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The Evidence for Hypovitaminosis D as a Cause of Cardiovascular Disease

Author
Weyland, Patricia G.

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The Evidence for Hypovitaminosis D as a Cause of Cardiovascular Disease

by

Patricia Weyland, MS, RN, FNP-BC, CDE, PhD(c)

DISSERTATION

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Dedication

This dissertation is dedicated to my family; my husband Scott, my daughter Vanessa, and my son Andrew. All of you mean more to me than you can possibly imagine and I am very grateful every day that all of you are part of my life. I have learned many things from you and I am a better person because of you. I hope I have inspired you to be life-long learners and to pursue your goals in life with the ultimate goal being happiness and health for yourself and all those around you.

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The Effect of Hypovitaminosis D on Risk for Cardiovascular Disease

Patricia G. Weyland, MS, RN, FNP-BC, CDE, PhD (c)

Abstract

Background: Serum 25-hydroxyvitamin D (25(OH)D) levels have been found to be inversely associated with both prevalent and incident cardiovascular disease (CVD) risk factors; dyslipidemia, hypertension and diabetes mellitus.

Objective: This review looks for evidence of a causal association between low 25(OH)D levels and increased CVD risk.

Method: We evaluated journal articles in light of Hill's criteria for causality in a biological system.

Results: The results of our assessment are as follows. Strength of association: many randomized controlled trials (RCTs), prospective and cross-sectional studies found statistically significant inverse associations between 25(OH)D levels and CVD risk factors. Consistency of observed association: most studies found statistically significant inverse associations between 25(OH)D levels and CVD risk factors in various populations, locations and circumstances. Temporality of association: many RCTs and prospective studies found statistically significant inverse associations between 25(OH)D levels and CVD risk factors. Biological gradient (dose-response curve): most studies assessing 25(OH)D levels and CVD risk found an inverse association exhibiting a linear biological gradient. Plausibility of biology: several plausible cellular-level causative mechanisms and biological pathways may lead from a low 25(OH)D level to increased risk for CVD with mediators, such as dyslipidemia, hypertension and diabetes mellitus. Experimental evidence: some well-designed RCTs found increased CVD risk factors with decreasing 25(OH)D levels. Analogy: the association between serum 25(OH)D levels and CVD

risk is analogous to that between 25(OH)D levels and the risk of overall cancer, periodontal disease, multiple sclerosis and breast cancer.

Conclusion: All relevant Hill criteria for a causal association in a biological system are satisfied to indicate a low 25(OH)D level as a CVD risk factor.

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Chapter 1

Introduction

Cardiovascular Disease

Cardiovascular disease (CVD) is a composite of diagnoses which includes coronary artery disease (CAD), congestive heart failure, cardiomyopathy, pulmonary heart disease, rheumatic heart disease, cardiac dysrhythmias, essential hypertension, hypertensive renal disease, and cerebrovascular disease (Miniño & Klein, 2010). CVD remains the leading cause of death in the US with ischemic heart disease and stroke the first and second leading causes of death, respectively (Kochanek, Xu, Murphy, Miniño, & Kung, 2011; World Health Organization, 2014). CVD has also become the leading cause of death in all other regions of the world (World Health Organization, 2014) with 80% of the burden of CVD in low and middle-income countries (Yusef et al., 2001).

Despite adequate screening and evidence-based treatment of known modifiable CVD risk factors, an estimated 70% of cardiac events will not be prevented (Kones, 2011). This finding suggests that there are other unknown CVD risk factors. Therefore, further research to identify additional risk factors for CVD is necessary and must be a priority. Adding to the urgency for on-going research to identify additional CVD risk factors is the prediction that the heart disease-related death rate may increase due to the increase in diabetes mellitus and obesity (Olshansky et al., 2005; Jones et al., 2012).

The identification of additional CVD risk factors and the provision of risk factor-modifying clinical preventive services could prevent tens of thousands of premature deaths in the US annually (Ford et al., 2007; Farley, Dalal, Mostashari, & Frieden, 2010) and many more worldwide (World Health Organization, 2014). Additionally, discovering unknown causes of known CVD risk factors including diabetes mellitus, obesity, hypertension, and dyslipidemia and

providing new treatments based upon these causes will also decrease the CVD-related death rate. Risk factor-modifying clinical preventive services should have benefits that outweigh potential treatment risks (Pletcher, Tice, Pignone, & Browner, 2004) and should be accessible to all.

Hypovitaminosis D and Increased CVD Risk

Scragg (1981) recognized a pattern in CVD morbidity and mortality; there was an increase in the winter, at lower altitudes, and at higher latitudes but there was no association with respiratory disease or temperature changes. Scragg (1981) hypothesized that the seasonal variation in solar ultra-violet radiation leading to fluctuations in vitamin D (D) metabolite status is a mechanism that contributes to the seasonality of CVD morbidity and mortality. The specific mechanism by which an increase in D metabolite status decreased CVD could be a direct effect on platelet activity, thrombus formation, or a change in calcium metabolism.

Several CVD risk factors and CVDs have been associated with Hypovitaminosis D, assessed by measuring serum 25-hydroxyvitamin D (25[OH] D) levels (DeLuca, 2004) including: lower high-density lipoprotein-cholesterol (HDL-c) and higher triglycerides (de Boer et al., 2009); an increase in blood pressure (Krause, Bühring, Hopfenmüller, Holick, & Sharma, 1998; Forman et al., 2007; de Boer et al., 2009; Zhao et al., 2010; Goel & Lal, 2011; Gupta, Brashear, & Johnson, 2011; Larsen, Mose, Bech, Hansen, & Pedersen, 2012); dysfunctional changes in the characteristics of plasma lipids (Schwartz, 2008; de la Lhera-Moya, 2010; Jorde et al., 2010); coronary artery calcium deposition (de Boer et al., 2009; Pletcher, Tice, Pignone & Browner, 2004; Taylor et al., 2005; Taylor et al., 2010); inflammation (Guasch et al., 2012), increased parathyroid hormone (van Ballegooijen et al., 2012); myocardial infarction (MI) (Giovannucci, Liu, Hollis, & Rimm, 2008; Wang et al., 2008; Kendrick, Targher, Smits, & Chonchol, 2009); CAD, DM, peripheral vascular disease, atrial fibrillation, transient ischemic

attack, ventricular tachycardia (Anderson et al., 2010); heart failure (Wang et al., 2008; Anderson et al., 2010); and stroke (Wang et al., 2008; Kendrick, Targher, Smits, & Chonchol, 2009; Anderson et al., 2010).

D deficiency or Hypovitaminosis D is defined in the *Endocrine Society* clinical practice guidelines (Holick et al., 2011) as a serum 25(OH) D level < 20 ng/mL and *insufficiency* as a level > 20 and < 30 ng/mL. The *Institute of Medicine* (IOM, 2010) guidelines define serum 25(OH) D *sufficiency* as > 20 ng/mL. Additionally, a committee composed of internationally-renowned D experts convened in Warsaw in 2012 to develop practice guidelines for serum 25(OH) D levels. The Warsaw committee, in contrast to the IOM, recommended that a serum 25(OH) D level of 30 to 60 ng/mL is needed to achieve and maintain optimal bone health as well as overall health and well-being (Pludowski et al., 2013).

Prevalence, Causes, and Treatment of Hypovitaminosis D

The prevalence of individuals with deficient levels of serum 25(OH) D in the United States is approximately 32% (Looker et al., 2011). It is estimated that the worldwide prevalence of low serum 25(OH) D, either deficiency or insufficiency, is one billion (Holick, 2007). The main causes of low serum 25(OH) D levels are strict sun protection and an inadequate amount of D in the diet or in supplement form (Vieth, 2006).

A low serum 25(OH) D level is easily corrected in the majority of individuals by supplementation with D_3 (cholecalciferol) (Houghton & Vieth, 2006; Heaney, Recker, Grote, Horst, & Armas, 2011). D_3 is approximately 87% more effective in raising and maintaining serum 25(OH) D levels and produces a two to three-fold greater amount of stored D than equivalent doses of D_2 (ergocalciferol) (Heaney, Recker, Grote, Horst, & Armas, 2011). A daily intake of up to 40,000 IU of D_3 per day is unlikely to result in D toxicity and there is an inverse

association between baseline serum 25(OH) D levels and post-supplementation serum 25(OH) D levels (Garland, French, Baggerly, & Heaney, 2011).

Levels of 25(OH) D can also be increased with sun exposure. Ultraviolet Beta (UVB) wave (290 to 315 nanometers) solar radiation is absorbed by *7-dehydrocholesterol* in the skin to form *pre-Vitamin D* with optimal photosynthesis at 295 to 300 nanometers. Pre-vitamin D is unstable and in the presence of heat it rapidly converts to *Vitamin D* (calciferol), moves out of the cells of the skin into the extra-cellular space, is drawn into the capillaries in the dermal layer of the skin by *vitamin D binding protein* (DBP) and proceeds to the general circulation (Holick, 2006). The synthesis of D in the skin accounts for approximately 80% of the D status of an individual (Rosen et al, 2012) and intoxication from D caused by over-exposure to sunlight is not possible. Once a critical amount of D is synthesized in the skin, sunlight then causes D to be photo-isomerized into lumisterol and tachysterol, two biologically inert compounds (Holick, MacLaughlin, & Doppelt, 1981).

D is a precursor compound and exerts no significant biological activity (Bikle, 2010) until it is hydroxylated by the liver with the enzyme *D-25-hydroxylase* to 25(OH) D and then the kidney with the enzyme *D-1α-hydroxylase* to 1α, 25-dihydroxyvitamin D (1α, 25$[OH]_2$ D), the biologically active form (Holick, 2006). A decrease in serum levels of 25(OH) D leads to an increase in parathyroid hormone secretion which increases renal D-1α-hydroxylase activity and a subsequent elevation in 1α, 25$(OH)_2$ D level (Bikle, 2010). Intra-cellular and extra-cellular calcium concentrations (Bikle, 2010), metabolism of calcium and phosphorus, down-regulation of renin production, stimulation of insulin production, and maintenance of normal cell proliferation and differentiation are regulated by 1α, 25$(OH)_2$ D (Holick, 2006).

Dietary sources of D including *Vitamin D_2* (ergocalciferol) derived from plants and yeast and *Vitamin D_3* (cholecalciferol) found in animal sources contribute to only a small portion of the total D status of an individual (Pilz et al., 2011). D is found naturally in oily fishes and in eggs. Artificial sources of D include mushrooms that are sun-dried, ultraviolet-irradiated foods, D-fortified foods such as milk and orange juice, and D supplements (Bikle, 2010).

D_3 is approximately 87% more effective in increasing and maintaining serum 25(OH) D levels and adipose tissue storage is two to three times greater than an equivalent amount of D_2. Vitamin D_3 is also less expensive than D_2 and should be the preferred type of D used when correcting insufficient or deficient states (Heaney, Recker, Grote, Horst, & Armas, 2011).

Dietary D is transported in a significantly different way than D that is synthesized in the skin which is mainly bound to DBP. When D_2 and D_3 from natural dietary sources, fortified foods, and supplements are ingested, the vitamins are first incorporated into *chylomicrons*, and then they are absorbed into the lymphatic system, and ultimately enter the cardiovascular (CV) circulation. Unlike D synthesized by UVB radiation, dietary D, after being incorporated into chylomicrons and entering the CV circulation, may be taken up by muscle and adipose tissue due to *lipoprotein lipase enzyme* activity (Holick, 2006).

D acquired from the diet is slowly transferred to DBP and D that remains in the chylomicron remnant is removed from the CV circulation by the liver (Jones, 2008). The D that becomes bound to DBP after entering the CV circulation, is then released from the DBP and is hydroxylated on C-25 by the D-25-OHases (Cytochrome P450 enzymes; CYP27A1, CYP3A4, CYP2R1, and CYP2J3) in the liver to 25(OH) D (Holick, 2006).

Lab Assays

High-Performance Liquid Chromatography

The high-performance liquid chromatography **(**HPLC) assay was developed in the 1930's and involves a process of separating dissolved compounds in a solution. It requires a solid phase extraction or liquid-liquid extraction with or without saponification to thoroughly purify and concentrate the sample. Reversed phase C_{18} hydrocarbon columns are used to separate closely related long chain hydrophobic compounds. Retinol (Vitamin A) often co-elutes with 25(OH) D_2 and so further purification is then required (Hymøller & Jensen, 2011).

A significant amount of controversy persists regarding the level of serum 25(OH) D that should be used to determine a "sufficient" quantity (Grant, 2010; Norman & Bouillon, 2010; Vieth, 2006). If the desired ranges for "sufficiency," "insufficiency," and "deficiency" are changed, the accuracy of the serum 25(OH) D assay may be affected. Cavalier et al. (2010) explored a concept they termed "measurement uncertainty." They defined the term as the dispersion of values that surrounds the *true* value. They found that for measured 25(OH) D levels near 80 nmol/L (32 ng/mL) using four different assays, including the HPLC and the DiaSorin RIA, the *true* level of 25(OH) D could be 11.5% to 27.5% lower or higher (95% CI), depending upon the assay used.

Cavalier et al. (2010) also found that due to measurement error it was necessary to have a serum 25(OH) D level of ≥ 100 nmol/L (40 ng/mL). This is because the true value would then be more likely to be at least ≥ 80 nmol/L (32 ng/mL) which is the desired minimum value for *sufficiency*. This illustrates how the accuracy of a measurement is related to the precision. The National Institutes of Standards and Technology worked with the *National Institutes of Health's Office of Dietary Supplements* and developed a standard reference material for serum 25(OH) D

level analysis. The purpose of standard reference material is to achieve accuracy in assays. It can also be used to validate newly developed analytic methods (Phinney, 2008).

Serum Free 25(OH) D Assay, Vitamin D Binding Protein, and D Receptors

Serum free 25(OH) D levels were measured with a new assay that *directly* measures serum *free* 25(OH) D levels (Future Diagnostics B.V., Wijchen, The Netherlands). Serum free 25(OH) D is a more accurate measure of *bio-available* 25(OH) D (Mendel, 1989) and therefore, it may be a more accurate measure of overall D status. This is because only free 25(OH) D is available for conversion to the *biologically-active* D metabolite, $1\alpha, 25(OH)_2$ D (Holick, 2006).

The current method available for determining the free 25(OH) D level is a calculated *indirect* measure and is less accurate than the directly-measured assay (Schwartz et al., 2014). The assay is able to determine both the amount of serum free 25(OH) D and the serum DBP level. The serum DBP level affects the amount of serum 25(OH) D that is bound and is affected by unique physiological characteristics such as pregnancy and liver disease (Rosmalen, 2011; Schwartz et al., 2014) which must be considered in order to optimize the accuracy and precision of a laboratory test.

D is a molecule that is lipophilic and is similar to its precursor, cholesterol. To be soluble in plasma it requires DBP which is a protein-carrier (Jones, 2008). Approximately 88% of the 25(OH) D in serum is bound to DBP, approximately 11% is bound to albumin, and only approximately 0.03% is unbound (Bikle et al., 1986). Because DBP is the major plasma protein carrier of 25(OH) D, it highly influences the level of free 25(OH) D in the circulation. Less than 5% of the DBP binding sites are occupied by 25(OH) D. The availability of unoccupied binding sites may act as a buffer when the concentration of D metabolites increases, may protect against D intoxication, and can act as a circulating reservoir for D metabolites. DBP also functions to

activate macrophages, scavenge actin, transport fatty acids, and promote chemotaxis (Speeckaert, Guangming, Delanghe, & Taes, 2006).

DBP also functions as a circulatory system transporter and is involved with the uptake by target cells of the serum D metabolites (Bikle et al., 1986). Once 25(OH) D binds to DBP, the new complex then binds to *megalin* on the renal tubule cell membrane and is transported into the cell. Inside the cell 25(OH) D is released and then converted in the mitochondria by CYP27B1 to form 1α, $25(OH)_2$ D (Holick, 2006).

There are similarities and differences between 25(OH) D and 1α, $25(OH)_2$ D. They differ in their protein-binding affinity constants for both DBP and albumin. They also differ in their free fractions, total concentrations, and free concentrations. The DBP and albumin affinity constants for 25(OH) D are 10 to 20 times greater than they are for 1α, $25(OH)_2$ D. Conversely, the percentage of 25(OH) D and 1α, $25(OH)_2$ D bound to DBP is similar; 88% and 85%, respectively (Bikle et al., 1986).

Vitamin D is actually more correctly categorized as a pre-hormone and the active metabolite, 1α, $25(OH)_2$ D, is the ligand for the Vitamin D receptors (VDR) (Rosen et al, 2012). VDRs are located within most cells and tissues of the body and the calcium and phosphorus homeostasis is maintained by the interaction of 1α, $25(OH)_2$ D with the VDR in the small intestinal cells (Holick, 2006). The skin contains the greatest number of VDRs of any organ. The nuclear VDR is a mediator of 1α, $25(OH)_2$ D on target gene expression. CYP24A1 inactivates 1α, $25(OH)_2$ D by 24-hydroxylation which is expressed in the skin and is an example of both autocrine and paracrine actions of D (Rosen et al., 2012).

Hypovitaminosis D and HDL

Several studies have found positive associations between serum 25(OH) D and HDL-c levels (de Boer et al., 2009; Jorde et al., 2010; Guasch et al., 2012; Vacek et al., 2012; Deleskog et al., 2013).

Dyslipidemia

Dyslipidemia is defined as a lipoprotein metabolism disorder and includes over-production of LDL-c and deficiency of HDL-c. Cholesterol is the principal sterol synthesized by animals and is formed predominantly in the liver. It is a precursor to steroid hormones, bile acid, and D biosynthesis. It is an essential structural component of animal cell membranes and is required for the maintenance of proper cell permeability (Hanukoglu, 1992). Cholesterol is carried by circulating plasma lipoproteins including; HDL, LDL, very low-density lipoprotein, intermediate-density lipoprotein, and lipoprotein (a). The lipoprotein classes are heterogeneous and each contains two or more subclasses (Kulkarni, 2006).

Dyslipidemia was first identified as a primary risk factor for CVD in 1847 (Vogel, 1847). Currently, it is the most prevalent CVD risk factor and the most important known risk factor for MI in all regions of the world and for all age groups (Yusef et al., 2004). Serum HDL-c has a strong inverse independent association with both CVD and coronary heart disease (CHD) mortality for both genders, all ages, and risk strata (Cooney et al., 2009). Gemfibrozil administration in males with CHD, low serum HDL-c levels, and without elevated LDL-c levels, led to an increase in serum HDL-c level, no decrease in serum LDL-c level, and reduced the risk of non-fatal MI or death from CHD by 22%, (Rubins et al., 1999). HDL inhibits the inflammatory process in the endothelium, inhibits aggregation of platelets and coagulation, and inhibits oxidation of LDL. HDL promotes nitric oxide production in the endothelium and also

maintains the integrity of the endothelium through anti-apoptotic effects (Umaerus, Rosengren, Fagerberg, Hurt-Camejo, & Camejo, 2012).

But not all HDL-c is atheroprotective, there are pro-inflammatory effects due to reduced *cholesterol carrying capacity* or decreased *cholesterol efflux capacity* from macrophages which is associated with an increase in CAD risk (Khera et al., 2011). HDL can be sub-classified by *size* of HDL particles and *small* HDL particles (HDL_3) but not *large* HDL particles (HDL_2) have been found to predict CHD events. Additionally, the *particle number* of both LDL (LDL-p) and HDL (HDL-p) is significantly and independently associated with new CHD events and may be a more accurate indicator of CHD risk. The *amount of cholesterol per particle,* or total LDL-c and total HDL-c does not predict CHD as well due to variation in composition; particle number, size, or amount of cholesterol per particle or a combination of these characteristics (Otvos et al., 2006).

The screening rate for dyslipidemia has improved to an estimated 90% in the US and when it is treated there is a significant decrease in the CVD-related death rate (Farley et al., 2010). Knowing all of the causes or factors that contribute to dyslipidemia will improve treatment success and will further decrease the CVD-related death rate. Therefore, further research to determine additional causes of dyslipidemia needs to be a priority in order to provide more successful treatments.

HDL_2 which is the large buoyant subclass of HDL was chosen as the mediating variable between low 25(OH) D level and increased CVD risk because of the atheroprotective qualities reported in research studies (Umaerus et al., 2012). Isolating HDL_2 and excluding HDL_3, which is not thought to be atheroprotective, from total HDL-c will remove a possible source of confounding. Using serum free 25(OH) D assay levels versus serum total 25(OH) D assay levels

and HDL $_2$ versus total HDL-c for the association assessment may lead to more accurate conclusions.

High Density Lipoprotein Subclass Assay-Vertical Auto Profile II Method

The Vertical Auto Profile II (VAP II) test was used to measure serum cholesterol levels. It is a comprehensive, highly sensitive, direct test for measuring the lipoprotein cholesterol profile using ultracentrifugation. The lipoproteins are physically separated and react with an enzymatic cholesterol reagent. All of the lipoproteins are directly measured including LDL. Direct measurement without the use of calculations or assumptions decreases error and therefore increases the accuracy of the assay (Kulkarni, 2006). The report includes the cholesterol concentrations for the four major classes and subclasses of lipoproteins. Because it includes more information it identifies more patients at risk for CVD than the standard lipid panel.

Person, Environment, Health, and Nursing

The antecedents to low serum 25(OH) D levels arise from conditions within the person or the environment or both. A person may possess characteristics that are associated with low serum 25(OH) D; D malabsorption, genetic factors, older age, or intake of medications that decrease serum 25(OH) D levels. Penetration of the epidermis by UVB photons determines the efficiency of D synthesis in the skin (Holick, 2006) and the efficiency of the production of D by exposure to sunlight decreases as the amount of melanin in the skin increases (Bikle, 2010). High levels of skin melanin which absorb UVB photons decrease D production by approximately 90% (Holick, 2006). Medications which can alter D metabolism include Cholestyramine, Orlistat, fat-soluble vitamins (McDuffie et al., 2002; Compston & Horton, 1978) and Phenytoin and Phenobarbital (Gough et al., 1986).

There may be an insufficient amount of UVB radiation in the environment due to residing at a low elevation or high latitude. Also, there may be an insufficient exposure to UVB radiation due to topical sunscreen application which reduces the production of D by approximately 90%. An insufficient intake of dietary or supplemental D is also a source of serum 25(OH) D insufficiency or deficiency (Holick, 2006).

Low serum 25(OH) D may have a negative effect upon a person's health as a consequence of multiple pathophysiological processes. The proposed physiological conceptual framework pathway has been limited to the hypothesized effect of a decrease in HDL_2 (Figure 1). The decrease in HDL_2 then leads to an increased risk of CVD because HDL_2 has been found to be atheroprotective. If low serum 25(OH) D is confirmed to be a primary risk factor for CVD and then corrected, results from epidemiological studies suggest that a significant decrease in the mortality rate both in the United States (Grant, 2009) and worldwide (Grant, 2011) could result.

Meleis (2007) suggests that there must be an integration of a variety of paradigms in order to fully answer questions that arise from advanced practice nursing. She also suggests that the focus of nursing care will require the integration of the biomedical model of healthcare with a focus on the environment and the person. In addition, systems which are open will be responsive to and influenced by societal needs. Nursing practice involves the formation and implementation of interventions that arise from the collection of subjective and objective data. These interventions will alleviate or mitigate the perceived problem if the relationship between variables is correctly assessed as causal and not merely associative. This is why it is necessary to know if the relationship is causal or merely an association so that interventions are appropriate and timely (Schumacher & Gortner, 1992).

Determining Causality

Hill's Criteria for Causality in a Biological System

A causal association is implied by the stated hypothesis that low serum 25(OH) D levels lead to an increase in CVD. Physiological models are recursive; the pathways are unidirectional and the links are causal. Due to the complexity of physiological pathways, it may not be possible to know with certainty that there is a causal association. Serum 25(OH) D may be associated with increased CVD because of some other variable or there may be reverse causation. It may be appropriate instead to state that there is a *probability* of CVD occurring in the future following a decrease in serum 25(OH) D, given a particular set of conditions, based upon the past occurrence of this sequence of events.

One approach used to determine if a cause and effect association likely exists between variables is to apply Sir Austin Bradford Hill's *Criteria for Causality in a Biological System* (Hill, 1965). Hill proposed nine criteria to be used to assess aspects of an association to determine the likelihood that it is causal. Hill states the criteria are useful as rules of evidence that are necessary and should be observed before determining that a cause and effect association exists. He also states that the nine criteria are guidelines and should not be construed to be sufficient and indisputable evidence for a cause and effect association.

Randomized Controlled Trials

Inherent in the modern view of science and the current process of conducting research is a theory of matter known as *Mechanism.* The *Mechanistic World View* combines the concepts of *causation* and *explanation* with the idea that matter is composed of very small parts and physical contact is necessary for an interaction to occur. Physical phenomena are explained in terms of

mechanical interactions. In the mid 1600's Robert Boyle stated that experimentation is the only occasion during which hypotheses that are causal should be proposed (Godfrey-Smith, 2003).

A natural progression, following a long-standing acceptance of the Mechanistic World View, was the development of the *Logical Positivism* movement. Logical Positivism is an extreme form of *Empiricism* and began in Vienna during the early 1900s. The original founders were theoretical physicists, mathematicians, and logicians. The movement's focus was on the development and elevation of all disciplines to the level of *Science*. The Logical Positivists viewed *Science* as a unified body with an emphasis on each of the disciplines using the same research methods (Godfrey-Smith, 2003).

Their process required that data be collected by performing experiments with subsequent analysis and theory development. The Logical Positivists believed that introducing or removing particular interventions or conditions would lead to a predictable change; producing or avoiding a particular outcome. *Constants* in theories were *observable*, such as laboratory results, or were *theoretical*, and required formal organization using calculus and logic. The purpose of *Science* was to explain phenomena and to control and predict outcomes with the use of theories. Theory was the only way to organize knowledge and required that a particular framework be used (Rodgers, 2005). The randomized controlled trial design is a way to test a causal theory that is consistent with Logical Positivism. Randomly assigning participants to an active intervention or to a placebo increases the likelihood that the two groups will not have different outcomes due to any variable other than the experimental variable (Hulley, et al., 2007).

Dissertation Aims

The purpose of this manuscript is to present the findings of the literature review and the secondary analysis studies which I conducted along with members of my Dissertation

Committee. These studies were performed in order to partially satisfy the requirements for the degree of Doctor of Philosophy in Nursing in the Graduate Division of the University of California, San Francisco. The overall aim of this dissertation is to begin the process of the scientific investigation of Hypovitaminosis D as a causal risk factor for CVD. The primary aims and their associated hypotheses are;

Primary Aim:

To determine the likelihood of a causal association between Hypovitaminosis D and CVD using Hill's criteria for causality in a biological system.

Secondary Aims:

1) To determine if serum total 25(OH) D levels are associated with serum HDL-c, HDL_2, or HDL_3 levels.

H1: There is a statistically significant positive association between serum total 25(OH) D levels and serum HDL-c, HDL_2, or HDL_3 levels.

2) To determine if serum free 25(OH) D levels are associated with serum HDL-c, HDL_2, or HDL_3 levels.

H2: There is a statistically significant positive association between serum free 25(OH) D levels and serum HDL-c, HDL_2, or HDL_3 levels.

3) To determine if HDL-c, HDL_2, or HDL_3 levels will increase in participants supplemented with Vitamin D compared to participants who received a placebo.

H3: HDL-c, HDL_2, or HDL_3 levels will increase in participants supplemented with Vitamin D compared to participants who received a placebo.

4) To determine if HDL-c, HDL_2, or HDL_3 levels will increase in participants supplemented with Vitamin D and who reported using an HMG-CoA RI, compared to participants who received D supplementation but reported not using an HMG-CoA RI.

H4: HDL-c, HDL_2, or HDL_3 levels will increase in participants supplemented with Vitamin D and who reported using an HMG-CoA RI, compared to participants who received D supplementation but reported not using an HMG-CoA RI.

Presentation of Dissertation

This dissertation is presented in five chapters. In this chapter, the importance of decreasing the incidence of cardiovascular disease (CVD) in order to decrease mortality rates, the effect that Hypovitaminosis D may have on increasing CVD risk as well as the prevalence, causes, and treatment of Hypovitaminosis D are discussed. Dyslipidemia as a mediator between Hypovitaminosis D and increased CVD risk as well as the relevant lab assays for the studies are also discussed. The nursing framework for the physiological theory (Figure 1)and ways to assess causation are included also. The selection process for inclusion of serum sample data for the secondary analyses is also included (Figure 2). Chapter 2 is a systematic review of peer-reviewed journal articles from PubMed regarding the evidence for a causal association between D status and CVD risk. The manuscript was published in the journal; *Nutrients* 2014, *6*, 3403-3430; doi: 10.3390/nu6093403. Chapter 3 is a cross-sectional secondary analysis utilizing serum sample data to determine if there is an association between either total or free 25(OH) D levels and either serum HDL-c or HDL subclasses. Chapter 4 is also a secondary analysis of serum sample data from a randomized double-blinded placebo-controlled trial. The purpose of this analysis was to determine if there was an increase in serum HDL-c or HDL subclasses with vitamin D supplementation. Both of the secondary analyses will be submitted in the future for

publication to peer-reviewed journals. Chapter 5 presents the conclusions that were drawn from this work as well as the implications for clinical practice, health policy, and future directions.

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Figure 1. Physiological conceptual framework for the interrelationships among; person, environment, health, nursing, low serum 25-hydroxyvitamin D and the pathway to increased CVD.

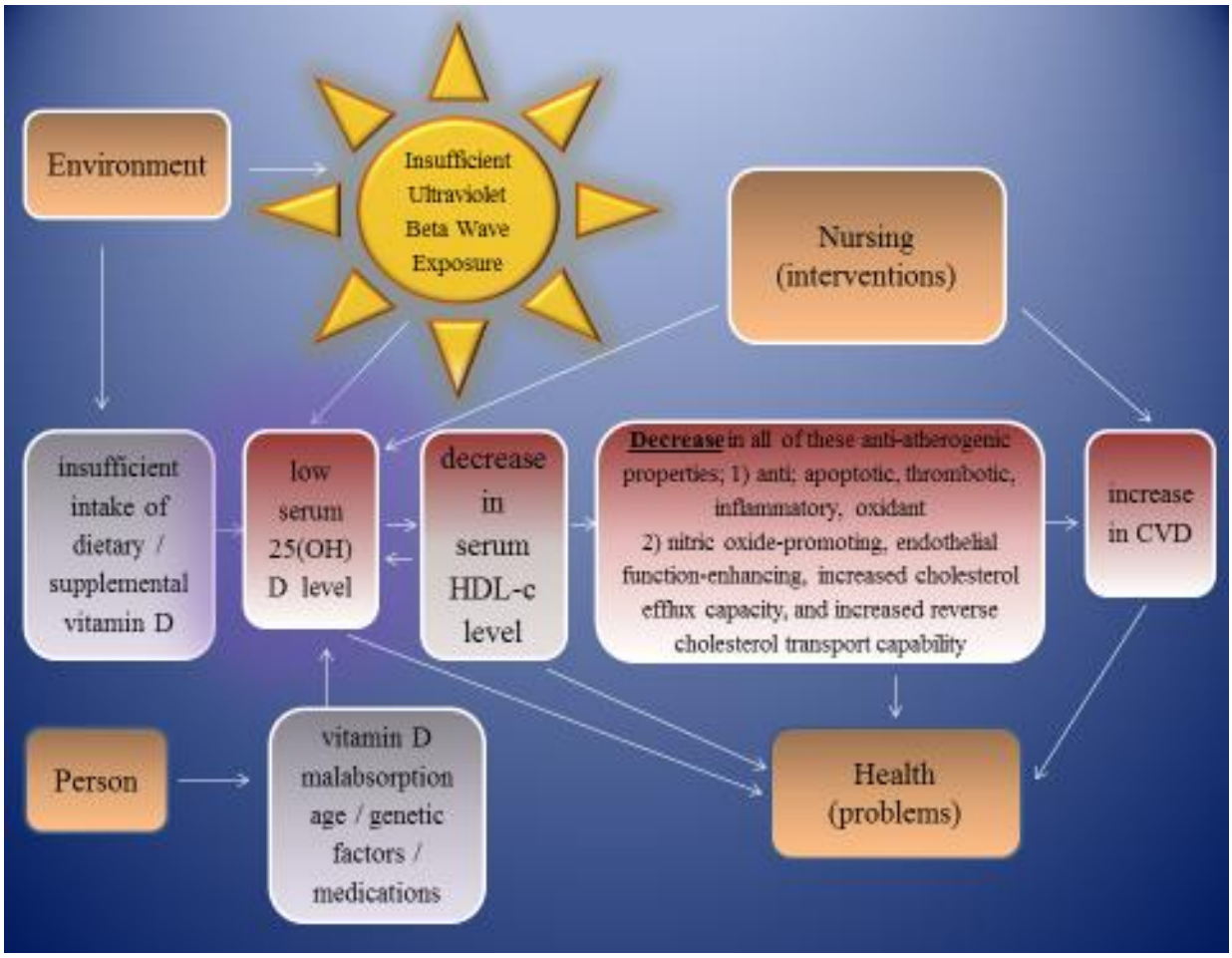
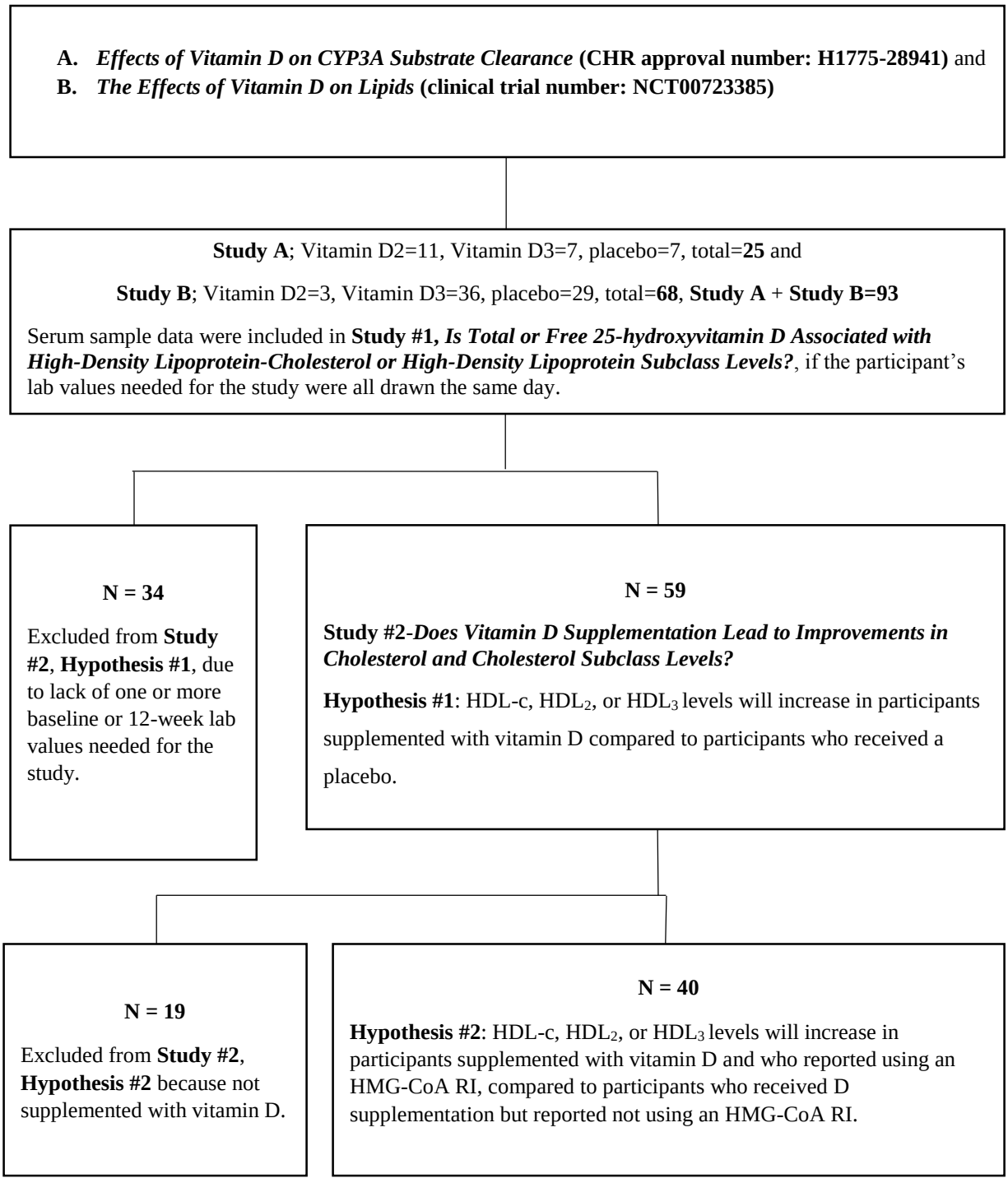


Figure 2. Selection process for inclusion of serum sample data for secondary analysis in Studies #1 and #2.



CYP3A = cytochrome-P450 enzyme family 3, sub-family A, HDL-c = high-density lipoprotein-cholesterol, HMG-CoA RI = 3-hydroxy-3-methyl-glutaryl-Co-enzyme A reductase inhibitor

Chapter 2

Does Sufficient Evidence Exist to Support a Causal Association between Vitamin D Status and Cardiovascular Disease Risk? An Assessment Using Hill's Criteria for Causality

Patricia G. Weyland, William B. Grant, and Jill Howie-Esquivel

University of California, San Francisco

Patricia G. Weyland, MS, RN, FNP-BC, CDE, PhD (c)
School of Nursing, Department of Physiological Nursing, UCSF
#2 Koret Way Box 0610
San Francisco, CA 94143
patricia.weyland@ucsf.edu

William B. Grant, Ph.D.
Sunlight, Nutrition, and Health Research Center
P.O. Box 641603
San Francisco, CA 94164-1603, USA
www.sunarc.org wbgrant@infionline.net

Jill Howie-Esquivel, RN, NP, PhD
School of Nursing, Department of Physiological Nursing, UCSF
#2 Koret Way Box 0610
San Francisco, CA 94143
jill.howie-esquivel@nursing.ucsf.edu

Abstract: Serum 25-hydroxyvitamin D (25(OH)D) levels have been found to be inversely associated with both prevalent and incident cardiovascular disease (CVD) risk factors; dyslipidemia, hypertension and diabetes mellitus. This review looks for evidence of a causal association between low 25(OH)D levels and increased CVD risk. We evaluated journal articles in light of Hill's criteria for causality in a biological system. The results of our assessment are as follows. Strength of association: many randomized controlled trials (RCTs), prospective and cross-sectional studies found statistically significant inverse associations between 25(OH)D levels and CVD risk factors. Consistency of observed association: most studies found statistically significant inverse associations between 25(OH)D levels and CVD risk factors in various populations, locations and circumstances. Temporality of association: many RCTs and prospective studies found statistically significant inverse associations between 25(OH)D levels and CVD risk factors. Biological gradient (dose-response curve): most studies assessing 25(OH)D levels and CVD risk found an inverse association exhibiting a linear biological gradient. Plausibility of biology: several plausible cellular-level causative mechanisms and biological pathways may lead from a low 25(OH)D level to increased risk for CVD with mediators, such as dyslipidemia, hypertension and diabetes mellitus. Experimental evidence: some well-designed RCTs found increased CVD risk factors with decreasing 25(OH)D levels. Analogy: the association between serum 25(OH)D levels and CVD risk is analogous to that between 25(OH)D levels and the risk of overall cancer, periodontal disease, multiple sclerosis and breast cancer. Conclusion: all relevant Hill criteria for a causal association in a biological system are satisfied to indicate a low 25(OH)D level as a CVD risk factor.

Keywords: association; cardiovascular disease; causation; Hill criteria; vitamin D

1. Introduction

Cardiovascular disease (CVD) is the leading cause of death in the United States and has been since the early 1900s [1]. CVD incidence peaked in the 1960s and then gradually declined over the next 50 years. From 1980 to 2000, the death rate for coronary heart disease (CHD) for men, adjusted for age, decreased from 543 to 267 per 100,000, and for women, the death rate decreased from 263 to 134 per 100,000. Almost half of the decline can be attributed to decreasing CVD risk factors, including hypertension (HTN), smoking and dyslipidemia [2]. The CVD death rate has now plateaued, but, alarmingly, may be increasing [1], reducing life expectancy for the first time [3]. To decrease CVD morbidity and mortality, we must identify and effectively treat all risk factors and their causes.

Robert Scragg [4] first hypothesized that increasing ultra-violet (UV)-related vitamin D status affords protection against CVD. The serum 25-hydroxyvitamin D (25(OH)D) level is the most widely used measurement to assess overall vitamin D status [5]. Serum 25(OH)D levels are inversely associated with several CVDs, including myocardial infarction (MI) [6,7], coronary artery disease (CAD), heart failure, atrial fibrillation, ventricular tachycardia [8], peripheral vascular disease (PVD) [8–11], stroke [8,12], incident coronary artery calcium (CAC) [13–16], cardiac valve and vascular calcification [17] and all CVDs [18].

Study findings have inversely associated risk factors for CVD with serum 25(OH)D levels, including lower serum high-density lipoprotein cholesterol (HDL-C) levels, higher serum triglyceride (TG) levels [15], diabetes mellitus (DM) [8,19], increased blood pressure (BP) [15,20–25], dysfunctional changes in the characteristics of plasma lipids [26–28], inflammation [29] and increased serum parathyroid hormone (PTH) levels [30].

Isolating primary risk factors that cause CVD is challenging, because the human body responds to disrupted homeostasis by up- and down-regulating cellular function. Multiple pathways may exist between a low serum 25(OH)D level and increased CVD risk. Some pathways may be direct and not include any intermediate factors, whereas others may be indirect and include an intermediate factor(s). Moreover, CVD is not a single diagnosis, but rather, according to the National Center for Health Statistics, a group of diagnoses, including CAD, heart failure, essential HTN, hypertensive renal disease, cardiac dysrhythmias, rheumatic heart disease, cardiomyopathy, pulmonary heart disease and cerebrovascular disease [31].

The level of sufficiency for serum 25(OH)D is still being debated. Two schools of thought exist regarding what constitutes a sufficient level: 20 ng/mL [32,33] and 30 ng/mL [34–37]. Approximately 32% of the U.S. population has a deficient serum 25(OH)D level (defined as <20 ng/mL) [38]. The worldwide prevalence of deficient serum 25(OH)D levels is approximately one billion [39]. The primary causes of low serum 25(OH)D levels are strict sun protection and inadequate dietary or supplemental vitamin D intake [40]. Levels are easily elevated by oral vitamin D supplementation [41]. A daily intake of 10,000–20,000 IU of cholecalciferol (vitamin D3) per day is unlikely to result in vitamin D toxicity [42]. Results from epidemiological studies suggest that if a low serum 25(OH)D level is a primary risk factor for CVD and then corrected, all-cause mortality could decrease significantly, both in the United States [43] and worldwide [44].

2. Approach and Rationale

The research studies used for this evaluation were located in the PubMed database by using the following search terms: Hill's criteria for causality, vitamin D, cardiovascular disease, randomized controlled trial, seasonality, hypertension, dyslipidemia, coronary artery calcium,

parathyroid hormone, inflammation, diabetes mellitus and high-density lipoprotein cholesterol. Studies were also sought in the references of the preceding studies. We evaluated studies for relevance to this assessment and being representative of current research. We included them regardless of whether they supported criteria for a causal association between serum 25(OH)D levels and CVD risk.

We evaluated the likelihood of a causal association between a low serum 25(OH)D level and increased risk for CVD by applying Sir Austin Bradford Hill's criteria for causality in a biological system [45] (see Table 1). Causality is multifaceted, and certain conditions must be met to determine that a causal association is likely. Hill stated that the criteria are useful, as we most often depend on observed events to detect relationships between sickness and its antecedents. Waiting to take action until research results explain the entire chain of events that lead to disease may not be necessary when discovering a few links in the chain may suffice.

The criteria relevant to this evaluation include all, except specificity and coherence. This evaluation does not include specificity, because evidence supports low serum 25(OH)D levels and increased risk of several other disease processes [43]. This evaluation does not include coherence, because of its similarity to plausibility (see Table 2), and the information would be redundant. Hill's criteria have been used to assess a causal association between serum 25(OH)D levels and cancer risk [46], periodontal disease [37], multiple sclerosis (MS) [47], breast cancer risk [48] and the most prevalent cancers [49].

To arrive at the most accurate conclusions and to intervene with the most effective treatments, a thorough understanding of causality and of the limitations inherent in how we determine whether a causal association exists is essential. No single type of study, including randomized controlled trials (RCTs), can evaluate each of Hill's criteria. This evaluation used

Hill's criteria, because it can consider the results of RCTs, prospective, cross-sectional and epidemiological studies.

3. Findings: Evaluation Using Hill's Criteria for Causality

The studies included in this criteria section and all of the studies in the subsequent criteria sections are ordered by design; first are the meta-analyses, then prospective, retrospective, cross-sectional, case-control and lastly ecological studies. They are then ordered from the highest to the lowest relative risk ratio (RR), hazards ratio (HR) or odds ratio (OR) when available.

3.1. Strength of the Association

The stronger the positive or negative association between two variables, the more likely the association is causal. However, this may not always be true. One must consider all that is known about the two variables before concluding that an association is causal. For example, a very strong association may exist between an exposure and a disease, but another unknown variable may mediate the two. Alternatively, an exposure may directly cause a disease, but only under certain, sometimes very limited, circumstances; therefore, the association between the exposure and the disease would be weak. Therefore, a strong association is neither necessary nor sufficient to determine the likelihood of a causal association.

Satisfying the strength of association criterion requires a thorough evaluation of the correlation between vitamin D status and CVD risk. To come as close as possible to determining the true strength of an association, one must determine and then consistently use the most accurate and precise measures of the exposure and the disease [102]. Most researchers agree that the serum 25(OH)D level is the most accurate measure of overall vitamin D status. Several investigators have found statistically significant associations between serum 25(OH)D levels and CVD risk factors or CVDs.

Correia and colleagues [51] performed a prospective study in which they examined the association between serum 25(OH)D levels and the incidence of CVD-related mortalities during hospitalization. Ten percent of their 206 participants were severely deficient, defined as serum 25(OH)D levels ≤10 ng/mL. Incident CVD-related mortality was much higher at 24% for the group of patients with severe serum 25(OH)D deficiency *versus* 4.9% in the group of patients with levels >10 ng/mL (RR 4.3, 95% CI, 1.8, 10, $p = 0.001$). These results are impressive, but the authors acknowledge that the CIs were very wide. Anderson and colleagues [8] completed a study with both cross-sectional and prospective data, which offered support for an association between serum 25(OH)D levels and CVD risk. The researchers examined 41,504 electronic health records and concluded from the cross-sectional data that there is an inverse association between prevalence of CVD risk factors and serum 25(OH)D levels. A significant increase in the prevalence of HTN (30% relative increase RI), DM (90% RI), PVD (53% RI) and hyperlipidemia (9% RI) was present in the group with serum 25(OH)D levels ≤15 ng/mL compared with the group with levels >30 ng/mL ($p < 0.0001$ for all, significant after Bonferroni correction for multiple comparisons). The authors acknowledge that selection bias may have been present, because only individuals who had serum 25(OH)D levels in their record were included in the study.

Researchers outside North America have also found inverse associations between serum 25(OH)D levels and risk factors for CVD, although sun exposure and diet may differ. Jang and colleagues [50] performed a cross-sectional study with 320 Korean girls whose average age was 13 years, 63.8% of whom had serum 25(OH)D levels <20 ng/mL. After adjusting for physical activity and BMI *Z*-score, the researchers found that serum 25(OH)D levels were negatively

associated with fasting blood glucose levels ($r = -0.1748$, $p = 0.0033$) and insulin resistance ($r = -0.1441$, $p = 0.0154$), both risk factors for metabolic disorders.

The 2013 study by Deleskog and colleagues [53] had mixed results. The researchers performed a cross-sectional study with 3430 participants, 8% of whom had deficient serum 25(OH)D levels defined as <51 nmol/L (<20 ng/mL), 82% had insufficient levels defined as 51–75 nmol/L (20–30 ng/mL) and 10% had sufficient levels defined as >75 nmol/L (>30 ng/mL). No independent association emerged between serum 25(OH)D level insufficiency and carotid intima media thickness. However, those with deficient levels were more likely to have CVD risk factors, including higher BP, blood glucose, TG levels and lower serum HDL-C levels. Additionally, they were more likely to have DM. Sun and colleagues [12] performed a case-control study in which they examined the association between ischemic stroke risk and serum 25(OH)D levels in 464 females with ischemic stroke and 464 female matched controls. The researchers compared participants in the lowest *versus* highest tertiles of serum 25(OH)D levels after adjusting for dietary and lifestyle covariates. Lower serum 25(OH)D levels were associated with an increased risk for ischemic stroke (OR 1.49, 95% CI, 1.01, 2.18, $p < 0.04$).

Scragg and colleagues [6] were one of the first research teams to examine the association between serum 25(OH)D levels and CVD. The researchers performed a case-control study with 179 MI cases with controls matched for age, sex and date of blood collection. They found an RR for MI of 0.43 (95% CI, 0.27, 0.69) for participants with serum 25(OH)D levels at or above their study median value of 32 nmol/L (12.8 ng/mL) *versus* below the median.

Deleskog and colleagues [52] included 774 participants in a case-control study to evaluate the association between serum 25(OH)D levels and premature MI (younger than 60

years). Serum 25(OH)D levels were analyzed twice as a categorical variable; insufficiency was defined as <50 nmol/L (20 ng/mL) and was compared with levels ≥50 nmol/L; a separate analysis defined insufficiency as <75 nmol/L (30 ng/mL), which was compared with levels ≥75 nmol/L. Neither of the definitions of serum 25(OH)D level insufficiency were independently associated with premature MI. Therefore, the results do not support the criterion. The researchers concluded that the serum 25(OH)D level insufficiency may promote risk factors that are already established and known to promote atherothrombosis.

The criterion strength of the association has thus been met for 25(OH)D levels and CVD or CVD risk factors, including MI, CVD-related mortality, ischemic stroke risk, HTN, DM, PVD, hyperlipidemia, elevated blood glucose and increased insulin resistance.

3.2. Consistency of the Association

An association is consistent if it is observed under different circumstances, at different times, in various places and by various researchers [45]. Consistency is also confirmed if the results of a study can be replicated with a different sample of participants with the same study design and analytic methods. Inconsistent study results may occur when differences exist in study design, lab assays, definitions of serum 25(OH)D level deficiency, insufficiency *versus* sufficiency and statistical methods. Confidence in the results of meta-analyses depends on an assessment of the comparability of all studies included in the analysis [103].

Parker and colleagues [54] carried out the study with the strongest support for the criterion of consistency. In their meta-analysis, they systematically reviewed 28 studies with a total of 99,745 participants. The researchers reported important variations among studies included in their review, including categories of serum 25(OH)D levels, study design and analyses. Despite these differences, 29 of 33 ORs from the 28 studies showed an inverse

association between serum 25(OH)D levels and the prevalence of cardio-metabolic disorders. One study demonstrated no effect, and three studies showed a positive association. Parker and colleagues [54] found a 43% reduction in cardio-metabolic disorders with the highest levels of serum 25(OH)D (OR 0.57, 95% CI, 0.48, 0.68).

The meta-analysis by Wang and colleagues [55] offers additional strong support. They included 19 prospective studies with a total of 65,994 participants, of whom 6123 developed CVD. The 19 studies included CVD, CVD mortality, CHD and stroke as outcomes. Wang and colleagues found an inverse linear association between serum 25(OH)D in the range 20–60 nmol/L (8–24 ng/mL) and the risk of CVD (RR, 1.03, 95% CI, 1.00, 1.06).

Giovannucci and colleagues [7] found results consistent with the previous studies. This prospective, nested, case-control study included 454 male participants who were CHD cases and 900 male controls matched for age, HTN, aspirin use, physical activity, serum TG and low-density lipoprotein cholesterol (LDL-C) levels, as well as alcohol use. The median values for each of the four categories of serum 25(OH)D levels were entered as continuous variables in a regression model. The researchers found a two-fold increase in risk for MI if the serum 25(OH)D level was less than 16 ng/mL compared with those with a level of at least 30 ng/mL (RR, 2.42, 95% CI, 1.53, 3.84; $p < 0.001$). They also found a 2.1% decreased risk of MI for every 1 ng/mL increase in serum 25(OH)D levels. Only including males in the study prevents the generalizability of the results to females.

Support for the consistency criterion is also evident in the prospective study by de Boer and colleagues [15] ($N = 1370$). At baseline, 723 (53%) had CAC. Over a three-year period, 135 participants developed CAC. The researchers adjusted for gender, age, ethnicity/race, location, season, activity level, smoking status, body mass index (BMI), DM, BP and serum lipid and C-

reactive protein (CRP) levels. They found that serum 25(OH)D levels were inversely associated with incident, but not prevalent, CAC; for every 10 ng/mL decrease in the serum 25(OH)D level, the risk of developing CAC increased by 23% (RR, 1.23, 95% CI, 1.00, 1.52, $p = 0.049$).

Finally, a cross-sectional study by Kendrick and colleagues [56] found similar supporting results by using data from 16,603 participants of the Third National Health and Nutrition Examination Survey (NHANES III). Serum 25(OH)D level deficiency, defined as <20 ng/mL, was associated with a 57% increased odds for prevalent CVD. After adjusting for gender, age, ethnicity/race, season, activity level, smoking status, HTN, DM, BMI, dyslipidemia, chronic kidney disease and vitamin D use, the odds decreased to 20% (OR, 1.20, 95% CI, 1.01, 1.36, $p = 0.03$).

A study-participant characteristic that should be included in the evaluation of the consistency criterion is ethnicity. A prospective study by Michos and colleagues [82] found that serum 25(OH)D levels less than 15 ng/mL were not associated with fatal stroke in blacks, but were associated with fatal stroke in whites. One limitation of this study is that because the median time to fatal stroke was 14.1 years and the serum 25(OH)D levels were only drawn once at baseline, there could have been undetected significant changes in serum 25(OH)D levels during the study. Differences in CHD events, including angina, MI, cardiac arrest or CHD death, by ethnicity were found in a prospective study by Robinson-Cohen and colleagues [66]. The researchers found an association between lower serum 25(OH)D levels and incident CHD events for white or Chinese, but not black or Hispanic participants. The same limitation is present in this study; only a baseline serum 25(OH)D level was drawn, and there was a median follow-up period of 8.5 years.

An unexplained difference by ethnicity was found by Gupta and colleagues [104], who performed a cross-sectional study. The researchers found significant associations between both pre-diabetes and pre-hypertension and gender, age and BMI in Mexican-Americans. However, they did not find an association between either pre-diabetes or pre-hypertension and serum 25(OH)D levels, as has been found for both non-Hispanic whites and non-Hispanic blacks. The authors stated that the reason for these results was unclear.

Results from a study performed by Rezai and colleagues [14] found that there was an association between serum 25(OH)D levels and left ventricular end-diastolic volumes for men of all ethnicities. The results of this cross-sectional study add support to the criterion of consistency, because low 25(OH)D levels showed the same association with poorer CV status for all ethnicities. This may mean that disparities in the prevalence of low vitamin D status among ethnicities may cause the disparities among ethnicities in the prevalence of CVD. Webb and colleagues [12] found that the pulse wave velocity (PWV) was higher in British South Asians of Indian descent than in white Europeans (9.32 m/s *vs.* 8.68 m/s, $p = 0.001$) using a cross-sectional design. They also found that the serum 25(OH)D level was independently associated with PWV, when adjusted for age, mean arterial pressure, sex, glucose, heart rate, vasoactive medications and South Asian ethnicity ($R2 = 0.73$, $p = 0.004$). The researchers concluded that vitamin D insufficiency may mediate an increase in aortic stiffness without a difference in the risk profile, including vascular disease.

The preceding studies have shown mixed results, and the reasons for the differences are multifaceted. One reason for the disparity in serum 25(OH)D levels among different ethnic groups is that vitamin D production is inversely proportional to skin pigmentation [105]. Skin pigmentation varies among members of the same ethnic group, and designing a study in which

skin pigmentation is objectively quantified and included as a variable may help to clarify differences between individuals *versus* groups. Studies that use ethnicity self-reporting or that have the investigator determine the ethnicity of the participants can also decrease the validity of the findings.

The consistency of the association criterion has thus been met due to the research results regarding the systematic review by Parker and colleagues and the smaller described supporting studies. The studies regarding ethnicity have mixed results. Parker and colleagues in their meta-analysis found overall associations between serum 25(OH)D levels and MI, stroke, ischemic heart disease, PVD, DM and metabolic syndrome.

3.3. Temporality

Temporality refers to the direction of influence in a sequence of events. An event or phenomenon cannot cause another event or phenomenon if the presumed cause does not precede the presumed effect. Determining whether a potential risk factor precedes a disease process is particularly difficult when the disease is chronic and progresses slowly [45]. Determining the temporal direction of influence of low serum 25(OH)D levels in relation to CVD risk by examining the results of prospective studies or meta-analyses that have included only prospective studies will help determine if the criterion of temporality has been met.

DM is a well-established risk factor for CVD, and the association between serum 25(OH)D levels and DM has been prospectively studied. Song and colleagues [57] included 21 prospective studies with 76,220 participants, 4996 incident type 2 DM cases and serum 25(OH)D in a meta-analysis. The researchers compared the highest to lowest serum 25(OH)D levels using categories and found that the summary RR for type 2 DM was 0.62 (95% CI, 0.54, 0.70). The statistical significance of the inverse association between DM risk and serum 25(OH)D levels

remained after controlling for sex, criteria for DM diagnosis, follow-up time, sample size and 25(OH)D assay type. Each 10 nmol/mL (4 ng/mL) increase in the serum 25(OH)D level was associated with a 4% lower risk of type 2 DM (95% CI, 3, 6; *p* linear trend = 0.0001). Therefore, low 25(OH)D levels may be a risk factor for CVD with type 2 DM as the mediator.

Wang and colleagues [55] examined the association between CVD mortality along with CVD risk and serum 25(OH)D levels in a meta-analysis of 19 prospective studies. Collectively, these studies had 65,994 participants, of whom 6123 developed CVD. The researchers used the median serum 25(OH)D levels, or if unavailable, they compared the mean or the midpoint of the upper and lower bounds in each of the 25(OH)D categories from each of the 19 studies to the category of the risk of CVD. Being in the lowest category was associated with a higher risk for all CVDs (pooled RR 1.52, 95% CI, 1.30, 1.77), for CVD mortality (pooled RR 1.42, 95% CI, 1.19, 1.71), for CHD (pooled RR 1.38, 95% CI, 1.21, 1.57) and for stroke (pooled RR 1.64, 95% CI, 1.27, 2.10) than the highest category.

Although the study by Anderson and colleagues [8] was included in both the Song and colleagues and Wang and colleagues meta-analyses, it is a landmark study, and the results are important to cite. This study offers strong support for temporality. The prospective study using electronic health records monitored participants for an average of 1.3 years and a maximum of 9.3 years. The prevalence of serum 25(OH)D levels ≤30 ng/mL was 63.6%. Participants without risk factors for CVD with serum 25(OH)D levels ≤15 ng/mL had a higher risk of incident HTN, dyslipidemia and DM than those with levels >30 ng/mL. Adjusted relative rates for death increased by 20% for serum 25(OH)D levels of 16–30 ng/mL and increased by 77% for serum 25(OH)D levels ≤15 ng/mL. The researchers concluded that these data provide support for low serum 25(OH)D level as a primary risk factor for CVD. Schöttker and colleagues [18], in a

prospective study with 9578 participants, found an increased risk of cardiovascular mortality associated with decreased serum 25(OH)D levels (hazards ratio (HR) 1.39, 95% CI, 1.02, 1.89).

Tsur and colleagues [58] conducted a prospective cohort study over a two-year period that assessed incident impaired fasting glucose (IFG) and DM type 2 in 117,960 participants. The researchers adjusted for several variables, including sex, age, BMI, serum LDL-C, HDL-C, TG levels, history of HTN, smoking status and CVD. Participants with a serum 25(OH)D level ≤25 nmol/L (10 ng/mL) had an OR for progression from normoglycemia to IFG of 1.13 (95% CI, 1.03, 1.24), from normoglycemia to DM of 1.77 (95% CI, 1.11, 2.83) and from IFG to DM of 1.43 (95% CI, 1.16, 1.76), compared with a serum 25(OH)D level >75 nmol/L (30 ng/mL). The researchers concluded that a low serum 25(OH)D level may be an independent risk factor for IFG and DM that can eventually lead to CVD.

The previously described meta-analyses of prospective studies and the additional prospective studies offer evidence that temporality is satisfied, because they all use serum 25(OH)D levels taken at the time of enrollment, which precedes the incident event or death. Furthermore, most reviewed individual prospective studies, and a meta-analysis of prospective studies showed an increased incidence of CVD or CVD risk factors with decreasing serum 25(OH)D. The CVDs or risk factors for CVD included CVD mortality, CHD, stroke, dyslipidemia, HTN, type 2 DM and IFG.

3.4. Biological Gradient (Dose-Response Relation)

In the context of this assessment, the biological gradient, or dose-response relation, refers to the change in the prevalence or incidence rate of CVD or risk factors for CVD as serum 25(OH)D levels change. The biological gradient criterion is satisfied when the value of the dependent variable (effect) can be predicted, with some degree of confidence, when the value of

the independent variable (cause) is known. Hill [45] states that securing a satisfactory quantitative measure to use for this purpose is often difficult.

Wang and colleagues [55] showed a biological gradient effect in their 2012 meta-analysis. They found a linear (graded) and inverse association between serum 25(OH)D levels of 20–60 nmol/L (8–24 ng/mL) and the risk of CVD. They found a linear trend for the RR = 1.03 (95% CI, 1.00, 1.06) for every 25 nmol/L (10 ng/mL) decrease in 25(OH)D ([55]; Figure 3). Wang and colleagues had similar results in an earlier study [60]. They examined low serum 25(OH)D levels and incident CVD prospectively in 1739 participants from the Framingham Offspring Study. A serum 25(OH)D level <15 ng/mL was associated with a two-fold increase in an age and sex-adjusted five-year incident rate for CVD compared with those with a level of ≥15 ng/mL (multivariable-adjusted HR = 1.62, 95% CI, 1.11, 2.36; $p = 0.01$). The researchers also found a graded increase in CVD risk for serum 25(OH)D levels of 10–14 ng/mL (multivariable-adjusted HR = 1.53, 95% CI, 1.00, 2.36; $p = 0.01$) *versus* levels <10 ng/mL (multivariable-adjusted HR = 1.80, 95% CI, 1.05, 3.08; $p = 0.01$).

Anderson and colleagues [8] performed a prospective study, which was included in the Wang and colleagues meta-analysis. The researchers found statistically significant and biologically-graded inverse associations between serum 25(OH)D levels and the prevalence of CVD and CVD risk factors, including PVD, HTN, DM and hyperlipidemia (all $p < 0.0001$). The researchers categorized serum 25(OH)D levels; levels of serum 25(OH)D ≤15 ng/mL *versus* those >30 ng/mL were associated with increased prevalence of DM (90% relative and 14% absolute) and HTN (30% relative and 12% absolute) (p trend for both <0.0001).

Vacek and colleagues [59] performed a retrospective study ($n = 10,899$) for a 68-month period. Using univariate analysis, the researchers found statistically significant ORs for vitamin

D deficiency, defined as <30 ng/mL, and CAD (OR, 1.16, 95% CI, 1.012, 1.334, $p = 0.03$), cardiomyopathy (OR, 1.29, 95% CI, 1.019, 1.633, $p = 0.03$) and HTN (OR, 1.40, 95% CI, 1.285, 1.536, $p \leq 0.0001$).

The criterion, biological gradient, or dose-response curve, has thus been met. Most reviewed studies used serum 25(OH)D levels as categorical or continuous variables and found strong evidence for a graded association between levels and CVD/CVD risk factors, including nonspecific CVD, PVD, HTN, DM, hyperlipidemia, elevated BMI, elevated serum LDL-C and TG levels and decreased serum HDL-C levels.

3.5. Plausibility

Biological plausibility can be confirmed when the suspected causation mechanism is consistent with the current knowledge of biology. The actual physiological pathway of the hypothesized causal association between low serum 25(OH)D levels and increased risk for CVD may include mediators that are known CVD risk factors or other unknown factors. Specific cellular-level causative mechanisms that explain the increase in CVD associated with low vitamin D status need to be identified in order to definitively state that the criterion, biological plausibility, has been met.

Several cellular-level causative mechanisms have been proposed. It should be taken into consideration that, in contrast to the causative agent of an infectious disease, these proposed causative mechanisms do not necessarily compete with one another and are not mutually exclusive. Some or all of the proposed mechanisms may be accurate. This is because CVD is a broad category of diseases, and each of the diseases has multiple causes.

An *in vitro* study by Oh and colleagues [17] found an inhibition of foam cell formation when macrophages from persons with type 2 DM exposed to modified LDL were cultured in the

bio-active form of vitamin D; 1α,25-dihydroxyvitamin D3 (1α,25(OH)2D3). They also found accelerated foam cell formation when the vitamin D receptors (VDRs) were deleted from the macrophages. A reduction in the formation of atherosclerotic lesions in mice with the administration of the vitamin D analog, calcitriol (1α,25(OH)2D3), was seen by Takeda and colleagues [32]. They hypothesize that calcitriol modulates the systemic and intestinal immune systems by inducing immunologically-tolerant dendritic cells and T-cells, both of which are anti-atherogenic.

Additionally, an *in vitro* study by Riek and colleagues [106] was performed in order to determine if vitamin D plays a role in monocyte migration and adhesion. The researchers examined monocytes from study participants ($n = 12$) with type 2 DM and obesity who were vitamin D deficient. The researchers found a 20% reduction in monocyte migration in monocytes incubated with 25(OH)D3 compared to vitamin D-deficient conditions ($p < 0.005$). They also found that, compared to monocytes maintained in vitamin D-deficient conditions, incubation with 25(OH)D3 also significantly decreased adhesion ($p < 0.05$). The researchers concluded that hydroxylation of 25(OH)D3 to 1,25(OH)2D3 at the cellular level may play a role in vitamin D anti-atherogenic effects.

VDRs were also found in human coronary artery smooth muscle cells (CASMC) by Wu-Wong and colleagues [107]. When CASMC were treated with the vitamin D analogs, calcitriol or paricalcitol (19-nor-1α,25(OH)2D2) there was an upregulation of 24-hydroxylase and also an upregulation of thrombomodulin (TM) mRNA. Downregulation of TM mRNA has been associated with atherosclerosis and thrombosis. Finding that upregulation occurred led the researchers to hypothesize that this is the mechanism that leads to a decrease in morbidity and mortality with vitamin D analog use in persons with chronic kidney disease.

Many studies have shown inverse associations between established CVD risk factors, such as dyslipidemia, HTN and DM [108] and serum 25(OH)D levels (see Table 2). The following research studies further assist in evaluating the plausibility of a causal association.

3.5.1. Dyslipidemia

A proposed causal mechanism for the association between low serum 25(OH)D levels and increased risk for CVD involves dysfunctional changes in the characteristics of plasma lipids, including metabolism or transport [26], the ability to promote macrophage efflux, [27] and changes in serum levels of total cholesterol (total-C), HDL-C, LDL-C and TGs [15,28].

Skaaby and colleagues [70] investigated the association between serum 25(OH)D levels at baseline and incident dyslipidemia over five years in a prospective study with 4330 participants. A serum 25(OH)D level of 10 nmol/L (4 ng/mL) higher at baseline was associated with decreased serum TG levels (β = −0.52, 95% CI, −0.99, −0.05, $p = 0.03$) and decreased serum very-low-density lipoprotein cholesterol (VLDL-C) levels (β = −0.66, 95% CI, −1.1, −0.2, $p = 0.005$). With the same higher serum 25(OH)D level at baseline, the OR for incident hypercholesterolemia was 0.94 (95% CI, 0.90, 0.99, $p = 0.01$). The researchers concluded that higher serum 25(OH)D levels may favorably change lipid profiles and therefore positively influence cardiovascular health.

Karhapää and colleagues [109] performed a cross-sectional study in which they examined the relationship between serum 25(OH)D levels and total-C, LDL-C, HDL-C and TG levels in a study that included 909 male participants. The researchers found a significant inverse association between serum 25(OH)D levels and total-C, LDL-C and TG levels (β = −0.15, −0.13 and −0.17, respectively; $p < 0.001$), which supports lower serum 25(OH)D levels leading to a less favorable lipid profile. However, they found no association between serum 25(OH)D and HDL-C levels,

which does not support an association between lower serum 25(OH)D levels and a more favorable lipid profile.

Jorde and colleagues [28] also examined the association between serum 25(OH)D levels and serum lipid levels by using both cross-sectional and longitudinal data collected over 14 years. The cross-sectional study included 10,105 participants, and the researchers found that with increasing quartiles of serum 25(OH)D levels, serum HDL-C and LDL-C levels increased and serum TG levels decreased. In the longitudinal study with 2159 participants, the researchers found that increasing quartiles of serum 25(OH)D levels were associated with decreased serum TG levels. These results, except for the increase in serum LDL-C levels, support associating higher serum 25(OH)D levels with a more favorable lipid profile.

Researchers have also conducted genomic and cytochrome P450 enzyme studies to determine mechanisms that cause low serum 25(OH)D levels to lead to dysfunctional changes in lipids. Shirts and colleagues [69], in a cross-sectional study with 1060 participants, investigated the influence of single-nucleotide polymorphisms on serum HDL-C, LDL-C and TG levels for gene-25(OH)D interactions. Participants with deficient levels of serum 25(OH)D were more likely to also have lower serum HDL-C levels ($p = 0.0003$). Chow and colleagues [110] incubated human hepatocytes with 1,25(OH)2D3 and found a reduction in cholesterol production due to an increase in cytochrome P450 enzyme 7A1 activation of the VDR.

Guasch and colleagues [29] found an association between low plasma 25(OH)D levels and atherogenic dyslipidemia after adjusting for BMI in a cross-sectional study with 316 participants. When the researchers introduced serum-ultrasensitive CRP levels as a covariable, an association was no longer present. They suggested that inflammation may mediate the effect of serum 25(OH)D levels on lipid profiles.

3.5.2. Hypertension

The cause of HTN is usually unknown. Researchers have investigated the association between serum 25(OH)D levels and both prevalent and incident idiopathic HTN and pre-HTN [20,22–25]. Carrara and colleagues [61] conducted a prospective interventional trial in which they administered 25,000 IU of oral cholecalciferol (vitamin D3) weekly over two months to 15 participants with essential HTN. There was neither randomization to different interventions or a placebo group. Because the researchers found reduced aldosterone ($p < 0.05$) and renin plasma levels ($p < 0.05$) after supplementation, they concluded that for persons with essential HTN and a low serum 25(OH)D level, vitamin D supplementation may help decrease BP.

Forman and colleagues [21] performed a prospective study with 1811 participants with measured plasma 25(OH)D levels. The researchers found that incident HTN was greater for participants with a plasma 25(OH)D level of <15 ng/mL compared to those with a level ≥30 ng/mL (RR 3.18, 95% CI, 1.39, 7.29). For men only ($n = 613$), the RR for the same comparison was much greater (RR 6.13, 95% CI, 1.0, 37.8) compared to women only ($n = 1198$) (RR 2.67, 95% CI, 1.05, 6.79). Forman and colleagues [62] also conducted a prospective study that included only women participants aged 32–52 years. The researchers found that incident HTN increased for the lowest quartile (6.2–21.0 ng/mL) *versus* the highest quartile (32.3–89.5 ng/mL) for 25(OH)D levels (OR, 1.66, 95% CI, 1.11, 2.48, $p = 0.01$).

Increased arterial stiffness may be an effect of low serum 25(OH)D level. Giallauria and colleagues [63], in a cross-sectional study with 1228 participants, found a statistically significant inverse association between serum 25(OH)D levels and arterial stiffness, measured with PWV (adjusted $R2 = 0.27$, $\beta = -0.43$; $p = 0.001$). Furthermore, measuring PWV, Mayer and colleagues [64] performed a cross-sectional study and found a negative association with serum 25(OH)D

level quartiles. The lowest serum 25(OH)D level quartile (<20 ng/mL) had the highest PWV score compared with the second, third or fourth quartile ($p = 0.0001$).

Three studies with only female participants had similar results. Pirro and colleagues conducted a cross-sectional study with 150 postmenopausal and serum 25(OH)D-insufficient (<30 ng/mL) participants [65]. The researchers found a significant association between arterial stiffness, measured with PWV and serum 25(OH)D levels, but not after controlling for logarithmically-transformed serum PTH levels. Serum PTH levels were associated with arterial stiffness ($\beta = 0.23$, $p = 0.007$). Reynolds and colleagues [66] in a cross-sectional study found a similar association between serum 25(OH)D levels and aortic stiffness (PWV scores) ($\beta = -0.0217$, 95% CI, −0.038, −0.005, $p = 0.010$) for 75 female participants with systemic lupus erythematosus. The authors did not state that serum PTH levels were measured and controlled for, and therefore, PTH levels may have mediated the association.

3.5.3. Diabetes Mellitus

DM is an important risk factor for CVD. Several studies have associated serum 25(OH)D levels and both prevalent and incident DM. Afzal and colleagues [19], in a prospective study with 9841 white participants, found an increased risk of type 2 DM for study participants with plasma 25(OH)D levels <5 ng/mL *versus* ≥20 ng/mL (HR, 1.22, 95% CI, 0.85, 1.74). The researchers also performed a meta-analysis of 13 studies and found a greater prevalence of type 2 DM for those in the lowest *versus* highest quartile for the serum 25(OH)D level (cut-points for the quartiles varied among the 13 studies) (OR, 1.39, 95% CI, 1.21, 1.58). Anderson and colleagues [8], found an adjusted RI in incident DM of 89% for very low (≤15 ng/mL) *versus* sufficient (>30 ng/mL) categories of serum 25(OH)D levels (HR, 1.89, 95% CI, 1.54, 2.33, $p < 0.0001$). Forouhi and colleagues [67] found in a prospective study with 524 participants that

baseline 25(OH)D levels were inversely associated with the 10-year risk of hyperglycemia (fasting glucose: $\beta = -0.002$, $p = 0.02$) and insulin resistance (fasting insulin $\beta = -0.15$, $p = 0.01$).

3.5.4. Metabolic Syndrome

Studies have been conducted to assess the association between both incident and prevalent metabolic syndrome and serum 25(OH)D levels. A prospective study by Gagnon and colleagues [74] found that 12.7% of 4164 participants developed metabolic syndrome over a five-year follow-up period. A higher risk of metabolic syndrome was present for those with serum 25(OH)D levels in the first quintile (<18 ng/mL) (OR = 1.41, 95% CI, 1.02, 1.95) and second quintile (18–23 ng/mL) (OR = 1.74, 95% CI, 1.28, 2.37) compared with the highest quintile (≥34 ng/mL). Serum 25(OH)D levels were inversely associated with fasting glucose ($p < 0.01$), homeostasis model assessment for insulin resistance ($p < 0.001$), TG ($p < 0.01$) and waist circumference ($p < 0.001$). No association with two-hour plasma glucose ($p = 0.29$), HDL-C ($p = 0.70$) or BP ($p = 0.46$) was evident at the five-year follow-up.

Another cross-sectional study conducted by Brenner and colleagues [72] with 1818 participants found an 8.9% prevalence of metabolic syndrome. The researchers found an inverse association between plasma 25(OH)D levels and the number of components for metabolic syndrome ($\beta = -0.1$, $p < 0.0001$). Components of metabolic syndrome included serum HDL-C level <40 mg/dL (males) or <50 mg/dL (females), serum TG level >1.7 mmol/L, fasting plasma glucose >110 mg/dL, BP > 130/85 mmHg and waist circumference >102 cm (males) or >88 cm (females). A lower OR (0.50, 95% CI, 0.24, 1.06) for metabolic syndrome was evident for study participants whose plasma 25(OH)D level was in the highest *versus* lowest quartile. After adjusting for age, sex, ethnicity, smoking status, physical activity and month of interview,

researchers found that a 10-nmol/mL (4 ng/mL) increase in the plasma 25(OH)D level was inversely associated with the homeostasis model assessment for insulin resistance score (β = −0.08, $p = 0.006$). Another cross-sectional study by Reis and colleagues [71] that included 1654 participants with DM assessed the prevalence of metabolic syndrome. The researchers divided serum 25(OH)D levels into quintiles and found an OR of 0.27 (CI, 0.15, 0.46; p trend <0.001) for metabolic syndrome for the highest quintile (median = 88 nmol/L (35 ng/mL)) *versus* the lowest quintile (median = 26.8 nmol/L (10.7 ng/mL)).

In a case-control study by Makariou and colleagues, 52 participants with metabolic syndrome had lower serum 25(OH)D levels (mean = 11.8 ng/mL, range = 0.6–48.3 ng/mL) than 58 controls (mean = 17.2 ng/mL, range = 4.8–62.4 ng/mL; $p = 0.027$) [75]. Serum 25(OH)D levels were inversely associated with serum TG levels ($r = −0.42$, $p = 0.003$) and small dense LDL-C ($r = −0.31$, $p = 0.004$).

The criterion for plausibility has thus been satisfied. There are several proposed biologically-plausible cellular-level mechanisms for the increase in CVD associated with low vitamin D status. Studies involving the assessment of an association between serum 25(OH)D levels and dyslipidemia, HTN, DM and metabolic syndrome have also been evaluated. Dyslipidemia, HTN, DM and metabolic syndrome are all plausible mediators between low serum 25(OH)D levels and increased risk of CVD. Specifically, the studies support increased serum LDL-C, VLDL-C and TG levels, decreased serum HDL-C levels, increased arterial stiffness, increased insulin resistance, hyperglycemia and increased incident metabolic syndrome as potentially plausible mediators.

3.6. Experiment

Researchers have conducted RCTs to assess the effect of serum 25(OH)D levels on CVD risk factors. However, vitamin D RCTs conducted to date have mixed results. The main reason is that vitamin D RCTs have been designed largely on the model used for pharmaceutical drugs, which assumes that the agent used in the trial is the only source of the agent and that a linear dose-response relation exists. Neither assumption is valid for vitamin D.

Another consideration is that chronic disease is caused by more than one risk factor and may occur only after long-term *versus* short-term vitamin deficiency. Vitamin supplementation studies are usually designed to assess the decrease in risk due to increasing vitamin intake to meet the minimum sufficiency level. Additional information would be gained from studies that also test the effects of supplementation on levels beyond those previously established for disease risk [111].

Robert Heaney [112] recently outlined the steps to design and conduct vitamin D RCTs: (1) start with the 25(OH)D level-health outcome relation; (2) measure the 25(OH)D levels of prospective participants; (3) enroll only those with low 25(OH)D levels; (4) supplement with enough vitamin D3 to increase 25(OH)D levels to the upper end of the quasi-linear region of the 25(OH)D level-health outcome relation; and (5) re-measure 25(OH)D levels after supplementation. For CVD, these recommendations would translate to enrolling people with 25(OH)D levels below about 15 ng/mL and then supplementing with 2000–4000 IU of vitamin D3 per day to raise 25(OH)D levels to >30–40 ng/mL.

The effect of vitamin D supplementation on CVD risk factors for women with polycystic ovarian syndrome was investigated by Rahimi-Ardabili and colleagues [113]. The study participants taking the vitamin D supplement had a statistically significant increase in serum vitamin D level and statistically significant decreases in serum total-C, TGs and VLDL-C levels

(all $p < 0.05$). They did not have any changes in serum levels of HDL-C, LDL-C, apolipoprotein-A1 (Apo-A1) or high-sensitivity C-reactive protein (hs-CRP). The placebo group had no changes.

Schnatz and colleagues [77] supplemented participants ($n = 600$) with 1000 mg of elemental calcium and 400 IU of vitamin D per day. The researchers found a 1.28 mg/dL decrease in LDL-C ($p = 0.04$) with a 38% increase in the 25(OH)D level. The researchers also found an increase in HDL-C and a decrease in TGs.

Breslavsky and colleagues [68] conducted an RCT, including 47 participants with type 2 DM, who were randomized into two groups. One group received cholecalciferol (vitamin D3) at 1000 IU per day for 12 months, whereas the other group received a placebo. After being similar at baseline, the group receiving cholecalciferol had significantly decreased hemoglobin A1c levels ($p < 0.0001$), but no change occurred in the placebo group.

Grimnes and colleagues [114] performed an RCT with 94 participants with low serum 25(OH)D levels. The participants were randomly assigned to receive a 20,000 IU supplement of oral D3 or a placebo twice weekly for six months. The supplement did not improve the lipid profile, which included total-C, LDL-C, HDL-C and TGs.

Ponda and colleagues [82] conducted a randomized, placebo-controlled, double-blinded trial. They randomized 151 vitamin D-deficient participants to receive oral D3 at 50,000 IU weekly for eight weeks or placebo and then examined the effect on serum cholesterol levels. In the supplemented group, serum 25(OH)D levels increased, serum PTH levels decreased and serum calcium levels increased. When participants were stratified by the change in serum 25(OH)D level and the serum calcium level, those whose response was greater than the median response had an increase in serum LDL-C of 15.4 mg/dL compared with those who had lower

than the median response. The analysis of the group receiving placebo did not show this relationship. Table 3 shows results from RCTs in order of serum 25(OH)D level at time of enrollment. This RCT does not support a beneficial effect on lipid status.

In a manuscript under preparation, it was found that for vitamin D RCTs related to CVD risk factors, the median baseline serum 25(OH)D level for the RCT with significant beneficial effects was 15 ng/mL, while the median baseline serum 25(OH)D for those without beneficial effects was 19 ng/mL (Grant, in preparation). This finding underscores the importance of having a low baseline serum 25(OH)D level when designing and conducting vitamin D RCTs to evaluate the findings of observational studies, as proposed by Heaney [112].

The criterion for experiment has thus been met. We reviewed RCTs that supplemented participants with vitamin D and found that most well-designed RCTs supported a causal association between serum 25(OH)D levels and CVD risk.

3.7. Analogy

The likelihood of a causal association between low vitamin D status and several diseases has been evaluated using Hill's criteria for causality in a biological system. Hill's criteria were met when Grant [46] evaluated overall cancer risk, when breast cancer risk was evaluated by Mohr and colleagues [48], when Grant and Boucher [37] evaluated periodontal disease and when Hanwell and Banwell [47] evaluated multiple sclerosis. Hanwell and Banwell found that all of the criteria were satisfied, except the criterion for disease prevention by intervention (experiment). The researchers state that fulfilling this criterion will be difficult because multiple sclerosis has a low incidence, the age of onset is highly variable and there is a lack of consensus regarding optimal vitamin D dose and the timing of treatment.

The criterion, analogy, has thus been met. Several assessments with various diseases have shown an analogous association to low serum 25(OH)D levels and CVD risk.

3.8. Confounding Factors

Potischman and colleagues [119] discussed the inadequacies of traditional causal criteria for assessing nutrients, but they acknowledged that they are necessary for public health recommendations. The authors stated that additional important considerations exist, such as confounding, errors in measurement and dose-response curves for nutrients.

Opländer and colleagues [88] discovered a potentially confounding factor for the association between production of vitamin D in the skin and a decrease in BP. UVB irradiation is responsible for vitamin D production and is associated with a decrease in BP, but UVA irradiation was found to also decrease BP. The effect was attributed to UVA irradiation-induced release of nitric oxide.

Beveridge and colleagues identified other confounders [91]. Associations in vitamin D studies may be confounded by the effects of other CVD risk factors in addition to those being studied, and confounding related to the possibility of reverse causality may also occur. Liberopoulos and colleagues [93] found that statins have different effects on the increase of serum 25(OH)D levels. Woodhouse and colleagues [94] found a seasonal variation in serum total-C, HDL-C and TG levels. These confounders can be controlled for with the use of appropriate statistical analyses, just as age, gender, ethnicity, BMI and smoking status are often controlled for in research studies.

Essential to the credibility of study results is the measurement and reporting of adherence to the intervention. The evaluation of adherence to oral vitamin D supplements given in a study may be either absent or inadequate. Furthermore, an inquiry about concurrent use of personal

oral vitamin D supplementation may differ across studies. Negative study results may simply be attributed to a lack of adherence to the intervention, because it leads to bias and a decrease in the statistical power.

4. Conclusions

Despite the identification and treatment of currently recognized CVD risks, CVD remains the leading cause of death. The focus of vitamin D research has recently expanded to include the effects of vitamin D status on CVD and CVD risk factors. Low serum 25(OH)D levels are associated with increased incidence [8], prevalence [56] and risk factors for CVD [15]. This assessment demonstrates that Hill's criteria were satisfied.

Potential benefits of decreasing the impact of a risk factor for CVD should outweigh potential risks. Repletion of vitamin D stores with a supplemental dose of 10,000 IU per day or less is unlikely to lead to toxic effects [39]. Repletion can be accomplished by a sensible increase in sun exposure [37] or by consuming vitamin D-rich foods, but this goal is most easily accomplished with oral supplementation. Furthermore, more severe deficiencies in serum 25(OH)D levels show a more rapid increase than less severe deficiencies [103]. Treatment for some CVD risk factors is expensive and may be difficult to access, but oral vitamin D supplements are readily accessible and reasonably priced. Other considerations for individualized treatment should include attention to skin melanin content, latitude and altitude of residence, dietary habits and amount of sun exposure.

The physiological mechanisms hypothesized to cause low vitamin D status to increase CVD risk have not yet been confirmed. Nearly all research studies regarding low vitamin D status and increased risk of CVD use observational study designs. More RCTs are needed that incorporate the complex pharmacokinetic and pharmacodynamic properties of vitamin D in the

study design: dose-response curve, half-life, avoidance of toxicity and use of the most accurate and precise serum assays.

Exposure to sunlight or vitamin D supplementation may be used in an RCT, although having a control group with a zero serum 25(OH)D level would not be possible. This approach is possible only in drug studies [120]. Nutrients are more appropriately studied in the context of proving negative causation: the absence of an antecedent caused the consequence. This study design would be consistent with research involving preventive healthcare strategies.

Current scientific evidence supports a causal association between serum 25(OH)D levels and increased risk for CVD on the basis of Hill's criteria for causality in a biological system. Only RCTs starting with low serum 25(OH)D levels found significant beneficial effects of vitamin D supplementation in reducing risk factors associated with CVD. However, evidence to date suggests that raising serum 25(OH)D levels to at least 30 ng/mL will reduce the risk of CVD.

Whether it is ethical to design a study in which a group of people is deprived of a known essential nutrient to measure an endpoint should be carefully determined. Furthermore, waiting for completion of long-term RCTs to change treatment recommendations, especially when risks are minimal, may adversely affect the health of countless individuals. According to Hill [45]: "All scientific work is incomplete—whether it be observational or experimental. All scientific work is liable to be upset or modified by advancing knowledge. That does not confer upon us a freedom to ignore the knowledge we already have, or to postpone the action that it appears to demand at a given time."

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Author Contributions

All of the authors contributed to the conception and design of this review as well as the analyses performed in order to determine if the criteria had been met. Patricia G. Weyland drafted the review with considerable assistance with the review of literature, organization and editing from Jill Howie-Esquivel and William B. Grant. All of the authors have approved all of the manuscript revisions as well as the final version prior to submission for publication.

Conflicts of Interest

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Table 1.Hill's criteria for causality in a biological system

Criterion	Defining question
Strength of the association	Is there a large difference in the outcome between exposed and non-exposed persons?
Consistency of the observed association	Has the outcome been observed by multiple researchers, in various circumstances, places, and at different times?
Specificity of the association	Are there specific persons or geographic locations associated with specific outcomes?
Temporality (temporal relationship of the association)	Does the cause always precede the effect?
Biological gradient	Is there a dose–response curve?
Plausibility of the biology	Is the suspected causation consistent with current knowledge of biology?
Coherence	Are there any serious conflicts with the biology or natural history of the disease?
Experiment (experimental or quasi-experimental evidence)	Has an observed association led to a preventive action that has prevented the outcome?
Analogy	Is there an analogous exposure and outcome?

Table 2.Studies used to evaluate causality between low D and increased risk of CVD

Criterion	Proposed mechanism	Reference	No effect	Satisfied?
Strength of association		[6,8,12,50–53]		Yes
Consistency		[7,15,54–56]		Yes
Temporality		[8,18,55,57,58]		Yes
Biological gradient		[8,55,59,60]		Yes
Plausibility	Blunts renin–angiotensin system	[61,62]		Yes
	Arterial stiffness (HTN)	[15,62–66]		
	Reduced risk of DM	[19]		
	Insulin resistance	[67]		
	Glucose regulation	[58,67,68]		
	Seasonal variations in serum 25(OH)D	[4]		
	Lipids	[69,70]		
	Metabolic syndrome	[71–75]		
	DM type 2 and its progression	[19,57,76]		
Experiment	RCTs	[77]	[78]	Yes
	Blood pressure reduction	[79]		
	Blunts renin–angiotensin system	[61]		
	Arterial stiffness (PWV)	[25]		
	Insulin resistance	[80,81]		
	Glucose	[80,81]		
	Lipids		[82–84]	
	Metabolic syndrome	[85,86]		
Analogy	Cancer	[46,87]		Yes
	DM type 2	[19]		
Confounding factors	Nitric oxide liberated by solar UV	[88–90]		
	Calcium supp.	[91]]		
	Reverse causation	[91]		
	CVD risk factors affect 25(OH)D levels (obesity)	[91]		
	Physical activity	[92]		
	Statins	[75,93]		
	Seasonal variations in temp.	[94,95]		
Concerns				
Excess vitamin D		[96]		
Hypercalcemia		[97]		
DM	Limited effect of vitamin D	[98–101]		

Table 3. Results of studies on D supplementation and CVD risk factors [ordered by mean serum 25(OH) D (ng/mL)]

Mean serum 25(OH)D (ng/mL)	**Vitamin D_3 dose (IU/d)**	**Increase in 25(OH)D (ng/ML)**	**Mean Age (years)**	**Health outcome of interest**	**Findings**	**Reference**
8.4	4000	19.6	42	Insulin sensitivity	5.9 vs. –5.9 (p=0.003)	[115]
8.4	4000	19.6	42	Fasting serum glucose	–3.6 vs. 1.1 (p=0.02)	[115]
13	400 or 1000	13	64	HDL-c, LDL-c, TG, ApoA1, ApoB100, HOMA-IR, hs-CRP, sICAM-1, IL-6	Not significant	[83]
<20	2000*			Total cholesterol, HDL-c, LDL-c, TGs	Not significant	[82]
14.7	1000	15	38	Total cholesterol, LDL-c, ApoA1, ApoA1:ApoB-100	Significant to $p<0.01$	[108]
14.7	1000	15	38	HDL-c, LDL-c:ApoB-100	Significant to $p<0.04$	[108]
14.7	1000	15	38	ApoB-100, lipoprotein(a)	Not significant	[108]
16.1	2857*	40	52	Insulin sensitivity	Not significant	[114]
16.3	0		51	Systolic BP	+1.7 mm	[79]
16.3	1000		51		–0.66 mm	[79]
14.5	2000		51		–3.4 mm	[79]
15.6	4000		51		–4.0 mm	[79]
19.6	4000	19.5	14.1	Insulin sensitivity	–1.36 vs. +0.27 (p== 0.03)	[116]
				Fasting insulin	–6.5 vs. +1.2 (p=0.03)	[116]
19.6	2857* or 5714*	40	52	HDL-c, LDL-c, TGs, ApoA1, ApoB, hs-CRP	Not significant	[84]
22.9	2857* or 5714*	22.8	50	TNF-α, IL-6, HOMA-IR, QUICKI	Not significant	[117]
23	1000	21	61	Systolic BP	–1.5 mm vs. +.4 mm (p = 0.26)	[25]
30.3	2500	16	64	Glucose, CRP, FMD, diastolic BP, systolic BP, PWV	Not significant	[118]

*Average daily oral intake from a bolus dose; FMD = flow-mediated dilation; QUICKI = qualitative insulin sensitivity check index; hs-CRP = high-sensitivity c-reactive protein; TNF-α = tissue necrosis factorα, IL-6 = interleukin 6; ApoA1 = apolipoprotein A1; ApoB = apolipoprotein B.

Chapter 3

Is Total or Free 25-hydroxyvitamin D Associated with High-Density Lipoprotein-Cholesterol or High-Density Lipoprotein Subclass Levels?

Patricia G. Weyland, MS, RN, FNP-BC, CDE, PhD(c) [1*]

Steven M. Paul, PhD [2]

Jill Howie-Esquivel, RN, PhD, NP [3]

[1] = PhD Candidate, Department of Physiological Nursing, School of Nursing, University of California, San Francisco

[2] = Principal Statistician, School of Nursing, University of California, San Francisco

[3] = Assistant Professor, Department of Physiological Nursing, School of Nursing, University of California, San Francisco

[*] Author to whom correspondence should be addressed; #2 Koret Way, Box 0610, San Francisco, CA 94143, USA, e-mail-patricia.weyland@ucsf.edu, tel.-831-420-7324, fax-831-713-5967

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ABSTRACT

Background: Cardiovascular disease (CVD) remains the leading cause of death in the US. Both the pre-hormone total 25(OH) D, a measure of vitamin D (D) status, and high-density lipoprotein-cholesterol (HDL-c) are inversely associated with incident and prevalent CVD. Free 25(OH) D may be a more accurate measure of D status and HDL-c has subclass levels, HDL_2 and HDL_3, which may be more accurate measures of atheroprotective lipoprotein. The associations between total and free 25(OH) D and HDL-c, HDL_2, and HDL_3 warrant further investigation.

Objective: To determine if either total or free 25(OH) D is associated with HDL-c or HDL subclasses.

Methods: A cross-sectional analysis utilizing serum sample data from ninety three community-dwelling adults in the San Francisco Bay Area was conducted. Total 25(OH) D was measured with liquid chromatography-tandem mass spectrometry, free 25(OH) D by immunoassay, and HDL-c, HDL_2, and HDL_3 by the Vertical Auto Profile II test. Analyses were completed using correlational and multiple regression analyses.

Results: Positive associations between total 25(OH) D and HDL-c, HDL_2, and HDL_3 and between free 25(OH) D and HDL-c and HDL_2 were found. After controlling for gender, body mass index (BMI), and estrogen use neither total 25(OH) D nor free 25(OH) D showed a statistically significant unique contribution to the total variance in HDL-c, HDL_2, or HDL_3. Gender and BMI uniquely contributed to the total variance in HDL-c, HDL_2, and HDL_3 in all models. Estrogen use uniquely contributed to the total variance in HDL_2 for both models.

Conclusions: Inclusion of gender, BMI, and estrogen use in the multiple regression models attenuated the already small associations between total 25(OH) D and HDL-c, HDL_2, and HDL_3 as well as the associations between free 25(OH) D and HDL-c and HDL_2. Study findings do not support the hypothesis that low D status leads to a decrease in atheroprotective lipoprotein.

Key words: cardiovascular disease, total 25-hydroxyvitamin D, free 25-hydroxyvitamin D, high-density lipoprotein-cholesterol, high-density lipoprotein subclasses, and estrogen

INTRODUCTION

A decline and subsequent plateau in the annual number of heart disease-related deaths in the US (Jones et al., 2012) has been credited to an increase in the detection and treatment of CVD risk factors (Ford et al., 2007). But this has not changed the status of cardiovascular disease (CVD) as the leading cause of death (Kochanek, Xu, Murphy, Miniño, & Kung, 2011). Also, new data suggest that CVD rates may now be increasing (Jones et al., 2012). Tens of thousands of premature deaths could be prevented by increasing risk factor-modifying clinical preventive services that reduce cardiovascular disease (CVD) (Farley, Dalal, Mostashari, & Frieden, 2010).

Scragg (1981) first recognized a pattern in CVD morbidity and mortality; the rates increase in the winter, at lower altitudes, and at higher latitudes. Additionally, he concluded that the CVD rates are not always associated with respiratory disease or temperature changes. Scragg (1981) hypothesized that the seasonal variation in solar ultra-violet radiation that causes a change in vitamin D (D) metabolite status may be a mechanism that contributes to CVD.

Vitamin D status is currently assessed by measuring the pre-hormone total 25-hydroxyvitamin D (25[OH] D). Looker et al. (2011) defined total 25(OH) D deficiency as < 20 ng/mL and determined the rate of deficiency in the US was approximately 32%. If D status is linked to CVD, a substantial decrease in morbidity and mortality could be possible with D deficiency correction. The estimated worldwide prevalence of deficient or insufficient total 25(OH) D defined as < 30 ng/mL is one billion (Holick, 2007), making D status an international concern.

A controversy persists regarding which gender has the higher prevalence of total 25(OH) D deficiency. Kendrick et al. (2009) found a higher prevalence of total 25(OH) D deficiency in

females, but conversely, de Boer et al. (2009) found that total 25(OH) D deficiency was associated with males. There may not be an inherent difference in the genders that would predispose to a difference in 25(OH) D levels. Investigators have speculated that occupational or clothing differences between the genders may account for the total 25(OH) D level differences (El-Menyar et al., 2012).

Inverse associations have been found between total 25(OH) D levels and CVD or CVD risk factors. Total 25(OH) D levels < 16 ng/mL were associated with twice the risk for myocardial infarction when compared with levels ≥ 30 ng/mL (Giovannucci, Hollis, & Rimm, 2008). Prevalent CVD was 57% greater when total 25(OH) D levels were < 20 ng/mL (Kendrick, Targher, Smits, & Chonchol, 2009). Anderson et al. (2010) found deficient levels of total 25(OH) D, defined as ≤ 30 ng/mL, were associated with a higher *prevalence* of CVD risk factors including diabetes mellitus (DM), hypertension, and hyperlipidemia ($p < 0.0001$). Additionally, the researchers found that for participants without CVD risk factors, total 25(OH) D levels ≤ 15 ng/mL were associated with a higher *incidence* of DM, hypertension, and hyperlipidemia ($p < 0.0001$).

Hyperlipidemia and low total high-density lipoprotein-cholesterol (HDL-c) levels are well-established CVD risk factors (Goff et al., 2014). In a randomized controlled-trial (RCT) Rubins et al. (1999) found that Gemfibrozil increased HDL-c and decreased the incidence of non-fatal myocardial infarction by 23% (95% CI, 4, 38, $p = 0.02$) for males with CHD and low HDL-c. These results were not due to increased levels of low-density lipoprotein-cholesterol (LDL-c) because only individuals with normal LDL-c levels were included in the study. An independent inverse association was found by Cooney et al. (2009) between HDL-c and both CVD and CHD mortality (adjusted hazard ratios per 0.5 mmol/l increase in HDL-c for women,

0.60, 95% CI, 0.51, 0.69 and for men, 0.76, 95% CI, 0.70, 0.83, for the CVD mortality endpoint). The proposed physiology of the atheroprotective qualities of HDL-c versus LDL-c include; anti-apoptotic, anti-thrombotic, anti-inflammatory, anti-oxidant, nitric oxide-promoting, endothelial function-enhancing, increased cholesterol efflux capacity, and increased reverse cholesterol transport capability (Movva & Rader, 2008; Khera et al., 2011).

The pathway from low D status to increased CVD needs to be established. One potential pathway is that low D status leads to a decrease in atheroprotective lipoprotein. Low total 25(OH) D levels were associated with low total HDL-c levels by Jorde, Figenschau, Hutchinson, Emaus, and Grimnes (2010) and Guasch et al. (2012). Total HDL-c may be sub-classified into large and buoyant (HDL_2) or small and dense (HDL_3) lipoproteins. The degree of atheroprotectivity for each of these subclasses remains controversial but investigators describe differing pro-inflammatory effects, cholesterol-carrying capacity, and efflux capacity (Otvos et al., 2006; Kim et al., 2014).

Otvos et al. (2006) found that HDL_3 particles but not HDL_2 particles predicted CHD events. As the number of HDL_3 particles increased, there was an increase in CHD events at baseline (odds ratio [OR], 0.82, 95% confidence interval [CI], 0.72, 0.94, $p = 0.004$) and during the study treatment (OR, 0.74, 95% CI, 0.64, 0.85, $p = 0.0001$) was found. In contrast, Kim et al. (2014) found HDL_3 was the only statistically significant negative predictor of prevalent carotid artery disease. The researchers performed a stepwise linear regression adjusting for age, smoking status, and DM and found that HDL_3 was the only subclass of HDL that had a statistically significant inverse association with prevalent carotid artery disease for both men ($n = 1201$, $p = 0.0011$) and women ($n = 524$, $p = 0.033$).

In addition to total 25(OH) D, a novel measure of Vitamin D status, *free* 25(OH) D (Future Diagnostics B.V., Wijchen, The Netherlands) may provide a more accurate measure of D status because it measures only the *bio-available* portion of the pre-hormone total 25(OH) D. Only *free* 25(OH) D can be converted to the *bio-active* hormone, 1α, 25-dihydroxyvitamin D (1α, 25[OH]$_2$ D) (Holick, 2006). The unbound form or *free* 1α, 25(OH)$_2$ D, as the *free hormone hypothesis* states (Mendel, 1989), is then able to cross the membrane of a cell to exert an effect. In order to potentially increase the accuracy of the assessment of the association between D status and atheroprotective lipoprotein, *free* 25(OH) D levels will be included as a variable in addition to total 25(OH) D levels and used in a model to assess for an association with HDL-c, HDL_2, and HDL_3.

Assessing for an association between both total and free 25(OH) D and total HDL-c, HDL_2, and HDL_3 levels may provide evidence for a mechanism of CVD development. This knowledge may then lead to more effective prevention strategies and to a reversal of existing CVD. Therefore, the purpose of this study is to determine if there is an association between D status measured by total or free 25(OH) D and atheroprotective lipoprotein measured by total HDL-c or the subclasses HDL_2 or HDL_3. We hypothesized that there is a statistically significant positive association between measures of D status and measures of atheroprotective lipoprotein. More specifically, the aims of this study are to:

1) Determine if serum total 25(OH) D levels are associated with HDL-c, HDL_2, or HDL_3 levels.

2) Determine if serum free 25(OH) D levels are associated with HDL-c, HDL_2, or HDL_3 levels.

MATERIALS AND METHODS

Research Design

This study is a cross-sectional analysis utilizing serum sample data from an RCT.

Participants

Socio-demographic and serum sample data were collected between 2008 and 2012 from study participants who were recruited from the San Francisco Bay Area for two multiple-phase studies; the *Effects of Vitamin D on CYP3A Substrate Clearance* (CHR approval number: H1775-28941) and the *Effects of Vitamin D on Lipids* (clinical trial number: NCT00723385). A wide range of ages and races were represented in the study. Health status included: hypertension (44.1%), coronary artery disease (16.1%), DM (10.8%), and liver disease (not including active cirrhosis) (12.9%). Medications included statins (43%), diuretics (26.9%), and oral contraception or estrogen use (7.5%).

Individuals with active cirrhosis or women who were pregnant were excluded from this study. These exclusion criteria were used to avoid *confounding* due to differences in the level of serum D binding protein in these individuals (Rejnmark et al., 2006; Schwartz et al., 2013; Schwartz et al., 2014). Multiple regression analyses were run controlling for estrogen use because of the effect of estrogen on total 25(OH) D (Buchanan et al., 1986), total and free 1α, $25(OH)_2$ D (Bikle et al., 1992) and HDL-c levels (Williams et al., 1993; Terauchi et al., 2012; Wang et al., 2014).

Informed consents were obtained from all study participants and both studies were approved by the Committee on Human Research at the University of California, San Francisco.

Laboratory Measurements

Serum total 25(OH) D levels were measured using a liquid chromatography tandem mass spectrometry assay (Mayo Clinical Laboratories, Rochester, MN, certified by Clinical

Laboratory Improvement Amendments). The coefficient of variation (CV) is approximately 10% at levels > 10 ng/mL. The internal standard used was from the National Institute of Standards and Technology quality assurance program for analysis of D metabolites in human serum, funded by the National Institutes of Health Office of Dietary Supplements.

Serum free 25(OH) D levels were measured by immunoassay (Future Diagnostics B.V., Wijchen, The Netherlands, http://www.future-diagnostics.nl/). This new procedure uses plasma or serum samples from patients and controls that are exposed to antibodies reactive with 25(OH) D. They are immobilized on a solid phase microtiter well or on superparamagnetic particles in a simple buffer and allowed to react. The antibody captures the free 25(OH) D during a 90 minute incubation period. A labeled analog of 25(OH) D then reacts with the remaining antibody during a second incubation after the solid phase is washed out. The signal is quantified after a third incubation and subsequent wash. The free 25(OH) D level in the sample is inversely proportional to the quantification of the signal. The assay measures free 25(OH) D levels ranging from 1.1-40 pg/mL (Rosmalen, 2011).

Serum HDL-c, HDL_2, and HDL_3 levels were included in the complete cholesterol assessment measured using the *Vertical Auto Profile II* (VAP II) test (Atherotech Diagnostics Lab). The VAP II test is a single vertical spin, inverted rate zonal, density gradient ultracentrifugation technique in which all of the cholesterol concentrations are measured simultaneously (Kulkarni, 2006). Levels of HDL_2 and HDL_3 are determined by the separation of the relative positions, the shapes of the curves, and the lipoprotein peaks in the density gradient. Kulkarni et al. (1997) reported a long-term between-rotor CV = 9% for HDL_2 and 5% for HDL_3.

Statistical Design and Data Analysis

The *IBM SPSS Statistics Version 22* program was used for all statistical calculations. Means and standard deviations are reported for continuous variable data and frequencies for categorical variable data. All data met the assumptions for the statistical tests that were used. The α-level was set at < 0.05, and all tests were two-tailed. The power of the study was 0.80 and an effect size of $r \geq 0.28$ was likely to be detected as significant (Hulley et al., 2007).

The Pearson Product Moment correlation was used to determine correlations between age, gender, body mass index (BMI), statin and estrogen use, serum total and free 25(OH) D levels, and serum HDL-c, HDL_2, and HDL_3 levels. Multiple regression analyses were performed to determine the degree to which independent variables uniquely contribute to the total variance of serum HDL-c, HDL_2, and HDL_3 levels in five models; serum total 25(OH) D and HDL-c, total 25(OH) D and HDL_2, total 25(OH) D and HDL_3, free 25(OH) D and HDL-c, and free 25(OH) D and HDL_2 levels, controlling for gender, BMI, and estrogen use.

RESULTS

Participants and Laboratory Measures

Serum sample data from ninety-three participants were included in the study. The participants' demographic characteristics and laboratory values are presented in Table 1. There were 50% more men than women in the study and there was a wide range of ages from 25 to 91 years old. The most prevalent race was Caucasian, next African-American, and then Asian. The means of both the total 25(OH) D and the HDL_2 levels were low at 29.0 ng/mL (sufficient ≥ 30 ng/mL) and 13.1 mg/dL (normal > 15 mg/dL), respectively.

There was a strong positive correlation between HDL-c and both HDL_2 and HDL_3. There was also a strong positive correlation between HDL_2 and HDL_3. Free 25(OH) D and total

25(OH) D were strongly positively correlated. HDL-c, HDL_2, and HDL_3 were all positively correlated with total 25(OH) D and female gender and they were all negatively correlated with BMI. Only HDL-c and HDL_2 were positively correlated with free 25(OH) D. Estrogen use was positively correlated with HDL-c, HDL_2, HDL_3, and free and total 25(OH) D. No statistically significant correlation was found between age and any other variable except statin use (see Table 2).

Serum *total* 25(OH) D and Cholesterol Measures of HDL-c, HDL_2, and HDL_3

In the analysis, HDL-c was used as the dependent variable and four independent variables were entered into the model (see Table 3). This model explained 46.6% of the total variance in HDL-c ($p < 0.001$). Female gender uniquely explained 13.5% ($p < 0.001$) and BMI uniquely explained 14.9% (< 0.001) of the total variance in HDL-c. In the next analysis HDL_2 was used as the dependent variable and four independent variables were entered into the model (see Table 4). The overall model explained 36.5% of the total variance in HDL_2 ($p < 0.001$). Female gender uniquely explained 9.9% ($p < 0.001$), BMI uniquely explained 10.2% ($p < 0.001$), and estrogen use uniquely explained 3.6% ($p = 0.029$) of the total variance in HDL_2. HDL_3 was also used as the dependent variable and four independent variables were entered into the model (see Table 5). This model explained 47.1% of the total variance in HDL_3 ($p < 0.001$). Female gender uniquely explained 14.5% ($p < 0.001$) and BMI uniquely explained 15.8% (< 0.001) of the total variance in HDL_3.

Serum *free* 25(OH) D and Cholesterol Measures of HDL-c and HDL_2

To examine *free* 25(OH) D levels HDL-c was used as the dependent variable and four independent variables were entered into the model (see Table 6). The overall model explained

47.0% of the total variance in HDL-c ($p < 0.001$). Female gender uniquely explained 15.1% ($p < 0.001$) and BMI uniquely explained 16.6% ($p < 0.001$) of the total variance in HDL-c. The regression was then performed with HDL_2 as the dependent variable and four independent variables were entered into the model (see Table 7). The overall model explained 38.0% ($p < 0.001$) of the total variance in HDL_2. Female gender uniquely explained 11.2% ($p < 0.001$), BMI uniquely explained 11.0% ($p < 0.001$), and estrogen use uniquely explained 2.9% ($p=0.046$) of the total variance in HDL_2.

DISCUSSION

This study is novel because directly-measured not calculated free 25(OH) D levels are included as a measure of D status. Free 25(OH) D may be a more accurate measure of D status than total 25(OH) D and the most accurate measure of free 25(OH) D is obtained from directly measuring serum levels (Schwartz et al., 2013). This study includes some findings that support and some findings that are contrary to other study findings.

Female gender was associated with HDL in all of the models. This result is consistent with the results of some previous studies. Freedman et al. (2004) found that females had twice the number of large HDL particles (HDL_2) than males ($p < 0.001$). Similarly, Johnson et al. (2004) also found that females had higher levels of large HDL particles (HDL_2) than males ($p < 0.0001$). Contrary to the results of our study Johnson et al. (2004) found that females had lower levels of small HDL particles (HDL_3) than males ($p < 0.0001$).

We also found that BMI was uniquely associated with HDL in all of the models. This has been found in other studies as well. Our findings are consistent with Da Costa et al. (2012) who also found that BMI was negatively correlated with HDL-c. Williams et al. (1993) found more complicated relationships between BMI and HDL subclasses. They found that higher BMI

in males and females that did not drink alcohol were associated with a higher HDL_{3b} ($p < 0.05$) and larger-diameter HDL_{3c} ($p < 0.05$) and a lower HDL_{2b} ($p < 0.05$).

Estrogen use uniquely contributed to the total variance of HDL_2. Nogueira-de-Souza et al. (2009) had similar results. The researchers found that after one year of estrogen therapy there was a significant increase in HDL-c levels ($p = 0.029$). Similarly, Williams et al. (1993) examined HDL subclasses and found that postmenopausal women on estrogen therapy had an increase in HDL-c and that 88% of the increase was within HDL_{3a} and HDL_{2a}.

Contrary to the results of some previous studies, age was not associated with total or free 25(OH) D, HDL-c, HDL_2, or HDL_3 in this study. Previously, Jorde et al. (2010) found that there was a positive association between age and increasing quartiles of total 25(OH) D ($p < 0.01$) and de Boer et al. (2009) also found that lower levels of serum 25(OH) D (< 15ng/mL) were associated with younger age. Williams et al. (1993) found a complex relationship between age and HDL by gender and estrogen therapy.

Because neither total nor free 25(OH) D uniquely contributed to the total variance in HDL-c, HDL_2, or HDL_3, this study does not provide support for the proposed physiological mechanism. Schwartz (2008) also had negative results. No difference in HDL-c levels with D supplementation was found ($n = 16$). Additionally, Kane et al. (2013) performed an RCT with D supplementation and found no association between either total or free 25(OH) D level and HDL-c or HDL subfraction level ($n = 49$). They did find that for study participants on statins, free but not total 25(OH) D levels were inversely associated with total cholesterol, LDL-c, and triglyceride levels.

Free 25(OH) D measures were included in this study because it is important to use the most accurate measure of D status in order to increase the validity of study results. Currently,

there is a consensus that total 25(OH) D is the most accurate D metabolite to measure when assessing overall D status (Holick, 2009). Because the *free hormone hypothesis* (Mendel, 1989) may apply to D metabolites; free 25(OH) D may be a more accurate measure of overall D status than total 25(OH) D.

Historically, free 25(OH) D levels were calculated. This was because an assay that directly measured serum free 25(OH) D levels had not been available. Today, the calculation is not currently being performed due to the increased cost of performing the multiple assays that are required and the complexity of the calculations. Using an assay that directly measures free 25(OH) D (Future Diagnostics B.V., Wijchen, The Netherlands) Schwartz et al. (2013) found that the association between total and free 25(OH) D levels varies depending upon the presence of clinical conditions such as hepatic cirrhosis. They also found that free 25(OH) D levels calculated using circulating D binding protein and albumin levels were less accurate when compared to directly-measured levels.

Schwartz et al. (2014) found that calculated levels were higher than directly-measured free 25(OH) D levels. Directly measuring versus calculating free 25(OH) D levels warrants further study to determine the clinical relevance in determining optimal D status in persons with various chronic and clinical conditions. After evaluation of the clinical significance of the differences in directly-measured versus calculated free 25(OH) D levels, the most accurate measure of free 25(OH) D may be the direct measurement.

HDL subclass measures were included in the analysis because it is also important to use the most accurate measure of atheroprotective lipoprotein in order to increase the validity of research results. Using total HDL-c or the subclasses HDL_2 or HDL_3 will lead to different results and different conclusions. Superko et al. (2012) performed a review of 80 studies and

found that neither HDL_2 nor HDL_3 differentiated atheroprotective properties of HDL subclasses. The researchers suggest that further description of the HDL particles would provide a better risk assessment for CVD.

Because deficient and insufficient total 25(OH) D levels are highly prevalent in the US and worldwide, it is of paramount importance to determine if low D status is a primary risk factor versus biomarker for CVD. Only further research will determine this important question. Further research may also provide more detailed explanations of the physiological mechanisms that lead from low D status to increased CVD. Knowledge of these physiological mechanisms may then be incorporated into clinical practice guidelines detailing the process of the diagnosis of low D status and the most effective treatment. Clinicians will then provide D supplementation, which is very safe and reasonably priced, more consistently in order to normalize 25(OH) D levels and thereby possibly decrease the mortality rate due to CVD.

To achieve this goal, additional D RCTs should be performed that are designed specifically to test a nutrient. Drug study designs are not inappropriate for nutrients. Heaney (2014) provides strong evidence to support designing nutrient studies in a way that takes into consideration the unique characteristics of a nutrient. He presents detailed guidelines including rules to follow in order to increase the validity of the research results and the subsequent conclusions that are drawn by the researchers. Additionally, Grant (2009) estimated that by increasing total 25(OH) D levels to at least 45 ng/mL there would be a significant decrease in the annual preventable death rate in part due to a decrease in CVD. Including this level as the target level in a study may lead to a greater benefit than lower levels.

Treatment for low D status should not wait for long-term RCT D studies. The preponderance of current research evidence supports a causal association between D status and

CVD (Weyland et al., 2014). Vitamin D supplements to normalize 25(OH) D levels are reasonably priced and no harm to individuals from doses as high as 10,000 to 20,000 IU of D_3 (cholecalciferol) per day has been reported (Garland et al., 2011).

Limitations

There are several limitations to this study. First, there was a relatively small number of participants included in the study. Future studies need to include a larger number of participants to increase the power and generalizability of the results. Second, cross-sectional studies cannot establish a *causal* association between variables. There are many published observational studies that show an association between 25(OH) D level and risk of CVD, but the *gold standard* for establishing *causation* is an RCT. Third, low 25(OH) D level may be representative of or a biomarker for CVD and therefore have no direct causal association. Fourth, lack of a more detailed analysis of HDL is a potential explanation for the negative findings in this study.

CONCLUSIONS

Small but statistically significant associations were found between total 25(OH) D and HDL-c, HDL_2, and HDL_3 as well as between free 25(OH) D and HDL-c and HDL_2. When gender, BMI, and estrogen use were included in the multiple regression models the associations were no longer present. The findings in this study do not support the hypothesis that low D status leads to a decrease in atheroprotective lipoprotein.

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Table 1. Study participant characteristics and laboratory values

	N (%)
Total Study Participants	93
Gender: Men	56 (60.2)
Women	37 (39.8)
Race: African-American	22 (23.7)
Asian	12 (12.9)
Caucasian	57 (61.3)
Other	2 (2.2)
Current Cigarette smoker	19 (20.4)
Alcohol drinker	52 (55.9)
Hypertension	41 (44.1)
Coronary Artery Disease	15 (16.1)
Diabetes Mellitus, Type 1 or 2	10 (10.8)
Liver disease (not including active cirrhosis)	12 (12.9)
Statin use	40 (43.0)
Diuretic use	25 (26.9)
Oral contraception or Estrogen use	7 (7.5)
	mean ± standard deviation
Age (years)	55.8 ± 16.4
Weight (kg)	82.0 ± 19.1
Height (cm)	169.2 ± 9.0
BMI (kg/m^2)	28.6 ± 6.0
Total 25(OH) D (sufficient ≥ 30 ng/mL)	29.0 ± 10.6
Free 25(OH) D (pg/mL)	5.1 ± 1.6
HDL-c (normal ≥ 40 mg/dL)	51.7 ± 14.9
HDL_2 (normal > 15 mg/dL)	13.1 ± 6.3
HDL_3 (normal > 25 mg/dL)	38.6 ± 9.3

BMI = body mass index, HDL-c = high-density lipoprotein cholesterol, 25(OH) D = 25-hydroxyvitamin D

Table 2. Pearson Correlation Coefficients, N = 93

	Female	BMI	Statin	Estrogen	Total D	Free D	HDL-c	HDL_2	HDL_3
Age	-.024	.018	.480**	-.176	-.103	-.093	-.039	.002	-.065
Female		.000	-.129	.351**	.135	-.032	.471**	.423**	.472**
BMI			.163	-.098	-.189	-.065	-.436**	-.363**	-.448**
Statin				-.083	.050	.053	-.196	-.176	-.193
Estrogen					.325**	.258*	.388**	.389**	.357**
Total D						.736**	.318**	.258*	.332**
Free D							.204*	.211*	.179
HDL-c								.936**	.970**
HDL_2									.823**

* = correlation is significant at the 0.05 level (2-tailed), ** = correlation is significant at the 0.01 level (2-tailed)

BMI = body mass index, HDL-c = high-density lipoprotein-cholesterol, D = 25-hydroxyvitamin D

Table 3. Simultaneous Multiple Regression, dependent variable = HDL-c, N = 93

Source	R^2	Beta	R^2-change (sr^2)	df	F	p-value
Overall	.466			4/88	19.18	< 0.001
Female		.394	.135	1/88	22.34	< 0.001
BMI		-.394	.149	1/88	24.59	< 0.001
Estrogen		.167	.022	1/88	3.68	0.058
Total-25(OH)D		.136	.016	1/88	2.65	0.107

BMI = body mass index, HDL-c = high-density lipoprotein-cholesterol, 25(OH) D = 25-hydroxyvitamin D

Table 4. Simultaneous Multiple Regression, dependent variable = HDL_2, N = 93

Source	R^2	Beta	R^2-change (sr^2)	df	F	p-value
Overall	.365			4/88	12.63	< 0.001
Female		.337	.099	1/88	13.79	< 0.001
BMI		-.327	.102	1/88	14.23	< 0.001
Estrogen		.212	.036	1/88	4.95	0.029
Total-25(OH)D		.082	.006	1/88	0.81	0.327

BMI = body mass index, HDL = high-density lipoprotein, 25(OH) D = 25-hydroxyvitamin D

Table 5. Simultaneous Multiple Regression, dependent variable = HDL_3 (N = 93)

Source	R^2	Beta	R^2-change (sr^2)	df	F	p-value
Overall	.471			4/88	19.59	< 0.001
Female		.407	.145	1/88	24.14	< 0.001
BMI		-.406	.158	1/88	26.37	< 0.001
Estrogen		.122	.012	1/88	1.96	0.165
Total-25(OH)D		.161	.023	1/88	3.74	0.056

BMI = body mass index, HDL = high-density lipoprotein, 25(OH) D = 25-hydroxyvitamin D

Table 6. Simultaneous Multiple Regression, dependent variable = HDL-c, N = 93

Source	R^2	Beta	R^2-change (sr^2)	df	F	p-value
Overall	.470			4/88	19.50	< 0.001
Female		.418	.151	1/88	24.98	< 0.001
BMI		-.411	.166	1/88	27.63	< 0.001
Estrogen		.163	.021	1/88	3.52	0.064
Free-25(OH)D		.149	.020	1/88	3.36	0.070

BMI = body mass index, HDL-c = high-density lipoprotein-cholesterol, 25(OH) D = 25-hydroxyvitamin D

Table 7. Simultaneous Multiple Regression, dependent variable = HDL_2 (N = 93)

Source	R^2	Beta	R^2-change (sr^2)	df	F	p-value
Overall	.380			4/88	13.49	< 0.001
Female		.361	.112	1/88	15.88	< 0.001
BMI		-.335	.110	1/88	15.69	< 0.001
Estrogen		.190	.029	1/88	4.10	0.046
Free-25(OH)D		.152	.021	1/88	2.99	0.087

BMI = body mass index, HDL = high-density lipoprotein, 25(OH) D = 25-hydroxyvitamin D

Chapter 4

Does Vitamin D Supplementation Lead to Improvements in Cholesterol and Cholesterol Subclass Levels?

Patricia G. Weyland, MS, RN, FNP-BC, CDE, PhD(c) [1*]

Steven M. Paul, PhD [2]

Jill Howie-Esquivel, RN, PhD, NP [3]

[1] = PhD Candidate, Department of Physiological Nursing, School of Nursing, University of California, San Francisco

[2] = Principal Statistician, School of Nursing, University of California, San Francisco

[3] = Assistant Professor, Department of Physiological Nursing, School of Nursing, University of California, San Francisco

[*] Author to whom correspondence should be addressed; #2 Koret Way, Box 0610, San Francisco, CA 94143, USA, e-mail-patricia.weyland@ucsf.edu, tel.-831-420-7324, fax-831-713-5967

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ABSTRACT

Background: Known risk factors for cardiovascular disease (CVD) include diabetes mellitus, hypertension, and dyslipidemia. Low 25-hydroxyvitamin D (25[OH] D) level is associated with an increase in both the prevalence and incidence of CVD and therefore may be a direct or indirect risk factor for CVD. Low 25(OH) D level that leads to a decrease in high-density lipoprotein cholesterol (HDL-c) or one of the subclasses, HDL_2 or HDL_3, is a plausible indirect mechanism for increased CVD risk. The effect of vitamin D (D) supplementation on HDL-c, HDL_2 and HDL_3 level is not known. The effect of HMG-CoA reductase inhibitor (HMG-CoA RI) use with D supplementation on HDL-c, HDL_2 and HDL_3 levels also remains unclear.

Aim: To test the hypotheses that:

1) HDL-c, HDL_2, or HDL_3 levels will increase in participants supplemented with Vitamin D compared to participants who received a placebo; and

2) HDL-c, HDL_2, or HDL_3 levels will increase in participants supplemented with Vitamin D and who reported using an HMG-CoA RI, compared to participants who received D supplementation but reported not using an HMG-CoA RI.

Methods: This is a secondary analysis of serum sample data obtained from fifty-nine San Francisco Bay Area community-dwelling adults from a randomized double-blinded placebo-controlled trial.

Results: No statistically significant difference in HDL-c, HDL_2, or HDL_3 levels was found for participants who received D supplementation for 12 weeks compared with participants who received a placebo. There was also no statistically significant difference in HDL-c, HDL_2, or HDL_3 levels for participants who received D supplementation and who reported using an HMG-CoA RI compared to participants who received D supplementation but reported not using an HMG-CoA RI.

Conclusions: The results of this study do not support the hypotheses. A small sample size and additional limitations to this study may have contributed to the negative findings.

Key words: cardiovascular disease, total 25-hydroxyvitamin D, high-density lipoprotein-cholesterol, and high-density lipoprotein subclasses

INTRODUCTION

Approximately 70% of cardiac events will not be prevented despite the application of evidence-based treatment of modifiable cardiovascular disease (CVD) risk factors (Kones, 2011). This finding suggests that there are additional undiscovered CVD risk factors or unexplained mechanisms for suspected risk factors. The incidence of CVD decreases when primary prevention is appropriately implemented thereby decreasing known modifiable risk factors such as hypertension (HTN), hypercholesterolemia, and smoking (Farley, Dalal, Mostashari, & Frieden, 2010; Cooney et al., 2009). Primary prevention is reserved for individuals that meet a certain level of increased risk, so that benefits outweigh potential treatment risks (Pletcher, Tice, Pignone, & Browner, 2004). It is critically important that clinicians identify all known modifiable CVD risk factors, prescribe treatments per published guidelines, and improve patient adherence in order to decrease the incidence of cardiac events (Kones, 2011).

Evidence from numerous research studies supports low vitamin D (D) status as an additional risk factor for CVD (Weyland et al., 2014). D status is assessed by measuring serum 25-hydroxyvitamin D (25[OH] D) (Holick, 2009). The prevalence of deficient 25(OH) D levels, defined as < 20 ng/mL, in the United States is approximately 32% (Looker et al., 2011). The estimated worldwide prevalence of deficient 25(OH) D (< 20 ng/mL) and insufficient 25(OH) D (< 30 ng/mL) levels is one billion (Holick, 2007). Therefore, it is of paramount importance to determine if deficient serum 25(OH) D is a primary risk factor for CVD versus merely a biomarker.

One plausible explanation for why 25(OH) D levels change was proposed by Scragg (1981). He hypothesized that solar ultra-violet radiation varies by season and this causes D

metabolite status to fluctuate and contribute to the pattern of an increased morbidity and mortality rate in winter. Subsequently, numerous cross-sectional and prospective studies have shown the prevalence of CVD and CVD risk factors increases as 25(OH) D levels decrease. The CVDs and risk factors for CVD include; prevalent myocardial infarction (MI), angina, or stroke (25[OH] D levels < 20 ng/mL versus ≥ 20 ng/mL, odds ratio [OR], 1.20, 95% confidence interval [CI], 1.01, 1.36, $p = 0.03$) (Kendrick et al., 2009), MI, stroke, and heart failure (25[OH] D levels < 10 ng/mL versus ≥ 15 ng/mL, $p < 0.001$) (Wang et al. (2008), MI (25[OH] D levels ≤ 15 ng/mL versus ≥ 30 ng/mL, relative risk [RR], 2.42, 95% CI, 1.53, 3.84, $p < 0.001$) (Giovannucci et al., 2008), incident HTN (25[OH] D levels < 15 ng/mL versus ≥ 30 ng/mL, RR, 3.18, 95% CI, 1.39, 7.29) (Forman et al., 2007), incident HTN, hyperlipidemia, DM, and peripheral vascular disease (25[OH] D levels ≤ 15 ng/mL versus > 30 ng/mL, all $p < 0.0001$) (Anderson et al., 2010), hyperglycemia (fasting glucose: $\beta = -0.002$, $p = 0.02$) and insulin resistance (fasting insulin $\beta = -0.15$, $p = 0.01$) (Forouhi et al., 2008), and higher BMI ($\beta = -0.25$, $p < 0.0001$) (Da Costa et al., 2012).

Low high-density lipoprotein-cholesterol (HDL-c) level is an additional risk factor for CVD but the exact mechanism for this effect remains controversial (Khera et al., 2011). One aspect of the controversy involves the decision to use total HDL-c or a subclass or subfraction of HDL-c as the most accurate measure of atheroprotective lipoprotein. The subclasses are identified by size, density, protein composition, and charge (Movva & Rader, 2008). Decreasing quartiles of serum 25(OH) D levels were associated with lower serum HDL-c levels ($p < 0.001$) (Jorde et al., 2010). Additionally, in a cross-sectional study, children who had been supplemented with D_3-containing milk at school had higher levels of 25(OH) D and HDL-c versus children who had not been supplemented (both $p = 0.01$) (Graham et al., 2008). This

evidence is supportive, but well-designed randomized controlled trials (RCT) will provide the best evidence for the determination of a causal association between 25(OH) D and HDL-c levels.

The number of RCTs that have examined this association using D supplement pills, nutritional D sources, or ultra-violet β-wave exposure has increased but the results are inconsistent. A study by Shab-Bidar et al. (2011) included only participants with type 2 DM who were given either a yogurt drink with 500 IU D_3 or without D_3 twice daily for 12 weeks. A larger mean change in HDL-c from baseline to 12-weeks was found in the group given D_3 ($p = 0.04$). Al-Daghri et al. (2012) recommended increases in dietary D intake as well as increased sun exposure to participants in their study and then measured 25(OH) D levels. The researchers found that after adjusting for age and body mass index (BMI), 25(OH) D levels were positively correlated with HDL-c levels ($p < 0.001$). An additional RCT with positive results was performed by Hirschler et al. (2013). Children were given D_3 5,000 IU weekly for eight weeks and measured one year later. The researchers found the treated group had an increase in 25(OH) D levels ($p < 0.001$) and a decrease in the prevalence of low HDL-c (< 35 mg/dL) ($p < 0.01$).

Negative results were found by Maki et al. (2011) when they compared the percent change in HDL-c levels in participants with an elevated waist circumference after eight weeks of 1,200 IU D_3 (% change = 2.8) versus a multivitamin with minerals without D_3 (% change = 1.7) ($p = 0.653$). A meta-analysis of RCTs (n = 12 total intervention groups in 10 studies) examining the effects of D supplementation on lipid profiles was performed by Wang et al. (2012). The researchers found the pooled estimate of effect for a change in HDL-c was not statistically significant (difference in means = -0.14 mg/dL, 95% CI, -0.99, 0.71 mg/dL).

Given the evidence that is inconclusive regarding an association between D status and atheroprotective lipoproteins, we aimed to test the hypotheses that;

1) HDL-c, HDL_2, and HDL_3 levels will increase in participants supplemented with Vitamin D compared to participants who received a placebo; and

2) HDL-c, HDL_2, and HDL_3 levels will increase in participants supplemented with Vitamin D and who reported using an HMG-CoA RI, compared to participants who received D supplementation but reported not using an HMG-CoA RI.

MATERIALS AND METHODS

Research Design

This is a secondary analysis of serum sample data obtained from a randomized double-blinded placebo-controlled trial.

Participants

Socio-demographic and serum sample data were collected between 2008 and 2012 from study participants who were recruited from the San Francisco Bay Area for two multiple-phase studies; the *Effects of Vitamin D on CYP3A Substrate Clearance* (CHR approval number: H1775-28941) and the *Effects of Vitamin D on Lipids* (clinical trial number: NCT00723385). Participants ranged in age, race, and health status. Informed consents were obtained from all study participants and both studies were approved by the Committee on Human Research at the University of California, San Francisco.

Laboratory Measurements

Serum total 25(OH) D levels were measured using a liquid chromatography tandem mass spectrometry assay (Mayo Clinical Laboratories, Rochester, MN, certified by Clinical Laboratory Improvement Amendments). The coefficient of variation (CV) is approximately 10% at levels > 10 ng/mL. The internal standard used was from the National Institute of

Standards and Technology quality assurance program for analysis of D metabolites in human serum, funded by the National Institutes of Health Office of Dietary Supplements.

Serum HDL-c, HDL_2, and HDL_3 levels were included in the complete cholesterol assessment measured using the *Vertical Auto Profile II* (VAP II) test (Atherotech Diagnostics Lab). The VAP II test is a single vertical spin, inverted rate zonal, density gradient ultracentrifugation technique in which all of the cholesterol concentrations are measured simultaneously (Kulkarni, 2006). Levels of HDL_2 and HDL_3 are determined by the separation of the relative positions, the shapes of the curves, and the lipoprotein peaks in the density gradient. Kulkarni et al. (1997) reported a long-term between-rotor CV of 9% for HDL_2 and 5% for HDL_3.

Statistical Design and Data Analysis

The *IBM SPSS Statistics Version 22* program was used for all statistical calculations. Means and standard deviations are reported for continuous variable data and frequencies for categorical variable data. All data met the assumptions for the statistical tests that were used. The α-level was set at < 0.05 and all tests were two-tailed. The power of the study was set at 0.80 and it was determined that an effect size of $r \geq 0.36$ was likely to be detected as significant for hypothesis #1 and an effect size of $r \geq 0.43$ was likely to be detected as significant for hypothesis #2 (Hulley et al., 2007).

The Pearson Product Moment correlation was used to determine correlations between age, gender, BMI, HMG-CoA RI-use, and baseline and 12-week levels for total 25(OH) D, HDL-c, HDL_2, and HDL_3 for both hypothesis #1 and hypothesis #2. Caucasian race versus non-Caucasian race was added to the Pearson Product Moment correlation for hypothesis #2 to determine if there was a statistically significant correlation with the baseline or Week 12 values for HDL-c, HDL_2, or HDL_3. Repeated-Measures Analysis of Variance (RM-ANOVA) tests

were performed to evaluate hypothesis #1 and hypothesis #2. The RM-ANOVA had one between-subjects factor; group, and one within-subjects factor; time. This analysis allowed for testing the main effect of group, the main effect of time, and the group by time interaction. The test of the interaction determined if the change from baseline to 12 weeks was different for the two study groups.

RESULTS FOR HYPOTHESIS #1

Participant Characteristics

Serum sample data from 59 participants were included in the analysis to test hypothesis #1 (Table 1). There were 19% more men than women in the study and ages ranged from 25 to 91 years old. The most prevalent race was Caucasian (69.5 %), then Asian (16.9 %), and then African-American (11.9 %). There were no statistically significant differences in the D supplementation group versus placebo group for gender ($p = 0.579$), race ($p = 0.368$), age ($p = 0.734$), or BMI ($p = 0.251$).

Laboratory Values

The baseline range of 25(OH) D levels was from 7.5 ng/mL to 48.0 ng/mL. There were 12 participants in the placebo group and 23 in the D supplementation group who had a deficient (< 20 ng/mL) baseline 25(OH) D level. There were 6 participants in the placebo group and 13 in the D supplementation group who had an insufficient (≥ 20 and < 30 ng/mL) baseline 25(OH) D level. There was 1 participant in the placebo group and 4 in the D supplementation group who had a sufficient (≥ 30 ng/mL) baseline 25(OH) D level. The means of the baseline 25(OH) D and baseline HDL_2 levels for all participants were low at 19.8 ng/mL (sufficient ≥ 30 ng/mL) and 13.0 mg/dL (normal > 15 mg/dL), respectively. No statistically significant difference in the

means of the baseline levels for 25(OH) D ($p = 0.765$) or cholesterol levels of HDL-c ($p = 0.427$), HDL_2 ($p = 0.565$), or HDL_3 ($p = 0.419$) was found for the D supplementation group versus the placebo group.

A statistically significant greater mean 12-week 25(OH) D level ($p < 0.001$) for participants in the D supplementation versus placebo group was found. There were 3 participants in the placebo group and 26 in the D supplementation group who had a sufficient ($\geq$ 30 ng/mL) 12-week 25(OH) D level. There were 8 participants in the placebo group and 12 participants in the D supplementation group who had an insufficient ($\geq$ 20 and < 30 ng/mL) 12-week 25(OH) D level. There were 8 participants in the placebo group and 2 participants in the D supplementation group who had a deficient (< 20 ng/mL) 12-week 25(OH) D level.

Statistical Tests

The Pearson correlations showed a strong positive correlation between baseline and 12-week HDL-c, baseline and 12-week HDL_2, and baseline and 12-week HDL_3 (Table 2). Baseline and 12-week HDL-c were also strongly positively correlated with both baseline and 12-week HDL_2 and baseline and 12-week HDL_3. There was also a strong positive correlation between baseline and 12-week HDL_2 and baseline and 12-week HDL_3. Baseline total 25(OH) D was positively correlated with 12-week total 25(OH) D. Baseline and 12-week HDL-c, baseline and 12-week HDL_2, and baseline and 12-week HDL_3 were all positively correlated with female gender and negatively correlated with BMI. No statistically significant correlation was found between age and any other variable except HMG-CoA RI-use. HMG-CoA RI-use was positively correlated with BMI.

RM-ANOVA with one between-subjects factor; group, and one within-subjects factor; time, showed no statistically significant difference in the mean change in HDL-c, HDL_2 or HDL_3 for the group of participants who received D supplementation compared to the group of participants who did not receive D supplementation (Tables 3 to 5).

RESULTS FOR HYPOTHESIS #2

Participant Characteristics

Only the participants that received D supplementation were included in the analysis for hypothesis #2 (n = 40) (Table 6). There were 35% more men than women in the study and the most prevalent race was Caucasian (70.0 %), then Asian (20.0 %), and then African-American (10.0 %). There were more Caucasians than Asians or African-Americans in the D supplementation group with HMG-CoA RI use ($p = 0.007$).

Laboratory Values

The range of ages was also 25 to 91 years. The mean of the baseline 25(OH) D levels was low at 20.0 ng/mL (sufficient $\geq$ 30 ng/mL) as was the mean of the baseline HDL_2 levels at 12.7 mg/dL (normal > 15 mg/dL).

Statistical Tests

The same variables were correlated with each other except that BMI and HMG-CoA RI use were not correlated and 12-week total 25(OH) D was weakly correlated with 12-week HDL-c and 12-week HDL_3. Caucasian race was strongly correlated with age and HMG-CoA RI use but was not correlated with baseline or 12-week HDL-c, HDL_2 or HDL_3 (Table 7).

RM-ANOVA tests with one between-subjects factor; group, and one within-subjects factor; time, showed no statistically significant difference in the mean change in HDL-c, HDL_2 or HDL_3 for the group of participants who received D supplementation and reported using an HMG-CoA RI compared to the group of participants who received D supplementation but reported not using an HMG-CoA RI (Tables 8-10).

DISCUSSION

There was equal representation of gender, race, age span, and BMI values for the D supplemented group versus the placebo group for hypothesis #1. For hypothesis #2, the gender and BMI values were equally represented, but races other than Caucasian were underrepresented ($p = 0.007$) and the average age was higher ($p = 0.003$) in the HMG-CoA RI-use group versus the HMG-CoA RI non-use group. Because there were no significant correlations between either Caucasian race or age and either the baseline or 12-week levels of HDL-c, HDL_2 or HDL_3 (Table 7), neither race nor age was a source of confounding in the group comparisons.

The results also do not appear to be due to the lack of an effect of the D supplementation intervention. An effect can be assumed because there was no statistically significant difference in the baseline means for the 25(OH) D levels ($p = 0.765$) but there was a statistically significant difference in the means for the 12-week 25(OH) D levels for the D supplementation group versus the placebo group ($p < 0.001$). There were participants who had a sufficient baseline 25(OH) D level and participants who had an insufficient or deficient 25(OH) D level at 12-weeks. Because of this, there may have been less of an effect on cholesterol levels after 12 weeks of D supplementation. More of an effect may have been seen if all of the participants had been deficient initially and all of the participants in the D supplementation group had reached sufficiency at 12 weeks.

The Pearson correlations found between the variables in this study are similar to other studies. Freedman et al. (2004) also found that women had larger HDL (0.5 nm) particles and twice the number than in men (8 versus 4 μmol/L). A similar association between HDL_2 and female gender was found by Johnson et al. (2004) but HDL_3 was associated with male gender ($p < 0.0001$ for both). Da Costa et al. (2012) also found that BMI was inversely associated with HDL-c ($\beta = -0.61$, $p < 0.0001$), but contrary to the results in this study, they also found that BMI was inversely associated with 25(OH) D ($\beta = -0.25$, $p < 0.0001$). When Williams et al. (1993) further sub-classified the HDL particles, there was a similar negative association between BMI and HDL_{2b} ($p < 0.05$), but in contrast to this study there were positive associations between BMI and both HDL_{3b} ($p < 0.05$) and larger-diameter HDL_{3c} ($p < 0.05$).

It is important to continue to examine the role of low D status as a primary risk factor for CVD using the RCT design. The number of RCTs that have examined this association using D supplement pills, nutritional sources, or ultra-violet β-wave exposure has increased but the results remain inconsistent. Jorde and Grimnes (2011) addressed the issue of inconsistent results in their meta-analysis of 10 RCTs and 22 cross-sectional studies which examined associations between serum 25(OH) D and lipid levels. They identified three factors which may have contributed to the negative findings in the vast majority of the RCTs versus the positive findings in all of the cross-sectional studies included in their meta-analysis. The RCTs lacked sufficient power, none had been specifically designed to test the effect of 25(OH) D levels on lipid levels, and they all lacked inclusion criteria for dyslipidemia.

Heaney (2014) has proposed guidelines to increase the standardization of individual nutrient RCT study design and interpretation and to provide guidance for pooling multiple

nutrient research studies in meta-analyses or systematic reviews. Grant (2014) in an editorial has also addressed the shortcomings of these RCT results due to less than optimal study designs.

Appropriately designed RCTs will provide results that can be used to update clinical practice guidelines including the minimum sufficient serum 25(OH) D level for optimal health. Grant (2009) determined there would be a significant benefit to increasing the current recommendation to at least 45 ng/mL. Doses as high as 100,000 IU at least every 2 months maintain adequate and safe levels of 25(OH) D in healthy individuals (Ilahi et al., 2008). Adverse effects or toxicity from vitamin D supplementation appear to be rare even with doses as high as 10,000 IU per day (Garland et al., 2011). Clinical guidelines should also include how to individualize safe sun exposure to maximize the benefit (Grant, 2009; Brøndum-Jacobsen et al., 2013) and decrease the risk as much as possible.

Limitations

There are several limitations in this study. First, there was a relatively small number of participants. Including a larger number of participants would increase the power of the study. Second, the mean 12-week 25(OH) D level for D supplemented participants was 33.3 ng/mL, which is just slightly above the sufficient level of 30 ng/mL with some participants' 12-week level remaining below sufficient status despite supplementation. Third, total HDL-c, HDL_2, and HDL_3 may not be the most accurate measures of cardioprotective lipoproteins and thus not the most accurate measure to use. Quantification of the more extensively characterized HDL particles requires further separation but using these values may lead to more accurate results (Superko et al., 2012; Kim et al., 2014).

CONCLUSIONS

The results of this study do not support either hypothesis in this study. The results do not appear to be due to confounding or the lack of an effect of the D supplementation intervention. The correlations between the variables are similar to other studies but the limitations of the study may have contributed to the negative findings.

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Table 1. Study participant characteristics and laboratory values, N = 59 (Hypothesis #1)

	Total	Vitamin D Supplementation Group	Placebo Group	p-value*
	N (%)	N (%)	N (%)	
Study Participants	59	40 (67.8)	19 (32.2)	
Gender: Men	32 (54.2)	23 (57.5)	9 (47.4)	
Women	27 (45.8)	17 (42.5)	10 (52.6)	.579
Race: Caucasian	41 (69.5)	28 (70.0)	13 (68.4)	
Asian	10 (16.9)	8 (20.0)	2 (10.5)	
African-American	7 (11.9)	4 (10.0)	3 (15.8)	.368
Other	1 (1.7)	0 (0)	1 (5.3)	
	mean ± SD	**mean ± SD**	**mean ± SD**	**p-value* Vitamin D Supplementation Group versus Placebo Group**
Age (years)	56.0 ± 17.4	56.5 ± 18.3	54.9 ± 15.9	.734
Weight (kg)	81.7 ± 21.1	84.3 ± 21.3	76.1 ± 20.1	.167
Height (cm)	168.1 ± 8.7	168.7 ± 9.3	166.7 ± 7.2	.392
BMI (kg/m^2)	28.8 ± 6.8	29.5 ± 6.8	27.4 ± 6.8	.251
Baseline total 25(OH) D (ng/mL)	19.8 ± 8.5	20.0 ± 9.0	19.3 ± 7.5	.765
Baseline HDL-c (mg/dL)	51.2 ± 15.2	50.1 ± 15.9	53.5 ± 13.6	.427
Baseline HDL_2 (mg/dL)	13.0 ± 6.4	12.7 ± 6.8	13.7 ± 5.4	.565
Baseline HDL_3 (mg/dL)	38.1 ± 9.5	37.4 ± 9.7	39.6 ± 8.9	.419

* Independent-samples t-test, p-value < 0.05 = significant and all p-values are 2-tailed, SD = standard deviation, BMI = body mass index, 25(OH) D = 25-hydroxyvitamin D, and HDL-c = high-density lipoprotein-cholesterol

Table 2. Pearson Correlation Coefficients, N = 59 (Hypothesis #1)

	Female gender	BMI	HMG-CoA RI	Total D BL	Total D week-12	HDL-c BL	HDL-c week-12	HDL_2 BL	HDL_2 week-12	HDL_3 BL	HDL_3 week-12
Age	-.050	.002	.427**	-.121	-.129	.068	.090	.128	.100	.028	.077
Female Gender		.012	-.207	-.158	-.071	.582**	.497**	.503**	.417**	.591**	.522**
BMI			.257*	-.170	-.119	-.366**	-.446**	-.407**	-.393**	-.309*	-.448**
HMG-CoA RI				.084	.117	-.122	-.104	-.085	-.068	-.136	-.120
Total D BL					.487**	.041	.129	.113	.063	-.009	.158
Total D week-12						.157	.177	.161	.110	.145	.202
HDL-c BL							.896**	.933**	.842**	.970**	.881**
HDL-c week-12								.853**	.945**	.858**	.977**
HDL_2 BL									.841**	.819**	.814**
HDL_2 week-12										.780**	.855**
HDL_3 BL											.862**

* = correlation is significant at the 0.05 level (2-tailed), ** = correlation is significant at the 0.01 level (2-tailed), BMI = body mass index, HMG-CoA RI = 3-hydroxy-3-methyl-glutaryl-Co-enzyme A reductase inhibitor, D = 25-hydroxyvitamin D, BL = baseline, HDL-c = high-density lipoprotein-cholesterol

Table 3. Differences in HDL-c between baseline and Week 12 by Groups (Hypothesis #1)

Vitamin D Supplementation				Placebo		
Time period	**n**	**M**	**SD**	**n**	**M**	**SD**
HDL-c Baseline	**40**	**50**	**15.93**	**19**	**53**	**13.59**
HDL-c 12-week	**40**	**50**	**15.43**	**19**	**53**	**13.50**
Source		**df**		**F**		**p-value**
Main Effect of Group		**1, 57**		**0.61**		**.438**
Main Effect of Time		**1, 57**		**.143**		**.706**
Group by Time Interaction		**1, 57**		**0.05**		**.824**

HDL-c = high-density lipoprotein-cholesterol

Table 4. Differences in HDL_2 between baseline and Week 12 by Groups (Hypothesis #1)

Vitamin D Supplementation				Placebo		
Time period	**n**	**M**	**SD**	**n**	**M**	**SD**
HDL_2 Baseline	**40**	**13**	**6.83**	**19**	**14**	**5.42**
HDL_2 12-week	**40**	**12**	**6.58**	**19**	**14**	**4.72**
Source		**df**		**F**		**p-value**
Main Effect of Group		**1, 57**		**.604**		**.440**
Main Effect of Time		**1, 57**		**.404**		**.528**
Group by Time Interaction		**1, 57**		**.280**		**.599**

HDL = high-density lipoprotein

Table 5. Differences in HDL_3 between baseline and Week 12 by Groups (Hypothesis #1)

	Vitamin D Supplementation			Placebo		
Time period	**n**	**M**	**SD**	**n**	**M**	**SD**
HDL_3 Baseline	**40**	**37**	**9.74**	**19**	**40**	**8.91**
HDL_3 12-week	**40**	**38**	**9.30**	**19**	**39**	**9.51**
Source		**df**		**F**		**p-value**
Main Effect of Group		**1, 57**		**.490**		**.487**
Main Effect of Time		**1, 57**		**.060**		**.807**
Group by Time Interaction		**1, 57**		**.302**		**.585**

HDL = high-density lipoprotein

Table 6. Study participant characteristics and laboratory values, n = 40 (Hypothesis #2)

	Total	Vitamin D Supplementation and HMG-CoA RI	Vitamin D Supplementation without HMG-CoA RI	p-value*
	N (%)	N (%)	N (%)	
Study Participants	40	18 (45.0)	22 (55.0)	
Gender: Men	23 (57.5)	13 (72.2)	10 (45.5)	
Women	17 (42.5)	5 (27.8)	12 (54.5)	.116
Race: Caucasian	28 (70.0)	17 (94.4)	11 (50.0)	
Asian	8 (20.0)	0 (0)	8 (36.4)	.007
African-American	4 (10.0)	1 (5.6)	3 (13.6)	
	mean ± SD	**mean ± SD**	**mean ± SD**	**p-value* Vitamin D Supplementation with versus without HMG-CoA RI**
Age (years)	56.5 ± 18.3	65.7 ± 13.3	49.0 ± 18.7	.003
Weight (kg)	84.3 ± 21.3	90.9 ± 20.1	78.9 ± 21.1	.075
Height (cm)	168.7 ± 9.3	170.5 ± 8.9	167.3 ± 9.5	.276
BMI (kg/m^2)	29.5 ± 6.8	31.3 ± 6.5	28.1 ± 6.8	.139
Baseline total 25(OH) D (ng/mL)	20.0 ± 9.0	21.0 ± 9.7	19.3 ± 8.5	.551
Baseline HDL-c (mg/dL)	50.1 ± 15.9	47.4 ± 14.5	52.2 ± 17.0	.351
Baseline HDL$_2$ (mg/dL)	12.7 ± 6.8	11.5 ± 7.0	13.6 ± 6.7	.342
Baseline HDL$_3$ (mg/dL)	37.4 ± 9.7	35.9 ± 8.3	38.6 ± 10.8	.379

* Independent-samples t-test, p-value < 0.05 = significant and all p-values are 2-tailed HMG-CoA RI = 3-hydroxy-3-methyl-glutaryl-Co-enzyme A reductase inhibitor, SD = standard deviation, BMI = body mass index, 25(OH) D = 25-hydroxyvitamin D, HDL-c = high-density lipoprotein-cholesterol

Table 7. Pearson Correlation Coefficients, N = 40 (Hypothesis #2)

	Female gender	Caucasian	BMI	HMG-CoA RI	Total D BL	Total D week-12	HDL-c BL	HDL-c week-12	HDL_2 BL	HDL_2 week-12	HDL_3 BL	HDL_3 week-12
Age	-.022	.484**	.027	.460**	-.010	-.078	.105	.070	.169	.100	.060	.043
Female Gender		-.099	.078	-.269	-.211	-.019	.491**	.393*	.435**	.340*	.503**	.404**
Caucasian			.135	.482**	-.156	-.168	-.155	-.143	-.141	-.180	-.108	-.032
BMI				.238	-.211	-.261	-.410**	-.531**	-.405**	-.449**	-.385*	-.552**
HMG-CoA RI					.097	.103	-.151	-.180	-.154	-.111	-.139	-.222
Total D BL						.486**	.017	.143	.117	.088	-.054	.169
Total D week-12							.303	.337*	.289	.275	.287	.356*
HDL-c BL								.892**	.945**	.839**	.972**	.886**
HDL-c week-12									.876**	.955**	.845**	.979**
HDL_2 BL										.850**	.844**	.854**
HDL_2 week-12											.775**	.876**
HDL_3 BL												.852**

* = correlation is significant at the 0.05 level (2-tailed), ** = correlation is significant at the 0.01 level (2-tailed)

BMI = body mass index, HMG-CoA RI = 3-hydroxy-3-methyl-glutaryl-Co-enzyme A reductase inhibitor,

D = 25-hydroxyvitamin D, BL = baseline, HDL-c = high-density lipoprotein-cholesterol

Table 8. Differences in HDL-c between baseline and Week 12 by Groups (Hypothesis #2)

D Supplementation/HMG-CoA RI				D Supplementation/No HMG-CoA RI		
Time period	**n**	**M**	**SD**	**n**	**M**	**SD**
HDL-c Baseline	**18**	**47**	**14.51**	**22**	**52**	**17.03**
HDL-c 12-week	**18**	**47**	**14.82**	**22**	**52**	**15.80**
Source		**df**		**F**		**p-value**
Main Effect of Group		**1, 38**		**1.134**		**.294**
Main Effect of Time		**1, 38**		**.025**		**.874**
Group by Time Interaction		**1, 38**		**.099**		**.755**

D = vitamin D, HMG-CoA RI = 3-hydroxy-3-methyl-glutaryl-Co-enzyme A reductase inhibitor, HDL-c = high-density lipoprotein-cholesterol

Table 9. Differences in HDL_2 between baseline and Week 12 by Groups (Hypothesis #2)

D Supplementation/HMG-CoA RI				D Supplementation/No HMG-CoA RI		
Time period	**n**	**M**	**SD**	**n**	**M**	**SD**
HDL_2 Baseline	**18**	**12**	**7.00**	**22**	**14**	**6.70**
HDL_2 12-week	**18**	**11**	**6.72**	**22**	**13**	**6.56**
Source		**df**		**F**		**p-value**
Main Effect of Group		**1, 38**		**.741**		**.395**
Main Effect of Time		**1, 38**		**.844**		**.364**
Group by Time Interaction		**1, 38**		**.294**		**.591**

D = vitamin D, HMG-CoA RI = 3-hydroxy-3-methyl-glutaryl-Co-enzyme A reductase inhibitor, HDL = high-density lipoprotein

Table 10. Differences in HDL_3 between baseline and Week 12 by Groups (Hypothesis #2)

D Supplementation/HMG-CoA RI				D Supplementation/No HMG-CoA RI		
Time period	**n**	**M**	**SD**	**n**	**M**	**SD**
HDL_3 Baseline	**18**	**36**	**8.32**	**22**	**39**	**10.81**
HDL_3 12-week	**18**	**36**	**8.31**	**22**	**40**	**9.85**

Source	df	F	p-value
Main Effect of Group	**1, 38**	**1.371**	**.249**
Main Effect of Time	**1, 38**	**.334**	**.567**
Group by Time Interaction	**1, 38**	**.716**	**.403**

D = vitamin D, HMG-CoA RI = 3-hydroxy-3-methyl-glutaryl-Co-enzyme A reductase inhibitor, HDL = high-density lipoprotein

Chapter 5

Conclusions

Objectives for the Dissertation

The objectives for this dissertation were to:

1) Perform a review of original research studies as well as meta-analyses in order to determine the likelihood of a causal association between Hypovitaminosis D and increased cardiovascular disease (CVD) risk.

2) Perform a secondary analysis of serum sample data examining the associations between both serum total and free 25-hydroxyvitamin D (25[OH] D) levels and serum high-density lipoprotein-cholesterol (HDL-c) and HDL subclass levels using a cross-sectional study design.

3) Perform an additional secondary analysis of serum sample data to determine if vitamin D (D) supplementation for 12 weeks leads to an increase in serum HDL-c or HDL subclass levels.

4) Describe the implications of the results of these studies for research, clinical practice, and health policy.

Purpose and Findings of the Review

The purpose of the review was to determine the likelihood of a causal association between Hypovitaminosis D and increased risk for CVD. Sir Austin Bradford Hill's criteria for causality in a biological system (Hill, 1965) were used to evaluate the results of current original research studies as well as meta-analyses. Hill's criteria were originally used to evaluate the likelihood of a causal association between an agent and an infectious disease but have also been

used to evaluate the likelihood of a causal association between low D status and overall cancer risk (Grant, 2009), breast cancer risk (Mohr et al., 2012), periodontal disease (Grant & Boucher, 2010), and multiple sclerosis (Hanwell & Banwell, 2011). The criteria that were considered to be relevant to this evaluation were: analogy, biological gradient, consistency, experiment, plausibility, strength, and temporality (Table 2.1). All of the relevant Hill's criteria were supported by research results and therefore current scientific evidence supports a causal association between low serum 25(OH) D levels and increased risk for CVD.

Purpose and Findings of the Secondary Analyses

The purpose of both the cross-sectional analysis and the D supplementation versus placebo study was to provide evidence for a mechanism of CVD development. Isolating primary risk factors that cause CVD is challenging because the human body responds to disrupted homeostasis by up- and down-regulation of cellular function. Several cellular-level causative mechanisms have been proposed including low serum 25(OH) D levels leading to decreases in the atheroprotective lipoproteins including HDL-c and the HDL subclasses. Causative mechanisms in this context do not necessarily compete with one another and are not mutually exclusive; some or all of the proposed mechanisms may be accurate.

In the cross-sectional analysis, positive associations between total 25(OH) D and HDL-c, HDL_2, and HDL_3 and between free 25(OH) D and HDL-c and HDL_2 were found. After controlling for gender, body mass index, and estrogen use neither total 25(OH) D nor free 25(OH) D showed a statistically significant unique contribution to the total variance in HDL-c, HDL_2, or HDL_3. Gender and body mass index uniquely contributed to the total variance in HDL-c, HDL_2, and HDL_3 in all models. Estrogen use uniquely contributed to the total variance

in HDL_2 for both models. These results do not support the study hypothesis that low D status leads to a decrease in atheroprotective lipoprotein levels.

In the D supplementation versus placebo study, there was no statistically significant difference in HDL-c, HDL_2, or HDL_3 levels for participants who received D supplementation for 12 weeks compared with participants who received a placebo. There was also no statistically significant difference in HDL-c, HDL_2, or HDL_3 levels for participants who received D supplementation and who reported using an HMG-CoA reductase inhibitor compared to participants who received D supplementation but reported not using an HMG-CoA reductase inhibitor. The results of this study do not support the study hypotheses.

Implications of Research

The review results imply that there is a causal association between low serum 25(OH) D levels and increased CVD risk but this is not the end-point for this determination. Hill's criteria for causality in a biological system were first used to detect causal associations by documenting occupational injury or illness and then looking for rare or occult antecedent hazardous work conditions. The criteria are equally useful when the goal is to determine a causal association between a disease and a physiological phenomenon but verification of these results using other study designs such as randomized controlled trials (RCT) designed to test nutrients is necessary. Results of future D RCTs will then confirm one or more physiological pathways for the progression from a low D status to increased CVD risk.

The negative results for the cross-sectional study and the D supplementation versus placebo study may be due to several design limitations. Both of the studies included a relatively small number of participants; a larger number of participants would have increased the power of each of the studies. Total HDL-c, HDL_2, and HDL_3 may not be the most accurate measures of

atheroprotective lipoproteins; the accuracy of the atheroprotective lipoprotein level for participants for both studies may have improved if there was a more detailed analysis of HDL. Quantification of the more extensively characterized HDL particles requires a more complex assay process but using these values may lead to more accurate results (Superko et al., 2012; Kim et al., 2014).

There were additional limitations for the D supplementation versus placebo study: the inclusion criteria did not include D deficiency; the mean 12-week serum 25(OH) D level for D supplemented participants was 33.3 ng/mL, which is just slightly above the sufficient level of 30 ng/mL; and some participants' 12-week serum 25(OH) D level remained below sufficient status despite supplementation. The implications of these results include: future studies should have an adequate number of participants to increase the power of the study to detect a significant difference; a deficient baseline serum 25(OH) D level should be an inclusion criterion; and a sufficient serum 25(OH) D level for a pre-specified period of time should be the study end-point.

Clinical Implications

The review results support a causal association between serum 25(OH) D levels and CVD risk. Therefore, serum 25(OH) D levels should be assessed and treated. The inclusion of all known risk factors for CVD into a risk assessment will improve the accuracy of the prediction of risk and lead to an increase in the efficacy and cost-effectiveness of primary prevention (Pletcher et al., 2004). There are many risk factors for Hypovitaminosis D including; older age, higher skin melanin content, higher latitude and lower altitude of residence, low dietary intake of D, certain medications, renal and liver disease, and either low levels of sun exposure or sunscreen use during sun exposure.

Results from epidemiological studies suggest increasing serum 25(OH) D levels to ≥ 45 ng/mL, which is approximately double the current recommendation, can significantly decrease

CVD-related mortality both in the US (Grant, 2009) and worldwide (Grant, 2011). Raising awareness of the availability of high-quality clinical practice guidelines will provide the clinician with additional resources for decision-making regarding the diagnosis and treatment of Hypovitaminosis D. Currently, there is a tendency for clinicians to rely on patient demand or information from casual conversations with colleagues and specialists (Epling et al., 2014) to test for and treat low 25(OH) D levels.

Potential benefits of the reduction of a risk factor for CVD should outweigh potential risks. Repletion of serum 25(OH) D levels can be accomplished by consuming D-rich foods, but this goal is most easily accomplished with D supplementation with doses as high as 10,000 IU per day being unlikely to lead to toxic effects (Holick, 2007). Furthermore, more severe deficiencies in serum 25(OH) D levels show a more rapid increase than less severe deficiencies (Lappe & Heaney, 2012). Treatment for some CVD risk factors is expensive and may be difficult to access, but D supplements are readily accessible and reasonably priced.

Health Policy Implications

The results of these studies have potential implications for health policy including: whether or not to fortify food with D; which foods to fortify; how much D to add; and whether to add D_3 (cholecalciferol) or D_2 (ergocalciferol). D can also be acquired by sun exposure or tanning bed use. Policies which regulate tanning bed use including: their use by minors; exposure-time restrictions; and the amount of taxation for tanning bed use, may change if further evidence suggests the health risks due to low 25(OH) D levels are greater than the risks due to tanning bed use. All of these policy decisions will remain controversial until several controversies surrounding low 25(OH) D levels are resolved: low 25(OH) D levels are confirmed to be a precursor to disease versus a biomarker for disease versus both a precursor to and a

biomarker for disease; a determination of a sufficient serum 25(OH) D level to prevent all associated diseases; and the level of serum 25(OH) D that is toxic for various ages and health conditions.

Future Directions

Further research is needed because there may be multiple direct and indirect pathways between Hypovitaminosis D and increased CVD risk. Additionally, CVD is a group of diagnoses, including CAD, heart failure, essential HTN, hypertensive renal disease, cardiac dysrhythmias, rheumatic heart disease, cardiomyopathy, pulmonary heart disease and cerebrovascular disease (Miniño & Klein, 2010) and there may be very distinct pathophysiological processes related to these various diagnoses. RCTs should also incorporate the complex pharmacokinetic and pharmacodynamic properties of D in the study design: the dose-response curve, the avoidance of toxicity, and the use of the most accurate and precise serum assays.

D is a nutrient and is more appropriately studied in the context of preventive healthcare strategies. Also, the most appropriate measure of D status needs to be determined. There is a consensus that of the currently available D metabolite assays, the most accurate one to use to determine overall D status is serum total 25(OH) D (Holick, 2009). But if the *free hormone hypothesis* (Mendel, 1989) applies to D metabolites, the serum free 25(OH) D assay (Future Diagnostics B.V., Wijchen, The Netherlands) not the total 25(OH) D assay may become regarded as the most accurate measure of overall D status.

It is also important that a consensus be reached regarding the definition of deficient serum 25(OH) D levels for use in research studies. Serum 25(OH) D levels can be statistically analyzed as a continuous variable or they can be grouped according to cut-points and analyzed as

a categorical variable. The comparison of research study results and the inclusion of studies in meta-analyses will be facilitated by a consistent definition of 25(OH) D deficiency as well as the use of consistently defined cut-points.

The credibility of study results is also dependent upon the presence and adequacy of a report of the adherence to the D supplementation intervention. Furthermore, an inquiry about concurrent use of personal D supplementation needs to be consistent across studies. Negative study results may simply be attributed to a lack of adherence to the intervention or to an excessive amount of personal D supplementation or both.

Future research regarding the beneficial effects of exposure to sunlight, the most effective way to increase serum 25(OH) D levels, will contribute to the creation of guidelines for sensible sun exposure (Pludowski et al., 2013). The development of guidelines for sun exposure should take into consideration sunburn risk, latitude and elevation of residence, and usual amount of sun exposure. An appropriate goal for serum 25(OH) D levels may be 40 ng/mL which is naturally achieved by individuals who spend more than the average number of hours outside in the late morning and early afternoon on a daily basis (Vieth, 2006).

D is a nutrient and the most important direction for future research will be the use of the recently proposed rules for individual nutrient RCT design and for meta-analysis or systematic review inclusion criteria for nutrient RCTs (Heaney, 2014). The rules are based on the nutrient dose-response curve; the dose is the serum 25(OH D level and the response is the change in the disease or other dependent variable. The adoption and consistent use of these guidelines will also facilitate the comparison of results among D studies.

Summary

Sir Austin Bradford Hill's criteria for causality in a biological system (Hill, 1965) were used to evaluate the results of current original research studies as well as meta-analyses

regarding low serum 25(OH) D levels and increased CVD risk. All of the relevant criteria were satisfied and therefore, it is likely that a causal association exists between low serum 25(OH) D levels and increased risk for CVD. The results of the secondary analysis studies did not support the study hypotheses but that may have been due to several limitations of the studies. The implication of the study results is that additional D RCTs designed for the study of nutrients should be conducted in order to add to the knowledge of the association between Hypovitaminosis D and increased CVD risk. Several implications for clinical practice and health policy were described and future directions were discussed.

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Appendix: University of California, San Francisco, Committee on Human Research

Original data were collected as part of the following grants for which Dr. Janice Schwartz was the primary investigator;

Pfizer IIR WS526663, Effects of vitamin D on lipids in atorvastatin treated patients

R56 AG15982, Older Persons and Drugs: race, gender and age effects

The *Effects of Vitamin D on Lipids* study was submitted to the CHR at UCSF as follows; *a 12 week study of the effects of supplementation with 800-1000 IU vitamin D on lipid and vitamin D concentrations. At baseline and after 6 and 12 weeks of vitamin D or placebo, vitamin D and its OH-metabolites and fasting lipid levels (cholesterol, LDL-cholesterol (directly measured), HDL-cholesterol, triglycerides, and Lipoprotein(a) and Hemoglobin A1c and highly specific C-reactive protein (CRP) concentrations will be measured. Dietary intake of vitamin D will be estimated by dietary recall questionnaire or analysis of three 24-hour dietary intake logs.*

The *Effects of Vitamin D on CYP3A substrate clearance* study procedure was submitted to the CHR at UCSF as follows; *to test the hypotheses that high/supplemented vitamin D intake will increase CL/F (Clearance/bioavailability) of atorvastatin and conversely, that removal of high/supplemented vitamin D intake will decrease atorvastatin CL/F. We will also measure lipid concentrations to evaluate the pharmacodynamic and clinical consequences of altered CL/F. The focus is on clinical populations including the elderly, frail, and minorities under steady-state conditions and with conditions reflecting "usual" clinical conditions with use of sparse sampling techniques and minimal impact on clinical care. We propose 1) study of atorvastatin CL/F and lipid concentrations (total cholesterol, LDL, HDL) in patients receiving atorvastatin and vitamin D at baseline (ON-D) followed by vitamin D discontinuation for 6 weeks and repeat studies*

(OFF-D) of atorvastatin CL/F and lipid concentrations (total cholesterol, LDL, HDL) and 2) study of atorvastatin CL/F and lipid concentrations (total cholesterol, LDL, HDL) in patients receiving atorvastatin without supplemental vitamin D at baseline (NO-D) followed by vitamin D administration for 6 weeks and repeat atorvastatin CL/F and lipid concentrations (total cholesterol, LDL, HDL) (ON-D). We will give the same formulations of vitamins and same atorvastatin formulation to 80 participants. Vitamin D dosage (800 IU/day) was made based on the current prescribing at the nursing home that routinely prescribes vitamin D and multivitamins (Centrum silver). We will use the same laboratory for all cholesterol determinations and assays for drug concentrations will be done at one laboratory. We have chosen the duration of study periods of six weeks as follows: intestinal CYP3A turnover is estimated at 3-4 days—based on reports of time required to return to basal CL rates after grapefruit juice ingestion. New steady-state levels of CYP3A should thus be present after about 10-15 days weeks of dosing with new steady-state concentrations of atorvastatin at about two days later and cholesterol changes are usually assessed after four weeks at a stable atorvastatin dosage.

Inclusion criteria: (for the *Effects of Vitamin D on Lipids Study*)

a) Age 18 years or older

b) Able to give written informed consent

c) Screening 25(OH) D level ≥ 10 ng / mL and < 25 ng / mL (Mayo Laboratories)

Exclusion criteria:

a) Currently prescribed lipid-lowering agents that are CYP3A metabolized with a narrow therapeutic index

b) Absorption / ingestion issues (needs pills crushed, feeding tube, history of an intestinal or gastric bypass, or Vitamin D allergy)

c) History of kidney stones, osteoporotic fracture, lack of birth control method for pre-menopausal women, or detectable viral load for subjects on protease inhibitors (if on antiretroviral therapy; current regimen ≤ 6 months, viral load > 40 (PCR) or > 75 (bDNA), lowest CD4 count before therapy > 200, and current CD4 count < 250)

d) Any medication dose change ≤ 2 weeks prior to the visit

e) Starting or discontinuing a medication ≤ 1 month prior to the visit

f) Recent illnesses, surgeries, sarcoidosis, or cancer

g) Severe renal failure (eGFR ≤ 28 mL / min / 1.73 m^2) or current renal dialysis

h) Recent transfusion and / or screening Hct < 30% for women, < 34% for men

i) Elevated serum calcium

Protection of Human Subjects

Risks to Human Subjects

a. Human Subjects Involvement and Characteristics

Human subjects are involved in all proposed studies and are patients that are clinically stable. It is anticipated that the research team would have contact with between 80-100 people in order to complete studies in in the target number of subjects (60). It is anticipated that about 70 people will have in person screening evaluation visits that involve questions as to medical history, demographics, medical information, and informational review of the study and blood

drawn for screening purposes (10-20 cc). Study involvement for an individual participant is described in detail below.

Pre-entry for all subjects: 1) Screening for eligibility, including questions as to medical history and demographic information-phone call of 10-20 minutes.

At Entry for all subjects: 1) Consent and HIPAA, 2) Medical review of history and medications, 3) Blood sampling (10-20 cc)-visit of 1-1.5 hours.

Study Participation

Week 0 research visit (CRC): Medication intake review and status update with height, weight, and blood pressure measured, blood draw for fasting lipids, vitamin D and vitamin D-OH metabolites, and blood drawn for atorvastatin concentrations before and 0, 5, 1, 2, 3, 8, 10 and 12 hours after dosing. Vitamin D or matching placebo dispensed.-visit of 16-20 hr. (Subset only for CYP3A study)

Week 1-6: daily intake of study capsule. Research team phone contacts every two weeks (phone call of 5-20 minutes). At the end of week 6, research visit with fasting blood sampling for 25-OH vitamin D and cholesterol and other lipid concentrations and weight and blood pressure measured, sunshine exposure (and Block dietary recall questionnaire per parent grant)-and muscle testing performed. Visit of 2 hours.

Week 7-12: daily vitamin D or placebo. Research team phone contacts every two weeks (phone call of 5-20 minutes). At the end of the week 1 (or 13), a research visit to the CRC occurs with repeats of all tests done at the week 0 research visit (fasting blood sampling for vitamin D and cholesterol concentrations, atorvastatin, blood sampling, muscle strength testing). Payment is processed and subject receives within 6 weeks of study completion. (Phase III VitDalone

participants had sparse sampling, single dose level drawn at baseline, week 6, and week 12 if on a statin or protease inhibitor).

Subjects are offered the opportunity to consent to optional "genotyping" at the time of study entry. If they consent, buffy coat from one or more of the blood draws would be obtained. Samples are collected and processed at the P.I. site and are assigned an experimental code i.d. and no subject names or information are on the samples for DNA extraction and storage. Goals for genetic analysis are not established at this time but an overall goal of the P.I. is to analyze genes responsible for drug or vitamin metabolism or responses. Depending on the results of the study, the vitamin D receptor genes or cholesterol metabolizing genes might be analyzed at a future date.

Exclusion criteria are those related to the clinical stability of the subject to avoid confounding of results by clinical instability or changes in therapies, and those that would increase the potential risk of vitamin D administration (hypercalcemia, active malignancy, narrow therapeutic index CYP3A substrate medications (protease inhibitors were allowed in Phase III VitDalone Study and viral load was monitored), severe renal failure) or the inability to provide informed consent. Per local policy, no students or staff of the investigator will be enrolled.

b. Sources of Material

Health information, physical examination materials and blood will be obtained from participants. Reviews of medical records will also be performed. Laboratory measures of lipids, Vitamin D concentrations, calcium, creatinine, will be made and results stored in research files. In diabetics, HbA1C will also be measured. Data are available only to research staff and reside in password protected computers or research files in locked offices.

c. Potential Risks

For the blood draws, risks are those of venipuncture and include minimal pain and discomfort, and rarely, infection. Blood draws will be performed /placed by trained and highly qualified personnel trained in venipuncture techniques in the elderly (blood pressure cuff instead of tourniquet)small gauge needles or butterfly or angiocatheter insertion, compression for > 2 minutes after venipuncture). Records of numbers of attempts to perform venipuncture are kept as are data regarding bruising, and phlebotomist, and insertion site at the end of the study (recorded on study data collection forms). Those not meeting standards (< three attempts per person, one bruise/month) are retrained or removed from this responsibility. Patients with anemia of clinical significance (hematocrit < 30% in women and < 34% in men) will be excluded. This is the cutoff that has been approved locally for participation in research studies with "relatively" sparse blood sampling such as those proposed (less than half of blood donation levels at no less than 2 week frequencies) and has been the criteria for all research studies of the P.I. to date.

Research staff will be in contact with participants at two week intervals throughout and will ask regarding side effects (specific questions: any new symptoms or health problems or changes in blood pressure visits to physicians, changes in medications or changes in muscle function or other body parts, is health the same or different from study entry)-any changes of more than one unit of self-reported health status will be further investigated as to potential causality of study medication (units are excellent, very good, good, fair, poor). Phone interview questions related to any changes in status, visits to doctors, change in medications are reviewed with the P.I. within 24 hours for reports of medication changes, visits to hospitals, or changes in health status, and weekly for other parameter changes.

The risk of excess vitamin D concentrations is low…(relevant to the participants receiving vitamin D).

Adequacy of Protection against Risk

a. Recruitment and Informed Consent

Informed consent will be obtained by the investigator or trained research personnel. All subjects will receive a full verbal explanation of the proposed study with the potential risks and benefits as well as being provided a full written copy of the consent form for review. There is no limit to the time prospective studies will be given to consider study participation. It is considered that the subjects understand the information provided when they can respond with the answers that:

a) the research is voluntary

b) involves review of their medical history and medications and some loss of privacy

c) will take at least twelve weeks of their time

d) will require blood sampling three times after study entry and will occur over 16-20 hr on two of the days (CYP3A subset participants only)

e) participation will involve 12 weeks of vitamin D or placebo capsules daily

Consent may be obtained in offices of the research team or clinic rooms of facilities. Consent will be obtained in writing. For patients speaking languages other than English, a translator will be available during the consent process (and during biweekly phone calls and research visits). HIPPA release forms are provided in the language of the participant. A copy of the signed consent form will be retained by the investigator, a copy will be provided for the

participant's permanent medical records, and a copy will be provided to the subject. During the conduct of the study, participation can be terminated at any time at the request of the subject.

b. Protections against risk

Interviews are held in private rooms, and medical records are obtained for review for community-dwelling subjects, electronic password protected databases are accessed upon authorization or institutional charts are reviewed in privacy by study personnel, obtained records are treated as confidential. All staff are required to complete training in the ethical conduct of research and are also trained in the requirements of health information privacy acts. Information will be confidential to the extent of the law and stored in secure databases on secure and password protected computers and secure facilities (guarded facility entrance, individual locked and entry controlled research offices). Hard copy files are identified only by subject research identifiers. Blood obtained from subjects will be used only for research purposes and storage labels are identified only with research identifiers. Data collected for research purposes are held as confidential. Research tests and results do not involve tests that could affect insurability or other social risk or risk to reputation.

This study will be performed by qualified health care professionals and involves minimal risk to participants. Twenty-four hour access to research personnel is available via phone or message service.

There is no financial risk as research study tests are not billed to subjects and subjects are paid for participation. Payment amounts are considered noncoercive and similar to those used in past studies and approved by the UCSF CHR.

Potential Benefits of the Proposed Research to human subjects and others

Study subjects will be able to receive information regarding their lipid levels and vitamin D status as well as dietary intake analyses at the end of the study. Participants will be asked if they wish to receive the cholesterol results or have their physician receive the results, or not. Results are then shared as requested. The same choices are available for the dietary results. Results of these measurements could prompt treatment or preventive therapies by their health care provider.

If vitamin D is found to lower cholesterol concentrations, it could provide a valuable addition or alternative to current pharmacological therapies. The order of magnitude of LDL-cholesterol lowering that we have previously observed is similar to the maximum achieved by drastic lifestyle modification, greater than that seen with doubling of statin therapy, similar to that of ezetimide added to statin therapy and similar to that of poorly tolerated bile acid sequestrant therapy. In addition, vitamin D lowers cholesterol, this would create a new pathway and mechanism for pharmacologic agents to treat hyperlipidemia.

If vitamin D lowers atorvastatin concentrations, the magnitude of change will be estimated and should stimulate investigations into the impact of changes of these magnitude on other CYP3A4 substrates that are amongst the most commonly administered medications. The results may identify a previously unrecognized clinical induction effect of vitamin D. This may have enormous impact since vitamin D status is being assessed with increasing frequency with most older adults and racial minority groups have inadequate vitamin D status. This may lead to widespread use of vitamin D and increase the chances of additional interactions with CYP3A substrate medications that may have clinical consequences.

Importance of the knowledge to be gained

Drug interactions are becoming a frequent cause of adverse drug events and interactions between CYP3A4 substrates are amongst the most common drug interactions. Potential inducing effects of vitamin D on CYP3A4 activity has not been previously recognized clinically (relevant to intervention participants).

If vitamin D is found to lower cholesterol concentrations, it could provide a valuable addition or alternative to current pharmacological therapies. The potential risks are minimal. There are limited data on vitamin D supplementation but a growing body of knowledge suggests effects are beyond those on bone and mineral metabolism. A role in cardiovascular physiology is hypothesized but the extent and mechanisms of any effects are not known. The risk of participation is minimal and justified in relation to the potential information to be gained.

Data and Safety Monitoring Plan

This is not a clinical trial and does not require a data safety and monitoring board. There is a data and safety monitoring plan. The project and plan have been approved for performance by the UCSF Committee on Human Research (CHR). The data and safety monitoring plan involves review of weekly progress, laboratory tests, and complication rates by the PI. Pre-entry and safety laboratory results are reviewed within 48 hours of testing (on-line access). Potential adverse events reported in the bi-weekly phone call to research nursing staff are discussed the same day with the PI. Any adverse event occurring during a CRC admission is also reported and reviewed separately by the CRC and CTSI. All adverse events are reported to the CHR per the timelines for mild, moderate, and severe adverse events. Additional reviews of the projects of Dr. Schwartz are performed by the Research Committee of the Jewish Home at quarterly meetings. The Jewish Home Research Committee is not a licensed IRB/CHR and its activities do not super cede requirements of the UCSF CHR.

Inclusion of Women and Minorities

Women and minorities will be enrolled. The goal is to enroll equal numbers of men and women. Recruitment will be limited to subjects not taking vitamin D or supplements containing vitamin D or fish oil supplements, those with replete vitamin D status and will exclude those with potentially increased risk of complications of vitamin D administration and those unable to provide informed consent. No other subgroups are excluded.

The PI has a track record of including women in significant proportions in her investigations and makes specific efforts to recruit ethnic minorities. She has had some success as indicated by enrollment in her published and ongoing population pharmacokinetic studies. Although efforts have been made to enroll minorities, enrollment appears to have varies from 21% for a recent population kinetic study of a lipid lowering drug to 61% for an earlier study of antihypertensive medications. There were 135 subjects enrolled in the studies of vitamin D effects in both atorvastatin and non-statin treated people (16 in the published and 119 in the aborted trials). Seventy-one were men and 64 were women, 89 were Caucasian, 29 were African American, 11 were Asian, five were Hispanic and one was self-identified as other.

Continued targeted efforts will be made to increase minority enrollment. Advertisements and recruitment flyers will be posted at health care facilities (San Francisco General Hospital, St. Luke's Hospital, North of Market Senior Center, Mission Health Center) and educational facilities serving areas of the SF Bay area with significant numbers of minorities (San Francisco State, Community College, UCSF Schools of Nursing and pharmacy). The worksite of the PI has a staff of over 700 employees, of whom sixty percent are of Asian ethnicity. Recruitment posters, in-services, and research presentations will be made at this site. The UCSF Clinical Translational Research Institute and the Committee on Human Research maintain clinical trial

information on publicly accessible websites to facilitate enrollment and post listings at each of the clinical research center sites where significant numbers of minorities are employees; they also have increased minority recruitment efforts and the research team will make presentations at community groups identified through this mechanism.

The PI has one Spanish speaking staff member, and two research pharmacy student volunteers that speak Asian dialects. HIPPA forms are available in multiple languages and are provided in the native language of all participants, and the Block dietary recall questionnaire is available in Spanish.

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Patricia Wayland — December 22, 2014

Author Signature — Date