The Effect Of Vitamin D3 Supplementation On Brachial Artery Flow Mediated Dilation In Healthy Older Adults With And Without Rheumatoid Arthritis

by

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ABSTRACT

Despite advancements in drug therapy, cardiovascular disease (CVD) is the leading cause of death in the United States. Given this, research has begun to seek out alternative approaches to reduce CVD risk. One of these approaches is Vitamin D supplementation. Research has shown a link between Vitamin D status and CVD risk. Among the possible mechanisms is a positive effect of Vitamin D on vascular endothelial function, which can be measured with noninvasive techniques such as flow-mediated dilation (FMD). This dissertation is comprised of two studies. The first examines whether Vitamin D supplementation can improve FMD in older adults within a time period associated with peak increases in plasma Vitamin D concentrations. The second examines the effect of Vitamin D supplementation in people with Rheumatoid Arthritis (RA). In the first study 29 Post-Menopausal Women received either 100,000 IU of Vitamin D3 or a Placebo. Their FMD was measured at baseline and 2 weeks after supplementation. After 2 weeks there was a significant increase in FMD in the Vitamin D group (6.19 $\pm$ 4.87 % to 10.69 $\pm$ 5.18 %) as compared to the Placebo group (p=.03). In the second study, 11 older adults with RA were given 100,000 IU of Vitamin D or a Placebo. At baseline and one month their FMD was examined as well as plasma concentrations of Vitamin D and TNF-α. They also underwent a submaximal exercise test on the treadmill for estimation of maximum oxygen uptake (VO_{2max}). There was no significant change in FMD in the Vitamin D group as compared to

the Placebo group (p=.721). There was no significant improvement in either plasma Vitamin D or TNF-α in the Vitamin D group. There was a significant improvement in predicted VO_{2max} in the Vitamin D group (p=.003). The results of these studies suggest that a single 100,000 IU dose of Vitamin D can enhance FMD within two week in older adults, but that a similar dose may not be sufficient to increase FMD or plasma Vitamin D levels in older adults with RA. A more aggressive supplementation regimen may be required in this population.

DEDICATION

This dissertation is dedicated to my father, for his unwavering love and support and for teaching me the value of hard work and to the memory of my mother who is a constant source of inspiration and love.

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Chapter 1

INTRODUCTION

Despite several medical and pharmaceutical advances, cardiovascular disease (CVD) is still the leading cause of death in the United States (Centers for Disease Control and Prevention [CDC], 2010). With almost 600,000 deaths annually attributed to CVD, it is clear that alternate strategies for improving CVD disease need to be developed. (CDC, 2010) What is know is that being physically fit and having a balanced diet will significantly reduce the risk the developing CVD. While large scale policy efforts to improve the physical fitness and nutritional status of our population may be needed to enact widespread change, current research suggests that supplementation of various vitamins may also be effective in reducing CVD risk. One such vitamin is vitamin D.

Lower levels of Vitamin D have been associated through cross-sectional studies with the pathogenesis of atherosclerosis, a condition that is directly correlated with an increase in CVD risk. Vitamin D has also been shown through supplementation studies in both healthy and diseased populations to improve CVD status. Most of these supplementation studies have utilized a technique called brachial artery flow mediated dilation to assess endothelial function,

which is a significant predictor of vascular disease status.

While some of the early research in this area appears to be promising there is still much to be examined. This dissertation attempts to help fill some the gaps that currently exist in the literature.

First, there is no clear understanding of the speed at which vitamin D acts in respect to improving endothelial function. This will be examined in the first study of this paper. Study 1 examines endothelial function, as measured by brachial artery flow-mediated dilation, two weeks after a large dose of Vitamin D (100,000 IU). Based on pharmacokinetic studies of Vitamin D, serum levels of Vitamin D appear to peak 10-14 days after a dose of 100,000 IU. Despite this knowledge, there are no studies to date that examine endothelial function at the two-week time point after supplementation. Therefore, Study 1 examined the effects of 100,000 IU of Vitamin D on endothelial function after two weeks. The goal of this study is to better understand the time course of which Vitamin D is able to act on endothelial function.

Second, while some special populations have been studied in terms of Vitamin D supplementation many have not been studied. One of these populations is persons with Rheumatoid Arthritis (RA). The leading cause of early mortality in this population is CVD, so it is a population that may potentially benefit from this

type of intervention. Study 2 will examine the effects of Vitamin D supplementation in this population.

Finally, it is not definitively understood what the mechanism is by which Vitamin D functions in improving endothelial function. One theory suggests that it may essentially be functioning as an anti-inflammatory agent. A few studies using rodent models or cell cultures found that Vitamin D may reduce inflammation by blocking Tumor Necrosis Factor – α (TNF-α) production. This, however, has yet to be shown in human studies. Therefore, in Study 2 the effect of Vitamin D on TNF-alpha will also be examined with the goal of better understanding the mechanism by which Vitamin D works. In addition, the relationship between Vitamin D status and inflammation will be examined with respect to fitness level.

Hopefully these two studies will help better illuminate the utility of Vitamin D in reducing CVD thereby allowing future research the ability to pinpoint the most appropriate manner in which to use Vitamin D.

Chapter 2

REVIEW OF LITERATURE

Cardiovascular Disease

Cardiovascular disease is a term that encompasses a number of heart and blood vessel diseases. (American Heart Association [AHA], 2012). Most of these diseases are related to atherosclerosis, a condition in which plaque builds up in the arteries causing the arteries to narrow making blood flow more difficult. (AHA, 2012). The ability to predict one's risk for an adverse cardiac event has become a focus of CVD research. One tool by which researchers have begun to assess CVD risk is by examining a person's endothelial function.

The endothelium is a thin single layer of cells that line the inside of blood vessels. The endothelial cells respond to various stressors to help maintain homeostasis within the vessels. However, too much oxidative stress can result in endothelial dysfunction. (Esper, Nordaby, Vilarino, Paragano Cacharron, 2006) This endothelial dysfunction allows for the progression of inflammation and is the pathogenesis of atherosclerosis. The inflammatory process associated with endothelial dysfunction will be discussed in a subsequent chapter. Another essential element in maintaining homeostasis within the endothelium is the presence of nitric oxide.

Nitric Oxide (NO) is synthesized within the endothelium from the amino acid L-arginine by an endothelium isoform of Nitric Oxide synthase (eNOS) and is a potent vasodilator. (Vallance and Chan, 2001) A loss of NO has also been associated with an increase in adhesion molecule expression again increasing the risk of atherosclerosis. The role that NO plays as a prognostic tool for endothelial function will be discussed in subsequent chapters.

Overall, maintaining healthy endothelial function and reducing inflammation are essential in reducing the risk of an adverse cardiac event (Tousoulis, Charakida, Stefanaidas, 2006). With the importance of a healthy endothelium well understood the next step is developing a non-invasive tool that has the ability to accurately assess endothelial function. Flow Mediated Dilation is a tool that is being used to do just that. This chapter will discuss the utility and mechanisms by which this assessment tool functions.

Endothelial Function and Inflammation

Endothelial dysfunction is associated with higher levels of pro-inflammatory biomarkers such as: C-Reactive Protein (CRP), Tumor Necrosis Factor-Alpha (TNF-α), Interleukin-6 (IL-6), Inter-cellular Adhesion Molecule (ICAM), Vascular Cell Adhesion Molecule (VCAM), E- Selectin, P-Selectin and Von Willebrand Factor (vWF) while adversely affecting Circulating Endothelial Cells

(CEC) (Dod, Bhardwaj, Sajj 2010, Foster, Shantsila, Carruthers, Lip, Blann 2009, Sioulis, Malindretos, Makedou, Makris, Grekas 2009). High levels of inflammation as measured by C-Reactive Protein (CRP) are positively correlated with VCAM, ICAM , E-selectin and P-selectin.(Dod et al. 2010, Foster et al. 2009) These adhesion molecules result in leukocyte adhesion and movement into the arterial wall. Activated leukocytes within the arterial wall secrete matrix metalloproteinases and growth factors that result in hypertrophy of the arterial media. This decreases the diameter of the arterial lumen therefore increasing local shear stress and damaging the arterial intima (Jarvisalo, Juonala, Rairakari 2006). A decreased diameter increases the risk of blood clots thereby increasing the risk of a heart attack or stroke. (AHA, 2012)

As mentioned, nitric oxide (NO) is synthesized from L-arginine within the endothelium. While NO is associated with vasodilation, NO production is also associated with a decrease in pro-inflammatory cytokines, such as TNF-alpha. (Esper et al. 2006) Additionally, NO has also been shown to inhibit the NF-kB receptor which has the ability to transcribe pro-inflammatory genes. (Tak and Firestein 2001) An elevated inflammatory profile may indicate endothelial dysfunction and vice versa, diagnosed endothelial dysfunction indicates an increased inflammatory process. Both endothelial dysfunction and increased inflammation are highly correlated with an increased risk of atherosclerosis and

thereby an adverse cardiac event. One method that is currently being used as a means to assess endothelial function is brachial artery flow- mediated dilation (FMD).

Flow Mediated Dilation

Flow mediated dilation (FMD) of the brachial artery is a well-validated, non-invasive *in vivo* method of measuring endothelial function (Coretti, Anderson, Benjamin, Celermajer, Charbonneau 2002, Yeboah, Folsom, Burke, Johnson, Polak 2009). This procedure has been shown to have a high predictive value with regard to cardiovascular events in patients with and without established coronary artery disease (Yeboah et al. 2009). It has been a primary outcome measure to assess endothelial function in various types of studies ranging from drug trials to exercise studies. It has also been used as a means to examine correlations between endothelial function and other disease risk predictors such as Vitamin D status. The guidelines for the procedure are established by the Brachial Artery Reactivity Task Force and are discussed in detail in the methods section of this paper. Several studies would suggest that the normal range for FMD is 7-15% and for people with established CVD it is 0-5%. (Clarkson, Celermajer, Powe, Donald, Henry et al. 1997, Perticone, Cerevalo, Pujia, Ventura, Iacopino et al 2001)

Flow mediated dilation is Nitric Oxide (NO) dependent. (Esper et al. 2006). This is because NO is produced in response to shear stress which is created by an increase in the velocity of blood flow. (Esper et al. 2006) In the flow mediated dilation technique baseline images of the brachial artery are obtained through ultrasound. After baseline images are obtained a blood pressure cuff is placed around the participants forearm and inflated to 240 mm/Hg at which the artery is occluded resulting in ischemia. Once the cuff is released the increase in blood flow stimulates the production of NO resulting in the dilation of the artery. In the case of endothelial dysfunction, less NO will be produced therefore limiting the ability of the artery to dilate. The dilation is expressed as a percentage.

While FMD is frequently used in research today, there are some that question its ability as a prognostic tool. (Peretz et al. 2007) In a study by Peretz and colleagues, they performed FMD with a variety of techniques and found that while it may correlate with CVD risk, the variability found with different techniques results in significant differences in results between techniques. Considering these findings and the different techniques used at different centers performing FMD's it may be difficult to compare results found in one study to another study. The authors of this study concluded that for FMD to be a viable measure it needs to be further standardized to reduce variability. (Peretz et al. 2007) The study by Esper et al. also concluded that while further evaluation of the

measure needs to occur it appears that if done well FMD has a high prognostic value.

Cardiovascular Disease-Current Medical Treatments

While more advanced prognostic tools are being developed to assess CVD, advances are also needed in the way CVD is being treated. Currently, medications aimed at lowering cholesterol or blood pressure, such as angiotensin converting enzyme inhibitors (ACE Inhibitors) or statins are most often used to reduce the risk of CVD (Mayo Clinic, 2012). However, in more serious cases surgical procedures are needed to remove the blockages that are present within the vessels. Both ACE Inhibitor and statin therapy have been shown to improve FMD.

A study by Kovacs and colleagues in 2006, showed that in a population with cardiac dysfunction, there was a beneficial effect of ACE Inhibitors. After 8 weeks participants who received the ACE Inhibitor saw an improvement in FMD from $2.95 \pm 0.42\%$ to $5.96 \pm 1.1\%$. (Kovacs, Toth, Tarjan, Koller 2006) In addition improvements in TNF-alpha and C- Reactive Protein were also noted. In a longer term study, Ghiadoni et al. saw similar results after 6 months. Using hypertensive patients, the participants who were put on ACE inhibitors displayed an improvement in FMD after 6 months from 5.1 ± 1.9 % to 6.4 ± 2.4 % , $p < .01$. (Ghiadoni, Magagna, Versari, Kardasz, Huang, et al. 2003)

Similarly, research with statin therapy has produced improvements in FMD. Rawlings and colleagues looked at the effect of 1 month of statin therapy of FMD in 30 Caucasian men with stable atherosclerosis. They found that one month of therapy was enough to statistically improve FMD from 7.4 $\pm$ 0.6 % to 9.3 $\pm$ 0.4 %, p = .003. (Rawlings, Nohria, Yen-Liu, Donnelly, Creager et al. 2009) In a 3 month study Alber et al., found similar results in 33 patients with stable coronary artery disease. While the placebo group did not change the group treated with statins saw an improvement in FMD from 6.7 $\pm$ 3.8 to 8.5 $\pm$ 4.4%, $p < .01$. (Alber, Frick, Sussenbacher, Dorler, Dichtl et al. 2007)

While these drug therapies have show clinical improvement in regards to FMD they have both been associated with adverse side effects. Therefore, since it is known that altering lifestyle factors can have an effect on reducing CVD risk it is important to understand whether FMD can be improved by changing lifestyle factors such as diet and exercise.

Endothelial Function - Current Lifestyle Therapies

Exercise and FMD

Exercise has been shown to be a safe and effective way to improve endothelial function in healthy and high-risk populations with and without cardiovascular disease by modifying the endothelial cell phenotype (Mestek, Westby, Van

Guilder, Greiner, Stauffer, DeSouza 2010, Tesake, Akima, Uehata, Ishihara, Kurita 2009, Metosis, Stravropoulos-Kalinoglu, Sandoo, Veldhuijzen van Zanten, Toms et al. 2010). Exercise increases blood flow, which increases endothelial nitric oxide, and results in an increase endothelial growth (Metosis et al. 2010). Hambrecht et al. (2003) demonstrated that patients with established coronary artery disease, who participated in a 4-week exercise program, showed a 56% improvement in their coronary artery FMD. (Hambrecht, Adams, Erbs, Linke, Krankel et al. 2003) However, benefits to FMD from exercise have been seen in as little as 2 weeks (Tinken, Thijssen, Black, Cable, Green 2008). While exercise programs have been used to improve FMD in healthy and some disease populations, however exercise programs have not been used in patients with Rheumatoid Arthritis as a means to improve FMD. As will be discussed in subsequent chapters, CVD is the leading cause of early mortality in an RA population, therefore interventions aimed at improving FMD in this population may be a critical component in improving the longevity of this population.

In addition to physical activity, there have been several studies that examined the effect that various dietary factors have on FMD.

Fiber

A few studies have examined the effect of a high fiber diet has on improving endothelial function. One such study was conducted by Brock and colleagues at the University of Virginia. Brock et al. (2006) compared the effect of a high carbohydrate, high fiber diet to a low carbohydrate, low-fiber meal on improving FMD. Twelve adults with metabolic syndrome were given both meals in a random order and FMD was measured at baseline and 4 hours after eating. A high carbohydrate, high fiber meal significantly improved FMD (8.46 $\pm$ 4.54 % to 11.87 $\pm$ 4.42%, p = .001) whereas a low carbohydrate, low fiber meal significantly impaired FMD (7.65 $\pm$ 4.09 % to 5.69 $\pm$ 3.43%, p = .03) (Brock, Davis, Irving, Rodriguez, Barrett, et al. 2006) This study indicates the potential of dietary sources to improve FMD in the same manner as many of the drug studies discussed earlier. Long-term studies are needed to examine the ability for a high fiber diet to maintain improvements in FMD.

Berries

Berries are another dietary source that have been shown in the literature to have a positive effect on CVD risk. The reduction in risk comes primarily from the presence of anthocyanins in the berries. Zhu and colleagues conducted 2 separate studies using a concentrated anthocyanin supplement derived from berries and in both studies gave it to hypercholesterolemic subjects. The first study which

involved 12 individuals was a short study in which they examined FMD at baseline, 1 hour post consumption and 2 hours post consumption. (Zhu, Xia, Yang, Liu, Li et al. 2011) They found that at the one hour time point FMD had significantly increased from 8.3 $\pm$ .06% to 11.0 $\pm$ .08%. At 2 hours FMD was still elevated from baseline at 10.1 $\pm$ .09%. These changes were seen after only one dose of the anthocyanins. The authors followed up this study with a longer term 12 week study. In this study, 150 participants were randomized into either the anthocyanin group (n=75) or the placebo group (n=75) and were given one dose every day. After 12 weeks they showed a 28.4% increase in FMD in the group which received the anthocyanins as compared to the placebo group which saw a 2.2% increase. (Zhu et al. 2011) Again, this study provides evidence for the utility of dietary sources to improve FMD.

Dark Chocolate

While this is by no means an exhaustive list of the dietary sources that may have a positive effect on FMD, the final one that will be discussed here is dark chocolate. Dark chocolate is thought to have positive effects on CVD disease risk because of the flavonoids that are found in dark chocolate. Hermann et al. conducted a study to examine if dark chocolate could improve FMD in 25 male smokers. (Hermann, Spieker, Ruschitzka, Sudano, Hermann 2006) Participants were given 40g of dark chocolate and then FMD was examined at baseline, 2 hours after consumption, 4

hours, 8 hours and 24 hours after consumption. At 2 hours post consumption FMD had significantly increased from 4.4 $\pm$ 0.9% at baseline to 7.0 $\pm$ 0.7% at 2 hours ($p = .026$). This improvement was maintained until the 8 hour time point. (Hermann et al. 2006) Once again this study demonstrates the potential effect of a dietary source to improve FMD, however as with the fiber studies it would be beneficial to have a long-term study similar to the anthocyanin study which demonstrates the sustained ability of the dark chocolate to improve FMD over time.

Post Prandial

Studies have shown that while some foods may have a beneficial effect on FMD, some also may have a negative effect. (Brock et al. 2006) Generally, low carbohydrate, low fiber foods and high fat meals have been shown to impair FMD. (Brock et al. 2006, Plotnick, Corretti, Vogel, Hesslink, Wise et al. 2003) However, research has suggested that there may be certain foods that if eaten in combination with low carbohydrate or high fat foods will blunt the negative effect on FMD. Plotnick and colleagues took this idea one step further to determine whether consistent supplementation with foods known to improve FMD would be able to blunt the effect of the consumption of a high fat meal. They took 38 healthy participants and randomized them into a fruit and vegetable juice group with an without and additional supplement (Vineyard) that contained: “arginine

hydrochloride, coenzyme Q10, L-carnitine, mixed tocopherols, ascorbic acid, dried berry juices and extracts, and multiple herbal extracts, including ginkgo biloba, hawthorn berry, grape skin, grape seed, and green tea, and fruit and vegetable plus Vineyard group or the placebo.

> "The fruit concentrate was derived from apples, oranges, pineapples, papaya, cranberries, and peaches. The vegetable concentrate was derived from carrots, parsley, beets, broccoli, kale, cabbage, spinach, and tomatoes." (Plotnick et al. 2003)

Participants were given a high fat meal and FMD was examined before consumption and 3 hours after that meal to obtain baseline measures. After the baseline meal participants took their supplement for 4 weeks. After 4 weeks participants were given the same high fat meal and FMD was once again examined. What was found was that those who had taken either, the fruit and vegetable juice or the fruit and vegetable juice plus Vineyard did not have the same impairment in FMD as they did at baseline. There was no difference between either of the juice groups but the placebo group saw the same impairment in FMD as they did at baseline. (Plotnick et al. 2003) This study is significant because it demonstrates that a healthy diet of fruits and vegetables may reduce the deleterious effect of a high fat meal that is consumed.

Overall, it appears that while more long-term data are needed both exercise and diet have the ability to improvement FMD as with medications.

Inflammation and Lifestyle Factors

In attempt to better understand the role that inflammation plays in the development of atherosclerosis several lifestyle factors have been looked at to determine their effect on reducing inflammation. Both exercise and dietary factors have been examined with respect to inflammation

Exercise and C-Reactive Protein:

Many epidemiological studies have focused on the association between fitness levels and CRP. Data from the Aichi Prefectural Center for Health Care in Japan (n=2722) and from the Aerobics Center Longitudinal Study (ACLS, n=722) found a strong inverse association between VO2max scores and CRP levels. In the ACLS study there was an 80% reduction in CRP between the most fit and least fit subjects (Kokkinos and Myers 2010). CRP levels from the National Health and Nutrition Examination Survey (NHANES) III (n=13,748) were examined and showed that active subjects had 50% reductions in CRP levels when compared to sedentary subjects (Kokkinos and Myers 2010). While these large epidemiological studies provide solid evidence of the relationship of CRP and exercise, recently

many resistance and aerobic exercise interventions have been conducted in both healthy and diseased populations to examine this relationship.

A recent review of resistance training studies concluded that there was on a whole significant improvement in CRP levels across a variety of subject populations (de Salles, Simao, Fleck, Dias, Kraemer-Aguiar, et al. 2010). While improvements were seen in young healthy subjects it was suggested that the largest improvements were seen in overweight and obese subjects, possibly due to higher initial levels of CRP (de Salles et al. 2010). It was also suggested that an effective resistance training protocol should consist of at least 16 weeks of training (2 days/week) at 80% intensity to produce significant reductions in CRP (de Salles et al. 2010). A study by Stewart et al. (2007) found similar results when combining aerobic training with resistance in both young and old populations. In this study, healthy participants were separated by age and activity levels. Both the young and old already active participants acted as the controls for the study and the inactive participants underwent a 12 week aerobic and resistance exercise program. From pre-test to post-test there was a 58% reduction in CRP collectively between the old and young participants and there was no longer a significant difference from the active participants (Stewart, Flynn, Campbell 2007). While combined resistance and aerobic training appears to be effective in reducing CRP, a study by Martins et al. (2010) compared the effectiveness of resistance versus aerobic exercise on CRP levels in 45 older adults (average age = 76), a non-exercising control group was also used for comparison. A 16 week aerobic or strength

training protocol was used followed by a 16-week off-training period. CRP measures were taken at baseline, 16 and 32 weeks. No changes were seen in the control group, however at 16 weeks there was a 10% and 11% reduction in CRP in the aerobic and strength training groups, respectively. At 32 weeks the aerobic group had 51% and the strength group a 39% reduction from baseline in high sensitivity CRP (Martins et al. 2010). While the previous study participants were mainly healthy, several interventions have been conducted on various disease populations.

Since inflammatory markers play a large role in atherosclerosis, several studies using participants with known CVD as well as other chronic conditions have been conducted. A study by Dod et al. (2010) examined physical activity with other lifestyle changes in participants with coronary artery disease (CAD) or multiple risk factors for CAD. 47 participants were randomized to a control group or into a 12 week intervention where they made dietary changes, participated in moderate intensity exercise and stress management. Significant decreases in CRP were seen after 12 weeks only in the experimental group ($p<.03$) (Dod et al. 2010). In a 2008 study by Parrinello et al. participants with congestive heart failure were assigned to either a control or 10-week walking physical training program. Again there were no changes in CRP levels in the control group, however there was a significant decrease in CRP levels ($p<.05$) in the physical training group (Parrinello et al. 2008).

Based on the studies presented it appears that there is a strong case for the use of exercise in reducing CRP levels.

Exercise and TNF-alpha:

While there is a robust body of evidence for the effectiveness of in reducing CRP, the literature on TNF-alpha is not as conclusive. Many of the same studies that looked at CRP levels also examined TNF-alpha. The review of resistance training programs by de Salles et al. found that none of the studies resulted in a reduction of TNF-alpha, and the study by Stewart and colleagues that found a 58% improvement in CRP levels found no significant differences in TNF-alpha levels during the same 12 week protocol (deSalles et al. 2010, Stewart et al. 2007). However, some studies have shown positive effects of exercise on TNF-alpha levels. Lambert et al., conducted a 12 week combined aerobic and resistance training program on older adults. The training involved 3 days a week of 30 minutes aerobic exercise at 75% heart rate max and 30 minutes of resistance training at 65-80% of the participants 1 repetition maximum (Lambert, Wright, Finck, Villareal 2008). Another combined aerobic and resistance program, this time over 12 months showed significant reductions in TNF-alpha in patients with type 2 diabetes (Balducci, Zanuso, Nicolucci 2010). Based on these studies is appears that while the data is mixed, combined aerobic and resistance exercises seem to be more effective that either aerobic or resistance exercises alone.

Exercise and IL-6

As with CRP, there is a strong body of literature supporting the use of exercise for reducing levels of IL-6. A 12-week aerobic study by Goldhamer et al. in which participants exercised 3 days a week for 45 minutes at 75% of their heart rate maximum found reductions of 42% in IL-6 levels (Goldhamer, Tanchilevitch, Maor, Beniamini, Rosencheim et al. 2005). In another 6-month aerobic exercise study using type 2 diabetic patients also found significant reductions in IL-6 (Kagdalou, Iliadis, Angelopoulou 2007). When using sedentary males reductions of IL-6 levels have been seen in as little as 4 weeks (Thompson, Markovitch, Betts, Mazzatti, Turner, et al. 2010). The same 12-month combined aerobic and resistance study by Balducci et al that showed reductions in TNF-alpha levels also showed that exercise was efficacious in reducing IL-6 levels (Balducci et al. 2010). While the evidence is strong for aerobic exercise and combined aerobic and resistance training programs the one area where the evidence is weak is when looking at resistance only training programs. The same review of resistance exercises by deSalles found that resistance training alone did not impact IL-6 levels.

While some studies have produced mixed results regarding the effectiveness of exercise on inflammation there is a growing body of literature that suggests that a wide variety of training protocols are important in reducing inflammatory markers.

While more research is needed on some of the biomarkers, exercise does seem to be a safe way to help improve at least some aspects of inflammation.

In addition to exercise, several dietary factors have been examined as a way to improve one's inflammatory profile.

Fiber

Recent public health campaigns have focused on the promotion of dietary fiber to improve health status. While many people will follow this advice put forth by several governing bodies such as the FDA, HHS and American Heart Association most will not understand that one of the underlying reasons for fiber being healthy is its strong anti-inflammatory property. Epidemiological data from NHANES looking at thousands of United States citizens showed an inverse relationship between fiber intake and C-Reactive Protein, a protein which rises, in large part in response to systemic inflammation. (Ajani, Ford, Mokdad 2004) Besides large-scale epidemiological data, interventional studies have also showed improvements in inflammatory biomarkers through the use of fiber in both healthy and diseases populations. The British Regional Heart Study looked at fiber intake with Type 2 Diabetic patients and found that a lack of dietary fiber resulted in an increase in CRP levels. (Wannamethee, Thomas, Whincup, Sattar 2009) In a study that combined two to three weeks of exercise and high-fiber consumption several

inflammatory markers, namely CRP were significantly improved. (Roberts, Won, Pruthi 2006)

Berries

One type of food that is high in fiber and has strong anti-inflammatory properties are berries. Besides the fiber content berries are also high in anthocyanins, which are pigments responsible for the color of the berry and have strong antioxidant qualities. The anthocyanins seem to have the greatest effect on TNF-alpha levels of all the inflammatory biomarkers. TNF-alpha, which is a cytokine involved in the inflammatory process, is the biomarker that many modern medicines are targeting as a way to slow inflammation caused by autoimmune diseases such as Crohn's Disease and Rheumatoid Arthritis. (Singh, Christensen, Wells, Suarez-Almazor, et al. 2009) Interventional studies have shown the effect of blueberries, cranberries, and tart cherries on reducing serum levels of TNF-alpha levels. (Chung, Claycombe, Song 2008, Seymour, Lewis, Urcuyo-Llanes, Tanone, Kirakosvan et al. 2009) One study which used overweight rats, found that ingesting tart cherries even while consuming a high fat diet resulted in reductions in both TNF-alpha and IL-6 levels. (Seymour et al. 2009)

Omega-3 Unsaturated Fatty Acids

Another dietary factor that has been shown to reduce TNF-alpha as well as IL-6 is omega-3 unsaturated fatty acids. Omega 3's as they are often referred to as, are found most commonly in fish. Again, the beneficial effect of Omega 3's has been seen in both healthy and diseased populations. Based on results from several interventional studies the American Heart Association recommends that people consume fish 2 days a week to improve cardiovascular health. (Kris-Etherton, Harris, Appel 2002) In a review by Endres et al. looking at the use of Omega 3's in patients with Crohn's disease, most studies found a significant clinical improvement inflammatory markers and disease status. (Endres, Lorenz, Loeschke, 2009) As with Crohn's disease, a review looking at Omega 3's and Rheumatoid Arthritis found significant results in number of inflamed joints and disease symptoms with the use of Omega 3's. (James and Cleland 1997)

Dark Chocolate

A final dietary item that has more recently been recognized for its potential anti-inflammatory qualities is dark chocolate. The results are mixed in terms of studies showing positive effects of dark chocolate on inflammatory biomarkers. A study by Mathur et al., demonstrated through a 6-week trial that while consumption of dark chocolate reduced LDL oxidizability it has no effect on CRP levels. (Mathur, Devaraj, Grundy, Jialal 2002) Conversely, a study by di Giuseppe et al., found

through epidemiological data there was a significant inverse relationship between dark chocolate consumption and CRP levels. (diGuiseppe, Castelnuovo, Centritto, Zito, DeCurtis, et al. 2008) While there is debate over the effect on biomarkers there is evidence that dark chocolate consumption may positively affect coronary function. (Flammer, Sudano, Hermann, Gay, Forster, et al. 2007)

While the dietary factors mentioned have overall anti-inflammatory properties, they might have a more specific effect and immediate effect on postprandial metabolism. The postprandial state, or state immediately following the consumption of a meal, is the state that people spend most of their time. (Margioris, 2009) Foods have been found to have acute pro or anti-inflammatory effects on the body immediately post consumption. An increased inflammatory response in the postprandial state has been associated with increased risk of atherosclerosis. (O'Keefe and Bell 2007) Several studies have suggested that consumption of a high fat meal promotes a pro-inflammatory response. After one meal in which over 50% of the calories came from saturated fat increases in both TNF-alpha and IL-6 were seen within 4 hours. (Nappo, Esposito, Cioffi 2002) This result was seen in both healthy subjects as well as those with Type 2 Diabetes. While most studies show a clear link between a high fat diet and increases in inflammation, there may be certain dietary factors that may help mitigate this response. One such strategy may be the introduction of vitamin C to

a high fat meal. In the same study mentioned above subjects consumed a high fat meal but then in addition took a vitamin C supplement. This time the expected increases in pro-inflammatory markers were not seen, but rather TNF-alpha, IL-6 and other biomarkers remained stable. (Nappo et al. 2002) So while it may not always be possible to avoid high fat meals, there may be ways to supplement one's diet to help prevent a pro-inflammatory postprandial response.

While these three dietary factors are by no means an exhaustive list of foods that have beneficial effects on inflammation, they present a strong rationale for the use of diet to reduce inflammation. The research on the long-term effects of certain dietary factors is strong, however the research into the postprandial anti-inflammatory of food while encouraging is fairly new and needs further study.

In addition to whole foods, several vitamins have begun to be studied to better understand the effect that they may have on improving CVD risk. One such vitamin is Vitamin D.

Cardiovascular Disease and Vitamin D

Recently, one dietary factor that has been used in research as a potential means to improve cardiovascular disease risk is Vitamin D. As discussed in previous chapters Vitamin D requires the presence of several other vitamins and minerals to maximize its function, however altering Vitamin D status on its own may be

beneficial. Many of these studies focus on an overweight population, such as studies by Sugden et al. (2007) and Harris et al. (2011) (Sudgen, Davies, Witham, Morris, Struthers 2007, Harris, Pedersen-White, Guo, Stallmann-Jorgensen, Keeton, et al. 2011). Sugden et al., gave participants a single dose of either 100,000 IU of Vitamin D2 or a placebo and followed up at 8 weeks. Compared to the placebo the participants that received the Vitamin D had significant improvements in their flow-mediated dilation (FMD), indicating improvements in endothelial function (Sudgen et al. 2007). While this study had significant findings it utilized Vitamin D2 as opposed to D3, which is generally accepted to be more bioavailable and helps correct deficiencies better than D2. Harris et al. also conducted a large single dose supplementation study this time using D3. For this a dose of 60,000 IU of Vitamin D3 was given once a month for 4 months. A single dose of 60,000 IU is approximately the same as taking 2,000 IU daily. At the 16 week follow-up they again found significant improvements in endothelial function with supplementation (Harris et al. 2011). Tarcin et al. (2009) used healthy subjects for a 3 month study in which they received 300,000 IU of Vitamin D. One significant difference in this study is that the Vitamin D was administered intramuscularly as opposed to orally as was done in the two previously mentioned studies (Tarcin, Yavuz, Ozben, Telli, Velioglu, et al. 2009). Despite the differences in administration of the vitamin, this study also found improvements in endothelial function with Vitamin D supplementation. Significant associations

between Vitamin D and endothelial function have also been seen in older populations. A 2010 study by Jablonski et al. found a strong inverse correlation between Vitamin D status and endothelial function in older adults average age 62 (Jablonski, Chonchol, Pierce, Walker, Seals 2011). One study however did not show any changes positively or negatively in endothelial function after single doses of either 200,000 IU or 300,000 IU of Vitamin D3 (Witham, Dove, Drybaugh, Sudgen, Morris, et al. 2010). The authors proposed that improvements may not have been seen because the subjects were on statins or ACE inhibitors, which can affect the absorption of Vitamin D. Despite this one study it appears as though Vitamin D supplementation may be an effective way to improve endothelial function.

Inflammation and Vitamin D

One proposed mechanism by which Vitamin D improves FMD is that D3 reduces systemic inflammation (Harris et al. 2011). Endothelial dysfunction is associated with higher levels of inflammatory biomarkers, and activation of the inflammatory process may be responsible for both the early development of atherosclerosis as well as in the pathogenesis of RA (Metosis et al. 2010, Dessein Joffe, Singh 2005). Therefore, Vitamin D's potential role as an anti-inflammatory agent may explain the improvements seen in both CVD and RA with Vitamin D supplementation. Several studies have looked at the role of Vitamin D as an anti-

inflammatory agent and have come up with several different pathways by which Vitamin D reduces inflammation. Multiple reviews of the Vitamin D literature suggest that insufficient Vitamin D results in increases in the inflammatory cytokines, CRP and IL-10. (Holick 2007, Lee, O'Keefe, Bell, Hensrud, Holick 2008) However, studies using rodent models have suggested that TNF-alpha is the pivotal cytokine through which Vitamin D is affecting the inflammatory cascade. Zhu and colleagues used mice with Inflammatory Bowel Disease (IBD) and showed that introducing 1, 25 D3 into the model suppressed TNF-alpha production thereby reducing symptoms of IBD. (Zhu 2004) Most recently Zhang et al. (2012) also suggested that TNF-alpha as well as IL-6 may be affected by Vitamin D status. Using cell cultures the researchers demonstrated that removing Vitamin D resulted in an increase in TNF-alpha and IL-6 production. In addition they were able to identify a novel pathway by which Vitamin D works to affect the inflammatory process. The authors suggest that Vitamin D targets MAPK Phosphatase-1. Activation of MAPK Phosphatase-1 inhibited the production of TNF-alpha and IL-6 thereby reducing the inflammatory response. (Zhang, Leung, Richers, Liu, Remigio 2012) While these studies are promising it is important to determine if the same response is seen in human studies. This current study will aim to in part answer that question by examining the effect of Vitamin D3 supplementation on TNF-alpha in people with Rheumatoid Arthritis.

Current Research

The research presented here will examine the ability of Vitamin D3 to improve FMD in both a healthy population and in people with Rheumatoid Arthritis. Two different studies were conducted one looking at FMD 2 weeks after supplementation and s second study looking at FMD 4 weeks after supplementation. Previous research that has indicated that both medications and exercise and FMD can be improved after only 2 weeks, therefore this study will examine whether Vitamin D will have similar effects.

Vitamin D

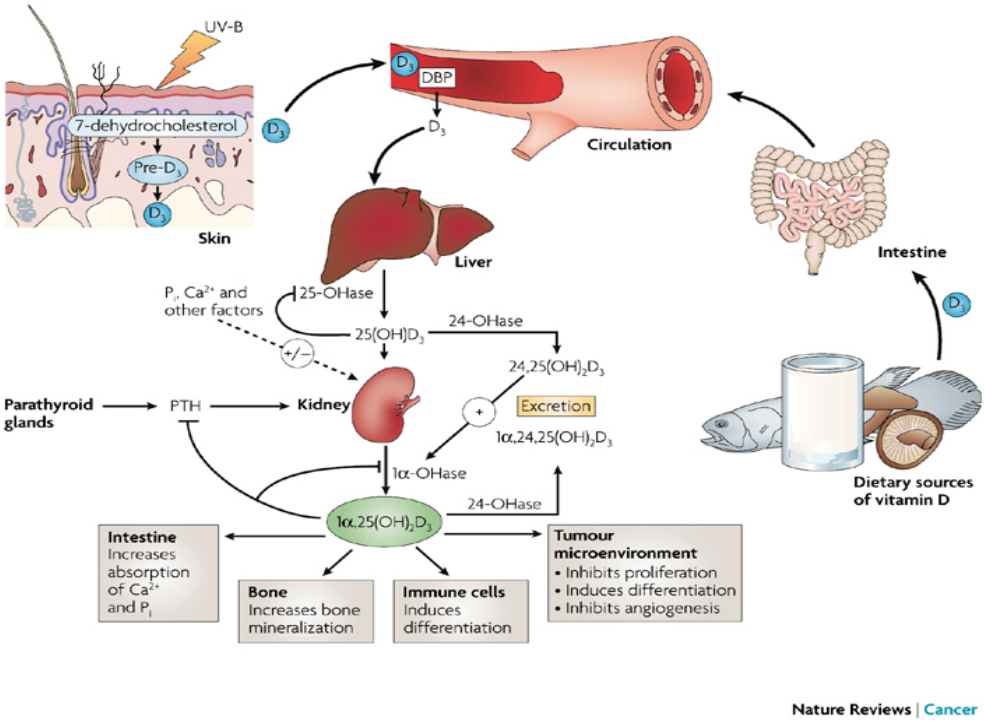
Vitamin D Metabolism

Vitamin D is a fat-soluble vitamin, which has generally been associated with aiding in the absorption of calcium to help maintain bone health. More recently it has been independently linked with many health conditions. It is estimated that the vast majority of Americans are Vitamin D deficient. (Melamed, Michos, Post, Astor 2008)

There are two forms of vitamin D that are used to help correct Vitamin D deficiency, ergocalciferol (D2) and cholecalciferol (D3). D2, which is derived from plant sources is not naturally produced by humans. D3, however, is produced by humans in the skin through a process of converting 7- dehydrocholesterol from ultraviolet radiation produced by the sun. D3 is also obtained from dietary sources, such as fish and dairy. However, adequate Vitamin D can not be obtained through dietary sources. D3 more effectively binds to receptor sites in the body than D2 and is therefore generally accepted to be more bioavailable and better utilized in the body than D2 (Leventis and Patel 2008). Some studies have demonstrated a 1.7 times greater effect of Vitamin D3 as compared to D2 (Trang, Cole, Rubin, Pierratos, Siu et al., 1998). For years D2 was the only form available for purchase and therefore much of the original research aimed at correcting Vitamin D deficiency was conducted using D2. However, today D3 is readily

available. Both Vitamin D2 and D3 are metabolized in the liver and are converted to 25-hydroxyvitamin D (25[OH]D), which may also be referred to as calcidiol. 25[OH]D is rapidly released from the liver and circulates in the blood, making serum 25[OH]D a strong indicator of one's Vitamin D status (Martins, Wolf, Pan, Zadshir, Tareen 2007). 25[OH]D is also metabolized in the kidney and is converted to 1,25 [OH]D that is used to maintain calcium balance in the body. The ability of the kidney to convert 25[OH]D to 1,25 [OH]D appears to decline over time that may partially explain bone loss that is often seen in older adults. (See Figure 1)

Figure 1



Deeb K, Trump D, Johnson C. Vitamin D signaling pathways in cancer: potential for anticancer therapeutics. *Nature Reviews Cancer* 2007; 7(9): 684-700

Co-Factors of Vitamin D

While Vitamin D helps regulate calcium metabolism, there are also other cofactors that help with the effectiveness of Vitamin D. Some of these cofactors include: magnesium, vitamin K, zinc and boron (Vitamin D Council, 2012).

Magnesium

Magnesium has been implicated in over 300 of the body's processes, one of which includes assisting in the metabolism of Vitamin D (Wester, 1987). Magnesium has been shown to have an effect on the body's ability to utilize Vitamin D (Carpenter 1988). Magnesium deficiency has often resulted in a decrease in production of 1,25 [OH], which in turn may affect the ability of Vitamin D to positively affect bone. (Saggese, Bertelloni, Baroncelli, Federico, Calisti et al., 1989) In one experimental study subjects were placed on a regular diet for 3 weeks or one that was deficient in magnesium. In addition to significant decreases in magnesium those in the experimental group experienced a significant decline in 1,25[OH]D from 55 Pmol/L to 43 Pmol/L ($p < .05$) (Fatemi, Ryzen, Flores, Endres, & Rude, 1991)

Recommended Daily Intake and Supplementation

While the RDI for Magnesium is 310-320 mg/day for women and 400-420 mg/day for men research suggests that to maintain optimal levels of magnesium a person should intake 3-5mg per pound of body weight per day ("Magnesium Deficiency

in the Pathogenesis of Disease. Early Roots of Cardiovascular, Skeletal, and Renal Abnormalities.," 1981). Magnesium can be obtained through various food sources such as: spinach, nuts and seeds, squash and beans among others. If sufficient amounts of magnesium can not be obtained through food sources supplementation may be necessary. While magnesium can be found in supplements in various forms magnesium malate and magnesium gycinate are considered to be the most bioavailable forms of Magnesium (Coudray et al., 2005)

Vitamin K

Vitamin K, a fat-soluble vitamin is primary responsible for clotting of blood and assisting with proper bone formation (Adams and Pepping, 2005; Hara and Akiyama, 2007). Research has indicated that adeqaute levels of Vitamin K are necessary when supplementing with vitamin D to prevent vascular calcification. Since the body's ability to absorb calcium is increased with vitamin D supplementation, adequate vitamin K is needed to ensure that calcium is properly delivered to bone as opposed to soft tissues (Fodor, Albu, Poantă, Porojan, 2010; Schurgers, Dissel, Spronk, Soute, Dhore et al., 2001)

Recommended Daily Intake and Supplementation

The amount of vitamin K that a person needs is dependent on their age. As one ages, the required amount of vitamin K increases. According to the National

Academies of Science the Adequate Intake (AI) for adult males it is 120 mcg/day and for females it is 90 mcg/day and since vitamin K is not stored in the body no upper level has been determined. Vitamin K can predominately be found in green leafy vegetables, such as kale. As with vitamin D, vitamin K exists in multiple forms, K1 and K2. It is generally accepted that K2 is superior for supplementation, since it is what interacts with vitamin D (Vitamin D Council, 2012).

Zinc

Zinc, a trace mineral found in muscle and bones is required for proper growth and development. In addition, zinc is essential in DNA synthesis and plays a role in maintaining the body's immune function. (Office of Dietary Supplements 2011, (Tomat, Costa, Arranz, 2011) Zinc works in conjunction with Vitamin D to support the immune system. T-cells, which help the body respond to foreign pathogens, are dependent on both zinc and vitamin D to function. If one is deficient in either zinc or vitamin D, this may slow the growth of T-cells therefore inhibiting the body's ability to fight disease or infections. (Chambers and Hawrylowicz, 2011; Dai, Phalen, & McMurray, 1998)

Recommended Daily Intake and Supplementation

Daily intake of zinc is required as it is not stored in the body. The recommended daily allowance for zinc is 8-11mcg for adults and 11-13 mcg for pregnant women to aid in the development of the fetus. (Office of Dietary Supplements, 2011) Food sources of Zinc include oysters, beans, nuts, among others. One consideration with zinc is its interaction with several medications, including immunosuppressant medications and prednisone both of which are extremely common for people with Rheumatoid Arthritis to be taking. Zinc inhibits the absorption of these medications so while maintaining zinc levels are important, supplementation may not be the appropriate choice for many people with RA. (Vitamin D Council, 2012)

Boron

A final mineral that has a direct effect on the body's utilization of Vitamin D is boron. Boron, a trace mineral, is known for its impact on calcium levels has also been discovered to be involved in several metabolic processes and may affect inflammatory levels. (McCoy, Kenney, Montgomery, Irwin, Williams et al. 1994; Naghii, Mofid, Asgari, Hedayati, Daneshpour, 2011; Samman, Naghii, Lyons Wall, Verus, 1998) Some research also suggests that boron plays an important role in RA. (Devirian and Volpe, 2003)

Boron also acts upon enzymes that affect the metabolism of Vitamin D, therefore low levels of Boron may limit the body's ability to utilize Vitamin D. (Devirian and Volpe, 2003)

Recommended Daily Intake and Supplementation

Currently there is no RDA for boron however considering that it is a water-soluble element the risk of toxicity is minimal. Boron can be obtained through food sources such as: honey, prunes as well as various other fruits and vegetables. (Vitamin D Council, 2012)

While vitamin D is an important vitamin in its own right it is important to understand the role that other vitamin's and mineral's have on improving the metabolism and effectiveness of Vitamin D.

Measuring Vitamin D

It is understood that vitamin D is essential to maintain many of the body's crucial functions, but it is equally as important to understand how to accurately measure one's vitamin D status. It is generally accepted that 25-hydroxy D (25[OH]D) is considered to be the gold standard for measuring Vitamin D according to the Institute of Medicine (Cashman and Kiely, 2011).. The reason being that 25[OH]D is the form of vitamin D that is released into the blood stream after it is

metabolized in the liver. 1,25 [OH]D which is the form that Vitamin D takes after it is metabolized in the kidneys has also been used in studies but is not considered as accurate. 25[OH]D measured in either ng/ml or nmol/l has been used in both healthy and diseased populations as the primary outcome measure when trying to improve Vitamin D status.

While opinions differ on the optimal level of serum 25[OH]D, most researchers would agree that serum concentrations should be above 50 nmol/l. Most, however would argue that for healthy populations an optimal concentration would be above 70 or 80 nmol/l. (Dawson-Hughes, Heany, Holick, Lips, Meunier 2005)

Vitamin D Supplementation

Daily Requirements

Currently there is much debate regarding how much and how often Vitamin D should be supplemented. Considering that one cannot get enough Vitamin D through diet, and that most people are deficient supplementation has become extremely important. (Vitamin D Council 2012) The RDA for Vitamin D for adults is currently listed at 600 IU. However, many experts would argue that this amount is extremely low. For example, the Vitamin D Council recommended that healthy adults get at least 5,000 IU per day of Vitamin D, significantly higher than the government recommendations. (Vitamin D Council 2012) It is estimated that the average American only consumes on average approximately 230 IU of Vitamin

D per day. (Lee, O'Keefe, Bell, Hensrud, Holick 2008) Supplementation through pill form is typically not needed if one has adequate exposure to the sun, however research has been shown that sunscreens have been extremely effective at inhibiting the amount of Vitamin D that can be produced by blocking the UV Rays. (Holick 2002) So while exposure to the sun can be enough to maintain sufficient Vitamin D, it is important to understand that sunscreen may significant blunt the effect of the sun on producing vitamin D.

While the daily required amount of vitamin D needed is still up for debate there have been a few supplementation studies that have examined how to raise 25[OH]D to healthy levels. In a study using healthy subjects that were Vitamin D deficient (below 30 nmol/l), supplementation of 28,000 IU per week (4,000 IU per day) for 6 months was enough to raise serum 25[OH]D levels to over 70 nmol/l which is considered in the healthy range (Dawson-Hughes et al. 2005). However, in diseased populations with severe deficiencies people may require large initial doses to begin the correct the deficiencies. Often mega doses as they are often referred to as are large single doses of over 50,000 IU are used in correcting deficiencies. Many of the studies discussed in this paper use multiple doses of greater than 100,000 IU. One special population that may require additional supplementation is older adults. Studies suggest that adults over the age of 70 have a 25% decrease in the ability to produce cholecalciferol as compared to healthy

younger adult. (Holick 2005) Cross sectional data in women aged 20-96 indicate that as women age 25[OH]D decreases. (Baker, Peacock, Nordin 1980) In general women see a greater decline in vitamin D status as they age then men. It has also been shown specifically in women that as they age the ability of the kidney to synthesize 1,25 D3 decreases which leads to a decrease in calcium metabolism therefore increasing the risk of osteoporosis and falling. (Tsai, Heath, Kumar, Riggs 1985; Leventis et al. 2008)

Due to the increased risk of Vitamin D deficiency in older adults, the current studies discussed in this paper will utilize a post-menopausal women and older male population.

Improving Endothelial Function and Disease Risk

Despite the growing number of studies using Vitamin D as a means to improve various outcomes of health, there is no consensus as to what the proper dosing should be to reduce disease risk. Specifically, there is not a standard dosing strategy in terms of improving endothelial function. Studies have ranged from oral doses as little as 400 IU per day to 300,000 IU over several weeks (Leventis et al. 2008; Sudgen et al. 2007). Studies that have looked at endothelial function in overweight individuals have shown that single doses of 60,000 IU are enough to maintain improvements for a month without additional supplementation (Harris et

al. 2011). With the large discrepancies in dosing suggestions it is important to understand the how serum levels of Vitamin D responds to these large doses.

Pharmacokinetics

One study, utilizing healthy older men, examined the pharmacokinetics of D3 showed that after a single dose of 100,000 IU, serum levels of Vitamin D reached their peak between 10-20 days after administration, than steadily declined after that point. In this study initial serum 25[OH]D concentrations averaged 70 nmol/l and increased to approximately 100 nmol/l after 10 days and remained there until close to 20 days. At the 30-day mark, levels had dropped to 90 nmol/l, which is still considered to be within a healthy normal range of 25[OH]D levels (Ilahi, Armas, Heany 2008). It is important to note that while these studies utilized older men as with the current studies, the men in the pharmacokinetics were healthy which could possibly effect the ability of Vitamin D to be absorbed in the body. Although there are discrepancies in the dosing of Vitamin D, what is most important is that studies have consistently shown that Vitamin D is extremely safe even at very high doses. Studies looking at the pharmacokinetics of Vitamin D3 toxicity have indicated that toxicity does not occur until a serum concentration of 750 nmol/l, although the authors advise against exceeding a level of 250 nmol/l. (Jones, 2008)

Multiple Dosing Studies

In addition to looking at the safety and pharmacokinetics of single doses, studies have examined the safety of multiple doses. Heaney et al. conducted a trial to examine the safety of high doses of Vitamin D. They used doses of both 5,000 IU and 10,000 IU daily for 20 weeks and reported no adverse affects (Ilhani 2008). Multiple studies have reported no adverse affects of single doses of 100,000 IU and higher in both healthy and diseased populations (Leventis 2008). Based on previous research looking at different dosing strategies in combination with current safety data and pharmacokinetic data, the studies reported in this paper will utilize a single dose of 100,000 IU of Vitamin D with the goal of improving endothelial function after both 2 weeks and 1 month.

Current Research

While there are many elements that are understood about Vitamin D, there are many elements that are not. One of the potentially most beneficial roles of Vitamin D from a public health standpoint is to improve chronic disease risk, namely cardiovascular disease. The current studies aim to examine the ability of Vitamin D to improve CVD risk in both healthy and diseased populations. While current research has examined both the correlation between one's current Vitamin D status and disease status there are far fewer studies that examine the effect that Vitamin D supplementation can have on improving CVD disease status. These

studies aim to examine the effect of supplementation on CVD risk in both healthy and diseased populations. These studies will also aim to better understand the mechanism by which Vitamin D works by examining the effect of TNF-alpha after supplementation.

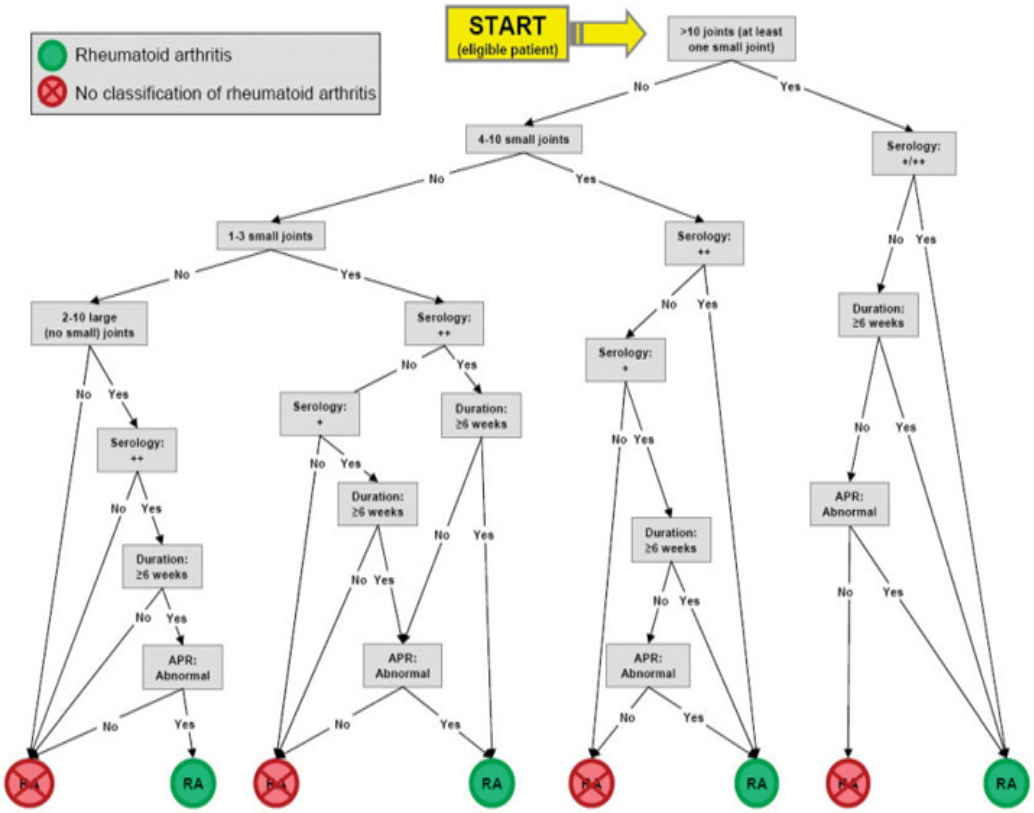
Rheumatoid Arthritis

Rheumatoid arthritis is a chronic debilitating inflammatory disease that primarily affects a person's joints in a symmetrical fashion. Most often the large joints such as the knees, shoulders and hips are the first to be affected however many of the smaller joints (hands, feet and wrists) are often involved. RA affects an estimated 1.3 million Americans and carries an annual estimated cost of $11,404.00 per person to treat (Joyce, Smith, Khandker, Melin et al. 2009). Current reports indicate that this number may in fact be on the rise. An on-going study in Olmsted County, Minnesota saw a rise in RA prevalence from .62% of the population in 1995 to .72% in 2007. (Myasoedova, Crowson, Maradit, Therneau, Gabriel 2010) While RA can affect both men and women it most often affects women and is usually diagnosed in the mid to late 20's. Recent studies suggest that the rate of women diagnosed with RA is sharply on the rise and it is estimated that up to 70% of the people diagnosed with RA are women. (Arthritis Foundation, 2012) As with many autoimmune diseases the prevalence increases in those with Eastern European Jewish heritage. Areas in northern latitudes have also been found to have higher rates of RA.

Rheumatoid Arthritis – Diagnosis Criteria

Diagnosis of RA is determined by criteria set forth by the American College of Rheumatology (ACR). The most recent diagnostic criteria for RA is as follows:

Figure 1
The 2010 Tree Algorithm for classifying definite RA (green circles) or for excluding its presence (red circles) among those who are eligible to be assessed by the 2010 ACR-EULAR RA Classification Criteria



APR = acute-phase response. Serology: + = low-positive for rheumatoid factor (RF) or anti–citrullinated protein antibody (ACPA); serology: ++ = high-positive for RF or ACPA; serology: +/++ = serology either + or ++

Aletaha D, Neogi T, Silman A, Funovits J, Felson D, et al. 2010 Rheumatoid Arthritis Classification Criteria: An American College of Rheumatology / European League Against Rheumatism Collaborative Initiative. Arthritis Rheum 2010;62:2569-81.

Once a diagnosis of RA is determined disease status is typically defined by a combination of blood work and a physical examination. One of these diagnostic tests is the Disease Activity State – 28 (DAS28) which utilizes a patients Erythrocyte Sedimentation Rate (ESR) or C-Reactive Protein (CRP) levels in combination with an examination of 28 joints, evaluation of tender points, redness and swelling. Most studies have shown that both the CRP and ESR method are in agreement when determining disease state.(Wells, Becker, Teng, Dougados, Schiff et al., 2008) However a few studies suggest that the CRP version of the DAS28 tends to underestimate one's disease state as compared to the ESR version. (Matsui, Kuga, Kaneko, Nishino, Eto et al. 2007)

The DAS28 yields a score from 0-9. A score of 0-2.6 indicates that a person is in remission, 2.7-3.2 indicates low disease activity, 3.3-5.1 moderate disease activity and anything above a 5.1 indicates severe disease activity. (Disease Activity Score Website, 2012) Recently, several other disease state measures have been developed such as the Clinical Disease Activity Index (CDAI), the Rheumatoid Arthritis Disease Activity Index (RADAI) and the Simplified Disease Activity Index (SDAI). However, research would suggest that more studies are needed evaluating the utility of these measures before concluding which is the most appropriate means to assess disease activity. (Dougados, Aletaha, van Riel 2007)

Rheumatoid Arthritis- Causes

While the rates of RA are on the rise, the underlying cause of the disease has yet to be clearly identified. Several proposed theories exist ranging from genetics to environmental factors with most researchers in agreement that it is most likely a combination of both genetic and environmental factors. (Arthritis Foundation, 2012) Before a diagnosis of RA is given, physicians will test for the Rheumatoid Factor (RF) in the blood. This is an antibody that acts against gamma globulin, and is often times present in people with RA. (Arthritis Foundation, 2012) However, the RF is not always present in people who develop RA, therefore the presence of RF is not necessary for a diagnosis of RA. Those without a positive RF that go on to develop RA are considered to have seronegative RA. There have also been a few genes that have been identified that are specifically associated with RA. Most commonly genes in the Human Leukocyte Antigen (HLA) region specifically the HLA-DRB1 and HLA-B27 genes have been associated with RA. (Weyand and Goronzy, 2000; Suzuki, Yamada, Chang, Tokuhiro, Sawada et al. 2003) Additionally, the peptidylarginine deaminases citrullinating enzymes that are encoded by PADI genes, specifically PADI type 4 has been linked to RA. (Suzuki et al. 2003) According to the Arthritis Foundation, a few other genes have been identified to play a role in RA: (Arthritis Foundation, 2012)

STAT 4: regulation and activation of the immune system

TRAF1: involved in chronic inflammation

C5: involved in chronic inflammation

PTPN22: associated with the development and progression of RA

Although there is promising and ongoing research to better understand the genes which play a key role in the development and progression, not all people with these identified genes develop RA and some who don't have these genes do develop RA. It is therefore important to attempt to determine what percentage genetics plays in the overall risk for the development of RA.

To determine genetic susceptibility, researchers in the United Kingdom conducted a study looking at the rates of concordance of RA between pairs of both mono and dizygotic twins. For many years it was believed that the concordance rate between monozygotic twins was 30%, however this study that looked at 91 pairs of monozygotic twins found a concordance rate of only 15%. In the same study the rate between 112 pairs of dizygotic twins was 3.6%. (Silman, Macgreggor, Thomson, Hooligan, Carthy, et al. 1993) The lower rates of concordance seen in this study, shed light on the need to better understand what may be alternative causes of RA if genetics potentially explain less than originally thought. A retrospective study conducted from 1980 -1995 in Sweden attempted to better understand some of the environmental factors that might have an effect on RA. This study determined that for both men and women, smoking status played a

significant role in the development of RA. In addition there was a positive dose response relationship between the number of packs per day a person smoked and their risk of developing RA ($p < .005$) There was also an inverse relationship seen between risk of RA and education level for both males and females. Specifically, for men an increased risk of RA was found in those people who were exposed to farm animals or mold while growing up. For females an increased risk was seen in those women who reported short fertile periods as well as prolonged exposure to hair dyes. Lastly, insulin treatment was associated with an increased risk of RA in women. (Reckner, Olsson, Skogh, Wingren 2001) As evident by this study, there are many factors that appear to play a role in the development in RA, however further research is needed to better understand how and why these factors increase the risk for RA. Another study which looked at the epidemiology of RA concluded that the main risk factors for RA are: genetic susceptibility, sex and age, smoking, infectious agents, hormonal, dietary, socioeconomic, and ethnic factors. (Alamanos and Drosos 2005) Given the complexity of the possible causes for RA, recently researchers have suggested that focusing prevention of the disease as is done with CVD, Type 2 diabetes and Cancer, may be a more effective approach that simply focusing on attempting to cure the disease once it has already developed. In a recent meta-analysis, the authors indicate that a large part of the risk of RA is due to smoking, but other modifiable factors such as dietary antioxidants and breastfeeding could also play a role. They also found that large

amounts of coffee and alcohol consumption were correlated with RA. All of the factors that this study identified as increasing the risk of RA are modifiable factors, meaning that they are within the control of a person. In the past it was somewhat assumed that if one was predisposed to RA through their genes that it was inevitable that they would develop the disease so the focus should be on trying to cure RA. However, given the number of modifiable risk factors that appear to increase the risk of RA the authors suggest that there may need to be a shift in the focus of RA to prevention instead of cure. (Lahiri, Morgan, Symmons, Bruce 2012)

Rheumatoid Arthritis – Role of Inflammation

While it is unclear exactly what causes RA, what is known is that in people with diagnosed RA their bodies are typically in a state of chronic inflammation. However, it is not fully understood how the hierarchy of cytokines is organized therefore making it difficult to determine which cytokine is the best target for clinical interventions. (See Figure 3) (McInnes and Schett 2007) Many macrophage derived cytokines (TNF-alpha, IL-1, IL-6, IL-15, IL-18) have been implicated in the pro-inflammatory response which leads to the activation of the transcription factor NF-kB. (McInnes and Schett 2007) Activation of NF-kB incites a greater pro-inflammatory response because of the ability of NF-kB to induce transcription of pro-inflammatory genes. (Tak and Firestein 2001) Given

the fact that many of these cytokines are responsible for several of the same processes in diseased tissue, some researchers suggest that medications that focus on only one cytokine may not be appropriate because blocking one cytokine may cause the others to take over its role. (Goronzy and Weyand 2001)

However, most recently studies have implicated TNF-alpha as the most appropriate cytokine to target. There a few findings that have lead researchers to that conclusion. First, in an experimental model researchers were able to show that an increase in TNF-alpha induced joint damage. (Goronzy and Weyand 2001) Secondly, again in experimental models it was shown that blocking TNF-alpha down regulated several other inflammatory cytokines such as IL-6 and Il-8, indicating that TNF-alpha may be the catalyst responsible for the cytokine cascade. (Goronzy and Weyand 2001) This finding was also replicated in arthritic mice. The results of these findings have lead to the development of several new biological drugs for RA that function to block TNF-alpha. These drugs have made a large impact on the RA population with many people achieving relief from their symptoms and some achieving clinical remission. These drugs however do not work for all persons with RA and most of the medications have a limited lifespan before a person builds up antibodies to the drug and it is no longer effective. For this reason, researchers are currently looking at other cytokines such as: IL-6, IL-15, IL-17 and IL-18 as targets for future medications. (McInnes and Schett 2007)

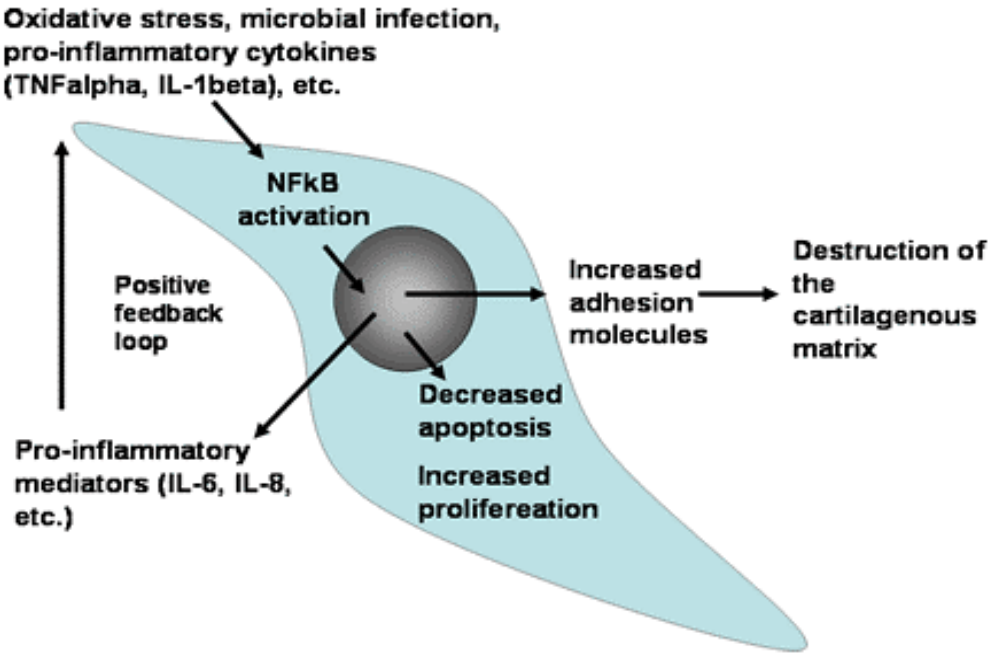


Figure 3: Inflammatory Cytokines in Rheumatoid Arthritis

Source: Okamoto (2005) The epigenetic alteration of synovial cell gene expression in rheumatoid arthritis and the roles of nuclear factor kB and Notch signalling pathways. Mod. Rheumatol. 15: 79-86.

Rheumatoid Arthritis – Cardiovascular Disease

Patients with Rheumatoid Arthritis have been found to have significantly impaired vascular endothelial function as well as increased levels of serum inflammatory markers (Lutzky, Hannawi, Thomas 2007; Metosis et al. 2010). Activation of the inflammatory process may be responsible for both the early development of atherosclerosis as well as in the pathogenesis of RA (Lutzky et al. 2007, Metosis et al. 2010). Endothelial dysfunction is associated with higher levels of

inflammatory biomarkers, which is also strongly correlated with adverse cardiovascular events (Dessein et al. 2005, Gonzalez-Juanatey, Testa, Garcia-Castelo, Garcia-Porrua, Llorca et al. 2003) and is the foundation for the pathogenesis of atherosclerosis. Since patients with RA have chronically higher levels of these inflammatory markers their risk of CVD has been estimated to be 50 percent greater than a healthy population (Anvina-Zubieta, Choi, Sadatsafavi, Etminana, Esdaile, et al. 2006).

As discussed in previous sections of this paper, FMD is a previously validated technique that has been used as a non-invasive means to assess endothelial function. Using FMD Arosio et al. (2007) in 65 women ages 41-52 with RA found that endothelial dependent dilation was significantly impaired compared to a control group ($p<.05$) and significantly correlated with CRP. (Arosio, De Marchi, Rigoni, et al. 2007).

Additionally other cross-sectional studies have also demonstrated that FMD is significantly impaired in the RA population. In a study using 68 women, 32 with RA and 36 healthy women, researchers found that the women with RA had significantly lower FMD's compared to the healthy women (5.6 ± 9.69 % to 23.24 ± 5.65 %, $p < .00001$). (Castro, Montenegro, Caravalho, et al. 2007) Gonzalez et al. demonstrated that not only do people with RA have impaired

FMD but that it continues to decline the longer they have been diagnosed with RA. They showed that is people who had RA from 1-7 years the average FMD was 7.4 $\pm$ 3.8% whereas the average for people who had had RA for over 20 years was 3.3 $\pm$ 4.4 (Gonzalez-Juanatey, Llorca, Gonzalez-Gay 2011). Given the significant problem that an increased CVD presents in this population, studies have begun to develop interventions aimed at improving FMD in RA patients.

Flammer et al. conducted an 8-week crossover trial with 11 RA patients to look at the effect of Angiotensin-Converting Enzyme (ACE) Inhibitors to improve FMD. Participants received 8 weeks of either an ACE Inhibitor or a placebo, after a sufficient washout period they received the other therapy. What was found was that FMD was significantly improved (2.85 $\pm$ 1.49 % to 4.00 $\pm$ 1.81 %, $p < .017$) in the ACE Inhibitor group while it remained unchanged in the placebo group. (Flammer, Sudano, Hermann, Gay, Forster et al., 2008) Another study looking at FMD and ACE Inhibitor therapy found that ACE inhibitors did not improve FMD in RA patients, however they found that statin therapy did improve FMD. 45 RA patients were given 8 weeks of an ACE inhibitor, a statin or a placebo. They found that of the three only the statins improved FMD (5.3 $\pm$ 1.1 % to 8.9 $\pm$ 1.4%, $p = .025$). The authors also showed decreased CRP and TNF-alpha levels in the statin group. (Tikiz, Utuk, Pirildar, Bayturan, Bayindir et al., 2005). In another study looking at statin therapy and its effect on FMD in RA patients,

Hermann and colleagues explored whether FMD could be changed in as little as 4 weeks. They found that they were able to significantly able to improve FMD with only 4 weeks of statin therapy as compared the placebo ($p < .02$). The study by Hermann is important because it showed that FMD maybe able to change faster than what had been shown in previous studies. (Hermann, Forster, Chenevard, Enseleit, Hurlimann, 2005)

A final study that examined FMD in RA patients looked at the effectiveness of TNF-alpha blocker therapy to improve FMD. As will be discussed shortly, TNF-alpha therapy represents the newest category of medications used to treat RA. In this study 11 RA patients were given 12 weeks of TNF-alpha therapy. Researchers found that FMD was significantly improved (3.2±0.4% to 4.1±0.5% $p = 0.018$). Additionally improvements in DAS28 scores and CRP levels were also seen. (Hürlimann, Forester, Noll, Enseleit, Chenevard et al., 2002) Gonzalez – Juanatey et al. (2008) also looked at the effect of TNF-alpha blockers on FMD, however in this study they examine changes 2 weeks after the patients first infusion and then 6 months later. In 6 RA patients at both the two-week and 6 month time point FMD was significantly improved from before the start of treatment. At the two week time point FMD improved from $3.35 \pm 1.58\%$ to $7.02 \pm 2.31\%$ ($p = .03$) This improvement was maintained at the 6 month follow-up. Again these findings were also correlated with improvements in CRP and DAS28

scores. (Gonzalez – Juanatey, Llorca, Vazquez-Rodriguez, Diaz-Varela, Garcia-Quiroga, Gonzalez-Gay 2008) Despite complications with these medications, it appears based on the results of this study that TNF-alpha blocker medications may be beneficial in reducing CVD risk in this population.

Rheumatoid Arthritis-Current Medical Treatments

Estimated costs of RA are 19.3 billion dollars annually and with the apparent increase in disease prevalence the question of how to treat the disease becomes more important. (Birnbaum, Pike, Kaufman, Marynchenko, Kidolezi 2010) While several mechanisms have been proposed, the underlying cause of RA still unknown, making treatment difficult. (Mikuls 2010) The most common trend in drug therapy over the last several years is biological therapy, namely TNF-alpha blockers. (Singh, Christensen, Wells, Suarez-Alamazor 2009) While many of these drugs have been shown to be efficacious they are extremely expensive and not without serious side effects. (Pichler 2006) Considering the potentially dangerous side effects of these biological agents, various lifestyle interventions using both diet and exercise have been used to help improve disease status in RA patients. This review will examine both the effectiveness and safety concerns of biological drug therapy in comparison with the effectiveness of lifestyle interventions on disease status as well as quality of life.

There are currently five approved TNF-alpha inhibitors, infliximab (Remicade), etancercept (Enbrel), adalimumab (Humira), certiolizumab (Cimzia), and golimumab (Simponi). (Singh et al. 2009) For the purpose of this short review, the focus will be on infliximab, certiolizumab and golimumab. While the full pathogenesis of RA is not understood one theory is that excess TNF-alpha at the site of the inflammation could be the driving force of disease activity, therefore blocking TNF-alpha formation could be beneficial in slowing the progression of the disease, hence the use of TNF-alpha inhibitors. (Metosis et al. 2007) The main outcome measures used to determine the effectiveness of these drugs is the Disease Activity Score-28 (DAS-28). The DAS-28 is a combination of a physical examination looking at the amount of inflammation present at the 28 joints of the shoulders, elbows, wrists, metacarpophalangeal joints, proximal interphalangeal joints and the knees and the Erythrocyte Sedimentation Rate (ESR) or C-Reactive Protein (CRP) levels. A simple calculation is used to determine a patients DAS28 score. Typically TNF-alpha blocker therapy is started when a DAS28 score exceeds 3.2. (Metosis et al. 2007)

Infliximab, which was one of the first TNF-alpha inhibitors approved, is administered intravenously in a hospital setting. The initial dosage is at week 0, week 2, week 6, then every 8 weeks after the 6-week point. (Yocum, Rahman, Han 2005) The START trial compared the effectiveness of infliximab (n=709) to a

placebo (n=354) on reducing the rheumatoid arthritis disease activity scores (DAS-28) and found significant reductions in DAS-28 scores in patients receiving infliximab ($p<.001$). (Yocum et al. 2005) Certiolizumab, unlike infliximab is self-administered by the patients through a subcutaneous injection in either their leg or stomach. After the initial pulse dose of injections every week, the normal dosage is once a month. This is an attractive treatment option for patients because it is only done once a month and can be done quickly at home as opposed to infliximab, which can take several hours in the hospital. As with the infliximab there have been several clinical trials showing efficacy for this drug. In the multi-center FAST4WARD trial 220 patients were given either a placebo (n=109) or 24 weeks of certiolizumab (n=111). (Fleischmann, Vencovsky, van Vallenhoven 2009) The baseline DAS28 score average was 6.3 ± 1.0 for all participants combined indicating high disease activity. After 24 weeks the group receiving the certiolizumab had a significant decrease ($p<.05$) in DAS28 scores while the placebo group did not. The treatment group had an average drop of 1.5 in their DAS28 scores. The most recently approved TNF-alpha blocker is golimumab. This drug, as with the certiolizumab is self-administered by the patient at their home every once a month. A 48-week double blind efficacy trial was recently completed looking at the effectiveness of golimumab versus a placebo on DAS28 scores. (Kremer, Ritchlin, Mendelsohn, Baker 2010) At week 14 of the trial the there were significant differences seen in decreases in DAS28 scores between the

golimumab group and the placebo (p=.001), however these differences were no longer seen at the 24 and 48 week time points. If the golimumab was combined with Methotrexate, which is another commonly used drug for RA, the differences in DAS28 scores were still significant. It appears that golimumab is only effective for long periods of time when combined with methotrexate.

While it appears that these drugs may be quite efficacious in regards to disease activity scores they are not without great risk. These drugs are all black box drugs according to the US Food and Drug Administration (FDA). Despite improvements in disease status through the use of these medications, the serious risks associated with these drugs may be strong enough that patients become leery of these drugs and start to seek out alternative therapies. One potential, under-utilized therapy is physical activity.

Rheumatoid Arthritis-Current Lifestyle Therapies

One potential explanation as to why physical activity may not be used often as a means to reduce disease symptoms is the perception that it may not be safe for the RA population. However, several studies have looked at the safety of exercise in this population and there have been no adverse effects of exercise training reported, even with high intensity aerobic or strength training exercise (Cairns and McVeigh 2009). Although exercise has been shown to be safe, patients with RA have been

shown to perform less daily physical activity as compared to a healthy population. (Van de Berg, de Boer, le Cessie, Breedveld, Vliet Vlieland 2007). Not only has exercise been shown to be safe, but exercise programs have also been shown to the beneficial on several clinical measures, such as inflammatory markers namely CRP as well as on multiple other cardiovascular risk factors (Metosis et al. 2010).

While many studies have been conducted looking at quality of life after exercise in RA patients, few studies have been done using DAS28 scores as an outcome. One strength based study, however looked at DAS28 scores and found that participants who underwent a strength training program had significant decreases in DAS28 scores compared with a placebo group ($p = .01$) and these finding persisted even at a 5 year follow-up. (Hakkinen, Sokka, Hannonen 2004) In aerobic training studies that have been conducted, there have been no significant changes in DAS28 scores. (Baillet, Zeboulon, Gossec, Combescure 2010) While, this could be viewed as negative it does indicate that there is not a detrimental effect of exercise on disease activity. Since the potential negative side effects of exercise pale in comparison to those of the medications discussed earlier it is clear that many more studies need to be conducted using exercise as a potential way of decreasing disease activity. It is possible that since so few studies have looked at DAS28 scores and exercise that the proper exercise protocols have not yet been employed. One other explanation for the lack of reduction in DAS28 scores in the

aerobic trials is that the participants already had higher disease levels. If regular physical activity was started immediately at diagnosis it is possible physical activity may keep DAS28 scores from raising to the point that TNF-alpha therapy need to be started.

As mentioned earlier, despite the limited amount of studies looking at exercise and DAS28 scores there have been many more studies looking at exercise and quality of life. The drug therapies were again effective with both the START and FAST4WARD showing significant improvements in quality of life. (Yocum et al. 2005; Fleischmann et al. 2009) However, exercise trials have also been shown to be effective.

Hankkinen et al. conducted one strength-based study that looked at quality of life through the Health Assessment Questionnaire. They examined the long-term effects that a dynamic strength program had on men and women with RA. The control group performed simple range of motion exercise while the exercise group performed home dynamic strength exercises, which utilized dumbbells and exercise bands for 12 months, two times per week. (Hankkinen et al. 2004) The participants in the exercise group were also encouraged to participate in other recreational activities in their spare time. After 1 year significant improvements were seen on the HAQ in the exercise group compared with the control group

(p=.01). However, without a continued exercise program the group differences went away at the 5-year follow-up. (Hankkinen et al. 2004) This has implications for the importance of continued promotion of physical activity in this patient population.

A study by Karatepe et al. employed a much shorter training protocol. The study had 44 participants perform range of motion exercises two times a day five days a week for the 4-week trial. All of the exercises were done at home and participants were given written instructions as well as weekly phone calls to remind them to perform the exercises. There was not a control group in this study. At the 4 week follow-up there were significant improvements on both the HAQ as well as on the Rheumatoid Arthritis Quality of Life survey (RAQoL), $p<.014$ and $p<.023$ respectively. Once the participants completed the four-week trial they were encourage to continue with their home based program for the next year, however they no longer received the reminder phone calls. Of the 44 participants that began the study only 28 returned for the follow up. However, at the one-year follow-up the improvements in the HAQ and RAQoL scores had been maintained. There were no significant differences between their scores at week 4 and week 52, in their HAQ scores ($p =1.00$) or their RAQoL scores ($p=1.00$). (Karapete, Gunaydin, Turkmen, Kaya 2009) This study indicates with the proper protocol

and education, it is possible to maintain exercise and improvements in quality of life over time.

De Jong et al., looked at the differences in functional ability measured by the McMaster Toronto Arthritis (MACTAR) Patient Preference Disability Questionnaire. Participants were assigned to either the usual care group, in which they only saw their physician for routine visits, or to the high intensity exercise group which involved an extensive 2-year twice a week training sessions that included 20 minutes on the bike, a 20 minute exercise circuit and 20 minutes of "sport or game". After 1 year, there were significant difference between the usual care group and the high intensity group on the MACTAR ($p=.034$) and after 2 years the improvements seen in functional ability were even greater between the 2 groups ($p=.017$). (de Jong, Munneke, Zwinderman, Kroon 2003) These findings support the notion that high intensity exercise is not only safe, but also beneficial for functional status of people with RA.

A final study examined the relationship between quality of life and fitness levels in patients with RA. Sixty-six participants, 10 male and 56 female, average aged 54 and 50 respectively underwent a maximal graded exercise test on a treadmill. (Chang, Chui, Hung, Lee 2009) The average VO2 max scores were 27.39 ± 8.09 for males and 22.60 ± 5.46 for females. The researchers took these scores and

correlated them with quality of life scores from the World Health Organization Quality of Life (WHOQOL) questionnaire, which was completed immediately after the maximal exercise test. This quality of life scale asks participants to assess the 2 weeks of their life prior to the study. (World Health Organization 1992) Researchers found that there was a significant positive correlation between aerobic fitness and quality of life ($p<.05$). (Chang et al. 2009) These findings provide an important message to RA patients who are looking to improve their quality of life.

The current modern drug therapies have shown efficacy in reducing disease levels and improving quality of life in the short term, however the question still remains about both the long-term efficacy and safety of these treatments. It is worth exploring other potential safe long-term options such as physical activity that may help keep disease levels low and quality of life high. Study two will examine the fitness levels of an RA population as well look at correlations between fitness level, Vitamin D status, FMD, inflammation and quality of life.

RA and Vitamin D

The exact etiology of RA is unknown leading to several proposed mechanisms for the pathogenesis of the disease. One current theory is that a lack of Vitamin D, may be associated with the development of the disease (Merlino, Curtis, Mikuls, Cerhan, Criswell et al. 2004). Several cross sectional studies have demonstrated a

link between serum 25[OH]D levels and the development of RA. Using data from the Iowa Women's Health Study, Merlino et al. demonstrated a significant inverse correlation between Vitamin D intake, as estimated by a validated food frequency questionnaire, and incidence of RA (Merlino et al. 2004). When using serum levels of 25[OH]D as opposed to self-reported food recalls, Oelzner et al. also found a significant inverse association between vitamin D status and RA disease activity (Oelzner, Muller, Deschner, Huller, Abendroth 1998). In addition to the association between vitamin D status and the development of RA, patients with diagnosed RA have been shown in general to be vitamin D deficient. One review suggests that the optimal level of serum vitamin D as measured by 25[OH]D for patients with RA is between 75-125 nmol/L, with most RA patients falling well below that range (Cutolo, Otsa, Uprus, Paolino, Seriolo 2007). Considering the low levels of Vitamin D in this population, Vitamin D supplementation has recently been explored. While suggestions have been made as to the proper way to correct Vitamin D deficiency through supplementation there have been very few studies that have solely used Vitamin D to improve RA disease status. A 1998 study by Cantora et al. showed that Vitamin D supplementation slowed the initiation and progression of inflammation related arthritis in rodents (Cantorna, Hayes, De Luca 1998). Using an analogue of Vitamin D, alphacalcidiol, Andjelkovic et al. showed that 3 months of 2 μg/day of alphacalcidol supplementation improved immunomodulatory effects in RA patients as measured

by lymphocyte proliferation. (Anjelkovic, Vojinovic, Pejnovic, Popvic, Dujic, et al. 1999) Also, using vitamin D analogues for one month Dottori et al. saw improvements in perceived pain in RA patients (Zittermann et al. 2003). There have been two randomized, double-blind, placebo-controlled studies with RA patients that used a combination of vitamin D3 and Calcium. Brohult and Johnson conducted a study showing that 2.5 μg daily improved pain symptoms in RA patients (Zittermann 2003). The second study, conducted by Buckley and colleagues was aimed at improving bone density and found significant improvements in this patient population with relatively small doses of vitamin D (500 IU daily) (Buckley, Leib, Cartularo, Vacek, Cooper 1996). While vitamin D supplementation seems to be effective for all patients with RA it may be of particular importance to post-menopausal women. Older women with or without RA are already at a higher risk of vitamin D deficiency due to their age. In addition to the problems with RA that may be associated with Vitamin D deficiency, this deficiency can also lead to an increased risk of osteoporosis and falling (Leventis et al. 2008). Besides the risk of both osteoporosis and falling being increased with a vitamin D deficiency these risks are also increased with a diagnosis of RA (Leventis et al. 2008). Given that information, particular attention should be paid to vitamin D levels in post-menopausal women with RA.

Current Research

While randomized controlled trials with vitamin D in this population are extremely limited, there have been several vitamin D supplementation studies that have been aimed at improving many of the complications that arise as a result of RA, namely CVD (Witham et al. 2010; Harris et al. 2011; Dessein et al. 2005; Melamed et al. 2008; Martins et al. 2007). This study will therefore examine the effect of Vitamin D supplementation on improving CVD and inflammation in order adults with RA.

DEFINITION OF TERMS

CVD: Cardiovascular Disease is a disease of the heart or blood vessels

RA: Rheumatoid Arthritis is an autoimmune disease that symmetrically attacks the body's joint tissues as well as other organ systems, can be both chronic and debilitating

Endothelial Dysfunction: improper functioning of the endothelial cells of blood vessels

Post Menopausal: Women no longer going through monthly menarche, for the purposes of the study women had not had menarche in the last year or had undergone a hysterectomy.

Body Composition: A measurement taken to determine total mass and fat free mass

Air Displacement Plethysmography: a technique to measure body volume by utilizing the inverse relationship between pressure and volume

RAOS: Rheumatoid and Arthritis Outcome Score: A 42-item questionnaire that addresses pain perception, functional state and quality of life specific to Rheumatoid Arthritis

Submaximal Exercise Test: For the purposes of this study a submaximal exercise test will use a modified Balke protocol. Participants will warm-up for 1 minute while the speed of the treadmill increased to a 3.3 mph at an incline of zero. After the warm-up they walked at a speed of 3.3 mph for one minute. After a minute the incline will be increased one percent and will increase one percent every subsequent minute until the participant reaches 85 percent of their age predicted maximum heart rate or they request to stop the test.

FMD: Brachial Artery Flow Mediated Dilation Brachial artery flow-mediated dilation is a well-validated, non-invasive method of measuring endothelium dependent flow mediated dilation. BAFMD has been shown to have a high predictive value with regard to cardiovascular events.

25[OH]D: 25 Hydroxyvitamin D, this is the form that Vitamin D takes after it has been metabolized in the liver. This is what will be measured from participant's plasma to determine their Vitamin D status.

TNF-α: Tumor Necrosis Factor –α, an inflammatory cytokine often associated with the pathogenesis of RA

ACR: American College of Rheumatology This is the governing body that sets the diagnostic criteria for Rheumatoid Arthritis

DAS28: Disease Activity Score – 28 A tool used to determine the current severity of a patients disease

Chapter 3

INTRODUCTION

Study 1: The Effect Of Vitamin D Supplementation On Brachial Artery Flow Mediated Dilation In Healthy Post Menopausal Women

With Cardiovascular disease (CVD) still the number one cause of death in the United States, researchers and physicians have begun to look past the typical drug therapies used to treat CVD. Much of this focus has been on physical activity. There is very strong evidence for the effectiveness of physical activity to improve CVD risk. (Blair 1989) However, getting people to exercise regularly has proven to be a difficult task. Dietary factors may be another alternative to drug therapies to reduce CVD risk. As discussed previously, several foods such as berries and dark chocolate have been studied as a means to reduce CVD. In addition to whole foods, supplementing with various vitamins and minerals may also reduce disease risk. Recently, vitamin D has been used in research as a potential means to improve CVD risk. Cross sectional studies have looked at the relationship between serum Vitamin D (25[OH]D) and FMD. Brachial artery flow mediated dilation (FMD) is a well-validated, non-invasive method of measuring nitric-oxide dependent vasodilation. FMD, which assesses endothelial function has been shown to have a high predictive value with regard to cardiovascular events (Yeboah et al. 2009). Yiu et al. examined this relationship and found in a study of 280 type 2 diabetic people that 25[OH]D was significantly associated with FMD.

(Yiu et al. 2011) In addition, vitamin D levels appear to decline with age. A review by Holick, suggested that by age 70 people have a 25% decline in their ability to metabolize vitamin D. (Holick 2007) A 2011 study by Jablonski et al. found a strong inverse correlation between vitamin D status and endothelial function in older adults average age 62 (Jablonski 2011).

While research suggests a link between Vitamin D and CVD risk there have not been many Vitamin D supplementation studies on endothelial function, measured by FMD. Many of these studies focus on an overweight population, such as studies by Sugden et al. (2007) and Harris et al. (2011) (Sudgen et al. 2007, Harris et al. 2011). Sugden et al., gave participants a single dose of either 100,000 IU of vitamin D2 or a placebo and followed up at 8 weeks. Compared to the placebo the participants that received the vitamin D had significant improvements in FMD, indicating improvements in endothelial function (Sudgen et al. 2007). While this study had significant findings it utilized vitamin D2 as opposed to D3, which is generally accepted to be more bioavailable and helps correct deficiencies better than D2. Harris et al. also conducted a large single dose supplementation study this time using D3. For this a dose of 60,000 IU of vitamin D3 was given once a month for 4 months. A single dose of 60,000 IU is approximately the same as taking 2,000 IU daily. At the 16-week follow-up they again found significant improvements in endothelial function measured by FMD with supplementation

(Harris et al. 2011). Tarcin et al. (2009) used healthy subjects for a 3 month study in which they received 300,000 IU of vitamin D. One significant difference in this study is that the vitamin D was administered intramuscularly as opposed to orally as was done in the two previously mentioned studies (Tarcin et al. 2009). Despite the differences in administration of the vitamin, this study too found improvements in endothelial function with vitamin D supplementation. One study however did not show any changes positively or negatively in endothelial function after single doses of either 200,000 IU or 300,000 IU of vitamin D3 (Witham et al. 2010). The authors proposed that improvements may not have been seen because the subjects might have been on medications, which can affect the absorption of vitamin D. Despite this one study it appears as though vitamin D supplementation may be an effective way to improve endothelial function. However, with all of these studies the shortest duration between supplementation and follow-up testing is 8 weeks. However, pharmacokinetics would indicate that after a single dose of 100,000 IU of vitamin D3, 25[OH]D concentration peaks in the blood between 10-20 days after supplementation. (Ilani et al. 2008) However while correlations have shown the relationship between endothelial function and 25[OH]D and supplementation studies have shown longer term utility of vitamin D supplementation no studies to date have measured FMD at the 2 week time point.

Considering previous data that has shown the relationship between vitamin D and FMD in addition to the data that show the significant association of decreasing 25[OH]D with age, the purpose of this study is to examine FMD at baseline and at 2 weeks after a large single dose of 100,000 IU of vitamin D3 in a post - menopausal population.

Specific Aim 1

Primary Aim: Determine the effectiveness of a single 100,000 IU dose of vitamin D3 has on improving endothelial function after two weeks

Hypothesis 1

Primary Aim : A single dose of 100,000 IU of vitamin D3 will significantly improve endothelial function after 2 weeks as compared to the control group, as measured by FMD

DELIMITATIONS

The dose response study was limited to post-menopausal women and men over the age of 50. Participants could have no known history of cardiovascular disease. Participants also were required to have their own form of transportation and must be able to read and write in English.

LIMITATIONS

This study utilized a convenience sample of volunteers. Participants were asked not to change their medications or supplement use for the 2 weeks of the study. This however, could not be validated except through verbal confirmation.

Chapter 4

STUDY DESIGN AND METHODS

Study 1: The Effect of Vitamin D Supplementation on Brachial Artery Flow Mediated Dilation in Healthy Post-Menopausal Women

STUDY DESIGN

A randomized, controlled, parallel-group design was utilized for this study.

RECRUITMENT

Post-menopausal women were recruited through flyers throughout the community as well as through email announcements.

ROLLING ENROLLMENT

Enrollment followed a rolling scheme. Participation lasted two weeks in duration. Participants were enrolled from October 2011 through February 2012.

ELIGIBILITY

Eligibility was restricted to non-smoking post-menopausal women without a history of kidney stones or current use of statin medication. Participants were required to provide their own transportation to and from the ASU Polytechnic Campus.

RANDOMIZATION

After signing informed consent, eligible participants were randomized to either the vitamin D or the placebo group.

METHODS

Once all consent forms were signed, participants were asked to fast for at least 8 hours prior to baseline testing. All testing occurred at the Healthy Lifestyles Research Center (HLRC) at the Arizona State University (ASU) Polytechnic Campus in Mesa, AZ.

ENDOTHELIAL FUNCTION

Once arriving at the laboratory participants underwent a brachial artery Flow Mediated Dilation (FMD) assessment of endothelial function using criteria set forth by the Brachial Artery Reactivity Task Force (Corretti et al. 2002). The sonographer was blinded to which group the participant was in. Participants were asked to lie quietly for 15 minutes on the ultrasound table before a sonographer obtained baseline images for 30 seconds from the participant's left arm. After the baseline images were acquired the sonographer inflated a blood pressure cuff on the participants forearm to 50 mmHg above systolic blood pressure for 5 minutes to completely occlude the artery. 30 seconds prior to cuff release the sonographer

began to record images again. At 5 minutes, the cuff was rapidly deflated while the sonographer continued to record images for the next 5 minutes. After all images were obtained, they were analyzed by a blinded researcher using previously validated, brachial artery edge-detection software. The FMD procedure took approximately 30 minutes. (Green 2009) FMD percentage is calculated by: (peak diameter-baseline diameter/baseline diameter) x 100. This is expressed as a percent change in baseline diameter.

VITAMIN D SUPPLEMENTATION

After baseline FMD testing was completed, participants were given a single oral dose of 100,000 IU of vitamin D3 (Bio Tech Pharmacal, Fayetteville, AR) or an identical placebo pill (Bio Tech Pharmcal, Fayetteville, AR). Participants did not know which pill they received. This dosage has been considered safe based on previous studies utilizing this or higher amounts of Vitamin D. (Harris et al. 2011; Sudgen 2010)

FOLLOW-UP TESTING

After 2 weeks participants returned to the ASU Polytechnic Campus, Healthy Lifestyles Research Center for follow-up testing that was identical to baseline testing.

SAMPLE SIZE CALCULATIONS

Using changes in FMD as the primary outcome measure, sample size was calculated based on findings of previous research (Sudgen et al. 2008, Harris et al. 2011) to see a mean absolute difference of 2.0% with 80% power. A sample size of 30 total subjects with 15 in each group was the recruitment goal for this study.

DATA ANAYLSIS

Paired t-tests in SPSS were used to analyze the effects that vitamin D had on changes in FMD. A One-Way ANOVA was run to examine the changes in vitamin D levels between treatment groups.

CHAPTER 5

RESULTS

Study 1: The Effect Of Vitamin D Supplementation On Brachial Artery Flow Mediated Dilation In Healthy Post Menopausal Women

Twenty-nine post-menopausal women were deemed eligible to participate in the study. Of the 29 women, 28 women completed the baseline and follow-up visits for the study. The one participant who dropped out had a family emergency and had to leave town before the follow-up testing. All statistics were run in IBM SPSS 19.

Descriptive data are presented in Table 1. There were no significant differences between groups at baseline.

Baseline Characteristics

Table 1

Group	Vitamin D	Placebo	p-value
Age	61.5 ± 11.0	61.0 ± 7.9	.901
Height (in)	64.0 ± 2.16	64.2 ± 2.42	.811
Weight (lb)	133.69 ± 31.0	143.92 ± 41.3	.489
BMI (kg/m^2)	23.0 ± 5.3	24.5 ± 7.0	.551
FMD	6.19 ± 4.87	10.16 ± 7.18	.133

There were no significant differences at baseline between the Vitamin D and Placebo group in regards to age or FMD.

Hypothesis 1

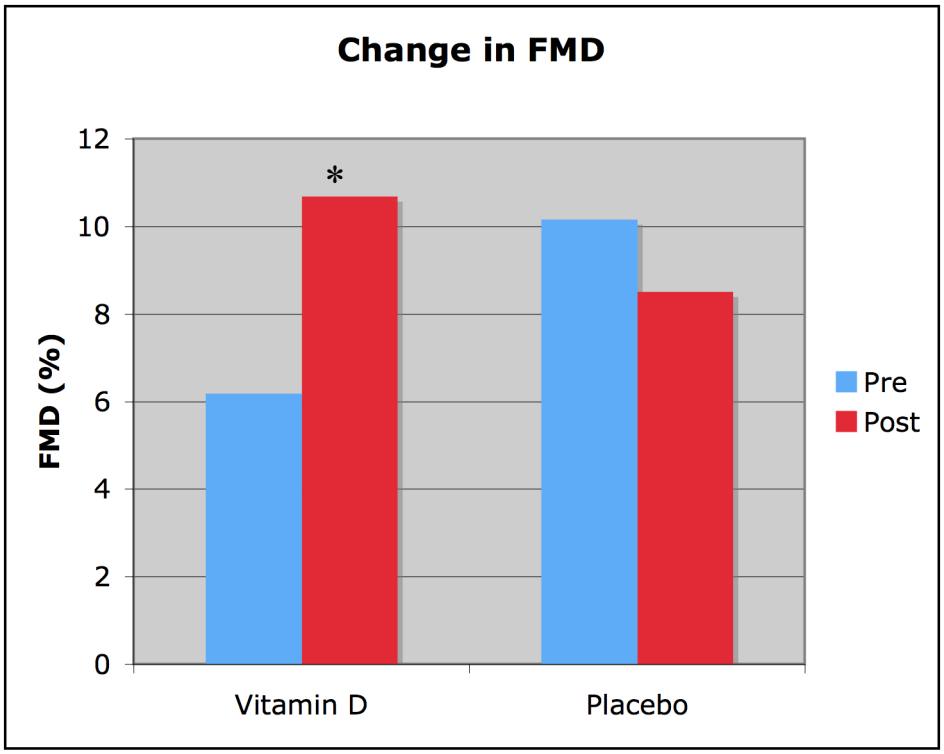
A single dose of 100,000 IU of vitamin D3 will significantly improve endothelial function as compared to the control group, as measured by Brachial Artery FMD. Paired T-tests were run to look at differences in FMD within both the vitamin D group and the Placebo group. (Table 2) Additionally a one-way ANOVA looking at the change in FMD was run to examine the between group differences. (Table 3)

Table 2: Change in FMD - Paired T-Tests

Condition	Pre	Post	p-value
Vitamin D	6.19 ± 4.87	10.69 ± 5.18	.031*
Placebo	10.16 ± 7.18	8.5 ± 5.67	.479

* Denotes a significant result at a level of $p < .05$

Figure 4



*Denotes a significant improvement $p < .05$

Paired T-Tests showed a significant improvement in FMD in the vitamin D group, $p = .031$. There was no significant difference in FMD in the Placebo group. These

data indicate a significant positive effect of vitamin D supplementation on FMD in postmenopausal women.

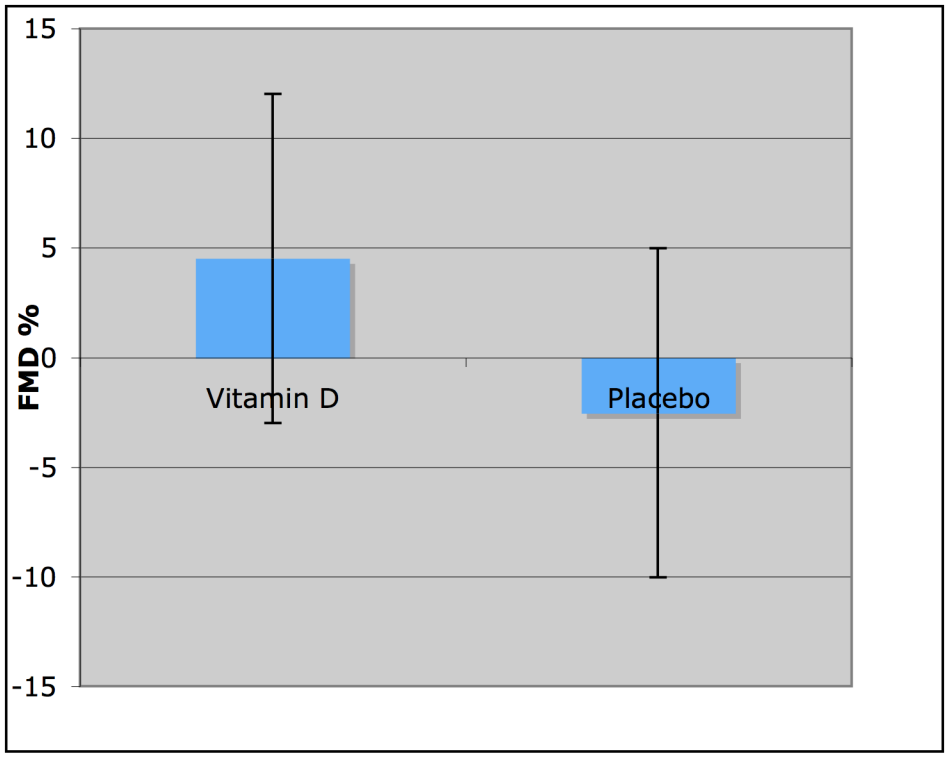
One-Way ANOVA: Change in FMD from baseline to post intervention

Table 3

Vitamin D	Placebo	p-value
4.51 ± 7.30	- 2.52 ± 8.75	.029 *

* Denotes a significant result at a level of $p < .05$

Figure 5



A One-Way ANOVA examining the change in FMD between groups showed a significant difference between the Vitamin D and the Placebo group. The increase that was seen in the Vitamin D group was significantly different than the change that was seen in the placebo group. Again these data indicate a positive effect of vitamin D on FMD in this population.

CHAPTER 6

CONCLUSION

Study 1: The Effect of Vitamin D Supplementation on Brachial Artery Flow Mediated Dilation in Healthy Post-Menopausal Women

DISCUSSION

The results of this study confirm what was hypothesized and what would have been predicted based on the results of previous research. The participants who received the 100,000 IU Vitamin D supplement saw significant increases in their FMD. Previous pharmacokinetic research has shown that after a single dose of 100,000 IU of Vitamin D, serum concentrations of 25[OH]D peak between 10 and 14 days after supplementation. (Ilahni et al. 2008) The follow-up FMD was obtained at the 14-day time point, which theoretically would be the point in which serum concentrations should be the highest. Given the fact that FMD and vitamin D have been previously correlated it was expected that FMD should be significantly improved at the 2-week time point. Additionally the correlation with vitamin D and FMD has been previously seen in the population used in this study, older females. (Jabolonski et al. 2011) Therefore the findings support the hypothesis. However, despite the fact that these findings were hypothesized, this study was the first to show that 100,000 IU of vitamin D could improve FMD after 2 weeks. Previous studies have only shown improvements after at least 8 weeks. This is important because pharmacokinetic studies would predict that if

FMD were correlated with vitamin D status then FMD would improve at the 2-week point. This study confirms that notion. In addition, the improvement from an FMD of 6% to 10% in the vitamin D is not only statistically significant but also clinically significant. Previous research would suggest that an FMD of over 7% would be considered in the healthy range so this study took a population that was slightly impaired into a range that is healthy (Clarkson et al. 1997; Perticone et al. 2001). A possible explanation for this finding is that vitamin D may essentially act an anti-inflammatory agent. Current research suggests that vitamin D may inhibit specific inflammatory cytokines, such as TNF-α (Zhang et al. 2012). This anti-inflammatory response to vitamin D needs further exploration but may be responsible for the results seen in this study. While still preliminary in nature, this finding has implications for the way that vitamin D dosing and supplementation could be administered.

Although there have been some suggestions made for supplementation to improve itamin D deficiency, currently, there is no standard dosing for improving FMD. Current strategies for improving vitamin D deficiency suggest that a person should be given 50,000 IU per week for 8 weeks. (Holick 2002) This is a significantly higher dose than what was used for this study. Considering that, a diagnosis of Vitamin D deficiency is becoming more common, it would be important to understand what the effects of that dosing strategy is on FMD. Since that dosing

strategy employs weekly dosing it would be interesting to examine FMD at baseline then after 2 weeks of the 50,000 IU. While the total Vitamin D administered would be 100,000 IU as was in this study, it would be interesting to see if there were differences between 1 dose of 100,000 IU or 2 doses of 50,000 IU on FMD. While the study helps to begin to understand the speed at which vitamin D can affect FMD, looking at different dosing strategies would enhance our knowledge of the speed at which vitamin D affects FMD.

As vitamin D deficiency is implicated in either the pathogenesis of various diseases or as a risk factor for the development of chronic diseases it is important that research attempts to develop standards for dosing for various conditions. For example, if 50,000 IU for 8 weeks helps in correcting for vitamin D deficiency but has an acute negative affect on FMD that needs to be determined. Additionally, if there are certain conditions, such as autoimmune diseases, for which more vitamin D is needed to correct deficiency or reduce disease symptoms, research is still needed for that as well. While the current study provides insight on how quickly vitamin D may act to improve FMD, significant further research is needed to better understand the role that vitamin D play in disease risk reduction.

Strengths

A strength of this study is that it has strong ecological validity. Participants were not asked to stop any of their medications or other supplements, they were only asked not to change what they were taking over the 2 weeks. This is different than previous studies in that most other studies asked participants to stop taking all other supplements. The current studies suggest that vitamin D may have an ability to improve FMD even in the presence of other medications and vitamins. Additionally, since the vitamin D was administered in the lab in one single dose it insured that the participants received the total dose. Finally, all participants resided in the same region and were tested during either late fall or early winter. This minimized the potential effects of variability in sun exposure.

Limitations

A limitation of this study is that serum levels of vitamin D were not taken. Previous literature suggests that serum vitamin D peaks approximately 10-14 days after a dose of 100,000 IU vitamin D3 and that vitamin D status and FMD are positively correlated (Ilhani et al. 2008). Due to the significant increase in FMD at the 2-week point it would be assumed that serum vitamin D levels would also be significantly increased, however that cannot be verified through this study. Another limitation is that this study only took place during one season with a very specific population. It would be important to understand if these

improvements would be seen in the Spring or Summer as opposed to the late fall, early winter which was when this study took place.

Potential Implications

This study has potential implications for the way CVD risk is approached, especially in a post-menopausal female population. Currently, statin therapy is a common way to attempt to reduce disease risk in older populations. Recently, this type of therapy has come under fire for both its effectiveness and safety. Understanding the ability of Vitamin D to help mitigate disease risk could prove to be an effective tool in reducing CVD risk. While these data are very preliminary they do set the stage for more research to better understand how Vitamin D can be used as both a preventive strategy as well as a treatment for CVD.

Future Research

Future research in the area of Vitamin D and CVD is needed to better understand proper dosage and timing of how and when Vitamin D is needed. This study is the first to demonstrate that FMD can change in only 2 weeks after 100,000 IU of Vitamin D. Up until this study 8 weeks was the shortest amount of time that had been examined. However, now knowing that FMD can change in 2 weeks future studies are needed to examine how often this dosage of Vitamin D needs to be taken. It would also be important to take blood levels along with multiple doses of

Vitamin D to better understand how we can affect serum concentrations as well as to see how strongly the correlations hold between Vitamin D and FMD.

It would also be important to replicate this study at various time points during the year to understand what effect seasonal variations may play in the effect of large doses of Vitamin D. It may be possible that in regions that have greater exposure to sun, there might not be as great as an effect of the Vitamin D supplement. Another strength of this study was that while it was done in the fall, it took place in Phoenix where sun exposure is greater than most other regions at that point in the year.

Finally, it would be important to look at this study with other populations including males and younger females. Post-menopausal women were used for this study because previous research suggests that they would be most at risk for Vitamin D deficiency and therefore an increase in CVD risk. Older men however would also be an ideal population for this type of intervention due to an increase in CVD risk as they age.

Overall, given the statistical and clinical significance of this study it provides solid groundwork to continue to explore the relationship of Vitamin D and CVD.

CHAPTER 7

INTRODUCTION

Study 2: The Effect Of Vitamin D Supplementation On Brachial Artery Flow Mediated Dilation, Plasma Vitamin D, Tumor Necrosis Factor – Alpha In Older Adults With Rheumatoid Arthritis

Rheumatoid Arthritis (RA) is an autoimmune disease that attacks the body's tissues, namely the synovium, a thin membrane that lines the joints. This disease which causes severe systemic joint pain can be both chronic and debilitating. (Lutzy et al. 2007) RA affects an estimated 1.3 million Americans. Current reports indicate that this number may in fact be on the rise. An ongoing study in Olmsted County, Minnesota saw a rise in RA prevalence from .62% of the population in 1995 to .72% in 2007. (Myasoedova et al. 2010) This disease which to date has no known cure carries an estimated medical cost of 19.3 billion dollars annually (Helmick, Felson, Lawrence, Gabriel, Hirsch et al. 2008; Birnbaum et al. 2010). This cost includes treating not only symptoms related to joint pain but also the many other complications that arise as a result of the disease. These complications can manifest in several of the body's systems ranging from lung and skin disorders to digestive tract and nervous system problems. However, the complication that results in the leading cause of early morbidity and mortality in this patient population is CVD (Gonzalez-Juanatey et al. 2008). Recent meta-analyses show that people with RA have a relative risk of 1.21 for CVD as compared with the general population (Crilly, Kumar, Clark, Scott, McDonald, et

al. 2009). The development of CVD has not only far reaching health consequences but it also significantly increases the cost of treating RA, placing a large financial burden on the patient. Joyce et al. (2009) reported that the annual cost of treating a patient with both RA and CVD is estimated to be $14,145.00, approximately $3,000 more per year per patient than those who have RA alone ($11,404.00) (Joyce et al. 2009). Currently, a common secondary prevention strategy in patients with RA and CVD is the use of statins (Bansback, Ara, Ward, Anis, Choi 2009). This therapy over a ten-year period results in an estimated cost of $10,650 per Quality Adjusted Life Years.

However, a possible common link between RA and CVD may be vitamin D. Vitamin D deficiency has been independently linked with the pathogenesis of both RA and CVD (Cutulo et al. 2007; Merlino et al. 2004; Leventis et al. 2008; Witham et al. 2010; Harris et al 2011). Using data from the Iowa Women's Health Study, Merlino et al. demonstrated a significant correlation between Vitamin D intake, as measured by a validated food frequency questionnaire, and incidence of RA (Merlino et al. 2004). When using serum levels of 25[OH]D as opposed to self-reported food recalls, Oelzner et al. also found a significant association between vitamin D status and RA disease activity (Oelzner et al. 1998). In addition to the association between vitamin D status and the development of RA, patients with diagnosed RA have been shown in general to be Vitamin D deficient.

In a cross-sectional study of 554 people, significant correlations were found between vitamin D status and arterial stiffness and endothelial dysfunction. (Al Mheid et al. 2011) Specifically, vitamin D supplementation has been associated with improvements in endothelial function. Endothelial dysfunction is strongly correlated with adverse cardiovascular events and is the foundation for the pathogenesis of arthrosclerosis (Dessein et al. 2005). Despite the association of vitamin D to both RA and CVD, vitamin D supplementation has yet to be used in RA patients as a means to improve a patients CVD risk profile. Therefore the purpose of this study is to do just that: Examine the effect that vitamin D supplementation has on endothelial function in RA patients.

Rationale

The etiology of RA is unknown, leading to several proposed mechanisms for the pathogenesis of the disease. One theory is that a lack of vitamin D may be associated with the development of the disease (Merlino et al. 2004). While suggestions have been made as to how to correct vitamin D deficiency there have been very few studies that have solely used vitamin D to improve RA disease status. A 1998 study by Cantora et al. showed that vitamin D supplementation slowed the initiation and progression of inflammation related arthritis in rodents (Cantora et al.1998). There have been two randomized, placebo-controlled studies with RA patients that used a combination of vitamin D3 and calcium. Brohult and

Johnson conducted a study showing that 2500 ng daily improved pain symptoms (Zitterman 2003). The second study was aimed at improving bone density and found significant improvements with relatively small doses of vitamin D (Buckley et al.1996). Vitamin D supplementation is effective for all patients with RA it is of particular importance to post-menopausal women. Older women with or without RA are at a higher risk of vitamin D deficiency due to their age that can lead to an increased risk of osteoporosis and falling (Leventis et al. 2008). Given that information, particular attention should be paid to vitamin D levels in post-menopausal women with RA.

While there have not many vitamin D supplementation studies done in the RA population, more studies have been conducted to look at the effect of supplementation on endothelial function, as measured by FMD. While not testing in regards to Vitamin D supplementation FMD, has been shown to be impaired in RA patients. (Kerekes, Szekanecz, Der, Sandor, Lakos 2008) Many of these studies focus on an overweight population, such as studies by Sugden et al. (2008) and Harris et al. (2011) (Sudgen et al. 2008; Harris et al. 2011). Sugden et al., gave participants a single dose of either 100,000 IU of Vitamin D2 or a placebo and followed up at 8 weeks. Compared to the placebo the participants that received the Vitamin D had significant improvements in their (FMD) (Sudgen et al. 2008). Harris et al. conducted a large single dose (60,000 IU per month for 4 months)

supplementation study using D3. At 16 weeks significant improvements in endothelial function were found with supplementation (Harris et al. 2011). Significant associations between Vitamin D and endothelial function have also been seen in older populations, showing a strong inverse correlation between Vitamin D status and endothelial function (Jablonski et al. 2011).

Despite positive findings in several studies the exact pathway that Vitamin D acts is unknown. One proposed mechanism by which Vitamin D improves FMD is that it reduces systemic inflammation (Harris et al. 2011). Endothelial dysfunction is associated with high levels of inflammatory biomarkers, and activation of the inflammatory process may be responsible for the development of atherosclerosis as well as in the pathogenesis of RA (Metosis et al. 2010, Dessein et al. 2005). Therefore, Vitamin D's potential role as an anti-inflammatory agent may explain the improvements seen in both CVD and RA. Specifically, a few studies using animal models have shown a relationship between Vitamin D supplementation and improvements in TNF-alpha levels. For that reason, this study will examine that relationship in humans.

This study will also examine the effect that Vitamin D supplementation has on estimated VO_{2max}. In a large cross-sectional study of 200 adults an inverse relationship between 25[OH]D and VO_{2max} was seen. (Ardestani, Parker, Mathur, Clarkson, Pescatello, et al. 2011) The authors of this study propose that a possible

reason for this association is that low levels of 25[OH]D may cause a decrease in cardiac output and an increase in peripheral vessel resistance which in turn will decrease VO_{zmax}. Due to this association, this study will examine the effects of Vitamin D supplementation on improving estimated VO_{2max} in people with Rheumatoid Arthritis.

Significance

This study aims to fill the gap in current research to examine the ability of Vitamin D to improve CVD in people with RA. In addition, by examining the inflammatory biomarker TNF-alpha this study will provide valuable information regarding the role of Vitamin D as an anti-inflammatory agent. If positive results are found this study will lay the groundwork for the use of Vitamin D as a novel therapy to treat CVD risk in the RA population.

Specific Aims

Primary Aim 1: Determine the effectiveness of a single dose of 100,000 IU Vitamin D3 has on improving endothelial function

Primary Aim 2: Determine the effectiveness that a single dose of 100,000 IU Vitamin D3 has on increasing serum 25[OH]D levels

Primary Aim 3: Determine the effectiveness that a single dose of 100,000 IU Vitamin D3 has on improving TNF-alpha

Secondary Aim 1: Determine the effectiveness a single dose of 100,000 IU Vitamin D3 on Quality of Life

Secondary Aim 2: Determine the effectiveness of a single dose of 100,000 IU of Vitamin D3 on improving estimated maximum aerobic capacity (VO_{2max})

Secondary Aim 3: Determine the correlation between change in Vitamin D status and change in endothelial function

Secondary Aim 4: Determine the correlation between Vitamin D status and estimated $VO2_{max}$

Secondary Aim 5: Determine the correlation between estimated $VO2_{max}$ and endothelial function

Hypotheses

Primary Aim 1: A single dose of 100,000 IU of Vitamin D3 will significantly improve endothelial function as compared to the control group, as measured by FMD

Primary Aim: A single dose of 100,000 IU of Vitamin D3 will significantly improve serum 25[OH]D levels as compared to the control group

Primary Aim 3: A single dose of 100,000 IU of Vitamin D3 will significantly improve TNF-alpha levels as compared to the control group

Secondary Aim 1: A single dose of 100,000 IU of Vitamin D3 will significantly improve quality of life scores as compared to the control group

Secondary Aim 2: A single dose of 100,000 IU of Vitamin D3 will significantly improve predicted VO_{2max} as compared to the control group

Secondary Aim 3: There will be a positive correlation between Vitamin D status and endothelial function

Secondary Aim 4: There will be a positive correlation between Vitamin D status and estimated VO_{2max}

Secondary Aim 5: There will be a positive correlation between estimated VO_{2max} and endothelial function

DELIMITATIONS

The dose response study was limited to post-menopausal women and men over the age of 50. The RA study was limited to post-menopausal women and men over the age of 50 with Rheumatoid Arthritis. They had to have been diagnosed with Rheumatoid Arthritis for at least one year and must be on stable medications for the duration of the one month study. Participants could have no known history of CVD. For both studies participants also were required to have their own form of transportation and must be able to read and write in English.

LIMITATIONS

For both studies it was a convenience sample of volunteers. For the dose response study participants were asked not to change their medications or supplement use for the 2 weeks of the study. In the RA study they were asked to stop all other vitamin supplementation for the month of the study, however this could not be validated with anything other than verbal confirmation. In this study participants were mainly recruited from one physician's office, which may limit the ability to generalize the results.

CHAPTER 8:

STUDY DESIGN AND METHODS

Study 2: The Effect Of Vitamin D Supplementation On Brachial Artery Flow Mediated Dilation, Plasma Vitamin D, Tumor Necrosis Factor – Alpha In Older Adults With Rheumatoid Arthritis

STUDY DESIGN

A randomized, controlled, parallel-group design will be utilized for this study.

Recruitment

Participants with rheumatoid arthritis were recruited and screened at routine office visits by Dr. Warren Rizzo, MD FACR or one of his research associates at Advanced Arthritis Care in Scottsdale, Arizona. (See Appendix D) Flyers with information regarding the study were placed in the Advanced Arthritis Care office. Dr. Rizzo also mentioned the study to eligible patients during routine office visits. Dr. Rizzo provided eligible patients information regarding the requirements of the study. Participation was then opened up to anyone in the general population with RA, to help meet enrollment requirements. These participants were given a physician consent form for their own Rheumatologist to complete prior to the start of the study. The same eligibility criteria were used for these participants as was used for the participants recruited from Dr. Rizzo's office. (See Appendix E)

Rolling Enrollment

Enrollment followed a rolling scheme. Participation lasted one month in duration. Participants were enrolled from October 2011 through January 30th 2012.

Eligibility

Participation eligibility was as follows: post-menopausal women or men over the age of 50 who have been diagnosed with Rheumatoid Arthritis for at least one year prior and have been on consistent drug therapy for at least 6 months. Their RA symptoms, as measured by the Disease Activity Score-28 (DAS28), needed to be well controlled with their current medication (DAS28 score of 3.2 or below). Patients also had to be able to speak and read English so that they could accurately fill out the RAOS as well as have their own method of transportation to the laboratory. Patients with a history of ischemic heart disease, coronary artery bypass grafting, pacemaker/implantable cardioverter defibrillator implantation, valvular heart disease, heart failure, cardiac transplantation, congenital heart disease, type 1 & 2 diabetes, asthma, lung disease, peripheral arterial disease, orthopedic problems or significant joint damage that limits exercise, uncontrolled hypertension, or positive stress test were excluded from the study.

Randomization

After signing informed consent, eligible participants were randomized to either the Vitamin D condition or the placebo group.

Methods

Once all consent forms were signed, participants were asked to fast for at least 8 hours prior to baseline testing. All testing occurred at the Healthy Lifestyles Research Center (HLRC) or Health Science Center (HSC) at the Arizona State University (ASU) Polytechnic Campus in Mesa, AZ.

Endothelial Function

Once arriving at the laboratory participants underwent an FMD assessment. This procedure addresses the participant's endothelial function through an ultrasound technique. The procedure for the FMD assessment followed the criteria set forth by the Brachial Artery Reactivity Task Force (Corretti et al. 2002). The sonographer was blinded to which condition the participant was in. Participants were asked to lie quietly for 15 minutes on the ultrasound table before a sonographer obtained baseline images for 30 seconds from the participant's left arm. After the baseline images were acquired the sonographer inflated a blood pressure cuff on the participants forearm to 50 mmHg above systolic blood pressure for 5 minutes to completely occlude the artery. 30 seconds prior to cuff

release the sonographer began to record images again. At 5 minutes, the cuff was deflated while the sonographer continued to record images for the next 5 minutes. After all images were obtained, they were analyzed by a blinded researcher using previously validated, brachial artery edge-detection software. This exam took approximately 30 minutes. (Green 2009) FMD percentage is calculated by: (peak diameter-baseline diameter/baseline diameter) x 100. This is expressed as a percent change in baseline diameter.

Blood Work

Following the BAFMD, baseline blood draws were carried out at the ASU Polytechnic campus, Health Science Center. The blood samples were used to examine Vitamin D levels, as measured by 25(OH)D as well as TNF-α levels. Plasma 25[OH]D levels were analyzed by a direct ELISA (ImmunoDiagnostics) (See Appendix H). TNF-α levels were also analyzed by the direct ELISA method. (See Appendix I) The blood draws were performed by a licensed phlebotomist.

Rheumatoid and Arthritis Outcome Score

After successful completion of the FMD and blood draw participants were given the Rheumatoid and Arthritis Outcome Score (RAOS) questionnaire. This is a 42-item scale that addresses pain levels, functional status as well as quality of life

specific to Rheumatoid Arthritis. This survey took approximately 10 minutes. (See Appendix J)

Body Composition

After the RAOS, participants had their body composition measurements assessed by the BodPod (Life Measurement, Inc.) prior to their fitness testing. Participants were asked to wear tight fitting clothing or a bathing suit for testing. The BodPod utilized air displacement plythsmography to obtain body fat percentages. Two measurements of body fat percentage were taken, and if the first two measures were highly variable a third was taken. This technique is considered to be a validated measure for assessing body fat percentage. (Fields D, Hunter G, Goran M, 2000) This exam takes approximately 10 minutes and is non-invasive.

Fitness Testing

The final portion of the baseline testing was a sub maximal exercise test for estimation of maximal aerobic capacity (VO_{2max}). For this study the sub maximal exercise test utilized a modified Balke protocol. This protocol is a graded walking test that was administered on the treadmill. Participants starting walking a 3.3 miles per hour at 0 percent incline. They warmed up for one minute while increasing the speed to 3.3. After the warm-up, the incline increased by one percent every minute until the participant reached 85% of their age predicted

maximum heart rate. Maximum heart rate was calculated using the equation 206.9 – (.67 x age). Participants could elect not to participate in the exercise portion if they felt uncomfortable with the procedure. The test was stopped if there is ≥10mmHg drop in blood pressure with increasing exercise intensity, angina, dizziness, syncope, symptoms of poor perfusion, or the subject has the desire to stop (Brubaker, Otto, Armstrong 2005). The test was stopped and subjects were excluded from the study if any of the above exercise exclusion criteria occurred or they could complete a satisfactory exercise test. All exercise tests were supervised by a qualified exercise physiologist. This test took approximately 15 minutes. Estimated VO_{2max} was calculated using the following equations that have been set forth by the American Council on Exercise. For men: 1.444 (time in minutes) + 14.99 and for women: 1.38(time in minutes) + 5.22. (American Council on Exercise, 2010)

Vitamin D Supplementation

After baseline testing was completed, participants were given a single oral dose of 100,000 IU of Vitamin D3 (Bio Tech Pharmacal, Fayetteville, AR) or an identical placebo pill (Bio Tech Pharmcal, Fayetteville, AR). Participants did not know which pill they received. This dosage has been considered safe based on previous studies utilizing this or higher amounts of Vitamin D. (Ilhani 2008, Tarcin 2009, Harris 2011)

Follow-up Testing

Participants were asked not to change anything in their normal routine for the next month. After 4 weeks participants returned to the ASU Polytechnic Campus, Healthy Lifestyles Research Center, and Health Sciences Center, Mesa AZ for follow-up testing that was identical to baseline testing.

Sample Size Calculations

Using changes in FMD as the primary outcome measure, sample size was calculated based on findings of previous research (Sudgen 2008, Harris 2011, Flammer 2008, Hermann 2005) to see a mean absolute difference of 2.0% with 80% power. A sample size of 14 total participants with 7 in each group was the recruitment goal for this study.

Data Analysis

A One Way ANOVA as well as paired T-tests were run in SPSS (IBM SPSS) to analyze the effect that vitamin D had on changes in FMD, 25[OH]D, TNF-alpha as well as estimated VO2 peak and quality of life. Pearson correlations were run to examine relationships between vitamin D and FMD, TNF-alpha and vitamin D, vitamin D and Quality of Life, FMD and Quality of Life, and VO2 and FMD and vitamin D.

CHAPTER 9

RESULTS

Study 2: The Effect Of Vitamin D Supplementation On Brachial Artery Flow Mediated Dilation, Plasma Vitamin D, Tumor Necrosis Factor – Alpha In Older Adults With Rheumatoid Arthritis

Thirteen subjects were enrolled in this study, 10 females and 3 males. Of the 13, 11 completed both baseline and follow-up testing. One person showed up and was not fasted so they dropped out of the study before any testing had occurred. The other completed the baseline testing but was sick for the follow-up testing and therefore was not able to complete that portion of the study. All statistics were run in IBM SPSS 19.

Baseline descriptive statistics are in Table 4

Descriptive Statistics

Table 4

	Vitamin D	Placebo	P-Value
Height (cm)	164.3 ± 9.8	163.5 ± 6.85	.75
Weight (lbs)	171.3 ± 41.5	177.2 ± 51.9	.84
Body Fat (%)	36.0 ± 9.2	40. 0 ± 12.7	.55
BMI	28.3 ± 4.2	30.5 ± 8.5	.61
FMD (%)	5.3 ± 3.97	6.18 ± 2.58	.69
Vitamin D (B/B_0%)	56.4 ± 12.37	51.4 ± 9.85	.48
TNF-alpha	.177 ± .31	.109 ± .12	.66
VO_2 (ml/kg/min)	22.33 ± 7.34	26.96 ± 3.01	.35
Quality of Life	67.7 ± 37.39	51.24 ± 19.96	.40

There were no significant differences between groups at baseline.

Primary Aim 1

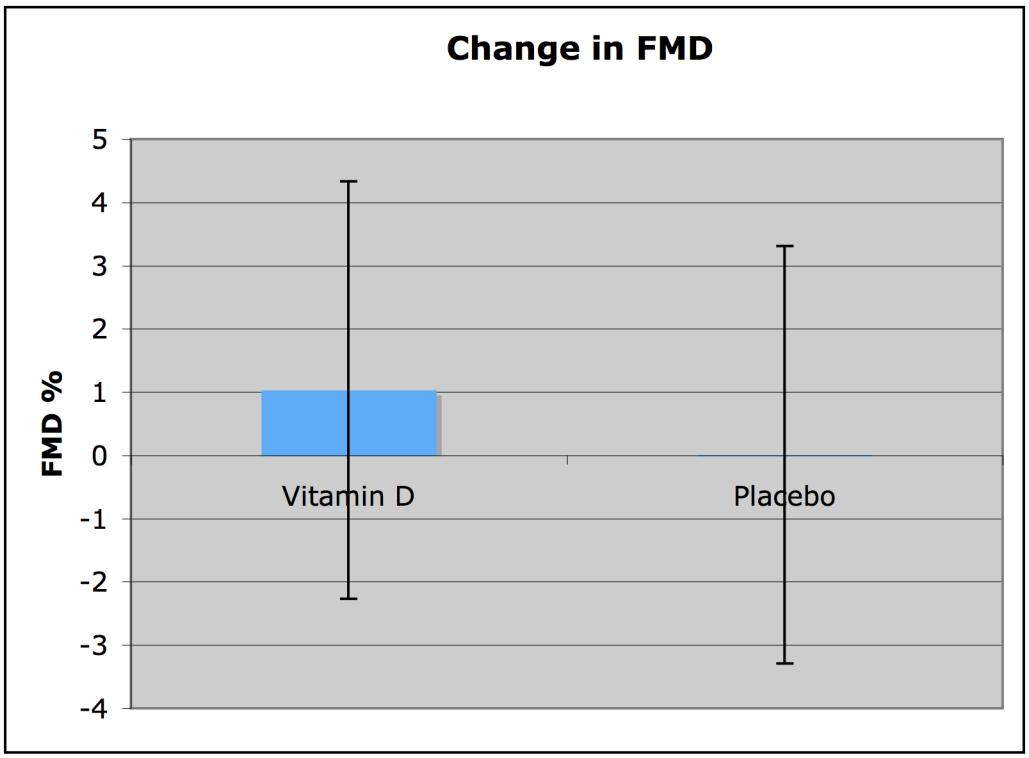
A single dose of 100,000 IU of Vitamin D3 will significantly improve endothelial function as compared to the control group, as measured by FMD.

Table 5

One-Way ANOVA: Change in FMD from baseline to 1 month

Vitamin D (mean ± SD)	Placebo (mean ± SD)	p-value
1.03 ± 5.25	.006 ± 4.18	.721

Figure 6



A One-Way ANOVA examining the change in FMD between both groups showed a significant difference between the Vitamin D and the Placebo group.

These data indicate that there was a significant positive effect of the Vitamin D on FMD.

Table 6

A Two-Way Repeated Measures ANOVA was run to examine the interaction of the treatment (Vitamin D and Placebo) and Time (Baseline and Follow-up)

Condition	Vit D Pre	Vit D Post	Placebo Pre	Placebo Post	p-value
Interaction	5.31 $\pm$ 3.97	6.33 $\pm$ 7.47	6.18 $\pm$ 2.57	6.19 $\pm$ 3.74	.721

The Two-Way Repeated Measures ANOVA did not show a significant interaction between treatment and time. These data again indicate a significant positive effect of Vitamin D on FMD.

Primary Aim 2

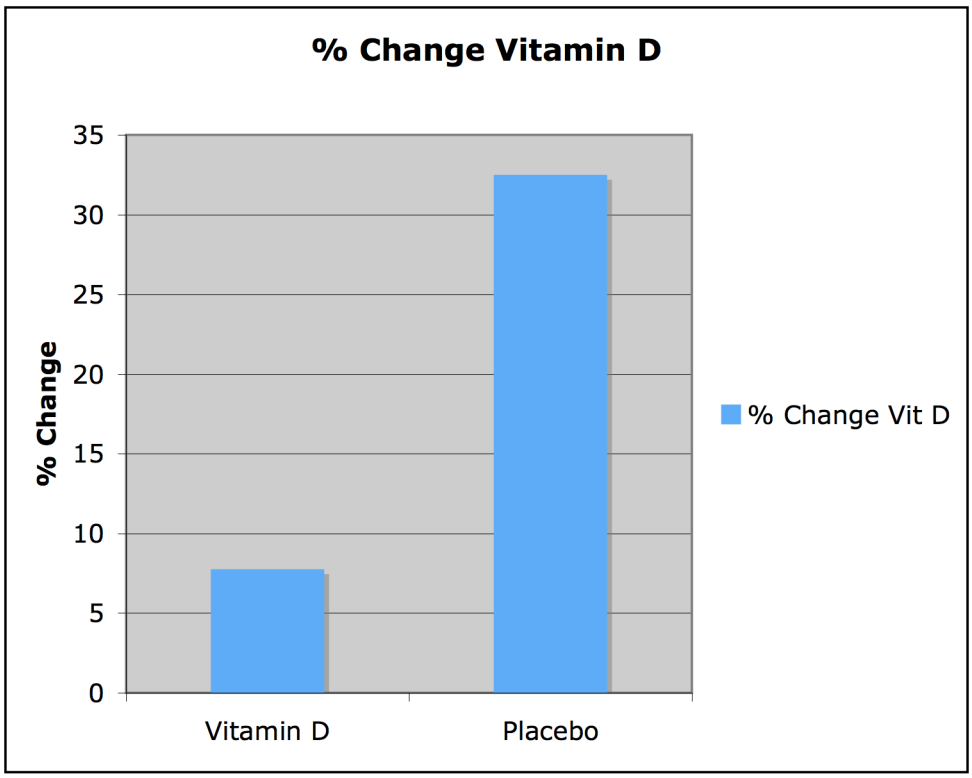
A single dose of 100,000 IU of Vitamin D3 will significantly improve plasma Vitamin D function as compared to the control group.

Table 7

One Way ANOVA: Percent Change in Plasma Vitamin D

	Vitamin D	Placebo	p-value
% Change in Vitamin D (Mean ± SD)	-7.7 ± 26.25	-32.5 ± 27.89	.164

Figure 7



A One-Way ANOVA did not show a significant difference between the changes in Vitamin D status of the group that received the Vitamin D as compared to the

placebo group. These data indicate that there was not significant effect of supplementation on plasma concentration

Table 8

Paired t-test: Vitamin D (B/Bo%)

Condition	Pre	Post	p-value
Vitamin D	56.4 $\pm$ 12.3	58.8 $\pm$ 10.58	.657
Placebo	51.38 $\pm$ 9.85	65.96 $\pm$ 4.7	.026*

* Denotes a significant change at $p < .05$

The values used for vitamin D represent the absorption rate of the competitor 25[OH]D that was added to the standards and samples. Therefore, the less absorption of the competitor 25[OH]D the greater the concentration of 25[OH]D that was present in the sample. A lower value indicates a higher level of plasma 25[OH]D. These data indicate that those participants in the placebo group saw a significant decrease in their plasma concentrations of 25[OH]D but there was no significant change in the Vitamin D group.

Primary Aim 3

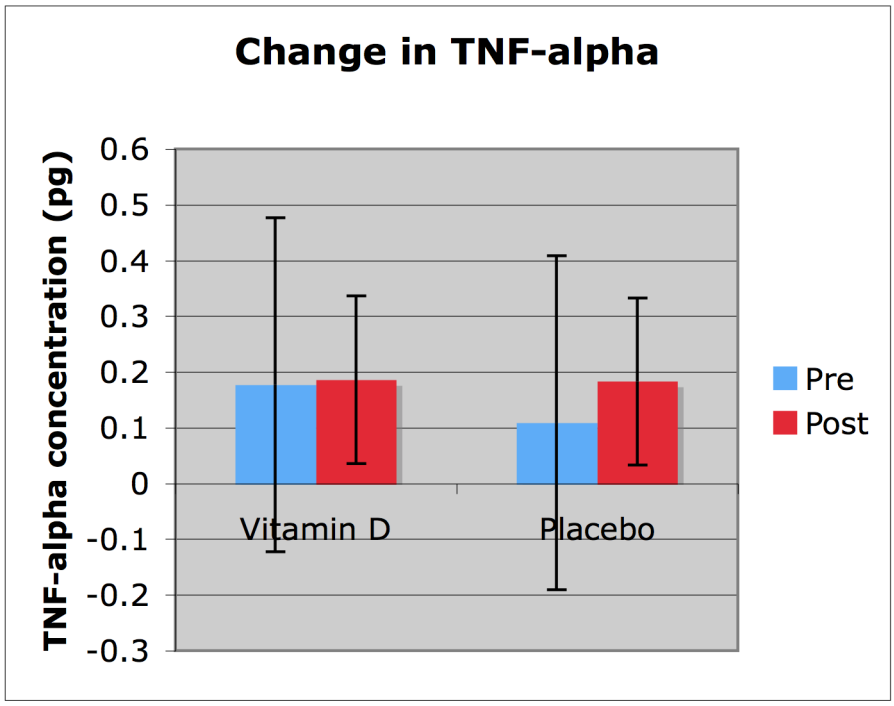
A single dose of 100,000 IU of Vitamin D3 will significantly improved TNF-alpha as compared to the control group.

Table 9

Paired T-tests: Change in Optical Density of TNF-alpha

Condition	Pre	Post	p-value
Vitamin D	.177 ± .31	.16 ± .296	.556
Placebo	.109 ± .126	.183 ± .209	.268

Figure 8



These data indicate no significant difference of either the Vitamin D or placebo on TNF-α levels.

Table 10

A One Way ANOVA was run on the percent change in TNF-α optical density from baseline to follow-up to examine the differences between groups

Vitamin D (mean ± SD)	Placebo (mean ± SD)	p-value
8.96 ± 47.9	172.8 ± 318.3	.240

Table 11

A two way repeated measures ANOVA was run to examine the interaction between treatment (Vitamin D and Placebo) and time (baseline and follow-up) for TNF -α.

	Vit D Pre	Vit D Post	Placebo Pre	Placebo Post	p-value
Interaction(pg)	.177 ± .31	.186 ± .296	.109 ± .126	.183 ± .209	.161

Again these data indicate no significant effect of either the Vitamin D or placebo on TNF - α levels.

Secondary Aim 1: A single dose of 100,000 IU of Vitamin D3 will significantly improve quality of life scores as compared to the control group.

Table 12

Paired T-tests

Condition	Pre	Post	p-value
Vitamin D	67.7 ± 37.39	73.95 ± 40.40	.296
Placebo	51.24 ± 19.95	51.23 ± 26.3	.999

These data indicate no effect of condition on quality of life as measured by the RAOS

Table 13

A Two - Way Repeated Measures ANOVA was run to examine the condition by time interaction

	Vit D Pre	Vit D Post	Placebo Pre	Placebo Post	p-value
Interaction	67.7 ± 37.39	73.95 ± 40.40	51.24 ± 19.95	51.23 ± 26.3	.43

Again there was no effect of the condition on quality of life

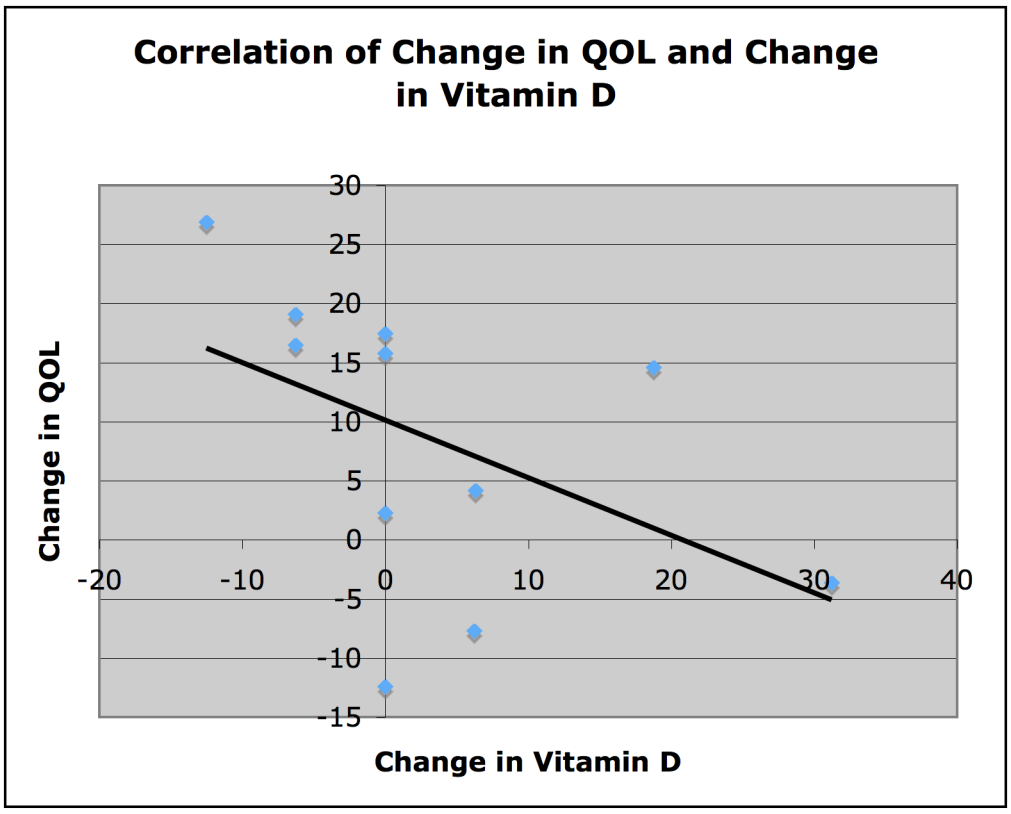
Table 14

	Pearson Correlation	p-value
Change in Vit D Status and Quality of Life	-.619	.042*

* denotes a significant difference at $p < .05$

There was a significant correlation between the change in Vitamin D status and change in Quality of Life. These statistics combined both the Vitamin D and control group. Those in the Vitamin D group whose serum concentrations of Vitamin D did not go up, also did not see an increase in Quality of Life.

Figure 9


Correlation of Change in QOL and Change in Vitamin D
Change in QOL
Change in Vitamin D
30
25
20
15
10
5
0
-5
-10
-15
-20
-10
0
10
20
30
40

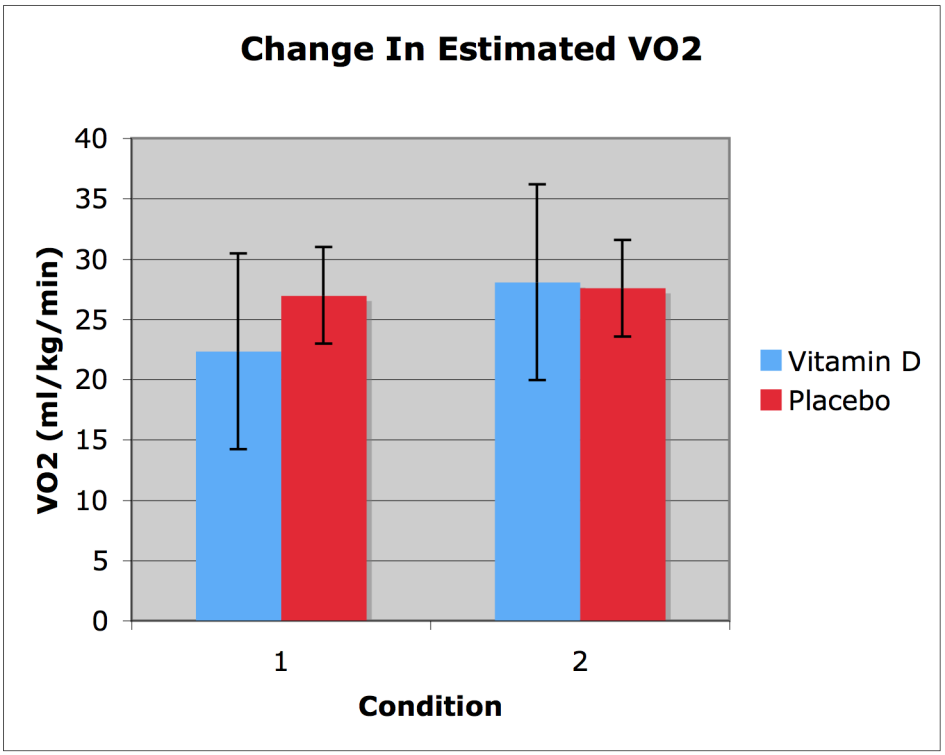
Secondary Aim 2: A single dose of 100,000 IU of Vitamin D3 will significantly improve estimated VO_{2max} as compared to the control group

Table 15 – Paired T-Tests

	Pre	Post	P-Value
Vitamin D (n=5) (ml/kg/min)	22.33 ± 7.35	28.08 ± 8.11	.003*
Placebo (n =3) (ml/kg/min)	26.96 ± 3.02	27.58 ± 4.56	.518

* denotes a significant difference at $p < .05$

Figure 10



T-tests demonstrated a significant improvement in predicted VO_2 in the Vitamin D group (p=.003) whereas there was no difference seen in the placebo group

A One Way ANOVA was run to examine the differences in VO_2 between the Vitamin D and placebo group

Table 16

	p-value
Differences in VO_2 between Vitamin D and Placebo	.030*

* denotes a significant difference at $p < .05$

A One Way ANOVA demonstrated that there was a significant difference seen between the Vitamin D and placebo group in terms of change in VO_2 from baseline to follow up testing. These data indicate that there was a significant affect of the Vitamin D supplementation on estimated VO_2.

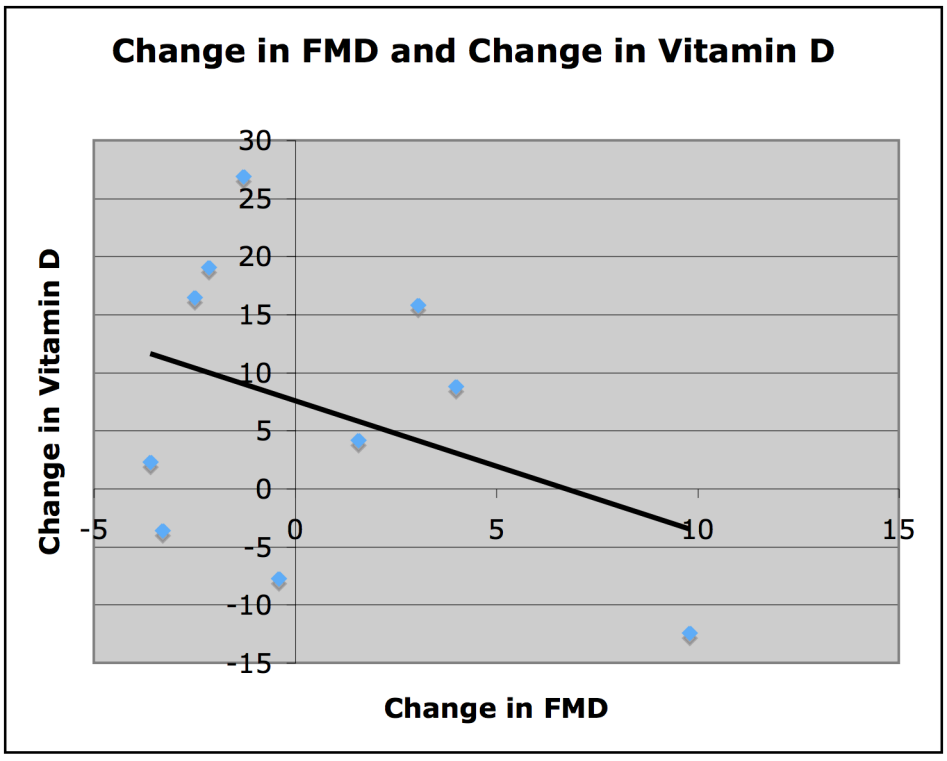
Secondary Aim 3: There will be a positive correlation between Vitamin D status and endothelial function

Table 17

	Pearson Correlation	p-value
Change in FMD and Change in Vitamin D Status	-.356	.312

There was a non-significant small correlation between change and FMD and change in Vitamin D status

Figure 11



Secondary Aim 4: There will be a positive correlation between Vitamin D status and estimated VO2 peak

Table 18

	Pearson Correlation	p-value
Correlation Between Baseline Vitamin D and VO_2	.343	.405

There was no significant correlation at baseline between Vitamin D status and predicted VO_2

Secondary Aim 5: There will be a positive correlation between estimated VO2 peak and endothelial function

Table 19

	Pearson Correlation	p-value
Correlation Between Baseline FMD and VO_2	-.561	.190

There was no significant correlation between FMD and VO_2. These data indicate that there was no significant correlation between FMD and estimated VO_2 at baseline.

Chapter 10:

CONCLUSION

Study 2: The Effect Of Vitamin D Supplementation On Brachial Artery Flow Mediated Dilation, Plasma Vitamin D, Tumor Necrosis Factor – Alpha In Older Adults With Rheumatoid Arthritis

Discussion

The results of this study in part supported the hypotheses as well as what would have been expected based on past research. At baseline, Vitamin D status and FMD were not correlated (Jablonski et al. 2011) . This may be due to the small sample size used in the study or it may have been the result of certain medications that subjects were on. In addition there was no significant increase in Vitamin D status in the group which received the Vitamin D, although 3 of the 6 people in the Vitamin D group did see an increase in their plasma levels of 25[OH]D. There was however a significant decrease in the Vitamin D status of those that were in the placebo group ($p=.02$). These findings while initially surprising, may be due to the fact that a single dose of 100,000 IU was simply not large enough to elicit change, but rather it was enough to keep levels from decreasing as was seen in the placebo group. The participants were tested from October to February, so it may be possible that as it got a month deeper into winter, sun exposure decreased which may be the explanation for the decreases seen in the placebo group. Therefore rather than increasing plasma levels of 25[OH]D the current dose was enough to maintain their current levels. In many cases of Vitamin D deficiency people are

given 50,000 IU per week of Vitamin D for 8 weeks to correct the deficiency, so it conceivable that a single dose of 100,000 IU was not enough. Another explanation for why no significant change was seen is that some research has shown that some medications such as prednisone may decrease Vitamin D absorption, giving further reason as to why 100,000 IU may not have been an efficacious dose. (Vitamin D Council, 2012) However, in the case of this study only one person in the Vitamin D group was on a stable dose on Prednisone and that person was one of the 3 to see improvements in plasma Vitamin D. (See Table 20) There does not appear to be any consistency in medications between those in the Vitamin D group who saw increases in plasma Vitamin D and those who did not. However, all of those that saw improvements were on some type of anti-TNF medication. In addition to medications possibly hindering the absorption of Vitamin D, it is also possible that the participants were low in any of the number of co-factors that affect Vitamin D metabolism. For example, if the participant was low in magnesium they might not have been able to properly utilize the Vitamin D that they received. Finally, pharmacokinetic data has shown in a healthy population that serum levels of 25[OH]D peak in the blood between 10-20 days after a single dose of 100,000 IU and start tapering off after that. In the healthy population the serum levels were still elevated at the 30 day mark, however it may be hypothesized that if there are absorption issues due to a chronic inflammatory state of this population, serum

levels may not have risen high and, in that case, may have tapered back toward baseline by the 30 day mark.

In addition to the non-significant improvements seen in the plasma concentrations, there was also no significant difference between the Vitamin D and placebo when examining the change in FMD from baseline to follow-up. This finding was somewhat surprising based on the finding from previous studies that demonstrated that Vitamin D supplementation improved FMD. (Harris et al. 2011, Sudgen et al. 2007) However, upon further consideration it is again possible that a dose of 100,000 IU was simply not enough to induce change. Since plasma Vitamin D did not change significantly and previous research has shown that Vitamin D and FMD are correlated, it is not surprising that FMD did not change. However, in Study 1, FMD did significantly change at the 2-week time point after the same 100,000 IU dose. It would be interesting to test the RA population at the 2-week time point as well to determine if the Vitamin D has an initial significant effect. If that were the case it would again lend support to the notion that 100,000 IU was simply not a large enough dose to sustain change over one month.

Additionally, it was expected that there would be a significant correlation between the change in Vitamin D status and FMD. The Vitamin D values that were used represented absorption values of the competitor 25[OH]D that was introduced to

the standards and samples, therefore a lower value indicated a higher level of Vitamin D. The reason for this was that the standards used in the ELISA kit did not produce the expected results. However, by looking at the baseline and follow-up values it was still possible to calculate the change in Vitamin D status. Therefore a significant correlation in this case would be a negative correlation, considering that a lower Vitamin D value in fact represented a higher plasma Vitamin D concentration. The results of this study showed a small to modest correlation of -.357 but failed to reach significance at $p = .311$. This lack of significance however again may be in part due to the small sample size of the study.

Plasma TNF-alpha levels did not differ from baseline to follow-up. Although, when looking at the interaction between condition and time a p-value of .240 was seen, while not significant it is again possible that a larger sample size could have elicited a significant change. While it was hypothesized that there would be changes in TNF-alpha in the Vitamin D group, this relationship has yet to be shown in human studies. In previous studies the relationship between Vitamin D and TNF-alpha has only been shown in rodent models or cell cultures. (Goronzy and Weyand 2001, Zhang 2012) There are a few explanations for why there was no change. First, it is again possible that 100,000 IU was too small of a dose to effect change on TNF-alpha. This seems even more plausible considering no significant change was seen in Vitamin D status. Secondly, it is possible that TNF-

alpha is not the cytokine that is largely responsible for the anti-inflammatory effect of Vitamin D. It is possible that other inflammatory cytokines such as IL-1 or IL-6 may be the key factor in the inflammatory cascade in terms of the effectiveness of Vitamin D. When looking at the medications of those in the Vitamin D group, all of those participants that saw improvements (5 of 6) were taking TNF-alpha blocking medications. (See Table 20)

Table 20 – Medications of Participant in Vitamin D Treatment Group

Subject	Medications	Change in FMD	Change in Vitamin D Status	Change in TNF-alpha
1	Not Available	Improved	Declined	Improved
2	Clonazepam Wellbutrin Folic Acid Seroquel Celebrex	Declined	Declined	Declined
3	Plaqunil Arava Rituxan Synthroid Fosamax Estrogen/Progestron	Improved	Improved	Improved
4	Humira Prednisone	Declined	Improved	Improved
5	Remicade Methotrexate Vytorin Omeprozole Metropolol	Declined	Improved	Improved
6	Enbrel Folic Acid Methotrexate Zyrtec	N/A	Declined	Improved

Interestingly, the data examining predicted VO_2 max did produce significant results. Participants receiving the vitamin supplement improved estimated VO_{2max} in comparison to the placebo. This improvement was a direct result of a longer exercise time on the post-test. The reasons for the increase in aerobic exercise capacity are speculative and this is the first study to examine estimated VO_{2max} as an outcome of vitamin D supplementation. However there are correlation data to suggest that there is a significant association between VO_{2max} and 25[OH]D in healthy populations (Mowry, Costello, Heelan 2009; Ardestani et al. 2011) As suggested in the article by Ardestani et al., the improvements seen in the Vitamin D group in estimated VO_{2max} could be due to improved cardiac output and decreased peripheral vessel resistance. (Ardestani et al. 2011) The improved exercise capacity may also be related to perceived Quality of Life, which only improved in the Vitamin D group. It is possible that the vitamin D in general made the participants feel better and as a consequence they were more mobile in the month between baseline and follow-up. Lastly, it has been shown in animal models that Vitamin D may affect skeletal muscle and in human studies Vitamin D has been shown to improve neuromuscular function and it is possible that these improvements may in turn improve performance. (Ceglia 2008; Pfeifer, Begerow, Minne 2002) While this study employed a small sample size the results are consistent with what cross sectional data would predict.

Lastly, there was a significant correlation when looking at the change in vitamin D status and the Quality of Life subscale of the Rheumatoid and Arthritis Outcome Score questionnaire ($p = .04$). This relationship has not previous been examined however, it was shown that those people who had an increase in their vitamin D status, significantly had an increase in their Quality of Life. This is an important relationship to understand especially when treating a patient population that is typically in chronic pain.

Overall, these findings provide some consistency with previous research but also open the door for several new research questions, especially aimed at a chronic disease population.

Strengths

There were several strengths of this study. First, it was the first of its kind to examine the role of Vitamin D supplementation in a population with Rheumatoid Arthritis. Additionally, this study attempted to correlate CVD risk through FMD with blood markers to better understand the association between serum inflammatory markers, CVD and FMD. While not all findings were significant, the results give an interesting look a novel dosage and timing of supplementation in this population. Since the dose of Vitamin D was given as a one-time mega dose while the participant was in the lab, it ensured that they received the complete

dose. This was also the first study to examine the effect of Vitamin D on Quality of Life. The significant findings in regards to Quality of Life are extremely important in this population. Having any sort of therapy that can improve Quality of Life is crucial in a population that suffers from chronic pain and therefore generally decreased quality of life. (Jakobsson and Hallberg 2002) This study also utilized the same sonographer for the FMD's in this study and all FMD's were conducted at the same time of day in the same room after at least 8 hours of fasting. Participants were all generally the same age, which should have limited the variability in FMD's that would be expected if a study was conducted using 20 versus 85 year olds. Another, strength of the study was that all but one of the participants came from the same physician's office, so they were generally living in the same region with the same sun exposure, which limited the variability that might be seen if this study had been conducted across several regions. Lastly, variability of sun exposure was also limited due to the short duration of the study. While it is possible that the changes seen in the Vitamin D status of placebo group were due to changes in sun exposure, the one month duration of the study minimized that risk.

Limitations

The main limitation of this study was the small sample size. This limited the ability potentially to see changes that might have otherwise been seen with a larger sample. Secondly, there was no midterm data collection. It would have benefited

the results of this study to repeat all tests at the halfway point to better understand the effect of Vitamin D overtime. Thirdly, considering the vast majority of the participants were from the same physician's office, and were all of similar age it is hard to generalize these results to other age groups. Also, all participants live in Arizona which experiences more sun exposure during the winter than most other places in the United States, it is possible that greater effects of Vitamin D supplementation may have been seen in a climate that was severely limited in their sun exposure. Lastly a limitation of this study is the variability of the FMD measure. As reported in previous sections of this paper, various techniques and software equipment can lead to significant changes in the FMD results. It would be important to replicate this study with a different sonographer in a different lab to determine if the findings are consistent between techniques.

Future Research

The results of this study suggest the need for more research in the area of Vitamin D supplementation in the RA population. First, it would be important to repeat this study with multiple arms; looking at both different dosing amounts as well as dosing frequency. Taking blood samples as well as FMD's at multiple time points throughout the study would help to understand which dose was working at each time point and at what point that effect began to taper off. It would also be important to again look at the role of Vitamin D and TNF-alpha in a larger sample but also include other cytokines such as IL-1 or IL-6. It would also be beneficial to

look at MAPK – Phosphatase 1 to determine if it was activated in the manner that was demonstrated in the cell cultures (Zhang et al. 2012). In addition, looking at participants calcium levels would also be important to understand the role of calcium status on the ability to change 25[OH]D status. The significant findings in regards to the improvements in VO_2 warrant further study, but support findings of previous cross-sectional data. It would be important to replicate this study to determine if this finding was seen again, since this was the first study to examine VO_2 after vitamin D supplementation. It would also be interested to see the potential changes in VO_2 over the course of a long-term study. Finally, it would be important to repeat this study with different age groups, at different times during the year as well as in different regions.

Potential Implications

This study while very preliminary in nature lays the ground work for future research in this area. It is clear from these data that there may be evidence for the use of Vitamin D as a strategy to improve CVD risk in the RA population, but further research is needed to better understand how Vitamin D before it should be implemented into clinical practice.

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APPENDIX A

STUDY 1: CONSENT FORM

The effects of Vitamin D3 supplementation on brachial artery flow-mediated dilation in post-menopausal women

INTRODUCTION

The purposes of this form are to provide you (as a prospective research study participant) information that may affect your decision as to whether or not to participate in this research and to record the consent of those who agree to be involved in the study.

RESEARCHERS

Glenn Gaesser, PhD, a professor in the Exercise Wellness Program, Healthy Lifestyles Research Center, at Arizona State University as well as two graduate students, Dana Ryan and Siddhartha Angadi hve requested your participation in a research study.

STUDY PURPOSE

The purpose of this research is to measure the effects of Vitamin D3 on the ability of your blood vessels to dilate in response to an increase in blood flow.

DESCRIPTION OF THE STUDY

If you are a smoker, or if you take statin medications, you cannot participate in this study.

If you decide to participate, then as a study participant you will be asked to come to the ASU Polytechnic campus on a total of two (2) occasions over a period of two (2) weeks. You must also have been on consistent medications and vitamin supplementation for 6 weeks prior to the start of the study and remain on these medications for the duration of the 2 weeks of the study.

Visit 1: You will report to the ISTB3 building on ASU's Polytechnic campus, Mesa, AZ in a fasted state. This requires that you not eat anything, or drink anything but water, for at least 8 hours prior to coming to the ISTB3 laboratory. All tests will be in the morning, typically between 7 and 10 AM, and will be arranged to suit your schedule.

Blood Pressure, Height and Weight

We will measure your height and weight, and then take your blood pressure after you have had a chance to rest quietly for 15 minutes.

Brachial Artery Flow Mediated Dilation (BAFMD):

This procedure involves taking ultrasound images of an artery in your upper arm before, during, and after a blood pressure cuff is inflated around your forearm. All measurements are made on your non-dominant arm. After lying quietly on a

padded ultrasound table for 20 minutes, a blood pressure cuff will be positioned on your forearm. After recording baseline ultrasound measures on your upper arm, the blood pressure cuff will be inflated to a pressure of 240 mmHg (enough to stop blood flow to your wrist and hand), and kept in place for 5 minutes. You may experience a tingling feeling in your hand, which is normal. The pressure cuff will then be deflated rapidly and ultrasound measures will be taken for 5 minutes.

Vitamin D Supplementation or Placebo

After the BAFMD you will receive either 100,000 IU of Vitamin D3 or an identical placebo. You will not know which you are receiving.

The total time required for visit 1 is about 45 minutes.

Visit 2:

After 2 weeks you will return to the ASU Polytechnic Campus. Visit 2 will be identical to Visit one, except you will not take any supplement or placebo pill.

The total time required for visit 2 is about 45 minutes.

POSSIBLE RISKS

There is minimal risk involved with taking the Vitamin D3 supplement:

- Fatigue
- Dizziness
- There is a very minimal increased risk of kidney stones in women who have been taking calcium supplements for over 7 years

There are no feasible alternative procedures available for this study other than choosing not to participate.

BENEFITS

There may be no direct benefits to you. However, possible benefits of your participation in the research include:

- Knowledge of vascular health
- Free Vitamin D3 Supplementation

NEW INFORMATION

If the researchers find new information during the study that would reasonably change your decision about participating, then they will provide this information to you.

CONFIDENTIALITY

All information obtained in this study is strictly confidential unless disclosure is required by law.

The results of this research study may be used in reports, presentations, and publications, but your name or identity will not be revealed. In order to maintain confidentiality of your records, Dr. Gaesser will use subject codes on all data collected, maintain a master list separate and secure from all data collected, and limit access to all confidential information to the study investigators. All data will be secured in locked filing cabinets in the ISTB3 building within the testing laboratory.

WITHDRAWAL PRIVILEGE

It is ok for you to say no. Even if you say yes now, you are free to say no later, and withdraw from the study at any time.

Your decision will not affect your relationship with Arizona State University or otherwise cause a loss of benefits to which you might otherwise be entitled.

Your participation is voluntary and if you decide not to participate or decide to withdraw from the study it will not affect your grade, treatment, care, employment status.

COSTS AND PAYMENTS

All study procedures will be provided to you at no cost to you.

You will be compensated $25 for completion of the study by either cash or gift certificate.

COMPENSATION FOR ILLNESS AND INJURY

If you agree to participate in the study, then your consent does not waive any of your legal rights. In case of injury you can expect to receive you will receive basic first aid and if necessary, 911 will be called. However, no funds have been set aside to compensate you in the event of injury.

VOLUNTARY CONSENT

Any questions you have concerning the research study or your participation in the study, before or after your consent, will be answered by or Dr. Glenn Gaesser (480-727-1884). If you have questions about your rights as a subject/participant in this research, or if you feel you have been placed at risk, you can contact the Chair of the Human Subjects Institutional Review Board, through the ASU Research Compliance Office, at 480-965 6788.

This form explains the nature, demands, benefits and any risks of the project. By signing this form you agree knowingly to assume any risks involved. Remember,

your participation is voluntary. You may choose not to participate or to withdraw your consent and discontinue participation at any time without penalty or loss of benefit. In signing this consent form you are not waiving any legal claims, right, or remedies. A copy of this consent form will be given to you.

Your signature below indicates that you consent to participate in the above study.

_____ _____

Subject's Signature Printed Name Date

INVESTIGATOR'S STATEMENT

"I certify that I have explained to the above individual the nature and purpose, the potential benefits and possible risks associated with participation in this research study, have answered any questions that have been raised, and have witnessed the above signature. These elements of Informed Consent conform to the Assurance given by Arizona State University to the Office for Human Research Protections to protect the rights of human subjects. I have provided the subject/participant a copy of this signed consent document."

Signature of Investigator_____

Date_____

APPENDIX B

STUDY 1: MEDICATION FORM

The effects of Vitamin D3 supplementation on brachial artery flow-mediated dilation in post-menopausal women

Subject Number _____

Age _____

Please list all current medications, dosage and how long you have been on each medication

DRUG, VITAMIN or HERBAL SUPPLEMENT	DOSAGE	LENGTH OF USE

APPENDIX C

STUDY 1: DATA RECORDING FORM

Subject Number _____

	Visit 1	Visit 2
Height		
Weight		
Blood Pressure		
FMD		

APPENDIX D

STUDY 2: PHYSICIAN SCREENING FORM

Eligibility Criteria:

Must be post menopausal women

Must have been diagnosed with Rheumatoid Arthritis for at least one year prior and be on consistent drug therapy

RA symptoms, as measured by the Disease Activity Score-28 (DAS28), should be well controlled with their current medication (DAS28 score of 3.2 or below).

Cleared to participate in physical activity.

Patients must also be able to speak and read English

Must have their own method of transportation to the ASU Polytechnic campus.

Exclusion Criteria

history of ischemic heart disease,

coronary artery bypass grafting,

cardiac catheterization, pacemaker/implantable cardioverter defibrillator implantation,

valvular heart disease, heart failure, cardiac transplantation, congenital heart disease,

type 1 & 2 diabetes,

asthma, lung disease

peripheral arterial disease,

orthopedic problems that limit exercise,

uncontrolled hypertension,

Patients on statins will be excluded. Smokers will also be excluded

APPENDIX E

STUDY 2: CONSENT FORM

CONSENT FORM

The effects of Vitamin D3 supplementation on brachial artery flow-mediated dilation in post-menopausal women and older men with rheumatoid arthritis

INTRODUCTION
The purposes of this form are to provide you (as a prospective research study participant) information that may affect your decision as to whether or not to participate in this research and to record the consent of those who agree to be involved in the study.

RESEARCHERS
Glenn Gaesser, PhD, a professor in the Exercise Wellness Program, Healthy Lifestyles Research Center, at Arizona State University, and Warren Rizzo, MD, a physician at Advanced Arthritis Care have requested your participation in a research study.

STUDY PURPOSE
The purpose of this research is to measure the effects of Vitamin D3 on the ability of your blood vessels to dilate in response to an increase in blood flow.

DESCRIPTION OF THE STUDY

If you decide to participate, then as a study participant you will be asked to come to the ASU Polytechnic campus on a total of two (2) occasions over a period of approximately four (4) weeks.

Visit 1: You will report to the ISTB3 building on ASU's Polytechnic campus, Mesa, AZ in a fasted state. You will be asked not to eat for 8 hours prior to arriving at the lab.

Rheumatoid and Arthritis Outcome Score
You will fill out the Rheumatoid and Arthritis Outcome Score questionnaire, which will ask questions about your pain levels and quality of life.

After completing the questionnaire, you will first have a resting measurement of the ability of an artery in your upper arm to dilate in response to an increase in blood flow called brachial artery flow-mediated dilation.

Brachial Artery Flow Mediated Dilation:

This procedure involves taking ultrasound images of an artery in your upper arm before, during, and after a blood pressure cuff is inflated around your forearm. All measurements are made on your non-dominant arm. After lying quietly on a padded ultrasound table for 20 minutes, a blood pressure cuff will be positioned on your forearm. After recording baseline ultrasound measures on your upper arm, the blood pressure cuff will be inflated to a pressure of 240 mmHg (enough to stop blood flow to your wrist and hand), and kept in place for 5 minutes. You may experience a tingling feeling in your hand, which is normal. The pressure cuff will then be deflated rapidly and ultrasound measures will be taken for 5 minutes.

Exercise Test

After completing the brachial artery flow mediated dilation, you will undergo a sub-maximal exercise test on a treadmill. The test begins with you walking at a comfortable pace on a level grade for one minute, after which the grade of the treadmill will increase until you reach a heart rate that is approximately 85% of your age-predicted heart rate. You will be monitored throughout the test by a trained exercise physiologist who will regularly ask you how you are doing as grade of the treadmill is progressively increased, and your heart rate will be recorded with an elastic chest strap (Polar heart rate monitor). You may stop the test at any time if you do not feel like you can continue.

Body Composition

After the submaximal test you will undergo a non-invasive body composition test. This test measures body fat percentage via the BodPod. This requires that you sit down in a fiberglass shell chamber (that looks something like a giant egg shell with a window), and rest quietly for a few moments. You are not required to do anything while in this shell other than rest quietly. The BodPod has a large window that allows you to see out into the room, and has an intercom that allows you to communicate with the investigator at all times. For this visit you will need to bring a tight-fitting bathing suit or similar clothing, such as tight-fitting lycra/spandex athletic shorts (and top, e.g., jog-bra).

Blood Draw

You will also undergo a blood draw to examine Vitamin D levels and TNF-alpha levels in your blood. A small amount of blood (less than 2 teaspoons) will be drawn from a forearm vein.

Vitamin D Supplementation

After the blood draw you will receive a Vitamin D3 pill or an identical placebo. You will not know which you are receiving.

The total time required for visit 1 is about 2 hours.

Visit 2:

Visit 2 will be identical to Visit one with the exception that you however you will not receive a Vitamin D or placebo pill.

The total time required for visit 2 is about 2 hours.

POSSIBLE RISKS

The risks involved with exercise and exercise testing include the chance of cardiac incident such as abnormal heart rhythms or a heart attack. However, this risk is very slight for someone without cardiovascular disease. Other risks include:

- Minor muscular soreness and stiffness following resistance exercise.
- Musculoskeletal injury – muscle or connective tissue strains or sprains.
- Physiological changes which may occur during exercise – abnormal blood pressure or heart rate, increased shortness of breath, and in rare instances a heart attack.
- Exercise may also result in nausea, faintness, dizziness, and shortness of breath.

You will be monitored by trained personnel who will oversee the exercise testing and watch for signs of distress, and stop testing if necessary All exercise testing procedures will comply with the guidelines for exercise test administration as recommended by the American College of Sports Medicine, National Strength and Conditioning Association and required by the Healthy Lifestyles Research Center at Arizona State University.

While performing resistance exercise you will be reminded during the exercises not to hold your breath and to exhale as you exert force against the resistance. You will be asked not to attempt any exercise that you feel is beyond your physical abilities. If you experience discomfort, feel you are unable to continue or wish to stop an exercise at any point, you are requested to inform the investigator immediately.

There is minimal risk involved with taking the Vitamin D3 supplement:

- Fatigue
- Dizziness

There are no feasible alternative procedures available for this study other than choosing not to participate.

BENEFITS

There may be no direct benefits to you. However, possible benefits of your participation in the research include:

- Knowledge of vascular health
- Free Vitamin D3 Supplementation
- Knowledge of fitness levels
- Knowledge of body composition

NEW INFORMATION

If the researchers find new information during the study that would reasonably change your decision about participating, then they will provide this information to you.

CONFIDENTIALITY

All information obtained in this study is strictly confidential unless disclosure is required by law.
Some information will be obtained from your medical record. The results of this research study may
be used in reports, presentations, and publications, but your name or identity will not be revealed. In
order to maintain confidentiality of your records, Dr. Gaesser and Dr. Rizzo will use subject codes
on all data collected, maintain a master list separate and secure from all data collected, and limit
access to all confidential information to the study investigators. All data will be secured in locked
filing cabinets in the ISTB3 building within the testing laboratory.

WITHDRAWAL PRIVILEGE

It is ok for you to say no. Even if you say yes now, you are free to say no later, and withdraw from the study at any time.

Your decision will not affect your relationship with Arizona State University or otherwise cause a loss of benefits to which you might otherwise be entitled.

Your participation is voluntary and if you decide not to participate or decide to withdraw from the study it will not affect your grade, treatment, care, employment status.

COSTS AND PAYMENTS

All study procedures will be provided to you at no cost to you.

You will be paid $50 for completion of the study by either compensation-check or gift certificate.

COMPENSATION FOR ILLNESS AND INJURY

If you agree to participate in the study, then your consent does not waive any of your legal rights. In case of injury you can expect to receive you will receive basic first aid and if necessary, 911 will be called. However, no funds have been set aside to compensate you in the event of injury.

VOLUNTARY CONSENT

Any questions you have concerning the research study or your participation in the study, before or after your consent, will be answered by or Dr. Glenn Gaesser (480-727-1884). If you have questions about your rights as a subject/participant in this research, or if you feel you have been placed at risk, you can contact the Chair of the Human Subjects Institutional Review Board, through the ASU Research Compliance Office, at 480-965 6788.

This form explains the nature, demands, benefits and any risks of the project. By signing this form you agree knowingly to assume any risks involved. Remember, your participation is voluntary. You may choose not to participate or to withdraw your consent and discontinue participation at any time without penalty or loss of benefit. In signing this consent form you are not waiving any legal claims, right, or remedies. A copy of this consent form will be given to you.

Your signature below indicates that you consent to participate in the above study.

_____ _____

Subject's Signature Printed Name Date

INVESTIGATOR'S STATEMENT

"I certify that I have explained to the above individual the nature and purpose, the potential benefits and possible risks associated with participation in this research study, have answered any questions that have been raised, and have witnessed the above signature. These elements of Informed Consent conform to the Assurance given by Arizona State University to the Office for Human Research Protections to protect the rights of human subjects. I have provided the subject/participant a copy of this signed consent document."

Signature of Investigator_____

Date_____

APPENDIX F

STUDY 2: HIPPA FORM

HIPAA AUTHORIZATION FORM

Protocol Title/ASU HS #: Effect of Vitamin D3 Supplementation of Flow Mediated Brachial Artery Dilation in Post Menopausal Women with Rheumatoid Arthritis

Principal Investigator: Glenn Gaesser, PhD and Warren Rizzo, MD

AUTHORIZATION TO COLLECT, USE, AND SHARE HEALTH INFORMATION FOR RESEARCH

By law, researchers must protect the privacy of health information about you. This form and the attached research consent form need to be kept together.

We are asking you to take part in the research described in the attached consent form. The researchers are not authorized to collect any health information about you unless that information is described in the consent form that you sign.

What is "health information"?

As used in this form, the phrase "health information" includes:

- Health information that identifies you.
- Information about you that is created during the research study. This might include the results of tests or exams that become part of the study records; diaries and questionnaires that you might be asked to fill out as part of the study and other records from the study.
- Information in your medical records that is needed for this research study. These might include the results of physical exams, blood tests, x-rays, diagnostic and medical procedures and your medical history.

The specific information that will be collected in this research is described in the attached consent form. For you to be in this research, we need your permission to collect and share this information.

Who will see the health information collected in this research?

If you agree to participate, you are giving permission for the researchers to share your health information with the following people and groups:

- Anyone listed in the informed consent document as a person or group that you agree may receive information about you,
- Anyone listed in a separate authorization for release of medical records or information that is signed by you,
- People at ASU who help with the research,
- People outside of ASU who are in charge of, pay for, or work with us on the research.
- Government agencies, review boards, and others who watch over the safety, effectiveness, and conduct of the research.
- Other researchers when a review board approves the sharing of the health information.
- Your health insurer if they are paying for care provided as part of the research study.
- Others, if the law requires.

The researchers cannot control what any of these persons or groups may do with the information they receive about you and the privacy of your information may no longer be protected by federal privacy rules after it is disclosed to them.

What if you don't want to participate in the research?

You do not have to sign this permission ("authorization") form if you do not want to be in the research. If you do not sign, then you will not be allowed to participate in the study. If you decide not to sign, it will not result in any penalty or loss of benefits to which you are entitled.

If you sign this form and then change your mind later, and do not want us to use and share your health information, you will need to send a letter to the researcher at the address listed on the attached consent form. The letter will need to say that you have changed your mind and do not want the ASU researcher to collect and share your health information. The researcher may still use the information they have already collected.

Will you get to see the health information collected about you?

Depending on the nature of the research, it is possible that you will not have access to health information about you that is created during the study until after the study is complete.

If you have any questions, please contact the researcher listed on the attached consent form. You may also call the ASU Office of Research Integrity and Assurance at 480-965-6788 with questions about the research use of your health information. Your researcher will give you a signed copy of this form.

I agree to the collection, use, and sharing of my health information for purposes of this research study.

- This permission will not expire unless you tell the researchers in writing that you have changed your mind and no longer want to participate.

_____ ___

Signature of research subject or subject's legal representative — Date

_____

Printed name of research subject or subject's representative's

---

Representative' relationship subject

APPENDIX G

STUDY 2: DATA RECORDING FORM

Subject Number _____

	Visit 1	Visit 2
Height		
Weight		
Blood Pressure		
FMD		
Plasma 25[OH]D		
TNF-alpha		
Body Fat %		
Submax Walking Test		
Quality of Life		

APPENDIX H

STUDY 2: 25[OH]D ELISA PROTOCOL

Procedure

Materials Provided

1. **CAL 0 - 6 – Calibrators (REF AC-5701A - AC-5701G):**
   Lyophilised buffered human serum containing 25-hydroxyvitamin D and <1% sodium azide (0.09% reconstituted). The exact value of each Calibrator is printed on the bottle label, 1 mL per bottle, 7 bottles per kit.

2. **MICROPLAT - Antibody Coated Plate (REF AC-5702W):**
   Microplate with 25-hydroxyvitamin D sheep polyclonal antibody linked to the inner surface of the polystyrene wells, 12 x 8 well strips in a foil pouch with desiccant.

3. **25-D BIOTIN 50x - 25-D Biotin Concentrate (REF AC-5703):**
   Lyophilised buffer containing 25-hydroxy-vitamin D labelled with biotin, and proprietary stabilisers, 1 mL per bottle.

4. **BUF - Buffer (REF AC-5703B):**
   Proprietary reagent for dissociating 25-hydroxy-vitamin D from binding proteins, 50 mL per bottle.

5. **ENZYMCONJ - Enzyme Conjugate (REF AC-5704):**
   Phosphate buffered saline containing avidin linked to horseradish peroxidase, protein, enzyme stabilisers and preservative. 22 mL per bottle.

6. **CTRL 1 - 2 – Controls (REF AC-5705A - AC-5705B):**
   Lyophilised human serum containing 25-hydroxy-vitamin D and <1% sodium azide (0.09% reconstituted), 1 mL per bottle, 2 bottles per kit.

7. **SUBS - TMB Substrate (REF AC-SUBS):**
   A proprietary aqueous formulation of tetramethylbenzidine (TMB) and hydrogen peroxide, 28 mL per bottle.

8. **HCL - Stop Solution (REF AC-STOP):**
   0.5M Hydrochloric Acid, 13 mL per bottle.

9. **WASHBUF 20x - Wash Concentrate (REF AC-WASHL):**
   Phosphate buffered saline containing Tween, 50 mL per bottle.

10. **Adhesive Plate Sealer**
    8 per kit.

11. **Documentation**
    Package Insert and QC report.

Materials Required but not Provided

1. Disposable 12 x 75 mm borosilicate glass or polypropylene tubes.
   Note: polystyrene tubes are not suitable. Do not reuse tubes.
2. Precision pipetting devices to deliver 25 µL and 200 µL.
3. Repeating pipettes to deliver 1 mL, e.g. Eppendorf Multipipette 4780, or similar.
4. Precision multi-channel pipettes to deliver 100 µL and 200 µL.
5. Vortex mixer.
6. Automatic microplate washer (optional).
7. Photometric microplate reader and data analysis equipment.

APPENDIX I

STUDY 2: TNF-ALPHA ELISA PROTOCOL

REAGENT PREPARATION

Bring all reagents to room temperature before use.

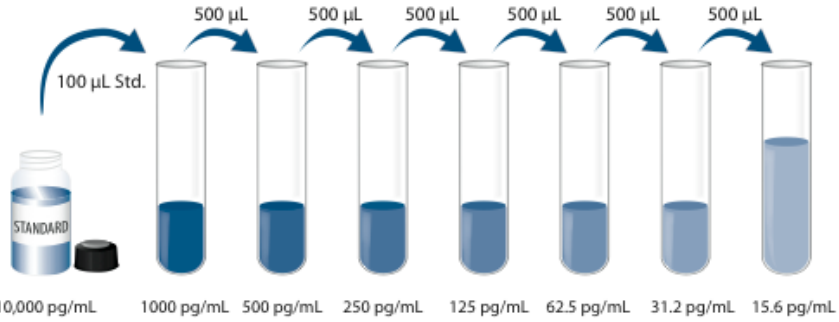
Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Dilute 20 mL of Wash Buffer Concentrate into deionized or distilled water to prepare 500 mL of Wash Buffer.

Calibrator Diluent RD6-35 (1X) (*for cell culture supernate samples*) - Add 10 mL of Calibrator Diluent RD6-35 to 40 mL of deionized or distilled water to yield 50 mL of Diluted Calibrator Diluent RD6-35.

Substrate Solution - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 200 µL of the resultant mixture is required per well.

TNF-α Standard - **Refer to vial label for reconstitution volume.** Reconstitute the TNF-α Standard with deionized or distilled water. This reconstitution produces a stock solution of 10,000 pg/mL. Allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

Pipette 900 µL of Calibrator Diluent RD6-35 (*for serum/plasma samples*) or Calibrator Diluent RD6-35 (1X) (*for cell culture supernate samples*) into the 1000 pg/mL tube. Pipette 500 µL of the appropriate Calibrator Diluent into the remaining tubes. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 1000 pg/mL standard serves as the high standard. The appropriate Calibrator Diluent serves as the zero standard (0 pg/mL).



ASSAY PROCEDURE

Bring all reagents and samples to room temperature before use. It is recommended that all standards, samples, and controls be assayed in duplicate.

1. Prepare all reagents and working standards as directed in the previous sections.
2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal.
3. Add 50 µL of Assay Diluent RD1F to each well. Assay Diluent RD1F will have a precipitate present. Mix well before and during use.
4. Add 200 µL of Standard, sample, or control per well. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature. A plate layout is provided to record standards and samples assayed.
5. Aspirate each well and wash, repeating the process three times for a total of four washes. Wash by filling each well with Wash Buffer (400 µL) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
6. Add 200 µL of TNF-α Conjugate to each well. Cover with a new adhesive strip.
   For Cell Culture Supernate Samples: Incubate for 1 hour at room temperature.
   For Serum/Plasma Samples: Incubate for 2 hours at room temperature.
7. Repeat the aspiration/wash as in step 5.
8. Add 200 mL of Substrate Solution to each well. Incubate for 20 minutes at room temperature. **Protect from light.**
9. Add 50 µL of Stop Solution to each well. The color in the wells should change from blue to yellow. If the color in the wells is green or if the color change does not appear uniform, gently tap the plate to ensure thorough mixing.
10. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

APPENDIX J

STUDY 2: RHEUMATOID AND ARTHRITIS OUTCOME SCORE QUESTIONNAIRE

Rheumatoid and Arthritis Outcome Score RAOS

Today's date: _____/_____/_____ Date of birth: _____/_____/_____

Name: _____

INSTRUCTIONS: This survey asks for your view about problems related to your hips, knees and/or feet. This information will help us keep track of how you feel about your hip, knee and/or foot problems and how well you are able to do your usual activities.
Answer every question by ticking the appropriate box, only one box for each question. If you are unsure about how to answer a question, please give the best answer you can.

Symptoms

These questions should be answered thinking of your hip, knee and foot symptoms during the **last week**.

S1. Do you have swelling in your hip, knee or foot?
Never Rarely Sometimes Often Always

S2. Do you feel grinding, hear clicking or any other type of noise when your hip, knee or foot moves?
Never Rarely Sometimes Often Always

S3. Does your hip, knee or foot catch or hang up when moving?
Never Rarely Sometimes Often Always

S4. Can you straighten your hip, knee or foot fully?
Always Often Sometimes Rarely Never

S5. Can you bend your hip, knee or foot fully?
Always Often Sometimes Rarely Never

Stiffness

The following questions concern the amount of joint stiffness you have experienced in your hip/knee/foot during the **last week**. Stiffness is a sensation of restriction or slowness in the ease with which you move your hip, knee or foot joint.

S6. How severe is your hip, knee or foot joint stiffness after first wakening in the morning?
None Mild Moderate Severe Extreme

S7. How severe is your hip, knee or foot stiffness after sitting, lying or resting **later in the day**?
None Mild Moderate Severe Extreme

Pain

P1. How often do you experience hip, knee or foot pain?

Never Monthly Weekly Daily Always

How much hip, knee or foot pain have you experienced the **last week** during the following activities?

P2. Twisting/pivoting on your hip, knee or foot (dancing, ball games, etc.)

None Mild Moderate Severe Extreme

P3. Straightening hip, knee or foot fully

None Mild Moderate Severe Extreme

P4. Bending hip, knee or foot fully

None Mild Moderate Severe Extreme

P5. Walking on flat surface

None Mild Moderate Severe Extreme

P6. Going up or down stairs

None Mild Moderate Severe Extreme

P7. At night while in bed

None Mild Moderate Severe Extreme

P8. Sitting or lying

None Mild Moderate Severe Extreme

P9. Standing upright

None Mild Moderate Severe Extreme

Function, daily living

The following questions concern your physical function. By this we mean your ability to move around and to look after yourself. For each of the following activities please indicate the degree of difficulty you have experienced in the **last week** due to your hip, knee or foot.

A1. Descending stairs

None Mild Moderate Severe Extreme

A2. Ascending stairs

None Mild Moderate Severe Extreme

For each of the following activities please indicate the degree of difficulty you have experienced in the **last week** due to your hip, knee or foot.

A3. Rising from sitting
None Mild Moderate Severe Extreme

A4. Standing
None Mild Moderate Severe Extreme

A5. Bending to floor/pick up an object
None Mild Moderate Severe Extreme

A6. Walking on flat surface
None Mild Moderate Severe Extreme

A7. Getting in/out of car
None Mild Moderate Severe Extreme

A8. Going shopping
None Mild Moderate Severe Extreme

A9. Putting on socks/stockings
None Mild Moderate Severe Extreme

A10. Rising from bed
None Mild Moderate Severe Extreme

A11. Taking off socks/stockings
None Mild Moderate Severe Extreme

A12. Lying in bed (turning over, maintaining leg position)
None Mild Moderate Severe Extreme

A13. Getting in/out of bath
None Mild Moderate Severe Extreme

A14. Sitting
None Mild Moderate Severe Extreme

A15. Getting on/off toilet
None Mild Moderate Severe Extreme

For each of the following activities please indicate the degree of difficulty you have experienced in the **last week** due to your hip, knee or foot.

A16. Heavy domestic duties (moving heavy boxes, scrubbing floors, etc)
None Mild Moderate Severe Extreme

A17. Light domestic duties (cooking, dusting, etc)
None Mild Moderate Severe Extreme

Function, sports and recreational activities
The following questions concern your physical function when being active on a higher level. The questions should be answered thinking of what degree of difficulty you have experienced during the **last week** due to your hip, knee or foot.

SP1. Squatting
None Mild Moderate Severe Extreme

SP2. Running
None Mild Moderate Severe Extreme

SP3. Jumping
None Mild Moderate Severe Extreme

SP4. Twisting/pivoting on your affected leg (dancing, ball games, etc)
None Mild Moderate Severe Extreme

SP5. Kneeling
None Mild Moderate Severe Extreme

Quality of Life

Q1. How often are you aware of your hip, knee or foot problem?
Never Monthly Weekly Daily Constantly

Q2. Have you modified your life style to avoid potentially damaging activities to your legs?
Not at all Mildly Moderately Severely Totally

Q3. How much are you troubled with lack of confidence in your hip/knee/foot?
Not at all Mildly Moderately Severely Extremely

Q4. In general, how much difficulty do you have with your hip/ knee/foot?
None Mild Moderate Severe Extreme

Thank you very much for completing all the questions in this questionnaire.

BIOGRAPHICAL SKETCH

Dana Meredith Ryan was born in San Diego, California. She recieved her high school education at The Bishop's School in La Jolla, CA. She completed her undergraduate studies at the University of Washington where she was also a 4-year member of the Women's Rowing Team. In addition to being a member of the team she was also a Pac-10 All-Academic Team Member. Upon graduation she moved back to San Diego to persue a Master's in Kinesiology at San Diego State University. During her tenure in San Diego, she was also the head coach of a high school rowing program. In 2009, she entered Arizona State University to begin her doctoral studies in Physical Activity, Nutrition and Wellness. During her time at ASU she was accepted to the Phi Kappa Phi Honor Society and served as the President of the Exercise and Wellness Graduate Club. She also recieved a grant from the Graduate and Professional Student Association for her dissertation research.