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Increasing Serum 25-Hydroxyvitamin D as a Possible Deterrent to the Onset of Multiple Sclerosis

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INCREASING SERUM 25-HYDROXYVITAMIN D LEVELS AS A POSSIBLE DETERRENT TO THE ONSET OF MULTIPLE SCLEROSIS

by

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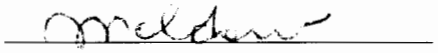
APPROVAL PAGE



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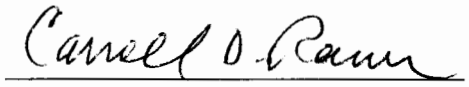

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Introduction

Multiple sclerosis (MS) is a chronic demyelinating disease of the central nervous system whose pathogenesis is unknown. Numerous possible causative agents/factors have been researched including environmental factors like climate, birth time, vitamin D intake and synthesis, hereditary factors, general levels of physiological substances, and autoimmune responses to viral, bacterial, and food proteins. (Hafler, 2004, McDonald and Ron 1999, Wehman-Tubbs et al., 2005) The most compelling data for possible causative agents come from research on Epstein Barr Virus (EBV) proteins and dairy product proteins like those found in cow's milk. Also, since MS is scarce along the equator and is much more prevalent at higher latitudes their seems to be a strong correlation between low ultra violet B (UVB) light exposure and low serum vitamin D levels, which may make a person more susceptible to developing MS. (Cantorna et al, 1996, Munger at el., 2004) Each of these three factors will be addressed in the proposed experiments that will be clearly explained at the end of this introduction.

It is clear that viruses can cause autoimmune disorders in animal models where myelin is targeted. The Theiler's murine encephalomyelitis virus (TMEV) is a mouse pathogen that leads to a progressive CD4+ T cell mediated demyelinating disease which is similar to human MS. The virus causes mice oligodendrocytes to lyse and it causes macrophages to undergo apoptosis. Both processes enable more viron to be created which results in more oligiodendrocyte destruction and inevitably leads to demyelination. (Gigoriadis and Hadjigeorgiou, 2006; Olson et al., 2001)

Since virus induced molecular mimicry mouse models for MS is available, it is plausible that the Epstein Barr Virus (EBV) can provide antigens that can cause a sustained cross-reactive immune response in humans. Because the EBV has a lifelong latency within B cells, that might explain the chronic deterioration that occurs in MS patients, and since it occasionally reactivates, perhaps each reactivation results in an exacerbation and further progression of the disease. The EBV virus or Herpes virus #4 has all the features required to produce a sustained cross-reactive immune response. (Cepok et al., 2005, Olson et al., 2001, Holmoy et al., 2004, Alotaibi et al., 2004, Christensen 2005)

It is well documented that more than 99% of people with MS have significantly elevated IgG antibodies against EBV proteins in both their CSF and in their blood serum when compared to healthy controls. (Pohl et al., 2006, Munch et al., 1998, Cepok et al., 2005, Ascherio et al., 2001, Wagner et al., 2004, Christensen 2005, Bray et al., 1992; Larsen et al., 1985) In fact, people with a history of severe EBV infection as evident by elevated anti-EBV IgGs in serum specifically, may be thirty times more likely to develop MS. (Levin et al., 2003)

The EBV proteins viral capsid antigen (VCA), epstein barr nuclear antigen 1 and 2 (EBNA1, EBNA2), early antigen (EA), and the tegument protein (BRRF2), have been studied thoroughly to determine if any or all of them are the antigens that cause the elevated anti-EBV IgGs seen in nearly 100 % of MS patients. Current research suggests that people with high serum antibody titers against the VCA, EBNA1, and EBNA2 EBV proteins provide the strongest predictors for developing MS. (Ascherio

et al., 2001; Levin et at., 2005; Levin et al., 2003, Sunstrum et al., 2004, Cepok et al., 2005)

It is also clear that MS is much more common in temperate latitudes when compared to tropical latitudes. In temperate latitudes, particularly in the western hemisphere, MS reaches 230+ per 100,000 people, whereas, at the equator the number of cases is remarkably small. (Beck et al., 2005; Sloka et al., 2005; Hayes et al., 1997) Scientists have determined that serum vitamin D levels in people in temperate zones are significantly reduced because they receive less ultra violet B (UVB) light. In fact, the Vitamin D metabolite 25-hydroxyvitamin D is significantly lower in the serum of MS patients when compared to healthy controls. (Nieves et al. 1994, Van der Mei et al., 2007, Munger et al., 2004) That is why many people with multiple sclerosis develop low bone density and are susceptible to small bone fractures. (Weinstock-Guttman et al., 2004, Cosman et al., 1998)

Recent data suggests that Vitamin D may be a natural inhibitor of MS, because it can inhibit murine EAE (Nashold et al., 2000, Cantorna et al.,1996, Cantorna et al., 2000), helps prevent the T cell proliferation seen in autoimmune responses, reduces the number of inflammatory proteins produced, and increases the number of anti-inflammatory cytokines produced. (Hayes CE 2000, Hayes et al.,1997, Deluca and Cantorna 2001, VanAmerongen et al., 2004) A recent, large epidemiological study showed that woman with the highest intakes of vitamin D (used supplements) had a 40% reduced risk of developing MS. (Munger et al., 2004)

Finally, there is a strong correlation between milk consumption and MS. In fact, regions where cow's milk consumption is high so is the incidence of MS. (Malosse

et al., 1993, Malosse et al., 1992) It is also interesting to note that dairy proteins and specifically butyrophilin found in cow's milk, can closely resemble myelin proteins and can cause autoimmune MS like disease in animal models. (Winer et al., 2001, Guggenmos et al., 2004, Stefferl et al., 2000)

In this proposed study, I will track eighty healthy females from Canada that all have specific qualities that (current research implies) may put a person at risk for the development of MS. Namely, they are all females (which make up approximately 70% of those affected with MS), they all come from Canada which according to current research makes them more likely to develop MS when compared to living near the equator, they all have elevated IgG concentrations against the EBNA and VCA EBV antigens (which research says is the greatest predictor for MS onset), and they all possess low serum 25-hydroxyvitamin D levels. It is reasonable to assume that the females in the study have low serum 25-hydroxyvitamin D levels because of a reduced exposure to UVB light away from the equator.

Forty of the eighty participants will take oral doses of vitamin D 400 IU, Calcium 1200 mg, and will be asked to eat a diet high in fish oils (fish oils are high in vitamin D) and are asked to avoid all dairy products (especially cow's milk). The forty participants will take two Caltrate tablets daily during the summer and winter months, however, during the winter months an additional 600 IU of vitamin D will be taken in tablet form to compensate for the reduced UVB exposure.

The other forty participants will act as controls and they will have no diet limitations and they will not be given the Caltrate supplements or vitamin D tablets. The unrestricted diet of the control group participants will be discussed to insure their

normal diet does not resemble the experimental group's diet. As time passes in the proposed study the serum 25-hydroxyvitamin D levels will be closely monitored in all eighty of the participants. If there is an increase in 25-hydroxyvitamin D levels (Due to vitamin D supplements) that should increase the quantities of the marker for the experiments, the anti-inflammatory cytokine transforming growth factor (TGF-β1). (Mahon et al., 2002; Cantorna et al., 1998)

As a marker for the proposed study the anti-inflammatory cytokine levels of TGF-β1 will be closely monitored in all eighty participant's serum. The proposed hypothesis states that if an anti-inflammatory cytokine can be produced in higher quantities (due to increasing serum 25-hydroxyvitamin D levels) that may result in a reduced likelihood of developing an inflammatory disease like MS. The TGF--β1 marker will be used because its increased production by regulatory T cells has been correlated with the inhibition of M.S. symptoms in mice with EAE. (Cantorna et al., 1998; Meyer et al., 2001) Also, it has been reported that increasing serum 25-hydroxyvitamin D levels can lead to an increased production of TGF-β1 in both human and mouse models. (Mahon et al., 2002; Cantorna et al., 1998; Meyer et al., 2001) Therefore, the anti-inflammatory cytokine TGF-β1 will be used in these proposed experiments as a marker whose increased production may result in a protective role against the development of MS.

In short, eighty healthy females that have elevated serum IgG concentrations against the EBNA and VCA EBV proteins and have low serum 25-hydroxyvitamin D concentrations are selected and split into two groups. The experimental group which consists of forty ladies will be given vitamin D supplements and are asked to avoid

dairy products, while the others forty females are not given the supplements and have no diet limitations. All eighty participants will have there serum 25-hydroxyvitamin D levels and TGF-β1 levels determined every six months. It is predicted that the experimental group participants will see an increased serum 25-hydroxyvitamin D level after receiving the vitamin D supplements which should result in an increased production of TGF-β1. It is also predicted that the increased TGF-β1 levels will produce a protective role against the onset of an inflammatory disease (MS). An internet survey will be used to monitor all participant's health and possible signs and symptoms of MS in between all serum testing.

Proposed Methods are Materials:

Initially two-hundred and fifty Canadian females from ages 30 – 45 were asked to provide blood samples for the proposed experiment. All samples underwent serum tests that looked for elevated IgG antibodies against EBNA1 and VCA EBV proteins, only those females (120 out of the 250) with elevated IgG titers to the EBV antigens were asked to continue the study. Of the one hundred and twenty females that had elevated IgG titers to the EBV antigens, only eighty also possessed a low serum 25-hydroxyvitamin D level. Therefore, the eighty females that had elevated IgG titers to the VCA and EBNA EBV antigens and low serum 25-hydroxyvitamin D levels were asked to participate in the study. ELISA's were used to calculate the specific serum IgG titers, and radioimmunoassay was used to calculate the serum 25-hydroxyvitamin D levels. (For the detailed procedures see below)

Detection of EBV EBNA1 and VCA specific antibodies

ELISA: six ml of serum from each participant is placed in an ARUP standard transport tube (the serum must be transported at 2 – 8 Celsius) and sent to ARUP Consult for ELISA completion of 00500235 and 0050245. (ARUP Consult is owned by the University of Utah) Between .5 and 1 ml of serum is required to run each ELISA. The first test, 00500235, will determine the quantity of EBV IgG antibody to VCA (Viral Capsid Antigen) that is present in serum.

High levels of IgG antibodies to this VCA antigen are seen frequently in MS patients, and they are important to examine. These IgG antibodies to the VCA appear within four to seven days and may persist a lifetime. (Pohl et al., 2006; Munch et al., 1998; Cepok et al., 2005; Ascherio et al., 2001; Christensen 2005; Bray et al., 1992) The IgM antibodies to VCA that will be produced are not of interest because they will generally appear after infection, spike, and then decline after just three months.

ELISA was then performed on the serum for the presence of IgG antibodies against EBV nuclear antigen EBNA1. The 0050245 test will complete this task. Although IgM antibodies to EBNA appear very quickly, often before VCA IgM, the EBNA IgG antibodies mark the transition between acute infection to convalescence, and can persist for a life time. Therefore, just EBNA IgG antibody titers will be considered. (ARUP's Laboratory Test Directory)

To determine whether or not serum antibodies are elevated, an excepted reference interval will be used from the ARUP Consult guide. (ARUP's Laboratory Test Directory) This reference interval will express if an individual is seropositive to EBV EBNA1 and VCA antigens, and at what titer that particular IgG antibody is in. Each

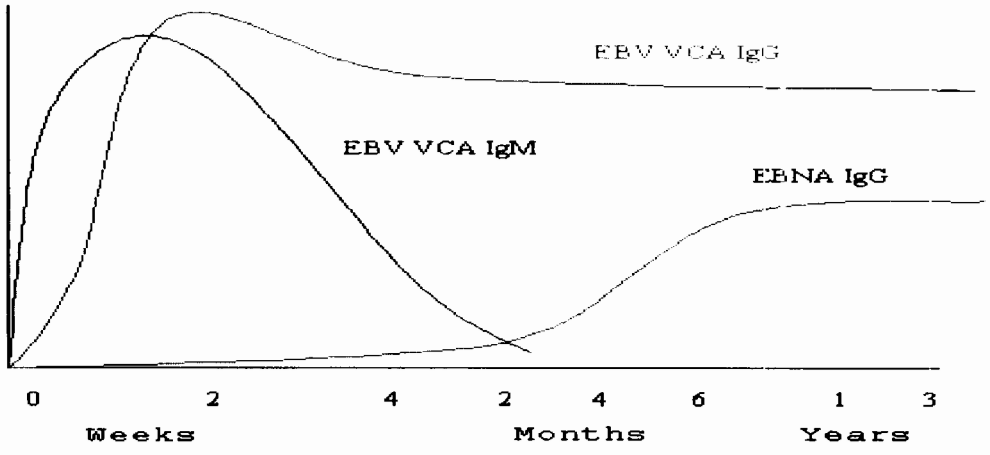
number value on the index value (IV) scale represents a specific quantity of antibody. Therefore, the scale represents specific quantities of antibody for every value. The number values that will be used to determine the parameters for the experiment are as follows:

0.90 IV or less: Negative – no significant level of detectable IgG antibody to EBV VCA or EBNA1 antigen.

0.91 – 0.99 IV: Equivocal – repeat testing usually done 10 – 14 days later.

1.00 IV or greater: Positive – IgG antibody to EBV VCA or EBNA1 is detected at a particular titer.

A histogram will be produced that will demonstrate the individual and her IgG levels for both antigens. The individuals with the highest average titers to the VCA and EBNA antigens combined will be selected for the study. These methods should provide the subjects necessary for the continuation of the study.



(Diagram taken from Nottingham University Hospitals Microbiology Services)

The next step in the experiment is to test all subjects that have an elevated IgG titer to VCA and EBNA1 antigens for low serum 25-hydroxyvitamin D levels.

Testing 25-hydroxyvitamin D serum levels in participants with elevated IgG titers

Serum concentrations of 25-hydroxyvitamin D are accepted as the best way to determine vitamin D status and the presence of deficiency. (Hanley and Davison 2005; Holick 2003; Hollis and Wagner 2005) Therefore, all participants that have elevated IgG titers against VCA and EBNA were further tested for serum 25-hydroxyvitamin D levels. The testing required collecting more serum after all participants had fasted for a day. The collected serum samples were sent to DiaSorin, Inc. (Stillwater, MN), where a commercially available radioimmunoassay was used to determine the 25-hydroxyvitamin D levels. The technique uses the competitive binding of the extracted forms of the serum 25-hydroxyvitamin D and a ^{125}I 25-hydroxyvitamin D tracer with a 25-hydroxyvitamin D specific goat antiserum. Addition of donkey anti-goat serum forms a precipitating complex permitting the quantitative determination of the 25-hydroxyvitamin D concentrations. (Kemp et al., 2007)

The normal range for 25-hydroxyvitamin D in serum for this test is between 20 – 56 ng/ml, while the optimal 25-hydroxyvitamin D levels are between 45 – 50 ng/ml. Although there is some debate as to what optimal levels should be, leaders in the field believe that the 25-hydroxyvitamin D levels should not fall below 32 ng/ml. (Holick 2000) However, some scientists believe that values between 20 and 32 should not be considered low, so to prevent confusion a value of below 25 ng/ml will be used to classify a low 25-hydroxyvitamin D serum level. (A value in which many patients with

MS have) The complete scale adopted by this proposed experiment is when serum 25-hydroxyvitamin D levels are below 25 ng/ml, that is considered low, 26 – 65 ng/ml is normal, and above 65 ng/ml is high.

In this proposed experiment, a 25-hydroxyvitamin D level below 25 ng/ml will be considered unwise, and may elevate your risk for developing MS. This is consistent with research done by leaders in the field. (Nieves et al. 1994, Van der Mei et al., 2007, Munger et al., 2004, Holick 2000) Therefore, each female that falls into this range (below 25 ng/ml) will be used in the study. All eighty females that possessed this condition while having elevated IgG titers to the VCA and EBNA EBV antigens were selected for the study. Every six months all eighty participants will have their 25-hydroxyvitamin D levels calculated in a similar manner to determine if the supplements are elevating the serum 25-hydroxyvitamin D levels into an acceptable range. Each participant will be informed of their reading and will be asked to keep a record of the calculated values.

Initial concentrations of TGF-B1 are determined

ELISA: The 80 participants with elevated IgG titers to the VCA and EBNA EBV antigens, and who have serum 25-hydroxyvitamin D levels below 25 ng/ml, were evaluated for serum TGF-β1 concentrations. Serum TGF-β1 was measured using commercial kits from Promega (Madison, WI). In the ELISA an anti-TGF-β1 coating antibody is adsorbed onto the microwells. The TGF-β1 present in the sample or standard binds to antibodies adsorbed to the microwells; a HRP-conjugated monoclonal anti-TGF-β1 antibody is added and binds to TGF-β1 captured by the first antibody. Following

incubation unbound enzyme conjugated anti-TGF-B1 is removed during a wash step and substrate solution reactive when HRP is added to the wells. The reaction is then terminated by addition of an acid. A colored product is formed in proportion to the amount of TGF-β1 present in the sample; therefore, the absorbance of each colored product is measured at 450 nm. A standard curve from seven TGF-β1 standard dilutions is generated and TGF-β1 sample concentration from each sample is determined. (DIACLONE issue 6-10-03/06) A serum TGF-β1 concentration of 7.5 ng/ml was determined to be the mean for all the ELISA tests. (Estimated values from a typical serum TGF-β1 ELISA) Therefore, since the mean is established after the initial series of ELISAs, TGF-β1 serum values can be checked every six months for changing levels by using the mean as a reference point.

It is important to the experiments that the TGF-β1 levels are monitored to determine if they become elevated when 25-hydroxyvitamin D becomes increased due to vitamin D supplements. It is predicted that the increase in TGF-β1 due to the increase in serum 25-hydroxyvitamin D levels may result in a protective role against the onset of the inflammatory disease MS.

Internet survey that will be completed every 3 months

An online survey of subject heath will be completed every 3 months. The survey will ask key questions as to the general health of the participants. Each question will be pertinent to the onset of M.S. MS has numerous signs and symptoms associated with its onset and it is useful to find out which females from which group are experiencing signs

or symptoms associated with MS. Also, it is essential to know if a participant is diagnosed with MS so I can determine if there is a correlation between serum 25-hydroxyvitamin D levels, TGF-β1 levels, and the onset of MS. The survey is simply a tool to assess the general health of each participant in the proposed study and to use that information to make predictions about 25-hydroxyvitamin D levels, TGF-β1 levels, and the onset of MS. The online survey (Or mail in survey) is as follows.

Please check either true or false for each question **Your name is:** **My original 25(OH) D level was** _____ **my latest is** _____. **DISCRIPTION OF POSSIBLE HEATH EVENTS**	**TRUE**	**FALSE**
I have been diagnosed with multiple sclerosis by a medical doctor during the last three months.		
I frequently experience paresthesia in my extremities.		
I have been diagnosed with optic neuritis by a medical doctor during the last three months.		
I have had episodes of vertigo which is new for me over the last three months.		
I have had balance problems over the last three months.		
I have experienced muscle weakness or a general sense of heaviness felt in my arms and/or legs.		
I feel as though I am in excellent health and I have experienced no unusual signs or symptoms that can be associated with an illness.		

Please state any unusual symptoms that you have experienced or say none. (Thank you and be well)

Anticipated Results for the Proposed Experiment

Initially I received serum samples from two-hundred and fifty Canadian females and their serum IgG levels to the EBV EBNA and VCA antigens were calculated by the ARUP Consult. (That two hundred and fifty females was eventually reduced to eighty based on their IgG titers and 25-hydroxyvitamin D levels) **Figure 1** was generated to select the best candidates for the study. Or in other words, of the two hundred and fifty females tested how many have elevated IgG titers to the VCA and EBNA antigens. The best candidates for the study are females that have elevated IgG titers to VCA and EBNA1 because nearly 100% of patients that have MS have elevated IgGs against the VCA and EBNA1 antigens.

Therefore, elevated IgG titers to the VCA and EBNA EBV antigens is one criteria that research suggests may place a person at risk for developing MS. Therefore, if a person had a summed and averaged (VCA + EBNA/2) IgG titer of less than 3.5 IV, they would be excluded from the experiment, and every person with an average IgG titer higher than 3.5, should have their serum analyzed for 25-hydroxyvitamin D levels. These index values are consistent with what the scientists conducting the 00500235 and 0050245 ELISAs have determined to be significantly high. Each number value on the IV scale represents a specific quantity of antibody. Of two-hundred and fifty initial volunteers one hundred and twenty of them had average IgG titers that were at 3.5 IV or

higher. **Figure 1** below shows the results for fifteen of the initial two-hundred and fifty females tested.

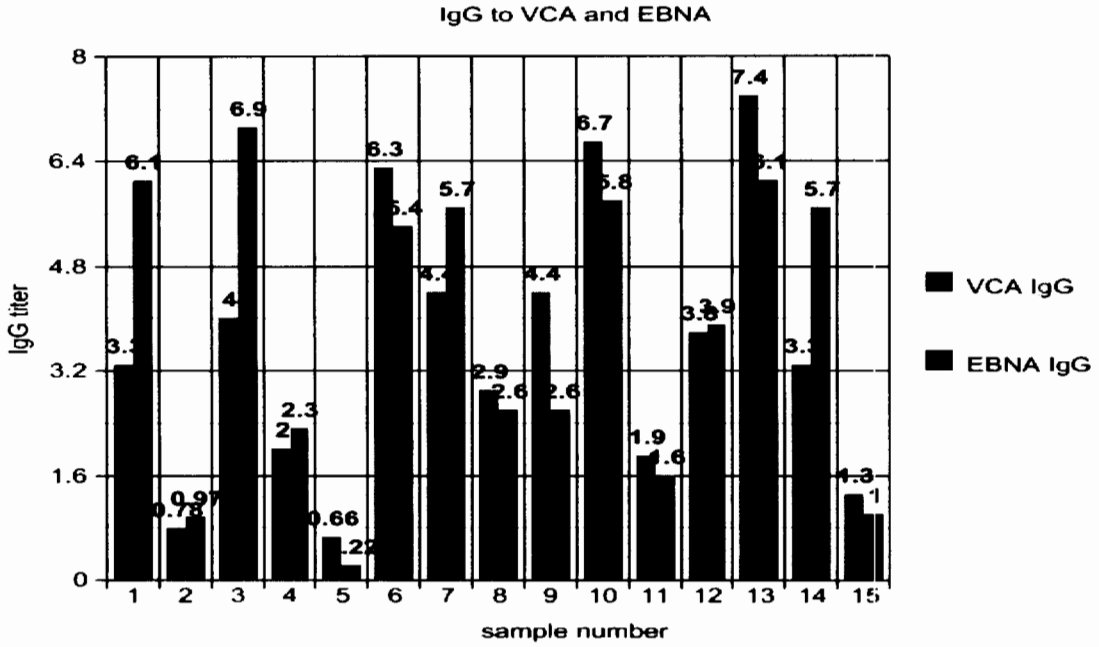


Figure 1: The data above represents the ELISA results for the serum samples of subjects 1 – 15. Similar data for all two-hundred and fifty initial female volunteers was received. Of the two-hundred and fifty volunteers, one hundred and twenty of them had significantly elevated IgGs to both the VCA, and EBNA EBV antigens. A summed and averaged index value of 3.5 or higher is considered to be at significantly high titers.

The summed average titers of VCA and EBNA IgG levels. (VCA + EBNA/2)

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
4.7	.88	5.4	2.1	.44	1.7	5.0	2.7	3.5	6.2	1.7	3.7	6.7	4.5	1.1

All one hundred and twenty females that had elevated (IV >3.5) IgG titers donated blood samples for the calculation of their serum 25-hydroxyvitamin D levels. The other females that did not have elevated IgG titers (IV < 3.5) were not asked to continue the study. The one hundred and twenty female (samples 1-120) serum samples that did have IV greater or equal to 3.5 were sent to DiaSorin, Inc. (Stillwater, MN) for

the most sensitive 25-hydroxyvitamin D test available. The data was simplified into line graphs like the one below. The results for participants 1-50 are shown in **figure 2** below.

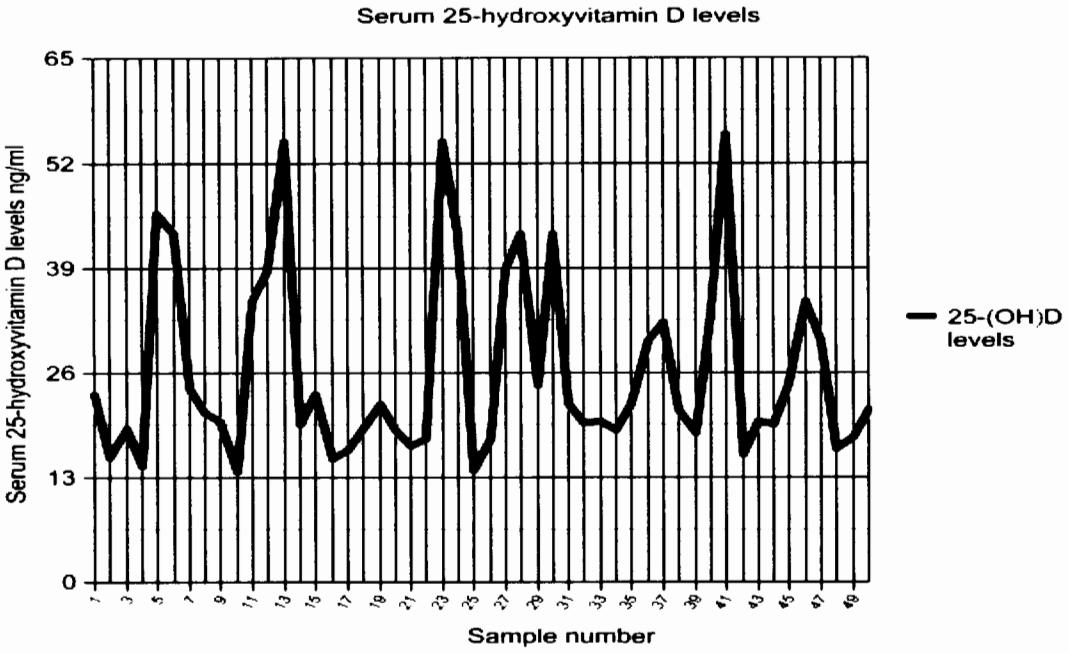


Figure 2: The figure above shows the initial total serum concentrations for subjects 1-50, all of which had an averaged IgG titer higher than 3.5 IV. All subjects that had 25-hydroxyvitamin D values below 25 ng/ml, were used for the experiment. All other participants were not asked to continue the experiment. However, they were informed of their 25-hydroxyvitamin D levels. Based on these results we now only have eighty females to participate in the experiments.

In the experiment to determine serum 25-hydroxvitamin D levels eighty females have serum 25-hydroxyvitamin D levels below 25 ng/ml. (They all also have elevated IgG titers to VCA and EBNA EBV antigens as well) Now that all eighty subjects possessed the desired characteristics that research implies put them at an elevated risk for developing MS, their blood samples were shipped to Promega (Madison, WI) where selected commercial kits were used to determine serum TGF-β1 levels. The results produced an over all average of 7.5 ng/ml of TGF-β1. TGF-β1 anti-inflammatory

cytokine was used as a marker to determine the affects of Vitamin D usage on deterring MS onset. If serum vitamin D levels (25(OH)D) are increased, that should result in an increase in TGF-β1, which is an anti-inflammatory cytokine. Since MS is an inflammatory disease of the CNS, any cytokine that reduces the likelihood of inflammation, should reduce the likelihood of acquiring an inflammatory disease. That is the predicted outcome of the experiments.

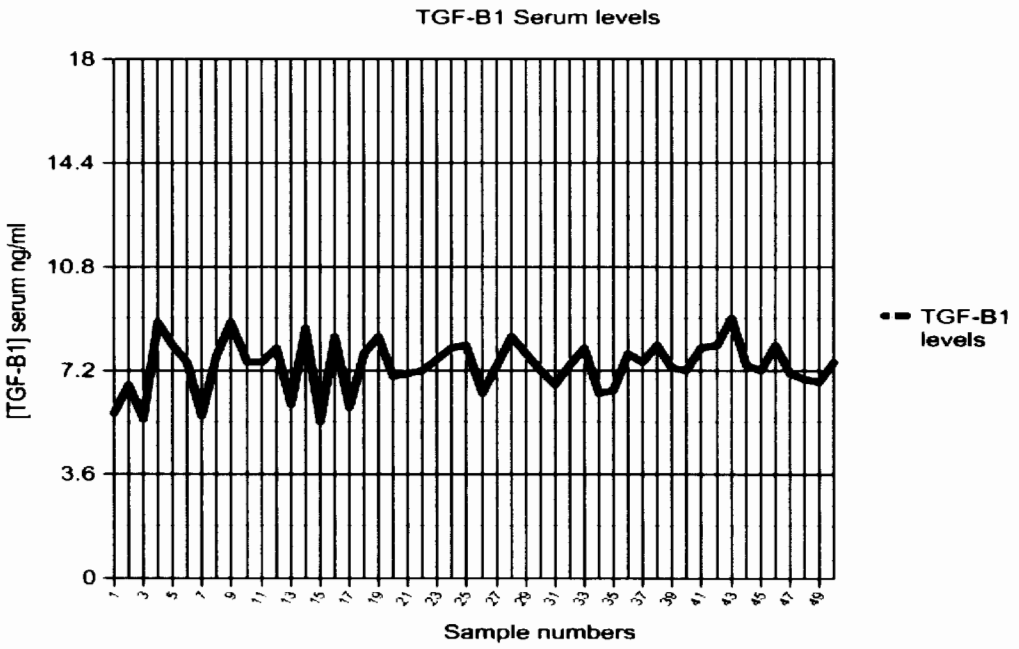


Figure 3: The figure above shows the initial serum TGF-β1 concentrations for fifty of the eighty females in the study. The mean for all eighty was 7.5 ng/ml of TGF-β1. The TGF-β1 anti-inflammatory cytokine will serve as a marker for vitamin D's ability to prevent inflammation, which may deter the onset of MS when in elevated quantities.

After six months the eighty females underwent serum testing for 25-hydroxyvitamin D and TGF-β1 levels as described earlier. The forty females in the experimental group that had dairy product limitations while taking Caltrate, showed an increase in both 25-hydroxyvitamin D levels and in TGF-β1 levels. However, the forty

females in the control group which had no diet limitations and are not taking the Caltrate, showed results very similar to their initial concentrations of both the 25-hydroxyvitamin D and TGF-β1 levels. After one year the trend continued for the forty females in the experimental group, while the forty females in the control group continued to produce similar results to their initial concentrations. The results for the one year TGF-β1 comparisons (**Figure 6**) and one year 25-hydroxyvitamin D serum level comparisons (**Figure 4**) for the forty females taking Caltrate are recorded below.

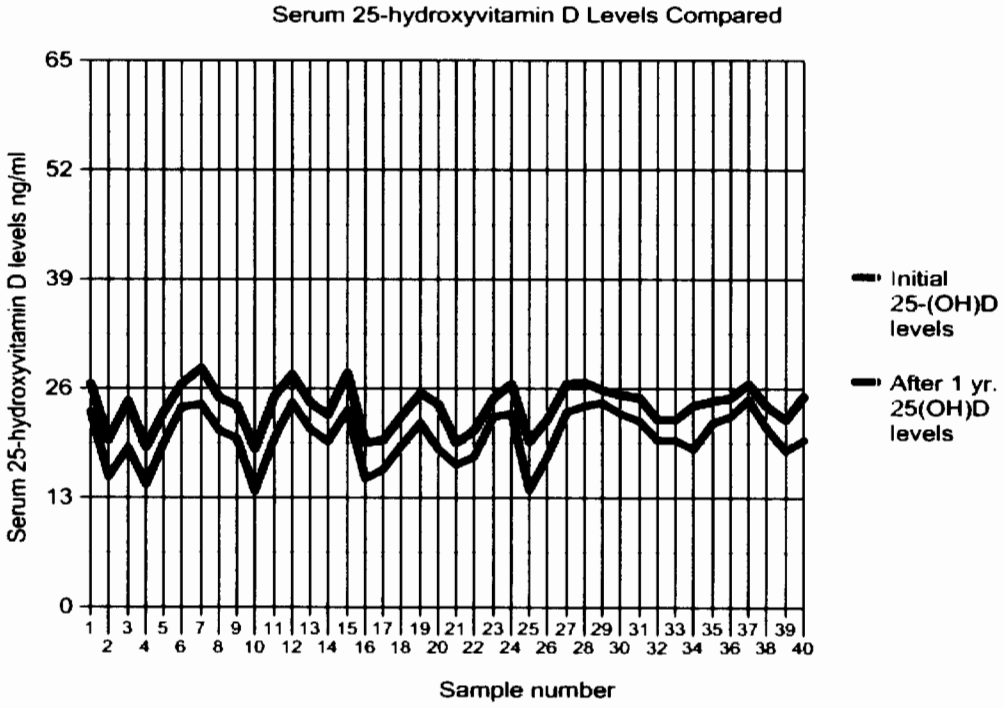


Figure 4: The figure above compares the initial 25-hydroxyvitamin D levels with the 25-hydroxyvitamin D levels after 1 year of taking Caltrate and additional vitamin D. Notice that there is a clear increase in 25-hydroxyvitamin D levels among most of the participants in the experimental group (40 females taking supplements). This increase should correlate with an increase in TGF-β1 serum levels.

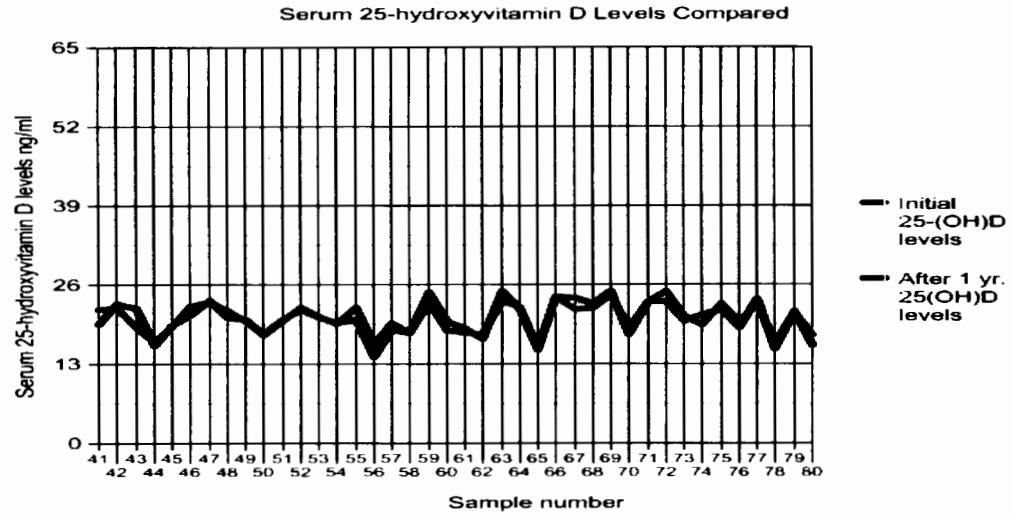


Figure 5: The figure above compares the initial 25-hydroxyvitamin D levels with the 25-hydroxyvitamin D levels after 1 year without taking the Caltrate, and they are permitted to eat dairy products. The forty members of this control group experienced readings very similar to their initial readings. Notice that the line does not change very much from participant to participant. This general tendency for the 25-hydroxyvitamin D levels to remain the same or decrease, should not lead to an increase in TGF -β1 serum levels.

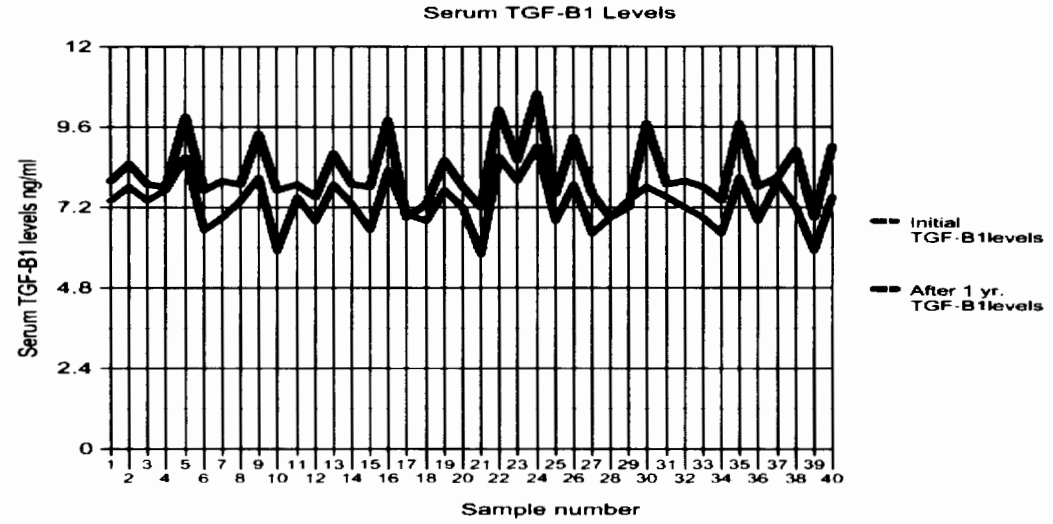


Figure 6: The figure above compares the initial concentrations of serum TGF-β1 levels with the serum TGF-β1 levels after 1 year for the 40 females in the experimental group. These females were given the Caltrate supplements and asked to avoid all dairy products. It is clear that the intake of the Caltrate leads to an increase in TGF-B1 serum levels. The increase in the anti-inflammatory cytokine could act as a deterrent to the onset of MS.

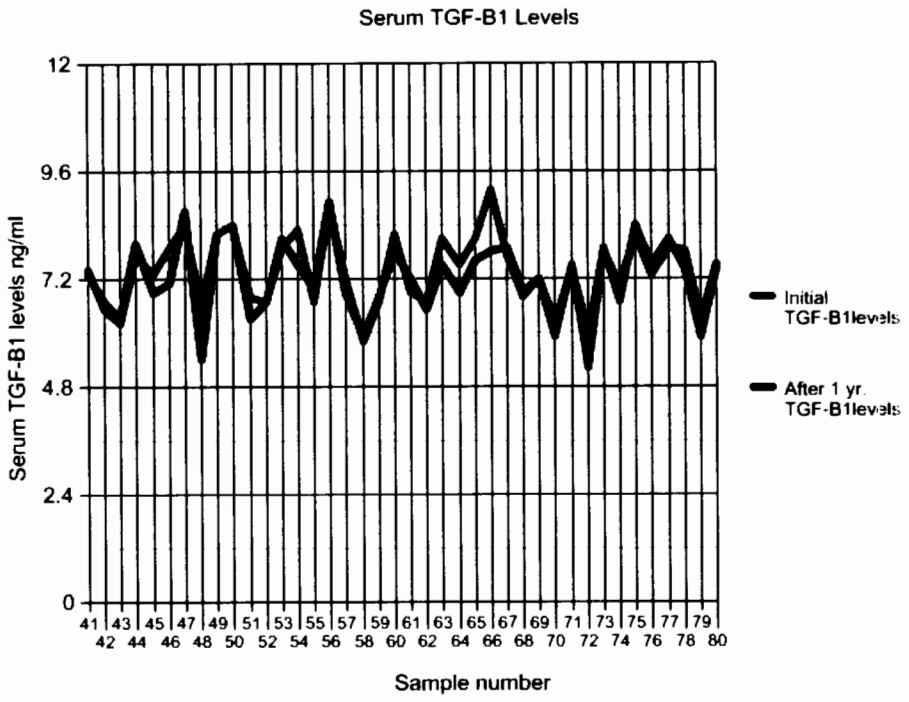


Figure 7: The figure above shows that among the females not taking the Caltrate, there is a slight decline, or stabilized level, of TGF-β1 in serum after one year. This may leave them slightly susceptible to an inflammatory disease like MS.

Discussion of Expected Results

All participants in the study were selected because they possessed four distinct characteristics, each of which research suggests may put them at an elevated risk of developing MS. 1) They all have elevated antibodies against EBV VCA and EBNA antigens. (Pohl et al., 2006; Munch et al., 1998; Cepok et al., 2005; Ascherio et al., 2001; Wagner et al., 2000; Christensen 2005; Bray et al., 1992) 2) They all have low serum 25-hydroxyvitamin D levels. (Nieves et al. 1994, Van der Mei et al., 2007, Munger et al., 2004) 3) They all live in Canada which is a country that is distant from the equator and declared to be a high risk zone for developing MS. (More than 200 per 100,000 people here have MS) (Beck et al., 2005; Sloka et al., 2005; Hayes et al., 1997) 4) They are all

females, which make up the majority of people who currently have MS. (Whitacre et al., 2001)

Current research has discovered a striking correlation that exists between EBV antigens and the development of MS. It is interesting to note that ninety percent of the world's population is seropositive to the EBV while close to one hundred percent (99.9) of MS patients are seropositive to EBV. In addition to the near one hundred percent seropositivity to EBV, MS patients also have significantly elevated IgG titers to the EBV VCA and EBNA proteins specifically, when compared to healthy controls. (Alotaibi et al., 2004; Levin et al., 2005; Ascherio et al., 2001; Pohl et al., 2006; Cepok et al., 2005; Sundstrom et al., 2004; Levin et al., 2003; DeLorenze et al., 2006) That is an important distinction to make because it directly links specific viral antigens to the etiology of MS.

Because current research has produced this correlation it was essential that all participants have elevated IgG antibodies against EBV VCA and EBNA antigens. It is theorized by various neuroscientists that the VCA and EBNA antigens can elicit an immune phenomenon called molecular mimicry, where VCA and EBNA antigens which are similar to self-antigens, cause the activation of autoreactive T-cells which results in demyelination. (Vaughan et al., 1995; Vaughan et al., 1995; Buljevac et al., 2005; Lang et al., 2002)

It is well documented that EBV can cause T cell cross recognition between its VCA and EBNA antigens and myelin antigens in animal models. (Wucherpfennig et al., 1995; Hemmer B, et al., 1997; Lang et al., 2002) Since the EBV enjoys a lifelong persistence in immunocompetent B cells and is periodically reactivated, the virus has all

the features which are required for a sustained cross-reactive autoimmune response. (Cepok et al., 2005)

Figure 1 presents the VCA and EBNA IgG titers for fifteen of the original two hundred and fifty females that volunteered for the experiment. All two hundred and fifty participants underwent serum ELISAs and there VCA and EBNA IgG levels were summed and averaged. The formula to determine who had elevated IgG levels against the EBV antigens was VCA + EBNA/2. This was done because both antigens have been consistently high in numerous MS studies and they both should have equal weight, even if one value far out stretches the other. (Pohl et al., 2006, Munch et al., 1998, Cepok et al., 2005, Ascherio et al., 2001, Wagner et al., 2000, Christensen 2005, Bray et al., 1992)

A summed and averaged index value of 3.5 or higher is considered to be at significantly high titers. Scientists at ARUP Consult who performed the ELISAs were contacted, and they determined that an index value of greater than 3.5 for either antigen is considered significantly elevated. Naturally, all volunteers that had a summed and averaged VCA and EBNA antigen level above 3.5 were considered extremely valuable to the study. In fact, all eighty of the participants in the study shared this distinction.

Not all females that had elevated IgG antibodies against the VCA and EBNA antigens also had low serum levels of 25-hydroxyvitamin D. If they did have high IgG titers to the EBV antigens, but, they had moderate to high levels of 25-hydroxyvitamin D, they were not asked to continue the study. Low levels of serum 25-hydroxyvitamin D was determined based on the most recent findings from leaders involved in Vitamin D

research. (Holick 1998; Nieves et al. 1994, Van der Mei et al., 2007, Munger et al., 2004; Hayes 2000)

A serum 25-hydroxyvitamin D level below 25 ng/ml was determined to be low, and was also a necessary characteristic of the eighty participants that participated in the study. (Holick 1998) Most scientists agree that the serum 25-hydroxyvitamin D serum levels should not drop below 32 ng/ml, however, all agree that it should not fall below 25 ng/ml. (Holick 1998) **Figure 2** shows the initial total 25-hydroxyvitamin D serum concentrations for subjects 1-50, all of which had an averaged IgG titer higher than 3.5 IV. Of the one hundred and twenty females with elevated IgG titers, eighty also possessed low (below 25 ng/ml) serum concentrations of 25-hydroxyvitamin D, and they all were asked to participate in the study.

The fourth distinction that all eighty females possess is that they all are born in a high risk area of the world. All participants live in Canada which is distant from the equator, and an area where MS reaches above 230 + per 100,000 individuals. (Beck et al., 2005; Sloka et al., 2005; Hayes et al., 1997) Because the female subjects are all from Canada they are exposed to reduced quantities of UVB light, especially in the winter months. Because Vitamin D is created in the skin when the skin is exposed to UVB light, people that are born in Canada often lack appropriate concentrations of vitamin D.

Once Vitamin D is created in the skin or ingested in select foods, it is transported to the liver where it is converted to 25-hydroxyvitamin D or (25(OH)D). The 25-hydroxyvitamin D is quickly released into the blood, and upon reaching the kidney, it is converted to the vitamin D hormone 1,25-dihydroxyvitamin D (calcitriol). The kidney

can synthesize 1, 25-dihydroxyvitamin D (calcitriol) when the parathyroid gland releases the parathyroid hormone (PTH), which is in turn regulated by the Ca+2 levels in the blood. (Hollis and Horst, 2007)

In the proposed experiments 25-hydroxyvitamin D levels are closely monitored, and provides us with a substance that can have dramatic immuno-regulatory functions. In fact, the lack of proper 25-hydroxyvitamin D levels is the explanation for why MS prevalence is nearly zero close to the equator, and is dramatically increased in the northern latitudes. (Willer et al., 2004; Hayes et al., 1997; Rosati, 2001; Schwartz, 1992) In comparison to Canada, Zimbabwe has .45 per 100,000, South Africa has 3.5 per 100,000, Brazil has 4 per 100,000, and Mexico has 8 per 100,000 people affected by MS.

It is apparent that 25-hydroxyvitamin D may be a natural inhibitor of M.S. Since vitamin D receptors can be found on CD8 T cells and on macrophages, vitamin D must have a regulatory effect on the immune system. (Bhalla et al., 1983; Provvedini et al., 1983) It is also well documented that M.S. is an inflammatory disease whose onset causes an increase in inflammatory cytokines like IL-2, TNF-alpha, and interferon-gamma, and a decrease in anti-inflammatory cytokines like TGF-β1 and IL-13. (Bertolotto et al., 1999; Killestein et al., 2001, Clerici et al., 2001).

It has been proven in EAE animal models that 25-hydroxyvitamin D can increase TGF-β1 titers (Cantorna et al., 1998; Mahon et al., 2003), and that can reduce the symptoms associated with EAE. (Nashold et al., 2000; Hayes 2000; Bemiss et al., 2002) Also, vitamin D consumption has helped prevent exacerbations among some individuals with MS. (Goldberg et al., 1986) Therefore, the beneficial affects of increasing 25-

hydroxyvitamin D levels is evident by the increased production of TGF-β1 anti-inflammatory cytokine.

Figure 4 compares the initial 25-hydroxyvitamin D levels with the 25-hydroxyvitamin D levels after 1 year of taking Caltrate. As expected, Caltrate consumption leads to an increase in serum 25-hydroxyvitamin D levels in all forty females in our experimental group. **Figure 6** shows the positive correlation that exists between 25-hydroxyvitamin D serum levels and TGF-β1 serum levels. Simply put, when serum 25-hydroxyvitamin D levels are elevated, serum TGF-β1 levels become elevated, which may result in a physiological protection against the development of an inflammatory process like MS. (That is the hope of the experiments)

Notice in **figure 5** that the forty females not taking the Caltrate supplements had 25-hydroxyvitamin D levels that remain relatively constant over the one year period. In the six month samples taken during the winter months 30 females had serum 25-hydroxyvitamin D levels that fell below there initial levels. After one years time, **figure 7** shows that since there was no increase in 25-hydroxyvitamin D levels, there was no increase in TGF-β1 levels among the forty females not taking the supplements, which may result in a reduced protection against the onset of an inflammatory disease. The positive correlation that exists between 25-hydroxyvitamin D levels, and TGF-β1 levels, has been reinforced greatly by this series of proposed experiments.

The increase in the TGF-β1 anti-inflammatory cytokine will serve as our primary marker for potential MS onset deterrence. The other potential markers are symptoms associated with the onset of MS, each of which is mentioned on the **internet survey** on

page 9. Figure 3 shows the initial concentrations of TGF-β1, which averaged 7.5 ng/ml among the eighty participants at the beginning of the study. **Figure 6** shows the results for the forty females taking Caltrate, the average TGF-β1 concentrations increased to 8.8 ng/ml. This is predicted to result in a protection against an inflammatory disease like MS. Therefore, it is predicted that 25-hydroxyvitamin D levels should be closely monitored, and if it is not, that may leave an individual more susceptible to the onset of an inflammatory disease.

Throughout the experiment the forty females selected for the experimental group were not only given oral doses of vitamin D 400 IU, Calcium 1200 mg (extra 600 IU of vitamin D during winter months) and asked to eat a diet high in fish oils (fish oils are high in vitamin D), they were also asked to avoid all dairy products, especially cow's milk. The vitamin D intake is increased by 600 IU during the winter months because there is even less UVB exposure which results in lower 25-hydroxyserum vitamin D levels. The Food and Nutrition Board of the Institute of Medicine has set the tolerable daily upper intake level for vitamin D at 50 μg (2,000 IU) for adults, or pregnant and lactating women. Therefore, all vitamin D quantities are safely below the toxicity levels for vitamin D.

The dairy product avoidance is suggested due to research involving dairy product proteins and autoimmune disorders. Since there is a correlation between milk consumption and MS, all individuals in the experimental group were asked to avoid milk, cheese, and yogurts. (Malosse et al., 1993, Malosse et al., 1992) Cow's milk in particular was to be avoided because butyrophilin can closely resemble myelin proteins

and can cause an MS like disease in animal models. (Winer et al., 2001, Guggenmos et al., 2004, Stefferl et al., 2000) There is no evidence of this in humans, however, experimental participants were asked to avoid it.

Summary of Proposed Experiment

It is plausible to think that if a substance can elevate anti-inflammatory cytokines it can help prevent or reduce the affects of an inflammatory process. The proposed experiment's data determined that Vitamin D is such a substance. When its serum concentrations were elevated by providing Caltrate and vitamin D tablets, the TGF-β1 anti-inflammatory cytokine was produced at a higher frequency. Real research confirms this possibility. (Mahon et al., 2002; Mahon, 2003; Cantorna et al., 1998; Meyer et al., 2001) Thus, vitamin D may have a preventive affect on the development of the inflammatory disease MS.

After one year of the proposed experiment the following expected results were produced. One individual called participant 48 has developed MS. Participant 48 is a member of the control group and did not attempt to elevate her serum 25-hydroxyvitamin D levels by taking the Caltrate and additional vitamin D. Participants number 54 and 56 have developed optic neuritis which may or may not be associated with the onset of MS. They are also among the control group females.

Thus far, all females in the experimental group enjoy good health. The internet surveys will continue for years to come as well as monitoring serum 25-hydroxyvitamin D and TGF-β1 levels. Naturally, after two or three years I expect both values to reach an acceptable level and remain there as time passes. Then I will check serum levels every two years and rely mostly on the internet surveys to draw conclusions.

The expected results from this proposed experiment shows that by elevating serum 25-hydroxyvitamin D levels an increase in TGF-β1 anti-inflammatory cytokine is produced. This may provide a protective role against an inflammatory disease like MS. Perhaps that is the explanation for why all females in the experimental group enjoy good health. The controls that did not elevate their 25-hydroxyvitamin D levels were likely at greater risk of developing MS than those that did take the supplemental vitamin D. This is evident by the female from the control group developing MS and two others from the control group developing symptoms common to MS onset.

Real scientific data points out an amazing truth about Canada; it is certainly a high risk zone for developing MS. If the expected results for my experiments are validated researchers could encourage vitamin D supplements throughout Canada to try to reduce the number of potential MS cases. The overall Canadian MS prevalence was 240 per 100,000, however by providence it ranged from 180 per 100,000 in Quebec, to 350 per 100,000 in Atlantic Canada. (Beck et al., 2005) Scotland has the ominous distinction of having the highest MS incidence in the world.

Real scientific research demonstrates a powerful correlation between vitamin D levels and the removal of inflammatory disease in mouse models. Also, it has slowed down the exacerbations that take place in human MS progression. (Nashold et al., 2000, Cantorna et al.,1996, Cantorna et al., 2000) Perhaps this occurs due to vitamin D's ability to elevate anti-inflammatory cytokines like TGF-β1. However, further studies should be geared toward finding other substances that can elevate additional anti-inflammatory cytokines, as well as, more studies on the immunological protective roles of Vitamin D. Finally, more emphasis needs to be placed on preventative strategies

against MS rather than just on treatments once a person is diagnosed. This proposed experiment showed two distinct characteristics that an extremely high percentage of MS patients posses. Namely, elevated serum IgGs to VCA and EBNA EBV antigens and low serum 25- hydroxyl vitamin D levels. Once that is determined in an individual, preventative vitamin D supplements could be administered to attempt to reduce the likelihood of developing MS by removing one significant risk factor while elevating an inflammatory cytokine that possibly can serve a protective role.

By administering supplemental vitamin D an increase in TGF-β1 is produced and perhaps that and other anti-inflammatory cytokines can help deter the onset of an inflammatory disease. (Diet is also important) This experiment introduces the possibilities that anti-inflammatory cytokines can be a target for the potential prevention of an inflammatory disease. If more are elevated perhaps that can play a preventative role in the onset of an inflammatory disease like MS.

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