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The Vitamin D Metabolite Ratio and Risks of Incident Cardiovascular Disease, Heart Failure,
and Cardiovascular Death: The Multi-Ethnic Study of Atherosclerosis

A Thesis submitted in partial satisfaction of the requirements
for the Master's Degree

in

Public Health

by

Jonathan Cheng

Committee in charge:

Professor Andrea Z. LaCroix, Chair
Professor Charles Ginsberg
Professor Lindsay Miller

2023

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University of California San Diego

2023

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LIST OF ABBREVIATIONS

CVD	Cardiovascular disease
VMR	Vitamin D metabolite ratio
MESA	Multi-Ethnic Study of Atherosclerosis
WFU	Wake Forest University
COL	Columbia University
JHU	Johns Hopkins University
UMN	University of Minnesota
NWU	Northwestern University
UCLA	University of California Los Angeles
HR	Hazard ratio
CI	Confidence interval
HF	Heart failure
BMI	Body mass index
CHF	Congestive heart failure
CRP	C-reactive protein
iPTH	Intact parathyroid hormone
FGF-23	Fibroblast growth factor 23
US	United States
SD	Standard deviation
N	Number
NSAID	Non-steroidal anti-inflammatory drug

HTN	Hypertension
CKD	Chronic Kidney Disease
eGFR	Estimated glomerular filtration rate
LDL	Low-density lipoprotein
HDL	High-density lipoprotein
IQR	Interquartile range
25(OH)D ₂	25-Hydroxyvitamin D ₂
25(OH)D ₃	25-Hydroxyvitamin D ₃
24,25(OH) ₂ D ₃	24,25-Dihydroxyvitamin D ₃

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ABSTRACT OF THE THESIS

The Vitamin D Metabolite Ratio and Risks of Incident Cardiovascular Disease, Heart Failure,
and Cardiovascular Death: The Multi-Ethnic Study of Atherosclerosis

by

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Master of Public Health

University of California San Diego, 2023

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Investigations into the link between vitamin D and cardiovascular disease (CVD) have yielded inconsistent results. The vitamin D metabolite ratio (VMR), the ratio of 24,25(OH)₂D to 25(OH)D, has shown stronger associations with fracture and mortality than 25(OH)D alone. Our study assessed the association between the VMR and CVD outcomes.

We evaluated a cohort of 6,313 participants from the Multi-Ethnic Study of Atherosclerosis (MESA), without pre-existing CVD, over 15 years. Utilizing Cox regression, we examined the associations of both the VMR and 25-hydroxyvitamin D [25(OH)D] with various cardiovascular events. Over the study, 800 participants developed CVD, including conditions such as myocardial infarction, resuscitated cardiac arrest, stroke, coronary heart disease death, and stroke death. Heart failure (HF) was observed in 398 participants, and 413 experienced cardiovascular mortality. Models were adjusted for factors including demographics, lifestyle, clinical conditions and medications, biomarkers, and kidney function.

Participants averaged 62 years (range 44-84), with 53% females. The mean (SD) 25(OH)D level was 22.7 (11.0) ng/mL, and the mean VMR was 15.2 (5.0). In fully adjusted models, a two-fold increase in VMR was associated with a 24% reduction in incident CVD (HR: 0.76, 95% CI: 0.65-0.88). However, there was no association between the VMR and HF (0.98, 0.78-1.24), or cardiovascular mortality (0.96, 0.77-1.21). 25(OH)D was not significantly associated with any CVD outcome.

In a diverse cohort, VMR was significantly associated with reduced incident CVD, but not HF or cardiovascular mortality. The results suggest that VMR may provide greater insight into vitamin D metabolism, compared with 25(OH)D alone.

CHAPTER 1: INTRODUCTION

1.1 Introduction to Cardiovascular Disease

Cardiovascular disease (CVD) is a broad term referring to a class of diseases involving the heart or blood vessels, including coronary artery disease, heart failure, and stroke.¹ As a leading cause of morbidity and mortality worldwide, it imposes a significant health and economic burden.² CVD accounted for 868,662 deaths, representing about 0.27% of the total US population, in 2017. It claimed more lives each year than all forms of cancer and chronic lower respiratory disease (CLRD) combined.³ Between 2015 and 2018, 126.9 million American adults had some form of CVD. Between 2016 to 2017, direct and indirect costs of total CVD were \$363.4 billion (\$216.0 billion in direct costs and \$147.4 billion in lost productivity/mortality).³

Atherosclerosis is the primary cause of CVD, a chronic inflammatory disease characterized by the accumulation of lipids and fibrous elements in the large arteries. This condition is associated with a number of risk factors, including advanced age, male gender, family history, tobacco smoking, hypertension, dyslipidemia, diabetes, obesity, and physical inactivity.⁴ Clinical manifestations of CVD depend on the specific type and may range from no symptoms to severe complications such as heart attacks (myocardial infarctions), strokes, or heart failure.⁵ Although medical and surgical treatments for CVD have improved, the most effective way to reduce its incidence and mortality is still to prevent CVD. This can be done by controlling risk factors such as physical inactivity and high cholesterol levels.⁶

Understanding the biological mechanisms underlying CVD is crucial for developing more effective prevention and treatment strategies. In recent years, vitamin D deficiency has emerged as a potential modifiable risk factor for CVD. However, the evidence supporting a link

between vitamin D deficiency and CVD is mixed, and there are still a number of limitations to the existing research. Nevertheless, the potential implications of the research on vitamin D and CVD for public health policy and clinical practice are significant.⁷

1.2 Understanding Vitamin D: Function and Role

Vitamin D, a fat-soluble vitamin, is known for its important role in key physiological processes. It is vital to bone health, modulates cell growth, facilitates neuromuscular function, and participates in managing the body's inflammatory response. The effectiveness of vitamin D hinges on a two-step activation process within our bodies.⁸

Following its intake either through diet, supplements, or ultraviolet B (UVB) radiation from sun exposure, vitamin D must be metabolically transformed to its active state.⁹ The liver undertakes the initial transformation, converting vitamin D into 25-hydroxyvitamin D [25(OH)D], also known as calcidiol. This form primarily serves as a marker for assessing vitamin D status in blood tests.

The final transformation primarily happens in the kidneys, where 25-hydroxyvitamin D is further metabolized into 1,25-dihydroxyvitamin D [1,25(OH)₂D], known as calcitriol. This is the physiologically active form of vitamin D. Once formed, calcitriol binds to vitamin D receptors (VDR) located in numerous tissues throughout the body, enabling it to regulate the wide assortment of physiological functions.¹⁰

Primarily, vitamin D oversees the regulation of calcium and phosphorus, a key factor in promoting bone health. It aids calcium absorption in the gut and maintains optimal calcium levels in our bloodstream. This not only helps prevent tetany, a condition marked by involuntary

muscle contractions from low calcium levels, but also promotes bone remodeling by osteoblasts, the cells responsible for bone formation.⁸

In addition to bone health, vitamin D also plays a critical role in the life cycle of cells. It's involved in processes such as cellular proliferation, differentiation, and apoptosis, which could have significant implications in the development of diseases like cancer.¹¹ In the context of neuromuscular function, vitamin D deficiency can lead to muscle weakness.¹² Lastly, vitamin D is important in the body's inflammatory response. Research indicates that it modifies the immune system by influencing the production and action of inflammatory cytokines. Thus, it may have potential therapeutic applications in conditions characterized by inflammation.¹³

1.3 Vitamin D Deficiency and Its Implications

Vitamin D deficiency, typically defined as serum 25(OH)D levels below 20 ng/mL, can have wide-ranging health impacts.⁸ In children, severe vitamin D deficiency can cause rickets, a disease characterized by delayed growth, bone pain, and skeletal deformities like bowed legs, resulting from poor bone mineralization.¹⁴ In adults, chronic deficiency can lead to osteomalacia, a condition characterized by softening of the bones due to defective bone mineralization, presenting symptoms such as diffuse bone pain and muscle weakness.¹⁵ Additionally, chronic vitamin D deficiency can lead to the development of osteoporosis, a condition that is characterized by a decrease in bone mass and a deterioration of bone tissue structure. The resulting fragile bones are more prone to fractures. This is particularly concerning for the elderly population, who are naturally more susceptible to falls and bone injuries.¹⁶ Adequate vitamin D levels are therefore crucial for maintaining overall bone health.

Vitamin D deficiency has also been linked to the increased risk of developing autoimmune disorders.¹⁷ Conditions such as multiple sclerosis, type 1 diabetes, and rheumatoid arthritis have all been associated with lower levels of vitamin D. These findings highlight the importance of maintaining adequate vitamin D levels for a broad spectrum of health outcomes.¹⁸

Additionally, vitamin D deficiency has been linked to certain cancers. Experimental research provides evidence that vitamin D can exert anti-carcinogenic effects, such as inhibiting cancer cell growth, reducing cancer cell migration, and inducing apoptosis in cancer cells.¹¹ Epidemiological studies also reinforce this link between vitamin D and cancer. These studies suggest that low vitamin D levels have been associated with an increased risk of developing breast, colorectal, and prostate cancers.¹⁹ These findings emphasize the potential of vitamin D not only in cancer prevention but also as a part of therapeutic strategies in oncology.

Finally, low vitamin D levels have been linked with cardiovascular diseases such as hypertension, coronary artery disease, and heart failure (HF).²⁰ Current theories suggest that vitamin D may contribute to cardiovascular health through the modulation of inflammatory responses, the renin-angiotensin-aldosterone (RAAS) hormone system, and direct actions on cardiac and vascular muscle cells.²¹ This opens up an interesting avenue for further exploration into the intricate relationship between vitamin D and cardiovascular health.

1.4 Vitamin D and Cardiovascular Disease

The association between vitamin D and CVD has been the focus of many scientific inquiries. Epidemiological studies have shown a correlation between vitamin D deficiency and increased CVD risk.²²

In the context of hypertension, low vitamin D status is thought to indirectly influence blood pressure regulation through the activation of the RAAS. Experimental studies suggest that vitamin D can inhibit renin expression, a key enzyme in the RAAS that regulates blood pressure. Therefore, vitamin D deficiency may lead to overactivation of the RAAS, contributing to hypertension.²³

Vitamin D is believed to exert protective effects against atherosclerosis through its anti-inflammatory properties. Research indicates that vitamin D can modify immune responses and curb the generation of pro-inflammatory cytokines, which are important players in the progression of atherosclerosis.²⁴

HF, characterized by the heart's inability to pump enough blood to meet the body's needs, has also been linked to vitamin D deficiency. This association is believed to initiate from the combined effect of vitamin D on blood pressure regulation, inflammation, and myocardial function. Additionally, vitamin D receptors are found in cardiac myocytes, indicating a direct role for vitamin D in heart function.²⁵

Despite these plausible mechanistic links, it's important to note that the exact relationships and underlying mechanisms connecting vitamin D to CVD, malignancy, and autoimmune conditions are still not completely understood. This complexity is illustrated by various findings. Meta-analyses, including observational and randomized control trials (RCTs), have shown that serum 25(OH)D levels are inversely correlated with risk of CVD events, hypertension, and all-cause mortality.²⁶⁻²⁹ An extensive meta-analysis, covering 73 cohort studies (n = 849,412) and 22 randomized control trials (n = 30,716), found that higher serum vitamin D levels were linked to lower risks of mortality from CVD and cancer, and that intervention with supplements significantly diminished mortality risk.²⁸ However, another meta-

analysis focusing solely on RCTs, encompassing 21 RCTs and including 83,291 patients with 41,669 receiving vitamin D and 41,622 receiving placebos, has shown that vitamin D supplementation was not related to a decrease in major adverse cardiovascular events, myocardial infarction, stroke, CVD mortality, or all-cause mortality.³⁰

1.5 The Vitamin D Metabolite Ratio (VMR)

The current conventional measure of Vitamin D status in clinical practice is the serum 25(OH)D concentration. However, this biomarker may not reflect the true Vitamin D stores due to the influence of vitamin D binding protein (VDBP).³¹ VDBP binds to circulating vitamin D metabolites, affecting their bioavailability and half-life.³² The majority of 25(OH)D (~85-90%) is bound to VDBP, with the remainder being either free (~1%) or albumin-bound (10-15%).³³ Additionally, VDBP can vary by over 30% across individuals, therefore, 25(OH)D measurements may reflect VDBP variability rather than free vitamin D hormone levels.³⁴ Consequently, relying solely on 25(OH)D may lead to an incomplete understanding of an individual's vitamin D status and metabolism.

In light of these limitations, the vitamin D metabolite ratio (VMR), the ratio of 24,25-dihydroxyvitamin D [24,25(OH)₂D] to 25(OH)D, is gaining attention as a potentially more accurate indicator of vitamin D status.^{35,36} 24,25(OH)₂D is formed when 25(OH)D is catabolized to 24,25(OH)₂D, a process stimulated by calcitriol binding to VDRs to help prevent vitamin D toxicity.³⁷ It is also important to note that the influence of VDBP on the VMR is hypothetically neutralized due to its proportional impact on both components, the numerator and denominator, of the VMR calculation. Studies have also shown that the VMR is independent of VDBP.^{38,39} Thus, the VMR delivers a perspective that is less confounded by VDBP concentrations.³⁸

Supporting the potential relevance of VMR, observational studies have shown a strong association between VMR and the risk of hip fracture and mortality. Specifically, a higher VMR was linked to an decreased risk of hip fractures and mortality, while the traditional measure, 25(OH)D, showed no such association.³⁶ This suggests that the VMR might provide valuable information beyond what 25(OH)D can offer, contributing to a more complete understanding of the role and status of vitamin D in health and disease.

1.6 Research Aims and Goals

Thus far, our understanding of the association between the VMR and cardiovascular outcomes is limited, with no studies to our knowledge directly examining this relationship. This gap in knowledge presents a barrier to fully appreciating the role of vitamin D metabolism in cardiovascular health. Current assessments primarily rely on serum 25(OH)D measurements, which may not accurately capture the full picture of an individual's vitamin D status due to factors like VDBP variability. Therefore, it's critical that we explore more comprehensive metrics, such as the VMR, to offer another perspective and understanding of vitamin D metabolism's associations on cardiovascular outcomes.

Hence, our research aims to fill this gap by describing the nature of individuals, from a generally healthy cohort without CVD, who exhibit a particular VMR and exploring the association of the VMR with incident cardiovascular disease, heart failure, and cardiovascular mortality. Additionally, we aim to identify any potential influence of sex, race, ethnicity, and estimated glomerular filtration rate (eGFR) on these relationships. This initial exploratory study sets the stage for future research focused on understanding the safety and potential risk or

benefits associated with certain VMR levels, thereby contributing valuable insights to both preventive and therapeutic strategies.

1.7 Acknowledgements

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CHAPTER 2: METHODS

2.1 Study Population

The Multi-Ethnic Study of Atherosclerosis (MESA) is an ongoing, community-based prospective cohort study of cardiovascular disease. Between 2000 and 2002, MESA recruited 6,814 adults without clinically apparent cardiovascular disease between the ages of 45 and 84 years from six communities located across the United States: Forsyth County, NC; Northern Manhattan and the Bronx, NY; Baltimore and Baltimore County, MD; St. Paul, MN; Chicago, IL; and Los Angeles County, CA. Institutional review boards at all participating centers approved the study, and all participants gave written informed consent.

2.2 Vitamin D Metabolite Measurements

All necessary measurements were performed at the University of Washington Nutrition Obesity Research Center. Measurements of 25(OH)D₃ and 24,25(OH)₂D₃ were made using liquid-liquid extraction and subsequent liquid chromatography-tandem mass spectrometry (LC–MS/MS).⁴⁰ These measurements were taken from baseline fasting serum samples collected from 2000 to 2002. Coefficient of variation for 25(OH)D₃ assays is <5.6% with an analytic range of 1–200 ng/mL.⁴¹ Original concentrations of 24,25(OH)₂D₃ were divided by two for proper calibration and traceability to NIST SRM 972a.⁴² The average observed coefficient of variation was 9.9–12.7% at 2.0–5.1 ng/mL with an analytic range of 0.1–100 ng/mL. All assays measuring 25(OH)D₃ and 24,25(OH)₂D₃ were calibrated to standards provided by the National Institute of Standards and Technology.⁴³ The VMR was calculated by dividing serum 24,25(OH)₂D₃ by serum 25(OH)D₃ and then multiplying by 1000.⁴⁴ As there was no spectroscopic evidence of

24,25(OH)₂D₂ the VMR was calculated using 24,25(OH)₂D₃ and 25(OH)D₃ only.

2.3 Outcomes

Incidence of Cardiovascular Disease: These events were a MESA-defined CVD composite. The composite included myocardial infarction, stroke, resuscitated cardiac arrest, angina leading to coronary revascularization, and cardiovascular-related death. The mean follow up time 14.5 years with 800 cases of cardiovascular disease over this time period. These outcomes were meticulously scrutinized and adjudicated by an expert panel from MESA. This panel was tasked with the comprehensive review of relevant medical records, laboratory data, and diagnostic imaging associated with each case.

Heart Failure Incidence: Our study also assessed the incidence of heart failure over a mean of 14.7 years of follow up with 398 cases of HF over this time period. The MESA determined the instances of heart failure with a dedicated committee of experts who examined all pertinent medical records, including diagnostic test results and cardiac imaging studies.

Cardiovascular Mortality: Over a mean follow-up period of 16.0 years, the MESA evaluated deaths attributed to cardiovascular causes. There were 413 cardiovascular deaths over this follow up period. This evaluation process included examining death certificates, relevant medical records, and available autopsy reports in accordance with the standard definitions provided by the National Center for Health Statistics and the World Health Organization.

2.4 Covariates

Baseline demographics, including race, ethnicity, smoking status, physical activity (defined by moderate or vigorous intensity exercise and number of minutes), and comorbidities

(smoking status, diabetes, hypertension, cholesterol levels, and BMI) were obtained at the initial MESA examination. This information was collected through a combination of self-administered questionnaires, standardized interviews administered by an interviewer, comprehensive in-person examinations, and laboratory tests. Participants self-identified as belonging to one of the four racial or ethnic groups: White, Black, Hispanic, or Chinese. Medications were logged in the MESA study through a combination of self-report and medication inventory.

The laboratory tests for the MESA study were conducted at the Central Laboratory (University of Pittsburgh Medical Center) and six Field Centers. The tests were conducted using standard operating procedures, quality control samples, and external quality assurance programs to ensure accuracy and consistency. Diabetes status was determined based on the following criteria: usage of an oral hypoglycemic medication or insulin, fasting blood glucose level of ≥ 126 mg/dL, non-fasting blood glucose level of ≥ 200 mg/dL, or a hemoglobin A1c level of $\geq 6.5\%$.⁴⁵ Body Mass Index (BMI) was calculated in units of kg/m^2 using measurements of weight and height taken during the examination. The Chronic Kidney Disease Epidemiology Collaboration equation was used to estimate the glomerular filtration rate (eGFR) from serum creatinine.⁴⁶

Laboratory measurements of serum calcium and serum phosphate were taken using a Beckman Coulter DxC autoanalyzer (Beckman Coulter Inc.), with methods including indirect potentiometry and timed-rate colorimetric reaction, respectively. The modified Doumas and Rodkey procedures were employed for urine albumin measurements. Urine creatinine was quantified using the Array 3600 CE Protein Analyzer (Beckman Coulter Inc.) via nephelometry. Albuminuria was determined as the ratio of albumin to creatinine from a single voided urine sample. Intact parathyroid hormone (iPTH) was measured with the Beckman Coulter Dxl

automated two-site immunoassay and intact fibroblast growth factor 23 (FGF-23) via the Kainos immunoassay using previously unfrozen serum.

2.5 Statistical Analysis

This section will describe the statistical methods used to analyze the data, including descriptive statistics, Cox proportional hazards regression, and interaction and stratified analyses.

Table 1: Baseline characteristics were summarized using descriptive statistics, including counts and proportions for categorical variables and means and standard deviations for continuous outcomes.

Table 2: Incident CVD outcomes, incident HF outcomes, and cardiovascular mortality were evaluated among MESA participants who had baseline vitamin D metabolite measurements, using Cox proportional hazards regression. To evaluate these outcomes, we examined the data using three progressively adjusted statistical models. Predictor variables were log-transformed when not normally distributed, including iPTH, FGF-23, CRP, triglycerides, and UACR. The reported hazard ratios (HRs) correspond to a two-fold increase (equivalent to doubling) in the VMR and 25(OH)D.

The first model was an unadjusted analysis and assessed the association between the VMR or 25(OH)D and incident CVD, HF, and cardiovascular mortality outcomes separately. The second model was adjusted for age, gender, race, and site of study. In the third and final model, it was adjusted for all of the prior variables plus physical activity, BMI, smoking status, diabetes, blood pressure, use of blood pressure medication, C-reactive protein (CRP) levels, cholesterol and triglyceride levels, use of cholesterol medication, eGFR, urine albumin to

creatinine ratio (UACR), iPTH levels, fibroblast growth factor 23 (FGF-23) levels, calcium, and phosphate levels.

Table 3: Multiplicative interaction terms of the VMR with gender, race and ethnicity, and eGFR were introduced into the fully adjusted models to assess whether the association between the VMR and CVD, HF, or cardiovascular mortality differed by these factors.

Figure 1: Finally, stratified analyses were performed in fully adjusted models to assess the association of the VMR and 25(OH)D with cardiovascular outcomes in different races.

All analyses were conducted using Stata SE version 15.1, with a p-value threshold of less than 0.05 set for statistical significance.

2.6 Acknowledgements

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CHAPTER 3: RESULTS

3.1 Participant Characteristics

The study included 6,473 participants, averaging 62 years of age, with 53% female, 38% White, 28% Black, 22% Hispanic, and 12% Chinese. Mean VMR was 152.1 with a standard deviation of 50.2, while the mean 25(OH)D level was 22.7 ng/mL with a standard deviation of 11.0 ng/mL. With higher VMR, a decrease was observed in the percentage of females, average BMI, prevalence of diabetes, and prevalence of CKD. Additionally, the higher quartiles corresponded to higher levels of physical activity and lower usage of anti-hypertensive and diabetes medications. In contrast, higher quartiles had higher percentages of White and lower percentages of Black participants. Median levels of vitamin D metabolites, including 25(OH)D₃, total 25(OH)D, and 24,25(OH)₂D₃, increased across quartiles.

Table 1: Participant Characteristics by VMR Quartile

	<u>Quartile 1</u> <u>(n= 1619)</u>	<u>Quartile 2</u> <u>(n=1618)</u>	<u>Quartile 3</u> <u>(n= 1618)</u>	<u>Quartile 4</u> <u>(n=1618)</u>
Range (pg/ng)	37.3-121.6	122.1-149.0	149.5-179.3	180.0-314.2
Age (years) ± SD	62.8 ± 10.4	62.1 ± 10.4	62.1 ± 10.2	61.8 ± 9.9
Female, n (%)	960 (59)	872 (54)	818 (51)	801 (50)
Race / Ethnicity, n (%)				
White	441 (27)	582 (36)	691 (43)	797 (49)
Chinese	167 (10)	207 (13)	219 (14)	195 (12)
Black	655 (40)	455 (28)	362 (22)	289 (18)
Hispanic/Latino	356 (22)	374 (23)	346 (21)	337 (21)
Site, n (%)				
WFU	242 (15)	232 (14)	237 (15)	260 (16)
COL	285 (18)	271 (17)	236 (15)	224 (14)
JHU	316 (20)	264 (16)	234 (14)	225 (14)
UMN	245 (15)	241 (15)	267 (17)	278 (17)
NWU	270 (17)	273 (17)	293 (18)	306 (19)

Table 1: Participant Characteristics by VMR Quartile (continued)

UCLA	261 (16)	337 (21)	351 (22)	325 (20)
BMI (kg/m ²) $\pm$ SD	30 $\pm$ 6	29 $\pm$ 5	28 $\pm$ 5	27 $\pm$ 5
Smoking status, n (%)				
Never or quit $\geq$ 12 months	1302 (81)	1377 (85)	1409 (87)	1409 (87)
Former $\leq$ 12 months	26 (2)	36 (2)	29 (2)	29 (2)
Current	282 (18)	201 (12)	179 (11)	174 (11)
Physical Activity, n (%)				
None	472 (29)	401 (25)	340 (21)	267 (17)
Intermediate (1–149 min/week moderate intensity or 1–74 min/week vigorous intensity or 1–149 min/week moderate/vigorous)	294 (18)	281 (17)	283 (18)	277 (17)
Ideal (150 min/week moderate intensity or 75 min/week vigorous intensity or 150 min/week)	844 (52)	933 (58)	994 (61)	1070 (66)
Diabetes, n (%)	257 (16)	227 (14)	146 (9)	93 (6)
Systolic BP (mm Hg) $\pm$ SD	130 $\pm$ 22	127 $\pm$ 22	125 $\pm$ 21	124 $\pm$ 20
Diastolic BP (mm Hg) $\pm$ SD	72 $\pm$ 11	72 $\pm$ 10	71 $\pm$ 10	72 $\pm$ 10
NSAID use, n (%)	286 (18)	276 (17)	273 (17)	289 (18)
On anti-HTN medications, n (%)	616 (38)	533 (33)	492 (30)	488 (30)
Diabetes medications, n (%)	231 (14)	193 (12)	119 (7)	78 (5)
Cholesterol medications, n (%)	267 (16)	268 (17)	240 (15)	275 (17)
Hormone Replacement, n (%)	374 (42)	370 (48)	423 (57)	417 (56)
CKD, n (%)	293 (18)	220 (14)	194 (12)	130 (8)
eGFR (ml/min/1.73m ²) $\pm$ SD	76 $\pm$ 18	78 $\pm$ 17	78 $\pm$ 15	79 $\pm$ 14
Total Cholesterol (mg/dL) $\pm$ SD	192 $\pm$ 37	194 $\pm$ 37	196 $\pm$ 34	194 $\pm$ 34
LDL (mg/dL) $\pm$ SD	117 $\pm$ 33	117 $\pm$ 32	118 $\pm$ 30	116 $\pm$ 30
HDL (mg/dL) $\pm$ SD	50 $\pm$ 14	51 $\pm$ 15	51 $\pm$ 15	52 $\pm$ 15
Triglycerides (mg/dL) $\pm$ SD	129 $\pm$ 81	136 $\pm$ 113	131 $\pm$ 79	131 $\pm$ 80
Albumin/Creatinine (mg/g), median [IQR]	6.2 (3.6-14.7)	5.6 (3.4-12.5)	4.9 (3.1-9.4)	5 (3.2-9.4)
Calcium (mg/dl) $\pm$ SD	9.6 $\pm$ 0.4	9.6 $\pm$ 0.4	9.6 $\pm$ 0.4	9.7 $\pm$ 0.4
Phosphate (mg/dl) $\pm$ SD	3.7 $\pm$ 0.5	3.7 $\pm$ 0.5	3.7 $\pm$ 0.5	3.7 $\pm$ 0.5
iPTH (pg/ml), median [IQR]	48.5 [36.4-64.5]	41.5 [32.6-53.2]	38.3 [30-49.8]	35.4 [27.8-45.6]

Table 1: Participant Characteristics by VMR Quartile (continued)

FGF-23 (pg/ml), median [IQR]	36.5 [29.0-45.2]	37.2 [30.0-46.1]	38.0 [30.8-46.5]	39.0 [32.2-48.4]
CRP (mg/L), median	2.5 [1.0-5.2]	2.0 [0.9-4.3]	1.8 [0.8-3.9]	1.5 [0.7-3.6]
25(OH)D ₂ (ng/mL), median [IQR]	0.3 [0.0-1.1]	0.6 [0.2-2.6]	0.6 [0.2-3.3]	0.6 [0.2-3.1]
25(OH)D ₃ (ng/mL), median [IQR]	14.3 [10.3-19.8]	20.1 [14.8-26.4]	24.0 [17.5-31.5]	28.0 [21.3-35.4]
Total 25(OH)D (ng/mL), median [IQR]	16.1 [11.3-22.8]	23.3 [16.9-30.2]	27.8 [20.9-34.2]	30.9 [24.3-37.5]
24,25(OH) ₂ D ₃ (ng/mL), median [IQR]	1.4 [0.9-2.1]	2.7 [2.0-3.6]	3.9 [2.8-5.2]	5.7 [4.3-7.4]

3.2 Cardiovascular Events

As detailed in **Table 2**, we observed 800 cases of CVD, 398 cases of HF, and 413 cases of cardiovascular mortality during a mean follow-up time of 14.5, 14.7, and 16.0 years, respectively.

3.3 Vitamin D Metabolism Markers and Cardiovascular Risk

A two-fold higher VMR was associated with a 24% reduction in incident CVD (95% confidence interval [CI] = 0.65-0.88, $p < 0.001$). There was no significant association between the VMR and incident HF (HR = 0.98, 95% CI = 0.78-1.24, $p = 0.891$) or between the VMR and cardiovascular mortality (HR = 0.96, 95% CI = 0.77-1.21, $p = 0.754$).

A two-fold increase in 25(OH)D was not statistically significantly associated with incident CVD (HR = 0.95, 95% CI = 0.85-1.06, $p = 0.395$), incident HF (HR = 1.03, 95% CI = 0.88-1.20, $p = 0.748$), or cardiovascular mortality (HR = 0.91, CI = 0.78-1.06, $p = 0.237$).

3.4 Interaction Analysis

No significant interaction effects were found between the VMR and race, gender, or

eGFR for CVD, HF, or cardiovascular mortality (p-values ranging from 0.068 to 0.747) (**Table 3**). Similarly, no significant interactions were found between 25(OH)D and race, gender, or eGFR for these same outcomes (p-values ranging from 0.271 to 0.962). Thus, the analyses showed no evidence that race, gender, or GFR modified the associations between VMR or 25(OH)D and CVD, HF, or cardiovascular mortality.

3.5 Stratification by Race and Ethnicity

While our interaction analysis yielded no significant findings, we performed a stratification analysis given the diverse population that make up the MESA. Among Whites, the VMR was significantly associated with a lower risk of CVD (HR: 0.71, CI: 0.52-0.95, $p = 0.02$). However, no significant associations were observed for HF (HR: 1.22, CI 0.80-1.84) or cardiovascular mortality (HR: 0.70, CI 0.45-1.10). For 25(OH)D, no significant associations were observed in any of the racial groups. In White participants, the hazard ratios (HRs) with 95% confidence intervals (CIs) for 25(OH)D with CVD, HF, and cardiovascular mortality were 0.71 (95% CI: 0.52-0.95), 1.22 (95% CI: 0.80-1.84), and 0.70 (95% CI: 0.45-1.10), respectively.

In Chinese participants, the HRs for VMR with CVD, HF, and cardiovascular mortality were 0.55 (95% CI: 0.30-1.01), 0.78 (95% CI: 0.30-2.07), and 1.13 (95% CI: 0.51-2.52), respectively. For 25(OH)D, the HRs were 0.92 (95% CI: 0.76-1.09), 0.95 (95% CI: 0.46-1.96), and 0.82 (95% CI: 0.48-1.38), respectively.

In Black participants, the HRs for VMR with CVD, HF, and cardiovascular mortality were 0.81 (95% CI: 0.64-1.04), 0.99 (95% CI: 0.70-1.39), and 0.98 (95% CI: 0.70-1.38), respectively. For 25(OH)D, the HRs were 0.99 (95% CI: 0.80-1.22), 1.17 (95% CI: 0.88-1.56), and 0.94 (95% CI: 0.72-1.22), respectively.

In Hispanic/Latino participants, the HRs for VMR with CVD, HF, and cardiovascular mortality were 0.80 (95% CI: 0.55-1.16), 0.85 (95% CI: 0.48-1.52), and 1.36 (95% CI: 0.76-2.45), respectively. For 25(OH)D, the HRs were 0.99 (95% CI: 0.78-1.26), 1.01 (95% CI: 0.71-1.45), and 1.04 (95% CI: 0.73-1.47), respectively.

Overall, the association between vitamin D metabolites and cardiovascular outcomes did not appear to be consistent across different races.

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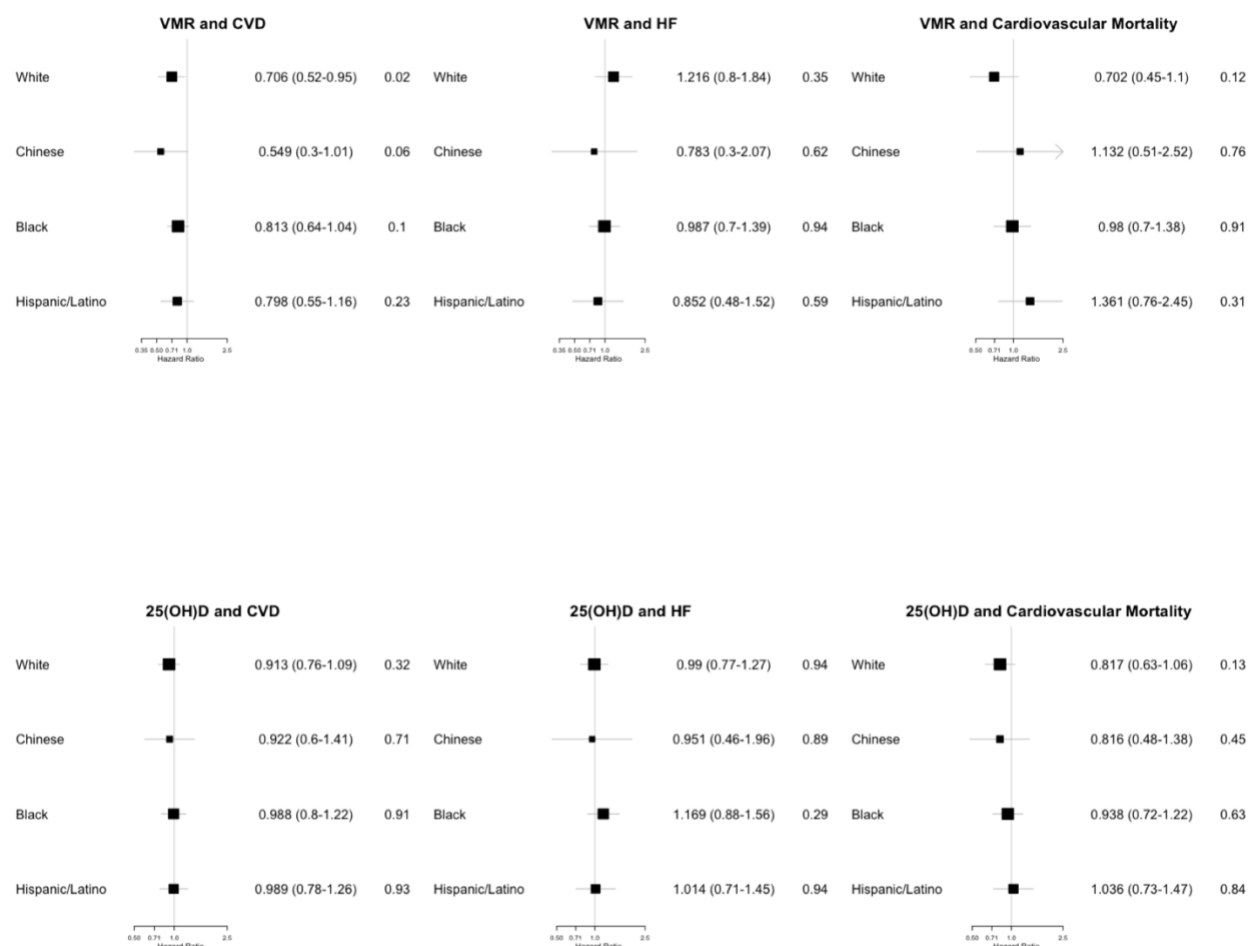


Figure 1: Forest Plot of Hazard Ratios for Cardiovascular Outcomes: Distribution of VMR and 25(OH)D, stratified by race, in fully adjusted models.¹

¹The stratified analysis was fully adjusted for age, gender, race, site of study, physical activity, BMI, smoking status, diabetes, blood pressure, use of blood pressure medication, C-reactive protein levels, cholesterol and triglyceride levels, use of cholesterol medication, eGFR, urine albumin to creatinine ratio, intact parathyroid hormone levels, fibroblast growth factor levels, calcium, and phosphate levels.

Table 2: Associations of Vitamin D Metabolites with Cardiovascular Events

Outcome	N Events / N at Risk	Mean Follow Up Time (years)	Model 1 HR (95% CI)	p-value	Model 2 HR (95% CI)	p-value	Model 3 HR (95% CI)	p-value
VMR								
CVD	800 / 6,303	14.5	0.71 (0.63, 0.81)	<0.001	0.70 (0.61, 0.80)	<0.001	0.76 (0.65, 0.88)	<0.001
HF	398 / 6,303	14.7	0.79 (0.65, 0.97)	0.021	0.79 (0.65, 0.97)	0.023	0.98 (0.78, 1.24)	0.891
Cardiovascular Mortality	413 / 6,303	16.0	0.78 (0.64, 0.94)	0.014	0.83 (0.68, 1.02)	0.074	0.96 (0.77, 1.21)	0.754
25(OH)D								
CVD	801 / 6,303	14.5	0.96 (0.87, 1.05)	0.362	0.90 (0.81, 0.99)	0.038	0.95 (0.85, 1.06)	0.395
HF	398 / 6,303	14.7	0.98 (0.86, 1.12)	0.807	0.92 (0.79, 1.06)	0.258	1.03 (0.88, 1.2)	0.748
Cardiovascular Mortality	414 / 6,303	16.0	0.90 (0.79, 1.02)	0.102	0.87 (0.76, 1.00)	0.056	0.91 (0.78, 1.06)	0.237

Table 3: P-values for Interaction Terms in Fully Adjusted Models of CVD Events

Interactions	CVD	HF	Cardiovascular Mortality
VMR x Race	0.577	0.192	0.068
VMR x Gender	0.431	0.305	0.444
VMR x eGFR	0.116	0.747	0.141
D25 x Race	0.584	0.851	0.271
D25 x Gender	0.675	0.733	0.766
D25 x eGFR	0.495	0.962	0.778

CHAPTER 4: DISCUSSION

4.1 Summary of Findings

In a large, community-based, multi-ethnic cohort of adults without baseline CVD, we examined the relationship between the VMR and 25(OH)D with incident CVD, incident HF, and cardiovascular mortality. Our findings demonstrated that the VMR was independently associated with incident CVD. This association persisted after adjusting for potential confounders, including demographics, lifestyle, clinical conditions and medications, biomarkers, and kidney function. In contrast, no significant associations were observed between 25(OH)D and cardiovascular events. These results suggest that the VMR may be a superior marker of functional vitamin D activity in relation to incident CVD events, as compared to 25(OH)D alone.

To our knowledge, this is the first study to evaluate the VMR with cardiovascular outcomes. Previous studies of vitamin D measures have typically used serum 25(OH)D levels, and the findings from these studies have been inconsistent. A meta-analysis of observational studies found that higher levels of 25(OH)D were associated with a lower risk of cardiovascular events, but a meta-analysis of randomized controlled trials evaluating vitamin D supplementation and cardiovascular events found no significant association.^{26–30} This discrepancy may be due to the fact that the majority of trials included in the RCT meta-analysis used low-dose vitamin D (~1,000 IU/day) and were not designed specifically to measure cardiovascular outcomes. Additionally, serum levels of 25(OH)D after supplementation were either not measured or not recorded from the included trials to assess for supplementation response.

While our study found an inverse association between the VMR and incident cardiovascular events, it did not investigate the underlying mechanisms of the association. The

possible mechanisms include the effects of vitamin D on the RAAS, inflammatory pathways, and endothelial function.²¹ Our prior study evaluating the VMR with coronary artery calcium (CAC), a non-invasive measurement of endothelial health, indicated that the mechanism of this relationship is likely not related to CAC. CAC is a measure of calcification in the coronary arteries, and it is a risk factor for cardiovascular events.⁴⁷ The findings of this study support the hypothesis that VMR may be a more accurate marker of vitamin D adequacy and functionality than 25(OH)D alone, as it is less influenced by vitamin D binding protein (VDBP).

In our investigation, we also placed particular emphasis on distinctions across various racial and ethnic groups. Our findings reveal a significant association between the VMR and a reduced risk of cardiovascular disease among Whites. However, it must be noted that no race/ethnicity interaction was detected, that the sample sizes and confidence intervals were wide in stratified analyses, and that the observed differences may have been due to imprecision or chance. Future research should probe deeper into these mechanisms to elucidate why the VMR exhibits a stronger association with cardiovascular disease among Whites. Our study found no statistical differences in the potential modifying effects of sex, race, ethnicity, and eGFR on the relationship between VMR and cardiovascular outcomes.

4.2 Strengths

Our study has several strengths. First, we used a large, multi-ethnic cohort with comprehensive baseline and follow-up data. This allowed us to adjust for a wide range of potential confounders, which increases the accuracy of our findings. Second, we used state-of-the-art LC-MS/MS techniques for the measurement of vitamin D metabolites. This ensured accurate quantification of both 25(OH)D and 24,25(OH)₂D₃, which is essential for studying the

relationship between the VMR and cardiovascular outcomes. Finally, our study is one of the first to examine this relationship in a large cohort. This provides novel insights into the potential utility of the VMR in clinical and research settings.

4.3 Limitations

Our study also has some limitations. First, we used a single baseline measurement of vitamin D metabolites, which may not accurately reflect long-term vitamin D status. Second, we cannot exclude the possibility of residual confounding due to unmeasured or imperfectly measured factors, such as dietary intake of vitamin D or sunlight exposure. Third, our study was observational, so future studies incorporating trial data is needed. Finally, our study was conducted in a predominantly middle-aged and older population, limiting the generalizability of our findings to younger individuals.

4.4 Conclusions

In conclusion, our study demonstrated that the VMR is significantly associated with a reduced incidence of CVD events in a healthy multi-ethnic cohort of middle-aged and older adults. This is in contrast to 25(OH)D alone which was not associated with any cardiovascular event. Further studies are needed to confirm our findings and to explore the potential utility of the VMR in clinical practice and public health initiatives aimed at reducing the burden of cardiovascular disease.

4.5 Future Directions

Future directions should focus on longitudinal assessments of the VMR and intervention studies targeting this marker. Future studies can also include younger study populations and longer follow-up periods to establish the prognostic value of the VMR in predicting cardiovascular events. Additionally, examination of other measures of cardiovascular disease such as endothelial health and its relationship to the VMR should be examined. More information about the VMR would offer additional evidence for clinical decision-making and inform preventive strategies for reducing the burden of cardiovascular disease.

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