence to support a dose-response relationship between vitamin D insufficiency and MS risk comes from studies evaluating serum 25(OH)D concentrations prior to and at the time of clinical onset of MS. In one study, risk of MS in mid-adulthood in young white adults (mean age 23 years) decreased significantly with increasing serum 25(OH)D concentrations: the odds ratio of MS associated with a 50 nmol/L (sic) increase in 25(OH)D was 0.59 (95% CI 0.36 to 0.97).193

When evaluating dose-response aspects of causation, it is important to consider whether the doses being evaluated are in the range relevant to the disease. A threshold effect may well exist, in which biological impact is notable only once this threshold is exceeded. For instance, Munger et al.193 reported a significantly lower risk of MS in white patients with serum 25(OH)D over 99.1 nmol/L but did not find a significant association between vitamin D status and risk of MS in the black or Hispanic patients (n= 109 MS patients, 218 controls). More than 66% of the black and Hispanic participants had serum 25(OH)D concentrations below 50 nmol/L and the highest serum 25(OH)D concentration was only 97.9 nmol/L and a protective effect of vitamin D was not observed. However, if circulating 25(OH)D concentrations needed to exceed 99 nmol/l to confer benefit, then a benefit of vitamin D would not be expected in these groups since the maximum 25(OH)D concentration was below 99 nmol/L. The ability to detect a dose-response requires study of populations that have serum 25(OH)D concentrations spanning the biologically relevant threshold of effect (Figure 2-4).
Cross-sectional study of participants with ranges of serum 25(OH)D concentrations at either the low 25(OH)D or the very high levels of 25(OH)D is unlikely to yield significant dose–response related data because both groups are on plateaus of the Biological Response Curve. Likewise, if a vitamin D intervention does not succeed in elevating participants' serum 25(OH)D concentrations beyond the lower biological response plateau, it is unlikely to elicit a significant response. A significant biological response is most likely to be observed when participants begin with insufficient vitamin D status and increase into the sufficient range. The circulating 25(OH)D concentrations defining sufficient vitamin D status remain unclear but expert consensus indicates that the minimum concentration is likely between 75 and 100 nmol/L.164
Dose-response or a biological gradient can also be considered in terms of the observed latitude gradient and varying amounts of UVR. The rate of first demyelinating events in Australia increased by 9.6% (95% CI 7.4 to 11.8) per higher degree of latitude, and in both North America and France, studies demonstrated that risk of MS increases with decreasing regional UVR. A recent study compiled global MS prevalence data from 54 studies and calculated the degree of risk contributed by numerous factors. The authors report a highly statistically significant inverse correlation between regional annual available UVR and MS prevalence; the relationship between UV and MS prevalence was so strong that it surpassed the effects of all of the other risk factors by at least 20-fold.

In a pooled analysis of data from Canada, Denmark, Great Britain and Sweden, the OR for increased risk of MS outcome in May births compared to November births was calculated. When the countries were examined individually, the season-dependent risk of MS outcome was proportional to MS prevalence in each country and, with the exception of Sweden, increased with the average latitude (hence, decreasing available UVB) of residence for the countries’ populations – with risk being highest in Scotland (OR 1.89, 95% CI 1.09 to 3.28), intermediate in Denmark (OR 1.22, 95% CI 1.08 to 1.38) and lowest in Canada (OR 1.13, 95% CI 1.05 to 1.22).

2.4.6 Plausibility

Clearly an important aspect of the Hill criteria is biological plausibility. What do we know about mechanisms that could be responsible for the relationship between vitamin D status and MS?
2.4.6.1 Animal studies:

Biological plausibility is often easier to study in-depth in animal models of disease than in humans, and an inducible model of CNS inflammation, termed experimental autoimmune encephalomyelitis (EAE), in mice or rats provides such an opportunity for exploring the effect of vitamin D and calcitriol on EAE induction, severity and amelioration.

Administration of calcitriol prior to EAE induction prevented signs of the disease from developing.\textsuperscript{228-232} Interestingly, an analog of calcitriol also demonstrated synergistic benefit when administered with interferon beta (IFN-\(\beta\))\textsuperscript{233} and additive effects with cyclosporine in the prevention of EAE.\textsuperscript{234} Calcitriol \textit{per se} has attenuated symptoms when administered after induction of EAE\textsuperscript{235} and has also alleviated established EAE.\textsuperscript{236} A variety of mechanisms underlying these effects have been proposed. Some of the calcitriol-related observations in EAE have been mediated via a reduction in monocyte activation,\textsuperscript{237} reduced macrophage accumulation within the CNS, reduced proliferation of self-reactive T lymphocytes in the CNS\textsuperscript{236} and increased apoptosis of pro-inflammatory cells.\textsuperscript{238} Also, one study of EAE demonstrated that IL-10 signaling was essential for the calcitriol-mediated inhibition (reduced incidence of EAE and lower peak clinical score) of EAE.\textsuperscript{231}

A recent set of experiments sought to evaluate the effect of relatively acute pre-induction UVR and post-induction UVR exposure on EAE.\textsuperscript{239} The authors concluded that UVR suppressed EAE independent of vitamin D\(_3\) production; however, they did not investigate whether the circulating 25(OH)D levels on the date of EAE disease induction influenced EAE disease severity. In the first experiment performed, 25(OH)D concentrations did not differ significantly across groups at the time of EAE induction despite differing pre-induction UVR protocols; all groups
experienced a similar EAE outcomes. In contrast, on the day of disease induction in the second experiment, the groups pre-treated with UVR did have higher 25(OH)D than the controls. Correspondingly, EAE was least severe in the UVR-treated group that had significantly higher 25(OH)D concentrations at the time of EAE induction. The observed difference in subsequent EAE severity persisted even though the 25(OH)D concentrations of the UVR-treated groups did not remain significantly higher than the control group post-induction. Taken together, these UVR exposure studies suggest that vitamin D status at the time of disease induction may be of more import than post-induction vitamin D status in terms of affecting subsequent EAE severity.

Furthermore, some EAE studies have demonstrated that the effects of supplementation with vitamin D per se differ based on the sex of the animal. Vitamin D3 supplementation prior to induction of EAE reduced signs of myelin basic protein (MBP)-induced EAE in female mice but not in males or ovariectomized females. In a follow-up study, administration of physiologically equivalent doses of 17β-estradiol (E2) restored the vitamin D3-mediated inhibition of myelin peptide (MBP- and MOG35–55) induced EAE in ovariectomized mice but did not reduced signs of EAE in the MOG35–55-induced males. The authors reported synergistic interactions of vitamin D3 and E2 as the potential mechanism underlying the findings: Circulating E2 was significantly elevated in the vitamin D3 supplemented intact female mice, E2 enhanced VDR expression within the central nervous system, and E2 decreased expression of the vitamin D degradation enzyme, CYP24A1. In light of reported differences in cytokine profiles of MS between male and female patients, significant sex-based differences in the relationship between latitude and incidence of first demyelinating events observed in Australia, and the well-recognized – and increasing – female preponderance in MS, these sex-specific aspects of vitamin D in EAE are intriguing. They also support the need for future studies to evaluate whether vitamin D insufficiency is of particular concern in female MS
patients, or whether vitamin D supplementation may be of greater benefit in females for both the prevention and treatment MS.\textsuperscript{244}

### 2.4.6.2 Biological Plausibility based on Vitamin D-Genetic Interactions in Humans:

As mentioned earlier in the section pertaining to genetics and MS risk, one of the strongest mechanistic links between vitamin D and MS comes from a recent study demonstrating that calcitriol modulates the expression of the particular HLA-DRB1 allele most consistently associated with increased risk of MS, HLA-DRB1*1501.\textsuperscript{74} Investigation of the major candidate genes, HLA-DRB1, HLA-DQA1 and HLA-DQB1 led to discovery of a conserved, functional vitamin D response element (VDRE) in the promoter region of the HLA-DRB1*1501 allele. Given that HLA-DRB1*15 was the only variant identified as having a functional VDRE in the promoter, expression of the other DRB1 variants would not be expected to be sensitive to vitamin D status. Among those carrying the vitamin D-responsive DRB1*15 allele, vitamin D deficiency or impaired vitamin D metabolism may lead to lower expression of the MHC Class II molecule.\textsuperscript{244} Reduced expression of MHC Class II molecules could impair presentation of self-antigens during negative selection, resulting in a lack of tolerance being established against those self-antigens. If the immune system fails to establish and maintain immune tolerance to molecules derived from the blood brain barrier (BBB) or CNS myelin, this could result in the type of demyelinating immune attacks observed in MS. Alternatively, it could be that the high levels of MHC present in the context of vitamin D sufficiency may contribute to activation-induced cell death of overly activated CNS-reactive cells; a decrease in MHC due to vitamin D
deficiency may weaken the strength of signal, and permit survival of cells that should be removed. On the other hand, this finding could even suggest a deleterious relationship whereby elevated vitamin D status increases expression of this risk gene, thus increasing antigen presentation and immune stimulation. However, this is not supported by the circumstantial evidence.\textsuperscript{244-248} While the functional consequence of this finding is yet to be determined, it does form a conceptual basis for a nutrient-gene interaction; thus connecting the genetic and environmental evidence implicating sunlight and vitamin D in the determination of MS risk.

### 2.4.6.3 Biological Plausibility based on Vitamin D Interactions with Human Cell Cultures:

Calcitriol down-regulates pro-inflammatory dendritic cell (DC) and T-helper lymphocyte 1 (Th1) activation and response, promotes an anti-inflammatory Th2 lymphocyte profile, suppresses the antigen-presenting capacity of macrophages and DCs, and decreases proliferation of pro-inflammatory T lymphocytes.\textsuperscript{198,244,247,249-257} In terms of cytokine profiles, calcitriol decreases production of pro-inflammatory cytokines such as IFN-\(\gamma\),\textsuperscript{257,258} IL-2,\textsuperscript{259-261} and TNF-\(\alpha\)\textsuperscript{254,257,262} while enhancing the secretion of the anti-inflammatory cytokine, IL-10.\textsuperscript{198,249}

Various \textit{in vitro} models have demonstrated that calcitriol also suppresses expression or reduces mRNA stability of matrix metalloproteinase 9 (MMP-9)\textsuperscript{263-269} which increases the permeability of the blood brain barrier to auto-reactive immune cells. MMP-9 is elevated in patients with MS, particularly RRMS and secondary progressive MS (SPMS)\textsuperscript{270-272} and is also elevated during MS relapses.\textsuperscript{273} This suggests that in addition to beneficial immune modulating effects, vitamin D could alter movement of immune cells from the circulation into the CNS.
Taken together, the biological plausibility of a role of vitamin D in the pathobiology of MS – in particular via immune-related mechanisms – is supported by evidence from research in animals, humans, and human cell lines.

2.4.1 Coherence

Any causal relationship should be relatively compatible with observations of the natural history and biology of the disease. Common mechanisms may even be identified that explain similar effects of different risk factors on MS. Regarding common mechanisms of risk factors in MS, Figure 2-5 illustrates plausible interactions between putative factors involved in the pathobiology of MS outlined in this section.

It is important to consider the vitamin D-related evidence in the context of other identified risk factors for MS, including the higher – and increasing – female to male ratio observed in adults and adolescents with MS, infection with EBV and exposure to tobacco smoke.
Figure 2-5: Determinants of low or impaired vitamin D status and hypothesized intermediary mechanisms underlying increased risk and severity of MS.\textsuperscript{180}

As discussed, female sex is clearly over-represented in adolescent and adult-onset MS and is apparently increasing.\textsuperscript{12,38,274} Although there are mixed findings regarding the effects of estrogen on the expression of VDR, VDBP and concentrations of circulating calcitriol\textsuperscript{275}, the effects of vitamin D supplementation on EAE susceptibility were found to be estrogen dependent: When exposed to vitamin D, both ovariectomized females given physiologic levels of estrogen and intact females were protected while males and estrogen-deficient ovariectomized females were not.\textsuperscript{240,241} Gender differences in cytokine profiles and vitamin D status in adults with MS (reviewed in \textsuperscript{276}) also highlight the possibility of sex-based differences in the relationship between vitamin D status and MS disease activity.

Immune reactivity to viral infection is a critical aspect of survival; however, dysregulation of immune response also contribute to stimulation of aberrant, autoimmunity. EBV is a type of
herpes virus that is responsible for infectious mononucleosis (IM); however, EBV infection is often clinically silent and thus, does not result in IM in everyone who has been infected. EBV infection as a potential risk factor for MS has received extensive interest and continues to be an active area of research. In adults, increased MS risk is strongly and consistently associated with EBV infection – as defined either by a history of IM or by EBV antigen seropositivity. Similarly, EBV seropositivity is also associated with increased risk of MS in children. An interaction between vitamin D status and infection with EBV in MS has been proposed based on the interaction of both EBV infection and vitamin D on the production of the anti-inflammatory cytokine, IL-10. Production of viral IL-10 by EBV may down-regulate human IL-10 production which could be further suppressed in the presence of vitamin D insufficiency. Overall, exposure or infection with EBV concurrent with vitamin D insufficiency could lead to an enhanced pro-inflammatory state. One study documenting an association between a history of infectious mononucleosis (IM)- an illness that represents a markedly exaggerated host response to EBV infection- and subsequent risk of MS found that the association was stronger if IM occurred prior to age 18y; immune responses to EBV during a time of impaired vitamin D status may be especially deleterious in terms of MS risk if occurring during the putative childhood window of MS susceptibility.

Exposure to tobacco smoke – whether passive or actively exposed – has been linked to increased MS risk in both adults and children, and with worse outcomes in adults with established MS. Smoking induces a pro-inflammatory milieu that may be exacerbated by concurrent vitamin D insufficiency. A combustion product from cigarette smoke, benzo[a]pyrene (B[a]P), enhanced in vitro breakdown of vitamin D in human macrophages, suggesting that smoking may exacerbate vitamin D insufficiency in immune cells (Figure 2-
5). That B[a]P is only produced when tobacco is smoked, may be one explanation for why tobacco smoking – not Swedish snuff use – was associated with increased risk of MS.²⁹²

Beyond environmental determinants, serum 25(OH)D concentrations are also under some genetic control.⁹⁹,²¹⁷,²⁹⁷⁻²⁹⁹ Studies of genes involved in vitamin D metabolism have revealed mixed findings regarding the relationship between certain variants and MS risk,²⁰⁵,³⁰⁰⁻³⁰⁷ although none have been poised to evaluate nutrigenomic interactions across the lifespan; that is, that vitamin D status at certain times of life may modify the relationship between the genetic variant and risk of MS.³⁰⁰ Further investigation of such genes in highly informative individuals – either those with markedly impaired vitamin D status or individuals diagnosed with MS despite residence in world regions with high ambient UVR – might provide novel information that may link specific aspects of vitamin D metabolism to MS.

2.4.2 Experiment

A causal association is considered to be one in which a change in the exposure results in a corresponding change in the outcome of interest. While double-blind, placebo-controlled experimental or intervention studies have the potential to produce the strongest evidence for a role of vitamin D in MS, they are limited in that it is obviously unethical to withhold an essential nutrient from patients in the placebo arm to determine whether low vitamin D increases MS risk or disease activity. Thus, in humans, experimental evidence for a causal role of vitamin D in reducing MS disease severity comes from vitamin D or calcitriol supplementation studies.

To date, primary prevention trials have not yet been attempted in humans to determine whether optimizing vitamin D status will reduce risk of MS. There are, however, a limited number of small studies that have explored vitamin D – and even calcitriol – supplementation in adults
with established MS; such studies primarily demonstrate the safety profile of vitamin D supplementation, and provide a preliminary view into efficacy.

In a double-blind, placebo-controlled trial, 17 adults with MS received 800 mg calcium plus 1,000 IU/d vitamin D over 6 months while 20 adults received calcium alone; only biochemical outcomes were reported. Vitamin D supplementation increased serum 25(OH)D levels from a mean of 42.5 nmol/L to 72 nmol/L while serum 25(OH)D did not change in the placebo group. Vitamin D supplementation increased TGF-β1 but did not change concentrations of the pro-inflammatory cytokines, TNF-α or IFN-γ, or the anti-inflammatory IL-13. The mean resultant serum 25(OH)D concentration in the vitamin D group did not reach the estimated minimum concentration for sufficiency (75nmol/L), which may have limited the ability to detect a significant effect (Figure 2-4). On the other end of the vitamin D status spectrum, a phase I (safety or dose-finding) study conducted by our colleagues administered 1200 mg elemental calcium plus doses of vitamin D₃ that increased from 4,000 IU/d to 40,000 IU/d to 12 patients with active MS over 28 weeks. Mean serum 25(OH)D concentrations at baseline were already within the estimated range of sufficiency at 78 nmol/L and they increased significantly to 386 nmol/L with no adverse events, changes in liver enzymes, electrolytes, or serum calcium, creatinine, or protein observed. The number of Gd-enhancing lesions decreased from a mean 1.75 to 0.83 per patient ($P = 0.03$) while relapse rate, EDSS scores and ambulation indices remained stable. A follow-up study - a 52-week open label, phase I/II study of 49 adults with relapsing-remitting MS was undertaken by our colleagues, Burton et al.: The patients in the treatment arm (n = 25) received 1000 mg calcium plus vitamin D₃ in doses escalating from 4,000 IU/d to 40,000 IU/d over 28 weeks, maintained at 10,000 IU for 12 weeks with a 12 week taper to the end of study. In the vitamin D treatment arm, mean serum 25(OH)D increased from 78 nmol/L to 413 nmol/L without adverse clinical or biochemical outcomes. Within the vitamin
D treatment group, neuronal antigen-induced T-cell proliferation was statistically significantly lower (i) after one year compared to baseline values and (ii) relative to age-, sex- and treatment-matched controls at one year. The vitamin D₃ intervention also resulted in a statistically significant decrease in annualized relapse rates (ARR) compared with the previous year; however, because of the open-label design of the study – hence, participants were aware of treatment status – and the somewhat subjective nature of ascertaining relapses, the reduction in ARR is regarded as a novel but preliminary finding. Goldberg et al.³⁰⁹ supplemented ten adult MS patients with a lower dose of vitamin D₃ (5,000 IU/d in cod liver oil) and body-weight defined doses of calcium, magnesium and demonstrated a statistically significant reduction in relapses by 12 to 24 months. Unfortunately, serum 25(OH)D concentrations were not reported at baseline or end of study.

Importantly, none of the aforementioned vitamin D intervention studies, particularly neither the Kimball et al.²¹⁶ nor the Burton et al.³⁰⁸ studies that administered doses up to 40,000 IU/d, reported adverse outcomes or biochemical indication of vitamin D toxicity – hypercalcemia or hypercalciuria – even though they provided calcium in addition to vitamin D at doses above the current North American Dietary Reference Intake’s (DRI’s) adult RDA of 600 IU/d and in excess of the current 4,000 IU/d “Tolerable Upper Intake Level” (UL).¹⁶⁰ Even so, these studies were of relatively brief duration and it is unclear whether the observed benefits could be replicated by providing vitamin D alone or whether vitamin D must be combined with a calcium supplement. Of note is that a recent Cochrane report on the subject of vitamin D interventions in MS determined that the only clinical trial that met the stringent Cochrane criteria for inclusion in meta-analysis was the Burton et al.³¹⁰
In a single trial that administered calcitriol, rather than vitamin D₃, a reduction in relapse rate of 27% was noted. However, in contrast to the vitamin D supplementation trials, this 48-week trial of calcitriol therapy led to mild hypercalcemia, even among patients compliant with the calcium-restricted diet protocol, highlighting the challenge and potential for toxicity in administering the non-nutrient, hormonal form of vitamin D.

2.4.3 Analogy

According to this criterion, a potential risk factor may be more readily accepted as a cause of a disease if a similar factor has already been shown to cause the same or related disease. As mentioned above under the criterion of specificity, vitamin D insufficiency is presently a candidate risk factor for other diseases, such as T1D, that bear the similarity of being immune-mediated inflammatory disorders. A recent vitamin D intervention study in MS patients revealed that selective T-Cell proliferative responses to both disease-associated, MS-relevant neuronal antigens and to T1D-relevant pancreatic antigens were suppressed in the vitamin D treatment group; this finding provides a common mechanism by which vitamin D insufficiency may contribute to immune dysregulation and autoimmunity. Thus, this co-existing interest in vitamin D as a common putative risk factor in numerous immune-mediated inflammatory diseases provides preliminary analogous evidence for a role of vitamin D in MS.
2.5 Summary of Evidence

The current evidence for a protective role of vitamin D in MS reasonably fulfills many of Hill’s criteria but additional evidence is required. In particular, sound experimental evidence of MS prevention or reduced risk of MS following intervention with vitamin D has not yet been reported, and the Temporality criterion would be greatly strengthened by additional evidence for low 25(OH)D concentrations preceding MS.

At present, the evidence for a protective role of vitamin D in MS has been deemed strong enough by some to warrant recommending vitamin D supplementation to individuals or populations at high risk of both vitamin D insufficiency and MS. Other investigators instead are advocating population-based primary prevention studies. Such studies have the potential to produce the strongest evidence for a protective role of vitamin D in MS. Some of the key considerations of such a primary prevention study will be discussed in the context of future directions in the concluding section of this thesis.

Until interventional studies are undertaken, evidence for a protective role of vitamin D in MS will continue to be derived largely from observational research. For instance, the observational evidence supporting a temporal relationship between circulating 25(OH)D concentrations and subsequent MS outcome presently only comes from a single study. In this nested case-control study, high circulating concentrations of 25(OH)D in young white American adults were associated with lower likelihood of subsequent MS diagnosis. While other studies do lend support for temporality, all have relied on proxies of vitamin D status such as supplemental and dietary intake, season of birth, latitude, available UVB, or relative sun exposure behaviours. These studies are limited in that they have not actually evaluated circulating 25(OH)D
concentrations prior to MS. Thus, further evidence for an inverse, dose-dependent or threshold-dependent relationship between circulating 25(OH)D levels and subsequent MS risk would add uniquely and importantly to the current evidence. In particular, it would be of interest to determine whether vitamin D insufficiency is an equivalent risk factor in spite of differing genetic or environmental exposures. Study of individuals differing in their region of residence and ancestry may enable further exploration of the contribution of vitamin D to MS biology in the context of variable risk factors. Whether a temporal relationship will be observed in pediatric-onset MS is also of interest because the drivers of expression of early-onset MS (i.e. MS in children and adolescents) remain elusive. Children with MS do not appear to have a higher burden of genes associated with increased MS risk, suggesting that it may be environmental risk factors mediating timing of disease expression. For instance, children with MS may experience more profound pre-diagnostic vitamin D deficiency (i.e. even lower 25(OH)D concentrations) than adults, or it may be that their early disease expression is driven by vitamin D insufficiency during the aforementioned childhood window of susceptibility. Thus, studies of vitamin D status in children at high risk of MS can uniquely inform our understanding of the contribution of vitamin D status to MS biology.
3 Thesis Rationale & Objectives

3.1 Rationale

Given the level of evidence indicating a protective role of vitamin D status in reducing MS risk and potentially modulating disease activity, the present thesis focused on defining whether serum 25(OH)D concentrations in children with ADS influenced MS outcome. Prior to commencement of the core study, methodological analyses were required to evaluate vitamin D laboratory metrics. Due to both the observed inter-methodological differences in 25(OH)D assays and the large number of samples needing to be analyzed for our primary study, we first sought to validate an automated method for assessing circulating 25(OH)D concentrations. Following the creation of rigorous standardized operating procedures for measuring of vitamin D status in our work, we proceeded with the core study objectives discussed below. We recognized that evaluation of vitamin D status as a predictor of MS outcome required appreciation of key genetic features (HLA-DRB1), vitamin D supplement use, and that the analyses would be predicated on careful clinical and MRI evaluation to define MS diagnosis in the pediatric cohort under study. In addition to the core pediatric population analyzed for this thesis, a unique opportunity arose to evaluate three female MS patients who also suffered from a rare genetic form of rickets – permitting evaluation of the relationship between a vitamin-D deficiency disease, HLA genetics and MS. Finally, we aimed to validate a simple sun exposure questionnaire for use in evaluating sun-related contributions to vitamin D status. The questionnaire was utilized in a collaborative study of Italian adults, and modifications for future suitability for collaborative international pediatric studies are underway.
3.2 Objectives

1) Evaluate the performance of automated chemiluminescent technologies for the quantification of circulating 25-hydroxyvitamin D concentrations.

2) Determine whether MS outcome is influenced by vitamin D status at the time of acute demyelination or by vitamin D-influenced genetic MS risk alleles by:
   a) Determining the contribution of 25-hydroxyvitamin D concentrations at ADS and subsequent diagnosis of MS.
   b) Evaluating whether HLA-DRB1*15 status modifies the relationship between 25-hydroxyvitamin D concentrations at ADS and MS outcome in children.
   c) Determine whether individuals with pseudo-vitamin D deficiency rickets (PDDR) – who were later diagnosed with MS – carry the vitamin D-responsive MS risk allelotype, \textit{HLA-DRB1*1501}.

3) Evaluate the performance of a simple recall-based sun exposure questionnaire as a predictor of serum 25-hydroxyvitamin D.

3.3 Hypotheses

1. An automated assay that detects both circulating 25-hydroxyvitamin D$_2$ and 25-hydroxyvitamin D$_3$, has comparable or superior precision \textit{vs.} the reference method (DiaSorin radioimmunoassay, RIA), and displays high agreement with the RIA will be suitable for use in studies with large populations and repeated measures.
2. Impaired vitamin D status or metabolism in early life is associated with higher risk of MS.

a) Serum 25-hydroxyvitamin D levels at the time of an initial demyelinating attack in childhood or adolescence will be inversely correlated to risk of MS.

b) The interaction of suboptimal vitamin D status during immune development may be particularly deleterious in terms of MS risk in those carrying HLA-DRB1*1501 allelotype, because its expression activated vitamin D is needed for its expression.

3. A brief focused questionnaire can be created that provides sufficient information on recent sun exposure to yield meaningful correlations with vitamin D status as defined by serum 25-hydroxyvitamin D concentrations.
4 Comparison of methods for analyzing circulating 25-hydroxyvitamin D concentrations

Adapted from:

4.1 Abstract

**Background:** Several methods exist for determination of the circulating biomarker of vitamin D status, 25-hydroxyvitamin D [25(OH)D]. Variability between methods and increased demand for analysis of circulating 25(OH)D concentrations drives demand for valid, high-throughput assays.

**Objectives:** To compare two new automated assays, DiaSorin “LIAISON 25 OH Vitamin D TOTAL”, and Roche Modular “Vitamin D3 (25-OH)”, with the well-established reference method, DiaSorin radioimmunoassay (RIA), for quantitation of serum total 25(OH)D concentrations.

**Methods:** Serum 25(OH)D concentrations were determined in samples from healthy human adults (n = 158) using DiaSorin RIA and the two automated platforms. Methods were compared by correlation, linear regression and Bland-Altman analyses.

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

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

The most reliable indicator of vitamin D status is measurement of 25-hydroxyvitamin D concentrations [25(OH)D] in serum or plasma. The liver produces 25(OH)D, the major circulating metabolite of vitamin D, by a hydroxylation of vitamin D at carbon 25. Two distinct forms of 25(OH)D exist: 25(OH)D₃, formed from vitamin D₃ (cholecalciferol), and 25(OH)D₂, produced from vitamin D₂ (ergocalciferol). Vitamin D₃ is synthesized naturally in skin exposed to UV radiation and also found in fatty fish. Vitamin D₂ is generated by UV irradiation of the plant sterol, ergosterol, and is generally accepted to be less potent than vitamin D₃. The Institutes of Medicine recently set the threshold of vitamin D sufficiency at 50 nmol/L for bone-related health related outcomes. However, beyond bone health, low circulating 25(OH)D concentrations have been associated with increased risk and progression of several diseases, including cancers, multiple sclerosis, and cardiovascular disease. Such research into the role of vitamin D beyond calcium homeostasis has substantially increased clinical interest in vitamin D.

The measurement of 25(OH)D is challenging because circulating 25(OH)D is highly lipophilic, bound strongly to the vitamin D binding protein, present in low (nanomolar) concentrations, and exists in two structurally similar forms, 25(OH)D₃ and 25(OH)D₂.

Measuring 25-hydroxyvitamin D in a clinical environment: challenges and needs.

Several published methods exist for determining 25(OH)D concentrations, including
competitive protein-binding assays, radioimmunoassay (RIA), High Performance Liquid Chromatography (HPLC), liquid chromatography-mass spectrometry (LC-MS), and the more recent automated immunoassays. In 1989, the International for Vitamin D External Quality Assessment Scheme metabolites (DEQAS, Northwest Thames, United Kingdom) was established to monitor the analytical reliability of 25(OH)D assays. However, several reports have demonstrated large inconsistency and variability in 25(OH)D measurements between methods and laboratories, emphasizing a need for appropriate reference materials and standardization of 25(OH)D assays.

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

Biological Samples

Human serum samples (n = 400) were obtained from a clinical trial in Toronto, Canada (latitude 43°N) in which healthy adults received either 28,000 IU vitamin D₃/week or a placebo for 8 weeks.³²⁷ Serum aliquots were stored at -80°C until analysis. Under these storage conditions, 25(OH)D is very stable in serum or plasma over a prolonged time and repeated freeze-thaw cycles.³²⁸,³²⁹ Quantitative determination of serum 25 (OH) D concentrations was performed in singleton by: DiaSorin “25-hydroxyvitamin D ¹²⁵I RIA” in April 2007 (n = 390), DiaSorin “LIAISON 25 OH Vitamin D TOTAL” CLIA in September 2007 (n = 390), and Roche Modular “Vitamin D3 (25-OH)” ECLIA in October 2007 (n = 158). The DiaSorin 25(OH)D RIA served as the reference method. Out of the 400 serum samples acquired, 390 samples were analyzed by both DiaSorin RIA and DiaSorin LIAISON TOTAL (10 samples had insufficient volume); only 158 samples were analyzed using the Roche Modular assay due to insufficient volumes and kits. Therefore, direct method comparisons are limited to the samples measured by all three assays (n = 158). The 158 samples were obtained from healthy adults at the beginning and end an 8-week trial of 20 subjects who received placebo and 59 subjects who received 4000 IU/d vitamin D₃.³²⁷ Therefore, 59 of the 158 samples were from subjects who had consumed the equivalent of 4000 IU/d for the previous 8 weeks.
Circulating 25(OH)D Assays

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

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

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

The LIAISON 25 OH Vitamin D TOTAL Assay is a direct competitive chemiluminescent immunoassay for human serum or plasma intended for use on the DiaSorin LIAISON automated analyzer. The assay uses magnetic particles (solid phase) coated with antibody against 25(OH)D and 25(OH)D conjugated to an isoluminol derivative (tracer). During the first incubation phase (10 minutes), 25(OH)D is dissociated from binding protein by buffer containing 10% ethanol and then binds to the anti-25(OH)D antibody on the solid phase. After another 10-minute incubation with the tracer, the unbound material is washed off and “starter” reagents are added to generate a flash chemiluminescent signal – inversely related to 25 (OH) D concentrations – which is measured by a photomultiplier.
This assay differs from its older version, “LIAISON 25 OH Vitamin D”, due to alterations in the on-board extraction procedure, the addition of a second incubation step, and the use of human serum-based calibrators instead of horse serum.

Roche Modular 25(OH)D ECLIA

The Roche Vitamin D3 (25-OH) assay is a direct competitive electrochemiluminescence immunoassay for human serum or plasma intended for use on Roche automated immunoassay analyzers. In this study, the Modular Analytics analyzer was used. The assay employs microparticles coated with streptavidin and a polyclonal sheep antibody against 25(OH)D, which is labeled with ruthenium. In the first incubation, 25(OH)D$_3$ in the sample competes with biotin labeled 25(OH)D for binding with the anti-25(OH)D antibody. In the second incubation, the biotin-25(OH)D/anti-25(OH)D antibody immunocomplex becomes bound to the microparticles via interaction of biotin and streptavidin. The microparticles are then magnetically captured onto the surface of an electrode. A voltage is applied to the electrode to produce a chemiluminescent emission, which is measured by a photomultiplier and is inversely proportional to 25 (OH) D concentrations.

Specifications for the three assays, as stated by the manufacturer, are listed in Table 4-1. According to the product inserts, hemolysis or lipemia at levels of that would be typically encountered in conventionally collected and prepared samples do not affect any of the analytical methods.

Quality assessment

All assays were performed in accordance with the manufacturers’ instructions. DiaSorin RIA
and LIAISON 25(OH)D results from our laboratory consistently fall within one standard
deviation (SD) of the group mean in the international DEQAS proficiency surveys. In the
January 2009 DEQAS results, the all methods mean ± SD (CV%) for a test sample was 47.2 ±
6.1 nmol/L (12.9%), compared to 46.7 ± 7.7 nmol (16.5%) for DiaSorin RIA, 46.6 ± 6.0 nmol/L
(12.9%) for DiaSorin LIAISON TOTAL, and 52.1 ± 5.9 nmol/L (11.3%) for the Roche assay.

Statistical Analyses

Serum 25(OH)D concentration data derived from each assay were summarized with descriptive
statistics and are given in nmol/L units. The three methods were compared by both linear
regression and Deming regression – which accounts for errors in both variables. The agreement
between each pair of assays was analyzed by the mean, difference method of Bland and
Altman. All data were analyzed with SPSS software (version 13.0) and Analyse-it for
Microsoft Excel. The criterion for significance was set at P < 0.05.

4.4 Results

Samples

Overall, 158 samples were evaluated by all three assays. None of the samples tested showed
visible signs of hemolysis or lipemia. Descriptive statistics of the 25(OH)D concentrations
measured by the DiaSorin RIA, DiaSorin LIAISON, and Roche Modular assays, respectively,
were: mean ± SD (76.4 ± 39.5, 75.5 ± 39.3, and 73.8 ± 31.2 nmol/L; P > 0.05), median (66.0,
67.0, and 68.4 nmol/L), 95% CI (70.2 to 82.6, 69.3 to 81.7, and 68.9 to 78.7 nmol/L), and range (16.0 – 183.0, 17.1 – 176.0, and 16.7 – 189.6 nmol/L).

**Precision**

The precision of the RIA, LIAISON, and Roche Modular assays was determined by using five human serum-based quality controls (kit, in-house pooled serum, and patient samples), spanning a 25(OH)D concentration range of 35–180 nmol/L. Each control sample was assayed in 2–6 replicates per run for 3–5 runs. Precision values are shown in Table 4-2. Precision ranges (CV%) for the RIA, LIAISON, and Roche Modular assays, respectively, were: within run (6.8–12.9%, 2.8–8.1%, and 1.9–5.5%), and total precision (7.4–14.5%, 7.3–17.5%, and 7.6–14.5%). These precision values fall within the CV ranges typically encountered with 25(OH)D methods in DEQAS (10–20%).

**Method correlations**

Regression parameters are shown in Table 4-3 and Deming regression plots of the 25(OH)D concentrations reported for each pair of assays are presented in Figure 4-1. Based on the regression analysis, the DiaSorin LIAISON platform correlated best with DiaSorin RIA (r = 0.918, n = 158). Furthermore, this correlation, based on samples measured by all 3 assays (n = 158), was essentially equivalent to the correlation between DiaSorin LIAISON and RIA 25(OH)D assays in the larger trial cohort (r = 0.917, n = 390). The Roche Modular method correlated reasonably well with DiaSorin RIA (r = 0.871, n = 158) and LIAISON (r = 0.862, n =
Method agreement

Bland-Altman analyses of the 25(OH)D methods are presented in Figure 4-2. LIAISON showed little bias when compared to DiaSorin RIA [bias +/- SD (95% CI) = -0.88 +/- 15.95 (-3.38 to 1.63) nmol/L]. Roche Modular demonstrated higher bias compared to DiaSorin RIA [-2.55 +/- 19.67 (-5.64 to 0.54) nmol/L] than to LIAISON [-1.67 +/- 20.14 (-4.83 to 1.50) nmol/L].
Table 4-1: Assay specifications, as stated in the manufacturers’ product inserts.

<table>
<thead>
<tr>
<th></th>
<th>DiaSorin 25-Hydroxyvitamin D 125I RIA</th>
<th>DiaSorin LIAISON 25 OH Vitamin D TOTAL</th>
<th>Roche Vitamin D3 (25-OH)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Assay format</strong></td>
<td>Extraction, equilibrium RIA</td>
<td>Direct, competitive, CLIA</td>
<td>Direct, competitive, ECLIA</td>
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<tr>
<td><strong>Platform</strong></td>
<td>Manual</td>
<td>Automated</td>
<td>Automated</td>
</tr>
<tr>
<td><strong>Analyzer(s)</strong></td>
<td>N/A</td>
<td>LIAISON</td>
<td>Elecsys, Modular Analytics, or Cobas</td>
</tr>
<tr>
<td><strong>Sample volume</strong></td>
<td>50 μL</td>
<td>25 μL</td>
<td>35 μL</td>
</tr>
<tr>
<td><strong>Sample type</strong></td>
<td>Serum or plasma (EDTA, Hep)</td>
<td>Serum or plasma (EDTA, Hep)</td>
<td>Serum or plasma (EDTA, Hep)</td>
</tr>
<tr>
<td><strong>Assay time</strong></td>
<td>110 min</td>
<td>20 min</td>
<td>18 min</td>
</tr>
<tr>
<td><strong>Analytical sensitivity</strong></td>
<td>3.75-NR</td>
<td>10–375 nmol/L</td>
<td>10–250 nmol/L</td>
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<table>
<thead>
<tr>
<th><strong>Analytical specificity</strong></th>
<th>% Cross-reactivity</th>
<th>% Cross-reactivity</th>
<th>% Cross-reactivity</th>
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<tr>
<td>Vitamin D3</td>
<td>0.8</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Vitamin D2</td>
<td>0.8</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>25(OH)D3</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>25(OH)D2</td>
<td>100</td>
<td>104</td>
<td>&lt;10</td>
</tr>
<tr>
<td>1,25(OH)2D3</td>
<td>11</td>
<td>17</td>
<td>Up to 100</td>
</tr>
<tr>
<td>1,25(OH)2D2</td>
<td>11</td>
<td>40</td>
<td>NR</td>
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<table>
<thead>
<tr>
<th><strong>Precision</strong></th>
<th>% CV</th>
<th>% CV</th>
<th>% CV</th>
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<tbody>
<tr>
<td>Within-run</td>
<td>8.6–12.5</td>
<td>2.9–5.5</td>
<td>3.5–4.9</td>
</tr>
<tr>
<td>Total</td>
<td>8.2–11.0</td>
<td>6.3–12.9</td>
<td>4.2–7.8</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th><strong>Method comparison</strong></th>
<th>NR</th>
<th>n=155, against RIA</th>
<th>n=291, against automated assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>(DiaSorin RIA is usually the reference method)</td>
<td>LIAISON=0.99 (RIA)+2.4</td>
<td>Roche=1.272 (other)−0.045</td>
<td></td>
</tr>
<tr>
<td>r=0.97</td>
<td>r=0.912</td>
<td></td>
<td>n=771 against LC-MS/MS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Roche=1.008 (LC-MS/MS)+0.045</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: CLIA, chemiluminescent immunoassay; CV, coefficient of variation; EDTA, ethylenediaminetetraacetic acid; Hep, heparin; LC-MS/MS, liquid chromatography tandem mass spectrometry; min, minutes; N/A, not applicable; NR, not reported; RIA, radioimmunoassay.
### Table 4-2: Within-run and total precision of the 25(OH)D assays

<table>
<thead>
<tr>
<th>Assay/Sample</th>
<th>n</th>
<th>Mean (nmol/L)</th>
<th>Within-run precision (%CV)</th>
<th>Total precision (%CV)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>DiaSorin RIA</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kit control 1</td>
<td>6</td>
<td>36.8</td>
<td>10.6</td>
<td>12.2</td>
</tr>
<tr>
<td>Kit control 2</td>
<td>5</td>
<td>164.6</td>
<td>6.8</td>
<td>7.4</td>
</tr>
<tr>
<td>In-house Level 1</td>
<td>15</td>
<td>41.2</td>
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**Table 4-3:** Methods comparison - regression (linear and Deming) and correlation parameters (Pearson and Spearman)

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<th>Assays</th>
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<th>Linear regression</th>
<th>Deming regression</th>
<th>Correlation</th>
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<td>158</td>
<td>LIAISON = 0.91 (RIA) + 5.75</td>
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<td>r = 0.92</td>
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<td></td>
<td></td>
<td></td>
<td>rho = 0.94</td>
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<tr>
<td></td>
<td>390</td>
<td>LIAISON = 0.91 (RIA) + 5.80</td>
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<td>r = 0.917</td>
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<td>rho = 0.92</td>
</tr>
<tr>
<td>Roche vs. RIA</td>
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<td>Roche = 0.76 (RIA) + 15.57</td>
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<td></td>
<td>rho = 0.90</td>
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<tr>
<td>Roche vs. LIAISON</td>
<td>158</td>
<td>Roche = 0.68 (LIAISON) + 22.20</td>
<td>Roche = 0.77 (LIAISON) + 16.04</td>
<td>r = 0.86</td>
</tr>
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<td></td>
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<td>rho = 0.90</td>
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Figure 4-1: Deming Regression of 25(OH)D quantitation comparison between DiaSorin RIA (reference method), DiaSorin LIAISON Total, and Roche Modular platforms. (A) RIA vs. LIAISON (left: n=158; right: n=390, full cohort). (B) RIA vs. Modular (n=158). (C) LIAISON vs. Modular (n=158). Pearson and Spearman correlation coefficients are indicated. Dotted lines indicate 95% confidence intervals.
Figure 4-2: Bland-Altman bias plots show differences in paired samples at their mean 25(OH)D concentrations for n = 158 paired samples.

(A) RIA vs. LIAISON, (B) RIA vs. Roche, and (C) LIAISON vs. Roche. Thick solid lines indicate bias (means of paired differences) and dashed lines show 95% limits of agreement.
4.5 Discussion

Given that the latest consensus document indicated that a serum 25(OH)D concentration of 75 nmol/L or greater is likely to be sufficient for optimal for health, a substantial proportion of the North American population would be classified as having some level of insufficiency or deficiency. Accumulating evidence of widespread vitamin D deficiency in apparently healthy pediatric and adult populations in Canada and beyond has, in part, driven both clinical and research interest in assessing circulating 25(OH)D concentrations. Therefore, the rapid, precise, and accurate measurement of circulating 25(OH)D concentrations is important.

The DiaSorin \(^{125}\)I-based RIA has been the method of choice for measuring 25(OH)D concentrations and was the primary method used in the studies upon which the estimates of optimal vitamin D status were based. However, the RIA is time-consuming, labour intensive, and employs radioactive compounds which pose a health hazard, are expensive to dispose of, and limit automation. The increasing demand for non-radioactive, high-throughput circulating 25 (OH) D measurements has led to development of several automated platforms. DiaSorin and Roche Diagnostics have introduced fully automated immunoassay systems employing chemiluminescent technology for 25 (OH) D concentration determinations in serum or plasma.

Here, we have compared these automated methods with the reference method, DiaSorin RIA. To our knowledge, the comparison of DiaSorin LIAISON Total, Roche Modular, and DiaSorin RIA methods for 25(OH)D has not been reported in the literature. We found that DiaSorin LIAISON demonstrated a stronger correlation (r = 0.918) and better agreement (bias = -0.88 nmol/L) with the DiaSorin RIA reference method than the Roche Modular assay (r = 0.871, bias = -2.55 nmol/L).
This study employed the second-generation and most recent version of the LIAISON assay, “LIAISON 25 OH Vitamin D TOTAL”. Our results indicate that this assay showed a higher correlation with DiaSorin RIA than reported previously with the older version of the assay, “LIAISON 25 OH Vitamin D”. Recently, Roth et al. evaluated the accuracy of several 25(OH)D methods, including the LIAISON and Roche assays presented here. However, they chose an LC-MS method as their reference method and made no direct comparisons between the automated methods and RIA; thus, it is difficult to compare the present study with that of Roth et al. We felt that the DiaSorin RIA was the more appropriate reference method due to its length of time in use, its performance in DEQAS, and its use in the majority of studies linking circulating 25(OH)D concentrations to health and disease outcomes and the establishment of reference values. In contrast, at the time of this methods evaluation, we were not aware of reference values for circulating 25(OH)D based upon LC-MS methods. Further complicating the use of LC-MS as a reference technology is the variety of LC-MS methods used to analyze vitamin D metabolites and – at the time of methods evaluation – lack of widely available standards. Nonetheless, in the Roth et al., study, both the LIAISON and Roche assays demonstrated good correlation and agreement with LC-MS. In the time since performing this methods evaluation, there have been many improvements made with respect to LC-MS methods of analysis of vitamin D metabolites and the analytical sensitivity of LC-MS has even enabled the quantitation of 25(OH)D in dried blood spots. However, the accuracy and agreement of the bloodspot methods have yet to undergo systematic external validation.

Leino et al. reported a similar correlation between the Roche 25(OH)D assay and DiaSorin RIA (r = 0.836, n = 163) to what we report here. However, our investigation using the baseline and final serum samples from a placebo-controlled, 4000 IU vitamin D per day dosing clinical trial, we were able to analyze a larger number of samples with a wider range of 25(OH)D
concentrations than Leino et al. A rigorous evaluation and comparison of methods should test a broad range of analyte concentrations; if the range of concentrations tested in a methods analysis is relatively small, one may only be able to speculate about the performance at either the high or low ends of the range of interest. This is particularly relevant for vitamin D because there is a wide distribution of 25(OH)D in the general population and it is important to accurately and precisely identify those with serum 25(OH)D levels suggesting either vitamin D deficiency or potential vitamin D intoxication.

Clinical diagnosis and treatment decisions related to vitamin D are based on assessment of total 25(OH)D concentrations. Therefore, given that both 25(OH)D$_2$ and 25(OH)D$_3$ contribute to total 25(OH)D levels, the analytical method of choice should detect both equally in order to report an accurate total 25(OH)D value. The major limitation of the Roche assay is its inability to detect 25(OH)D$_2$. As stated in the product insert (Table 4-1), the Roche assay has <10% cross-reactivity with 25(OH)D$_2$. In contrast, the DiaSorin RIA and LIAISON assays claim 100% cross-reactivity with 25(OH)D$_2$ and 25(OH)D$_3$ on an equimolar basis. Consumption of supplements or foods containing vitamin D$_2$ (e.g. mushrooms) will contribute to total 25(OH)D concentrations; however, this contribution would be underestimated by the Roche assay. It is unlikely that vitamin D$_2$ significantly affected the total 25(OH)D concentrations measured in our Canadian subjects because vitamin D$_3$ is more commonly used in supplements and fortified foods in Canada. However, this problem would be more pronounced in the US, where vitamin D$_2$ is commonly used. Furthermore, the lack of sensitivity to 25(OH)D$_2$ would also pose an issue for patients receiving pharmaceutical preparations of high-dose vitamin D, which presently only exist as vitamin D$_2$ (e.g. Calciferol or Drisdol). However, the lower correlation and agreement of the Roche assay with the reference method is more likely related to the assay itself than to its insensitivity to 25(OH)D$_2$. As shown in Figure 4-2B, the Roche Modular assay tended
to overestimate 25(OH)D at low concentrations (< 50 nmol/L) and underestimate 25(OH)D at high concentrations (> 100 nmol/L). For example, when DiaSorin RIA reference values of 25 nmol/L and 150 nmol/L are applied to the Deming regression equations, the corresponding DiaSorin LIAISON concentrations are 24.3 nmol/L and 148.1 nmol/L while the Roche Modular values are 34.6 nmol/L and 129.6 nmol/L, respectively. Furthermore, following our analysis, we noticed the same discrepancy in the January 2009 DEQAS results. For example, the DiaSorin RIA mean ± SD for a “low” DEQAS test sample was 26.5 ± 4.2 nmol, compared to 22.1 ± 3.5 nmol/L for DiaSorin LIAISON TOTAL, and 44.8 ± 9.9 nmol/L for the Roche assay. If the Roche method reports higher levels at the lower 25(OH)D concentrations, this would be clinically important in that vitamin D deficiency would be not be detected in some participants, and thus, would remain untreated. In contrast, the DiaSorin RIA mean ± SD for a “high” DEQAS test sample was 79.3 ± 13.8 nmol, compared to 73.3 ± 8.8 nmol/L for DiaSorin LIAISON TOTAL, and 53.0 ± 6.1 nmol/L for the Roche assay. The discordant accuracy at the lower and upper end of the measuring range may be related to the extraction procedure, the antibody used, or matrix effects. Given the relatively accurate performance of the other automated CLIA, the LIAISON, it is likely the accuracy of the Roche assay could likely be improved by modifying the method to correct the potential problems outlined.

Within-run precision was generally higher in the automated LIAISON and Roche Modular assays compared to the manual RIA. The Roche method displayed the best within-run precision, however, it is notable that the range of its 25(OH)D quality controls (~60 – 180 nmol/L) begins at a relatively high concentration compared to the ranges encompassed by the DiaSorin RIA and LIAISON quality controls (~40 – 180 nmol/L) which are more representative of concentrations observed in the general population. Total precision did not substantially different among the three assays. Of note, our precision values were slightly lower than those reported in the product
inserts (Table 4-1) but are still not ideal. The large inter-assay differences observed in the 25(OH)D data for many of the paired samples (Figure 4-2 A, B, and C) are disconcerting in that clinical decisions may differ substantially depending on which assay was used. Thus, further work is needed to establish more precise assay methods. A more comprehensive evaluation of precision performance would have used a greater number of replicates and runs than those used in the present study; however, we ran our sample in singleton on each assay because of limited sample volumes and limited kits.

Overall, the variation among 25(OH)D methods observed in the present study was smaller than previously reported but our results illustrate the need for standardization of 25(OH)D assays. We conclude that, relative to the DiaSorin RIA, the DiaSorin LIAISON 25 OH Vitamin D TOTAL assay is a reasonably accurate and relatively precise tool for the determination of 25(OH)D. The LIAISON assay exhibited better correlation and agreement with the reference RIA method than the recently introduced Roche assay which is further limited by its inability to detect 25(OH)D₂. Automated, accurate 25(OH)D methods provide greater speed and convenience, and improve work flow and efficiency in the high-throughput clinical laboratory as it continues to meet increasing demand for 25(OH)D testing. Bearing all of these considerations in mind, and the limitations of the methods at hand, we have elected to utilize the LIAISON 25 OH Vitamin D TOTAL assay for assessing vitamin D among the children enrolled in the Canadian Pediatric Demyelinating Diseases Program (Chapter 6).

4.6 Acknowledgements

We thank DiaSorin for donating the LIAISON 25 OH Vitamin D TOTAL integrals and Roche Diagnostics for donating the Vitamin D3 (25-OH) kits.
5 Vitamin D and multiple sclerosis outcome in children with acquired demyelinating syndromes

5.1 Abstract

**Context:** Acute demyelinating syndromes (ADS) may represent monophasic disease or the sentinel attack of multiple sclerosis (MS). Epidemiological data implicate (i) childhood as the period of MS risk determination, (ii) environmental and genetic factors in disease etiology; and (iii) vitamin D as protective.

**Objective:** To evaluate vitamin D status at the earliest possible clinically identifiable point in the MS disease process (first attack, ADS) in children.

**Design, Setting and Participants:** Consecutive participants (<16 y) were enrolled at presentation with ADS and prospectively evaluated at 23 Canadian centres. MS was diagnosed according to established criteria. Vitamin D status was defined by serum 25-hydroxyvitamin D (25(OH)D) concentrations.

**Main Outcome Measure:** Cox proportional hazards modeled risk of MS outcome as a function of serum 25(OH)D tertiles at ADS, accounting for age, sex, season, and human leukocyte antigen (HLA)-DRB1*15 status.

**Results:** Serum 25(OH)D concentrations were measured in serum samples obtained a median of 9 days from ADS clinical onset (interquartile range, 5-17; maximum 36) in 211 children subsequently observed for a median time of 3.3 years (IQR 1.9-4.8). MS was diagnosed in 41 (20%) after a median time of 3.7 mos. (3.1-7.3). Risk of MS was substantially lower for children in the highest 25(OH)D tertile at ADS (≥74 nmol/L) compared to those in the lowest tertile (<50 nmol/L) (adjusted hazard ratio 0.41; 95% confidence interval 0.18 - 0.97).
**Conclusions:** Children with higher serum 25(OH)D levels at an initial demyelinating episode are less likely to be diagnosed with MS even after accounting for other features associated with MS risk (female sex, HLA-DRB1*15 status, and older age at onset). Given that only one of 29 children receiving vitamin D supplements prior to acute demyelination was diagnosed with MS, strategies to increase compliance with recommended vitamin D intakes in children may have implications for MS risk.

### 5.2 Introduction

Prevalence of MS increases with distance from the equator, implicating latitudinal influences on UVB exposure and subsequent cutaneous synthesis of vitamin D as etiologic contributors to MS.

MS risk in migrant populations is influenced by region of birth and residence during childhood, implicating higher vitamin D status during childhood with lower MS risk. Retrospective case-control studies indicate that greater sun exposure during childhood is associated with a reduced risk of adult-onset MS.

In a national prospective population-based pediatric cohort, we evaluated whether vitamin D status, defined by serum 25-hydroxyvitamin D (25(OH)D) concentration, at the time of initial acute demyelination predicts MS outcome in a population experiencing demyelination in childhood – within the putative window of MS risk acquisition.
5.3 Materials and Methods

**Participants:** Consecutive pediatric participants under 16 years with first presentation of ADS (features described in Table 5-1) at one of 23 participating sites were enrolled between September 2004 and June 2010 (data closure date). Research Ethics Boards at all sites approved this study. Written informed assent and consent was obtained from all participants and guardians.

The half-life of serum 25(OH)D concentration is estimated to be approximately two months in humans. To evaluate 25(OH)D concentrations reflecting vitamin D status at ADS onset, only children for whom serum was obtained within 40 days of ADS onset were included in the present analyses.

All participants underwent standardized assessment at enrolment, at 3, 6, and 12 months from ADS onset, annually thereafter, and at second demyelinating attack, if it occurred. ADS presentations were classified according to established criteria. Children were excluded if they had culture-proven bacterial meningitis, viral encephalitis, demyelination of the peripheral nervous system (i.e., Guillain-Barré syndrome, chronic inflammatory demyelinating polyneuropathy), biochemical or radiologic suspicion of inherited or genetically defined leukodystrophy, metabolic, or mitochondrial disease, systemic and laboratory features suggestive of systemic lupus erythematosus or connective tissue disease, or radiation- or chemotherapy-associated white matter damage. Exclusion was determined by the reporting physician. Race and ancestry were determined by parental interview using standard methods and classifications. Age- and sex-appropriate body mass index (BMI) percentiles were calculated from height and weight measures using the Centers for Disease Control growth charts. Serial research MRI scans were obtained according to a standardized protocol.
diagnosis was conferred using consensus criteria.\textsuperscript{25,345} Date of MS diagnosis was the date of confirmed second attack or MRI evidence of new lesions meeting criteria for dissemination in time,\textsuperscript{25} whichever came first. Children were excluded if they met criteria for relapsing or multiphasic acute disseminated encephalomyelitis (ADEM),\textsuperscript{345} neuromyelitis optica (NMO)\textsuperscript{349} or if they experienced isolated relapses of optic neuritis or transverse myelitis in the absence of clinical or MRI evidence of involvement of other CNS areas. All others were considered monophasic ADS as of the date of data closure.

Participants reporting intake of any vitamin D-containing supplements (e.g. vitamin D supplements, multivitamins, and cod liver oil) for more than 14 days within 3 months prior to ADS onset were classified as “exposed” while all other participants were classified as “unexposed”.

**Biological Measures:** Blood samples were obtained at ADS presentation, shipped on the same day as procurement, and processed using standardized protocols at the study biorepository in the Experimental Therapeutics Program at the Montreal Neurological Institute (Montreal, Canada). Serum sample aliquots were stored at -80°C until 25(OH)D concentrations were analyzed (Mount Sinai Hospital, Toronto, Canada). Samples were analyzed blinded to clinical data. Serum 25(OH)D concentrations were determined using the automated chemiluminescent Liaison “25-OH Vitamin D TOTAL” assay (DiaSorin, Stillwater, MN) previously validated in-house.\textsuperscript{350} Control samples (low and high 25 (OH) D concentrations) were analyzed in duplicate or triplicate in each run to determine assay reliability. Intra-assay and inter-assay coefficients of variation (CV) were 8.0% and 9.2% at 37 nmol/L, and 7.4% and 9.4% at 120 nmol/L.
Vitamin D sufficiency (≥75 nmol/L) and insufficiency (<75 nmol/L) were defined *a priori* based on published consensus definitions. Recently proposed definitions for vitamin D sufficiency (≥50 nmol/L) and insufficiency (<50 nmol/L) were also evaluated.

Total genomic DNA, extracted from whole blood, was used to type HLA-DRB1 alleles by an allele-specific PCR amplification method as described in our prior publication from the present cohort. The presence of at least one DRB1*15 allele was classified as “DRB1*15 positive”.

**Statistical Analysis:** Demographic and biochemical data were summarized with descriptive statistics. All continuous variables were assessed for normality by the Shapiro-Wilk method. Continuous variables are described as medians with interquartile range (IQR) and were evaluated using Spearman’s correlation, Mann-Whitney U and Kruskal-Wallis tests as appropriate. Unrelated categorical variables were evaluated using Pearson’s Chi square test. Vitamin D status is reported as unadjusted serum 25(OH)D concentrations in nmol/L. The BMI percentile of those diagnosed with MS was compared to that of the monophasic group using ANCOVA, with age at onset as a covariate. Season of ADS sample collection was categorized as: Winter (Jan–Mar), Spring (Apr–Jun), Summer (Jul–Sep), and Autumn (Oct–Dec). Ordinal trend analyses of the association between serum 25(OH)D tertiles at ADS and categorical variables, such as MS outcome, were conducted using the Somers’ *d* measure. Cox proportional hazards regression models assessed whether serum 25(OH)D at ADS presentation was associated with subsequent MS diagnosis, with zero time being the date of initial symptom onset (ADS) and MS outcome being the earliest time point at which MS diagnosis was confirmed. Monophasic participants were censored at date of database lock (n=210) or last study visit prior to withdrawal (n = 1). Model assumptions – namely, proportionality of the data – were tested using graphical methods (comparison of Kaplan-Meier plots) and time-dependent covariates.
Modeled continuously, serum 25(OH)D concentrations did not meet proportional hazards (PH) assumption but tertiles of serum 25(OH)D did and were thus used in the Cox PH models.

Covariates included sex, age at ADS presentation, season of ADS presentation, and HLA-DRB1*15 status.

To evaluate vitamin D supplement intake as a predictor of MS risk, vitamin D supplement use prior to ADS onset was substituted into the model in lieu of 25(OH)D tertiles.

We tested for additive or multiplicative interactions between vitamin D insufficiency or sufficiency (using our *a priori* cut point of 75 nmol/L) and DRB1*15 status by including indicator variables in the model which represented the four potential states: vitamin D sufficient and DRB1*15 negative; vitamin D sufficient and DRB1*15 positive; vitamin D insufficient and DRB1*15 negative; and vitamin D insufficient and DRB1*15 positive.

To address the possibility of race- or ancestry-related effects on serum 25(OH)D concentrations or MS outcome, multivariable analyses were also restricted to children of European ancestry, 100% of whom also identified their race as Caucasian. Ancestry and race were determined by parental interview.

As the likelihood of MS outcome may differ by initial clinical presentation, we conducted multivariable analyses in children considered at higher risk (children with brain lesions at onset) excluding children considered to have a lower risk for MS (ADEM or optic neuritis or transverse myelitis with normal brain imaging).
Two-sided $P$ values less than 0.05 were considered statistically significant. Statistical analyses were carried out using SPSS 16.0 (SPSS Inc, Chicago, IL) and SAS V9.2 (SAS Institute Inc., Cary, NC).

### 5.4 Results

Of 332 participants enrolled, 211 had serum samples obtained within 40 days of onset (median of 9 days from ADS clinical onset (IQR 5-17; maximum 36); Figure 5-1). These 211 participants are described in Table 5-2. Participants were subsequently observed for a median interval of 3.3 years (IQR 1.9-4.8), with 193 (93%) observed for at least 1 year from ADS onset, 157 (75%) for at least 2 years, 123 (60%) for at least 3 years and 77 (37%) for at least 4 years.

Forty-one children (19%) were diagnosed with MS; this diagnosis was made after a median of 3.7 mos. (IQR 3.1–7.3) from ADS onset. Length of observation did not differ between children with monophasic ADS and those subsequently diagnosed with MS ($P = 0.082$). No child was exposed to MS-specific immunomodulatory therapies prior to confirmation of MS diagnosis.

Consistent with published literature, children subsequently diagnosed with MS were older at ADS (median ages 13.7 years (IQR 10.5–14.6)) compared to those remaining monophasic (9.7 years (IQR 5.7–13.1), $P<0.0003$). Of the 129 children considered at lower risk of MS outcome (ADEM or normal brain MRI at ADS) only four (3.1%) were diagnosed with MS.

Among the 138 (n = 26 MS) participants with BMI percentile data at ADS, 26% (n = 36) met criteria for being overweight or at risk of overweight (defined as BMI $\geq 85^{th}$ percentile for age and sex). These children were older ($P = 0.0002$), and age and BMI percentile positively correlated ($\rho = 0.27, P = 0.001$). Although 46% (n = 12) of the 26 children with MS had BMI $\geq 85^{th}$ percentile, compared to only 21% of the monophasic ADS group ($P = 0.010$), after adjusting for age, BMI was not associated with MS outcome ($P = 0.15$).
**Vitamin D Related Analyses:** Serum 25(OH)D concentrations correlated negatively with age (rho = -0.25, \( P = 0.0002 \)) and BMI percentile (rho = -0.22, \( P = 0.009 \)) but did not differ by sex (\( P = 0.16 \)). Serum 25(OH)D concentrations were higher amongst Caucasians of European ancestry (n = 136; 63.5 (49.8–82.1) nmol/L) relative to the other participants (n = 75; 50.3 (30.9–81.1) nmol/L; \( P = 0.008 \)).

Median (IQR) serum 25(OH)D concentrations at time of ADS were lower in the 41 children subsequently diagnosed with MS (49.2 (34.2–68.5) nmol/L), compared to the 170 children with monophasic ADS (64.0 (44.7–84.2) nmol/L; \( P = 0.003 \)). The proportion of participants diagnosed with MS decreased with increasing serum 25(OH)D tertile (Table 5-2 and Figure 5-2; \( P \) trend = 0.05). Cox proportional hazards analyses revealed a lower risk of MS among children who had 25(OH)D concentrations in the highest tertile (≥74 nmol/L) vs. the lowest 25(OH)D tertile (<50 nmol/L) (Table 5-3). Only 20% (n = 8) of those diagnosed with MS had sufficient serum 25(OH)D concentrations (≥75 nmol/L) at ADS compared to 35% (n = 59) of those with monophasic ADS (\( P = 0.061 \)). Based upon the more conservative definition of vitamin D sufficiency (≥ 50 nmol/L), the proportion of vitamin D sufficient children was lower among those diagnosed with MS (20 of 41, 49%) compared to those with monophasic ADS (120/170, 71%, \( p = 0.008 \)).

Among the 29 (14%) children who regularly consumed vitamin D-containing supplements during the 3 months prior to ADS onset, the median (IQR) serum 25(OH)D concentration was higher than that of non-vitamin D-supplement users (72.8 (58.4–97.7) nmol/L vs. 59.8 (38.9–78.4) nmol/L; \( P = 0.008 \)). Only 1 child out of 29 (3%) was diagnosed with MS; the lower risk of MS diagnosis among vitamin D supplement users approached significance (unadjusted HR 0.14, 95% CI 0.02 to 1.04; \( P = 0.055 \)).
Median serum 25(OH)D concentrations did not differ by season of ADS presentation (Figure 5-3). The proportion of patients subsequently diagnosed with MS also did not differ by season of ADS presentation (Table 5-2).

As expected, the proportion of participants diagnosed with MS was higher among the 73 children who were HLA-DRB1*15 positive as compared to the 135 children who were HLA-DRB1*15 negative (30% vs. 14%; \( P = 0.005 \)) (Figure 5-2). Amongst the DRB1*15 positive participants, the proportion of individuals diagnosed with MS decreased with increasing 25(OH)D tertile (Figure 5-2; \( P \) trend = 0.024). For the subgroup of DRB1*15 negative participants, the relationship between 25(OH)D tertile and MS was not statistically significant (\( P \) trend = 0.086), but the power of this subgroup analysis is low.

**Multivariable Analyses:** The relationship between 25(OH)D tertiles and MS risk remained significant after adjustment for sex, age, and season (Table 5-3). When DRB1*15 status was added to the multivariable model (\( n = 208 \)), the lower risk of MS amongst those in the highest 25(OH)D tertile vs. the lowest tertile persisted (Figure 5-4 and Table 5-3). There was no evidence of additive or multiplicative interaction between DRB1*15 status and vitamin D status on risk of MS (data not shown).

Analysis restricted to Caucasians of European ancestry (\( n = 136 \)) revealed an even more robust relationship between 25(OH)D tertiles and risk of MS (highest vs. lowest tertile; HR 0.12, 95% CI 0.31 to 0.48).

When the children considered to be at lower MS risk were excluded, the relationship between 25(OH)D tertiles and risk of MS (highest vs. lowest tertile; HR 0.34, 95% CI 0.13 to 0.89) was
similar to that found for the entire cohort. In addition, those in middle tertile also had a reduced risk of MS (HR vs. lowest tertile 0.37, 95% CI 0.17 to 0.82).

**Figure 5-1:** Enrollment of Children Presenting with Acquired Demyelinating Syndromes
The proportion of MS patients decreased with increasing 25(OH)D tertile in both the overall sample \( (P_{\text{trend}} = 0.005) \) and amongst the subgroup of patients carrying one or more DRB1*15 allele \( (P_{\text{trend}} = 0.024) \). The proportion of children with MS was higher in the overall DRB1*15 positive group compared to the DRB1*15 negative group \( (P = 0.005) \) and, when analyses were stratified by 25(OH)D tertile, also in the lowest tertile of 25(OH)D \( (<50\text{nmol/L}; P = 0.043) \). * \( p<0.05 \), ** \( p<0.01 \).

**Figure 5-2:** Multiple sclerosis diagnosis stratified by DRB1*15 status and 25(OH)D tertiles at ADS amongst 73 DRB1*15 positive patients and 135 the DRB1*15 negative patients.
Concentrations of 25(OH)D did not differ between any seasons in the overall sample (P = 0.06) nor did they differ between seasons in either subgroup of children (MS, P = 0.23; monophasic ADS, P = 0.12). Overall median serum 25(OH)D concentrations were significantly higher among those with monophasic ADS vs. those with MS (P = 0.003) and, when analyzed within each season separately, also during the spring (*, P = 0.014).

**Figure 5-3:** Serum 25(OH)D concentrations of monophasic ADS and MS patients in each season of ADS presentation.
The Hazard Ratios with 95% Confidence Intervals are adjusted for age of onset, sex, season, and DRB1*15 status.

**Figure 5-4:** Time to MS diagnosis stratified by serum 25(OH)D tertiles at ADS (n = 208; 41 diagnosed with MS, 167 with monophasic ADS).

The Hazard Ratios with 95% Confidence Intervals are adjusted for age of onset, sex, season, and DRB1*15 status.
### Table 5-1: Clinical Features of Acquired Demyelinating Syndromes (ADS)

<table>
<thead>
<tr>
<th>Optic Neuritis (ON)(^{353})</th>
<th>One or more of:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>• acute and painful loss of vision</td>
</tr>
<tr>
<td></td>
<td>• decreased visual acuity</td>
</tr>
<tr>
<td></td>
<td>• reduced perception of colour vision</td>
</tr>
<tr>
<td></td>
<td>• abnormal visual evoked potential findings</td>
</tr>
<tr>
<td>PLUS:</td>
<td>• Absence of CT or MRI evidence of optic nerve compression, infiltration or trauma.</td>
</tr>
</tbody>
</table>

| Transverse Myelitis (TM)\(^{354}\) | • Acute motor/sensory spinal deficits |
|                                   | • Bilateral symmetric or asymmetric |
|                                   | • Defined spinal sensory level |
|                                   | • Progression to maximal deficit 4 hours and 21 days |
| PLUS one of:                     | • CSF pleocytosis |
|                                   | • increased IgG index |
|                                   | • Gadolinium-enhancement of cord |
| AND                              | • neuroimaging exclusion of spinal compression |

<p>| Acute Disseminated Encephalitis (ADEM)(^{345}) | A first clinical attack with a presumed inflammatory or demyelinating cause, with acute or subacute onset, which affects multifocal areas of the CNS. The clinical presentation is polysymptomatic and includes encephalopathy that may consist of one or more of the following: |
|                                                | • Behavioral change, e.g. irritability, lethargy |
|                                                | • Alteration in consciousness, e.g. somnolence, coma |
|                                                | • Seizures |
| Neuroimaging should show focal or multifocal lesion(s), predominantly involving white matter, without radiological evidence of previous destructive white matter changes | |
| • Usually shows multifocal, hyperintense, at least one or more large (≥ 1-2 cm) bilateral, usually asymmetric lesions in the supratentorial or infratentorial regions. | |
| • Subcortical white matter, as well as gray matter (e.g. the basal ganglia and thalamus), is also frequently involved | |
| • Spinal cord MRI may show a large confluent | |</p>
<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Criteria</th>
</tr>
</thead>
</table>
| intramedullary lesion(s) with variable enhancement | Attack should be followed by improvement, either clinically or on MRI or both, but there may be residual deficits  
- No history of a clinical episode with features of a prior demyelinating event  
- No other etiologies (e.g. known infections, TIA or migraine with aura) can explain the event |
| Monofocal Demyelination      |  
- Acute onset of neurological deficits  
- Deficits localizable to a single CNS site  
- Extrinsic to the optic nerve or spinal cord |
| Polyfocal Demyelination       |  
- Acute onset of polyfocal neurological deficits  
- Absence of encephalopathy |
| Multiple Sclerosis (MS)^25,345 | Less than 18 years of age at time of first attack  
- 2 clinical attacks disseminated in time and space  
- 1 attack plus McDonald MRI criteria for space and time  
OR  
- Positive for “dissemination in space” if 2 lesions plus positive CSF  
NOTE  
- If first attack meets ADEM criteria- cannot be considered as contributing to space or time: requires 2 further non-ADEM episodes for MS diagnosis |
| Neuromyelitis Optica (NMO)^249 |  
- Optic Neuritis  
- Transverse Myelitis  
PLUS 2 of:  
- MRI lesion spanning > 3 spinal segments  
- Onset brain MRI not meeting criteria for MS  
- Serum antibodies against aquaporin 4 |
| Recurrent ADEM^245            | A second episode of demyelination in a child with a past ADEM episode provided that:  
- The second episode meets criteria for ADEM  
- The symptoms are the same as initial ADEM episode  
- MRI demonstrates the same regions of involvement as were present on the initial ADEM episode |
| Multiphasic ADEM^245          | A second episode of demyelination in a child with a past ADEM episode provided that: |
- The second episode meets criteria for ADEM
- Clinical or MRI features involve new areas of the central nervous system
- Attacks are separated by more than 3 months
Table 5-2: Participant Demographics - Overall and within each tertile of serum 25-hydroxyvitamin D at presentation with acquired demyelinating syndromes (ADS).

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Overall</th>
<th>Lowest Tertile 25(OH)D (&lt;50 nmol/L)</th>
<th>Middle Tertile 25(OH)D (50-73.9 nmol/L)</th>
<th>Highest Tertile 25(OH)D (&gt;74 nmol/L)</th>
<th>P-trend across Tertiles a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample size, No. (%)</td>
<td>211</td>
<td>70 (33%)</td>
<td>70 (33%)</td>
<td>71 (34%)</td>
<td>0.995</td>
</tr>
<tr>
<td>Age at baseline, median (IQR), yr.</td>
<td>10.6 (6.2–13.7)</td>
<td>12.8 (9.1–14.5)</td>
<td>9.8 (5.9–13.6)</td>
<td>9.6 (5.4–12.3)</td>
<td>0.002</td>
</tr>
<tr>
<td>Female sex, No. (%)</td>
<td>106/211 (50%)</td>
<td>41/70 (59%)</td>
<td>32/70 (46%)</td>
<td>33/71 (46%)</td>
<td>0.150</td>
</tr>
<tr>
<td>BMI &gt;85th percentile, No. (%)</td>
<td>36/138 (26%)</td>
<td>21/48 (44%)</td>
<td>7/40 (18%)</td>
<td>8/50 (16%)</td>
<td>0.009</td>
</tr>
<tr>
<td>HLA-DRB1*15 positive, No. (%)</td>
<td>73/208 (35%)</td>
<td>27/69 (39%)</td>
<td>22/68 (32%)</td>
<td>24/71 (34%)</td>
<td>0.517</td>
</tr>
<tr>
<td>Race, No. (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.372</td>
</tr>
<tr>
<td>Black</td>
<td>9/211 (4%)</td>
<td>5/70 (10%)</td>
<td>1/70 (1%)</td>
<td>3/71 (4%)</td>
<td>0.315</td>
</tr>
<tr>
<td>Caucasian</td>
<td>173/211 (82%)</td>
<td>51/70 (73%)</td>
<td>62/70 (89%)</td>
<td>60/71 (85%)</td>
<td>0.009</td>
</tr>
<tr>
<td>Mixed</td>
<td>10/211 (5%)</td>
<td>2/70 (3%)</td>
<td>5/70 (7%)</td>
<td>3/71 (4%)</td>
<td>0.315</td>
</tr>
<tr>
<td>Oriental</td>
<td>17/211 (8%)</td>
<td>11/70 (16%)</td>
<td>1/70 (1%)</td>
<td>5/71 (7%)</td>
<td>0.315</td>
</tr>
<tr>
<td>Unknown</td>
<td>2/211 (1%)</td>
<td>1/70 (1%)</td>
<td>1/70 (1%)</td>
<td>0</td>
<td>0.315</td>
</tr>
<tr>
<td>Presenting phenotype, No. (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.315</td>
</tr>
<tr>
<td>ADEM</td>
<td>57/211 (27%)</td>
<td>12/70 (17%)</td>
<td>23/70 (33%)</td>
<td>22/71 (31%)</td>
<td>0.009</td>
</tr>
<tr>
<td>Monofocal Optic Neuritis</td>
<td>51/211 (24%)</td>
<td>19/70 (27%)</td>
<td>13/70 (19%)</td>
<td>19/71 (27%)</td>
<td>0.009</td>
</tr>
<tr>
<td>Monofocal Other</td>
<td>26/211 (12%)</td>
<td>9/70 (13%)</td>
<td>12/70 (17%)</td>
<td>5/71 (7%)</td>
<td>0.009</td>
</tr>
<tr>
<td>Monofocal Transverse Myelitis</td>
<td>39/211 (19%)</td>
<td>14/70 (20%)</td>
<td>15/70 (21%)</td>
<td>10/71 (14%)</td>
<td>0.009</td>
</tr>
<tr>
<td>Polyfocal</td>
<td>38/211 (18%)</td>
<td>16/70 (23%)</td>
<td>7/70 (10%)</td>
<td>15/71 (21%)</td>
<td>0.315</td>
</tr>
<tr>
<td>Normal brain MRI at ADS</td>
<td>75/208 (36%)</td>
<td>31/70 (44%)</td>
<td>19/69 (28%)</td>
<td>35/69 (51%)</td>
<td>0.333</td>
</tr>
<tr>
<td>Percent receiving supplemental vitamin D at ADS, No. (%)</td>
<td>29/211 (14%)</td>
<td>6/70 (9%)</td>
<td>9/70 (13%)</td>
<td>14/71 (20%)</td>
<td>0.054</td>
</tr>
</tbody>
</table>
Characteristics | Overall | Lowest Tertile 25(OH)D (<50 nmol/L) | Middle Tertile 25(OH)D (50-73.9 nmol/L) | Highest Tertile 25(OH)D (>74 nmol/L) | P-trend across Tertiles a |
--- | --- | --- | --- | --- | --- |
**Season of baseline visit, No. (%)** | | | | | 0.250 |
Spring (Apr–Jun) | 56/211 (27%) | 21/70 (30%) | 21/70 (30%) | 14/71 (20%) | |
Winter (Jan–Mar) | 60/211 (28%) | 24/70 (34%) | 20/70 (29%) | 16/71 (23%) | |
Autumn (Oct–Dec) | 52/211 (25%) | 14/70 (20%) | 15/70 (21%) | 23/71 (32%) | |
Summer (Jul–Sep) | 43/211 (20%) | 11/70 (16%) | 14/70 (20%) | 18/71 (25%) | |
MS, No. (%) | 41/211 (19%) | 21/70 (30%) | 12/70 (17%) | 8/71 (11%) | 0.005 |
Time from ADS onset to MS diagnosis, median (IQR), mos. | 3.7 (3.1–7.3) | 3.3 (2.9–4.2) | 6.9 (4.2–12.9) | 3.1 (2.3–31.6) | 0.208 |

**Abbreviations:** 25(OH)D, 25-hydroxyvitamin D; ADEM, acute disseminated encephalomyelitis; ADS, acquired demyelinating syndromes; BMI, body mass index; HLA, human leukocyte antigen; IQR, inter-quartile range; MS, multiple sclerosis; SD, standard deviation.

a Non-ordinal variables with more than two categories (race, presenting phenotype, and season of baseline) were evaluated with Pearson’s Chi Square.

b Of 332 children enrolled, 83 did not have samples obtained within 40 days of ADS, 2 were diagnosed with NMO, 6 experienced relapsing optic neuritis or transverse myelitis without involvement of other CNS areas, 18 were diagnosed with non-demyelinating disorders following accrual of further clinical or laboratory data, and 12 were excluded due to protocol violation (i.e. late enrolment, failure to confirm demyelination, or age > 16 years at ADS onset). Serum was evaluated for antibodies directed against aquaporin 4 for all 211 participants and was positive only in the two children diagnosed with NMO.

c Children with BMI ≥ 85th percentile for age and sex are overweight or at risk of overweight.

d Seventy-three children did not have both height and weight data recorded at ADS presentation.

e Three children did not have HLA-DRB1 status for analysis.

f “Race” (Black, Caucasian, Oriental, Native Canadian, or Mixed) was determined by interview with the parent, according to published guidelines.

g Focality was determined by neurological examination only, without consideration of MRI findings. Monofocal refers to clinical features localizable to a single site in the central nervous system, while polyfocal infers that multiple central nervous system sites are involved based on the clinical features.

h Three children did not have brain MRI available for analysis.

i Reported intake of vitamin D containing supplement more than 14 days in the previous 3 mos.
Table 5-3: Hazard ratios and 95% confidence intervals for tertiles of 25-hydroxyvitamin D status at presentation with acquired demyelinating syndromes (ADS) and risk of MS.

<table>
<thead>
<tr>
<th>Model 1: Unadjusted Vitamin D Status</th>
<th>All Participants</th>
<th>MS / n</th>
<th>Hazard Ratio (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum 25(OH)D Tertile (nmol/L)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 50</td>
<td>41/211</td>
<td>21/70</td>
<td>1.00</td>
</tr>
<tr>
<td>50–73.9</td>
<td>12/70</td>
<td>0.51 (0.25 to 1.03)</td>
<td></td>
</tr>
<tr>
<td>≥ 74</td>
<td>8/71</td>
<td>0.32 (0.14 to 0.73)</td>
<td></td>
</tr>
</tbody>
</table>

| Model 2                             |                  |        |                       |
| Serum 25(OH)D Tertile (nmol/L)      | 41/211           |        |                       |
| < 50                                | 21/70            | 1.00   |                       |
| 50–73.9                             | 12 /70           | 0.60 (0.29 to 1.23) |
| ≥ 74                                | 8 /71            | 0.37 (0.16 to 0.87) |
| Age (years)                         |                  | 1.15 (1.05 to 1.26) |
| Male Sex                            |                  | 1.59 (0.83 to 3.04) |
| Season                              |                  |        |                       |
| Spring (Apr–Jun)                    | 10/56            | 1.00   |                       |
| Winter (Jan–Mar)                    | 9/60             | 0.69 (0.28 to 1.70) |
| Autumn (Oct–Nov)                    | 13/52            | 1.34 (0.58 to 3.10) |
| Summer (Jul–Sep)                    | 9/43             | 1.82 (0.70 to 4.74) |

| Model 3                             |                  |        |                       |
| Serum 25(OH)D Tertile (nmol/L)      | 41/208           |        |                       |
| < 50                                | 21/69            | 1.00   |                       |
| 50–73.9                             | 12 /68           | 0.63 (0.31 to 1.30) |
| ≥ 74                                | 8 /71            | 0.41 (0.18 to 0.97) |
| Age (years)                         |                  | 1.17 (1.06 to 1.28) |
| Male sex                            |                  | 0.60 (0.31 to 1.16) |
| Season                              |                  |        |                       |
| Spring (Apr–Jun)                    | 10/54            | 1.00   |                       |
| Winter (Jan–Mar)                    | 9/60             | 0.52 (0.21 to 1.31) |
| Autumn (Oct–Nov)                    | 13/51            | 1.19 (0.51 to 2.77) |
| Summer (Jul–Sep)                    | 9/43             | 1.02 (0.40 to 2.60) |
| HLA-DRB1*15 Positive               | 22/208           | 2.77 (1.47 to 5.24) |

Abbreviations: 25(OH)D, 25-hydroxyvitamin D; ADS, acquired demyelinating syndromes; CI, Confidence Interval; MS, multiple sclerosis.
5.5 Discussion

We have demonstrated an inverse, dose-dependent relationship between vitamin D status at the time of an initial acute demyelinating attack and MS risk in children. Our findings remain robust even after accounting for other important determinants of MS risk, including sex, age, and HLA-DRB1*15 status, and for important determinants of vitamin D status such as season and ancestry. When analyses were restricted to children considered at particularly high risk of MS, our findings became more robust, suggesting that the presence of low circulating 25(OH)D concentrations at ADS associates with MS-specific pathobiology rather than contributing to acute demyelination per se.

Reverse causation – in which disease-related symptoms (e.g. fatigue, disability, or Uhthoff’s phenomenon) modify behaviours that in turn alter vitamin D status – has challenged interpretation of the contribution of vitamin D status in prevalent pediatric and adult cohorts. A key aspect of our work is the evaluation of vitamin D in samples collected in close proximity (median 9 days) to the first demyelinating event. All children in our study were in good health prior to ADS and free of co-morbidity or medication-related influences on vitamin D. Thus, the 25(OH)D concentrations can be reasonably assumed to reflect their premorbid vitamin D status.

Our work expands the body of literature on vitamin D as an environmental contributor to MS. In the only study to explore vitamin D status prior to the first attack of MS, 25(OH)D concentrations in young adult military recruits were inversely related to subsequent MS risk. Serum 25(OH)D levels were lower in adults with acute demyelination relative to healthy controls. Lower 25(OH)D levels associate with clinical disease activity in both adult and
pediatric MS populations.\textsuperscript{197,201,357} Taken together with our finding that, within a cohort of patients with CNS demyelination, low 25(OH)D at ADS was specifically associated with MS risk, this supports an etiologic role for vitamin D in MS biology.

Numerous studies demonstrate an inverse association between BMI and serum 25(OH)D concentrations in both children\textsuperscript{358} and adults.\textsuperscript{359-361} Interestingly, obesity in late adolescence is associated with subsequent increased risk of MS in females – a finding that is postulated to relate to lower serum 25(OH)D concentrations in obese individuals.\textsuperscript{362} Supporting this concept, we observed an inverse correlation between BMI percentiles and serum 25(OH)D among the 138 participants for whom we were able to calculate BMI percentiles. Although median BMI percentiles were higher among those diagnosed with MS, age adjustment rendered this observation non-significant. Study of larger cohorts may clarify relationships between pediatric obesity and vitamin D status and MS risk.

MS is influenced by both environmental and genetic factors.(reviewed in \textsuperscript{58}) As discussed in previous sections of this thesis, a gene most strongly and consistently associated with MS is \textit{HLA-DRB1*1501},\textsuperscript{74} the expression of which is influenced by vitamin D.\textsuperscript{74} Vitamin D also confers numerous beneficial immune-related effects;\textsuperscript{251,255,363} suggesting it influences MS biology via both epigenetic and immune-related mechanisms.\textsuperscript{256} Figure 5-1 illustrates the heightened MS risk in children who are both HLA-DRB1*15 positive and in the lowest vitamin D tertile. Although, we did not find a statistical interaction between circulating 25(OH)D levels at ADS and HLA-DRB1*15 on MS outcome this does not negate a potential important biological interaction, or an interaction occurring during very early immune system development.\textsuperscript{248}
We considered models for seasonal adjustment of 25(OH)D concentrations, as have been employed in two studies of vitamin D in established MS.\textsuperscript{201,364} A key assumption of these models is that seasonal 25(OH)D values change in a relatively consistent manner among participants; and by inference, that individuals remain in the same 25(OH)D ranking or tertile across seasons. However, a recent study of Canadian adults showed marked inter-personal variation in seasonal changes in serum 25(OH)D suggesting that such seasonal vitamin D modeling was not appropriate in our population.\textsuperscript{133} Furthermore, since the measured 25(OH)D concentrations are of biological interest, statistical adjustment of 25(OH)D concentrations may obscure the relationship between vitamin D status at ADS and MS outcome. Thus, we accounted for season in our multivariable models but did not adjust 25(OH)D values. Neither median serum 25(OH)D concentrations, the number of children presenting with ADS, nor the likelihood of MS outcome differed as a function of season of ADS presentation. As season was not related to vitamin D status or MS outcome, it is unlikely to have confounded our findings.

The new Recommended Dietary Allowance for vitamin D is 600 IU per day for those aged 1-70 years.\textsuperscript{160} However, Canadian children and adolescents only consume approximately 200-300 IU vitamin D per day.\textsuperscript{365} Thus, vitamin supplements, which generally contain between 200 IU – 1000 IU of vitamin D, may contribute substantially to vitamin D status. The 29 children (14\%) who reported supplemental vitamin D intake prior to ADS had higher circulating 25(OH)D levels, and nearly all (n = 28, 97\%) remained monophasic. In both uni- and multivariable analysis, the lower risk of MS associated with pre-ADS vitamin D supplementation approached significance. While requiring evaluation in a larger cohort, these findings are consistent with a report wherein supplemental vitamin D intake was associated with reduced risk of adult-onset MS.\textsuperscript{314}
A limitation inherent to our prospective design is that some children currently classified as having monophasic ADS will be diagnosed with MS. Mitigating this concern is the brief interval between ADS and MS diagnosis found for the patients diagnosed to date in the present study (median time to MS, 3.7 mos. (IQR 3.1–7.3) after ADS onset), similar to the short inter-attack interval reported in pediatric MS patients. The continued, prospective 8-year nature of the study will permit evaluation of whether children who experience a longer interval from ADS to confirmation of MS differ as a function of vitamin D status from those diagnosed in relatively close proximity to their ADS event. Our study was designed to evaluate predictors, at ADS onset, of subsequent MS outcome. We did not evaluate vitamin D supplement use following ADS, and thus cannot infer that vitamin D supplementation will reduce MS risk in children who have already experienced CNS demyelination. However, it is plausible that improving vitamin D status in childhood might reduce MS risk.

5.6 Acknowledgements

The site coordinators and site investigators of the Canadian Paediatric Demyelinating Disease Network provided invaluable assistance in data collection (Appendix 7).
6 Pseudovitamin D–Dependent Rickets, HLA-DRB1, and Multiple Sclerosis

Adapted From: Ramagopalan SV*, Hanwell HEC*, Giovannoni G, et al. Vitamin D Dependent Rickets, HLA-DRB1 and Risk of Multiple Sclerosis. Archives of Neurology. 2010;67(8):1034-1035. (*These authors contributed equally to original publication)
6.1 Introduction

Multiple sclerosis (MS) is a common inflammatory disease of the central nervous system characterized by myelin loss, axonal pathology, and eventual progressive neurological dysfunction. The cause of MS is not yet conclusively known; however, it is clear that genetic and environmental components are important. Genes play a relatively small but important role in MS, with extended major histocompatibility complex haplotypes, especially those containing HLA-DRB1*15, exerting the strongest effect. The involvement of the environment is also inescapable, and the geographical distribution of MS suggests that low sunlight—and thus impaired vitamin D production—may be a key environmental risk factor for the disease.

Evidence to support a role for vitamin D in MS comes from a number of studies including investigations that showed that vitamin D deficiency increases the risk of MS. Furthermore, pseudovitamin D deficiency rickets (PDDR, also known as vitamin D–dependent rickets type I; OMIM 264700) has recently been described as an MS risk factor. Three Norwegian patients diagnosed in childhood with PDDR rickets caused by mutations in the CYP27B1 (OMIM 609506) gene were later diagnosed with MS. CYP27B1 encodes 25-hydroxyvitamin D-1 alpha-hydroxylase, the enzyme that produces active vitamin D (calcitriol).

Recent evidence has highlighted the pleiotropic actions of calcitriol on immune and central nervous system development and function. The recently identified and functionally active vitamin D response element in the HLA-DRB1 promoter region suggests direct interactions between HLA-DRB1, the main susceptibility locus, and vitamin D, a strong candidate for mediating the environmental effect in MS etiology. Here we sought to obtain human leukocyte antigen (HLA) profiles of the Norwegian patients to see if vitamin D–HLA interactions might contribute to MS development in individuals with PDDR.
6.2 Materials and Methods

After approval by the institutional ethics committees, blood samples were collected from the patients for genomic DNA extraction. *HLA-DRB1* genotyping and sequencing has been described elsewhere. The methods used to quantitate serum calcitriol are unknown.

6.3 Results

All 3 patients carried the MS risk allele *HLA-DRB1*15, with the previously described vitamin D response element present in the promoter. Patients 1 and 2 – female siblings – (as coded previously) were homozygous for *HLA-DRB1*15.

6.4 Discussion and Conclusion

The frequency of PDDR in the general population is very low; thus, chance comorbidity of MS and PDDR is unlikely but further studies to assess this are warranted. The high frequency of vitamin D insufficiency in the general population has led experts to consider vitamin D supplementation as a measure to reduce the prevalence of diseases that may be associated with vitamin D insufficiency, such as MS. However, the time period when supplementation would be most effective – if at all – is still under debate. All three patients in this case series had exceptionally impaired vitamin D status prior to their PDDR diagnosis (discussed below),
suggesting that impaired vitamin D status may exert its influence on MS susceptibility during very early childhood or the prenatal period. This is in line with the recent finding of HLA-DRB1 underlying the month-of-birth effect in patients with MS, maternal effects in the disease, and place of birth influencing MS risk.

Two patients were treated with 30 000 IU of ergocalciferol per day (AFI-D2 Forte; Nycomed Pharma, Zurich, Switzerland), and 1 patient received 0.25 µg of calcitriol per day (Rocaltrol; Roche, Basel Switzerland). The efficacy of ergocalciferol therapy in PDDR, however, is limited by the impaired or ablated conversion of vitamin D to calcitriol due to mutations in the CYP27B1 gene. As such, while 25(OH)D concentrations soared (672 nmol/L), the ergocalciferol regimen failed to elevate circulating calcitriol (24 pmol/L) to within the reference range. Thus, vitamin D-related signaling –more aptly called calcitriol-dependent signaling – was likely not optimized in the patients treated with ergocalciferol. Given that the post-diagnostic ergocalciferol therapy did not restore calcitriol to within the reference ranges, it is unlikely that this therapy restored optimal calcitriol-dependent signaling. If vitamin D status does indeed modify MS risk, this finding allows for the possibility that impaired vitamin D status could influence MS risk at later time periods.

In the patient who received calcitriol, it is likely that calcitriol-dependent signaling was, to a large extent, restored post-diagnosis because their post-Rocaltrol circulating calcitriol (42 pmol/L) was within the reference range (38-150 pmol/L), albeit at the lower end. Given that circulating calcitriol was restored to reference levels with calcitriol therapy, then it would seem that this supports the hypothesis that – if vitamin D modifies MS risk – it was the impaired calcitriol-related signaling prior to diagnosis and treatment of PDDR that was important for determining MS risk.
While diagnosis and treatment began at some point in childhood, the exact age of diagnosis and start of calcitriol therapy are unknown; extrapolating from the patient’s age (54 years old in 2008) and the first commercial availability of the prescribed Rocaltrol (1973), this patient could not have been younger than 19 years old at the time of calcitriol initiation. Thus, aberrant vitamin D metabolism at any point in her life from birth until at least the end of her adolescence could have contributed to her risk of MS.

A further point of consideration is whether or not calcitriol therapy does indeed restore all necessary vitamin D- and calcitriol-related signaling in patients with PDDR. The uptake and metabolism of the various metabolites of vitamin D by various cell types is still incompletely understood. Many types of immune cells possess the calcitriol-generating CYP27B1 enzyme – which is controlled by autocrine or paracrine signaling within the cell; thus, it is plausible that immune cells may preferentially take up 25(OH)D to activate it *in situ* rather than taking up circulating calcitriol. If this indeed the case, then individuals with PDDR treated with calcitriol may still be at a disadvantage because even though their immune cells are capable of taking up 25(OH)D, the PDDR-related defects in the CYP27B1 enzyme ablate or impair its ability to respond to immune-related autocrine or paracrine activation signals to catalyze conversion of 25(OH)D to calcitriol *in situ*. If this is the case, then PDDR patients administered calcitriol may have normal circulating calcitriol concentrations but the function of the cells controlled by intracellular vitamin D-related signaling may not be restored to normal. In other words, when calcitriol is provided to those with PDDR at a dose that brings circulating calcitriol concentrations within the reference range, there is a possibility that their vitamin D-related signaling remains impaired indefinitely. If intracellular activation of vitamin D metabolites is necessary for optimal immune function, then even calcitriol-replete PDDR patients may
continue to experience disturbances of immune function that could contribute to the pathobiology of MS.

Case studies detailing both short-term and long-term outcomes in patients with PDDR indicate that (i) treatment with calcitriol results in variable correction of traits, (ii) early timing of the intervention is critical to optimizing long-term bone-related outcomes such as height, and (iii) calcitriol therapy initiated in infancy prevents skeletal abnormalities, suggesting that local production of calcitriol is not necessary for bone development.\textsuperscript{107,368} This finding cannot necessarily be extrapolated, however, to other cell types such as immune cells that – in contrast to bone cells – locally activate 25(OH)D to calcitriol in response to cytokines rather than mineral concentrations. Non-skeletal outcomes have not been well-described in patients with PDDR; thus, whether or not therapy with calcitriol optimizes of vitamin D-dependent signaling in non-skeletal systems such as the CNS and immune system remains to be seen.

The presence of MS risk associated allele in all three PDDR patients later diagnosed with MS supports the possibility of biological interactions between vitamin D and HLA-DRB1*15 in the pathobiology of MS. The incidence of MS is on the rise, and it seems increasingly probable that vitamin D is a major environmental risk factor. Studies of MS disease prevention through provision of vitamin D seem timely.
7 Sun exposure questionnaire predicts circulating 25-hydroxyvitamin D concentrations in Caucasian hospital workers in southern Italy

7.1 Abstract

**Introduction:** Solar ultraviolet B (UVB)-catalyzed cutaneous synthesis of vitamin D is a key contributor to a person’s vitamin D status as measured by circulating concentrations of 25-hydroxyvitamin D [25(OH)D]. Thus, recent sun exposure should correlate with circulating 25(OH)D.

**Methods:** A sun exposure score was calculated for healthy adults using a recall questionnaire assessing daily Time in Sun (<5min; 5-30min; >30min) and Skin Exposure (face/hands; face/hands and arms; face/hands and legs; and “bathing suit”) for 1 week in each of the winter and summer (n = 47 and 23, respectively; n = 18 participated in both). Concentrations of 25(OH)D were measured by DiaSorin RIA on end-of-week sera.

**Results:** Mean serum 25(OH)D was higher in summer than winter (58.6 ± 16.5 vs. 38.8 ± 29.0 nmol/L, respectively, \( P = 0.003 \) unpaired). The calculated Sun Exposure Score correlated strongly with serum 25(OH)D during summer (Spearman’s rho = 0.59, \( P = 0.003 \)); based on the Pearson coefficient of determination, summer Sun Exposure Score explained 38% of the variability in summer serum 25(OH)D. The Sun Exposure Score did not correlate with 25(OH)D in the winter (rho = 0.19, \( P = 0.210 \)). The summer correlation was largely explained by the Time In Sun (rho =0.58, \( P = 0.004 \)) rather than area of Skin Exposed (rho = 0.10, \( P = 0.660 \)). Although there was a correlation between winter and summer Sun Exposure Scores (rho = 0.63, \( P = 0.005 \)), there was no summer vs. winter correlation in serum 25(OH)D (rho = 0.08, \( P = 0.76 \)).
Conclusion: This simple 1-week sun exposure recall questionnaire predicted summer serum 25(OH)D concentrations, accounting for 38% of the variability in 25(OH)D among healthy Italian adults.

7.2 Introduction

In humans, vitamin D is primarily derived through the interaction of the sun’s ultraviolet B (UVB) radiation with a cutaneous cholesterol precursor, 7-dehydrocholesterol. Vitamin D from both cutaneous synthesis and dietary or supplemental intake undergoes hepatic hydroxylation to form 25-hydroxyvitamin D (25(OH)D), the circulating concentrations of which are the objective biomarker of vitamin D status.

The importance of sun exposure for vitamin D status is highlighted by the observations from temperate regions where circulating 25(OH)D concentrations fluctuate annually, lagging approximately two months behind the incident solar radiation. 369 129,370 Interestingly, serum 25(OH)D concentrations among those living in temperate regions are commonly below the established range for optimal vitamin D status,102 not only during winter and early spring, but also throughout much of the year.370-372 It is possible that algorithms may be developed that will direct serum 25(OH)D analyses towards those at highest risk of impaired vitamin D status. We hypothesize that people spending little time outdoors during the day or exposing very little skin to the sun are at an elevated risk of having low serum 25(OH)D concentrations. The present study evaluates this hypothesis by evaluating the relationship
between responses to a simple sun exposure recall questionnaire and concurrent serum 25(OH)D concentrations during the winter and summer seasons in healthy adults living in Southern Italy.

### 7.3 Materials and Methods

**Participants:** Healthy adult Italian Caucasian hospital workers in southern Italy (latitude: 40 degrees N) volunteered for this study, providing signed informed consent. Participants were eligible if physical examinations and routine laboratory tests of renal and hepatic function were normal. Participants were excluded if they had conditions associated with impaired vitamin D metabolism or used medication(s) known to affect calcium metabolism. The ethics committee of the “Casa Sollievo della Sofferenza” Hospital, San Giovanni Rotondo (FG), Italy, approved this study.

**Data Collection:** The study was carried out during January and February 2003 (“winter” visit), and July and August 2003 (“summer” visit). Fasting blood and random urine samples were collected between 07.00 to 09.00 hours, and stored at -70°C until assayed as part of the assessment of the biochemical parameters related to bone and mineral metabolism. Serum 25(OH)D concentrations were analyzed by radioimmunoassay (RIA; DiaSorin, Stillwater, MN, USA) with intra- and inter-assay CVs < 7.2% and < 12%, respectively. Serum ionized calcium (iCa) was assessed via ion-specific electrode (AVL LIST GmbH Medizintechnik, Graz, Austria).

During the sample collection visit, participants’ recollection of daily sun exposure over the previous week was assessed via a questionnaire administered during the sample collection visit.
There were three choices for the amount of time spent outdoors each day (0 = < 5 min, 1 = 5 min - 30 min, 2 = and ≥ 30 min) and four choices for clothing or skin exposure while outdoors (1 = face & hands only; 2 = face, hands and arms; 3 = face, hands and legs; and 4 = “bathing suit”). A score to estimate of their mean weekly sun exposure was calculated: The product of the amount of time spent outdoors and the amount of skin exposed was calculated for each day to create a daily Sun Exposure Score (min = 0, max = 8). All seven days’ Sun Exposure Scores were summed to equal the weekly Sun Exposure Score (min = 0, max = 56). Similarly, the weekly Time in Sun Score and weekly Skin Exposure Score were each calculated by summing the seven daily scores (respectively, min = 0, max = 14 and min = 7, max = 28).

**Statistics:** All statistical analyses were carried out using SPSS 13.0 (SPSS Inc., Chicago, IL). Means and standard deviations describe parametric data; medians and interquartile ranges describe non-parametric data. The Pearson coefficient of determination and Spearman rho describe relationships between the weekly Scores and 25(OH)D, separately for each season. Paired t-tests (for comparisons of parametric variables between winter and summer for the n = 18 participants completing both visits), unpaired t-tests (for comparisons of all participants, n = 43 in winter and n = 27 in summer) and the Fisher exact test were used to evaluate categorical variables. \( P < 0.05 \) was set as the limit of significance.

### 7.4 Results

Participant characteristics are presented in **Table 7-1**. Mean serum 25(OH)D concentrations were higher in summer than winter (respectively, 58.6 ± 16.5 nmol/L and 38.8 ± 29.0 nmol/L, \( P = 0.003 \) unpaired). The correlation between the serum 25(OH)D and weekly Sun Exposure
Score was significant in summer but not winter (respectively, Spearman rho = 0.59 and 0.19, $P = 0.003$ and 0.212; **Figure 7-2A**). Interestingly, all 47 participants reported skin exposure only in the lowest category (face and hands) during winter; hence the distribution in **Figure 7-2C**.

In summer, the correlation between the weekly “Time in Sun” score was almost as strongly correlated with serum 25(OH)D as the weekly Total Sun Exposure Score (rho = 0.58, $P = 0.004$; **Figure 7-2B**). In contrast, the amount of skin exposed to the sun (Skin Exposure) did not correlate significantly with serum 25(OH)D (rho = 0.10, $P = 0.66$).

A subgroup of 18 participants (9 males and 9 females) attended both study visits. In these 18 participants, there was no significant correlation between seasons for serum 25(OH)D concentrations (rho = 0.08, $P = 0.76$). The weekly Sun Exposure Scores and Time in Sun Scores were, however, correlated between the summer and winter (respectively, rho = 0.63, $P = 0.005$ and rho = 0.84, $P < 0.001$).
Table 7-1: Participant characteristics at the winter and summer visits

<table>
<thead>
<tr>
<th></th>
<th>Winter (n=47)</th>
<th>Summer (n=23)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age (y)†</td>
<td>45.6 ± 13.5</td>
<td>42.2 ± 9.0</td>
<td>0.288</td>
</tr>
<tr>
<td>Sex (Males : Females )</td>
<td>17 : 30</td>
<td>10 : 13</td>
<td>0.607</td>
</tr>
<tr>
<td>Body Mass Index (kg/m²)</td>
<td>25.5 ± 4.5</td>
<td>24.0 ± 3.4</td>
<td>0.126</td>
</tr>
<tr>
<td>Serum ionized Calcium (mmol/L)</td>
<td>1.21 ± 0.03</td>
<td>1.24 ± 0.2</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Vitamin D Status

<table>
<thead>
<tr>
<th></th>
<th>Winter (n=47)</th>
<th>Summer (n=23)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean serum 25(OH)D (nmol/L)</td>
<td>38.8 ± 29.0</td>
<td>58.6 ± 16.5</td>
<td>0.003</td>
</tr>
<tr>
<td>25(OH)D &lt; 75 nmol/L (n, %)</td>
<td>41 (87%)</td>
<td>18 (78%)</td>
<td>0.485</td>
</tr>
<tr>
<td>25(OH)D &lt; 30 nmol/L (n, %)</td>
<td>25 (53%)</td>
<td>1 (4%)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

† Results presented as means ± SD or n participants with percent (in brackets).
Shown in gray are the values ascribed to each category and the method for deriving the Sun Exposure Score (sum of the daily products of Time Outdoors and Skin Exposure). Each day’s Time in Sun and Skin Exposure results were multiplied; the sum of all 7 days resulted in derivation of the weekly Sun Exposure Score. Sun Exposure Score ranges from 0 (lowest amount of time spent outdoors and lowest amount of skin exposed) to a maximum score 56 (outdoors for more than 30 minutes in bathing suit every day).

**Figure 7-1:** Layout and scoring for the weekly sun exposure recall questionnaire.

<table>
<thead>
<tr>
<th></th>
<th>Time Outdoors</th>
<th></th>
<th>Amount of Skin Exposed</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&lt;5 min</td>
<td>5-30 min</td>
<td>&gt;30 min</td>
<td>Hands</td>
<td>Hands, face</td>
<td>Hands, face, arms</td>
<td>Hands, face, legs</td>
</tr>
<tr>
<td>Monday</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Tuesday</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Wednesday</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Thursday</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Friday</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Saturday</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Sunday</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
</tbody>
</table>
Figure 7-2: Serum 25(OH)D concentrations in summer (black open circles) and winter (small grey circles) as a function of (A) Sun Exposure Score which is derived from 1-week recall of (B) Time in Sun and (C) Skin Exposure. Regression line (with regression equation, $r^2$ and $P$-value) and the 95% Confidence Interval displayed for the summertime data only.
7.5 Discussion and Conclusions

The present study demonstrates that a simple questionnaire assessing both the amount of time spent outdoors and amount of skin exposed in a given week for healthy Caucasian adults living at latitude 40°N offers a good prediction of 25(OH)D concentrations in summer. This questionnaire involves minimal participant burden but includes a timeframe long enough to estimate typical activity for an individual. This questionnaire could serve as a screening tool to identify patients at increased risk of low sun exposure who might benefit from a serum 25(OH)D determination.

The strength of the relationship detected between sun exposure in the summer and serum 25(OH)D, highlights the key role of summer sun exposure in determining vitamin D status in a Caucasian population at 40° N latitude. The present study did not collect information regarding type of clothing worn, use of sunscreens, or vacation travel, nor was dietary or supplemental intake assessed to quantify exogenous sources of vitamin D. While the relationship might have been improved by inclusion of such additional measures, it also would have increased the complexity of the questionnaire, making routine clinical use less feasible and recall less accurate. Furthermore, skin pigmentation affects the rate of cutaneous vitamin D synthesis, and thus, the relationship between the Sun Exposure Score and circulating 25(OH)D may vary with skin pigmentation. Since all participants were native Italians, future use of this questionnaire in ethnically diverse populations should also objectively query participant skin pigmentation to evaluate its potential influence on the relationship between sun exposure and serum 25(OH)D.

A comparable study of men working outdoors in Nebraska, USA focused more intently on the type and quality of clothing in addition to time spent outdoors.373 Despite more precise
description of skin exposure in that study, the correlation between sun exposure and serum 25(OH)D concentrations was weaker than in the present study. The stronger summertime correlation observed in the present study may be partly explained by the relative ethnic and cultural homogeneity of the Italian participants. Also, the present correlation was almost entirely driven by Time Spent in Sun, not the amount of exposed skin, suggesting that the categories for time spent in the sun used in this study (<5 min, 5-30 min, >30min) are easily recalled and likely to be of biological significance. The timeframes were selected based upon unpublished preliminary study in this population and the 30-minute time frame used for the highest category is only slightly longer than the estimated time needed to achieve maximal dermal synthesis of vitamin D in fair skinned individuals with arms and legs exposed to large-angle incident sunshine. The summer Skin Exposure score alone did not correlate significantly with summer 25(OH)D concentrations. One possible explanation for this finding is that participants with moderately high skin exposure when in the sun did not report spending much time in the sun. People who spend very little time in the sun may be expected to have low 25(OH)D; if these participants wear light clothing (arms and/or legs uncovered) most days but are outdoors less than 5 minutes each day of the week, their 25(OH)D status would be expected to correspond with their low Time In Sun Scores and low overall Sun Exposure Scores but would not correspond with their high Skin Exposure Scores. It is in these sorts of instances that the importance of querying both Time In Sun and Skin Exposure becomes obvious, as the derived Sun Exposure Score reflects actual sun exposure habits more accurately than querying either variable alone.

In contrast to the summer data, the Sun Exposure Score did not correlate with serum 25(OH)D in the winter. Given that there is insufficient solar UVB to generate vitamin D in the skin at latitudes > 40°N during the winter months, the participants would not produce vitamin D
during time spent outdoors at that time. It would be of interest to investigate the strength of the relationship between winter sun exposure and serum 25(OH)D at varying latitudes and explore this relationship in other populations of varied ethnicities with cultural practices that may affect the relationship between sun exposure and serum 25(OH)D concentrations.

Winter sun exposure habits, particularly time spent in the sun, strongly reflected the summer sun exposure habits. However, in the winter, all subjects indicated that they exposed only their face and hands, resulting in identical responses for the Skin Exposure variable. This finding limited statistical analyses but also highlighted the uniformly minimal wintertime skin exposure in this group of Italian adults.

While the present observations may apply only to a Caucasian population and, therefore, need to be confirmed in other populations, these findings emphasize the importance of outdoor activity as a way to maintain adequate vitamin D nutritional status. In spite of its limitations, the present study offers a useful tool for clinicians to administer to patients to estimate circulating 25(OH)D concentrations.
8 Thesis Discussion and Conclusions

“All scientific work is incomplete - whether it be observational or experimental. All scientific work is liable to be upset or modified by advancing knowledge. That does not confer upon us a freedom to ignore the knowledge we already have or postpone the action that it appears to demand at a given time.” – Sir Austin Bradford Hill, 1965

Our work contributes to a rapidly growing body of evidence suggesting a role for vitamin D in the pathobiology of multiple sclerosis. In the context of Bradford Hill’s criteria for assessing evidence for causation (Figure 2-3),¹ the results of our pediatric study are (i) consistent with the body of literature to date – in that higher vitamin D status was associated with lower risk of MS, and (ii) provide evidence of Temporality and Biological Gradient in that we report – for the first time in the published literature – a dose-dependent relationship between vitamin D status at the earliest clinically identifiable manifestation of pediatric demyelination and the likelihood of subsequent MS diagnosis.

In this final section, the findings and limitations of the research presented herein will be placed in the context of the present literature, and potential avenues for further studies will be discussed. As mentioned in the summary of the Hill Criteria, to satisfy the requirements of the Hill Criteria, experimental evidence demonstrating that vitamin D reduces the incidence of MS is still required. The logistics of such work are daunting and no such studies have been attempted yet in humans. Support for a primary prevention study is gaining interest in the
medical research community; thus, the concluding portion of this section will detail some important considerations for vitamin D as a primary prevention strategy for MS.

**Measurement of vitamin D metabolites**

When this doctoral research began in 2007, the most commonly used method for measuring circulating 25(OH)D concentrations was the DiaSorin RIA; this was the method used in the majority of human vitamin D-related studies that vitamin D-intake and 25(OH)D concentration recommendations have been based on. However, the RIA is limited by a slow-throughput methodology requiring highly trained personnel and radio-iodinated reagents. The requirement for measuring serum 25(OH)D concentrations in the large cohort enrolled in the Canadian Paediatric Demyelinating Diseases program motivated the validation of a high-throughput assay against the RIA. Thus, we first compared the performance of the DiaSorin RIA against new automated high-throughput methods for the rapid analysis of circulating 25(OH)D concentrations in serum samples. The LIAISON “25 OH Vitamin D TOTAL” assay agreed strongly with the RIA method across a broad, biologically relevant range of 25(OH)D concentrations while also providing superior precision. As such, the LIAISON was selected for analysis of circulating 25(OH)D concentrations in the children enrolled in the Canadian Paediatric Demyelinating Diseases program.

While the LIAISON immunoassay served well for our serum analyses, liquid chromatography, tandem mass spectrometry (LC-MS/MS) technology has the potential to afford a higher level of precision than immunoassays such as the LIAISON. The wide acceptance and use of LC-MS/MS for analysis of vitamin D metabolites has been challenged in part by infrastructural
costs and the lack of standardized extraction and analytical methodologies. Furthermore, internal standards are needed in order to quantify target analytes using LC-MS/MS. The National Institute of Standards and Technology (NIST) has only recently developed serum-based standard reference materials for vitamin D metabolites.\textsuperscript{374} Previously, comparisons of results produced by differing LC-MS/MS methods were limited by the lack of calibrated standards. Therefore, wide use of the new NIST standards will likely serve to improve inter-lab LC-MS/MS analytical agreement. A systematic comparison of the LIAISON “25 OH Vitamin D TOTAL” with LC-MS/MS methods currently in use at the Hospital for Sick Children is planned. To facilitate this, the NIST standards and a common set of samples representing 25(OH)D across a broad, biologically relevant range could be analyzed using both the LIAISON and LC-MS/MS technologies.

Also, since the publication of our methods analysis,\textsuperscript{350} there has been growing interest in not only high-throughput methods for assessing circulating serum 25(OH)D but also the assessment of other vitamin D metabolites – such as 24,25(OH)\textsubscript{2}D. The analysis of 24,25(OH)\textsubscript{2}D concurrent with 25(OH)D has been enabled by the use of LC-MS/MS technology. Given that quantitation of the 24,25(OH)\textsubscript{2}D metabolite provides additional insight into vitamin D metabolism – which may be altered in children at risk for MS – future studies include evaluating 24,25(OH)\textsubscript{2}D by LC-MS/MS in our pediatric cohort.

Another research direction that has been enabled by LC-MS/MS technology is the assessment of circulating 25(OH)D concentrations by analysis of dried blood spots.\textsuperscript{339,341,375} Blood spot cards for assessment of vitamin D status are a very attractive option for population-based studies for numerous reasons. The cards and a small kit of materials and instructions for blood spot collection can be mailed out to participants at low cost. Completed cards can be returned via
mail, eliminating the need for participants to attend study visits, and reducing costs related to phlebotomy personnel and equipment for collection. Furthermore, the vitamin D metabolites are relatively stable in blood spot cards.\textsuperscript{339,340} Cost and level of coordination required to transport and store blood spot cards would likely be many fold lower than that of samples collected via venipuncture which require insulating packaging, specific handling, timing, temperatures, and higher freight charges. Blood cards are stable at room temperature, avoiding the requirement for the prompt processing of samples derived from venipuncture and eliminating the expensive – 70°C or –80°C storage required for serum samples. As such, blood cards could prove ideal for large population-based studies and for studies involving sites in multiple countries.

Of interest is the validation of recently published methods for analyzing 25(OH)D in dried blood spots.\textsuperscript{339} Furthermore, the feasibility of quantitating both 25(OH)D as well as 24,25(OH)\textsubscript{2}D levels in dried blood spots should be explored by systematically comparing the performance of serum-based LC-MS/MS methods to those of dried blood spots for analysis of 25(OH)D and 24,25(OH)\textsubscript{2}D. Although 24,25(OH)\textsubscript{2}D does circulate at nanomolar concentrations, levels are generally only around 10 to 40% of circulating 25(OH)D.\textsuperscript{376} Thus, whether 24,25(OH)\textsubscript{2}D would be below the limit of detection in dried blood spots from participants with low 25(OH)D concentrations will be of particular concern.

**The Importance of Timing**

We elected to assess vitamin D status within 40 days of ADS onset. ADS is the earliest possible clinically identifiable time point in the MS disease process. Given the relatively long half-life of 25(OH)D in the circulation, we felt that the 25(OH)D levels measured within the 40 day
window provided a reasonable estimate of the child’s vitamin D status concurrent with their acute illness. We also felt that the measure was unlikely to be confounded by demyelinating disease or with medications prescribed for acute ADS treatment. Consistent with a protective role of vitamin D, we did observe that children with higher serum 25(OH)D concentrations at the time of ADS were less likely to be diagnosed with MS. I propose three possible interpretations of this finding: (1) Low 25(OH)D concentrations observed at ADS indicate chronic vitamin D insufficiency which contributes to the subclinical pathobiological development of MS; (2) Low 25(OH)D concentrations specifically at ADS propagate immune dysregulation leading to perpetual immune targeting of the CNS resulting in MS, and (3) Children with low 25(OH)D concentrations at ADS are more likely to continue to have low 25(OH)D levels following ADS which contribute to continued pathologic dysregulation of the inflammatory response leading not only to an MS outcome but also to increased MS relapse rate.

The first hypothesis – that chronic vitamin D insufficiency contributes to MS outcome in children with ADS – presupposes a subclinical phase of MS disease activity prior to a sentinel clinical attack. This concept is supported by MRI and CSF features at ADS which provide evidence of established MS biology. Specifically, MRI evidence of even a single clinically silent brain lesion at ADS was strongly associated with subsequent MS diagnosis. The presence of oligoclonal bands in the CSF – a biomarker of chronic intrathecal B cell activation – also predicts MS outcome.\textsuperscript{377} Together, these findings indicate that MS risk has already been determined at the time of ADS, rendering the third hypothesis – where MS risk is determined in part by vitamin D status post ADS – less likely. However, 25(OH)D levels post-ADS may influence MS disease activity. Recent work describing an inverse relationship between relapse rate and 25(OH)D concentrations obtained in a cross-sectional manner in prevalent pediatric MS
patients indicates that vitamin D status may influence time to relapse or relapse-rates. Thus, future studies should include evaluating relationships between serial 25(OH)D concentrations and measures of disease activity such as relapse rates.

Our work to date does not permit evaluation of whether vitamin D status in infancy, early childhood, or in children prior to ADS onset influences risk ADS or MS. Studies demonstrating that birth season, childhood sun exposure, and migration before adulthood to world regions of high MS prevalence can affect subsequent MS risk, suggest that vitamin D status as early as the prenatal time period may be relevant. In addition, data regarding adolescent diet and weight, serum 25(OH)D status and symptomatic infection with EBV (infectious mononucleosis), suggest that MS risk may be modifiable until late into the second decade of life. To illuminate our understanding of vitamin D status across the lifespan and potentially the time of life within which vitamin D status affects MS biology, a questionnaire is being developed to be administered to participants in the Canadian Paediatric Demyelinating Disease program and in an international study of children with ADS (pilot project ongoing).

This questionnaire queries the participants’ skin type, and both lifetime and recent sun exposure habits and oral intake of vitamin D; it also queries the participants’ mother regarding her skin type, and her sun exposure and oral vitamin D intake during pregnancy and lactation (if applicable). The sun exposure component of this questionnaire utilizes the simple format of the sun exposure questionnaire described in Chapter 6 which highlighted the important contribution of summertime sun exposure to vitamin D status; it also queries sunscreen use and head covering. Responses to these questionnaires will help us to determine whether particular vitamin D-related exposures during certain stages of life are related to risk of pediatric MS.
Pathobiological Mechanisms

This thesis explored one aspect of potential mechanisms by which vitamin D-related biology may be involved in MS – exploration of an interaction between vitamin D status at ADS and the vitamin D-responsive MS risk gene, \textit{HLA-DRB1}*15 on MS outcome. In Chapter 5, circulating 25(OH)D levels at ADS and HLA-DRB1*15 positivity were both significant predictors of MS outcome. For this, DRB1*15 status was evaluated dichotomously (positive: DRB1*15 heterozygotes and homozygotes vs. negative: no DRB1*15 allele). This dichotomized stratification may not be sufficient to tease out interactions between vitamin D status and DRB*15. For instance, in large epidemiological studies, DRB1*15 homozygotes have two fold higher risk of adult-onset MS than DRB*15 heterozygotes.\textsuperscript{65} Thus, ideally DRB1*15 heterozygotes and homozygotes would be evaluated separately. The rarity of DRB1*15 homozygotes (n = 13, n = 5 MS) and our sample size precluded such analyses in the Canadian Paediatric Demyelinating Diseases cohort.

In addition to evaluating DRB1*15 homozygotes and heterozygotes separately, it is also relevant to consider that more complex HLA-related analyses may be warranted. Genetic studies have revealed that the association between HLA-DRB1 alleles and adult-onset MS is complicated by epistasis – wherein the effects of one gene or one allele are modified by another.\textsuperscript{65} Notably, the risk conferred by an HLA-DRB1*15 allele is strongly modified by the protection afforded by co-existence of an HLA-DRB1*14.\textsuperscript{381} Although the DRB1*14 variant was not found to harbour a functional, conserved vitamin D response element, epistatic interactions between DRB1*14 and DRB1*15 could confound the associations between vitamin D status, DRB1*15 status, and MS outcome. Further genetic analyses accounting for epistatic
interactions may be required to fully appreciate the interactions between vitamin D status and HLA-DRB1*15. These analyses would require large populations.

Furthermore, to evaluate potential interactions between vitamin D status and HLA-DRB1*15 on MS outcome, HLA-DRB1*15 heterozygotes and homozygotes would be analyzed separately, circulating 25(OH)D concentrations at a certain times of life would be factored in, as would variants in key genes encoding products involved in the vitamin D metabolic pathway. Two recent studies have evaluated relationships between MS risk, HLA-DRB1*15 status (positive vs. negative), and single nucleotide polymorphisms (SNPs) in several vitamin D-metabolizing and signaling genes. The SNPs were selected based upon previous work that associated these variants with serum 25(OH)D concentrations; however, neither study actually measured actual 25(OH)D concentrations. In one study of healthy adults and adults with MS, SNPs (selected based on their association with higher serum 25(OH)D concentrations in prior studies) were associated with lower risk of MS. Interestingly, the lower MS risk conveyed by the SNPs was attenuated among those with one or more HLA-DRB1*15 allele. These findings suggest that genetic predictors of higher vitamin D status are less protective against MS among those carrying HLA-DRB1*15. A second study exploring vitamin D pathway SNPs did not observe significant relationships between any variants in vitamin D pathway genes and MS risk nor interactions with HLA-DRB1*15 status (positive vs. negative). Both studies are limited in that they also evaluated DRB1*15 homozygotes and heterozygotes together, but also in that neither study assessed (i) environmental predictors of 25(OH)D or (ii) actual circulating 25(OH)D levels at any life stage. Interpretation of the study findings must consider that (i) genetic prediction of vitamin D status does not account for the substantial influence of the environment on vitamin D status across the lifespan and (ii) the effects of genetic variants in vitamin D pathways on MS outcome may depend upon vitamin D status. If susceptibility to MS
relating to vitamin D biology is ultimately driven by insufficient cellular exposure to calcitriol or by impaired calcitriol-related signalling, it is possible that certain vitamin D pathway gene variants may only be relevant to MS outcome in the context of suboptimal vitamin D concentrations. For instance, in Tasmanian adults, a common variant in the vitamin D receptor was associated with MS only among those who reported low levels of winter sun exposure in childhood and presumably would have had low 25(OH)D concentrations at that time.300

Future research could select variants in vitamin D pathway genes previously associated with either MS outcome (e.g. vitamin D receptor) or vitamin D status (vitamin D binding protein).217,298,300 As an exploratory, hypothesis-generating study, our future research may include evaluating variants within the alternate, membrane-bound calcitriol receptor, 1,25D3-MARRS receptor which has hitherto not been studied in association with either 25(OH)D levels nor MS outcome. The 1,25D3-MARRS receptor is apparently multifunctional and could be of interest to MS because (i) it is part of the major histocompatibility complex class I peptide-loading complex and, thus, is capable of influencing MHC class I antigen presentation,382 and (ii) impaired activity of the 1,25D3-MARRS receptor results in dysregulation of the signal transducer and activator of transcription-3 (STAT3) signalling pathway that is involved in inducing naive T cells to differentiate into pro-inflammatory Th17 cells.383,384

Thus, to further clarify the contribution and interaction of vitamin D biology and HLA-DRB1*15, we are interested in evaluating MS outcome as it relates to the interactions between HLA-DRB1*15 status, serum 25(OH)D levels at ADS, and a carefully chosen selection of vitamin D pathway genetic variants.
Vitamin D as a Therapeutic or Primary Prevention Strategy for MS

In the present thesis, evidence has been presented to support (i) a causal link between vitamin D insufficiency and MS and (ii) the biologic plausibility of this association. The next important questions are whether provision of vitamin D would modify MS disease activity or risk of MS. As discussed in the background (Chapter 2), some preliminary work has been conducted in the area of vitamin D interventions as a therapeutic strategy for modifying disease activity in established MS. However, further information on dose and efficacy is needed. In the meantime, there is already good rationale to encourage MS patients to consume vitamin D supplements: As previously discussed, low 25(OH)D levels are frequently observed in patients with established MS. Low levels of 25(OH)D exacerbate bone loss and increases risk of fractures and many MS patients have low bone mineral density, increased risk of fracture, and possess multiple risk factors for osteoporosis.

In terms of investigating the efficacy of vitamin D for prevention of MS, this would require a primary prevention study – defined as a study designed to prevent disease by altering susceptibility or reducing exposure for susceptible individuals. To determine the feasibility of studying vitamin D status as a primary preventative strategy for MS, there are considerations to take into account such as the (i) timing, (ii) nature, and (iii) estimated efficacy of intervention, as well as the (iv) target population for intervention.

Timing of Intervention

If a causal relationship between vitamin D status and MS risk is assumed – the literature indicates that the window of risk determination may start as early as the prenatal period and last
until the end of adolescence. Thus, to maximize likelihood of success, one strategy could involve enrolling female participants in a vitamin D supplement study prior to and during conception. However, if enrolment was restricted to pregnant females or females planning to become pregnant in the near future, this would necessitate a period of observation of the offspring – who may not elect to continue supplementation – lasting at least 30 to 40 years in order to ascertain the majority of MS diagnoses (occurring primarily between the ages of 18 and 40 years).

Alternatively – given other evidence indicating a broad window of MS susceptibility – intervention with vitamin D commencing at any time prior to the end of the second decade may afford protection. In this model, participants would include women who plan to conceive, expectant mothers, infants, children and adolescents (<20 years). Participants would begin supplementation at varying stages of life, and because the precise period of life within which vitamin D may protect against MS is unknown, statistical analyses would have to account for the ages at which participants were exposed to vitamin D supplementation. Also critical is consistent compliance with vitamin D supplementation from the point of study entry forward, or, at the very least, until the participants were no longer within the window of risk determination.

Another key consideration related to timing or duration of intervention is the concept of a pre-clinical phase of MS disease activity (latency), which may be operative for an unknown amount of time preceding ADS. Vitamin D supplementation in individuals who have entered this sub-clinical phase of the disease may be ineffective in preventing MS but may reduce disease activity. Thus, in considering the statistical analyses of the outcomes of an intervention,
one strategy would be to focus on the data gathered starting a set number of years – perhaps 3 to 5 years – from the initiation of intervention.

**Intervention Type: UVB or Vitamin D**

Thus far, MS clinical trials have only (i) evaluated the effects of oral vitamin or calcitriol in adult patients with established RRMS – ergo, not in the context of primary prevention, and (ii) utilized oral vitamin D or calcitriol supplementation rather than UVR exposure. Much of the epidemiologic studies cited as providing evidence for a role of vitamin D in the biology of MS pertain to latitudinal and UVR-related exposures. While UVB-catalyzed synthesis of vitamin D in the skin is a major source of vitamin D, it is important to consider that UVR stimulates neuroendocrine and immune-modulating pathways that may either function independently of or in concert with vitamin D produced in the skin. Therefore, ingested vitamin D does not completely reproduce the effects of UVR exposure. It is, thus, plausible that achieving a particular range of circulating 25(OH)D via controlled UVR exposure could result in significantly different immune-related and clinical outcomes as compared to the same 25(OH)D levels achieved via oral vitamin D supplementation. Whether or not UV-stimulated vitamin D-independent mechanisms do also contribute to the apparent benefit conferred by UVR on MS risk remains unclear.

The risks associated with both acute and chronic UVR exposure (e.g. sun burn, melanoma, etc.) challenge the use of UVR as a means to resolve vitamin D insufficiency. Also, establishing a UVR dose to safely produce and maintain a certain level of circulating 25(OH)D presents a formidable challenge.
Rationale for supplementation with oral vitamin D comes from a nested case control study that reported an association between higher intakes of dietary or supplemental vitamin D and lower odds of MS.314 Interestingly, children who regularly consumed vitamin D-containing supplements prior to ADS (Chapter 5; at least 14 days during in the three months prior to ADS onset) were less likely to be diagnosed with MS. Thus, not only is oral supplementation with vitamin D a more pragmatic approach for intervening to improve vitamin D status than with UVB, our very preliminary finding suggests that oral supplementation specifically in childhood could be of benefit.

Oral vitamin D₃ per se would be preferable to vitamin D₂ because vitamin D₃ is the form of vitamin D that is synthesized endogenously and vitamin D₂ is not as effective as vitamin D₃.315,316,394 Also, the established safety profile of oral vitamin D₃ makes vitamin D₃ preferable to calcitriol, which bears a much higher risk of inducing hypercalcemia 311 in healthy adults and adults with MS, vitamin D₃ was safe at doses several fold higher than the current Institute of Medicine (IOM) Dietary Reference Intake’s tolerable upper intake limit (4,000 IU/d).154,216,308 Although the safety profile of vitamin D in pediatrics is less well defined, a recent review of the available literature indicates that vitamin D intakes above the current IOM recommendation of 600 IU/d are safe and may be necessary for optimizing growth and bone health from infancy though adolescence.104 Among the highest doses of vitamin D reported in the pediatric literature are 50,000 IU per week (~7,100 IU/d) for 6 weeks in infants and toddlers,395 100,000 IU administered bimonthly (~1,600 IU/d) for 6 months in adolescents,396 either 14,000 IU per week or 2,000 IU/d in infants, toddlers, and pre-schoolchildren for up to 12 months,397-400 and 30,000 IU per month (~1,000 IU/d) for 12 months in toddlers.401 All of these studies indicate that treatment was well tolerated, with no instances of vitamin D toxicity reported.
Relevant to consideration of vitamin D dose is recognizing that there remains uncertainty regarding the optimal range for circulating 25(OH)D concentrations. As stated previously, the 2010 IOM Adequate Intake (AI) for vitamin D is 400 IU for infants from birth to 1 year, and the Recommended Dietary Allowances (RDAs) for vitamin D are 600 IU for everyone up to age 70 years and 800 IU for those ≥71 years of age.\textsuperscript{160} These doses are based upon the intakes needed for 97.5% of the healthy population to attain a circulating 25(OH)D concentration of 50 nmol/L – the current target set by the IOM for bone-related health related outcomes.\textsuperscript{160} However, the Canadian Pediatric Society and other expert consensus reports have indicated that a minimum 25(OH)D level of 75 nmol/L is needed to optimize a variety of health outcomes in both children and adults.\textsuperscript{102,104,402} Furthermore, the IOM DRIs target healthy populations of BMI <25.0 kg/m\textsuperscript{2}; however, there is a high prevalence of overweight (BMI 25.1 -29.9 kg/m\textsuperscript{2}) or obesity (BMI ≥30.0 kg/m\textsuperscript{2}) and the effective dose of vitamin D needed to raise serum 25(OH)D concentrations increases with BMI.\textsuperscript{403} Thus, for a primary prevention study, doses of vitamin D given should be higher than what is currently outlined in the Institute of Medicine AI and RDA to ensure achievement and maintainance of circulating 25(OH)D concentrations above 75 nmol/L in the majority (≥97.5%) of participants.

The need to provide adequate doses must be balanced with the necessity of also minimizing risk of toxicity in all participants. Regarding age-specific doses, there is very little evidence to guide long-term vitamin D dosing for infants, toddlers, and pre-pubsecent children and some pilot work may need to be conducted to define age-appropriate doses in these children. In the absence of such evidence, 800 IU/d for infants and toddlers and at least 1000 IU/d for those aged 4 to 9 y – are reasonable doses in terms of elevating and maintaining 25(OH)D concentrations within the target range (75-225 nmol/L)\textsuperscript{402} while also having an acceptable safety profile.\textsuperscript{104,395,401} For older children and adolescents aged 10-17 years, a minimum dose of 2,000 IU/d vitamin D is
proposed because of its demonstrated ability to elevate and maintain approximately 90-95% of participants’ 25(OH)D concentrations within the proposed optimal 75-225 nmol/L range.\textsuperscript{399,400} For adults and pregnant women, a minimum dose of 4,000 IU/d is proposed because this dose is reasonably effective at raising 25(OH)D concentrations above 75 nmol/L in the majority of adults, irrespective of baseline 25(OH)D level, and it is also safe.\textsuperscript{154,404,405}

But how would oral vitamin D\textsubscript{3} be provided to participants? Vitamin D could be provided as a dietary supplement; however, supplement interventions are challenged by the expense of long-term provision and distribution of the supplements and by the possibility of attrition – either from participant dropout, loss to follow-up or decreased compliance over the duration of the study. Adherence to supplementation could likely be enhanced by protocols for weekly rather than daily administration of vitamin D.\textsuperscript{406}

While administration of supplemental vitamin D would enable age-appropriate dosing as discussed above, oral supplementation may be most feasible in studies of a limited size and duration. On the other hand, if a multi-year or multi-decade, population-level intervention was required to demonstrate benefit of vitamin D, an evidence-based food-fortification strategy would represent a much more feasible alternative. An evidence-based strategy for an effective vitamin D fortification strategy could be enabled through (i) collection of dietary information via validated food frequency questionnaires\textsuperscript{407,408} from a nationally representative, population-based sample, and (ii) using those dietary data, modelling estimated vitamin D intakes if either new types of foods (e.g. flour, non-dairy milk, oils, cereals) were mandatorily fortified with vitamin D or if the amount of vitamin D in currently mandatorily-fortified foods was elevated.

Overall, the means of provision of oral vitamin D – whether by supplements or via an improved national food fortification strategy – will depend upon the objective and scope of the study. A
Efficacy of Vitamin D Intervention

A high prevalence of vitamin D insufficiency is observed in many populations with high MS prevalence. For instance, approximately 60% or more United Kingdom (UK) residents are estimated to be vitamin D insufficient and, according to the most recent population-based estimates, so are approximately 60% of Canadians. Thus, a substantial proportion of the population of the UK and Canada is considered “exposed” to the risk factor of vitamin D insufficiency or deficiency. Targeting vitamin D interventions within populations with high prevalence of vitamin D insufficiency would result in a large proportion of the population increasing their vitamin D status and thus, having the highest likelihood of producing an observable reduction in MS risk. Germaine to defining target population or sample size is the concept of Population Attributable Fraction (PAF) – defined as the proportion of disease cases that would be prevented over a specified time following elimination of the exposure of interest. PAF assumes a causal relationship between an exposure – in this case, exposure to vitamin D insufficiency (25(OH)D concentrations < 75 nmol/L) – and reduced risk of MS. The PAF for vitamin D insufficiency and risk of MS estimated from the Munger et al. nested case-control study of young adults entering the US military is approximately 0.3 (Appendix 3). What this means is that, if the relationship between vitamin D status and MS outcome is causal, then approximately 30% of MS cases could be prevented by eliminating vitamin D insufficiency in the population of interest – ergo, raising serum 25(OH)D concentrations above 75nmol/L. While an approximate PAF of 30% is certainly meaningful in the context of reducing risk of MS, this
estimate does have its limitations. First, the serum 25(OH)D measures reported by the Munger et al.\textsuperscript{193} were collected in late adolescence and early adulthood – in other words, nearing the end or even past the putative window of MS susceptibility. If vitamin D status earlier in life is key, then the serum 25(OH)D concentrations measured by Munger et al.\textsuperscript{193} may only be rough approximates of the participants’ vitamin D status at the earlier critical time. Thus, the PAF of 0.3 estimated from Munger et al.\textsuperscript{193} may underestimate the true protection afforded by vitamin D sufficiency. Another limitation of the estimated PAF is that it is predicated upon the assumption that a vitamin D intervention will eliminate vitamin D insufficiency in the population of interest. Given (i) the high interpersonal variability in circulating 25(OH)D responsiveness to oral vitamin D intake\textsuperscript{298} and either (ii) the improbability of 100\% compliance with supplementation, or (iii) inadequate vitamin D intake from fortified foods due to uncommon dietary habits resulting in lower than estimated intake of vitamin D for the duration of the study, it is unlikely that 100\% of participants will achieve and maintain circulating 25(OH)D over 75 nmol/L. Thus, while the PAF of 0.3 may underestimate the true efficacy of optimizing vitamin D status in reducing risk of MS, it is still a useful estimate for use in the context of sample size determination.

Consideration of the PAF is, however, only one component of estimating the sample size needed to adequately power a study to detect a statistically significant effect of vitamin D intervention in reducing risk of MS. If the desired power (0.80) and two-sided alpha for significance (0.05) for a proposed intervention are held constant, then the sample size required is a function of the PAF, incidence rate, and length of proposed intervention and observation. Table 8-1 reveals the person years required to detect an effect of a vitamin D intervention given the estimated PAF of 0.3 discussed above and also given varying incidence observed in countries such as Scotland, the UK, Canada, and the US. Higher PAF estimates (e.g. 0.5 and 0.7) are listed in the table.
because of the possibility that eliminating vitamin D insufficiency may actually be more
effective in reducing the cases of MS than the Munger et al.\textsuperscript{193}-based PAF data would suggest as
discussed above.

Table 8-1 indicates that very large sample sizes would be needed for a vitamin D intervention. Table 8-1 also highlights the importance of incidence rate and planned length of study on the sample size needed for an adequately powered intervention. The final consideration in planning a primary prevention study is to address the issues of incidence rate and study duration by defining the target population.
### Table 8-1: Primary Prevention of MS by Vitamin D Intervention: Estimated Sample Sizes and Study Observation Lengths

<table>
<thead>
<tr>
<th>Scenario</th>
<th>Cases Prevented By Vitamin D (%)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Annual Incidence Rate (per 100,000)</th>
<th>Post-Vitamin D Intervention Incidence Rate (per 100,000)</th>
<th>Number of Person Years Needed to Detect Significant Effect</th>
<th>Participants needed for 10 year study</th>
<th>Participants needed for 20 year study</th>
<th>Power</th>
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<td>10</td>
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<td>84.7</td>
</tr>
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<sup>a</sup> See Appendices 3 and 4 for basis of calculations. Models assume the following: (i) The population of interest is within a period of risk modification and disease expression, (ii) Vitamin D Intervention is 100% efficacious in eliminating vitamin D insufficiency in the population, and (iii) 2-sided alpha <0.05 (validated using 412 and 413).

<sup>b</sup> Population Attributable Fraction (PAF): The number of cases reduced by eliminating vitamin D insufficiency, expressed as a percent, where PAF of 0.3 = 30%, 0.5 = 50%, and 0.7 = 70%.
Target Populations

The preceding sections and data from Table 8-1 demonstrate that, regardless of the estimated PAF or incidence rate, a primary prevention vitamin D intervention would require thousands of participants followed for many years. As previously discussed, MS is relatively rare at the population level: Global prevalence is estimated at 30/100,000\(^5\) while estimated MS incidence rates range from 0.28 to 12/100,000 population per year.\(^{414,415}\) In order to adequately power a vitamin D intervention, interventions could be focused on populations at highest risk of MS. One population that could be targeted would be first degree relatives of people with MS who, on average, have an approximately 5\% lifetime risk of MS. This represents a risk that is 16 to 20 fold that of the general population in Scotland, the UK in general, or Canada. Thus, interventions could be restricted to women of childbearing age who have MS or to women planning to bear children with an affected male, or infants, children and adolescents up to age 20 years who have siblings or parents with MS.\(^{416}\) However, these studies would still require the enrollment of thousands of participants. Furthermore, regardless of the MS incidence rate, intervention and observation would have to extend over an absolute minimum of 10 years because (i) the putative window of opportunity – or the time of life within vitamin D intervention is likely to be effective – ranges from the prenatal time through to the end of adolescence, and (ii) the age of MS diagnosis typically extends from age 18 through 45. Thus, in order to ascertain the majority of MS diagnoses, participants would need to be observed from before age 20 past the average age of MS diagnosis. The highest probability of observing an effect of vitamin D on reducing MS risk would come from a study that would extend observation to 30 or 40 years such that the participants who received interventions beginning at the earliest time points in life (e.g. prenatal or infancy) would be observed into and past the time
of life within which they are most likely to be diagnosed with MS. The logistics of conducting such a large, long-term intervention are daunting.

As discussed previously, interventions using evidence-based vitamin D food fortification strategies may serve as an alternative model. Such a strategy would be ideally explored in a country that has a high MS prevalence, high frequency of vitamin D insufficiency, and no current fortification policy. Scotland is ideal candidate for vitamin D intervention for several reasons (represented in Table 8-1 Scenario 1A, 2A, and 3A; discussed in Appendix 4): (i) MS incidence is approximately 10/100,000 per year and lifetime risk of MS may be as high as 0.3% at the population level; (ii) vitamin D insufficiency is prevalent; and (iii) there is currently no national food fortification strategy for vitamin D.

Food fortification is limited in that it would not provide specific doses targeted at each age range. However, an evidence-based approach to fortifying foods with vitamin D could reasonably optimize the oral vitamin D intake of the majority of the population. Fortification strategies could be developed by or in collaboration with the Scottish government by (i) examining the efficacy of fortification policies in other countries, (ii) collecting dietary intake data from a representative sample of the population to estimate food intakes across the lifespan and across various cultural backgrounds, and (iii) modeling the effects of various fortification strategies – both the type of fortified foods and the amounts of vitamin D added – on projected vitamin D intakes by taking into account both the estimated dose-response and the food patterns of those living in Scotland.
Although incidence of MS is lower in Canada, a similar approach could be taken in terms of revising fortification policy. Given that Canadians are not meeting their recommended intake of vitamin D fortified foods nor are Canadians ingesting the recommended vitamin D supplement dosing,\textsuperscript{365,417} the majority of Canadians are vitamin D insufficient.\textsuperscript{332} The Institute of Medicine has very recently revised the vitamin D Dietary Reference Intakes so this is an optimal time to revisit and update vitamin D food fortification policy in Canada.

Although food fortification at the population level does not afford the same level of precision, control and detailed observation that a targeted primary prevention study would, it does provide a realistic opportunity to study or observe the MS risk-reducing potential of vitamin D. If a standardized strategy of capturing incident MS diagnoses could be established and implemented prior to initiation of evidence-based national vitamin D fortification, researchers could compare MS incidence before and after fortification. Although this method has many limitations, it may represent the most pragmatic, equitable and broadly beneficial means of improving population vitamin D status for what may be numerous health outcomes, including MS. Thus, it is reasonable to conceive that the governments of Scotland and Canada could be convinced of the merits of adopting evidence-based vitamin D food-fortification practices given that (i) other autoimmune diseases such as type 1 diabetes mellitus – which has a higher incidence rate than MS and is typically diagnosed during childhood – are also associated with vitamin D status, and a positive impact of vitamin D intervention on reduction of type 1 diabetes could be detected in a shorter time interval; and (ii) disorders that predominantly occur in older adults or the elderly – a portion of the population that is increasing – may also be reduced by vitamin D supplementation;\textsuperscript{90,320,418,419} In addition to the benefit of improved quality of life and health, there could be substantial economic benefits of reducing incidence of diseases such as MS, diabetes, cancer and osteoporosis.
Conclusion

The present work reviews and adds to the growing body of evidence supporting a role of vitamin D in MS biology. The characterization of vitamin D status in a population-based prospective study of Canadian children and adolescents experiencing an initial CNS demyelination attack (ADS) provides direct and novel evidence of an inverse association between vitamin D status at ADS and MS risk in children. Further research will clarify the contribution of – and potential interactions between – vitamin D status, variants in vitamin D pathway genetics, and variants in MS-related vitamin D-responsive genes, such as HLA-DRB1*15 with respect to MS outcome. Considering the high prevalence of vitamin D insufficiency among the Canadians, evidence-based modifications to Canadian vitamin D food fortification practices for the prevention of MS seem timely.
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8.1 Appendix 1: HEC Hanwell’s Contributions

1. Introduction and Background:

Conceived and drafted it in its entirety, including figures and tables (unless reproduced with permission and indicated as such).

2. Comparison of methods for analyzing circulating 25-hydroxyvitamin D concentrations

Co-conceived study design; ran samples on the LIAISON and Roche assays; selected and performed statistical analyses; produced Figures 2A-C and tables except Table 1; interpreted data; provided major manuscript revisions; and inserted additional commentary and updated literature beyond that of the published manuscript for thesis.

3. Pseudovitamin D–Dependent Rickets, HLA-DRB1, and the risk of multiple sclerosis

Interpreted biochemical results including those not discussed in published manuscript; assisted in drafting manuscript; provided critical manuscript revisions; and inserted additional commentary and discussion beyond that of the published manuscript.

4. Vitamin D and multiple sclerosis outcome in children with acquired demyelinating syndromes

Devised study concept and design (to be implemented within Dr. Banwell’s pre-existing national Paediatric Demyelinating Diseases research program); analyzed serum samples for 25-hydroxyvitamin D; computed BMI percentile data; conceived and performed statistical analyses; produced figures and tables; interpreted results; drafted manuscript with major contributions from Dr. Banwell; in revision.

5. Sun exposure questionnaire predicts circulating 25-hydroxyvitamin D concentrations in Caucasian hospital workers in southern Italy

Conceived plan of data analysis; performed statistical analyses; produced figures; interpreted results; and prepared, submitted, and revised manuscript for publication.
8.2 **Appendix 2**: Publications arising from thesis

**Published:**


**Accepted:**

1. Kimball SK, **Hanwell H**, and Banwell B. Vitamin D and Multiple Sclerosis. *Functional Food Reviews*. (Invited Review; Submitted June 10, 2011 and Accepted June 20, 2011)

**In Preparation:**

### 8.3 Appendix 3: Estimated Population Attributable Fraction

<table>
<thead>
<tr>
<th>Country</th>
<th>USA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Population</td>
<td>Young American adult military personnel</td>
</tr>
<tr>
<td>Study design</td>
<td>Nested case-control</td>
</tr>
<tr>
<td>Sample size</td>
<td>MS n = 257 Healthy controls(^2), n = 514</td>
</tr>
</tbody>
</table>

| Estimated Relative Risk (RR) based upon Odds Ratio (OR) | 2.07 (< 75 vs. ≥ 100 nmol) |
| (RR-1)/RR | (2.07-1)/2.07 |
| Proportion of cases exposed to risk factor at that level (p\(d_e\)) | 70/148 |
| Population Attributable Fraction (PAF, p\(d_e\)(RR - 1)/RR\(^1\)) | 0.47*0.51 |

\(^1\) This table outlines a method of estimating the contribution of vitamin D insufficiency to risk of multiple sclerosis (a.k.a. the population attributable fraction, PAF) via extrapolation of data from the single published study that has evaluated vitamin D status (circulating serum 25-hydroxyvitamin D (25(OH)D) concentrations) prior to MS diagnosis. PAF assists in estimating the proportion of cases that could be prevented by eliminating the exposure, which, in this case, is vitamin D deficiency/insufficiency as defined by serum 25(OH)D < 75 nmol/L.

\(^2\) Healthy controls were matched to MS patients by age (±1 year), sex, race/ethnicity (non-Hispanic white, non-Hispanic black, Hispanic, or other), dates of sample collection (±30 days, except for the sample collected after the date of MS onset), and branch of military service (Army, Navy, or Marines).
8.4 Appendix 4: Poisson Distribution & Calculations

Prior to undertaking a study of primary prevention of multiple sclerosis via vitamin D supplementation, one must determine the minimum number of cases needed to detect a significant effect of vitamin D intervention.

\[
d \text{ value (t value for infinite degrees of freedom)} = \frac{|\lambda_o - ((\lambda_o + \lambda_a)/2)| / 2 - 0.5}{\sqrt{((\lambda_o + \lambda_a)/4})}
\]

In Scotland, the event rate without intervention is approximately 10/100,000 annually. Thus, 10 new MS cases would be expected in a 1-year intervention. Because events are rare, independent, and occur with a known average rate (i.e. approximately 10 new MS diagnoses per 100,000 per year, \(\lambda_o\)) it is appropriate to use a Poisson distribution – which is a discrete probability distribution that expresses the probability of a number of independent events occurring within a fixed period of time.

Based upon calculations from data presented in the Munger et al. 2006 paper, intervening with vitamin D to eliminate exposure (i.e. elevate all 25(OH)D levels > 75nmol/L) reduces risk by an estimated 30%. Thus, the expected event rate with vitamin D would be 7 per 100,000 per year (\(\lambda_a\)). The time of life wherein vitamin D supplementation could reduce risk of MS may extend from the prenatal period through to the end of the second decade while the time of life wherein MS is most likely be diagnosed is from ages 18 through 40 years of age. Thus, the minimum time to intervene and prospectively monitor MS outcomes would be 20 years. Supplementation would begin at various stages of life ranging from the prenatal time period through adolescence and continue as they enter into the time of life when they are most likely to be diagnosed with MS. How many people would need to be enrolled for a 20-year study where a difference between 10 per 100,000 (\(\lambda_o\)) and 7 per 100,000 (\(\lambda_a\)) can be detected as statistically significant (2-tailed alpha < 0.05 and power = 80%); \(d\) value must be \(\geq 1.96\).

If PAF = 30% then \(\lambda_a = 0.7\lambda_o\)

To determine the minimum number of cases needed to detect a significant effect of vitamin D intervention, substitute 0.7\(\lambda_o\) for \(\lambda_a\) and solve for \(\lambda_o\)

\[
1.96 = \frac{|\lambda_o - 1.7\lambda_o/2| - 0.5}{\sqrt{(1.7\lambda_o/4)}}
\]

\(\lambda_o = 80\) cases

Incidence = 10/100,000 per year so a one year study would need 800,000 participants to observe 80 cases in an untreated population. Thus, a 20-year study would need to enroll 40,000 participants to observe 80 cases in an untreated population.

Therefore, if PAF is 30% and annual incidence is 10/100,000 a 20 year intervention study needs to enroll 40,000 participants to detect a significant effect of vitamin D intervention on risk of MS.

NOTE: To explore the effect of substituting other estimates of PAF, incidence rates, or varying lengths of observation, substitute new values into the equations (see Table 8-1).
8.5 Appendix 5: Banwell et al. Lancet Neurology Paper
Clinical, environmental, and genetic determinants of multiple sclerosis in children with acute demyelination: a prospective national cohort study

Brenda Banwell, Amit Bar-Or, Douglas L Arnold, Dessa Sadovnick, Sridar Narayanan, Melissa McGowan, Julia O’Mahony, Sandra Magalhaes, Heather Hanwell, Reinhold Vieth, Raymond Teller, Thierry Vincent, Giulio Disanto, George Ebers, Katherine Wambera, Mary B Connolly, Jerome Yager, Jean K Mah, Fran Booth, Guillaume Sebire, David Callen, Brandon Meaney, Marie-Emmanuelle Dilenge, Anne Lortie, Daniela Pohl, Asif Doja, Sunita Venketaswaran, Simon Levin, E Athen MacDonald, David Meek, Ellen Wood, Noel Lowry, David Buckley, Conrad Yim, Mark Awuku, Pamela Cooper, François Grand’Maison, J Burke Baird, Virender Bhan, Ruth Ann Marrie

Summary

Background HLA-DRB1*15 genotype, previous infection with Epstein-Barr virus, and vitamin D insufficiency are susceptibility factors for multiple sclerosis, but whether they act synergistically to increase risk is unknown. We aimed to assess the contributions of these risk factors and the effect of established precursors of multiple sclerosis, such as brain lesions on MRI and oligoclonal bands in CSF at the time of incident demyelination, on development of multiple sclerosis in children.

Methods In our prospective national cohort study, we assessed children who presented with incident CNS demyelination to any of the 16 paediatric health-care facilities or seven regional health-care facilities in Canada. We did univariate and multivariable analyses to assess contributions of HLA-DRB1*15, Epstein-Barr virus, vitamin D status, MRI evidence of brain lesions, and CSF oligoclonal bands as determinants of multiple sclerosis. We used classification and regression tree analyses to generate a risk stratification algorithm for clinical use.

Findings Between Sept 1, 2004, and June 30, 2010, we screened 332 children of whom 302 (91%) were eligible and followed-up for a median of 3·14 years (IQR 1·61–4·51). 63 (21%) children were diagnosed with multiple sclerosis after a median of 127 days (99–222). Although the risk of multiple sclerosis was increased with presence of one or more HLA-DRB1*15 alleles (hazard ratio [HR] 2·32, 95% CI 1·25–4·30), reduced serum 25-hydroxyvitamin D concentration (HR per 10 nmol/L decrease 1·11, 1·00–1·25), and previous Epstein-Barr-virus infection (HR 2·04, 0·99–4·20), no interactions between these variables were detected on multivariate analysis. Multiple sclerosis was strongly associated with baseline MRI evidence of one or more brain lesion (HR 37·9, 5·26–273·85) or CSF oligoclonal bands (6·33, 3·35–11·96), suggesting established disease. One patient diagnosed with multiple sclerosis had a normal MRI scan, and therefore sensitivity of an abnormal MRI scan for multiple sclerosis diagnosis was 98·4%.

Interpretation Risk of multiple sclerosis in children can be stratified by presence of HLA-DRB1*15 alleles, remote Epstein-Barr virus infection, and low serum 25-hydroxyvitamin D concentrations. Similar to previous studies in adults, brain lesions detected on MRI and CSF oligoclonal bands in children are probable precursors to the clinical onset of multiple sclerosis. Children with a normal MRI are very likely to have a monophasic illness.

Funding Canadian Multiple Sclerosis Scientific Research Foundation.

Introduction HLA-DRB1*15 genotype, remote infection with Epstein-Barr virus, and vitamin D insufficiency are possible predisposing factors to multiple sclerosis, but have not previously been assessed in one cohort. Such an investigation would allow their interrelations and relative contributions to be established. Because risk of multiple sclerosis is strongly affected by country of residence during childhood, the contribution of environmental factors to development of this disease can be uniquely explored in children living in an area of high prevalence who have incident demyelination during the time of risk factor acquisition. In addition to consideration of these predisposing factors, clinical, MRI, and laboratory findings at presentation provide predictive information about the likelihood of subsequent disease—although the relative contribution of these features in prediction of multiple sclerosis outcomes in the paediatric population is less well defined than it is for the adult population. Improved identification of children who are very likely to be diagnosed with multiple sclerosis would justify clinical and MRI monitoring for diagnostic confirmation and would enable prompt initiation of targeted treatment. Conversely, identification of children in whom multiple sclerosis is unlikely would substantially reduce concern for the patients, parents, and care providers. We aimed to assess the contribution of predisposing environmental factors and clinical and laboratory findings on development of multiple sclerosis in a national cohort of children in Canada.
Methods

Participants and study design

In our prospective national cohort study of incident demyelination in children, we obtained comprehensive clinical, laboratory, and MRI data to examine the contribution and interrelationships of \( HLA-DRB1*15 \), remote Epstein-Barr-virus infection, and vitamin D status as predisposing factors and clinical features, MRI images, and oligoclonal bands as predictors of multiple sclerosis. We developed a decision tree to aid in counselling regarding multiple sclerosis risk.

All 16 Canadian paediatric health-care facilities and seven additional regional health-care facilities (located >3 h from a paediatric facility) participated in this study, following ethics approval from ethics boards at every site. Children aged younger than 16 years were eligible if they presented to one of the facilities with neurological deficits and MRI findings that were consistent with an acute demyelinating syndrome (defined in webappendix pp 1–2), and were enrolled within 90 days of symptom onset. Guardians of all children and children who were old enough to comprehend the consent process (typically ≥12 years) provided informed written and verbal consent; parents or legal guardians of younger children provided assent. Children aged 16–17 years provided assent with informed written and verbal consent. We obtained assent from younger children.

We developed a decision tree to aid in counselling regarding multiple sclerosis risk.

Procedures

To ensure consistency of the data, site investigators (paediatric neurologists or paediatricians) attended training sessions provided by BB, AB-O, DLA, and DS, and used standardised case report forms to record clinical data. Data were entered centrally by trained staff at The Hospital for Sick Children (Toronto, ON, Canada). One investigator (BB) used a-priori criteria (based on the neurological examination and without reference to neuroimaging features) to delineate whether the clinical features of acute demyelinating syndrome were attributable to one site within the CNS (clinically monofocal disease) or more than one CNS site (clinically polyfocal disease), or whether the child met criteria for acute disseminated encephalomyelitis (polyfocal deficits plus encephalopathy).

Serum and DNA blood samples were obtained up to 90 days before symptom onset, shipped on the day of procurement, and processed centrally with standardised protocols. Serum samples were stored at −80°C until batched analysis, which was done masked to clinical data. We established concentrations of serum 25-hydroxyvitamin D (the biomarker of vitamin D status) with the automated chemiluminescent LIAISON 25-hydroxyvitamin D total assay (DiaSorin, Stillwater, MN, USA). To establish vitamin D status at the time of acute demyelination, we assessed samples obtained within 40 days of symptom onset. We categorised the season of sample collection as winter (Jan 1–March 31), spring (April 1–June 30), summer (July 1–Sept 30), or autumn (Oct 1–Dec 31).

We detected serum IgG antibodies directed against Epstein-Barr virus capsid antigens, nuclear antigens (EBNA1), and early antigens using standardised ELISA kits (DiaSorin). Remote Epstein-Barr-virus infection was defined by the presence of antibodies against Epstein-Barr virus capsid antigens and EBNA1, and anti-EBNA1 IgG titres were established as previously described. We assayed neuromyelitis optica IgG antibodies by indirect immunofluorescence using primate cerebellar sections with diluted sera (1 in 50) following the manufacturer’s instructions (Instrumentation Laboratory, Lexington, MA, USA), and aquaporin-4 antibodies were quantified using a cell-based assay. Total genomic DNA was extracted from whole blood. We established \( HLA-DRB1*15 \) alleles by use of an allele-specific PCR amplification method. When clinically indicated, lumbar punctures were done and CSF was analysed locally. We recorded total CSF white-blood-cell count, presence of oligoclonal bands (defined as those not present in concurrently processed serum), and the method of oligoclonal band analysis. CSF IgG index was not obtained with sufficient consistency to be included.

For radiological assessment, standardised MRI protocols were optimised at all sites. All participants were offered brain MRI at baseline, 3 months, 6 months, and 12 months, and at a second demyelinating event. We also obtained data from brain and spine scans that were done for clinical reasons.

Identifiable participant characteristics on scans were removed and the anonymous scans were analysed centrally at the McConnell Brain Imaging Centre and Montreal Neurological Institute (Montreal, QC, Canada). Baseline brain MRI scans were scored for the presence or absence of T2 lesions and serial scans were scored for presence of new T2 lesions or gadolinium-enhancing T1 lesions according to criteria for lesion dissemination in time. We assessed spinal cord images for focal lesions or longitudinally extensive lesions that spanned more than three spinal segments.

All participants were examined quarterly in the first year, and once per year thereafter, and at the time of second demyelinating event if applicable. The primary outcome was a diagnosis of multiple sclerosis. Diagnoses of neuromyelitis optica, relapsing demyelination at a single site (optic nerves or spinal cord), and recurrent or multiphasic acute disseminated encephalomyelitis were conferred according to established criteria (see webappendix pp 1–2).

Statistical analysis

We estimated a sample size of 300 participants would be needed on the basis of the assumption that 25% of participants would be diagnosed with multiple sclerosis (β=0.80, α=0.05, seven-to-nine independent variables of interest and a low-to-medium multiple correlation coefficient between variables of 0·2–0·3). Categorical variables are reported as frequency (%) and continuous variables as mean (SD) or median (IQR). For univariate
analyses, we did Kaplan-Meier analysis, χ² tests, Fisher’s exact tests, t tests, and Wilcoxon or Kruskal-Wallis tests as appropriate. For multivariable analysis, we constructed Cox proportional hazards models, where zero time was initial symptom onset and the event of interest was multiple sclerosis (defined at the earliest date of MRI or clinical confirmation). We censored all other participants at the date of database lock for analysis, the last study visit before withdrawal, or loss to follow-up, whichever came first. The proportional hazards assumption was tested using time-dependent covariates and graphical methods. We assessed linearity of continuous variables with Martingale residuals and model fit by analysis of residuals. We report unadjusted and adjusted hazard ratios (HRs) and 95% CI as measures of association between multiple sclerosis and age at symptom onset (modelled continuously with age in years), sex, phenotype at presentation, presence or absence of one or more T2 lesions on baseline brain MRI, presence or absence of one or more positive HLA-DRB1*15 alleles, serum 25-hydroxyvitamin D concentration (modelled continuously), and remote Epstein-Barr virus exposure. We report the c statistic for the comparable logistic regression models as measures of model discrimination.

We used classification and regression-tree analysis (CART), which uses a non-parametric binary recursive partitioning method to produce a decision tree that identifies homogeneous subgroups of patients at different risks of a disease state (ie, multiple sclerosis). For a continuous variable, CART searches through the full range of values and finds the best cutpoint. The decision tree stratified all patients according to the presence or absence of T2 brain lesions at onset, the age chosen as the optimal cut-point by the CART programme (≤11·85 years or >11·85 years), and clinical presentation with or without acute disseminated encephalomyelitis. Remote Epstein-Barr-virus infection, HLA-DRB1*15 status, and serum 25-hydroxyvitamin D concentration were not retained in the decision tree, which is consistent with baseline clinical and MRI features that suggest a pathobiology already influenced by previous exposure to these prognostic factors. We used the Gini index as our splitting criterion, needing improvements of 0.001 or more to split the nodes. To reduce the risk of overfitting, we used leave-one-out cross-validation. Statistical analyses were done with SAS version 9.2 and PASW version 18.0.

Role of the funding source
This study was funded by the Multiple Sclerosis Society of Canada Scientific Research Foundation. The funding source had no role in study design, data collection, data interpretation, or data analysis, in the writing of the report, or in the decision to submit for publication. BB and RAM had full access to all of the data in the study and BB had final responsibility for the decision to submit for publication.

Figure 1: Participant characteristics
Children who have an initial event that meets criteria for ADEM have to have two other non-ADEM events to confer a diagnosis of multiple sclerosis. ADEM=acute disseminated encephalomyelitis. *Lasting >24 h and within 365 days of initial symptom onset. †Lesions on baseline brain MRI or new lesion accrual on serial MRI. ‡New MRI lesions >3 months after acute demyelination, as per criteria for dissemination in time. §Diagnosed on the basis of optic neuritis, longitudinally extensive (lesion spanning >3 spinal segments) transverse myelitis, or both, and serological evidence of antibodies directed against aquaporin-4. ¶Optic neuritis >3 months after ADEM, but no subsequent non-ADEM events. ‖Dissemination in time.

Results
Between Sept 1, 2004, and June 30, 2010, we enrolled 332 children (figure 1), of whom 302 were eligible and followed up for more than 3 years (table 1). Six (2%) participants withdrew from the study after a median of 93 days (IQR 85–187) of follow-up, and data were censored at time of withdrawal. Of 302 eligible patients with acute demyelinating syndrome, 63 (21%) were diagnosed with multiple sclerosis (24 by MRI evidence of dissemination in time only). Median time to a second clinical event or change in the status of onset was 127 days (IQR 91–222). 50 (79%) of these 63 participants were diagnosed with multiple sclerosis within 365 days of onset. 245 (81%) of 302 participants were treated with corticosteroids, but this did not differ between the 49 (78%) of 63 children diagnosed with multiple sclerosis and the 191 (83%) of 231 children who were not (p=0·17). No participants were treated with disease-modifying therapies before confirmation of their diagnosis of multiple sclerosis.
Female sex and older age at symptom onset were associated with an increased risk of multiple sclerosis. However, although we explored the contribution of race, ancestry, and family history to multiple sclerosis risk, very large populations would be required to fully assess such determinants (table 1). The likelihood of subsequent multiple sclerosis diagnosis differed by phenotype at presentation (table 1, webappendix p 7). Compared with children who presented with polyfocal deficits but no encephalopathy, those children with polyfocal deficits and encephalopathy (ie, acute disseminated encephalomyelitis) were less likely to be diagnosed with multiple sclerosis (HR 0·10; 0·03–0·29). Mean age at onset of acute disseminated encephalomyelitis was 6·46 years (SD 4·2) compared with 10–7 years (4·1) for polyfocal disease (p<0·0001) and 10–6 years (4·1) for monofocal disease (p<0·0001). Mean follow-up in children who had acute disseminated encephalomyelitis at onset was 2·82 years (1·55), compared with 2·82 years (1·53) for polyfocal disease and 3·06 years (1·65) for monofocal disease. Only four (5%) of 77 children with acute disseminated encephalomyelitis were diagnosed with multiple sclerosis (HR for children with vs without acute disseminated encephalomyelitis 0·18, 95% CI 0·07–0·50).

On univariate analysis, the presence of one or more T2 lesions on initial brain MRI was strongly associated with multiple sclerosis (table 2). After adjustment for age at onset, presence of one or more T2 lesions remained associated with increased risk of multiple sclerosis (HR 44·39, 95% CI 6·16–319·98; χ² statistic 0·85). Only one of the 61 patients diagnosed with multiple sclerosis who underwent baseline MRI had normal brain imaging at onset; the sensitivity of an abnormal MRI for a diagnosis

<table>
<thead>
<tr>
<th></th>
<th>Overall (n=302)</th>
<th>Monophasic ADS (n=231)</th>
<th>Multiple sclerosis (n=63)</th>
<th>Hazard ratio (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Follow-up (years)</td>
<td>3·14 (1·61–4·51)</td>
<td>3·06 (1·48–4·53)</td>
<td>3·51 (2·51–4·53)</td>
<td>NS</td>
</tr>
<tr>
<td>Age at onset (years)</td>
<td>9·55 (4·5)</td>
<td>8·85 (4·5)</td>
<td>12·0 (3·8)</td>
<td>1·18 (1·10–1·27)</td>
</tr>
<tr>
<td>Age at multiple sclerosis diagnosis (years)</td>
<td>12·6 (3·9)</td>
<td>1·0 (reference)</td>
<td>1·0 (reference)</td>
<td></td>
</tr>
<tr>
<td>Phenotype at ADS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Monofocal</td>
<td>173 (57%)</td>
<td>122 (57%)</td>
<td>36 (57%)</td>
<td>1·0 (reference)</td>
</tr>
<tr>
<td>Polyfocal</td>
<td>52 (17%)</td>
<td>29 (13%)</td>
<td>23 (37%)</td>
<td>2·34 (1·39–3·95)</td>
</tr>
<tr>
<td>ADEM</td>
<td>77 (25%)</td>
<td>70 (30%)</td>
<td>4 (6%)</td>
<td>0·23 (0·08–0·65)</td>
</tr>
<tr>
<td>Female</td>
<td>156 (52%)</td>
<td>112 (48%)</td>
<td>41 (65%)</td>
<td>1·87 (1·11–3·14)</td>
</tr>
<tr>
<td>Female-to-male ratio</td>
<td>1·1</td>
<td>1·0 (reference)</td>
<td>1·0 (reference)</td>
<td></td>
</tr>
<tr>
<td>Place of birth†</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Canada</td>
<td>277 (94%)</td>
<td>212 (94%)</td>
<td>58 (94%)</td>
<td>0·91 (0·33–2·52)</td>
</tr>
<tr>
<td>Other</td>
<td>18 (6%)</td>
<td>13 (6%)</td>
<td>4 (6%)</td>
<td>1·0 (reference)</td>
</tr>
<tr>
<td>Parental place of birth‡</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mother</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Canada</td>
<td>201 (67%)</td>
<td>153 (67%)</td>
<td>43 (69%)</td>
<td>1·12 (0·66–2·33)</td>
</tr>
<tr>
<td>Other</td>
<td>99 (33%)</td>
<td>72 (33%)</td>
<td>13 (31%)</td>
<td>1·0 (reference)</td>
</tr>
<tr>
<td>Father</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Canada</td>
<td>198 (67%)</td>
<td>154 (68%)</td>
<td>40 (65%)</td>
<td>0·89 (0·53–1·50)</td>
</tr>
<tr>
<td>Other</td>
<td>98 (33%)</td>
<td>72 (32%)</td>
<td>22 (35%)</td>
<td>1·0 (reference)</td>
</tr>
<tr>
<td>Familial origin§</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>European ancestry</td>
<td>194 (66%)</td>
<td>151 (67%)</td>
<td>39 (63%)</td>
<td>1·13 (0·67–1·89)</td>
</tr>
<tr>
<td>Non-European ancestry</td>
<td>101 (34%)</td>
<td>73 (33%)</td>
<td>23 (37%)</td>
<td>1·0 (reference)</td>
</tr>
<tr>
<td>Race¶</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>White</td>
<td>251 (85%)</td>
<td>193 (85%)</td>
<td>52 (84%)</td>
<td>1·03 (0·52–2·03)</td>
</tr>
<tr>
<td>Non-white</td>
<td>46 (15%)</td>
<td>34 (15%)</td>
<td>10 (16%)</td>
<td>1·0 (reference)</td>
</tr>
<tr>
<td>Family history of multiple sclerosis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Any</td>
<td>42 (14%)</td>
<td>32 (14%)</td>
<td>10 (16%)</td>
<td>1·14 (0·58–2·24)</td>
</tr>
<tr>
<td>First-degree relative</td>
<td>3 (9%)</td>
<td>3 (9%)</td>
<td>2 (3%)</td>
<td>1·0 (reference)</td>
</tr>
<tr>
<td>Second-degree relative</td>
<td>14 (5%)</td>
<td>11 (5%)</td>
<td>3 (5%)</td>
<td>1·0 (reference)</td>
</tr>
<tr>
<td>Third-degree relative</td>
<td>27 (9%)</td>
<td>21 (9%)</td>
<td>6 (10%)</td>
<td>1·0 (reference)</td>
</tr>
</tbody>
</table>

Data are median (IQR), mean (SD), or n (%), unless otherwise stated. ADS=acute demyelinating syndrome. NS=not significant. ADEM=acute disseminated encephalomyelitis (ie, polyfocal deficits with encephalopathy). *Eight children with recurrent demyelination did not meet criteria for multiple sclerosis (figure 1) and are not included in the monophasic acute demyelinating syndrome or multiple sclerosis columns. No data for five participants; other group consists of three children born in the USA, two in Bangladesh, and one in each of China, Ecuador, Egypt, France, India, Israel, Kazakhstan, Mexico, Philippines, Saudi Arabia, South Korea, Sri Lanka, and Sweden. †No data for three mothers and six fathers. ‡No data for seven families. ¶No data for five participants.
of multiple sclerosis was 98·4%. Two (2%) of 103 children with normal brain MRI scans at onset had recurrent optic neuritis and two had recurrent transverse myelitis with persistently normal brain MRI scans. Of 223 children who underwent MRI and who were not diagnosed with multiple sclerosis or recurrent non-multiple sclerosis demyelination, 98 had normal brain MRI (43·9% specificity). After exclusion of children with initial presentations that met criteria for acute disseminated encephalomyelitis, 56 of 116 children with one or more lesion on brain MRI were diagnosed with multiple sclerosis (48·3% positive predictive value), corresponding

<table>
<thead>
<tr>
<th>All*</th>
<th>Monophasic ADS</th>
<th>Multiple sclerosis</th>
<th>Hazard ratio (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Brain MRI</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥1 T2 lesion</td>
<td>188/291 (65%)</td>
<td>125/223 (56%)</td>
<td>60/61 (98%)</td>
</tr>
<tr>
<td>Spine MRI†</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Any lesion</td>
<td>77/98 (79%)</td>
<td>60/74 (81%)</td>
<td>15/21 (71%)</td>
</tr>
<tr>
<td>Focal lesion(s)</td>
<td>17/77 (22%)</td>
<td>15/60 (25%)</td>
<td>11/15 (73%)</td>
</tr>
<tr>
<td>Longitudinal extension</td>
<td>44/77 (57%)</td>
<td>44/60 (75%)</td>
<td>4/15 (27%)</td>
</tr>
<tr>
<td><strong>CSF oligoclonal bands‡</strong></td>
<td>44/170 (26%)</td>
<td>18/123 (15%)</td>
<td>24/60 (60%)</td>
</tr>
<tr>
<td><strong>CSF white blood cell count</strong></td>
<td>225/291 (77%)</td>
<td>178/223 (80%)</td>
<td>29/61 (48%)</td>
</tr>
<tr>
<td>Cells per μL</td>
<td>44·8 (70·7)</td>
<td>49·2 (76·6)</td>
<td>25·9 (35·7)</td>
</tr>
<tr>
<td><strong>Serum sample</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Obtained within 40 days</td>
<td>214/242 (90%)</td>
<td>175/189 (92%)</td>
<td>41/48 (83%)</td>
</tr>
<tr>
<td>Obtained within 90 days</td>
<td>240/242 (99%)</td>
<td>188/189 (99%)</td>
<td>47/48 (98%)</td>
</tr>
<tr>
<td><strong>Epstein-Barr virus serology</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Remote infection</td>
<td>120/240 (50%)</td>
<td>82/185 (44%)</td>
<td>37/49 (76%)</td>
</tr>
<tr>
<td>Recent infection</td>
<td>7/240 (3%)</td>
<td>4/184 (2%)</td>
<td>3/49 (6%)</td>
</tr>
<tr>
<td><strong>Epstein-Barr virus nuclear antigen</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Titre (AU/mL)§</td>
<td>178·1 (17·3–234·0)</td>
<td>153·8 (6·9–223·2)</td>
<td>212·0 (104·2–251·6)</td>
</tr>
<tr>
<td>Tertiles</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤76·6</td>
<td>56/170 (33%)</td>
<td>45/125 (36%)</td>
<td>8/42 (19%)</td>
</tr>
<tr>
<td>76·7–213·8</td>
<td>57/170 (34%)</td>
<td>42/125 (34%)</td>
<td>15/42 (37%)</td>
</tr>
<tr>
<td>≥213·9</td>
<td>57/170 (34%)</td>
<td>38/125 (30%)</td>
<td>18/42 (44%)</td>
</tr>
<tr>
<td><strong>HLA-DRB1*15</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>One allele</td>
<td>82/279 (29%)</td>
<td>54/212 (25%)</td>
<td>26/60 (43%)</td>
</tr>
<tr>
<td>Both alleles</td>
<td>15/279 (5%)</td>
<td>10/212 (5%)</td>
<td>5/60 (8%)</td>
</tr>
<tr>
<td>One or more alleles</td>
<td>97/279 (35%)</td>
<td>64/212 (30%)</td>
<td>31/60 (51%)</td>
</tr>
<tr>
<td><strong>Neuromyelitis optica IgG</strong></td>
<td>2/279 (1%)</td>
<td>0/212</td>
<td>0/59</td>
</tr>
<tr>
<td><strong>Serum 25-hydroxyvitamin D concentrations within 40 days of onset</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean concentration (nmol/L)¶</td>
<td>63·9 (28·6)</td>
<td>66·4 (20·9)</td>
<td>52·3 (23·1)</td>
</tr>
<tr>
<td>Tertiles (nmol/L)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤49·8</td>
<td>72/216 (33%)</td>
<td>51/175 (29%)</td>
<td>21/41 (51%)</td>
</tr>
<tr>
<td>49·9–74·0</td>
<td>72/216 (34%)</td>
<td>60/175 (34%)</td>
<td>12/41 (29%)</td>
</tr>
<tr>
<td>≥74·1</td>
<td>72/216 (33%)</td>
<td>64/175 (37%)</td>
<td>8/41 (20%)</td>
</tr>
</tbody>
</table>

Data are number of positive tests/number of participants for whom a specific investigation was done and available for centralised assessment (%), mean (SD), or median (IQR), unless otherwise stated. ADS=acute demyelinating syndrome. AU=arbitrary units. *For 12 participants, only clinical examination and clinically obtained MRI studies were recorded, and four patients consented to clinical follow-up only, but their characteristics did not differ from other participants, eight children with recurrent demyelination did not meet criteria for multiple sclerosis (figure 1) and are not included in the monophasic ADS or multiple sclerosis columns, but are summarised in webappendix p 6. †Spine MRI was available for 98 participants with clinical transverse myelitis or for whom spinal cord imaging was done as part of their assessment for demyelination; compared with focal transverse myelitis, longitudinally extensive transverse myelitis was associated with a lower risk of MS (hazard ratio 0·17, 95% CI 0·05–0·59), even after adjustment for age (the mean age of children with ≥1 longitudinally extensive spinal lesion was 8·28 years (SD 4·9) compared with 12·1 years (3·8) for children with focal lesions only [p=0·001]). ‡We did CSF analysis with isoelectric focusing in 130 children, with immunofixation in 14, and by protein electrophoresis in 23; in ten children, the method of analysis was not specified, in centres without access to isoelectric focusing methods, the frequency of oligoclonal bands might be underestimated. §We analysed anti-Epstein-Barr-virus nuclear antigen titres for all children with serological evidence of remote Epstein-Barr-virus infection and sufficient serum for analysis. ¶Hazard ratios per 10 nmol/L increase in 25-hydroxyvitamin D concentration.

Table 2: Laboratory and imaging features at presentation: univariate analyses
to a more than 60-fold increased risk (HR 61.23, 95% CI 8.47–442.57). Conversely, of these 116 children without acute disseminated encephalomyelitis, 96 had a normal brain MRI, of whom 95 were not diagnosed with multiple sclerosis (98.9% negative predictive value).

Baseline MRI status was a more robust correlate of multiple sclerosis than was clinical phenotype (webappendix p 7).

44 (26%) of 170 children tested had CSF oligoclonal bands (table 2), which were most common in children with polyfocal demyelination (16 [44%] of 36 children), followed by children with monofocal demyelination (22 [27%] of 108 children), and then children with acute disseminated encephalomyelitis (eight [19%] of 42 children, p=0.04). Of those tested, 24 (60%) of 40 children diagnosed with multiple sclerosis had oligoclonal bands compared with 18 (14%) of 123 of those not diagnosed with multiple sclerosis (HR 6.33; 95% CI 3.35–11.96). 24 (57%) of 42 children with positive oligoclonal bands were diagnosed with multiple sclerosis.

Compared with 49 children with negative bands and no lesions on MRI, the 39 children with positive oligoclonal bands and MRI lesions had a much greater likelihood of diagnosis of multiple sclerosis (HR 45.89, 95% CI 6.18–340.58). In 51 children with no lesions on MRI, only two (4%) had positive oligoclonal bands, but neither was diagnosed with multiple sclerosis.

146 (68%) of 216 participants analysed had serum 25-hydroxyvitamin D concentrations of less than 75 nmol/L. Concentrations seemed to be lower in the winter (median 53.8 nmol/L, IQR 37.8–71.2) and spring (59.2, 42.3–74.8) than they were in the summer (71.5, 49.8–89.7) and autumn (68.7, 42.6–87.0), but this was not a significant difference (p=0.06). Risk of multiple sclerosis did not differ by season of presentation of acute demyelinating syndrome.
Age at onset >11.85 years predicted risk of 28.1%. Those under the age 11.85 years have a 60.6% predicted risk of multiple sclerosis, whereas those older than 11.85 years have a 28.1% predicted risk of multiple sclerosis. For children with a presentation of an acute demyelinating syndrome other than acute disseminated encephalomyelitis or normal brain MRI, our multivariable models showed that HLA-DRB1*15 alleles were associated with development of multiple sclerosis, and interactions between these two predisposing factors were detected. After adjustment for age at onset, the presence of HLA-DRB1*15 alleles remained associated with development of multiple sclerosis, although this percentage will probably increase with time, these three factors are unlikely to be wholly sufficient for the development of multiple sclerosis.

Discussion
In our nationwide prospective cohort study of children with incident demyelination, the presence of HLA-DRB1*15 alleles, remote Epstein-Barr-virus infection, and low 25-hydroxyvitamin D concentrations were independently associated with development of multiple sclerosis, although this percentage will probably increase with time, these three factors are unlikely to be wholly sufficient for the development of multiple sclerosis. Although we cannot exclude possible biological interactions, we did not detect additive or multiplicative interactions between HLA-DRB1*15 genotype, remote Epstein-Barr-virus infection, and low serum 25-hydroxyvitamin D concentrations on multivariate analysis. A larger sample size might show such interactions. In a post-hoc analysis we assessed risk of multiple sclerosis in children with all three factors versus those with none; the absence of all three predisposing factors conveyed a notably low risk of multiple sclerosis, and they distinguish children with monophasic demyelination from those with a sentinel multiple sclerosis attack, and that they convey risk independently of one another. Webappendix pp 3–4 compares our study with previously published work that explored risk determinants for multiple sclerosis in children.

We generated the algorithm by classification and regression-tree analysis (CART). Use of the algorithm provides risk estimates of 1.9–60.6%. The classification accuracy is 83.7%. ADEM=acute disseminated encephalomyelitis.

Figure 2: Multiple sclerosis risk stratification algorithm for children presenting with an acute demyelinating syndrome
We generated the algorithm by classification and regression-tree analysis (CART). Use of the algorithm provides risk estimates of 1.9–60.6%. The classification accuracy is 83.7%. ADEM=acute disseminated encephalomyelitis.
need replication, but raise the possibility that vitamin D status either modifies the effect of other prognostic factors or that vitamin D status influences the degree of disease activity or time to relapse. Webappendix p 5 summarises data for the presence or absence of every predisposing factor in children diagnosed with multiple sclerosis.

Our approach to the data was to assess predisposing factors as predictors of multiple sclerosis. MRI evidence of brain lesions and CSF oligoclonal bands were also assessed as predictors of multiple sclerosis, but were not regarded as predisposing factors. Our first statistical model of the eventual diagnosis of multiple sclerosis was designed to show an association with the predisposing factors (table 3, webappendix p 8). Because abnormal brain MRI and CSF oligoclonal bands were assumed to show an already established multiple sclerosis biology, the predictive power of these variables should be strong. The good association that we noted between abnormal brain MRI and subsequent diagnosis of multiple sclerosis, and the nearly 99% negative predictive power of a normal brain MRI at initial presentation, coupled with the increased prevalence of oligoclonal bands in children diagnosed with multiple sclerosis (HR 6.33, 95% CI 3.35–11.96) support this assertion and its clinical relevance. Separate models should show associations between the predisposing factors and the presenting clinical and paraclinical characteristics. In a logistic-regression model adjusting for age at onset, remote Epstein-Barr-virus infection was associated with a decreased risk of an acute disseminated encephalomyelitis compared with non-acute disseminated encephalomyelitis presentation (OR 0.91, 95% CI 0.98–3.73). Finally, predisposing factors might initiate a disease process that is initially subclinical, thus influencing clinical and MRI features and the likelihood of CSF oligoclonal bands at the time of presentation with an acute demyelinating syndrome. If so, we would expect that inclusion of predisposing factors and clinical or paraclinical characteristics in the same model would result in the clinical and paraclinical features being so powerfully associated with the outcome that the contribution of predisposing factors could be obscured. In support of this concept, when MRI was included in the multivariate model, HLA status was no longer retained as a variable (table 3), and when clinical presentation was included (specifically with or without acute disseminated encephalomyelitis), remote Epstein-Barr-virus infection was no longer retained. However, vitamin D status was retained even in the presence of clinical and paraclinical data.

Special consideration needs to be given to children with acute disseminated encephalomyelitis. In our study, this group was younger than children with polyfocal or monofocal acute demyelinating syndrome. Only four children (6%) who were diagnosed with multiple sclerosis had acute disseminated encephalomyelitis. The frequency of multiple sclerosis in children presenting with acute disseminated encephalomyelitis in our study differs from a French study" of 132 children with acute disseminated encephalomyelitis, of whom 24 (18%) were diagnosed with multiple sclerosis (mean observation 5.4 years [SD 3.3]). Our requirement of clear documentation of encephalopathy as a useful criterion for diagnosis of acute disseminated encephalomyelitis (and all acute demyelinating syndrome presentations) on the basis of clinical features only (as is permitted in the diagnostic criteria"), might have contributed to these differences. Compared with children presenting with polyfocal deficits without encephalopathy, children with polyfocal deficits with encephalopathy (ie, acute disseminated encephalomyelitis) were less likely to be diagnosed with multiple sclerosis, supporting the requirement for encephalopathy as a useful criterion for diagnosis of...
monophasic disease. Our decision not to assess MRI features as part of the adjudication of clinical presentation was intended to allow determination of the role of MRI as an independent correlate of multiple sclerosis outcome.

Finally, we created a simple decision tree to help clinicians to stratify multiple sclerosis risk on the basis of clinical and MRI information that would be readily available at presentation with acute demyelinating syndrome (figure 2). Although the presence of T2 lesions is straightforward to adjudicate on standard imaging, more complex scoring of MRI scans for lesion dissemination in space6 might have added specificity, but such criteria are not commonly used by paediatric health providers and can be described incorrectly.29 Our proposed decision tree will also help to provide appropriate counselling of families and selection of children for surveillance such as serial MRI.

Our study has several limitations. Although we aimed to enrol all eligible Canadian children, those with mild symptoms might not have reported to paediatric health-care facilities. However, we believe that few children with key symptoms were missed because every Canadian paediatric health-care facility participated. Moreover, for 3 years, we used the reporting system of the Canadian Paediatric Society (CPS) to establish the incidence of acute demyelination in Canadian children.21 In the final year of the CPS study, enrolment at all 23 sites in the present study occurred concurrently, and we obtained data for 98% of children reporting to the CPS.21 Although every effort was made to obtain all clinical, laboratory, and MRI data at all times and according to the rigorous protocol parameters designed for the study, we did not succeed for all participants, reflecting the challenges inherent in such work in the paediatric context. This issue was particularly important when considering spinal fluid analyses. We did not do lumbar punctures for research, but were allowed access to information about CSF oligoclonal bands for patients for whom such testing was done on clinical indication. Therefore, children from whom spinal fluid was obtained differed clinically from those whose clinical status at presentation of acute demyelinating syndrome did not prompt lumbar puncture. We also acknowledge that some of the children presently classified as having monophasic demyelination will ultimately be reclassified as having multiple sclerosis. Apart from the 16 children with brain MRI lesions who are presently classified as having monophasic demyelination, the absence of baseline lesions or new lesions on serial MRI over the first year, and published data indicating a short interval between the first and second attacks (typically <12 months) in paediatric multiple sclerosis,19–24 reduces the likelihood of misclassification of outcome in our cohort. Moreover, such misclassification would bias our findings towards the null hypothesis. We used CART to develop our decision tree, which is a data-driven technique and is sensitive to the actual dataset, Thus, assessment of the decision tree in an independent cohort is necessary. Finally, we did not assess all putative predisposing factors, but focused on HLA-DRB1*15, Epstein-Barr virus, and vitamin D status because sufficient evidence exists to implicate these factors as biologically plausible.

The presence of HLA-DRB1*15 alleles is an inherited risk factor for multiple sclerosis, whereas vitamin D insufficiency and exposure to Epstein-Barr virus are acquired environmental risks that might contribute through altering expression of immune-related genes25 or immune behaviour through, for example, viral persistence in human B cells and subsequent viruses-influenced immunological responses.14,15,26,27 T cell and B cell responses to myelin and other antigens are raised in children with acute demyelinating syndrome and confirmed multiple sclerosis,19,23 but studies incorporating knowledge of the HLA-DRB1*15, Epstein-Barr virus, and vitamin D status of the child have not been done. Such studies might yield new insights into how predisposing factors contribute to multiple sclerosis biology.

Contributors BB and RAM were responsible for the core design and content of the report and had access to all aspects of the data. AB-O provided comprehensive editorial and content review. BB, AB-O, RAM, DLA, and DS served as the principal investigators of the Canadian Paediatric Demyelinating Disease study, were responsible for obtaining the grant funding, reviewed all aspects of the data, and provided in-depth edits to the final report. SN was involved in the neuroimaging analyses. MMG, SM, and JO’M have participated in data analyses in their capacity as study staff. GE and GD were responsible for the genetic HLA analyses. TV did the neuroradiology optica assays, and RD did the viral studies. HH did the vitamin D assays as a component of her doctoral work, with RV and BB as supervisors. BB, KW, MBC, JY, JKM, FB, GS, DC, BM, M-ED, AI, DP, AD, SV, SJ, EAMD, DM, EW, NI, DB, CY, MA, PC, FG’M, JBB, and VB were site investigators, were responsible for enrolment of participants at their sites, and have reviewed and approved the content of the manuscript.

Conflicts of interest BB, AB-O, DLA, DS, and RAM serve as lead investigators and funds from the study grant (Multiple Sclerosis Scientific Research Foundation) have supported work done at their institution. None of the investigators receives personal salary support from the study sponsor. Funds from the study grant have supported travel for presentations at national and international meetings. BB has received speaker’s honoraria from Merck-Serono, Biogen-IDEC, Bayer Healthcare, and Teva Neuroscience, and serves as an adviser on paediatric therapies for Biogen-IDEC, Merck-Serono, and Genzyme. BB and AB-O are supported by the Multiple Sclerosis Society of Canada (MSSC) and the Canadian Multiple Sclerosis Scientific Research Foundation, and by a New Emerging Team Grant in Autoimmunity supported by the Canadian Institutes of Health Research and MSSC. AB-O has received consultancy fees from Bayhill Therapeutics, Berlex, Biogen-IDEC, BioMS, Diogenix, Eli-Lilly, Genentech, GlaxoSmithKline, Guthy-Jackson/GGF, Merck-Serono, Novartis, Roche, Teva Neuroscience, and Wyeth. AB-O has received speaker’s honoraria from Bayer, Bayhill Therapeutics, Berlex, Biogen-IDEC, BioMS, Diogenix, Eli-Lilly, Genentech, GlaxoSmithKline, Guthy-Jackson/GGF, Merck-Serono, Novartis, Ono, Roche, Teva Neuroscience, and Wyeth. AB-O has funding from the US National Institutes of Health, Canadian Institutes of Health Research, the Canadian Multiple Sclerosis Society, Biogen-IDEC, and Teva Neuroscience for research unrelated to the present project. DLA has served as a speaker at meetings or as a consultant for Bayer, Biogen,
Articles

Elan, GlaxoSmithKline, Roche, and Teva Neuroscience. DLA receives financial remuneration and stock options from NeuroRx Research.

DS has received speaker’s honoraria from Biogen, Merck-Serono, Teva Neuroscience, and Bayer and has grant support (unrelated to the present grant funding) from Canadian Institutes of Health Research, the Multiple Sclerosis Society of Canada and the Canadian Multiple Sclerosis Research Foundation. SN has received consultancy fees or speaker’s honoraria from Teva Neuroscience and NeuroRx Research. MMG, SM, and J’OM served as administrative staff and received salary support from the funding agency (Multiple Sclerosis Scientific Research Foundation).

HH received a doctoral salary award from the Canadian Institute of Health Research. RV is a paid consultant for Ortho Clinical Diagnostics and Merck Serono, holds a grant from Dairy Farmers of Canada, receives payment in connection to a patent for a vitamin D supplement, has received payment for lectures including service on speaker’s bureaus from Merck, Carlsons Laboratories, and DiaSorin. GD has received funding from the Multiple Sclerosis Society of Canada. FG’M has received consultancy fees from Biogen-IDEC, Teva Neuroscience, and Novartis, travel reimbursement from Teva Neuroscience and Biogen-IDEC, payment for lectures by Teva Neuroscience, and payment for manuscript preparation (unrelated to the present project) by EMD Serono and for educational presentations by Novartis. RAM has funding from the Canadian Institute of Health Research, the Multiple Sclerosis Society of Canada, Manitoba Health Research Council, HSC Foundation, Public Health Agency of Canada, Rx&D Research Foundation, and Sanofi-Aventis for research unrelated to the present project. RT, TV, and GE report no disclosures. KW, MBC, JY, JKM, FB, GS, DC, BM, M-ED, AL, DP, AD, SV, SI, EAMD, DM, EW, NL, DB, CY, MA, PC, FG’M, JBB, and VB served as site investigators and received funds to their institution for study costs. None of the site investigators received personal salary support and none reports any other disclosures.

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References

8.6 Appendix 6: Wagner et al., In Press
The ratio of serum 24,25-dihydroxyvitamin D₃ to 25-hydroxyvitamin D₃ is predictive of 25-hydroxyvitamin D₃ response to vitamin D₃ supplementation

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ABSTRACT

24,25-Dihydroxyvitamin D (24,25VD₃) is a major catabolite of 25-hydroxyvitamin D (25VD) metabolism, and may be physiologically active. Our objectives were to: (1) characterize the response of serum 24,25VD₃ to vitamin D₃ (25VD₃) supplementation; (2) test the hypothesis that a higher 24,25VD₃ to 25VD₃ ratio (24,25:25VD₃) predicts 25VD₃ response.

Serum samples (n = 160) from wk 2 and wk 6 of a placebo-controlled, randomized clinical trial of 25VD₃ (28,000 IU/wk) were analyzed for serum 24,25VD₃ and 25VD₃ by mass spectrometry.

Serum 24,25VD₃ was highly correlated with 25VD₃ in placebo- and D₃-treated subjects at each time point (p < 0.0001). At wk 2, the 24,25:25VD₃ ratio was lower with D₃ than with placebo (p = 0.035). From wk 2 to wk 6, the 24,25:25VD₃ ratio increased with the D₃ supplement (p < 0.001) but not with placebo, such that at wk 6 this ratio did not significantly differ between groups. After correcting for potential confounders, we found that 24,25:25VD₃ at wk 2 was inversely correlated to the 25VD₃ increment by wk 6 in the supplemented group (r = -0.32, p = 0.02) but not the controls.

There is a strong correlation between 24,25VD₃ and 25VD₃ that is only modestly affected by D₃ supplementation. This indicates that the catabolism of 25VD₃ to 24,25VD₃ rises with increasing 25VD₃. Furthermore, the initial ratio of serum 24,25VD₃ to 25VD₃ predicted the increase in 25VD₃. The 24,25:25VD₃ ratio may therefore have clinical utility as a marker for D₃ catabolism and a predictor of serum 25VD₃ response to D₃ supplementation.

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1. Introduction

Vitamin D (VD) has received considerable attention because of associations between low VD status and increased risk for several diseases, including osteoporosis, cancers, multiple sclerosis, diabetes, cardiovascular disease, and microbial infections [1–7]. The determinants of serum 25-hydroxyvitamin D (25VD), the classic measure of VD status, include environmental (e.g. season, latitude, sunlight, diet) [8,9], demographic (e.g. ethnicity, body mass index [BMI]) [10], and genetic factors (e.g. polymorphisms in metabolism and transport genes) [11–13]. However, the factors that modify response to VD supplementation warrant further study, especially in view of the large inter-individual variation that has been reported in serum 25VD response to supplementation with identical doses of VD [12,14,15]. An analysis of 24,25-dihydroxyvitamin D (24,25VD), the major metabolite of 25VD, could provide clinically relevant information that may shed light on these inter-individual differences.

25VD is produced via 24-hydroxylation of 25VD by the cytochrome P450 24-hydroxylase enzyme (CYP24A1; V_max = 0.088 mol/min/mol P450, K_m = 160 nM) [16]. In addition, CYP24A1 catalyzes the side-chain metabolism of 1,25-dihydroxyvitamin D (1,25VD), considered to be the primary active metabolite. CYP24A1 is expressed in many tissues [17–20] but the biological activity of 24,25VD remains controversial. The general view is that 24,25VD production is the first step to inactive 24,25-dihydroxylated metabolites of VD, thus regulating synthesis of 1,25VD [21,22]. However, there is considerable evidence demonstrating that 24,25VD has unique biological properties, including physiological roles in embryogenesis, cartilage development, and
fracture repair [23–29]. Recently, Larsson et al. demonstrated that 24,25VDD binds to Catalase, suggesting that 24,25VD-mediated signal transduction may occur through modulating hydrogen peroxide production [30].

Few clinical studies have reported circulating 24,25VDD concentrations [31–37], likely because its measurement is technically challenging and its physiological role is unclear. Liquid chromatography–tandem mass spectrometry (LC–MS/MS) has received increased attention because it is capable of measuring 25VDD, and 25VD2 separately, but the capability for measuring 24,25VDD has not been widely exploited. Furthermore, the effects of VD3 supplementation on serum 24,25VDD concentrations in humans are unknown. Here, we characterize the biochemical response of serum 24,25VDD to VD3 supplementation in healthy adults using a highly sensitive and specific LC–MS/MS assay for simultaneous determination of serum 25VDD and 24,25VDD concentrations. We hypothesized that a higher 24,25VDD:25VD3 ratio (24,25:25VD3) would predict a smaller serum 25VD3 response to an increased VD3 intake because a relatively higher 24,25VDD would indicate higher catabolism.

2. Materials and methods

2.1. Study samples

Human serum samples (n = 160) were obtained from a randomized, double-blind, placebo-controlled clinical trial carried out in Toronto, Canada (latitude 43° N). Healthy young adults, half of whom were female, received either 28,000IU VD3/wk as a supplement or fortified cheese of equivalent bioavailability (n = 60), or a placebo (n = 20), for 8 weeks during the winter months [38]. Serum aliquots were stored at −80°C until analysis. Under these storage conditions, VD metabolites are stable in serum or plasma over a prolonged time and repeated freeze–thaw cycles [39,40]. Samples were available from subjects at wk 2 (n = 80) and wk 6 (n = 80) of the dosing protocol. The study protocol was approved by the Research Ethics Boards of the University of Toronto and of Mount Sinai Hospital (Toronto, Canada).

2.2. 25VD and 24,25VD assays

Aliquots of 200 μL serum were spiked with 50 μL of d6-25-hydroxyvitamin D3 [d6-25VD3] (Medical Isotopes Inc., Pelham, NH, USA) internal standard and extracted with 1 mL of methyl-tert-butyl ether. The upper ether phase was transferred to a clean borosilicate tube and the solvent evaporated under a stream of nitrogen gas at 40°C. The residue was dissolved in 1 mL of 4:1 methanol:water and 1 mL of heptane was added. The methanol phase was transferred into clean borosilicate tubes and evaporated to dryness under a stream of nitrogen gas at 40°C. The residue was dissolved in 100 μL of 1:1 methanol:water and transferred into an HPLC auto-sampler vial. A 20 μL aliquot was analyzed by LC–MS/MS.

The chromatographic separation of 25VDD, 25VD2, and 24,25VDD was carried out using an Agilent Technologies 1200 series HPLC system in linear gradient mode at a flow rate of 0.80 mL/min on an Eclipse C8 column (50 mm × 3.0 mm, 1.8 μm) employing a mobile phase consisting of methanol–water (37:63) increasing to 100% methanol over 4 min and maintained at 100% methanol for 1 min. The column was re-equilibrated with methanol-water (37:63) for 1 min. The column temperature was maintained at 50°C. The total chromatographic run time for each sample was 6.5 min and typical retention times for 24,25VDD, 25VDD, 25VD3, and 25VD2 were 2.92, 3.65, 3.66, and 3.72 min, respectively.

An API 5000 mass spectrometer (Applied Biosystems/Sciex, Concord, ON, Canada) was equipped with an atmospheric pressure chemical ionization (APCI) source and operated in the positive mode. The ion source temperature was maintained at 400°C, the corona current adjusted to 3.0 μA, and collision gas, nebulizer gas and collision pressure set to 5, 40, and 30, respectively, the collision energy set to 24 V and the declustering potential set to 100 V. The ion-transitions of m/z 417.4 → 399.4, 407.5 → 399.4, 401.4 → 383.4, and 413.4 → 395.4 were monitored to detect and quantify 24,25VDD, 25VDD, 25VD3, and 25VD2, respectively. The dwell time per transition was set to 50 ms.

Analyst software (version 1.4.2) mediated data acquisition, peak-area integration and comparison against the standard curve to calculate the concentration of unknowns. The standard curve was derived from calibrators of 25VDD, 25VD2, and 24,25VDD (Sigma–Aldrich) prepared in 100% ethanol that were analyzed within the same analytical run. The absolute concentrations of the calibrators were assigned using the Agilent 8453 E ultraviolet/visible spectrophotometer and calculated using the Merck Index molar absorptivity of 18,300 AU mol−1 L−1 at 265 nm.

Serum 25VD was also determined by Diasorin “25-hydroxyvitamin D 125i Radioimmunoassay (RIA)” and Diasorin “LIAISON 25 OH Vitamin D TOTAL” chemiluminescent immunoassay (LIA), as reported previously [41], and used for confirmatory analyses. Serum 24,25VDD and 25VD3 concentrations measured by LC–MS/MS are presented, unless otherwise indicated.

2.3. LC–MS/MS method evaluation

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

The limit of detection (LOD) and limit of quantification (LOQ) are defined as the peaks that give signal to noise ratios of 3:1 and 10:1, respectively, and were determined by running the calibration curve in triplicate with the following calculations: LOD = (3 × SD0_calibration) / slopecurve, LOQ = (10 × SD0_calibrated) / slopecurve. Functional sensitivity was evaluated by diluting L1 and measuring it 5 times to determine the concentration that gives a coefficient of variation (CV) near 20%.

The specificity of the LC–MS/MS assay to measure 24,25VDD and 25VD3 separately was evaluated by spiking pooled serum with either 25VDD (∼500 nmol/L), 24,25VDD (∼50 nmol/L), or both, and assaying as described above. Samples were run in triplicate on 2 separate days. The method was evaluated for potential interference of high bilirubin, hemoglobin, and lipemic conditions by spiking separate control plasma pools with bilirubin (800 μmol/L), hemoglobin (3 g/L), and lipids (100 mmol/L), and assaying as described above.

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

2.4. Other biochemical measurements

Calcium, phosphate, and creatinine in serum and urine, as well as serum parathyroid hormone (PTH), were measured on the modular Analytics Serum Work Area (Roche) as previously described [36]. Glomerular filtration rate (GFR) was estimated from serum
creatinine using the Modification of Diet in Renal Disease (MDRD) Study equation [42].

2.5. Statistical analyses

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

3. Results

3.1. LC–MS/MS method evaluation

All data were normally distributed, as indicated by the Kolmogorov–Smirnov test. LC–MS/MS assay performance characteristics are shown in Table 1. Total imprecision for all VD metabolites (CV = 7.3–14%) was comparable to immunoassays (5–15%) [40]. Linearity was confirmed across the analytical measurement range for all VD metabolites. The functional sensitivity for all VD metabolites (<1 nmol/L) was lower (i.e. higher sensitivity) than immunoassays (<10 nmol/L). 25VD₃ was not detected in any sample. Specificity experiments indicated no cross-reactivity (i.e. complete resolution) between 24,25VD₃ and 25VD₃. Bilirubin, hemolysis, and triglycerides did not interfere with measurement of VD metabolites. Lastly, serum 25VD₃ concentrations determined by LC–MS/MS correlated well with those measured by RIA (r = 0.915, p < 0.0001) and LIA (r = 0.907, p < 0.0001). However, both the RIA and LIA 25VD₃ methods demonstrated significant positive bias compared to LC–MS/MS (15.0 and 13.6 nmol/mL, respectively, p < 0.0001), likely because these immunoassays have 100% cross-reactivity with 24,25VD₃ [34].

3.2. Biochemical responses

Linear regression analysis indicated that serum 24,25VD₃ and 25VD₃ were highly correlated in the total sample (Fig. 1), and separately in the placebo- and VD₃-treated sub-groups at wk 2 (r = 0.81, r = 0.86, respectively; p < 0.0001) and wk 6 (r = 0.92, r = 0.81, respectively; p < 0.0001). LOESS fitting supported these findings but suggested slight deviation from linearity at 25VD₃ concentrations >100 nmol/mL. All correlations persisted when serum 25VD₃ values previously measured by RIA and LIA were used (p < 0.0001). On average, serum 24,25VD₃ values were 14% of 25VD₃ concentrations.

In the VD₃ group, serum 24,25VD₃ and 24,25:25VD₃ ratio also correlated with serum creatinine at wk 2 (r = −0.46, r = −0.39, respectively; p < 0.005) and wk 6 (r = −0.39, r = −0.39, respectively; p < 0.005). However, neither serum 24,25VD₃ nor 24,25:25VD₃ ratio correlated significantly with estimated GFR, nor with PTH, calcium, or phosphate in serum or urine.

Table 2 shows the absolute 25VD₃ and 24,25VD₃ concentrations at wk 2 and wk 6. After 2 wk of treatment, both serum 25VD₃ and 24,25VD₃ concentrations were significantly greater in the VD₃ group (69.6 ± 17.5 and 8.9 ± 3.1 nmol/L, respectively) compared to placebo (40.2 ± 17.2 and 5.9 ± 2.5 nmol/L, respectively) (p < 0.0001). By wk 6, serum 25VD₃ and 24,25VD₃ had increased to 90.5 ± 19.7 and 12.8 ± 3.6 nmol/L with VD₃ supplementation.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>24,25VD₃</th>
<th>25VD₃</th>
<th>25VD₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imprecision (%CV)</td>
<td>7.3–9.6</td>
<td>5.3–6.5</td>
<td>7.5–13</td>
</tr>
<tr>
<td>Between-day</td>
<td>5.2–7.4</td>
<td>4.7–6.6</td>
<td>5.3–7.0</td>
</tr>
<tr>
<td>Within-run</td>
<td>9.1–12</td>
<td>7.3–8.5</td>
<td>10.0–14.0</td>
</tr>
<tr>
<td>Total linearity</td>
<td>LOESS-fit line: y = 0.0204x + 0.0000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Analytical measurement</td>
<td>0–60</td>
<td>0–437</td>
<td>0–403</td>
</tr>
<tr>
<td>Range (nmol/L)</td>
<td>Linear calibration curve</td>
<td>y = 0.0297x + 0.0003</td>
<td></td>
</tr>
<tr>
<td>Limit of detection</td>
<td>0.24</td>
<td>0.25</td>
<td>0.17</td>
</tr>
<tr>
<td>Limit of quantification</td>
<td>0.8</td>
<td>0.83</td>
<td>0.57</td>
</tr>
<tr>
<td>Functional sensitivity</td>
<td>1.13</td>
<td>0.59</td>
<td>1.14</td>
</tr>
<tr>
<td>Specificity (nmol/L)</td>
<td>Cross-reactant</td>
<td>5 ± 0.1</td>
<td>31 ± 0.2</td>
</tr>
<tr>
<td>-25VD₃ (≤50 nmol/L)</td>
<td>4 ± 1.0</td>
<td>534 ± 10</td>
<td></td>
</tr>
<tr>
<td>-24,25VD₃ (≤50 nmol/L)</td>
<td>66 ± 9.0</td>
<td>30 ± 1.0</td>
<td></td>
</tr>
<tr>
<td>Both</td>
<td>69 ± 2.0</td>
<td>491 ± 23.0</td>
<td></td>
</tr>
<tr>
<td>Interference (nmol/L)</td>
<td>Interferant</td>
<td>10.6</td>
<td>75.3</td>
</tr>
<tr>
<td>-Bilirubin (800 μmol/L)</td>
<td>12.1, +14%</td>
<td>77.5, +2.9%</td>
<td>12.1, +14%</td>
</tr>
<tr>
<td>-Hemoglobin (3 g/L)</td>
<td>11.3, +6.6%</td>
<td>79.6, +5.7%</td>
<td>11.3, +6.6%</td>
</tr>
<tr>
<td>-Triglyceride</td>
<td>10.9, +2.8%</td>
<td>71.7, –4.8%</td>
<td>10.9, +2.8%</td>
</tr>
<tr>
<td>(100 nmol/L) Method comparison</td>
<td>Not reported</td>
<td>LC–MS/MS = 0.82</td>
<td></td>
</tr>
<tr>
<td>Regressions/correlation</td>
<td>No reference</td>
<td>LC–MS/MS = 0.82</td>
<td></td>
</tr>
<tr>
<td>method for</td>
<td>(RIA) = 0.32</td>
<td></td>
<td></td>
</tr>
<tr>
<td>24,25VD₃</td>
<td>r = 0.92, n = 160</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Agreement</td>
<td>Not reported</td>
<td>RIA bias = 15.0 ± 13.4</td>
<td></td>
</tr>
<tr>
<td>method for</td>
<td>LC–MS/MS = 0.82</td>
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<td></td>
</tr>
<tr>
<td>24,25VD₃</td>
<td>LIA bias = 12.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LIA bias = 16.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RIA bias = 0.3 ± 0.1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LIA bias = 13.6 ± 13.8</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2

Serum 25VD₃ and 24,25VD₃ concentrations over time in subjects consuming placebo (n = 20) or VD₃ (n = 60).^a

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Placebo-treated</th>
<th>Vitamin D-treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>25VD₃ (nmol/L)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>wk 2</td>
<td>40.2 ± 17.2</td>
<td>69.6 ± 17.5 †</td>
</tr>
<tr>
<td>wk 6</td>
<td>39.2 ± 17.1</td>
<td>90.5 ± 19.7 †</td>
</tr>
<tr>
<td>Change</td>
<td>−1.1 ± 4.1</td>
<td>21.2 ± 9.1 †</td>
</tr>
<tr>
<td>24,25VD₃ (nmol/L)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>wk 2</td>
<td>5.9 ± 2.5</td>
<td>8.9 ± 3.1 †</td>
</tr>
<tr>
<td>wk 6</td>
<td>5.6 ± 2.6</td>
<td>12.8 ± 3.6 †</td>
</tr>
<tr>
<td>Change</td>
<td>−0.3 ± 1.3</td>
<td>4.1 ± 2.0 †</td>
</tr>
<tr>
<td>24,25:25VD₃ (nmol/L)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>wk 2</td>
<td>0.155 ± 0.05</td>
<td>0.127 ± 0.02 †</td>
</tr>
<tr>
<td>wk 6</td>
<td>0.146 ± 0.04</td>
<td>0.142 ± 0.02 †</td>
</tr>
<tr>
<td>Change</td>
<td>−0.009 ± 0.04</td>
<td>0.016 ± 0.02 †</td>
</tr>
</tbody>
</table>

^a Values are means ± SD.
† p < 0.05 (compared to placebo).
** p < 0.001 (compared to placebo).
* p < 0.001 (compared to wk 2).

respectively \((p < 0.0001)\), but remained unchanged in the placebo group \((p = 0.26)\). The increases in serum 25VD3 and 24,25VD3 during this time period was significantly greater in the supplemented group \((21.2 ± 9.1 \text{ and } 4.1 ± 2.0 \text{ nmol/L, respectively})\) than in controls \((-1.1 ± 4.1 \text{ and } -0.3 ± 1.3 \text{ nmol/L, respectively})\) \((p < 0.0001)\).

At 2 wk of treatment, the ratio of 24,25VD3 to 25VD3 was lower in the VD3 group \((0.127 ± 0.02)\) than in the placebo group \((0.155 ± 0.05)\) \((p = 0.03)\). From wk 2 to wk 6, the 24,25:25VD3 ratio increased with the VD3 supplement to 0.142 ± 0.02 \((p < 0.001)\) but remained unchanged in controls \((6: wk 6: 0.146 ± 0.04; p = 0.34)\). At wk 6, the 24,25VD3 to 25VD3 ratio did not differ significantly between VD3- and placebo-treated subjects \((p = 0.61)\). These results were confirmed when using RIA and LIA serum 25VD3 values. When stratifying by gender in the VD3 group, the 24,25:25VD3 ratio was not significantly different between genders at wk 2 \((\text{females: } 0.131 ± 0.02; \text{males: } 0.122 ± 0.02; p = 0.20)\) but at wk 6 it was significantly higher in females \((0.149 ± 0.02)\) compared to males \((0.134 ± 0.02)\) \((p = 0.01)\).

Linear regression indicated that the 24,25:25VD3 ratio at wk 2 and wk 6 was significantly inversely correlated with the change in serum 25VD3 \((\text{i.e. wk 6-wk 2})\) in the VD3 group \((r = -0.38, p = 0.0044; r = -0.30, p = 0.03; \text{respectively})\) but not in the placebo group \((r > 0.40)\) \((\text{Fig. 2})\). These correlations were essentially the same, irrespective of 25VD measurement method \((p < 0.05, \text{data not shown})\). Significant correlation between 24,25:25VD3 and 25VD3 response to VD3 persisted at wk 2 and wk 6 after controlling for serum 25VD3 \((\text{wk 2 and 6})\), 24,25VD3 \((\text{wk 6})\), BMI, age, gender, PTH, calcium, phosphate, and creatinine \((p < 0.05)\). After controlling for baseline serum 25VD3 \((\text{as measured by RIA and LIA})\) and 24,25VD3 at wk 2, the association between 24,25:25VD3 and 25VD3 response was attenuated at wk 6 \((r = -0.21, p = 0.14)\) but not at wk 2 \((r = -0.35, p = 0.01)\). The 24,25:25VD3 ratio at wk 2 also correlated significantly with the overall change in serum 25VD3 \((\text{i.e. wk 8-wk 0})\) \((r = -0.40, p = 0.003)\) in the VD3 group but not in the placebo group \((p = 0.22)\).

4. Discussion

Our data suggest a new clinical indication utility for measuring serum 24,25VD3, the major metabolite of 25VD3, by a novel LC–MS/MS assay for simultaneous determination of 25VD3 and 24,25VD3. The developed LC–MS/MS method was highly sensitive, specific, and the first to quantify 24,25VD3 in serum. Investigators should therefore exploit the capability of LC–MS/MS methods to measure both serum 24,25VD3 and 25VD3 simultaneously. Indeed, 24,25VD3 is the most abundant 25VD metabolite and its roles in fracture healing and cartilage growth [25–29] support its physiological relevance beyond VD3 metabolism.

We found that serum 24,25VD3 concentrations were highly correlated with serum 25VD3, indicating that the catabolism of 25VD3 into 24,25VD3 rises with increasing 25VD3 concentrations. This is consistent with the findings of other investigators [34,36,37]. In our study, the correlation between these variables was remarkably strong; indeed, 82% of the variation in serum 24,25VD3 could be explained by 25VD3 concentrations. Furthermore, serum 24,25VD3 increased in parallel with 25VD3 levels during the 4 weeks of 28,000 IU/wk VD3 supplementation. In fact, the two variables are so closely related that one might argue that serum 24,25VD3 could serve as an alternative marker of VD status. Taken together, the strong correlation and similar response of serum 24,25VD3 with 25VD3 indicate that 24,25VD3 measurement provides clinically useful information pertaining to VD status and supplementation.

Since 24,25VD3 concentration changed in proportion to that of 25VD3, we normalized serum 24,25VD3 response by calculating the ratio of 24,25VD3 to 25VD3. This ratio served as an index of VD3 clearance since 24,25VD3 is the major initial catabolite of 25VD3 metabolism. Interestingly, the 24,25:25VD3 ratio at wk 2 was significantly lower in the VD3 group than placebo, indicating a possible lag in 24-hydroxylation during the early phase of supplementation. We speculate that this lag effect is the result of: (1) the large incremental increase in 25VD3 observed during the first 2 wk of dosing, which was greater than that observed at any other time interval, and (2) the slower reaction kinetics of CYP24A1 [turnover number \(\text{[TN]} = 2–20 \text{min}^{-1}\)] compared to CYP27A1 \([25\text{-hydroxylase}; \text{TN} = 40–50 \text{min}^{-1}]\) [16,19]. By wk 6, however, the 24,25:25VD3 ratio had increased significantly with supplementation, as a response to the VD3 loading. Overall, these results suggest that catabolism is induced with VD3 supplementation but these adaptations may occur over weeks not days. Indeed, in vitro studies indicate that a variety of molecular mechanisms may be involved, including gene expression up-regulation and enzyme trafficking [43]. Future studies should investigate the genetic influences of CYP24A1 genotypes on VD catalytic activity and biochemical response.
Our data provide insight on the in vivo effects of this altered expression and kinetic behaviour of the CYP24A1 enzyme. Firstly, the correlation in the VD$_3$-treated group of serum 24,25VD$_3$ and 24,25:25VD$_3$ ratio with serum creatinine, a measure of renal function, is supportive of the idea of variable renal CYP24A1 action in 25VD$_3$ metabolism. Accordingly, the 24,25:25VD$_3$ ratio may be useful in monitoring kidney function during VD$_3$ supplementation but this needs to be studied directly. The increase in 24,25:25VD$_3$ over time is consistent with the induction of renal CYP24A1 catabolic capacity with increasing VD$_3$ loading. The concept of induction proportional to load is also supported by the LOESS fit line (Fig. 1), which appears to become more curvilinear at serum 25VD$_3$ concentrations exceeding 100 nmol/L. Lastly, we found that the 24,25:25VD$_3$ ratio was significantly higher in supplemented women compared to men at wk 6. This suggests that females were catabolising 25VD$_3$ at a slightly faster rate than males during the later parts of VD$_3$ supplementation, an effect that may be related to estrogens. Further research is needed to elucidate the regulation of CYP24A1 activity by gender and varying 25VD$_3$ concentrations.

A major finding of this study was that the 24,25:25VD$_3$ ratio alone predicted the magnitude of the serum 25VD$_3$ change resulting from VD$_3$ supplementation. This inverse correlation remained significant at wk 2 after controlling for other variables that may affect serum 25VD$_3$ response, including baseline 25VD$_3$, BMI, gender, serum PTH, and serum calcium. Although moderate ($r = -0.38$), this correlation was similar to those commonly reported with more conventional correlates of vitamin D response and status, including BMI ($r = -0.41$) [10] and PTH ($r = -0.34$) [38]. Taken together, these results suggest that relative 24,25VD$_3$ concentration, as assessed by a ratio of circulating 24,25VD$_3$ to 25VD$_3$ early after dosing commences, is a potentially important determinant of serum 25VD$_3$ response to supplementation. Consequently, this ratio may assist in identifying individuals who are more likely to experience a lower serum 25VD$_3$ response and thereby require more VD$_3$ due to a higher 24,25:25VD$_3$ ratio (i.e. higher 25VD$_3$ catabolism) during the early loading stage (i.e. wk 2) of the supplementation protocol.

Data on 24,25VD$_3$ can also be evaluated from the perspective of the biological activities of the VD metabolites. Differential 24,25VD$_3$ production and 25VD$_3$ response may impact bioactive 1,25VD$_3$ levels, particularly in extra-renal 1,25VD$_3$ synthesis, which may well depend on 25VD$_3$ substrate supply, and in the renal failure population, which exhibit abnormalities in renal VD metabolism. Also, 25VD$_3$ itself has been reported to be a functional ligand of VDR and to exert genomic actions independent of 1,25VD$_3$ [44]. Therefore, differences in serum 25VD$_3$ responses due to increased VD catabolism or other factors might directly affect 25VD$_3$-mediated responses such as cell growth regulation. Lastly, there is substantial evidence supporting unique biological properties for 24,25VD$_3$, particularly with respect to bone and cartilage [23,25–27,29]. In fact, preliminary evidence for the presence of a unique, non-nuclear membrane receptor for 24,25VD$_3$ has been reported [45]. The availability of robust LC–MS/MS methods for simultaneous determination of 25VD$_3$ and 24,25VD$_3$, like the one presented here, will also help elucidate the functional role of 24,25VD$_3$ in human physiology. Furthermore, the 24,25:25VD$_3$ ratio may indicate not only metabolic differences in serum 25VD$_3$ response but also differential functioning of 24,25VD$_3$ between individuals and/or target tissues. For instance, the 24,25:25VD$_3$ ratio (i.e. local or systemic), may be important in investigating the rate of putative 24,25VD$_3$-dependent processes, such as fracture healing, whereby a higher ratio could hypothetically indicate faster healing.

Several limitations bear mention. Serum 24,25VD$_3$ concentrations at baseline and end-of-study were not available. However, baseline 24,25VD$_3$ levels in the VD$_3$ group would, in all probability, be similar to those at wk 2 in the placebo group, particularly since baseline 25VD$_3$ concentrations did not differ significantly between groups. However, end-of-study (wk 8) 24,25VD$_3$ determination may have provided additional meaningful data. The relatively small increment in 25VD$_3$ in the VD$_3$ group from 6 to 8 wk is certainly compatible with the notion of proportional catabolism, but a direct test of this supposition is warranted. Nonetheless, our evidence indicates that metabolic clearance rate at wk 2 appears to be the key determinant of 25VD$_3$ response, such that the inclusion of baseline or end-of-study 24,25VD$_3$ measurements would not have substantially changed our findings.

In conclusion, the measurement of serum 24,25VD$_3$ in conjunction with 25VD$_3$ shows promise as a novel marker of VD$_3$ catabolism and predictor of serum 25VD$_3$ response to VD$_3$ supplementation. It should be emphasized that LC–MS/MS assay methods can be modified to measure both serum 25VD$_3$ and 24,25VD$_3$ simultaneously, thus providing more comprehensive data regarding VD status and repletion. Moreover, further in vivo evidence may confirm the biological activity of 24,25VD$_3$ in physiological processes such as fracture repair, making its measurement ever more important. Future research should continue to explore the clinical utility of 24,25VD$_3$ measurement in VD testing. Ultimately, this information may aid clinicians in adjusting VD$_3$ dose for optimum individual benefit, thus contributing to the goal of personalized medicine and nutrition.

Acknowledgments

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References


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8.7 Appendix 7: Members of the Canadian Paediatric Demyelinating Disease Network

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K Wambera, MD; Victoria General Hospital, Victoria, BC
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J Yager, MD; Stollery Children’s Hospital, Edmonton, AB
C Yim, MD; Trillium Health Centre, Mississauga, ON