NON-CLASSICAL ROLE OF VITAMIN D AND DEVELOPMENT OF OPTIMAL VITAMIN D CUTOFFS FOR CARDIOMETABOLIC HEALTH OUTCOMES

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Abstract

Over the last decade, vitamin D deficiency has emerged as a potential risk factor for the development of cardiometabolic diseases. However, the evidence from epidemiological studies and randomized controlled trials (RCT) has yielded conflicting results. Moreover, vitamin D guidelines by the Institute of Medicine and the Endocrine Society have led to substantial disagreement about what defines optimal levels of vitamin D status, owing in part to the inter-laboratory differences in the measurement of vitamin D status (as measured by total 25-hydroxyvitamin D [25(OH)D]) and the inconsistent findings from epidemiological and RCT data in relation to non-skeletal health outcomes. For non-skeletal health outcomes, disagreement still exists about whether the optimal level of 25(OH)D is higher than the currently recommended levels of 25(OH)D for bone health. Therefore, the objectives of this dissertation were; i) to assess the dose-response relationship between standardized total 25(OH)D levels and cardiometabolic health outcomes; ii) to develop optimal vitamin D cutoffs in relation to cardiometabolic health, and; iii) to assess the clinical utility of total 25(OH)D as a biomarker for adverse cardiometabolic health outcomes. Studies 1 and 2 used cross-sectional data from the National Health and Nutrition Examination Survey (NHANES, 2001-2010), and studies 3 and 4 used prospective data from NHANES III (1988-1994) mortality follow-up. Standardized total 25(OH)D data was used in all four studies.

In study 1, results showed that a higher total 25(OH)D was inversely associated with cardiometabolic disease, irrespective of race/ethnicity. In study 2, the optimal total 25(OH)D associated with normal glucose and insulin homeostasis was estimated at 60 nmol/L overall, but differed by race/ethnicity (non-Hispanic whites: 68 nmol/L, non-Hispanic blacks: 41 nmol/L, and Mexican-Americans: 54 nmol/L). In study 3, low total 25(OH)D (<50 nmol/L) exacerbated the risk of cardiometabolic mortality associated with metabolic dysfunction in normal-weight and obesity groups. Finally, in study 4, a single measurement of total 25(OH)D <30 nmol/L in middle- to older-aged adults was associated with high lifetime risk of cardiometabolic mortality, particularly among those with ≥2 major traditional CVD risk factors. Taken together, these findings suggest that low total 25(OH)D is a strong risk marker of adverse cardiometabolic health outcomes.

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List of Abbreviations

7-DHC: 7-dehydrocholesterol

$1,25(OH)_2D$: 1,25-dihydroxyvitamin D

$24,25(OH)_2D$: 24,25-dihydroxyvitamin D

AI: adequate Intake

BMI: body mass index

BP: blood pressure

CDC: Centers for Disease Control and Prevention

CI: confidence interval

CIF: cumulative incidence function

CM: cardiometabolic mortality

CRP: C-reactive protein

CVD: cardiovascular disease

CV: coefficient of variation

CYP24A1: 24-hydroxylase

CYP27A1: mitochondrial 25-hydroxylase

CYP27B1: 1-alpha-hydroxylase

CYP2R1: microsomal 25-hydroxylase

DCA: decision curve analysis

DEQAS: Vitamin D External Quality Assessment Scheme

DHCR7: 7-dehydrocholesterol reductase

DRI: dietary reference intake

EAR: estimated average requirement

eGFR: estimated glomerular filtration rate

ES: Endocrine Society

FFA: free fatty acid

FPF: false-positive fraction (1-specificity)

FPG: fasting plasma glucose

FRS: Framingham risk score

HOMA-IR: homeostasis model assessment of insulin resistance

HDL-C: high-density lipoprotein cholesterol

HEI: healthy eating index

HR: hazard ratio

ICD-10: tenth revision of the International Classification of Diseases

IDI: integrated discrimination improvement

IOM: Institute of Medicine

IR: insulin resistance

IU: international unit

KM: Kaplan-Meier

LC-MS/MS: liquid chromatography-tandem mass spectrometry

LDL-C: low density lipoprotein cholesterol

LR: likelihood ratio

LTPA: leisure-time physical activity

MA: Mexican-Americans

MEC: mobile examination centre

MET: metabolic equivalent

MetS: metabolic syndrome

MHNW: metabolically healthy normal-weight

MHO: metabolically healthy obesity

MHOW: metabolically healthy overweight

NCEP ATP III: National Cholesterol Education Program Adult Treatment Panel III

NCHS: National Center for Health Statistics

NDI: National Death Index

NHANES: National Health and Nutrition Examination Survey

NH-white: non-Hispanic white

NH-black: non-Hispanic black

NRI: net reclassification index

OR: odds ratio

PA: physical activity

PH: proportional hazard

PIR: poverty to income ratio

PTH: parathyroid hormone

PR: prevalence ratio

RAAS: renin-angiotensin-aldosterone system

RCS: restricted cubic spline

RCT: randomized controlled trial

RDA: recommended dietary allowance

RF: risk factor

RIA: radioimmunoassay

ROC: receiver operating characteristic curve

RR: relative risk

RXR: retinoid x receptor

SNP: single nucleotide polymorphisms

SZA: solar zenith angle

TC: total cholesterol

TG: triglyceride

TNF-alpha: tumor necrosis factor alpha

TPF: true-positive fraction (sensitivity)

UL: tolerable upper intake level

UVB: ultraviolet B radiation

vDBP or DBP: vitamin D binding protein

VDR: vitamin D receptor

VDRE: vitamin D response element

VDSP: vitamin D standardization program

WC: waist circumference

Chapter 1: Background

The role of vitamin D in calcium metabolism and skeletal health has been well established [1,2]. Over the last decade, vitamin D deficiency has emerged as a potential risk factor for type-2 diabetes and cardiovascular disease (CVD) [3,4]. In epidemiological studies, vitamin D deficiency has been inversely associated with insulin resistance, metabolic syndrome (MetS), type-2 diabetes, and CVD [3,4]. However, the evidence from observational studies has not been consistent. Moreover, randomized controlled trials (RCTs) assessing the effects of vitamin D supplementation on glycaemia, incident type-2 diabetes, and CVD have not supported a beneficial role of vitamin D supplementation in the prevention of type-2 diabetes and CVD [3,4]. However, most trials to date have been underpowered with relatively short duration and were not properly designed for glycemic and CVD outcomes [5,6]. Therefore, the current evidence on the role of vitamin D in non-skeletal health remains inconclusive and insufficient to determine the potential role of vitamin D in the prevention and treatment of cardiometabolic health outcomes and further research is needed to more fully understand the role of vitamin D in human health.

The assessment of vitamin D status is based on measurement of circulating total 25-hyroxyvitamin D (25(OH)D) concentration, which reflects both food intake and endogenous production of vitamin D [2]. Accurate and precise measurement of 25(OH)D is required to estimate vitamin D deficiency at the population level and to study its association with various disease outcomes [7,8]. Over the last 10 years, studies assessing the relationship between serum 25(OH)D and disease risk have used unstandardized laboratory assays for serum 25(OH)D. There has been a growing concern over the commercially available assays for 25(OH)D, either lacking accuracy (i.e., the ability of a test to return the 'true' value) or precision (i.e., the ability of a test to return the same result with repeated testing) [9,10]. Inaccurate or imprecise measurement of 25(OH)D may lead to null findings of associations with health outcomes [8]. In response to these concerns, the Vitamin D Standardization Program (VDSP) was established in 2010 to promote the standardization of 25(OH)D laboratory procedures worldwide, recognizing the necessity for accurate and reliable measurement of 25(OH)D for research into health risks/benefits, clinical management of vitamin D deficiency, and reliable estimation of vitamin D

deficiency at the population level [7,11]. Given the limitations of previous studies, a systematic re-evaluation of previous research using standardized measurement of total 25(OH)D is necessary. Furthermore, previous studies assessing the relationship between serum 25(OH)D and cardiometabolic health outcomes have predominantly included populations of White European origin and the role of vitamin D in other ethnic subpopulations is not well characterized. There is some evidence that suggests substantial ethnic differences in the relationship between vitamin D status and cardiometabolic health outcomes [12–15]; as such, results from prior studies conducted in predominantly white/European populations may not be generalizable to other ethnic subpopulations. Taken together, the objective for the first study of this thesis was to examine the cross-sectional association between standardized serum 25(OH)D levels and cardiometabolic health outcomes (as measured by homeostatic model assessment of insulin resistance, metabolic syndrome, and Framingham 10-year CVD risk) and to further explore ethnic variation using data from the National Health and Nutrition Examination Survey (NHANES, 2001-2010) (Chapter 4).

In addition to the standardization issue, the optimal vitamin D status (as measured by total circulating 25(OH)D concentration) remains controversial and there are discrepancies between national guidelines for recommended levels of total 25(OH)D in relation to bone health. Current guidelines by the Health and Medicine Division (HMD) of the National Academies (formerly known as the Institute of Medicine, IOM) and the Endocrine Society (ES) recommend different thresholds for serum 25(OH)D. Specifically, the IOM defines vitamin D deficiency as a 25(OH)D level < 30 nmol/L, vitamin D insufficiency as a level of 30-50 nmol/L, and vitamin D sufficiency as > 50 nmol/L [2]. On the other hand, the ES defines serum 25(OH)D < 50 nmol/L as deficient, levels between 50-74 nmol/L as insufficient, and levels $\geq$ 75 nmol/L as sufficient [16]. In addition, other national guidelines also advocate for various cutoffs, ranging from 30 nmol/L to > 100 nmol/L [17]. The discrepancy among national guidelines is largely due to the differences in laboratory assays for 25(OH)D and the inconsistent findings from epidemiological and RCT data in relation to skeletal and non-skeletal health outcomes. For non-skeletal health outcomes, disagreement still exists about whether the optimal level of 25(OH)D is higher than the currently recommended levels of 25(OH)D for bone health [18]. Moreover, although circulating total 25(OH)D concentration is accepted as the best marker of vitamin D status (i.e.,

biomarker of exposure), in their 2011 report, the IOM raised concerns over the utility of serum 25(OH)D as a biomarker of effect (i.e., whether the level of total 25(OH)D is causally related to, and is a reliable predictor of, a health outcome of interest) [2]. Several studies have shown a significant inverse association between serum 25(OH)D levels and cardiometabolic disorders, however a significant association does not necessarily support the clinical utility of total 25(OH)D as a screening biomarker for an underlying health state in asymptomatic adults [2,19–21]. Therefore, using standardized measurement of 25(OH)D from NHANES 2001-2010, the objective for the second study of this thesis was to assess the clinical utility of serum 25(OH)D in the diagnosis of insulin resistance and to estimate the optimal 25(OH)D level associated with normal glucose and insulin homeostasis in asymptomatic, ethnically diverse U.S. adults (Chapter 5).

In addition to sun exposure behaviors, dietary vitamin D intake and ethnic background, it has been recently shown that serum level of 25(OH)D is also largely determined by body composition [22]. Obesity is a risk factor for vitamin D deficiency [23] and individuals with overweight and obesity have consistently lower serum 25(OH)D levels compared to individuals with normal weight [22]. However, the clinical consequences of lower serum 25(OH)D in individuals with overweight/obesity are not fully understood. Moreover, within the obesity category, the MHO (metabolically healthy obesity) phenotype displays low to intermediate risk for cardiometabolic health outcomes [24]; however, the biomarkers underlying the healthy metabolic profile of the MHO phenotype are not fully elucidated. Vitamin D has been hypothesized to play a key role in preserving the healthy metabolic profile of MHO phenotype [25]; however, conflicting evidence exists [26,27]. Moreover, while previous studies have investigated the individual contributions of obesity, metabolic health, and vitamin D status in relation to cardiometabolic health outcomes, their joint associations are largely unknown. Therefore, using a harmonized definition of MHO phenotype recently proposed by Ortega et al. [28], the third study of this thesis investigated the prognosis of MHO phenotype in relation to all-cause and cardiometabolic mortality and further evaluated the joint association of metabolic health phenotype and vitamin D status as measured by standardized 25(OH)D level using data from the Third National Health and Nutrition Examination Survey (NHANES III, 1988-1994) with follow-up through December, 2011(Chapter 6).

Lastly, in the context of long-term disease risk prediction, national guidelines suggest that clinicians should consider a patient's risk factor burden within the context of lifetime risk [29,30]. The predictive ability of traditional CVD risk factors (i.e., smoking, dyslipidemia, diabetes, and hypertension) in relation to lifetime risk for CVD has been well established [31,32]. However, the long-term predictive ability of total 25(OH)D is not well characterized. Previous studies have established an independent and inverse association between low serum 25(OH)D levels and CVD events among individuals with and without CVD at baseline [33,34]. However, a significant association does not necessarily support the predictive ability of a biomarker in the context of long-term disease risk prediction at the population level [19–21]. Moreover, previous studies have primarily assessed the relative risk associated with vitamin D deficiency; however, the relative risk reduction reported in previous studies has limited interpretation (i.e., relative risk reduction varies by choice of 25(OH)D cutoff). Therefore, to quantify the magnitude of absolute risk associated with vitamin D deficiency at the population level, an estimation of the absolute risk according to serum 25(OH)D cutoffs recommended by the IOM and ES would clarify the predictive value of these widely used cutoffs in relation to lifetime risk for cardiometabolic mortality. Therefore, the last study of this thesis evaluated i) whether serum 25(OH)D measured in middle to older-aged adults is associated with lifetime risk for cardiometabolic mortality, and ii) what the combined effect of serum 25(OH)D and traditional CVD risk factors might be on the remaining lifetime risk of cardiometabolic mortality. Effect modification by level of adiposity was further explored via stratification of analyses according to body mass index (BMI) categories. Data from NHANES III (1988-1994) with follow-up through December, 2011 was used for this study (Chapter 7).

Chapter 2: Literature Review

2.1 Global Cardiometabolic Risk

2.1.1 Background

The term "global cardiometabolic risk" is an umbrella term that refers to a comprehensive list of factors that contribute to risk of cardiovascular disease (CVD) and metabolic abnormalities such as type-2 diabetes [35]. The terminology was created to acknowledge the limitations of metabolic syndrome (clustering of risk factors such as abdominal obesity, dyslipidemia, hypertension, and dysglycemia), which was often used to evaluate individual risk for type-2 diabetes and CVD [36]. In response to controversies surrounding the application of metabolic syndrome in clinical practice (see section 2.1.3 'identification of cardiometabolic risk' below for detailed discussion), the concept of global cardiometabolic risk and its utility in clinical practice was described by Després and Lemieux in 2006 [37], in which they emphasized the need for development of a global algorithm that incorporates both traditional and emerging risk factors known to clinically affect risk of CVD and the underlying metabolic abnormalities such as type-2 diabetes (see figure 2-1 below). The concept of global cardiometabolic risk is analogous to the bench researcher's goal of discovering and identifying all known mechanisms when studying the pathogenesis of a disease. As such, assessment of global cardiometabolic risk provides a comprehensive assessment of CVD and metabolic abnormalities by incorporating both traditional and nontraditional risk factors. As our understanding of the pathophysiological process underlying cardiometabolic diseases evolves, one should also anticipate the identification of emerging risk factors/markers that contribute to cardiometabolic risk and this will allow clinicians to take early preventive actions in the early stages of CVD and type-2 diabetes.

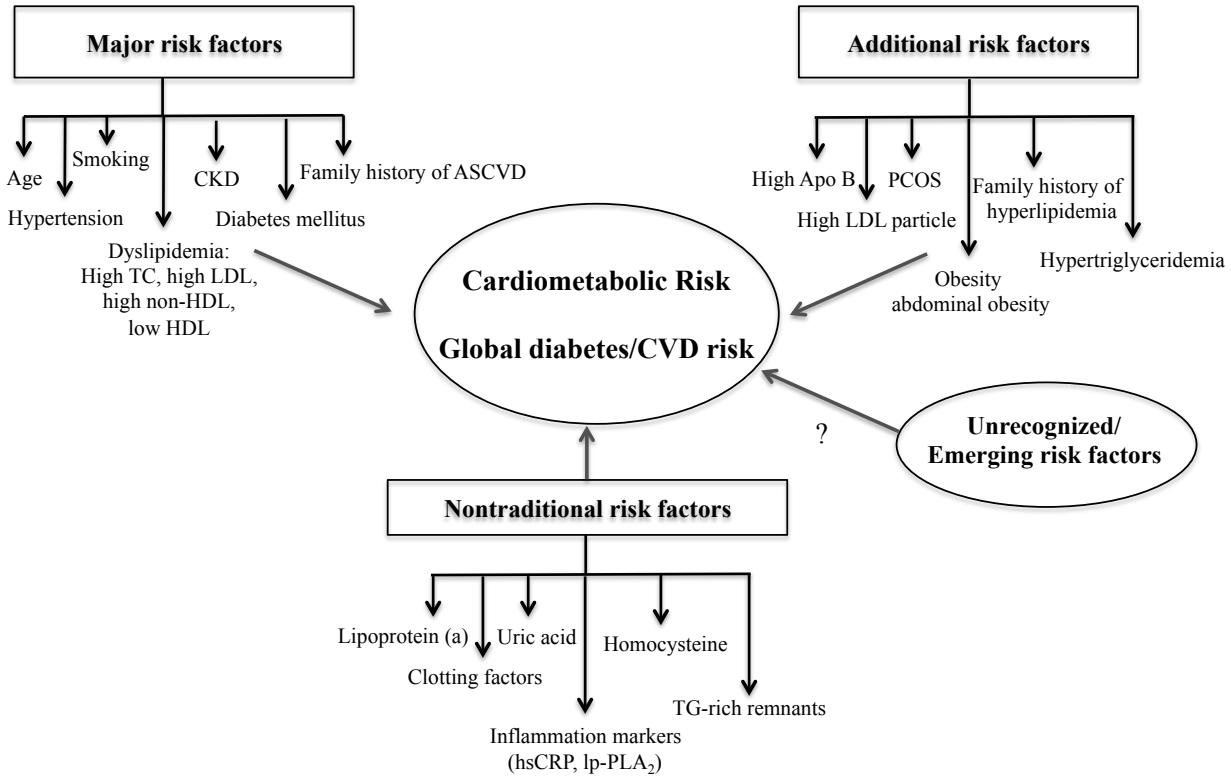

Figure 2. 1 A comprehensive list of risk factors that contribute to global cardiometabolic risk Modified from Jellinger PS et al. [38].

2.1.2 Pathophysiology

The pathophysiological bases of CVD and type-2 diabetes are complex, with some factors affecting both CVD and type-2 diabetes and while others are distinct. At the landmark Banting Lecture award in 1988, Gerald Reaven proposed that insulin resistance predisposes individuals to hyperlipidemia, hypertension, and type-2 diabetes and thus is the underlying cause of most CVD outcomes [39]. While there is ongoing debate regarding the direct contribution of insulin resistance to global cardiometabolic risk [36], insulin resistance is strongly associated with components of atherogenic, prothrombotic, and inflammatory states in individuals with cardiometabolic risk factors. At the initial stages of insulin resistance, there is a compensatory mechanism that increases the level of insulin secretion in order maintain normal blood glucose levels [39]. However, as insulin resistance progresses over time (due to genetics and environmental factors), this compensatory mechanism is reduced or impaired and the resulting

beta-cell dysfunction leads to reduced insulin production by the pancreas [39]. This reduction in insulin production is also associated with increased free fatty acid (FFA) levels in the circulation [39]. Thus, reduced insulin production coupled with increased FFA levels leads to elevation of blood glucose concentration resulting in type-2 diabetes [39]. In the liver, the increase in FFA flux increases the production of glucose, triglycerides, and very low-density lipoprotein (vLDL) and in the muscle, FFAs reduce glucose uptake by the muscle, resulting in hyperglycemia [35,40]. The increase in FFAs flux secondary to increased lipolysis in the adipose tissue also decreases glucose uptake in the adipose tissue, which has been implicated as a key component of cardiometabolic risk [35,40] In addition, hyperinsulinemia and increased FFAs flux may alter sodium reabsorption in the kidney and increase sympathetic nervous system activity leading to hypertension [35,39,40].

Prothrombotic and proinflammatory mechanisms are also involved in the etiology of CVD and type-2 diabetes. Visceral adipose tissue is a major source of local and systemic inflammatory molecules that exacerbate tissue level insulin resistance, cause vascular injury and promote atherosclerosis [41]. Specifically, inflammatory cytokines secreted by the adipose tissue (i.e., interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-alpha)) exacerbates insulin resistance in the liver, muscle, and vascular endothelial tissue by increasing lipolysis of triglyceride stores in the adipose tissue (i.e., resulting in increased FFAs flux), which ultimately leads to type-2 diabetes and CVD [41–43]. Furthermore, adiponectin (anti-inflammatory, insulin-sensitizing protein) levels are also reduced in state of insulin resistance [44,45]. In response to the vascular injury (due to increased secretion of cytokines and FFAs flux), the liver increases the production of fibrinogen, CRP, and plasminogen activator-inhibitor (PAI-1), which results in a prothrombotic state [35,40]. Moreover, TNF-alpha, IL-6, and CRP have also been shown to stimulate endothelial production of adhesion molecules (i.e. E-selectin, intercellular adhesion molecule-1, and vascular adhesion molecule-1) which are mediators of endothelial dysfunction in capillary and arteriolar endothelium [46].

In summary, the pathophysiological basis of CVD and type-2 diabetes risk is complex and involves a complex interplay among various tissues (i.e., liver, muscle, and adipose tissue), leading to clustering of risk factors that constitute the global cardiometabolic risk, namely

abdominal obesity, insulin resistance, glucose intolerance, hyperlipidemia, hypertension, prothrombotic state, and inflammatory state.

2.1.3 Identification of Cardiometabolic Risk

Cardiometabolic risk refers to the sum of risk factors that increase an individual's risk of CVD and type-2 diabetes. Numerous risk algorithms and clinical tools have been developed to assess an individual's risk for CVD or metabolic abnormalities, using either relative or absolute risk algorithms. Identification of cardiometabolic risk is mainly determined by assessment of traditional CVD risk factors such as hypertension, dyslipidemia, or smoking, by nontraditional risk factors such as insulin resistance, and by other risk factors including genetics and other environmental factors. It is well recognized that there is interplay among these risk factors and there is a large overlap between the main outcomes (i.e., people with type-2 diabetes are at a higher risk for CVD events). The goal of early screening is to obtain a comprehensive understanding of an individual's risk for cardiometabolic events through assessment of various risk factors known to clinically affect risk of CVD or metabolic abnormalities such as type-2 diabetes, thereby enabling appropriate individual preventive measures to be taken.

In relation to the assessment of insulin resistance, there are different laboratory techniques and indices available to measure insulin resistance in primary research and epidemiological studies (i.e., hyperinsulinemic euglycemic clamp [47], frequently sampled intravenous glucose tolerance test [48], homeostatis model assessment of insulin resistance (HOMA-IR) [49], whole body insulin sensitivity (IS) index [50]); however, measurement of insulin resistance remains a challenge in clinical practice. In 2001, the National Cholesterol Education Program Adult Treatment Panel III (NCEP ATP III) proposed metabolic syndrome (MetS) as a simple clinical tool to identify individuals likely to be characterized by insulin resistance and its related cluster of metabolic abnormalities [51]. At the time, assessment of MetS was deemed useful for establishing relative risk of both CVD and type-2 diabetes, where it has been estimated that the presence of MetS increases the risk of CVD by 1.5 to 2.0-fold and increases the risk of type-2 diabetes by 3 to 5-fold [52–56].

The MetS clinical criteria describe a constellation of risk factors originally known as "syndrome X" and it captures abnormalities in blood glucose, blood pressure (BP), triglycerides (TG), and/or low high lipoprotein (HDL) cholesterol, which are known to be down-stream effects of insulin resistance coupled with visceral or ectopic fat deposition [57]. While different cutoff values and inclusion of risk factors have been proposed by different research groups, the general consensus on the key features of MetS include visceral obesity, dysglycemia, hypertension, elevated TGs, and low HDL-cholesterol [58,59]. In 2009, the International Diabetes Federation Task Force on Epidemiology and Prevention, the National Heart, Lung and Blood Institute, the American Heart Association, the World Heart Federation, the International Atherosclerosis Society, and the International Association for the Study of Obesity agreed on a harmonized set of criteria for MetS, which categorizes MetS based on the presence of at least three of the following criteria: elevated waist circumference (population and country specific definitions, ≥ 102 cm for men and ≥ 88 cm for women in United States, Canada, and Europe), elevated triglycerides (≥ 150 mg/dL or drug treatment), reduced HDL cholesterol (< 40 mg/dL for males and < 50 mg/dL for females or drug treatment), elevated BP (systolic ≥ 130 mmHg and/or diastolic ≥ 85 mmHg, or drug treatment), and elevated fasting glucose (≥ 100 mg/dL or drug treatment) [60]. While MetS is useful for the assessment of *relative risk* of CVD and type-2 diabetes, assessment of MetS does not capture *absolute risk* and therefore, it does not assess global cardiometabolic risk [57]. Moreover, MetS does not weigh the severity of a risk factor (i.e., mild hypertension is classified in the same category as treated severe hypertension) and therefore the presence of MetS implies that relative risks for CVD and type-2 diabetes are increased irrespective of the severity or particular cluster of risk factors [61].

In clinical practice, it is important to estimate the absolute risk of cardiometabolic risk in high-risk individuals to implement appropriate prevention or treatment strategies. Numerous risk algorithms have been developed and validated to predict future risk of type-2 diabetes and CVD. There are few risk algorithms that predict future risk of type-2 diabetes [62]; however, there is no strong evidence of a clinical benefit to support a strategy of population-based screening for type-2 diabetes using risk algorithms [63]. On the other hand, numerous risk algorithms have been developed and validated to predict short-term (i.e., 10-year) CVD risk and these include the Framingham risk Score (FRS) [64,65], Reynolds Risk Score (RRS) [66,67], Multi-Ethnic Study

of Atherosclerosis (MESA) 10-year atherosclerotic CVD risk [68], and the Pooled Cohort Risk Assessment equation for atherosclerotic CVD, which is currently endorsed by the 2013 American College of Cardiology(ACC)/American Heart Association (AHA) guidelines [29]. All of these risk algorithms were developed to assess an individual's 10-year risk of atherosclerotic CVD and, importantly, they weigh the severity of risk factors (i.e. treated versus untreated hypertension). However, there are several shortcomings that should also be recognized with all 10-year risk assessment algorithms such as the FRS and other risk assessment algorithms that are currently endorsed by the U.S. and Canadian national guidelines. First, short-term risk algorithms are highly dependent on the patient's age such that older individuals are more likely to be targeted for therapy and therefore 10-year risk algorithms are not suited to capture CVD risk in younger populations with low short-term risk (< 10% in 10 years) [69,70]. Prior research has shown that 82% of US adults have low short-term risk (< 10% in 10 years); however, two thirds of these adults (an estimated 87 million) have a high residual lifetime risk for CVD [71]. Therefore, a lag time in risk factor development and heterogeneity in risk factor burden makes short-term risk prediction tools less effective, particularly in younger populations with low short-term risk [70,72]. The most recent guidelines in the US and Canada recommend that clinician should consider patients' risk factor burden within the context of residual lifetime risk to capture the total cumulative burden of CVD with advancing age, particularly in adults 20 to 59 years of age who are not at high short-term risk [29,30]. Second, short-term risk algorithms do not incorporate additional and nontraditional risk factors [i.e., obesity, triglycerides, Apo B, CRP, coronary artery calcification (CAC)] which have been shown to be strongly associated with CVD risk. The most recent guidelines by American Association of Clinical Endocrinologist and American College of Endocrinology recommend use of additional screening tools, such as the RRS which incorporates information on family history of heart attack and inflammation (i.e., CRP) or the MESA 10-year atherosclerotic CVD risk, which incorporates information on family history of heart attack and CAC [38]; however, it should be noted that these risk algorithms, similar to the FRS, only assess 10-year CVD risk and are not suitable for assessing risk in low to intermediate-risk individuals.

2.1.4. Clinical Utility of Biomarkers: Statistical Assessment

In a clinical setting, measurement of biomarkers allows clinicians to better identify high-risk individuals, diagnose certain disease conditions and predict prognosis of a disease. Advances in biomarker research related to CVD and type-2 diabetes over the past 30 years have led to development of more sensitive screening tools and improved treatments for high-risk individuals. The previous section described clinical tools and risk algorithms that have undergone various statistical assessments and the clinical utility of these tools has been well established. The use of emerging biomarkers for different stages of CVD and type-2 diabetes remains an active area of research and there is increasing interest in utilizing emerging biomarkers in the assessment of global cardiometabolic risk. This section will provide a general overview of current concepts related to statistical assessment of biomarkers and risk algorithms and the practical considerations that are prerequisite to their clinical use.

Clinicians often use a set of biomarkers as a clinical tool to optimally manage a patient. In 2001, the definition of a biomarker was standardized by National Institute of Health as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to a therapeutic intervention" [73]. Biomarkers can be classified on the basis of their intended use as screening biomarkers (identifying a subclinical disease), diagnostic biomarkers (identifying an underlying disease condition), or prognostic biomarkers (predicting future disease course, including recurrence and response to therapy) [73]. A biomarker can be measured using a biosample (i.e., blood or urine or tissue test), obtained from a recording (i.e., blood pressure), or an imaging test (i.e., echocardiogram). Subsequently in 2009, the American Heart Association established criteria for how biomarkers should be evaluated in a standardized fashion before they can be used in a clinical setting [74]. In order for biomarkers to be routinely used in clinical practice, there are certain characteristics of biomarkers that increase their clinical value. A biomarker needs to be measured using a standardized protocol that produces accurate and reliable measurement of the biomarker, has a high sensitivity (i.e., identifying true positives), specificity (i.e., identifying true negatives), and predictive values for the outcome of interest. It must also be easy to interpret by clinicians, have a measurement technique that is acceptable to the patient, and consistently

predict the outcome of interest independent of established predictors in multiple studies so as to translate into better patient outcomes [75].

Establishment of a significant association between a biomarker and a health outcome of interest is the first step in the assessment of a new biomarker; however, statistical significance alone is not sufficient to determine the clinical significance of a biomarker. Therefore, several metrics such as accuracy, discrimination, calibration, and reclassification of risk are used to assess the performance of biomarkers or risk algorithms [76]. Using the receiver operating characteristic (ROC) curve, the accuracy of a biomarker is evaluated in terms of its sensitivity (i.e., true positives) and its specificity (i.e., true negatives) at select cut-points [77]. The ROC curve displays the tradeoff between sensitivity and specificity of a biomarker or a risk algorithm in clinically identifying those with disease. Each point on the ROC curve represents the conditional probability of obtaining a positive test result from a diseased individual compared to a non-diseased individual [76]. Moreover, using information on sensitivity and specificity at select cut-points, the likelihood ratio (LR) can be calculated and may be more useful to clinicians in answering the following questions: *what is the likelihood of obtaining a positive test result in a patient with disease compared to a patient without disease (LR+)?*, and *what is the likelihood of obtaining a negative test result in a patient with disease compared to a patient without disease (LR-)?* [76]. The performance of biomarkers and risk algorithms can also be assessed in terms of their discriminative ability to distinguish cases from non-cases in cross-sectional studies or distinguish those who will develop disease from those who will not develop disease in prospective designs. The concordance index (or c-statistic) is used to measure discrimination and is equivalent to the area under the ROC curve [74]. It is important to point out that the c-statistic provides a metric for overall performance of biomarkers and risk algorithms and, therefore, it is possible for two different biomarkers and risk algorithms to have the same c-statistic, yet their accuracy may differ substantially in terms of sensitivity and specificity.

Although the c-statistic measures the discriminative ability of a biomarker and risk algorithm, it does not test whether the risk prediction is precise or well calibrated. Therefore, calibration is another metric for performance evaluation and it measures the ability of a biomarker and risk algorithm to accurately predict risk by comparing the predicted and observed frequency of

events. The Hosmer-Lemeshow goodness-of-fit statistic is often used as an indicator of model calibration [78]. The test divides the sample into deciles of risk and the observed number of events is compared with the expected number of events. Importantly, if a biomarker or risk algorithm underestimates or overestimates risk in certain subgroups, it can be re-calibrated in a population of interest (i.e., in other ethnic subpopulations).

Newer statistical methods have been developed to evaluate the clinical utility of new biomarkers beyond traditional measures such as detecting an increase in the c-statistic. In fact, the c-statistic has been shown to be a relatively insensitive measure when evaluating the incremental value of new biomarkers in a risk prediction model [20,79]. In response to criticisms and limitations of the c-statistic, the net reclassification index (NRI) and the integrated discrimination improvement (IDI) have been proposed by Pencina et al. to assess the degree to which a new biomarker improves risk classification [80,81]. The NRI and IDI tests measure the extent of risk reclassification by assessing whether the new biomarker correctly reclassifies risk upward for individuals who experience the event of interest and correctly reclassifies risk downward for individuals who do not experience the event of interest. While it has been shown that the NRI and IDI are more sensitive metrics than the c-statistic in evaluating the incremental value of new biomarkers, the underlying methodological approaches for both NRI and IDI have also been criticized [82–85]. Specifically, the NRI and IDI metrics have been criticized for applying equal weights to false-negative and false-positive cases [86] and for artificially inflating risk reclassification [84]. In response to these criticisms, decision analytic approaches have been proposed by Vickers et al. [87]. Specifically, Vickers at al. proposed the decision curve analysis (DCA) to assess the clinical utility of measuring a biomarker in a clinical setting. DCA estimates the net benefit by taking the difference between true-positive results and false-positive results. DCA analysis allows clinicians to assess the clinical consequences of having the biomarker information by plotting the net benefit (i.e., observing an increase in the proportion of true-positive cases for a given false-positive rate) across a range of risk threshold probabilities.

In summary, no single statistical measure can adequately assess all the pertinent characteristics of biomarkers and risk algorithms, and while several statistical approaches are available to statistically assess the performance of biomarkers and risk algorithms, the optimal test strategy is

a randomized controlled trial that assesses whether the use of the biomarker or risk algorithm in a clinical setting improves clinical outcomes, and whether the improvement in clinical outcomes is sufficient to justify the additional costs and subsequent treatments [76].

2.1.5 Obesity and Cardiometabolic Risk

Obesity is a progressive chronic disease with genetic, environmental, and lifestyle determinants. Obesity is arguably one of the strongest predictors of type-2 diabetes and CVD and is strongly associated with metabolic abnormalities characterized by insulin resistance [63,88]. In the Nurses' Health Study, 61% of the incident type-2 diabetes cases were attributable to overweight and obesity (defined as BMI $\geq$ 25 kg/m^2) [89]. Moreover, abdominal obesity (either assessed by waist circumference or waist-to-hip ratio) also predicts type-2 diabetes risk independent of BMI [90]. Furthermore, obesity is also associated with increased CVD events [91] and increased overall lifetime CVD risk [92]. While overweight and obesity are significant risk factors for type-2 diabetes and CVD, there are substantial individual differences in the cardiometabolic risk profile of individuals within the same BMI category [93]. A subset of individuals with obesity, termed 'metabolic healthy obesity (MHO)', has been identified based on healthy metabolic features such insulin sensitivity, high HDL-cholesterol, and low levels of fasting glucose and triglycerides [94–96]. Earlier studies reported that the MHO phenotype is not associated with increased risk of CVD events [95]. However, the clinical utility of the MHO phenotype has been controversial and some recent studies have challenged the existence of the MHO phenotype by showing that obesity, irrespective of the underlying metabolic health, is associated with an increased risk of CVD events [97–103]. While study-specific factors such as sample size, age, genetics, ethnicity, cardiorespiratory fitness, and follow-up time may explain some of the discrepancies among studies, the lack of a harmonized definition for the MHO phenotype largely accounts for these discrepancies [28]. Table 2-1 below presents the differences in the available definitions for the MHO phenotype, including the recently harmonized definition by Ortega et al. [28]. Prior to the harmonized definition proposed by Ortega et al., most studies included at least one metabolic abnormality to define MHO [97–101,103], yet the type of metabolic dysfunction varied widely, which could have affected the long-term risk estimates differently across studies. In contrast to previous definitions of the MHO phenotype (which allowed the presence of at least 1 risk factor), the newly harmonized criterion defines MHO as a BMI $\geq$ 30 kg/m^2 with zero

metabolic abnormalities. The newly harmonized definition of the MHO phenotype would allow researchers to assess whether obesity or metabolic health is a more important predictor of long-term health outcomes.

Table 2. 1 Various criteria for metabolically healthy obesity

	Meigs et al. [95]	Stefan et al. [96]	Aguilar-Salinas et al. [104]	Karelis et al. [105]	Wildman et al. [106]	Ortega et al. [28]
Obesity component	BMI ≥ 30 kg/m^2	BMI ≥ 30 kg/m^2	BMI ≥ 30 kg/m^2	BMI ≥ 30 kg/m^2	BMI ≥ 30 kg/m^2	BMI ≥ 30 kg/m^2
Metabolic components						
WC (cm)	≥ 102 (M) ≥ 88 (F)					
BP (mmHg)	≥ 130/85 or treatment		< 140/90 and no treatment		≥ 130/85 or treatment	≥ 130/85 or treatment
FPG (mg/dL)	≥ 100 or treatment		< 126 and no treatment		≥ 100 or treatment	≥ 100 or treatment
TG (mg/dL)	≥ 150 or treatment			< 150	≥ 150	≥ 150 or treatment
HDL-c (mg/dL)	< 40 (M) < 50 (F)		≥ 40	≥ 50 and no treatment	< 40 (M) < 50 (F) or treatment	< 40 (M) < 50 (F) or treatment
HOMA-IR				< 1.95	> 90th percentile	
Other		IS > 75th percentile		TC < 200 mg/dL LDL < 100 mg/dL and no treatment	hsCRP > 90th percentile	
MHO criteria	< 3 of the above	All of the above	All of the above	4 of the above	< 2 of the above	0 of the 4 above

Modified from Velho et al. [107]. BMI, body mass index; WC, waist circumference; F, female; M, male; BP, blood pressure; FPG, fasting plasma glucose; TG, triglycerides; HDL-c, high-density lipoprotein cholesterol; HOMA-IR, homeostasis model assessment of insulin resistance; WBISI, insulin sensitive; TC, total cholesterol; LDL, low-density lipoprotein.

2.1.6 Interventions to Reduce Cardiometabolic Risk

As described in previous sections, there is a complex interplay between genetics, epigenetics, and lifestyle factors in the pathophysiology of cardiometabolic disorders. While genetics may predispose some individuals to type-2 diabetes and CVD, modifiable factors such as obesity, sedentary lifestyle, and poor diet significantly contribute to the incidences of both CVD and type-2 diabetes [89,108]. Therefore, lifestyle interventions such as weight loss, diet, and physical activity remain the cornerstone of CVD and type-2 diabetes prevention and treatment.

The key components of lifestyle intervention include a healthy diet (i.e. primarily plant-based diet high in polyunsaturated and monounsaturated fatty acids, with limited intake of saturated fat and avoidance of trans fat), regular physical activity (i.e., at least 150 minutes/week of moderate-intensity exercise and 2 days/week strength training), weight loss in patients with overweight and obesity (i.e., 5-10%), and smoking cessation [29,30]. Suboptimal diet has been shown to be one of the strongest risk factors for cardiometabolic mortality; Micha et al. [109] recently reported that almost half of all cardiometabolic deaths (heart disease, stroke and type-2 diabetes) in the USA were attributable to unhealthy dietary patterns (i.e., diets high in sodium, low in nuts or seeds, high in processed meats, low in omega-3 fats, low in vegetables and fruits, and high in sugar-sweetened beverages). While lifestyle therapy is recommended for all patients, adherence to dietary and exercise interventions is often challenging at a community level [110]. A recent study using data from the National Health and Nutrition Examination Survey (NHANES) reported that < 2% of US adults meet all cardiovascular health metrics (i.e., nonsmoking status, optimal blood pressure, cholesterol levels, BMI, and glucose levels, being physically active, and having a healthy diet) [111].

Moreover, although lipid lowering agents such as statins have been proven to be effective in reducing the rate of CVD events in high-risk individuals, the residual risk of CVD remains high among patients prescribed high-dose statins, despite significant reductions in LDL cholesterol [112,113]. Furthermore, among patients prescribed statins, there is poor long-term adherence with non-adherence estimated at ~46% (where non-adherence is defined as taking less than 80% of the prescribed medication) [114]. Therefore, identification of cost-effective, alternative or

complimentary therapeutic options at the population level is needed to reduce the residual risk and decrease the burden of cardiometabolic health outcomes.

2.2 Vitamin D

2.2.1 Background

Vitamin D, also known as the sunshine vitamin, was first identified as a vitamin in the 20th century; however, it is now recognized as a fat-soluble prohormone. Vitamin D refers to a group of fat-soluble seco-sterols (i.e., its molecular structure is similar to steroid hormones but two of the B-ring carbon atoms (C9 and C10) are not joined) [115]. The two major forms are vitamin D_2 (ergocalciferol) and vitamin D_3 (cholecalciferol). Vitamin D is a 27-carbon secosteroid and the only difference between the two forms is that vitamin D_2 has an extra double bond between carbons 22 and 23, and a methyl group on carbon 24. Vitamin D_2 is produced in fungi and plants, whereas vitamin D_3 is produced in the skin after sunlight exposure and is found in animal products (e.g., fish oils and cod liver).

Vitamin D has been mainly recognized for its role in calcium metabolism and skeletal health. Vitamin D promotes calcium absorption from the intestine and aids in mineralization of newly formed osteoid tissue in the bone. It is well established that prolonged vitamin D deficiency leads to rickets in children, which is characterized by growth retardation and skeletal deformities, such as bowed legs or knocked knees [1]. In adults, severe vitamin D deficiency leads to osteomalacia, a condition characterized by aching bone pain and muscle weakness [1]. In the 1930s and 1940s, vitamin D fortification of milk was widely introduced in North America and Europe along with the promotion of sun exposure, which significantly eliminated rickets in children [116]. However, in the early 1950s, a limited number of cases of hypercalcemia and supravalvular aortic stenosis were reported in children, which were suspected to be due to vitamin D intoxication from milk [117,118]. Although several of the hypercalcemia cases from the 1950s were likely to be a result of an inherited disease (Williams syndrome), at that time, these cases were linked to vitamin D intoxication due to evidence from animal studies showing hypercalcemia and vascular calcification in animals treated with high pharmacological doses of vitamin D [118]. Therefore, vitamin D food fortification was banned in most European countries for some decades. It was not until the early 1980s when Robert Scragg challenged the notion of

vitamin D toxicity by introducing the hypothesis that the increase in cardiovascular disease in the winter might be a result of low vitamin D levels as a consequence of limited sunlight exposure [119].

Currently vitamin D is well recognized for its role in skeletal health; however, the non-skeletal health effects of vitamin D are less understood and there is ongoing debate in relation to vitamin D requirement and the impact of vitamin D status on several disease states including cardiovascular disease, type-2 diabetes, cancer, and autoimmune disorders [2,16]. The existing controversy has led to segregation of opinions on the significance of vitamin D in health and disease [120]. To date, there has been no consensus on optimal vitamin D status, mainly due to lack of standardized measurement of vitamin D and lack of well-designed RCTs in relation to non-skeletal health outcomes [8,121]. Currently, vitamin D recommendations are based on skeletal health outcomes; however, it has been argued that "one size fits all" recommendations for vitamin D are not sufficient and, instead, vitamin D recommendations should be disease-specific [18]. In addition, other risk factors such as ethnicity and obesity influence vitamin D levels and should be taken into consideration [120]. A more detailed discussion of these issues will be addressed in the following sections.

2.2.2 Sources of Vitamin D

Most people obtain vitamin D through skin exposure to sunlight, particularly solar ultraviolet B (UVB) radiation (290-315 nm) [122]. During sunlight exposure, 7-dehydrocholesterol (7-DHC), in the epidermis and dermis layers of the skin, is converted to previtamin D_3. Non-enzymatic (heat) isomerization of previtamin D_3 to vitamin D_3 is the last step in the synthesis of vitamin D_3. Synthesis of vitamin D_3 in skin is dependent on the amount of UVB radiation reaching the skin and substrate availability of 7-DHC [122]. A variety of other environmental and endogenous factors influence vitamin D_3 synthesis including latitude, season of the year, skin pigmentation, clothing, and use of sunscreen [122]. These factors are described in more detail in section 2.2.7 entitled "Factors affecting serum 25(OH)D levels".

Vitamin D can also be obtained through the diet, although to a smaller extent (~20%). Vitamin D_2 (ergocalciferol) can be obtained from UV irradiation of the plant sterol, ergosterol, which is found in fungus [1]. A major source of naturally occurring vitamin D_2 is sun-dried shitake

mushrooms (1600 IU of vitamin D_2 per 3.5 oz serving) [1]. On the other hand, vitamin D_3 (cholecalciferol) can be obtained from animal sources, such as cod liver oil (~400 IU of vitamin D_3 per teaspoon) and oily fish (e.g., wild caught salmon, 600 IU to 1000 IU; mackerel, 250 IU of vitamin D_3 per 3.5 oz serving) [1]. Given that there are a few naturally-occurring food sources of vitamin D, most countries including US and Canada fortify dairy products with vitamin D [123]. Fluid milk is voluntarily fortified with 385 IU/L of vitamin D_3 in the United States [123]. In Canada, under the Food and Drug Regulation, vitamin D_3 fortification of fluid milk (400 IU/L) and margarine (530 IU/100 G) is mandated [123]. Other voluntary vitamin D (D_2 or D_3) fortification practices include yogurts, bread products, cereals, orange juice, plant milk products, and infant formulas [123].

In addition to diet and sunlight exposure, vitamin D can be obtained from supplements. Vitamin D supplements can be in the form of either vitamin D_2 or vitamin D_3 [120]. Although vitamin D supplements can be vitamin D_2 or D_3, it has been shown that vitamin D_3 is more effective in raising blood 25(OH)D concentrations [124]. As such, it is generally recommended that vitamin D_3 formulation be used for nutritional and clinical purposes [2,124]. In the United States, vitamin D supplements can be found in multi-vitamin and mineral formulations (usually containing 400 IU per dose) as well as a single over-the-counter supplement (400 IU to 5,000 IU or up to 50,000 IU per dose) [2]. In Canada, vitamin D supplements can also be found in multi-vitamin formulations (400 IU per dose) and as a single over-the-counter supplement with dosage levels ranging from 400-1,000 IU and dosages above 1,000 IU can be obtained by a prescription [2].

2.2.3 Metabolism of Vitamin D

When vitamin D is obtained from the diet, it is absorbed with other dietary fats in the small intestine and is incorporated into chylomicrons, which are then transported into the circulation via the lymph system. Vitamin D can also be obtained through sun exposure, which involves the conversion of its precursor 7-DHC to previtamin D_3, which then undergoes a temperature-sensitive isomerization (i.e., rearrangement of three double bonds) to form vitamin D_3 [122]. Vitamin D, whether endogenously synthesized or ingested, is an inactive prohormone and must undergo a two-step hydroxylation to become fully activated [125]. Vitamin D is transported in the blood mainly bound to vitamin D-binding protein (vDBP) and is first transported to the liver

where vitamin D is enzymatically hydroxylated by the cytochrome P450 enzyme, 25-hydroxylase (CYP2R1 and/or CYP27A1), on carbon 25 to form 25-hydroxyvitamin D (25(OH)D, calcidiol) [125]. Serum 25(OH)D is the major circulating form of vitamin D and is used to measure vitamin D status [2]. 25(OH)D is then transported to the kidney where it is enzymatically hydroxylated by the enzyme 1α-hydroxylase (CYP27B1) on carbon 1 to form the active vitamin D metabolite, 1,25-dihydroxyvitamin D ($1,25(OH)_2D$, calcitriol) [125]. While the conversion of vitamin D to 25(OH)D in the liver is not tightly regulated, synthesis of $1,25(OH)_2D$ is strictly regulated by renal CYP27B1 activity to maintain a homeostatic range [125]. $1,25(OH)_2D$ is feedback regulated by serum calcium, phosphorous, parathyroid hormone (PTH), and fibroblast growth factor-23 (FGF-23) [125]. Furthermore, to prevent excess synthesis, $1,25(OH)_2D$ induces its own catabolism by stimulating the catabolic enzyme 24-hydroxylase (CYP24A1) [125]. 24-hydroxylase restricts the availability of $1,25(OH)_2D$ in two ways; i) by catalyzing the conversion of $1,25(OH)_2D$ into 24-hyroxylated, biologically inactive, water-soluble calcitrioic acid (1,24,25-hydroxyvitamin D) targeted for excretion by the kidney; ii) by converting 25(OH)D to 24,25-hydroxyvitamin D to decrease the amount of 25(OH)D available for 1α-hydroxylation [125]. A schematic representation of the vitamin D metabolic pathway is illustrated in Figure 2-2 below.

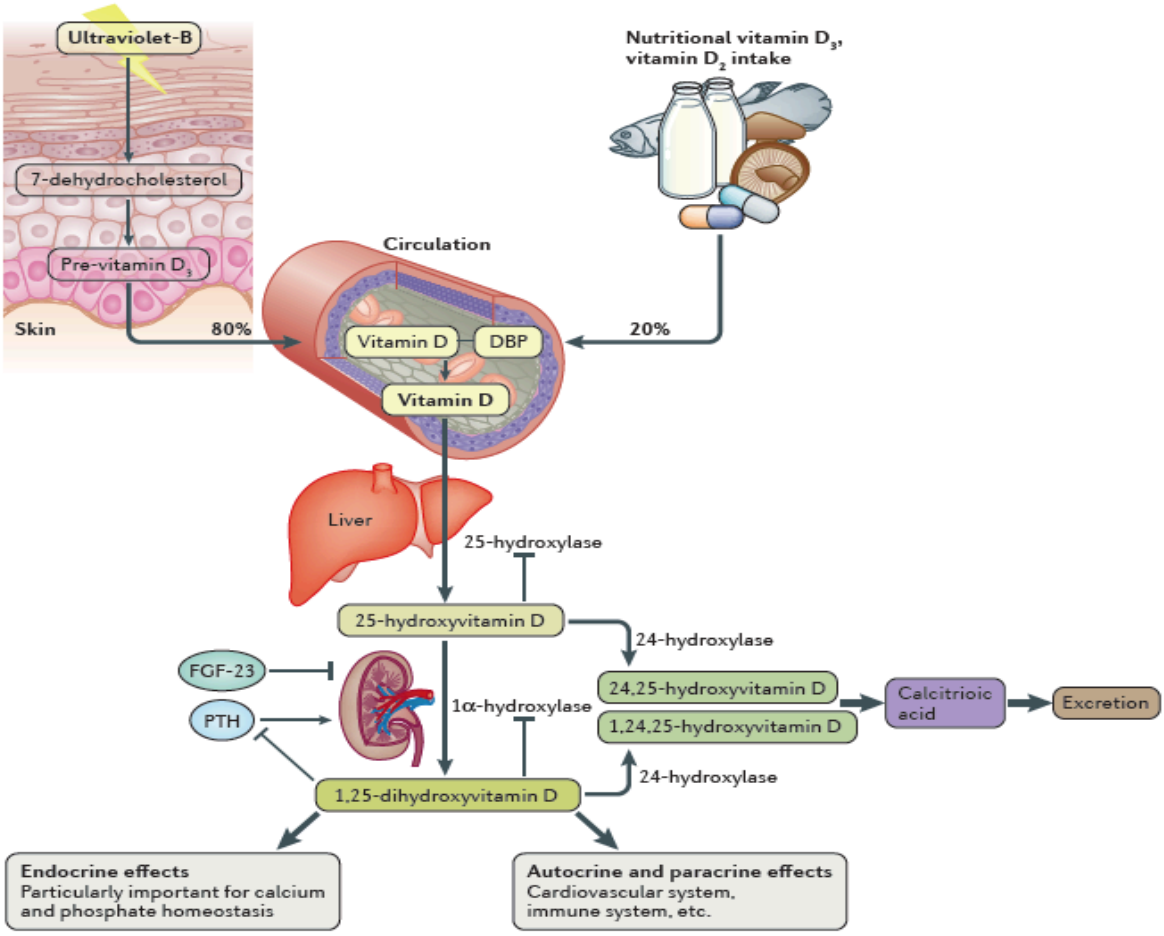

Figure 2. 2 Human metabolism of vitamin D.

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In addition to the renal production of $1,25(OH)_2D$, the 1α-hydroxylase is also expressed in other vitamin D target tissues and cells including skin, pancreas, brain, prostate, and immune cells [125], which results in the extra-renal production of $1,25(OH)_2D$ metabolite. While the $1,25(OH)_2D$ metabolite can be locally synthesized by extra-renal tissues, the regulation of the non-renal $1,25(OH)_2D$ remains an active area of research.

2.2.4 Vitamin D Mechanism of Action

The biological actions of $1,25(OH)_2D$ are mediated by the vitamin D receptor (VDR) [125]. The VDR belongs to the steroid receptor family which includes receptors for sex hormones, adrenal steroids, thyroid hormone and retinoic acid [125]. VDR is expressed in the classic vitamin D organs involved in calcium metabolism and homeostasis (i.e., intestine, bone, kidney); however, VDR has also been identified in other tissues and cells (i.e., heart, breast, colon, prostate and pancreas) [125].

The $1,25(OH)_2D$ hormone can enter the cell by diffusion, which is facilitated by binding of DBP to a cell surface receptor (i.e. megalin in the kidney) or can be locally synthesized (i.e., extrarenal conversion of 25(OH)D to $1,25(OH)_2D$ by CYP27B1) [125]. $1,25(OH)_2D$ then binds to VDR in the cytoplasm and this induces a conformational change to interact with retinoic acid receptor (RXR) to form a heterodimer [125]. The activated $1,25(OH)_2D$-VDR-RXR heterodimer then translocates to the nucleus and binds to specific DNA sequences [i.e., vitamin D response elements (VDREs)] in and around target genes to regulate their expression [125].

The classical function of $1,25(OH)_2D$ is to maintain serum calcium levels within a normal range and to optimize bone health. There are three different mechanisms by which $1,25(OH)_2D$ maintains calcium homeostasis. The first mechanism is the well-established role of $1,25(OH)_2D$ by increasing intestinal calcium absorption. This mechanism is based on the observation that the mineral and skeletal deformities of patients with hereditary vitamin D-resistant rickets (HVDRR, characterized by hypocalcemia, hyperparathyroidism, early-onset rickets, and organ resistance to $1,25(OH)_2D$) were reversed when these patients were given intravenous or high oral calcium [126]. In addition, when VDR null mice, representing an animal model of HVDRR, are fed a rescue diet high in calcium, rickets and osteomalacia are prevented [127,128]. These observations demonstrate that a defect in VDR signaling will result in impaired intestinal calcium absorption, which in turn impairs proper bone mineralization. The second mechanism involves active calcium reabsorption by the kidneys (in the distal tubules), which is regulated by $1,25(OH)_2D$ and PTH [125]. When serum calcium levels drop below the normal range, PTH levels are elevated and this in turn increases the synthesis of $1,25(OH)_2D$ in the kidney, which

then increases calcium reabsorption at the distal tubules [125]. The third mechanism involves bone remodeling. Calcium is a major constituent of the bone and bone structural integrity relies on sufficient calcium levels from the circulating blood. Blood supply of calcium is dependent on intestinal calcium absorption and renal calcium reabsorption. In adults, the bone is under continuous remodeling, where bone resorption is in balance with bone formation. Under conditions of inadequate dietary calcium supply and increased renal calcium loss, $1,25(OH)_2D$ works in conjunction with PTH to stimulate bone resorption, which in turn increases serum calcium levels [125]. In addition to mobilizing calcium from the bone, $1,25(OH)_2D$ also inhibits bone matrix mineralization during a negative calcium balance and thereby preserves normal serum calcium levels [129].

The biological effects of $1,25(OH)_2D$ are not limited to the maintenance of calcium homeostasis. The VDR is found in cells of organs not typically associated with calcium or bone metabolism [125]. In addition, the 1α-hydroxylase (CYP27B1) enzyme is present in numerous non-renal cells and tissues including skin, brain, pancreas, prostate and macrophages [125], resulting in local synthesis of $1,25(OH)_2D$ from circulating 25(OH)D. Over the course of the last decade, the non-classical action of vitamin D has been an active area of research.

2.2.5 Assessment of Vitamin D Nutritional Status

The assessment of vitamin D nutritional status is based on measurement of circulating total 25(OH)D (sum of $25(OH)D_2$ and $25(OH)D_3$) concentration in serum or plasma, which reflects both food intake and endogenous production of vitamin D [2]. Although other biomarkers of vitamin D metabolism and status (i.e., $1,25(OH)_2D$, serum cholecalciferol, free/bioavailable 25(OH)D, vDBP, 3-epi-25(OH)D, and $24,25(OH)_2D$) have been considered [130,131], total 25(OH)D is the accepted measure of vitamin D status [2]. In comparison to other vitamin D metabolites, serum 25(OH)D has a long half-life (~2 weeks), is directly linked to vitamin D substrate availability, and is widely studied in relation to various disease outcomes at the population level [2]. Therefore, total 25(OH)D is a reliable biomarker of vitamin D status at the population level [2,132].

2.2.6 Measurement of 25-hydroxyvitamin D

Accurate and precise measurement of total 25(OH)D levels is required to estimate the prevalence of vitamin D deficiency at the population level and to establish robust cut-offs linked to various health outcomes [8]. Although total 25(OH)D is considered the best available biomarker of vitamin D status, measurement of total 25(OH)D has been problematic despite substantial improvement in precision and accuracy of most commercial assays [133,134]. It has become clear that the older assays used in many of the vitamin D clinical trials lacked both accuracy (i.e., the ability of a test to return the 'true' value) and precision (i.e., the ability of a test to return the same result with repeated testing) [132]. There are various commercially available assays that quantify total circulating 25(OH)D, ranging from immunoassays and chemiluminescence assays to liquid chromatography tandem mass spectrometry (LC-MS/MS) (currently the accepted gold-standard method) [135]. However, these assays vary in their quantification of total 25(OH)D, with an estimated 20% variability between assays (even when the same samples are retested) [8–10,136]. Given the availability of several assays for the quantification of total 25(OH)D, the International External Quality Assessment Scheme for Vitamin D metabolites (DEQAS) was established in 1989 to monitor the variability and reliability of 25(OH)D assays [9]. DEQAS and other studies have documented the large inter-assay and inter-laboratory variability in the measurement of total 25(OH)D [7,9,137]. Due to considerable imprecision and inaccuracy among different assays, the Office of Dietary Supplements of the U.S. National Institute of Health (NIH) launched the vitamin D standardization program (VDSP) in 2010 in an effort to standardize the laboratory measurement of serum 25(OH)D worldwide and to provide standardized reference measurement procedures and reference materials to assay manufacturers and clinical laboratories [7,138]. The VDSP performance limits for an assay require a mean bias of ≤ 5% and a coefficient of variation (CV) of ≤ 10% [138]. Most importantly, the availability of standardized serum 25(OH)D levels will allow pooling of 25(OH)D data across different studies, which will improve our understanding of the role of vitamin D in health and disease [7].

2.2.7 Factors Affecting Serum 25(OH)D Levels

Numerous factors can affect cutaneous vitamin D synthesis and total circulating 25(OH)D levels. The solar zenith angle (SZA) is an important factor mediating the association between sun

exposure and cutaneous vitamin D synthesis [139]. The SZA is a function of time of day, year (season), and latitude; therefore, endogenous synthesis of vitamin D is minimal when the SZA is the largest (i.e., early morning and late afternoon, winter season from October to April, high latitude as measured by distance from the equator: living above 35 north latitude in the northern hemisphere) [140].

Advancing age has also been associated with reduction in the synthesis of vitamin D, due in part to decrease in 7-dehydrocholesterol levels and alterations in skin morphology [141]. Skin pigmentation is another factor influencing cutaneous vitamin D synthesis. Individuals with darker skin have a higher melanin content, which decreases UVB radiation reaching the skin [142]. In addition, sun avoiding behaviors as such as wearing clothing that covers most of one's body and use of sunscreen also reduces the amount of UVB radiation reaching the skin will significantly reduce cutaneous vitamin D synthesis [143,144] Other variables, such as air pollution, cloud cover, and sedentary behavior/physical inactivity can also impede sun exposure and thus reduce endogenous source of vitamin D. Genetic factors can also contribute to variability in total circulating 25(OH)D levels; however, genetics account for < 5% of the variance in 25(OH)D levels [145].

Body composition or level of adiposity is also another significant determinant of total circulating 25(OH)D levels. Populations with overweight and obesity have been consistently reported to have lower total 25(OH)D levels across age, ethnicity, and geography [22]. Moreover, a recent Mendelian randomization study confirmed the causal link between higher BMI and lower total 25(OH)D levels [23]. Ekwaru et al. observed substantial differences in serum 25(OH)D levels across categories of BMI and by absolute body weight (i.e, an average of 8.0 nmol/L and 20 nmol/L differences in participants with overweight and obesity relative to normal weight individuals) [146]. Initially, it was hypothesized that the lower 25(OH)D levels observed in patients with higher levels of adiposity was due to the increased sequestration of vitamin D in adipose tissue [147,148]. However, the most plausible hypothesis for the lower serum 25(OH)D levels observed among individuals with obesity is the greater volumetric dilution into fat, muscle, liver, and other tissues, and all of these compartments are increased in obesity [22,149]. Although individuals with overweight and obesity have significantly lower total 25(OH)D levels

than normal-weight individuals, the lower serum 25(OH)D levels may not necessarily reflect true vitamin D deficiency since most of the vitamin D is stored in adipose, muscle, and liver tissues and thus the amount distributed to serum is less [22]. Therefore, it is still not clear whether lower 25(OH)D levels observed among participants with overweight and obesity are associated with adverse health outcomes.

2.2.8 Recommended 25(OH)D Levels

Currently, there is no universal agreement in the definition of vitamin D deficiency, insufficiency, and sufficiency, as measured by serum concentration of total 25(OH)D, and there is discrepancy between two key guidelines from the Institute of Medicine (IOM) [2] and the Endocrine Society Task Force (ESTF) [16] (see Table 2-2 below). Serum concentrations for total 25(OH)D ranging from < 25 to < 75 nmol/L have been used as cut-off measures for vitamin D deficiency. The cut-off measures for vitamin D deficiency and sufficiency remains controversial mainly due to the unstandardized assays reporting great variability in outcomes and the lack of sufficient causality evidence from RCTs in relation to extra-skeletal health outcomes.

Table 2. 2 Reference cut points for total 25(OH)D levels according to IOM [2] and ESTF [16]

	Institute of Medicine	**Endocrine Society**
Deficiency	< 30 nmol/L	< 50 nmol/L
Insufficiency	30-< 50 nmol/L	52.5 - 72.5 nmol/L
Sufficiency	≥ 50 nmol/L	≥ 75 nmol/L

In 2011, the IOM updated the 1997 Dietary Reference Intakes (DRIs) for calcium and vitamin D and concluded that the evidence on skeletal, but not on non-skeletal health outcomes, was sufficient to provide a sound scientific recommendation for vitamin D intake requirements [2]. Therefore, the new DRIs for vitamin D were set in relation to skeletal health outcomes only (i.e., in relation to calcium absorption, bone mineral density, osteomalacia/osteoporosis, and rickets) [2]. In setting the new DRIs for vitamin D, the IOM used serum concentration of total 25(OH)D as a biomarker of vitamin D status and set reference levels for vitamin D under the assumption of minimal or no UVB exposure [2]. Given that there is no sufficient data by which to assess the dose-response relationships between vitamin D intakes and health outcomes, the IOM used total 25(OH)D as a biomarker of exposure (under the assumption of minimal or no sunlight exposure). In doing so, they set the estimated average requirement (EAR: median requirement meeting the

needs of 50% of the general population) at 40 nmol/L and the recommended dietary allowance (RDA: meeting the needs of 97.5% of the general population) at 50 nmol/L [2]. After setting the EAR and RDA, the committee then estimated the vitamin D intake required to achieve these target total 25(OH)D levels. To estimate the required vitamin D intakes, the committee performed a meta-regression analysis using aggregate data from RCTs that were performed during winter seasons at northern latitudes in Europe (> 49.5 °N) and in Antarctica (78 °S), and assessed the dose response relationship between the desired serum concentration of total 25(OH)D (i.e., RDA of 50 nmol/L) and vitamin D intake [2]. For infants, due to the insufficient evidence in estimating the EAR, adequate intakes (AI) were recommended instead of RDAs [2]. In addition to estimating the EAR, RDA, and AI for vitamin D, the committee also estimated the tolerable upper intake level (UL), which is the highest average daily intake that is likely to pose no risk of adverse health outcomes to almost all individuals in the general population [2]. Table 2-3 below provides a summary of the DRIs for vitamin D by life-stages.

Table 2. 3 Dietary Reference Intakes for vitamin D by life-stage

Life-stage Group	AI	EAR	RDA	UL
0 to 16 mo	400 IU	-	-	1,000 IU
6 to 12 mo	400 IU	-	-	1,500 IU
1-3 y	-	400 IU	600 IU	2,500 IU
4-8 y	-	400 IU	600 IU	3,000 IU
9-13 y	-	400 IU	600 IU	4,000 IU
14-18 y	-	400 IU	600 IU	4,000 IU
19-30 y	-	400 IU	600 IU	4,000 IU
31-50 y	-	400 IU	600 IU	4,000 IU
51-70 y	-	400 IU	600 IU	4,000 IU
>70 y	-	400 IU	800 IU	4,000 IU
Pregnant or lactating				
14-18 y	-	400 IU	600 IU	4,000 IU
19-30 y	-	400 IU	600 IU	4,000 IU
31-50 y	-	400 IU	600 IU	4,000 IU

Modified from Institute of Medicine 2011 report [2]

In addition to the vitamin D guidelines published by the IOM and ESTF, other guidelines recommend different target levels for total circulating 25(OH)D, depending on the population of interest and health-outcome. The recommended target levels for total 25(OH)D range from >25-30 nmol/L (Health Council of the Netherlands [101], Scientific Advisory Committee on Nutrition (SACN) in UK [102]), ≥ 50 nmol/L (European Food Safety Authority (EFSA) [103]), ≥ 75 nmol/L (International Osteoporosis Foundation (IOF) [104], American Geriatrics Society (AGS) [105]), and ≥ 100 nmol/L (vitamin D council and a few experts [17,155]) with a corresponding recommended daily average intake ranging from 200 IU to 2000 IU for most adults [17]. In deriving the recommended daily average intake for vitamin D, a major limitation of the DRIs set by the nutritional guidelines is the use of aggregate data meta-regression analysis, which only captures between-study variability, but not between-individual variability, which may lead to inaccurate estimation of the DRIs for vitamin D intake. To address this limitation, Cashman et al. [156] recently performed a meta-regression analysis using both aggregate data and individual participant data (IPD, considered the gold-standard) from seven vitamin D RCTs (total 882 participants) conducted during wintertime. Cashmen et al. found that the vitamin D intake needed to achieve a 25(OH)D level of at least 50 nmol/L in ≥ 97.5% of the population was 560 IU/d using aggregate data, whereas it was 1,040 IU/d when estimated using the IPD methodology [156]. Therefore, the choice of meta-regression analysis can significantly affect the estimates for DRIs and the general consensus is that IPD meta-regression should be used when estimating the dose-response curves for nutritional guidelines [156].

Lastly, although the IOM set 4,000 IU/d as an upper daily dose of vitamin D that is likely to pose no risk of adverse health effects in adults, there is no consensus on the upper safe limit of serum 25(OH)D levels. Nonetheless, serum 25(OH)D >250 nmol/L is generally considered the upper limit that is likely to increase the risk of toxicity [2,132]. This cutoff is in line with serum 25(OH)D levels of East Africans living in tropical weather conditions whose serum 25(OH)D do not exceed 250 nmol/L [157].

2.3 Vitamin D and Cardiometabolic Disease Risk

2.3.1 Background

In addition to the well-recognized role of vitamin D on skeletal health, emerging evidence suggests a potential role of vitamin D in cardiometabolic health outcomes, including insulin resistance, MetS, type-2 diabetes, and CVD. The earliest evidence on the link between vitamin D and CVD risk came from an ecological study by Robert Scragg in the early 1980s reporting a significant increase in CVD events in wintertime, the results of which led to the hypothesis that the increase in CVD events in winter might be a result of low serum 25(OH)D levels as a consequence of diminished sunlight exposure [119]. Early animal studies have also generated evidence on the link between vitamin D deficiency and impaired insulin secretion, a risk factor for type-2 diabetes [158–160]. Therefore, evidence from these earlier studies has generated new hypotheses and stimulated substantial research interest on the potential role of vitamin D in cardiometabolic health outcomes. Over the past decade, there have been numerous published studies on the non-skeletal health effects of vitamin D. The following sections will discuss the evidence from observational studies, Mendelian randomization studies, and randomized clinical trials (RCTs) of vitamin D supplementation that highlight research gaps.

2.3.2 Evidence From Mechanistic Studies

Insight into the role of vitamin D in the cardiovascular system came from animal studies investigating the effects of systemic and tissue-specific knockout of the VDR and 1α-hydroxylase enzyme (CYP27B1). Systemic VDR and CYP27B1 null mice exhibit hypertension and cardiac hypertrophy [161,162]. VDR activation has been shown to suppress the activity of the renin-angiotensin-aldosterone system [RAAS], a regulator of blood pressure and fluid balance [163]. Cardiomyocyte-selective VDR null mice also exhibit cardiac hypertrophy independent of the RAAS activity, thus indicating a direct antihypertrophic effect of $1,25(OH)_2D$ in vivo [164]. Mice with a systemic knockout of VDR also develop increased thrombogenicity [165]. Moreover, mice with knockout of VDR specifically in endothelial cells also develop endothelial dysfunction resulting from impaired blood vessel relaxation due to reduced endothelial expression of nitric oxide (NO) synthesizing enzyme, effects that are independent of the changes in RAAS activity [166,167]. In addition, the therapeutic role of $1,25(OH)_2D$ has

been tested in hypertensive, heart-failure prone rat models, where treatment with $1,25(OH)_2D$ in rats fed a high-salt diet reduced cardiac hypertrophy, an important factor in the progression of congestive heart failure [168].

Animal studies have also supported the role of vitamin D in pancreatic β-cell function, insulin resistance, and systemic inflammation, which are major risk factors for type-2 diabetes. The antidiabetic properties of vitamin D have been supported in *in vitro* and *in vivo* studies showing vitamin D deficiency impairs insulin secretion in rat pancreatic β-cells [158,159,169] and vitamin D supplementation improves impaired insulin secretion [158,160]. VDR is expressed in pancreatic β-cells, and vitamin D may directly affect β-cell function through binding of $1,25(OH)_2D$ to the VDR [170]. Moreover, the 1α-hydroxylase enzyme (CYP27B1) is also expressed in pancreatic β-cells, thereby allowing local synthesis of the active $1,25(OH)_2D$ metabolite [171]. Furthermore, in mice lacking a functional VDR, insulin secretion is impaired due to a reduction in insulin biosynthesis [172]. In humans, low serum 25(OH)D has been associated with impaired insulin secretion [173–175]; however, this observation has not been consistent across all studies [176]. The antidiabetic properties of vitamin D has also been linked to improving insulin sensitivity in insulin-responsive tissues [177]. $1,25(OH)_2D$ stimulates the expression of insulin receptors via binding to a vitamin D response element (VDRE) in the insulin receptor gene promoter [178–180]. Vitamin D may indirectly influence insulin sensitivity via regulation of calcium homeostasis, PTH, and fat infiltration in the liver and skeletal muscle [177,181–183]. Systemic inflammation is also involved in the pathogenesis of diabetes mellitus and CVD. $1,25(OH)_2D$ down regulates nuclear factor kappa-light-chain of activated B cells (NF-kappaB), which is a major transcription factor for TNF -alpha and other inflammatory mediators [184]. $1,25(OH)_2D$ may also protect against inflammation-induced diabetes risk and atherosclerosis through inhibition of foam cell formation and cholesterol uptake in macrophages [185,186]. Other immune-modulating effects of $1,25(OH)_2D$ include inhibition of dendritic cell differentiation, inhibition of lymphocyte proliferation, and enhanced regulatory T-lymphocyte development [187].

2.3.3 Evidence From Observational Studies

Low circulating serum 25(OH)D has been associated with indices of insulin resistance [12,188–191]; however, this association has not been consistent [176,192]. On the other hand, evidence on the association between vitamin D deficiency and incident type-2 diabetes has been more consistent; in a meta-analysis of 21 longitudinal cohorts (76,220 participants; 4996 incident diabetes cases), Song et al. reported a 38% relative risk reduction in type-2 diabetes for participants in the highest versus the lowest category of serum 25(OH)D [193]. Importantly, Song et al. showed that the dose-response relationship between serum 25(OH)D and type-2 diabetes risk was linear, where risk of diabetes declined monotonically with increasing serum 25(OH)D levels with no evidence of a threshold effect [193]. In relation to hypertension risk, a meta-analysis of prospective cohort studies including a total of 283,537 individuals and 55,816 incident cases of hypertension showed a 12% relative risk reduction in hypertension with every 25 nmol/L higher baseline 25(OH)D level [pooled RR=0.88 (0.81-0.97)] [194]. In relation to atherogenic lipid profile, the association between vitamin D deficiency and dyslipidemia has been mainly reported in cross-sectional study designs, where serum 25(OH)D has been shown to be positively associated with HDL-cholesterol and negatively associated with triglycerides [195]. Moreover, vitamin D deficiency has been also associated with inflammation and chronic kidney disease [196,197].

For CVD events, most of the observational studies support the link between vitamin D deficiency and increased risk of CVD (including myocardial infarction, stroke and CVD mortality) [4]. Although the inverse association between serum 25(OH)D and CVD risk has been consistent, some studies reported a J-shaped association, where both low (< 70 nmol/l) and high total 25(OH)D levels (> 125 nmol/l) were associated with increased risk of CVD [198]. However, in a recently updated meta-analysis of longitudinal cohorts (180,667 participants; 9,170 CVD deaths, 7,074 ischemic heart disease cases, 3,127 stroke cases, and 3,037 heart failure cases), Zhang et al. reported a 10% (RR=0.90, 95% CI: 0.86, 0.94) and 12% (RR=0.88, 95% CI: 0.80, 0.96) relative risk reduction in total CVD events and CVD mortality, respectively, per 25 nmol/L higher baseline 25(OH)D level [33]. Moreover, the highest risk for CVD was observed with 25(OH)D <60nmol/L and there was evidence of a non-linear association between

serum 25(OH)D and total CVD events and CVD mortality, although no evidence of a J-shaped association was observed [33]. In relation to the J-shaped association, it has been hypothesized that the increased CVD risk observed among those with high serum 25(OH)D levels > 125 nmol/L might be due to initiation of vitamin D supplementation or increased sun exposure as a consequence of a diagnosis of vitamin D deficiency or a clinical disease [199]; however, other unknown factors could also play a role.

While the link between vitamin D deficiency and CVD risk has been mostly consistent across observational studies, observational data is subject to residual confounding despite careful adjustments for confounders. Many disease conditions will reduce physical mobility and subsequently sun exposure, which may lead to reverse causality [200]. Moreover, the older assays used in previous studies assessing the association between serum 25(OH)D and cardiometabolic health outcomes are problematic because they lacked both accuracy and reliability in the measurement of total 25(OH)D. Specifically, unreliable and inaccurate measurement of 25(OH)D results in misclassification of individuals with respect to their serum 25(OH)D levels, which can lead to null associations in some subgroups (i.e., type-II statistical error) [8]. Moreover, while observational studies are useful in determining the dose-response relationship, pooling unstandardized 25(OH)D data in observational studies may lead to erroneous conclusions regarding the shape of the dose-response relationship and the inaccurate measurement of 25(OH)D prevents defining the absolute level of 25(OH)D that is associated with increased risk. Therefore, population based studies with available standardized measurement of 25(OH)D are needed to accurately assess the nature of dose-response relationship (e.g., linear versus non-linear relationship) as well as defining the absolute level of 25(OH)D that is associated with increased cardiometabolic disease risk. In addition to these limitations, most observational studies have been conducted in predominantly white/European populations and the role of vitamin D in other ethnic subpopulations is not well characterized. Although it is well known that there are large differences in serum concentrations of 25(OH)D between ethnic subpopulations (particularly in black and dark-skinned ethnic populations living in northern latitudes) [2,142], only a few studies have assessed the differences in 25(OH)D levels in relation to cardiometabolic health outcomes. Using unstandardized measurement of serum 25(OH) from NHANES data (1988-1994 and 2001-2006), ethnic-specific differences in diabetes

risk have been reported, where a significant negative association between 25(OH)D and type-2 diabetes risk was observed in Mexican-Americans and non-Hispanic whites, but not in non-Hispanic blacks [12,201]. Similarly, the association between serum 25(OH)D and heart failure and stroke has been found significant in whites but not in blacks [14,15]. Lastly, Robinson-Cohen et al. also reported an increased risk of coronary heart disease in vitamin D deficient whites and Chinese, but not in black or Hispanic subpopulations [13]. Therefore, results from studies conducted in predominantly white/European populations may not be generalizable to other ethnic subpopulations and it is important to further assess ethnic-specific variations using standardized measurement of 25(OH)D.

2.3.4 Evidence From Mendelian Randomization Studies

Mendelian randomization studies use information on genetic variations in single nucleotide polymorphisms (SNPs) that are involved in vitamin D metabolism to assess whether genetic variants affecting serum 25(OH)D concentrations are associated with a disease outcome [202]. By design, Mendelian randomization studies are not limited by confounding factors or reverse causation and therefore can provide a high level of evidence comparable to RCTs. In addition, Mendelian randomization studies capture the long-term exposure of vitamin D status (i.e. lifelong) and thus are considered to have an advantage over RCTs that are usually conducted for a short period of time (i.e., < 5 years).

To date, genome-wide association studies have identified five genetic loci that are associated with 25(OH)D concentrations [145,203]. These identified genes encode proteins that are involved in vitamin D metabolism, such as 7-dehyrocholesterol reductase (DHCR7), 25-hydroxylase (CYP2R1), 1α -hydroxylase (CYP27B1), 24-hydroxylase (CYP24A1), and vDBP (GC, group component) [145,202,203]. The genes encoding these proteins have been studied in Mendelian randomization studies to assess whether genetic variants affecting the synthesis and metabolism of vitamin D are associated with cardiometabolic health outcomes.

In relation to cardiovascular risk factors, a Mendelian randomization study reported a causal relationship between high 25(OH)D levels and a favorable lipid profile [204]. In another study, the causal relationship between genetically determined 25(OH)D was significant only in relation

to non-fasting remnant cholesterol (i.e., triglyceride-rich lipoproteins); however, no significant association in relation to HDL or LDL cholesterol was found [205]. In relation to markers of inflammation, a Mendelian randomization study did not support a causal association between genetically determined 25(OH)D and CRP levels [206]. For hypertension, a Mendelian randomization study using genes encoding vDBP levels as a proxy for 25(OH)D concentration found no causal association in relation to hypertension [207]; however, in another larger study, using genes encoding the 25-hydroxylase (CYP2R1) and DHCR7 as a proxy for 25(OH)D concentration, the authors found that a 10% increase in genetically determined 25(OH)D was associated with a significant decrease in diastolic BP (-0.29 mm Hg, 95% CI -0.52 to -0.07), systolic BP (-0.37 mm Hg, 95% CI -0.730 to 0.003), and 8.1% relative reduction in odds of hypertension (OR 0.92, 95% CI 0.87-0.97) [208].

In relation to type-2 diabetes risk factors, genes encoding the vDBP were not causally associated with fasting glucose or insulin levels [207]. For incident type-2 diabetes, a study reported a causal association between genetically determined 25(OH)D and type-2 diabetes risk [209]; however, this finding has not been supported by other Mendelian studies [210,211]. For type-1 diabetes, genes encoding CYP27B1, CYP2R1, and DHCR7 have been shown to be causally related to type-1 diabetes risk, supporting a genetic etiological role of vitamin D deficiency in type-1 diabetes [212]. Lastly, a small Mendelian study supported a causal link between serum 25(OH)D and adiponectin levels (considered to protect against cardiovascular diseases) [213].

In relation to CVD events, Mendelian randomization studies using genotypes of SNPs in vDBP levels, CYP2R1 or DHCR7 do not support a causal relationship between genetic variants affecting 25(OH)D concentration and coronary artery disease, stroke, or cardiovascular mortality [207,214–216]. In a large Mendelian study by Afzal et al., the odds ratio for a 20 nmol/L lower baseline 25(OH)D concentration was associated with an increased risk of cardiovascular mortality (OR 1.13, 95% CI 1.03-1.24), however, genetically determined 25(OH)D was not associated with cardiovascular mortality (OR 0.77, 95% CI 0.55-1.08) [217].

Although Mendelian randomization studies are considered to have a high-level of evidence and to have the same advantages as RCTs in avoiding confounding and reverse causation, there are

certain assumptions in Mendelian studies that may not apply to vitamin D. Specifically, the tested genetic variants account for < 5% of the variation in 25(OH)D levels and may be further confounded by pleiotropic effects of genetic variants. Most importantly, Mendelian randomization studies are limited by the assumption of a linear association between genetic variants of 25(OH)D levels and a disease outcome [3,5]; this assumption may not hold for vitamin D because the association between serum 25(OH)D and CVD has been shown to be non-linear [33,218]. Therefore, Mendelian randomization studies may not be suitable for establishing causality in certain disease outcomes if the relationship between vitamin D and a disease outcome is nonlinear.

2.3.5 Evidence From Randomized Controlled Trials

There are several RCTs and meta-analyses of RCTs that show a beneficial effect of vitamin D supplementation on blood pressure in adults, specifically in those with vitamin D deficiency and elevated blood pressure [219–222]. However, in a large meta-analysis published in 2015 by Beveridge et al. that included 46 RCTs (4,541 participants), there was no evidence of a beneficial role for vitamin D supplementation in lowering blood pressure [223]. Moreover, results from a recent RCT showed that monthly, high-dose vitamin D supplementation (200,000 IU initially and 100,000 IU/month thereafter) for ~1.1 years (median; range 0.9-1.5 years) did not significantly reduce blood pressure in the overall sample; however, in a subgroup analysis of participants with 25(OH)D <50 nmol/L, there was a significant reduction in aortic systolic BP (-7.5 mm Hg, 95% CI -14.4 to -0.6) and significant reductions in indices of arterial stiffness including augmentation index (-5.7 mm Hg, 95% CI -10.8 to -0.6), pulse wave velocity (-0.3 m/s, 95% CI, -0.6 to -0.1), peak reservoir pressure (-8.6 mm Hg, 95% CI -15.4 to -1.9), and backward pressure amplitude (-3.6 mm Hg, 95% CI -6.3 to -0.8) [224]. In another recent RCT, Raed et al. found a significant improvement in arterial stiffness in African American participants (BMI ≥ 25 kg/m^2) who were deficient in vitamin D (25(OH)D < 50 nmol/l) at baseline, with greatest improvements observed among those who supplemented with 4,000 IU/d of vitamin D_3 for 16 weeks [225]. In summary, although the evidence from RCTs seems to be inconsistent, research to date does suggest a beneficial role of vitamin D supplementation in hypertension, particularly in individuals with 25(OH)D < 50 nmol/L.

In relation to type-2 diabetes risk, the majority of the trials have reported null findings, with some reporting small effects on indices of insulin resistance and glucose parameters in patients with pre-diabetes and type-2 diabetes. In a meta-analysis of 35 RCTs (a total of 43,407 participants), vitamin D supplementation had no significant effect on fasting glucose or HbA1c, insulin resistance, or incident type-2 diabetes among those with normal glucose tolerance [226]. In the same meta-analysis by Seida et al, nearly significant improvements in fasting glucose and HbA1c were detected for individuals with prediabetes receiving vitamin D supplementation [226]. In another recent meta-analysis of 10 RCTs targeting those with prediabetes, vitamin D supplementation significantly reduced fasting plasma glucose and HbA1c levels [227]. On the other hand, the largest RCT in Tromso, Norway for the prevention of type-2 diabetes did not support a beneficial role of vitamin D supplementation (20,000 IU/week for 3 years) for decreasing the risk of type-2 diabetes in pre-diabetic patients, despite a seemingly lower rate of incident diabetes throughout the study period [HR 0.90, 95% CI 0.69-1.18] [228]. Therefore, the results from the Tromso trial remain inconclusive because the trial was underpowered to detect small but clinically significant reductions in diabetes risk [228]. Among patients with type-2 diabetes, a meta-analysis of 15 RCTs published in 2012 by George et al. reported a significant reduction in fasting glucose (-5.76 mg/dL or -0.32 mmol/L) and improvement in insulin sensitivity in patients given vitamin D supplementation versus placebo [229]. Since this meta-analysis, three new meta-analyses have been published in 2017 [230–232]. In the first, among patients with type-2 diabetes, Wu et al. reported significant reductions in HbA1c (standardized mean difference (SMD) -0.25, 95% CI -0.45 to -0.05) with vitamin D supplementation, however, no significant reduction in fasting glucose was found (SMD -0.14, 95%CI -0.31 to 0.03) [230]. However, among patients with baseline total 25(OH)D < 50 nmol/L, Wu et al. reported significant reductions in HbA1c (SMD -0.39, 95% CI -0.67 to -0.10) and fasting glucose (SMD -0.27, 95% CI -0.46 to -0.07) [230]. In the second, Mirhosseini et al. reported significant reductions in fasting glucose [-4.9 mg/dL, 95% CI -8.1 to -1.6 mg/dL], HbA1c [-0.30%, 95% CI -0.45% to -0.15%], and insulin resistance assessed by HOMA-IR [-0.66, 95% CI -1.06 to 0.26] [231] among patients with type-2 diabetes who supplemented with vitamin D, with a minimum dose of 4,000 IU/day [231]. Lastly, the meta-analysis by Krul-Poel et al. did not find significant reductions in HbA1c levels after vitamin D supplementation, except within a subgroup of studies (n=4) with a mean baseline HbA1c ≥ 8% wherein vitamin D supplementation significantly

decreased fasting glucose [232]. Taken together, vitamin D supplementation is unlikely to provide additional benefits in people with normal glucose tolerance [226]. However, to date, only one such RCT [233] reported significant improvements in insulin resistance in vitamin D deficient (25(OH)D < 50 nmol/L) South Asian women receiving 4000 IU/d of vitamin D_3 for 6 months. Evidence also remains inconclusive for the effects of vitamin D supplementation in preventing the progression of pre-diabetes to diabetes [228,234–242], although vitamin D supplementation may help improve glycemic control and insulin sensitivity in patients with type-2 diabetes [229–232].

Trials have also reported inconsistent results on the relationship between vitamin D and inflammatory markers, such as CRP and tumor necrosis factor [243–245]. In the context of favorable lipid profile, the majority of RCTs do not support a role of vitamin D supplementation [246,247].

Results of RCTs for cardiovascular events have also been inconsistent and remain inconclusive [248–252]. Results from the RECORD trial (Randomized Evaluation of Calcium or Vitamin D) including 5,292 participants showed that vitamin D supplementation (800 IU/d) in older adults (age ≥ 70 years) with a previous history of fracture significantly reduced risk of heart failure (HR 0.75, 95% CI 0.58 to 0.97); however, no significant effects were detected for myocardial infarction (HR 0.97, 95% CI 0.75 to 1.26) or stroke (HR 1.06, 95% CI 0.8 to 1.32) [251]. In the same study, the authors also conducted a meta-analysis of 21 RCTs (total of 13,033 participants) and found that vitamin D supplementation had no effect on heart failure (HR 0.82, 95% CI 0.58-1.15), myocardial infarction (HR 0.96, 95% CI 0.83-1.10), or stroke (HR 1.07, 95% CI 0.91-1.29) [251]. In another meta-analysis of RCTs of vitamin D supplementation with or without calcium, vitamin D had no effect on the incidence of myocardial infarction/ischemic heart disease or stroke/cerebrovascular disease [252]. The results remained similar when RCTs with or without calcium supplementation were analyzed separately [252]. A Cochrane review in 2014 including 56 RCTs with 95,286 participants showed that vitamin D_3 supplementation significantly reduced all-cause mortality (RR 0.94, 95% CI 0.91 to 0.98) and cancer-mortality (RR 0.88, 95% CI 0.78 to 0.98); however, no beneficial effects were observed for cardiovascular mortality (RR 0.98, 95% CI 0.90 to 1.07) [253]. Lastly, results from the Vitamin D Assessment

(VIDA) study conducted in New Zealand also did not support a beneficial role of vitamin D_3 supplementation (initial dose of 200,000 IU followed a month later by monthly doses of 100,000 IU, median 3.3 years) in relation to incident CVD and CVD mortality, and results were similar in a subgroup analysis of individuals with 25(OH)D < 50 nmol/L [254].

Although most RCTs to date have failed to show a beneficial role of vitamin D supplementation in relation to cardiometabolic health outcomes, the inconclusive evidence from RCTs should not be interpreted as 'evidence of absence of a beneficial effect' [255]. It has been widely recognized in the literature that the design of most RCTs of vitamin D supplementation to date have had significant shortcomings [5,256]; most RCTs have not included CVD related outcomes as their primary endpoint, used low doses of vitamin D or insufficient doses to appreciably increase 25(OH)D levels, have been short in duration (< 5 years), and used different formulations of vitamin D (D_3 versus D_2) and dosage schedules (i.e. steady versus intermittent dosing schedules). In relation to dosing schedules, there is currently no consensus regarding the optimal vitamin D dosing schedule and numerous RCTs to date have administered bolus intermittent supplementation strategy (i.e., monthly, once every 3-12 months) in order to increase participant compliance. However, Bruce Hollis has previously suggested that steady intake of vitamin D (i.e., daily) may be more beneficial than intermittent bolus intake because of the difference produced in serum vitamin D (cholecalciferol) and 25(OH)D concentrations [257]. For instance, a large bolus dose results in a large spike in both serum vitamin D and 25(OH)D concentrations and an immediate drop-off in serum vitamin D concentration followed by a more gradual but pronounced drop in 25(OH)D [258]. In contrast, daily dosing results in less pronounced increases in serum vitamin D and 25(OH)D levels and maintains both serum vitamin D and 25(OH)D at steady levels over a longer period of time [258]. Without a steady supply of vitamin D, serum 25(OH)D levels fluctuate greatly with bolus dosing and individuals might still be vitamin D deficient in spite of increase in serum 25(OH)D level [258,259]. In line with this hypothesis, it has been shown that daily supplementation is more beneficial than intermittent dosing schedule for the prevention of respiratory infections [260] which might also be the case for other health outcomes such as CVD. Therefore, vitamin D dosing schedule is an important variable to consider when assessing the totality of evidence of causality for the role of vitamin D

supplementation in cardiometabolic health outcomes and more research in this area is needed to ascertain the optimal dosing schedule.

Another significant shortcoming of RCTs is the recruitment of vitamin D sufficient participants (i.e., if the non-linear association holds true for cardiometabolic health outcomes then including individuals with 25(OH)D level above the critical inflection point dilutes any beneficial effect of vitamin D supplementation and decreases the power of the study to detect statistically significant effects) [6]. To date, none of the RCTs of vitamin D supplementation in relation to incident CVD outcomes have targeted individuals with vitamin D deficiency. In line with this hypothesis, Brenner et al. recently showed that if RCTs recruited vitamin D deficient individuals only, then the power of the study would be sufficient to detect significant effects [261].

Furthermore, previous studies (observational and RCTs) have used unstandardized serum 25(OH)D data and variations in assay performance have contributed to the misclassification of individuals with respect to their serum 25(OH)D levels [8]. It is well recognized that misclassification often yields null associations (i.e., type-II statistical error) [8]. Due to the assay measurement issues, most RCTs to date have relied on vitamin D intake or supplementation dose rather than measuring achieved serum 25(OH)D levels. Numerous factors can affect serum 25(OH)D levels and therefore it is important to monitor serum 25(OH) levels throughout the study to ascertain a target level that is associated with a disease outcome. Moreover, the low response rates observed in RCTs weaken their external validity and there is large uncertainty as to whether results from RCTs are generalizable to the general population [5]. In addition, RCTs are susceptible to contamination and unblinding (i.e., participants in the placebo group have easy access to over the counter vitamin D supplements and blood testing which could dilute the effect of the vitamin D supplementation in the intervention group) [5]. Therefore, it is crucial to monitor serum 25(OH)D throughout the trial to prevent bias due to contamination and unblinding in the placebo group.

2.4 Summary of Research Gaps in the Literature

Although most underpowered RCTs have failed to show a beneficial role of vitamin D supplementation in relation to cardiometabolic health outcomes, emerging evidence from well-

designed longitudinal studies, RCTs and some Mendelian randomization studies suggest a potential role for vitamin D in reducing cardiometabolic risk and its underlying pathophysiological disorders such as insulin resistance and hypertension. Currently, it is widely accepted that circulating total 25(OH)D levels is the best available marker of vitamin D status, reflecting both sun exposure and dietary vitamin D intake. However, the measurement of total 25(OH)D has been problematic, especially with regard to accuracy and precision of commercially available older assays [7,9]. Imprecise and inaccurate measurement of serum concentrations of 25(OH)D may lead to misclassification of individuals [8], which may result in inconsistent findings across studies. Therefore, many of the studies conducted to date have often been limited by imprecise and inaccurate measurement of circulating total 25(OH)D levels. The VDSP was launched in 2010 to promote the standardization of 25(OH)D laboratory procedures worldwide, recognizing the necessity for accurate and precise measurement of 25(OH)D in clinical and research laboratories. Moreover, the optimal vitamin D status (as measured by serum 25(OH)D) remains controversial and there is discrepancy between national guidelines for recommended levels of total 25(OH)D. The discrepancy between national guidelines is partly due to the differences in laboratory assays for 25(OH)D and the inconsistent findings from epidemiological and RCT data in relation to non-skeletal health outcomes. For non-skeletal health outcomes, disagreement still exists about whether the optimal level of 25(OH)D is higher than the currently recommended levels of 25(OH)D for bone health [18]. It has been previously proposed that a 25(OH)D level of > 75-100 nmol/L is the optimal cutoff for cardioprotective properties of vitamin D [4]. On the other hand, some studies have suggested a J-shaped association of vitamin D with CVD risk, whereby lower (< 70 nmol/L) and higher (> 125 nmol/L) levels of vitamin D are associated with increased CVD mortality [198]. Therefore, the desired level of 25(OH)D in relation to cardiometabolic health outcomes is largely unknown and more research in this area is needed to assess the shape of the dose-response relationship between serum 25(OH)D and cardiometabolic health outcomes, using standardized measurement of total 25(OH)D concentration.

Many studies have also been conducted in predominantly white/European populations and the role of vitamin D in other ethnic subpopulations is not well characterized. Although it is well known that there are large differences in serum concentrations of 25(OH)D between ethnic

subpopulations (particularly in black and dark-skinned ethnic populations living in northern latitudes) [2,142], only few studies have assessed differences in 25(OH)D levels in relation to cardiometabolic health outcomes. Of interest, Robinson-Cohen et al. reported an increased risk of coronary heart disease in vitamin D deficient whites and Chinese, but not in black or Hispanic subpopulations [13]. Similarly, the association between serum 25(OH)D and heart failure and stroke was found to be significant in whites but not in blacks [14,15]. Therefore, results from studies conducted in predominantly white/European populations may not be generalizable to other ethnic subpopulations and it is important to further study the role of vitamin D in other ethnic subpopulations, who are at a higher risk of vitamin D deficiency compared to whites/European populations.

In addition to sun exposure behaviors, dietary vitamin D intake, and skin pigmentation, it has been recently shown that serum level of 25(OH)D is also largely determined by body composition [22] and that obesity is a risk factor for vitamin D deficiency [23]. The relationship between vitamin D status and cardiometabolic health outcomes may be confounded by adiposity levels. Individuals with overweight and obesity have consistently lower serum 25(OH)D levels compared to individuals with normal-weight [22]; however, whether the lower serum 25(OH)D levels observed in in these populations reflects a clinical problem is largely unknown [22]. In addition, within the obesity category, the MHO (metabolically healthy obesity) phenotype displays low to intermediate risk for cardiometabolic health outcomes; however, the biomarkers underlying the healthy metabolic profile of the MHO phenotype are not fully elucidated. Vitamin D has been hypothesized to play a key role in preserving the healthy metabolic profile of MHO phenotype [25]; however, conflicting evidence exists in the literature [26,27]. Moreover, while previous studies have investigated the individual contributions of overweight and obesity, metabolic health, and vitamin D status in relation to cardiometabolic health outcomes, their joint associations are largely unknown. Therefore, assessment of the joint contributions of vitamin D and metabolic health phenotype will be useful for evaluating whether vitamin D status plays a significant role in modifying the relationship between different metabolic health phenotypes and cardiometabolic health outcomes. Identification of novel risk markers may therefore help to identify cost-effective interventions that could be beneficial for helping individuals transition from a high-risk metabolically unhealthy state to a metabolically healthy state [24].

Lastly, although circulating total 25(OH)D concentration is accepted as the best marker of vitamin D status (i.e., biomarker of exposure), in their 2011 report, the IOM raised concerns over the utility of serum total 25(OH)D as a biomarker of effect (i.e., whether levels of 25(OH)D is causally related to and is a reliable predictor of the health outcome of interest) [2]. Several studies have shown a significant inverse association between serum 25(OH)D levels and cardiometabolic disorders; however such associations are not necessarily causal or predictive of the health outcome of interest [2]. Therefore, a significant association does not necessarily support the clinical utility or the predictive value of a biomarker in the context of long-term disease risk prediction at the population level [19–21]. Moreover, the clinical manifestation of vitamin D deficiency occurs over a long period of time (i.e., osteoporosis in elderly) and it is not known whether prolonged exposure to low vitamin D levels increases the residual lifetime risk of adverse cardiometabolic health outcomes. In the context of long-term disease risk prediction, national guidelines suggest that clinicians should consider patients' risk factor burden within the context of lifetime risk [29,30]. The association between traditional CVD risk factors (i.e., smoking, dyslipidemia, diabetes, and hypertension) and lifetime risks for CVD has been well established [31,32]. However, the effect of serum 25(OH)D status on the lifetime risk of CVD events has not been evaluated. Therefore, this thesis will also attempt to assess the clinical utility and predictive ability of serum 25(OH)D independently and jointly with other traditional risk factors in relation to cardiometabolic health outcomes (i.e., insulin resistance and cardiometabolic mortality).

In summary, although RCTs are the gold-standard in evidence based medical research, it is well recognized that RCTs are more difficult to conduct properly and test the right dose-response in a targeted population for a sufficient time period [262]. Due to time, cost, and challenges related to proper design of RCTs, evidence on the role of vitamin D in relation to non-skeletal health outcomes is likely to continue to emerge from a range of study designs including well-designed cohort studies with available standardized 25(OH)D data [5]. Therefore, this thesis will attempt to address some of the knowledge gaps mentioned above, specifically by assessing the association of standardized serum 25(OH)D data with cardiometabolic health outcomes (i.e., insulin resistance, MetS and Framingham 10-year CVD risk) to: i) further explore variation by

ethnicity (Chapter 4); ii) assess the clinical utility of serum 25(OH)D along with traditional risk factors in the diagnosis of asymptomatic individuals with insulin resistance and estimate the optimal threshold level associated with normal glucose and insulin homeostasis (Chapter 5); iii) evaluate the joint association of metabolic health phenotype (across BMI categories) and vitamin D status in relation to cardiometabolic mortality (Chapter 6), and iv) evaluate the association between vitamin D deficiency (independently and jointly with traditional CVD risk factors) and residual lifetime risk for cardiometabolic mortality both overall and across the spectrum of BMI (Chapter 7).

Chapter 3: Research Objectives

Objective 1

The first objective of this thesis was to examine the association between standardized total 25(OH)D and cardiometabolic health outcomes in an ethnically diverse U.S population cohort (NHANES, 2001-2010).

Aim 1a- To assess the cross-sectional association of standardized total 25(OH)D with indices of cardimetabolic health outcomes (i.e., HOMA-IR, MetS and Framingham CVD risk).

Aim 1b- To assess the ethnic-specific association of standardized total 25(OH)D with indices of cardiometabolic disorders (HOMA-IR, MetS, and CVD risk) in Mexican-Americans, non-Hispanic whites, and non-Hispanic U.S. blacks.

Objective 2

The second objective of this thesis was to assess the clinical utility of standardized total 25(OH)D in the diagnosis of insulin resistance and estimate the optimal total 25(OH)D level using cross-sectional data from NHANES, 2001-2010.

***Aim 2a*:** To assess the clinical utility of measuring total 25(OH)D along with traditional risk factors in the detection of insulin resistance in asymptomatic adults without diagnosed diabetes.

Aim 2b: To estimate the optimal level of serum 25(OH)D associated with normal glucose and insulin homeostasis.

Objective 3

The third objective of this thesis was to evaluate the separate and combined associations of obesity, vitamin D, and metabolic health with all-cause and cardiometabolic mortality using data from the Third NHANES (1988-1994) with mortality follow-up data through December 31, 2011.

Aim 3a: To investigate the relative contribution of obesity in the absence of metabolic dysfunction on the incidence of all-cause and cardiometabolic mortality.

***Aim 3b*:** To assess the joint association of metabolic health phenotype and vitamin D with all-cause and cardiometabolic mortality.

Objective 4

The fourth objective of this thesis was to assess the association between vitamin D status and lifetime risk of cardiometabolic mortality using data from NHANES III (1988-1994) with mortality follow-up data through December 31, 2011.

Aim 4a: To assess whether a single measurement of total 25(OH)D in middle- to older-aged adults is associated with lifetime risk for cardiometabolic mortality and to further explore variation by BMI.

***Aim 4b*:** To evaluate the combined effect of total 25(OH)D and traditional cardiovascular disease risk factors on the remaining lifetime risk of cardiometabolic mortality and to further explore variation by BMI.

Chapter 4: Study 1- Standardized serum 25-Hydroxyvitamin D concentrations are inversely associated with cardiometabolic disease in U.S. adults: A cross-sectional analysis of NHANES, 2001-2010.

Chapter 4 has been previously published.

Al-khalidi B, Kimball SM, Rotondi MA, Ardern CI. Standardized serum 25-Hydroxyvitamin D concentrations are inversely associated with cardiometabolic disease in U.S. adults: A cross-sectional analysis of NHANES, 2001-2010. *Nutrition Journal (2017)* 16:16. DOI 10.1186/s12937-017-0237-6.

Authors' contribution:

The authors' responsibilities were as follows: data acquisition (BA), conception (BA, CIA), statistical analysis (BA), drafting of manuscript (BA), critical review, editing, and interpretation of results (BA, SMK, MAR, and CIA).

4.1 Abstract

Background: Previously reported associations between vitamin D status, as measured by serum 25-hydroxyvitamin D [25(OH)D] concentrations, and cardiometabolic risk factors were largely limited by variability in 25(OH)D assay performance. In accordance with the Vitamin D Standardization Program, serum 25(OH)D measurement was recently standardized in the National Health and Nutrition Examination Survey (NHANES) to reduce laboratory and method related differences in serum 25(OH)D results. We evaluated the overall and ethnic-specific associations between the newly standardized serum 25(OH)D concentrations and cardiometabolic risk in U.S. adults.

Methods: This study examined standardized 25(OH)D data from five cycles of the NHANES (2001-2010). The total sample included 7,674 participants (1,794 Mexican-Americans, 4,289 non-Hispanic whites, and 1,591 non-Hispanic blacks) aged ≥ 20 years who were examined in the morning after overnight fasting. Serum 25(OH)D was directly measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS) in 2007-2010, and was predicted from LC-MS/MS equivalents for 2001-2006. Serum 25(OH)D levels were categorized into quartiles (< 43.4, 43.4-58.6, 58.7-74.2, ≥ 74.3 nmol/L). Cardiometabolic risk was defined by the homeostatic model assessment of insulin resistance (HOMA-IR), metabolic syndrome (MetS), and Framingham cardiovascular disease (CVD) risk. Prevalence ratios and 95% confidence intervals were calculated using modified Poisson regression.

Results: After full adjustment for confounders, serum 25(OH)D ≥74.3 nmol/L was associated with lower cardiometabolic risk compared to 25(OH)D < 43.4 nmol/L in the overall sample [HOMA-IR: 0.70 (0.59, 0.84); MetS: 0.82 (0.74, 0.91); CVD risk: 0.78 (0.66, 0.91)]. These associations remained significant in Mexican-Americans [HOMA-IR: 0.54 (0.35, 0.82); MetS: 0.73 (0.55, 0.96)], non-Hispanic whites [HOMA-IR: 0.81 (0.68, 0.96); MetS: 0.84 (0.73, 0.95); CVD risk: 0.78 (0.64, 0.93)]; and in non-Hispanic blacks [HOMA-IR: 0.67 (0.45, 0.99); MetS: 0.75 (0.56, 0.97); CVD risk: 0.58 (0.41, 0.81)].

Conclusions: Low vitamin D status is a significant risk factor for cardiometabolic disease in U.S. adults based on standardized serum 25(OH)D results, irrespective of ethnic background. Future studies using standardized 25(OH)D data are needed to confirm these results, particularly amongst U.S. blacks with 25(OH)D concentrations above 75 nmol/L.

4.2 Background

A role in the renin-angiotensin aldosterone system (RAAS) and extensive immunomodulatory properties have identified vitamin D as a potential modifiable risk factor in cardiometabolic disorders [218,249,263]. Measurement of vitamin D status is based on circulating total 25-hydroxyvitamin D [25(OH)D] concentrations, which reflect both food intake and endogenous production of vitamin D. Low serum 25(OH)D levels have been linked to a range of non-skeletal health conditions in adults, including metabolic disorders and cardiovascular diseases [264–268]. In addition, vitamin D is thought to play a protective role against the development of type-2 diabetes by improving the insulin secretion of pancreatic beta cells and by maintaining glucose homeostasis [173,189,269–271]. However, studies investigating the relation between vitamin D status and cardiometabolic disorders are inconsistent [272–275]. Among the possible explanations for this discrepancy include the substantial heterogeneity among definitions for vitamin D deficiency, different age and ethnic distributions, and large variations in the performance of serum 25(OH)D assays.

Previous analyses of the NHANES have relied on unstandardized serum 25(OH)D measured by the Diasorin radioimmunoassay (RIA) kit, a method that has been criticized for its lack of precision and documented bias [11,276]. In accordance with the vitamin D Standardization Program (VDSP), the National Center for Health Statistics (NCHS) of the Centers for Disease Control and Prevention (CDC) recently released the standardized serum 25(OH)D data files in October, 2015 [277]. The standardized 25(OH)D data provide the most reliable estimates of serum 25(OH)D concentrations using the ultra-high performance liquid chromatography-tandem mass spectrometry (LC-MS/MS) method [278]. The LC-MS/MS method has improved sensitivity and specificity for serum 25(OH)D metabolites compared to previous immunoassay methods, and the standardization of serum 25(OH)D data allows for comparison across different survey cycles of the NHANES, providing sufficient power to study risk associated with varying concentrations of serum 25(OH)D. Thus, previously reported associations between serum 25(OH)D with cardiometabolic disorders using unstandardized serum 25(OH)D data from previous cycles of NHANES (1988-1994, and 2001-2006) were likely affected by method-related variations in serum 25(OH)D assays.

In addition to the assay-related differences in serum 25(OH)D results, ethnic variations in the relationship between vitamin D status and cardiometabolic disorders have been documented, with mixed results. For example, ethnic-specific differences in diabetes risk by serum 25(OH)D status have been confirmed in previous NHANES cycles (1988-1994, and 2001-2006), where an inverse relationship between unstandardized 25(OH)D concentrations and type-2 diabetes risk was observed in Mexican-Americans and non-Hispanic whites, but not in non-Hispanic blacks [12,201]. Similarly, 25(OH)D concentrations were significantly associated with fatal stroke and heart failure in NHANES III, with increased risk seen in white participants with low 25(OH)D, but not in black participants [14]. Finally, in a prospective study, an increased risk of coronary heart disease events was reported in white or Chinese participants with low serum 25(OH)D, but not in blacks or Hispanics [13].

Previous analyses using unstandardized data were likely confounded by large variations in serum 25(OH)D results, and ethnic-specific analyses were further constrained by small sample sizes in underrepresented populations, such as U.S. blacks, adding more uncertainty to these estimates. Accurate assessment of the overall and ethnic-specific variations in vitamin D is therefore crucial, and dependent upon the use of standardized data with sufficient statistical power to examine ethnic differences in the relationship between vitamin D status and cardiometabolic risk. Thus, the purpose of this study was to provide a comprehensive assessment of cardiometabolic risk including insulin resistance, metabolic syndrome, and cardiovascular disease risk in relation to serum 25(OH)D levels in U.S. adults, and to estimate the ethnic-specific associations using the newly standardized serum 25(OH)D data from NHANES 2001-2010.

4.3 Subjects and Methods

4.3.1 Participants

Conducted by the NCHS, NHANES is a series of stratified, multistage probability surveys designed to collect cross-sectional data on the health and nutritional status of the civilian, non-institutionalized U.S. population. NHANES is an ongoing survey and data are reported in 2-year intervals, which are available for public use [279]. NHANES oversamples certain under-represented groups in the population, including Mexican Americans, blacks, older adults and

those of lower socioeconomic status. Each survey cycle consists of an in-home interview, physical examinations and laboratory tests. Descriptions of the standardized protocols used for data handling during the interview, laboratory, and physical examinations have been previously published [280].

We initially identified 23,968 adults ≥ 20 years with available standardized serum 25(OH)D data from 2001-2010. We excluded 10,939 participants who fasted < 8 hours, 466 pregnant women, 1,355 participants in "other Hispanic" or "other race" category, 73 with serum albumin < 2.9 g/dL, 1,081 with estimated glomerular filtration rate (eGFR) < 60 mL/min/1.73 m^2, and 2,380 participants with missing covariate information. Those in the "other Hispanic" category were excluded due to a change in the 2007-2010 NHANES sampling design, where Hispanics were oversampled instead of only the Mexican-American population [281]. As such, we only included those in the "Mexican-American" category. The final analytical sample included 7,674 adults ≥ 20 years who fasted for ≥ 8 hours and self-identified themselves as Mexican-American (MA), non-Hispanic white (NH-white), or non-Hispanic black (NH-black). A flowchart for study sample derivation is shown in **Figure 4.1**. NHANES was approved by the NCHS institutional board and all adults provided written informed consent [282].

4.3.2 Measurement of Serum 25(OH)D

For 2007-2010, serum 25(OH)D metabolites were analyzed by the CDC laboratory using the LC-MS/MS method and total serum 25(OH)D (nmol/L) was calculated as the sum of 25(OH)D_3 and 25(OH)D_2, excluding the C3-epi-25(OH)D_3 metabolite. For 2001-2006, serum 25(OH)D levels were initially measured using the Diasorin RIA kit (Stillwater, MN); however, due to concerns about bias and imprecision for the Diasorin RIA assay, the CDC developed regression equations to convert RIA values to LC-MS/MS equivalents for NHANES 1988-1994 and 2001-2006 [278]. In order to combine serum 25(OH)D measurements, we used the predicted LC-MS/MS equivalent total serum 25(OH)D data from 2001-2006 and the total serum 25(OH)D data from 2007-2010.

4.3.3 Outcome Variables

Insulin Resistance

Insulin resistance was estimated by the homeostatic model assessment-insulin resistance index (HOMA-IR), calculated as the product of the fasting insulin concentration (μU/ml) and the fasting plasma glucose concentration (mmol/L), divided by 22.5 [49]. We defined insulin resistance as HOMA-IR $\geq 75^{th}$ percentile (sex- and ethnic-specific). Fasting glucose concentrations were measured by a Hexokinase enzymatic method, and serum insulin concentrations were measured by a radioimmunoassay in 2001-2002, a two-site immunoenzymometric method in 2003-2004 and an ELISA two-site enzyme immunoassay method in 2005-2010.

Metabolic Syndrome

We used the revised U.S. National Cholesterol Education Program Adult Treatment Panel III (NCEP/ATPIII) report to define MetS [58] as 3 or more of the following 5 criteria: 1) waist circumference (WC) $\geq$ 102 cm in men or $\geq$ 88 cm in women; 2) triglycerides $\geq$ 1.7 mmol/L or medication; 3) high density lipid (HDL) cholesterol < 1.0 mmol/L in men or < 1.3 mmol/L in women or medication; 4) blood pressure (BP) $\geq$ 130/85 mmHg or treatment for hypertension; 5) fasting blood glucose $\geq$ 5.6 mmol/L or treatment for diabetes. WC and BP data were collected during physical examinations and descriptions of the standardized protocol are provided in the NHANES Anthropometry Procedures Manual [283]. Up to four BP readings were obtained during the physical examination and the average BP was estimated using the BP protocol provided by NHANES [284]. Triglycerides were measured enzymatically in serum samples from the morning session. HDL-cholesterol was measured using either the Heparin manganese precipitation method or a direct HDL-cholesterol immunoassay method. Prescription medication use was self-reported during the in-home interview. Using the standardized generic prescription drug codes, medication use was classified into four drug categories; HDL-specific medications, lipid medications, anti-hypertensive medications, and anti-hyperglycemic medications.

Framingham CVD Risk

The Framingham CVD risk score was used to estimate the 10-year composite risk for coronary heart disease, stroke, peripheral artery disease, and heart failure [65]. In the first step of the algorithm, point scores for age, sex, total and HDL cholesterol, SBP, treatment of hypertension, smoking, and diabetes status were assigned [65]. CVD risk scores were subsequently computed for each participant based on age- and sex-specific criteria (range for men: -3 to ≥ 18; women: -3 to ≥ 21), which were then translated into a participant's absolute 10-year risk for a CVD event. The specific details on the point scores for the CVD risk algorithm are provided in Tables 5-8 in reference [65]. In our study, an absolute risk of ≥ 15% was defined as "high" and an absolute risk of < 15% as "low" for 10-year predicted risk. These cutoffs were calibrated to approximate the absolute CVD risk associated with insulin resistance (12.6%) and MetS (14.9%) in our study sample.

Total cholesterol was measured enzymatically at Johns Hopkins lipid laboratory (Baltimore, MD). Treatment of hypertension was established from self-reported anti-hypertensive medications. Smoking status (0=nonsmokers, 1=current smokers) was self-reported during the in-home interview. Diabetes status was defined as a fasting glucose ≥ 126 mg/dL (7.0 mmol/L) or current use of diabetes medication.

4.3.4 Confounders

Age, sex, ethnicity, smoking status, educational attainment, physical activity (PA), dietary supplement intake and use of medications were self-reported by questionnaire during the in-home interview. Educational attainment was categorized as less than high school, high school graduate, and some college or college graduate or higher. The PA questionnaire included a series of questions related to participant's daily activities, leisure-time activities, and sedentary activities at home. Participants self-reported the number of days in the past month or in a typical week they engaged in daily and leisure time activities and the average duration for these activities. A metabolic equivalent (MET) of 4.0 for moderate, and 8.0 for vigorous intensity PA were used to estimate the MET minutes/week for each participant. Supplemental intake of vitamin D for 2007-2010 was obtained from 24-hour dietary recall, and total daily vitamin D intake was estimated across all supplement sources for 2001-2006. Vitamin D supplementation

was categorized as “any” versus “nonusers”. Season corresponds to season of blood draw, which was reported by NHANES as winter months (November-April) and summer months (May-October). Standing height and weight were measured during the physical examination and were used to calculate body mass index (BMI: kg/m^2).

4.3.5 Statistical Analysis

For descriptive statistics, weighted means for continuous variables and weighted percentages for categorical variables along with 95% confidence intervals (CIs) were used. Differences in descriptive statistics across ethnic groups were assessed by survey-weighted Wald-F-Test and Chi-square tests to examine the independence of means and frequencies, respectively.

Modified Poisson regression models with robust error variances were used to estimate the prevalence ratios (PRs) and 95% CIs for cohort studies [285,286]. Serum 25(OH)D concentrations were categorized into quartiles (< 43.4, 43.4-58.6, 58.7-74.2, ≥ 74.3 nmol/L). To calculate the PRs for each outcome, the lowest 25(OH)D quartile (<43.4 nmol/L) was considered as the referent group (PR=1.00). The models were adjusted for confounders such as age, sex, ethnicity, education, season of blood draw (winter, summer), survey cycle (corresponding to survey year), BMI, vitamin D supplement use, smoking, total PA and relevant medication use (lipid-lowering and anti-hyperglycemic medications). The adjustment of these confounders in the models was based on the association of the confounder variables with both the outcome measures and serum 25(OH)D levels. Pairwise interactions with ethnicity and age were explored by including the product terms in the models (i.e. 25(OH)D*ethnicity and 25(OH)D*age). Models for insulin resistance, MetS, and CVD risk were subsequently stratified by ethnicity, and the CVD risk model was further stratified by age groups (i.e. 20-59 and ≥ 60 years). To explore the possibility of differential bias by comorbid conditions, a series of sensitivity analyses was conducted after the exclusion of participants with physician diagnosed diabetes and CVD (congestive heart failure, coronary hearts disease, angina, heart attack, and stroke), and relevant medication use (i.e. lipid, blood pressure, and anti-hyperglycemic medications).
To ensure the representativeness of the data, clinical fasting weights were applied to account for survey cluster design, oversampling, and nonresponse. Statistical analysis was performed using SAS 9.4 (Cary, NC) survey procedures, which appropriately accounted for cluster sampling and

the complex sample design of NHANES. All statistical tests were two-tailed at the alpha=0.05 level of significance.

4.4 Results

4.4.1 Participant Characteristics

Baseline characteristics of the participants are shown in **Table 4.1**. Overall, the mean age of the sample was 45.3 (44.7, 45.9), and the majority of the sample was NH-white (55.9 % NH-white, 23.4 % MA, and 20.7 % NH-black). Of the 7,674 participants, 22.5% (21.0, 24.1) were taking vitamin D supplements, with supplement use significantly higher in NH-whites [24.8% (22.8, 26.7)] compared to MAs [13.0% (11.0, 15.0)] and NH-blacks [14.7% (12.1, 17.3)]. The prevalence of MetS in the total sample was 36.1% (34.6, 37.6), and NH-whites had the highest prevalence of MetS [36.9% (35.0, 38.8)] compared to MAs [34.8% (31.5, 38.1)] and NH-blacks [31.6% (29.0, 34.0)]. The absolute CVD risk in the total sample was 7.92% (7.63, 8.20), and NH-Whites had significantly higher absolute CVD risk [8.26% (7.93, 8.59)] compared to MAs [5.85% (5.40, 6.31)] and NH-blacks [7.19% (6.77, 7.61)].

4.4.2 Serum 25(OH)D and HOMA-IR

The adjusted PRs for cardiometabolic risk by 25(OH)D quartiles in the total sample are shown in **Table 4.2.** After adjusting for age, sex, BMI, PA, ethnicity, season, survey cycle, education, smoking, and use of vitamin D supplement, lipid-lowering and anti-hyperglycemic medications, the highest 25(OH)D quartile (≥ 74.3 nmol/L) had a 30% relative risk reduction in HOMA-IR [0.70 (0.59, 0.84)] compared to those in the lowest quartile (< 43.4 nmol/L). The associations remained significant across all ethnic groups (**Table 4.3A**). The highest relative risk reduction was observed in MAs [0.54 (0.35, 0.82)], followed by NH-blacks [0.67 (0.45, 0.99), and NH-whites [0.81 (0.68, 0.96)] with serum 25(OH)D ≥ 74.3 nmol/L compared to the lowest 25(OH)D quartile (< 43.4 nmol/L).

4.4.3 Serum 25(OH)D and MetS

Overall, serum 25(OH)D levels ≥ 74.3 nmol/L were associated with a 18% risk reduction in MetS [0.82 (0.74, 0.91)] compared to the lowest 25(OH)D quartile (**Table 4.2**). The association of serum 25(OH)D with MetS remained significant across all ethnic-groups (**Table 4.3A**). For

MAs, the second 25(OH)D quartile (Q2: 43.4-58.6 nmol/L) had a 20% relative risk reduction in MetS [0.80 (0.66, 0.97) and the highest quartile a 27% risk reduction in MetS [0.73 (0.55, 0.96)] compared to the lowest quartile. Significant associations were observed only in the highest 25(OH)D quartile in NH-whites [0.84 (0.73, 0.95)] and NH-blacks [0.74 (0.56, 0.97)] compared to the lowest quartile.

4.4.4 Serum 25(OH)D and CVD risk

In the overall sample, the relative reduction in CVD risk was 14% in the second 25(OH)D quartile [0.86 (0.76, 0.98)], and 22% in the highest versus lowest 25(OH)D quartile [0.78 (0.66, 0.91)] (**Table 4.2**). In ethnic-specific analyses, significant associations were observed amongst the highest versus lowest quartiles for NH-whites [0.78 (0.64, 0.93)] and NH-blacks [0.58 (0.41, 0.81)], but not in MAs [0.90 (0.69, 1.16)] (**Table 4.3A**).

There was a significant interaction between serum 25(OH)D and age in the CVD risk model. This is consistent with the Framingham CVD risk because age is the most heavily weighted variable in the algorithm, as shown in **Figure 4.2.** Therefore, we further stratified the association of serum 25(OH)D with CVD risk by age groups (20-59 and ≥ 60 years). **Table 4.4** shows the adjusted PRs of CVD risk associated with 25(OH)D quartiles by age category. Overall, stratification by age resulted in similar results compared to the main model, except that the PRs for CVD risk in participants for 20-59 years old were lower compared to participants ≥ 60 years old. Age stratification did not affect the observed non-significant association of 25(OH) with CVD risk in MAs. For NH-blacks, significant associations were attained in participants ≥ 60 years for the highest quartile [0.63 (0.45, 0.870], but no significant association was found in participants 20-59 years old.

4.4.5 Sensitivity Analyses

After excluding participants with comorbid conditions, the estimates of PRs for the adjusted associations of 25(OH)D with HOMA-IR, MetS and CVD risk remained statistically significant (**Table 4.2**). Similarly in the ethnic-specific analyses, the adjusted associations of 25(OH)D with HOMA-IR, MetS and CVD risk were numerically similar in MAs and NH-Whites after excluding participants with comorbid conditions, although the associations were attenuated for NH-Blacks due to significant reductions in the number of events, particularly in the highest

serum 25(OH)D quartile (**Table 4.3B**). Overall, the sensitivity analyses revealed the stability of the PRs estimates in the original models.

4.5 Discussion

In this study, we have shown that standardized serum 25(OH)D concentrations were inversely associated with insulin resistance, MetS and CVD risk in a nationally representative sample of U.S adults. These associations remained significant across all ethnic subgroups, except for CVD risk in MAs. Overall, the results of our study suggest that serum 25(OH)D concentrations ≥ 75 nmol/L may be the optimal threshold in relation to cardiometabolic risk. To our knowledge, this study provides the most recently updated and comprehensive estimate of the association between standardized serum 25(OH)D levels and cardiometabolic risk, and unlike previous studies, our findings demonstrate that low 25(OH)D is a significant risk factor for cardiometabolic risk in U.S. blacks.

The inverse associations between vitamin D status and insulin resistance, MetS and CVD risk are aligned with previous epidemiologic studies. Of note, previous analyses of NHANES have found an inverse association between unstandardized serum 25(OH)D levels with insulin resistance and pre-diabetes [269,287], MetS [264], and CVD mortality [288]. Similarly, prospective studies have shown an inverse association between serum 25(OH)D and insulin resistance [191] and incident CVD in the Framingham Offspring Study [265], and incidence of MetS in a cohort of non-diabetic adults [268].

Previous studies have shown ethnic differences in the association of serum 25(OH)D with cardiometabolic risk factors. Ethnic differences between serum 25(OH)D and risk of diabetes were found in NHANES III [12], and confirmed in NHANES 2001-2006 with significant inverse associations in MAs and NH-whites, but not in NH-Blacks [201]. In a prospective study, Robinson-Cohen et al. [13] reported a significant increase in the risk of coronary heart disease events with low 25(OH)D levels in whites and Chinese, but not in black or Hispanic participants from the Multi-Ethnic Study of Atherosclerosis. Lutsey et al. [289] also observed that low 25(OH)D levels were a stronger risk factor for the development of heart failure in whites than black participants from the Atherosclerosis Risk in Communities study. Conversely, our results

showed a significant inverse association of standardized 25(OH)D with cardiometabolic risk in MAs, NH-Whites, and NH-blacks.

Our results are in contrast to the vitamin D paradox observed in blacks, where prior studies have suggested that even though blacks have circulating 25(OH)D in the deficient range (< 50 nmol/L), a compensatory mechanism exists in relation to bone health [290,291]. At this time, it is not fully understood whether other compensatory mechanisms exist in relation to cardiovascular health outcomes, but it has been speculated that vitamin D binding protein (DBP) levels in blacks are significantly lower compared to whites to compensate for lower total serum 25(OH)D in blacks. It has also been suggested that total 25(OH)D may not be the best biomarker of vitamin D status in blacks [292]. However, the monoclonal immunoassay used by Powe et al. [292] has been criticized for its lack of sensitivity to DBP polymorphisms, which provides erroneous results for vitamin D metabolites [293]. Using a novel LC-MS/MS method, Henderson et al. [294] recently showed that levels of DBP do not vary between whites and blacks. Therefore, it is possible that the use of immunoassays in a population with a low range of vitamin D metabolites results in inaccurate total 25(OH)D concentrations in blacks and that the results from existing immunoassays might be insufficient to allow identification of a significant association within these populations.

Previous attempts to assess the ethnic-specific association between cardiometabolic disorders and 25(OH)D have been limited due to reliance on samples with a low number of blacks within the upper range of 25(OH)D concentrations (i.e. ≥ 75 nmol/L). In comparison with prior work, our results suggest significant risk reductions in NH-blacks, which we speculate is due to reduced variation from the standardization of 25(OH)D measurements across all NHANES survey cycles [278]. Moreover, the lowest levels of 25(OH)D were observed in NH-blacks in our study, supporting the hypothesis that black Americans have reduced cutaneous synthesis of vitamin D due to increased skin pigmentation and decreased sun exposure [142]. In support of this hypothesis, Alzaman et al. [295] did not observe any systemic difference in the absorption or bioavailability of oral vitamin D metabolites between US blacks and whites, neither do their results support the paradoxical clinical correlates of vitamin D in US blacks [295]. Taken

together, our results add to the current evidence by demonstrating that total circulating 25(OH)D is a significant biomarker of cardiometabolic risk in U.S. blacks.

Although mechanisms relating vitamin D deficiency and cardiometabolic risk factors are not fully elucidated, several important mechanisms warrant discussion. Insulin resistance is the core trait of the metabolic disturbances observed in MetS, type-2 diabetes and CVD risk [296]. The presence of vitamin D receptors (VDRs) in pancreatic β-cells suggest that the active metabolite 1-α-25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) could directly influence insulin secretion [297]. Since insulin secretion is a calcium dependent process, it is possible that $1,25(OH)_2D_3$ modulates insulin secretion by increasing intracellular calcium levels [297]. Moreover, vitamin D deficiency promotes secondary hyperparathyroidism, stimulates the RAAS, which in turn increases the secretion of aldosterone [298]. Both low serum 25(OH)D and high PTH levels are related to arterial stiffness and vascular dysfunction, which are significant elements of hypertension and CVD risk [299]. It is possible that lower 25(OH)D and higher serum PTH levels could synergistically or independently play a role in the pathogenesis of developing hypertension and future CVD [300]. Due to the lack of data in our study, we were not able to elucidate the role of PTH in our analyses, however, the exclusion of participants with eGFR < 60 mL/min/1.73 m^2 reduces the potential confounding of secondary hyperparathyroidism caused by chronic renal failure and the impaired vitamin D metabolism by the diseased kidneys. Future studies using standardized serum 25(OH)D data are needed to re-evaluate the optimal 25(OH)D thresholds that maximally suppress PTH levels [7]. The complexity of vitamin D and PTH metabolism makes it challenging to fully disentangle the individual contributions of these factors and warrants further investigation.

In addition, the relationship between vitamin D deficiency and metabolic traits of these diseases is confounded by adiposity where individuals with obesity have lower serum 25(OH)D levels compared to normal-weight individuals and this is consistent across different age and ethnic groups [301–304]. Hence, serum 25(OH)D concentrations are related to fat mass and changes in serum PTH, and adipokines, such as leptin and adiponectin, may be crucial in elucidating the relationship between vitamin D, PTH and metabolic disturbances [305,306]. It is possible that normal vitamin D metabolism may be disrupted in obesity due to elevated levels of serum leptin,

which has been shown to suppress the conversion of 25(OH)D to $1,25(OH)_2D_3$ [307]. Although we were not able to further adjust for leptin levels, we adjusted for BMI, which is a proxy for fat mass and leptin levels [308]. Moreover, given that obesity and metabolic diseases are associated with low-grade inflammation, the anti-inflammatory and immunomodulatory properties of vitamin D have been suggested and supported by previous studies showing an inverse, but inconsistent, relationship between vitamin D and inflammatory markers such as C-reactive protein (CRP) [309–311]. A recent Mendelian randomization study found no causal relationship between vitamin D and CRP [206], which suggests that it is unlikely that vitamin D deficiency directly contributes to increased inflammation or vice versa. In our study, we found no evidence of the potential confounding effect of CRP levels on the relationship between serum 25(OH)D and all outcome measures (data not shown). Taken together, experimental and observational studies suggest that the relationship between vitamin D and cardiometabolic disorders are multimodal and mediated through direct and indirect pathways. Further, it is important that the results of this study are interpreted according to variation in vitamin D metabolism genes, which could explain the heterogeneity of responses to vitamin D deficiency across ethnic groups [312].

Although the evidence from previous observational studies and our results suggest that serum 25(OH)D levels ≥ 75 nmol/L are associated with lower risk of cardiometabolic disorders compared to 25(OH)D ≤ 50 nmol/L, these findings have not been consistent in clinical trials [313–316]. Among the possible explanations for these differences include the substantial heterogeneity in the definition of vitamin D deficiency, different age structure and target population (i.e., vitamin D deficient versus sufficient individuals), primary versus secondary prevention of CVD, differences in the available assays used to measure vitamin D metabolites, as well as the inclusion of different confounders in their analyses. Although the relationship between optimal vitamin D status and cardiovascular health remains to be elucidated, the standardization of serum 25(OH)D data and the large sample size of our study allowed a comprehensive estimate of the association between 25(OH)D and cardiometabolic risk in US adults using a nationally representative survey sample. Given that many risk factors for CVD are clustered in insulin resistance and MetS, it is reasonable to speculate that low 25(OH)D is a significant risk factor in their development, which may ultimately contribute to increased CVD risk. Future studies must be sufficiently powered to estimate the overall benefit, as well as study

the risks and benefits associated with varying circulating 25(OH)D concentrations in ethnic subpopulations.

There are several limitations to this study. Due to the cross-sectional design, we are unable to rule out the possibility of reverse causation. Although we have adjusted for potential confounders, we cannot rule out residual confounding or the effect of unmeasured confounders. Further, seasonality of serum 25(OH)D levels in NHANES must be considered a confounder because of the sampling design wherein data is collected from northern states in the summer and southern states in the winter. This sampling design results in higher average 25(OH)D and an increased range of 25(OH)D concentrations in northern states. Despite these limitations, our study provides national estimates of the association between serum 25(OH)D and cardiometabolic risk in the U.S. population and in ethnic subgroups using standardized serum 25(OH)D data from 2001-2010.

4.6 Conclusion

In this large national sample, standardized serum 25(OH)D concentrations were significantly associated with cardiometabolic risk including insulin resistance, MetS and CVD risk in U.S. adults, and low 25(OH)D is a significant risk factor for cardiometabolic risk in MAs, NH-whites, and NH-blacks. Standardization of serum 25(OH)D allows accurate assessment of vitamin D status, and future studies are needed to re-evaluate these risks in black Americans with serum 25(OH)D concentrations above 75 nmol/L.

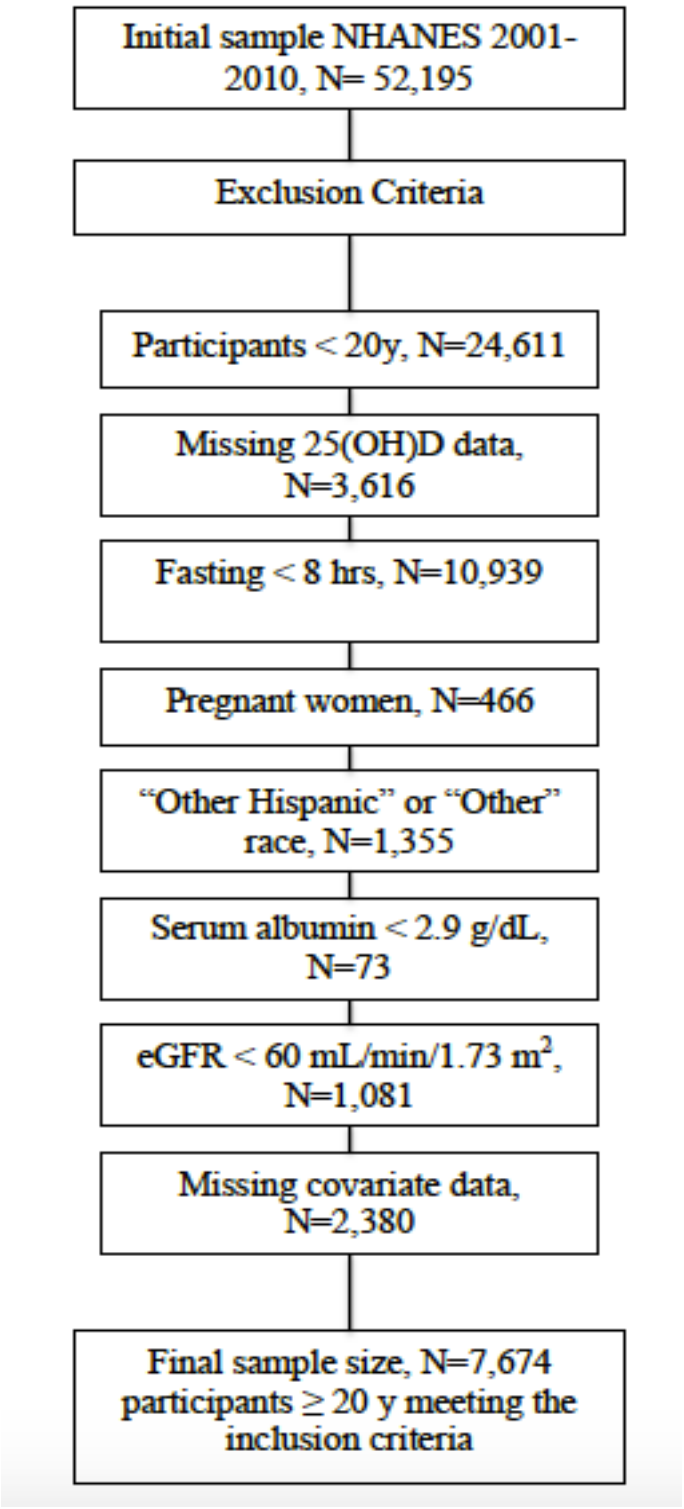

Figure 4. 1 Flowchart showing the exclusion criteria for sample derivation, NHANES 2001-2010.

Table 4. 1 Population Characteristics of U.S. Adults (≥ 20 years) in NHANES 2001-2010.

	All (N=7674)		Mexican Americans (N=1794)		Non-Hispanic Whites (N=4289)		Non-Hispanic Blacks (N=1591)		*P-value
Age (years)	45.3	44.7, 45.9	38.7	37.8, 39.6	46.6	45.9, 47.3	41.7	40.9, 42.6	< 0.0001
BMI (kg/m^2)	28.5	28.3, 28.6	28.8	28.5, 29.2	28.2	27.9, 28.4	30.1	29.7, 30.5	< 0.0001
WC (cm)	97.7	97.3, 98.2	97.1	96.2, 98.0	97.8	97.2, 98.3	98.3	97.3, 99.3	0.225
25(OH)D (nmol/L)	65.5	64.2, 66.9	53.9	52.4, 55.4	70.2	68.9, 71.5	42.2	40.3, 44.0	< 0.0001
Winter †	59.4	57.6, 61.3	52.3	50.6, 54.0	65.8	63.9, 67.8	39.8	38.2, 41.3	< 0.0001
Summer ‡	69.4	67.9, 70.9	58.4	55.8, 60.9	72.3	70.8, 73.8	44.9	41.8, 48.1	< 0.0001
Supplement nonusers	62.5	61.0, 64.0	52.9	51.3, 54.4	67.4	65.9, 69.0	39.9	38.1, 41.7	< 0.0001
Supplement users	75.9	74.2, 77.5	60.5	58.2, 62.7	78.5	76.9, 80.2	55.4	50.8, 59.9	< 0.0001
Vitamin D Supplement Use (%)	22.5	21.0, 24.1	13	11.0, 15.0	24.8	22.8, 26.7	14.7	12.1, 17.3	< 0.0001
HOMAIR	3.06	2.97, 3.16	3.76	3.52, 4.00	2.93	2.81, 3.05	3.4	3.20, 3.59	< 0.0001
MetS (%)	36.1	34.6, 37.6	34.8	31.5, 38.1	36.9	35.0, 38.8	31.6	29.0, 34.0	< 0.01
Absolute CVD risk (%)	7.92	7.63, 8.20	5.85	5.40, 6.31	8.26	7.93, 8.59	7.19	6.77, 7.61	< 0.0001
Self-reported Diabetes (%)	7.4	6.69, 8.10	8.76	7.33, 10.2	6.85	5.97, 7.74	10.1	8.42, 11.8	< 0.01
Self reported CVD (%)	6.34	5.61, 7.08	3.66	2.60, 4.71	6.66	5.79, 7.53	6.34	5.10, 7.59	< 0.0001
Medication Use (%)									
Blood Pressure	22.2	20.8, 23.7	9.88	8.26, 11.5	23.4	21.7, 25.1	24.2	22.1, 26.4	< 0.0001
Lipid	13.4	12.2, 14.5	6.06	4.65, 7.47	15	13.6, 16.3	8.31	6.90, 9.71	< 0.0001
Diabetes	5.2	4.58, 5.82	6.12	5.00, 7.24	4.8	4.06, 5.55	7.24	5.85, 8.61	< 0.01
Current smokers (%)	23.8	22.3, 25.4	19.1	16.7, 21.4	24.2	22.4, 26.1	24.8	22.2, 27.4	< 0.01
Education (% College)	58	55.8, 60.2	28.1	25.0, 31.1	62.6	59.7, 65.4	50.5	47.5, 53.4	< 0.0001

Values are weighted means or frequencies (%), and 95% CIs. **P* values are based on Wald F-Test or Chi-square test, which test the independence of means and frequencies across ethnic groups.

† Participants sampled from November – April. ‡ Participants sampled from May – October.

Table 4. 2 Prevalence ratios of cardiometabolic risk associated with serum 25(OH)D status in U.S. adults, NHANES 2001-2010.

	HOMAIR				Metabolic Syndrome				CVD Risk (≥ 15%)			
	Yes	No	PR	95% CI	Yes	No	PR	95% CI	Yes	No	PR	95% CI
Overall Sample												
25(OH)D quartiles (nmol/L)												
< 43.4	656	1231		**Ref**	853	1034		**Ref**	487	1400		**Ref**
43.4 - 58.6	529	1365	1.02	0.89, 1.17	825	1069	1.04	0.95, 1.13	495	1399	0.86‡	0.76, 0.98
58.7-74.2	444	1528	0.89	0.76, 1.04	835	1137	1.00	0.90, 1.10	553	1419	0.90	0.79, 1.02
≥ 74.3	289	1632	0.70‡	0.59, 0.84	630	1291	0.82‡	0.74, 0.91	430	1491	0.78‡	0.66, 0.91
***Healthy Sample**												
25(OH)D quartiles (nmol/L)												
< 43.4	347	890		**Ref**	361	876		**Ref**	150	1087		**Ref**
43.4 - 58.6	272	993	0.88	0.73, 1.06	350	915	0.99	0.84, 1.15	139	1126	0.85	0.64, 1.14
58.7-74.2	192	1076	0.83	0.66, 1.03	330	938	0.97	0.80, 1.17	141	1127	0.77	0.57, 1.04
≥ 74.3	108	1145	0.60‡	0.45, 0.81	193	1060	0.65‡	0.52, 0.80	119	1134	0.64‡	0.47, 0.87

Models are adjusted for age, sex, education, ethnicity, season of blood draw, survey cycle, smoking, BMI, total physical activity, vitamin D supplement use, and lipid and anti-hyperglycemic medications.

*Healthy Sample excludes those with self-reported diabetes, CVD, and taking medications (lipid, diabetes, blood pressure). Healthy sample models are adjusted for age, sex, education, ethnicity, season of blood draw, survey cycle, smoking, BMI, total physical activity, and vitamin D supplement use. ‡ $P < 0.05$.

Table 4. 3A Ethnic-specific prevalence ratios for cardiometabolic risk according to serum 25(OH)D status, NHANES 2001-2010.

	HOMAIR				Metabolic Syndrome				CVD Risk (≥ 15%)			
	Yes	No	PR	95% CI	Yes	No	PR	95% CI	Yes	No	PR	95% CI
Mexican Americans												
25(OH)D quartiles (nmol/L)												
< 43.4	175	346		**Ref**	263	258		**Ref**	122	399		**Ref**
43.4 - 58.6	156	445	0.9	0.70, 1.15	253	348	0.80‡	0.66, 0.97	132	469	0.85	0.66, 1.09
58.7-74.2	94	366	0.82	0.63, 1.07	188	272	0.95	0.78, 1.16	109	351	0.87	0.67, 1.14
≥ 74.3	25	187	0.54‡	0.35, 0.82	67	145	0.73‡	0.55, 0.96	45	167	0.90	0.69, 1.16
Non-Hispanic Whites												
25(OH)D quartiles (nmol/L)												
< 43.4	178	274		**Ref**	255	197		**Ref**	156	296		**Ref**
43.4 - 58.6	290	623	1.33	0.94, 1.36	452	461	1.07	0.95, 1.21	271	642	0.86	0.73, 1.01
58.7-74.2	314	998	0.93	0.77, 1.24	565	747	1.00	0.88, 1.14	378	934	0.88	0.75, 1.04
≥ 74.3	290	1322	0.81‡	0.68, 0.96	528	1084	0.84‡	0.73, 0.95	364	1248	0.78‡	0.64, 0.93
Non-Hispanic Blacks												
25(OH)D quartiles (nmol/L)												
< 43.4	246	668		**Ref**	327	587		**Ref**	209	705		**Ref**
43.4 - 58.6	80	300	0.89	0.71, 1.11	115	265	1.00	0.84, 1.19	92	288	0.83	0.65, 1.05
58.7-74.2	53	147	1.03	0.78, 1.38	78	122	1.11	0.89, 1.37	66	134	0.98	0.78, 1.23
≥ 74.3	18	79	0.67‡	0.45, 0.99	34	63	0.74‡	0.56, 0.97	21	76	0.58‡	0.41, 0.81

Models are adjusted for age, sex, education, season of blood draw, survey cycle, smoking, BMI, total physical activity, vitamin D supplement use, and lipid and anti-hyperglycemic medications. ‡ $P < 0.05$.

Table 4.3B Ethnic-specific prevalence ratios for cardiometabolic risk according to serum 25(OH)D status, NHANES 2001-2010*

	HOMAIR				Metabolic Syndrome				CVD Risk (≥ 15%)			
	Yes	No	PR	95% CI	Yes	No	PR	95% CI	Yes	No	PR	95% CI
Mexican Americans **25(OH)D quartiles (nmol/L)**												
< 43.4	98	273		**Ref**	135	236		**Ref**	41	330		**Ref**
43.4 - 58.6	89	343	0.99	0.72, 1.37	124	308	0.82	0.63, 1.07	32	400	0.49‡	0.29, 0.82
58.7-74.2	56	289	0.98	0.69, 1.38	106	239	1.08	0.82, 1.41	37	308	0.81	0.46, 1.44
≥ 74.3	15	143	0.53‡	0.31, 0.91	31	127	0.63‡	0.44, 0.91	17	141	1.27	0.83, 1.94
Non-Hispanic Whites **25(OH)D quartiles (nmol/L)**												
< 43.4	86	166		**Ref**	98	154		**Ref**	46	206		**Ref**
43.4 - 58.6	146	436	0.92	0.73, 1.19	184	398	0.98	0.79, 1.22	83	499	0.78	0.54, 1.13
58.7-74.2	124	692	0.76‡	0.58, 0.99	198	618	0.89	0.71, 1.12	92	724	0.68‡	0.47, 0.97
≥ 74.3	105	931	0.61‡	0.44, 0.83	156	880	0.61‡	0.47, 0.80	96	940	0.56‡	0.39, 0.80
Non-Hispanic Blacks **25(OH)D quartiles (nmol/L)**												
< 43.4	126	488		**Ref**	128	486		**Ref**	63	551		**Ref**
43.4 - 58.6	39	212	0.89	0.64, 1.24	42	209	1.05	0.77, 1.45	24	227	1.05	0.70, 1.58
58.7-74.2	19	88	1.1	0.69, 1.76	26	81	1.49	1.06, 2.10	12	95	1.26	0.66, 2.38
≥ 74.3	5	54	0.73	0.32, 1.70	6	53	0.55	0.25, 1.21	6	53	0.72	0.33, 1.58

*Excluding participants with self-reported diabetes, CVD, and those taking relevant medications. Models are adjusted for age, sex, education, season of blood draw, survey cycle, smoking, BMI, total physical activity, vitamin D supplement use. ‡ $P < 0.05$.

Table 4. 4 Adjusted prevalence ratios of cardiovascular risk (≥15%) associated with serum 25(OH)D by age, NHANES 2001-2010.

		CVD risk (≥ 15%)							
Serum 25(OH)D quartiles (nmol/L)		**Q1 (< 43.4)**	**Q2 (43.4-58.6)**		**Q3 (58.7-74.2)**		**Q4 (≥ 74.3)**	
Overall	20- 59 (y)	Ref	0.78	0.60, 1.02	0.93	0.70, 1.23	0.65‡	0.47, 0.92
	≥ 60 (y)	Ref	0.99	0.89, 1.09	0.89‡	0.80, 0.99	0.82‡	0.73, 0.92
*Mexican Americans	20- 59 (y)	Ref	0.70	0.49, 1.01	0.80	0.50, 1.27	0.75	0.44, 1.29
	≥ 60 (y)	Ref	0.93	0.79, 1.10	0.92	0.78, 1.08	0.81	0.63, 1.03
*Non-Hispanic Whites	20- 59 (y)	Ref	0.70	0.48, 1.02	0.89	0.62, 1.29	0.61‡	0.40, 0.93
	≥ 60 (y)	Ref	1.01	0.88, 1.16	0.90	0.79, 1.04	0.82‡	0.74, 0.97
*Non-Hispanic Blacks	20- 59 (y)	Ref	1.03	0.75, 1.43	0.97	0.69, 1.37	0.51	0.19, 1.40
	≥ 60 (y)	Ref	0.88	0.75, 1.04	0.86	0.71, 1.04	0.63‡	0.45, 0.88

Models are adjusted for age, sex, education, ethnicity, season of blood draw, survey cycle, smoking, BMI, total physical activity, vitamin D supplement use, and lipid and anti-hyperglycemic medications. ‡ $P < 0.05$.

*The ethnicity covariate is excluded in the ethnic-specific models.

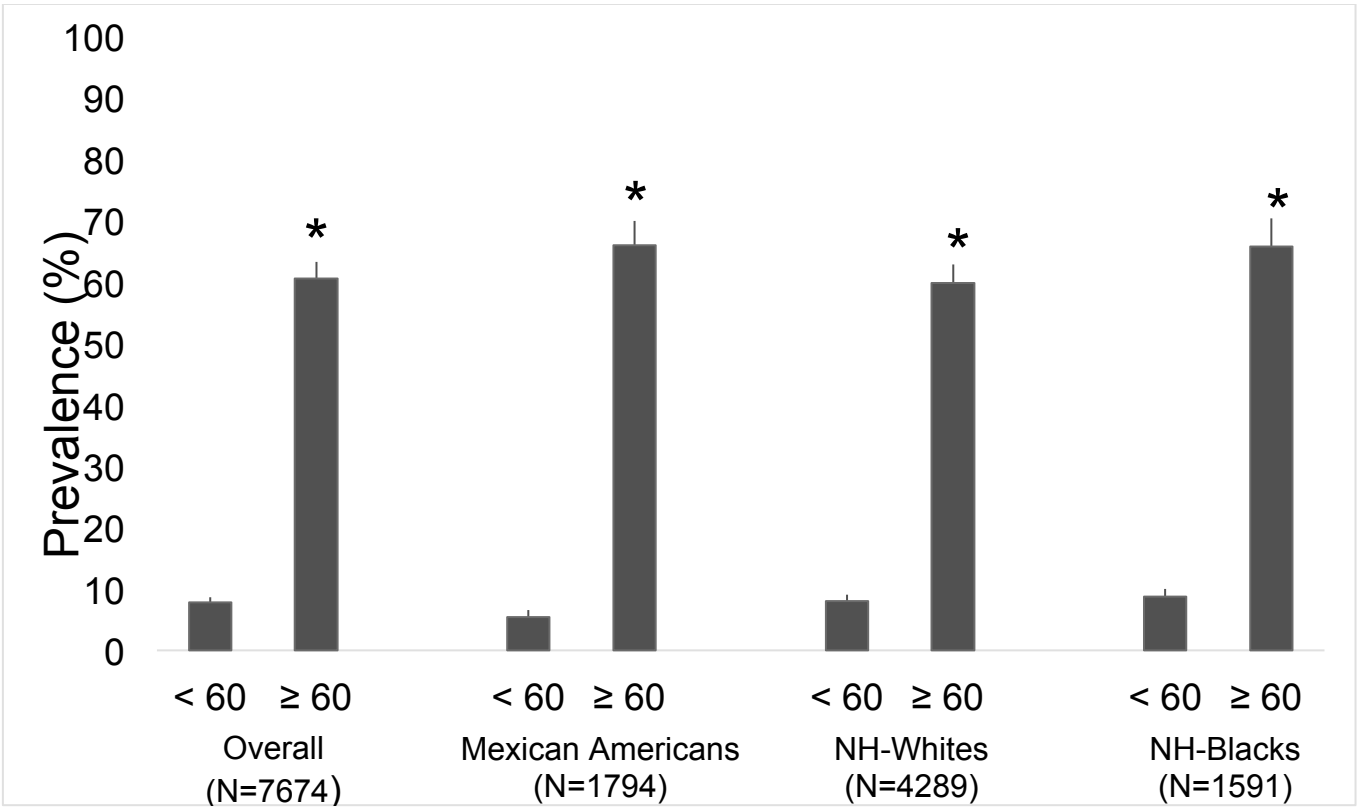

Figure 4. 2 Prevalence of cardiovascular disease risk (≥15%) by age status in participants ≥ 20 years, NHANES 2001-2010.

*$P < 0.05$.

Chapter 5: Study 2- Clinical utility of serum 25-hydroxyvitamin D in the diagnosis of insulin resistance and estimation of optimal 25-hydroxyvitamin D in U.S. adults

Chapter 5 has been previously published:

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Authors' contribution:

The authors' responsibilities were as follows: data acquisition (BA), conception (BA, CIA), statistical analysis (BA, MAR), drafting of manuscript (BA), critical review, editing, and interpretation of results (BA, SMK, MAR, and CIA).

5.1 Abstract

Aims: To assess the clinical utility of measuring serum 25-hydroxyvitamin-D [25(OH)D] along with traditional risk factors in the diagnosis of insulin resistance (IR) and to estimate the optimal 25(OH)D level associated with normal glucose and insulin homeostasis.

Methods: A cross-sectional analysis of 6,868 adults aged ≥ 20 years without diagnosed diabetes in the National Health and Nutrition Examination Survey, with available standardized 25(OH)D data (2001-2010). IR was defined by the homeostatic-model-assessment of insulin resistance (HOMA-IR; ≥ 75th percentile, sex-specific: 3.9 in men or 3.6 in women). Using logistic regression, two risk models were developed to estimate the risk of IR: Model 1 included established risk factors, and Model 2 additionally included serum 25(OH)D. Predictiveness curves and decision-curve analysis were used to assess differences in IR detection among models. Receiver-operating-characteristic curves were used to estimate the lower threshold for 25(OH)D. Results were validated in a testing sample.

Results: Model 2 marginally improved detection of IR: at a risk threshold of 0.2, adding 25(OH)D would identify an additional 2 to 4 cases per 1,000 people. Overall, the lower 25(OH)D threshold was estimated at 60 nmol/L, however, the threshold differed by ethnicity (Mexican-Americans: 54 nmo/L, non-Hispanic whites: 68 nmol/L, and non-Hispanic blacks: 41 nmol/L).

Conclusion: Addition of serum 25(OH)D to traditional risk factors provided small incremental improvement in detection of IR in asymptomatic adults. The optimal 25(OH)D threshold was estimated to be at least 60 nmol/L, however, the thresholds may differ by ethnic-background. Further research is needed to validate these results in other populations.

5.2 Introduction

In the United States, an estimated 86 million adults have pre-diabetes, and among an estimated 28.9 million adults with diabetes, 25% remain undiagnosed [317]. Type-2 diabetes is the clinical consequence of advanced stage insulin resistance (IR) and may take up to 10 years to develop. Despite several well-known risk factors, such as obesity and physical inactivity, the underlying causes of IR are not fully understood. Moreover, because IR is a clinical syndrome rather than a specific disease, early detection of IR in routine practice is often challenging [318]. Therefore, identification of novel risk factors is important for understanding the etiology as well as the ability to recognize IR prior to the development of type-2 diabetes so that lifestyle interventions can be implemented.

Vitamin D deficiency has been shown to be a significant risk factor for IR [233–235,319–321]. Vitamin D deficiency is plausibly related to IR through multiple interdependent mechanisms. The presence of vitamin D receptor (VDR) in pancreatic β-cells suggests a direct role of vitamin D in insulin secretion and through indirect mechanisms it may influence insulin sensitivity [297]. Furthermore, emerging research has revealed a novel role of the skeleton as an endocrine regulator of glucose and insulin metabolism [322]. Vitamin D deficiency is known to cause impairment in bone remodeling and fracture risk is double in type-2 diabetes due to compromised bone quality [323,324]. Therefore, disturbances in vitamin D metabolism and bone remodeling may be related to abnormal glucose metabolism in IR and type-2 diabetes, although the precise mechanisms related to these abnormalities is currently an active area of research [325,326].

Assessment of vitamin D status is based on circulating total 25-hydroxyvitamin-D [25(OH)D] concentration, which reflects both food intake and endogenous production of vitamin D. Current guidelines by the Health and Medicine Division (HMD) of the National Academies (formerly known as Institute of Medicine) and the Endocrine Society (ES) recommend thresholds for serum 25(OH)D in relation to bone health. Specifically, HMD recommends a threshold of 50 nmol/L, but ES advises at least ≥ 75 nmol/L for optimal levels [2,16]. As it stands, the optimal level for vitamin D status is still largely debated and it may differ depending on outcome. In

addition, inter-laboratory differences in the measurement of 25(OH)D and the lack of standardized data have impeded the development of reliable thresholds [7,11].
Serum 25(OH)D is currently accepted as a biomarker of exposure, however, it is not clear whether 25(OH)D also functions as a biomarker of effect (i.e., ability to quantify an existing clinical condition) [2]. Most studies have evaluated the association between serum 25(OH)D and cardiometabolic disorders, however, a significant association of a biomarker with a disease does not necessarily improve disease risk prediction or increase the discrimination ability between high and low risk individuals [20]. In addition, the threshold for a sufficient 25(OH)D level may differ according to health outcome and the current guidelines do not consider potential variation in health risk amongst multi-ethnic subpopulations. Thus, it is not clear whether the current thresholds for serum 25(OH)D are applicable to cardiometabolic health and whether these thresholds differ by racial/ethnic background.

Therefore, using standardized measurement of serum 25(OH)D from the National Health and Nutrition Examination Survey (NHANES 2001-2010), this study evaluated the clinical utility of serum 25(OH)D in the diagnosis of IR, and estimated the optimal 25(OH)D level associated with normal glucose and insulin homeostasis in asymptomatic U.S. adults.

5.3 Materials and Methods

5.3.1 Study Participants

NHANES is an ongoing cross-sectional survey on the health and nutritional status of the civilian, non-institutionalized U.S. population [279]. Descriptions of the complex survey design, standardized protocols used for data handling during the interview, laboratory and physical examinations have been previously published [280]. NHANES was approved by the National Center for Health Statistics institutional board and all participants provided written informed consent [282].

This study included 5 cycles of NHANES from 2001 to 2010. We initially identified 23,968 adults ≥ 20 years old with available standardized serum 25(OH)D data. Exclusions were based on the following: those who fasted < 8 hours, pregnant women, diagnosis of diabetes or unknown diabetes status, “other Hispanic” or “other race” categories, serum albumin < 2.9 g/dL,

estimated glomerular filtration rate (eGFR) < 60 mL/min/1.73 m^2, and those with missing data for study covariates. A flowchart for sample derivation is shown in **Figure 1 (Chapter 5: supplementary data)**.

5.3.2 Measurement of Serum 25(OH)D

From 2007 to 2010, serum 25(OH)D metabolites were analyzed by the CDC laboratory using the liquid chromatography-tandem mass spectrometry (LC-MS/MS) method. For 2001-2006, the CDC developed regression equations to convert the original RIA values to LC-MS/MS equivalents [278]. The retrospective standardization of serum 25(OH)D values from 2001-2006 has been shown to be robust and reliable [7]. In order to combine serum 25(OH)D measurements, we used the predicted LC-MS/MS equivalent 25(OH)D data from 2001-2006 and the total serum 25(OH)D directly measured by LC-MS/MS from 2007-2010.

5.3.3 Outcome Measure

IR was estimated using HOMA-IR, calculated as the product of fasting insulin (μU/ml) and fasting plasma glucose (mmol/L), divided by 22.5 [49]. We defined IR as HOMA-IR ≥ 75^{th} percentile (sex-specific HOMA-IR values: 3.9 and 3.6 for men and women, respectively). Fasting glucose concentrations were measured by a hexokinase enzymatic method, and insulin concentrations were measured by a radioimmunoassay in 2001-2002, a two-site immunoenzymometric method in 2003-2004 and an ELISA two-site enzyme immunoassay method in 2005-2010.

5.3.4 Risk Models

We randomly split the dataset into training and testing samples (ratio of 3:2, respectively), using the surveyselect procedure (SAS Institute, Cary, NC). Logistic regression was used to estimate the risk for IR in the training sample and results were subsequently validated in the testing sample.

To select the appropriate covariates in the models, the following risk factors were included in the initial model; serum 25(OH)D, age, sex, ethnicity [Mexican-American (MA); non-Hispanic white (NH-white); non-Hispanic black (NH-black)] , waist circumference (WC), fasting plasma glucose (FPG), serum triglyceride (TG), high-density lipoprotein (HDL) cholesterol,

hypertension (≥ 140/90 mmHg or on medication for hypertension), physical activity (PA), family history of diabetes, smoking, C-reactive protein levels (CRP), and anti-hyperglycemic and lipid-modifying medications. These variables were selected on the basis of the suggested risk factors in asymptomatic individuals by the American Diabetes Association that were also available in NHANES 2001-2010 [327]. Age, sex, ethnicity, smoking status, PA, family history of diabetes, and medications were self-reported by a questionnaire during the in-home interview. A metabolic equivalent (MET) of 4.0 for moderate, and 8.0 for vigorous intensity PA were used to estimate the MET minutes/week for engagement in daily and leisure-time PA. Medications were classified into four categories; lipid-modifying agents (i.e., HMG-CoA reductase inhibitors, fibric acid derivatives, bile acid sequesterants, and cholesterol absorption inhibitors), HDL-increasing medications (i.e. fibric acid derivatives), anti-hypertensive, and anti-hyperglycemic medications. It should be noted that for lipid medications, NHANES does not report the specific drug indication (i.e. whether participants were prescribed HMG-CoA reductase inhibitors to lower their triglycerides or low density lipoprotein (LDL) cholesterol or a combination of both is uncertain), instead only a therapeutic indication for classes of drugs is provided. WC, blood pressure, TG, HDL-cholesterol, and CRP data were collected during physical examinations using standardized protocols [280].

We used the backward elimination approach with a significance level of alpha = 0.10 to select the final risk factors in the models [328]. In the final model, the following risk factors were retained; serum 25(OH)D, age, sex, ethnicity, FPG, TG, HDL-cholesterol, WC, family history of diabetes, PA, and anti-hyperglycemic and lipid-modifying medications. To assess different models of IR detection, we computed two risk models: Model 1 included the selected risk factors excluding serum 25(OH)D and Model 2 included selected risk factors in Model 1 plus serum 25(OH)D (nested model). There was no evidence of multicollinearity among the selected risk factors. Significant interactions between FPG and anti-hyperglycemic medication, serum 25(OH)D and ethnicity, and serum 25(OH)D and age were observed. Risk models were subsequently stratified by categories of ethnicity and age; however, due to the small number of participants taking anti-hyperglycemic medications, we were unable to further stratify by medication use. Therefore, we retained the interaction term between FPG and anti-hyperglycemic medication in the final models. Models further stratified by ethnicity and age

subgroups were recalibrated by using ethnic- and age-specific coefficient estimates in the risk prediction models. We also included survey cycle in the final risk models to account for the potential effect of different analytic methods for serum 25(OH)D, FPG and insulin across survey cycles from 2001 to 2010.

5.3.5 Statistical Analysis

For descriptive statistics, serum 25(OH)D was categorized into quartiles as "< 44.1", "44.1-59.1", "59.2-75.3", and "≥ 75.4" nmol/L. Survey-weighted means or frequencies along with 95% CIs were calculated for continuous and categorical variables, respectively. Weighted Wald-F and Chi-square tests were used to examine the independence of means and frequencies across serum 25(OH)D categories.

Hosmer-Lemeshow goodness-of-fit statistics were used to calculate the models' fit and calibration, and receiver operating characteristics (ROC) curves were used to report the C-statistics [77,329]. Weighted predictiveness curves were constructed to display the distribution of the predicted IR risks using the two models [330]. Classification performance of the models was plotted as a function of true-positive fraction (TPF; sensitivity), and false-positive fraction (FPF; 1-specificity) corresponding to the estimated risk thresholds from logistic regression models [330].

To assess the clinical utility associated with addition of serum 25(OH)D to established risk factors, we used decision curve analysis (DCA) to estimate the net benefit of the models by taking the difference between the TPF and FPF weighted across risk thresholds [331]. With the DCA analysis, clinicians can assess the clinical utility of a new diagnostic test compared to a standard test or compare different risk prediction models with new biomarkers by quantifying the number of true-positive cases identified without an increase in false-positive cases (i.e. where a patient would be treated unnecessarily). For a given risk threshold, risk prediction models offer different predictive accuracies (i.e. sensitivity versus specificity), and it is not sufficient to know which model offers better sensitivity or specificity for a given outcome. DCA analysis allows clinicians to incorporate the clinical consequences of having the biomarker information by plotting the net benefit (i.e. observing an increase in the proportion of true-positive cases for a

given false-positive rate) across a range of risk threshold probabilities. The difference in net benefit between two clinical risk prediction models represents the extra number for true-positive cases identified without an increase in the false-positive fraction. In an effort to emphasize the application of the risk models in a clinical-setting, covariates were categorized as high glucose (FPG ≥ 100 mg/dL or medication), abdominal obesity (WC ≥ 102 cm in men or ≥ 88 cm in women), high triglycerides (TG ≥ 250 mg/dL or lipid-modifying agents), low HDL-cholesterol (< 40 mg/dL in men or < 50 mg/dL in women or HDL-increasing medications), and PA (Inactive; 0 MET minutes/week; Somewhat Active; < 500 MET minutes/week; Active; ≥ 500 MET minutes/week). Age and serum 25(OH)D were retained as continuous covariates. We calculated and plotted the net benefit curves for Model 1 (reference), and serum 25(OH)D augmented Model 2. We also calculated the net benefit if we assume "all at risk" (i.e., all have IR), and "none at risk" (i.e., zero benefit).

Model 2 was used to estimate the lower threshold for 25(OH)D concentration that predicted low risk of IR. Since there is no established "risk threshold" for clinical use in identifying higher- or lower-risk patients for IR, a risk threshold of 0.25 was selected as it corresponded to zero net benefit observed for "all at risk" in the DCA analysis, with a sensitivity of 82% and a specificity of 80% for the diagnosis of IR (Model 2). ROC analysis was used to estimate the optimal serum 25(OH)D cutoff for discriminating between individuals in the high and low risk categories for IR. The sensitivities and specificities corresponding to HMD and ES cutoffs of 50 and 75 nmol/L were also assessed through ROC analysis.

Hypertension did not meet the model inclusion criteria in our study (p-value > 0.10), however, the ADA identifies hypertension as one of the risk factors for prediabetes and diabetes in asymptomatic adults [327]. Therefore, we conducted a sensitivity analysis where we included hypertension (140/90 mm Hg and above or on BP medication) in the risk models.

5.4 Results

5.4.1 Study Sample

The total sample included 6,868 adults aged ≥ 20 years without diagnosed diabetes. Characteristics of participants by serum 25(OH)D quartiles are summarized in **Table 5.1**.

Overall, the mean age of the sample was 44.7 (44.0, 45.3) and the prevalence of IR was 22.3% (20.9, 23.6). The prevalence of IR decreased significantly from 34.8% (31.6, 37.9) to 14.7% (12.4, 17.1) across serum 25(OH)D quartiles ($P < 0.0001$). FPG, TG, HDL-cholesterol, WC, family history of diabetes, PA, and lipid-modifying medications were all significantly associated with serum 25(OH)D concentrations.

5.4.2 Model Performance and Predictiveness Curves

Results for the models' fit and weighted C-statistics in the training and testing samples are provided in **Table 5.2**. Model 1 and 2 demonstrated similar goodness-of-fit and weighted C-statistics, suggesting the adequate fit, and good discrimination of the models in the training and testing datasets.

Figure 5.1 plots the weighted predictiveness curves and the overall discrimination performance of the models by plotting TPF (sensitivity) and FPF (1-specificity) across risk thresholds for both models in the training (Figure 5.1A) and testing samples (Figure 5.1B). From the predictiveness curves, the estimated risks for IR were identical for Model 1 and 2 in the training and testing samples, such that the risk for IR begins to increase at a risk threshold of 0.20. In the training sample, an estimated 41% (Model 1) or 40% (Model 2) of the population have predicted risks $\geq$ 0.20. Similar results were obtained in the testing sample, where 40% (Model 1) or 39% (Model 2) have predicted risks ≥ 0.20. Both models reasonably discriminated between higher and lower risk patients for IR (as demonstrated by a good separation between TPF and FPF in Figure 1) and the FPF decreases substantially at a risk threshold of 0.20. Specifically, at this threshold in the training sample, Model 1 had a TPF of 85.6% and FPF of 26.1%, whereas Model 2 had a TPF of 85.5% and FPF of 25.4% for the diagnosis of IR. Similar results were obtained in the testing sample (Figure 5.1B).

5.4.3 Decision Curve Analysis

Figure 5.2 plots the net benefit curves across risk thresholds for both models in the training (Figure 5.2A) and testing (Figure 5.2B) samples. The net benefits associated with Model 1 and 2 were higher compared to the scenario if we assume "all at risk" or "none at risk". The net benefit curves derived from Model 2 (versus Model 1) were marginally higher across most risk

thresholds. For example, in the training-sample comparing Model 2 with Model 1 at a 0.20 risk threshold, the difference in net benefit equaled 0.17 (training sample) and 0.42 (testing sample) per 100 people, indicating approximately 2 to 4 extra cases of IR would be detected per 1,000 people using Model 2. At a different threshold, for example, the 0.54 risk threshold, the difference in net benefit between Model 2 and Model 1 equaled 0.24 (training sample) and 0.49 (testing sample) per 100 people, and again this indicates that approximately 2 to 5 extra cases of IR would be detected per 1,000 people screened using Model 2.

When hypertension was included in the DCA analysis, the results of the sensitivity analysis were identical to the main DCA analysis (i.e., hypertension excluded) (**Chapter 5: supplementary data, Figure 1**).

5.4.4 Serum 25(OH)D Thresholds

Results for the estimation of 25(OH)D thresholds in the overall sample and by age and race/ethnicity categories are shown in **Table 5.3**. The ROC analysis revealed an overall optimal serum 25(OH)D level above 60.6 nmol/L and 60.0 nmol/L in the training and testing samples, respectively. The thresholds varied slightly (by 1-5 nmol/L) according to age categories. Using the HMD cutoff of 50 nmol/L, sensitivities ranged from 71% to 75%, however, an estimated 43% to 68% of individuals with IR would be misclassified as low risk. Conversely, using the ES threshold of 75 nmol/L, the sensitivities were lower (30%-33%), but only 10% to 26% individuals with IR would be misclassified as having low risk.

The estimated 25(OH)D thresholds varied considerably by race/ethnicity. The lower thresholds for 25(OH)D were estimated at 54.2, 68.3, and 40.9 nmol/L for MAs, NH-whites, and NH-blacks, respectively in the training sample. Applying the HMD cutoff of 50 nmol/L resulted in a higher true-positive fraction compared to the ES cutoff of 75 nmol/L, however, the ES threshold resulted in a lower number of false-positive cases. Specifically, an estimated 25% (NH-blacks) to 75% (NH-whites) of individuals with IR would be misclassified as low risk using HMD's cutoff of 50 nmol/L, but only 5% (MAs and NH-blacks) to 27% (NH-whites) of individuals with IR would be misclassified using ES' cutoff of 75 nmol/L.

Inclusion of hypertension in a sensitivity analysis did not change the estimates for the optimal serum 25(OH)D (Chapter 5: supplementary data, Table 1).

5.5 Discussion

This study evaluated the clinical utility of measuring serum 25(OH)D along with traditional risk factors in the diagnosis of IR and estimated a threshold for optimal serum 25(OH)D levels in asymptomatic U.S. adults. We found that adding serum 25(OH)D to established risk factors resulted in a small incremental improvement in detection of IR in asymptomatic adults. Comparing model 2 to model 1 at a risk threshold of 0.2, the estimated net benefit for measuring serum 25(OH)D would translate into detecting approximately 2 to 4 extra cases of IR per 1,000 adults without an increase in false-positive cases using model 2. In the overall sample, an optimal 25(OH)D level was estimated to be at least 60 nmol/L. The threshold for serum 25(OH)D varied slightly by age groups (1-5 nmol/L), but differed substantially for different racial/ethnic backgrounds. Specifically, the minimum threshold was estimated to be at least 54 nmol/L for MAs, 68 nmol/L for NH-whites, and 41 nmol/L for NH-blacks. Taken together, our study provides further insight into the clinical utility of measuring serum 25(OH)D above and beyond traditional risk factors associated with IR in asymptomatic adults and our results suggest that the optimal 25(OH)D level associated with normal glucose and insulin homeostasis may be at least 60 nmol/L, although the threshold may differ by racial/ethnic background.

To date, relatively few studies have evaluated the clinical utility of serum 25(OH)D in the context of cardiometabolic disease risk prediction. In a study of patients with hypertension (1078 diabetics and 508 non-diabetics), addition of 25(OH)D to the Framingham Risk Score improved coronary heart disease (CHD) risk prediction, such that 33% of patients were reclassified according to CHD risk groups [332]. The Ludwigshafen Risk and Cardiovascular Health (LURIC) study also found that serum 25(OH)D was a significant predictor of CVD mortality and addition of 25(OH)D along with a biomarker panel consisting of interleukin-6, neutrophils, von Willebrand factor offered significant improvements in predicting CVD mortality compared to conventional risk factors [333]. However, in another study of 2975 patients undergoing coronary catheterization, 25(OH)D provided little prognostic value for predicting all-cause mortality above and beyond established risk factors [334]. Although these studies have evaluated the

prognostic value of serum 25(OH)D according to the American Heart Association guidelines for the assessment of novel biomarkers [76], use of statistical methods such as Net-Reclassification-Improvement (NRI) and Integrated-Discriminant-Improvement (IDI) have limitations, and have been challenged [335]. Nonetheless, the DCA analysis offers a simple alternative approach for assessing the clinical consequences of incorporating novel biomarkers or risk models in terms of net benefits captured over a wide range of threshold probabilities [331,336]. Although DCA analysis does not directly collect information on cost and effectiveness, it offers a simple approach for examining clinical usefulness of measuring a biomarker by showing whether a net benefit curve for a risk model is greater or lesser than the other alternative testing strategies. For any given probability threshold, the preferred model is the one with the highest net benefit without a significant increase in false-positive cases compared to other risk models [331,336]. In our study, addition of serum 25(OH)D to established risk factors provided small incremental net benefit in the detection of IR in asymptomatic U.S. adults. The ADA currently recommends regular screening in asymptomatic adults who have one or more of the traditional risk factors for prediabetes/diabetes, such as being physically inactive, having family history of diabetes, hypertension (i.e. 140/90 mmHg and above or on BP medications), dysglycemia (HDL cholesterol below 35 mg/dL or TG above 250 mg/dL), and having other conditions associated with IR [327]. Although it is well established that low serum 25(OH)D is significantly associated with IR, current screening practices do not monitor serum 25(OH) in high-risk individuals. While additional studies are needed to fully validate our results, monitoring serum 25(OH)D in high-risk individuals may provide clinicians with further information to effectively prioritize and implement lifestyle interventions that may prevent or delay the onset of type-2 diabetes and its long-term complications.

Previous studies on the estimation of thresholds for serum 25(OH)D have also been limited. The study by Bischoff-Ferrari [337] was the first to estimate optimal thresholds in relation to multiple health outcomes, reporting a wide range between 40 (lower-extremity function) to 90 nmol/L (BMD, dental health, risk of falls, fractures, and colorectal cancer). Heaney et al. [338] reported a similar range between 40 to 90 nmol/L for optimal insulin responsiveness and blood pressure in a cohort of nondiabetic Canadian adults. Similarly, Sorkin et al. [339] found that a threshold of 25(OH)D above 65 nmol/L supported normal glucose metabolism in black and white

postmenopausal women with obesity. In another study, using data from the Longitudinal Aging Study Amsterdam, Sohl et al. [340] reported a range between 46 to 68 nmol/L for optimal parathyroid hormone and blood pressure, respectively. Although most of these previous studies supported the idea that the optimal level for 25(OH)D should be above the currently recommended cutoff of 50 nmol/L, the use of unstandardized measurement of 25(OH)D in these studies greatly reduces the reliability of these thresholds. Specifically, previous studies relying on unstandardized measurements of 25(OH)D may have biased estimates and misclassified individuals with respect to their vitamin D status. A recent analysis of NHANES-III found that the prevalence of individuals below 30, 50, and 75 nmol/L increased from 4% to 6%, 22% to 31%, and 55% to 71% after serum 25(OH)D values were standardized [7]. Therefore, it is difficult to compare our results to previous studies using unstandardized data and different study populations with different study outcomes. To our knowledge, this is the first study to estimate an optimal vitamin D status associated with normal glucose and insulin homeostasis using standardized measurements of serum 25(OH)D in a representative U.S. population.

Although causality has not been established, the strongest evidence for the extra-skeletal role of vitamin D is in relation to insulin sensitivity in randomized clinical trials [233–235,319]. There are several important mechanisms that warrant further discussion relating to how measurement of serum 25(OH)D and other known risk factors can potentially prove to be beneficial for early recognition of IR in asymptomatic adults. Vitamin D status is a major determinant of skeletal muscle function [341–343], and low serum 25(OH)D has been linked to fat infiltration in the muscle [183]. In the IR state, fat infiltration impairs the normal glucose uptake by the muscle and low serum 25(OH)D could be indicative of muscle fat infiltration and impaired glucose metabolism. Moreover, abdominal obesity and low serum 25(OH)D levels often cluster with IR [271] and serum 25(OH)D levels could help to further characterize the underlying metabolic dysfunction. It has been suggested that 25(OH)D may be a key factor for the underlying healthy metabolic profile of a subset of individuals characterized by metabolically healthy overweight/obesity (MHO) phenotype [25], however, this hypothesis remains to be confirmed [27,344]. In addition, new emerging research has revealed an important role of the skeleton in regulating glucose and insulin homeostasis [322]. Vitamin D deficiency increases susceptibility to impaired bone remodeling and early bone aging in adults [323]. Impaired bone remodeling has

been linked to bone marrow adiposity in individuals with type-1 and type-2 diabetes [322,324]. Therefore, impaired bone remodeling could further interfere with glucose metabolism, and vitamin D may be a key factor, but is as of yet unconfirmed [326]. Although vitamin D supplementation has been recommended in individuals with type-2 diabetes to reduce the risk of diabetes-induced bone fragility [324], recommendation for vitamin D supplementation prior to development of type-2 diabetes is less certain but remains an active area of research.

Although we estimated the lower threshold for serum 25(OH)D status associated with normal glucose and insulin homeostasis, the optimal threshold for 25(OH)D remains an open question. It is possible that optimal 25(OH)D levels differ by racial/ethnic background; therefore, the underlying mechanisms as to why vitamin D needs vary among different ethnic groups needs further study. On the other hand, differences in the thresholds could reflect the lower mean 25(OH)D levels observed in MAs and NH-blacks compared to NH-whites. Complicating this further is that the estimation of optimal thresholds is particularly challenging in populations with high prevalence of vitamin D deficiency. Contrary to our results, optimal thresholds may not differ by racial/ethnic background [339]. This hypothesis is supported in our study by the substantially lower false-positive cases identified when applying the ES single cutoff of 75 nmol/L. These results suggested that application of the ES threshold of 75 nmol/L may reduce misclassification of individuals with high risk of IR as low risk, and this was evident across all age and race/ethnicity categories. Therefore, the optimal serum 25(OH)D required to support glucose and insulin homeostasis may be at least 75 nmol/L, irrespective of age and ethnic background. Future prospective studies are needed to estimate the optimal threshold in populations with higher serum 25(OH)D concentrations and confirm whether vitamin D needs vary substantially by ethnicity. Well-designed clinical-trials are also needed to assess the benefit associated with increasing serum 25(OH)D levels above 75 nmol/L in ethnically and geographically diverse populations. To date, most trials of vitamin D supplementation have reported negative results, but have been confounded by the inclusion of healthy individuals and those with type-2 diabetes, meaning that there is only a small window of opportunity to improve insulin sensitivity in individuals who are already healthy, whereas the pathologic changes in the pancreas of individuals with type-2 diabetes may be difficult to reverse in the later stages of the disease [345].

The findings of this study are subject to limitations. The cross-sectional design of NHANES precludes causality. Although our study had extensive exclusion criteria to include only those with early-stage IR and prediabetes, we are unable to completely rule-out the possibility of reverse-causation and the effect of unmeasured confounders (such as chronic alcohol consumption). Although we minimized the acute effect of alcohol by excluding participants who fasted < 8 hours, we were unable to account for the chronic effect of alcohol consumption in our study. NHANES asked participants about their alcohol consumption behaviors in the past 12 months, however, self-reported data is affected by recall bias and there was a large number of missing data (n=2,209, after all other study exclusions). Moreover, binge drinking (i.e., consumption of ≥ 5 (men) or ≥ 4 (women) drinks in a 2-hour period) is not captured in NHANES until years 2013-2014. Therefore, the self-reported nature of the data does not guarantee robust adjustment for the dose-response relationship between chronic alcohol consumption and IR. In addition, HOMA-IR is a proxy measure for IR and its use is limited in individuals with significant hyperglycemia, however, HOMA-IR has been shown to correlate well with the hyperinsulinemic euglycemic clamp in normoglycemic individuals and in persons with prediabetes [346]. In our study, we excluded individuals with diagnosed diabetes, and the 95th and 99th percentiles for FPG levels were 6.8 mmol/L and 9.0 mmol/L, respectively. Thus, cases of hyperglycemia were minimal in our study cohort. Lastly, the challenging nature of the estimation of a precise optimal cutoff using endemic 25(OH)D data is another limitation in our study. However, use of NHANES data ensures the generalizability of our findings to the ethnically diverse U.S. population.

In conclusion, measurement of serum 25(OH)D along with traditional risk factors in asymptomatic adults offered small incremental improvement in the detection of IR and an overall optimal threshold for serum 25(OH)D may be at least 60 nmol/L, however, the optimal level may vary considerably by racial/ethnic background. Future studies are needed to validate the clinical utility of serum 25(OH)D in the early detection of IR, and to ascertain the optimal serum 25(OH)D thresholds required for normal glucose and insulin homeostasis in ethnically and geographically diverse populations.

Table 5. 1 Characteristics of participants by serum 25(OH)D quartiles, NHANES 2001-2010

	Overall (N=6868)	25(OH)D < 44.1 nmol/L (N=1717)	25(OH)D 44.1-59.1 nmol/L (N=1692)	25(OH)D 59.2-75.3 nmol/L (N=1734)	25(OH)D ≥ 75.4 nmol/L (N=1725)	P-value
Age (years)	44.7 (44.0, 45.3)	42.2 (41.3, 43.1)	43.8 (42.9, 43.2)	45.1 (44.2, 46.0)	46.1 (45.2, 47.0)	<.0001
Serum 25(OH)D (nmol/L)	66.1 (64.7, 67.4)	33.0 (32.6, 33.5)	52.1 (51.9, 52.4)	67.0 (66.7, 67.2)	93.5 (92.4, 94.5)	<.0001
Gender, %						<.0001
Males	49.8 (48.8, 50.8)	43.2 (40.5, 45.9)	54.3 (51.4, 57.1)	53.9 (51.2, 56.6)	46.6 (44.0, 49.2)	
Females	50.2 (49.2, 51.2)	56.8 (54.1, 59.4)	45.7 (42.9, 48.5)	46.1 (43.4, 48.8)	53.4 (50.8, 55.9)	
Ethnicity, (%)						<.0001
Mexican Americans	9.2 (7.51, 10.9)	16.1 (12.4, 19.7)	12.7 (10.5, 15.0)	8.57 (6.85, 10.3)	3.40 (2.43, 4.36)	
Non-Hispanic Whites	79.8 (77.3, 82.2)	45.6 (40.7, 50.4)	76.3 (73.2, 79.3)	87.0 (85.0, 88.9)	94.8 (93.5, 96.0)	
Non-Hispanic Blacks	11 (9.48, 12.6)	38.3 (34.2, 42.4)	11.0 (9.02, 12.9)	4.44 (3.51, 5.36)	1.84 (1.19, 2.48)	
Prevalence of IR, %	22.3 (20.9, 23.6)	34.8 (31.6, 37.9)	26.8 (24.2, 29.4)	19.0 (16.8, 21.1)	14.7 (12.4, 17.1)	<.0001
Fasting plasma glucose (mmol/L)	5.47 (5.44, 5.50)	5.57 (5.50, 5.64)	5.51 (5.45, 5.57)	5.47 (5.42, 5.51)	5.38 (5.35, 5.41)	<.0001
Serum triglyceride (mmol/L)	1.53 (1.48, 1.58)	1.64 (1.47, 1.80)	1.64 (1.56, 1.73)	1.54 (1.47, 1.61)	1.37 (1.31, 1.42)	<.0001
HDL-Cholesterol (mmol/L)	1.40 (1.38, 1.41)	1.34 (1.32, 1.36)	1.32 (1.30, 1.34)	1.38 (1.35, 1.41)	1.50 (1.47, 1.53)	<.0001
Waist Circumference (cm)	96.9 (96.5, 97.4)	101.2 (100.0, 102.4)	98.9 (97.9, 99.9)	96.7 (95.8, 97.6)	93.3 (92.4, 94.1)	<.0001
Family history of diabetes, %	42.0 (40.2, 43.9)	49.4 (46.5, 52.2)	44.3 (40.7, 47.9)	40.0 (36.9, 43.1)	38.1 (35.4, 40.8)	<.0001
Physical activity						<.0001
Inactive	14.4 (13.1, 15.8)	24.1 (21.6, 26.6)	15.1 (12.9, 17.3)	11.9 (10.1, 13.8)	10.8 (9.05, 12.5)	
Somewhat active	17.8 (16.8, 18.9)	21.9 (19.2, 24.6)	19.8 (17.7, 21.9)	17.6 (15.1, 20.2)	14.4 (12.5, 16.2)	
Active	67.7 (65.9, 69.5)	53.9 (50.8, 57.1)	65.0 (62.4, 67.6)	70.4 (67.5, 73.3)	74.8 (72.2, 77.4)	
Medication Use						
Lipid-modifying, %	10.3 (9.29, 11.3)	7.54 (5.46, 9.63)	9.20 (7.57, 10.8)	10.7 (8.88, 12.5)	12.2 (10.5, 13.8)	0.004
Anti-hyperglycemic, %	0.35 (0.20, 0.50)	0.66 (0.08, 1.24)	0.16 (0.03, 0.30)	0.38 (0.11, 0.65)	0.28 (0.04, 0.52)	0.21

Values are survey-weighted means or frequencies (%), and 95% C.I. in parenthesis. *P*-values are based on Wald F-Test and Chi-square tests, which test the independence of means and frequencies, respectively, across serum 25(OH)D quartiles.

Table 5. 2 Comparison of models' fit and weighted C-statistics in the training and testing samples, NHANES 2001-2010.

	Training Sample (N=4120)		Testing Sample (N=2748)	
Overall	**Model 1**	**Model 2**	**Model 1**	**Model 2**
Goodness of fit*	12.6 (0.13)	11.9 (0.15)	5.80 (0.67)	7.16 (0.52)
Weighted C-statistics	0.880 (0.870-0.891)	0.883 (0.873-0.893)	0.869 (0.855-0.883)	0.874 (0.860-0.887)

*Hosmer-Lemeshow goodness of fit test; the numbers are χ^2, with *P* values in parentheses. *Model 1* included age, sex, ethnicity (Mexican-American; non-Hispanic white; non-Hispanic black), fasting plasma glucose, triglyceride, HDL-Cholesterol, waist circumference, family history of diabetes, physical activity, and medication use (anti-hyperglycemic and lipid-modifying medications). *Model 2* included selected risk factors in Model 1 plus serum 25(OH).

Table 5. 3 Serum 25(OH)D thresholds in relation to normal glucose and insulin homeostasis in the training and testing datasets overall and by age and racial/ethnic background, NHANES 2001-2010.

		Estimated 25(OH)D thresholds			HMD threshold (50 nmol/L)		ES threshold (75 nmol/L)	
	AUC (95% CI)	25(OH)D (nmol/L)	Sensitivity	Specificity	Sensitivity	Specificity	Sensitivity	Specificity
Training sample (N=4120)								
Overall	0.62 (0.60-0.63)	60.6	55	62	72	43	31	84
20-39 y	0.66 (0.63-0.69)	58.1	57	64	71	50	31	89
40-59 y	0.62 (0.60-0.65)	60.3	58	60	74	41	33	84
≥ 60 y	0.57 (0.54-0.61)	61.0	57	56	73	32	32	78
MAs	0.62 (0.59-0.66)	54.2	56	64	64	51	17	95.5
NH-Whites	0.63 (0.60-0.65)	68.3	55	62	86	25	43	73
NH-Blacks	0.54 (0.50-0.58)	40.9	50	58	31	71	5.0	94
Testing sample (N=2748)								
Overall	0.64 (0.62-0.66)	60	56	65	72	48	31	85
20-39 y	0.70 (0.67-0.74)	55.7	63	69	71	57	30	90
40-59 y	0.64 (0.60-0.67)	60.1	55	64	71	50	31	85
≥ 60 y	0.56 (0.52-0.61)	61.7	55	55	75	34	33	74
MAs	0.66 (0.62-0.70)	53.2	59	65	64	57	18	95
NH-Whites	0.64 (0.61-0.67)	67.0	59	62	87	31	44	73
NH-Blacks	0.59 (0.54-0.64)	40.6	52	59	35	75	8.0	95

Abbreviations: AUC, Area Under Curve; HMD, the Health and Medicine Division of the National Academies (formerly known as the Institute of Medicine); ES, the Endocrine Society; MAs, Mexican-Americans; NH-Whites, non-Hispanic whites; NH-Blacks, non-Hispanic blacks.

Sensitivity, proportion of true positive cases [true positive/(true positive + false negative)]. Specificity, proportion of true negative cases [true negative/(true negative + false positive).

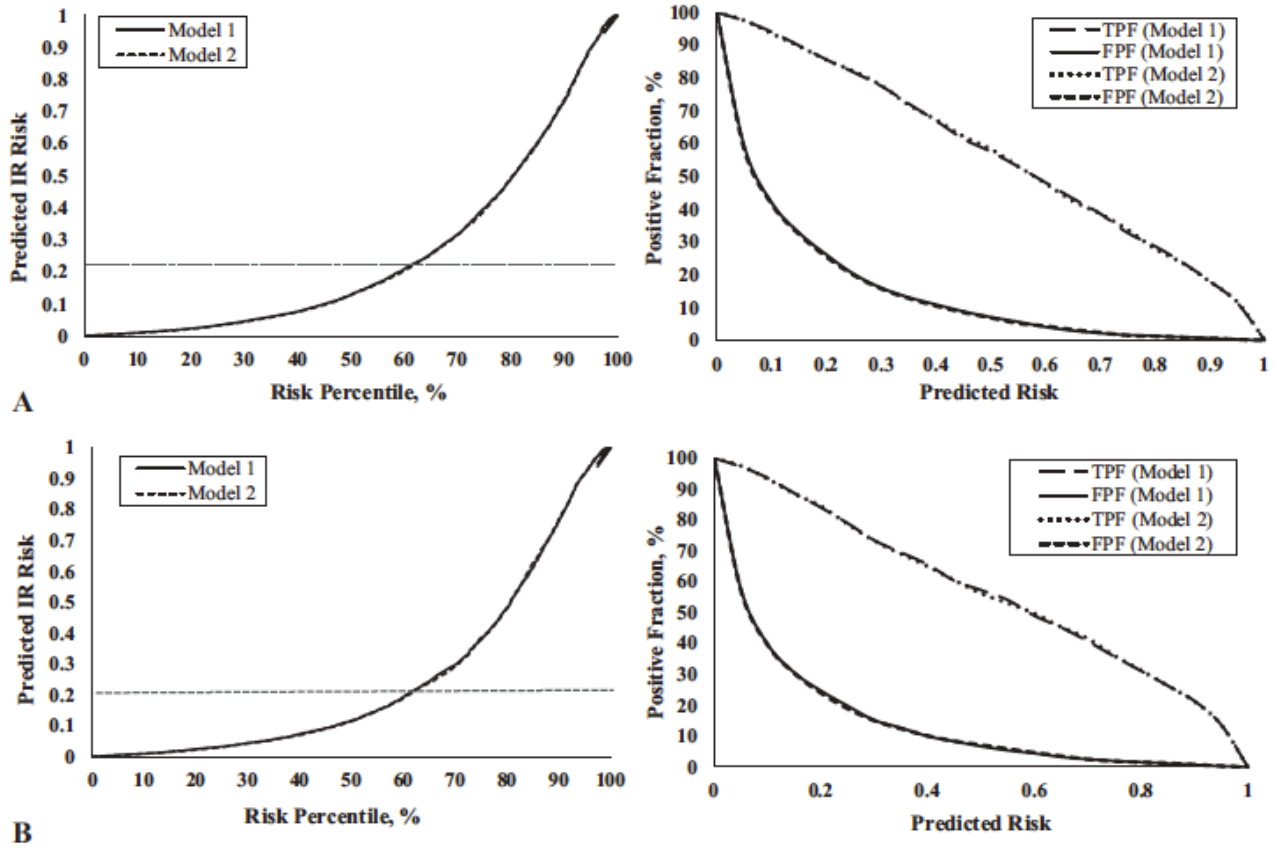


Figure 5. 1 Weighted predictiveness curves.

Weighted predictiveness curves (left panel) and true-positive fraction (TPF, sensitivity) and false-positive fraction (FPF,1-specificity) (right panel) in the training (A) and testing (B) datasets for Models 1 and 2, NHANES 2001–2010. The horizontal grey dashed lines in predictiveness curves indicate the prevalence of insulin resistance in the training (22.6%) and testing (21.8%) datasets. Model 1 included age, sex, ethnicity (Mexican-American; non-Hispanic white; non-Hispanic black), fasting plasma glucose, triglyceride, HDL-Cholesterol, waist circumference, family history of diabetes, physical activity, and medication use (anti-hyperglycemic and lipid-modifying medications). Model 2 included selected risk factors in Model 1 plus
serum 25(OH).

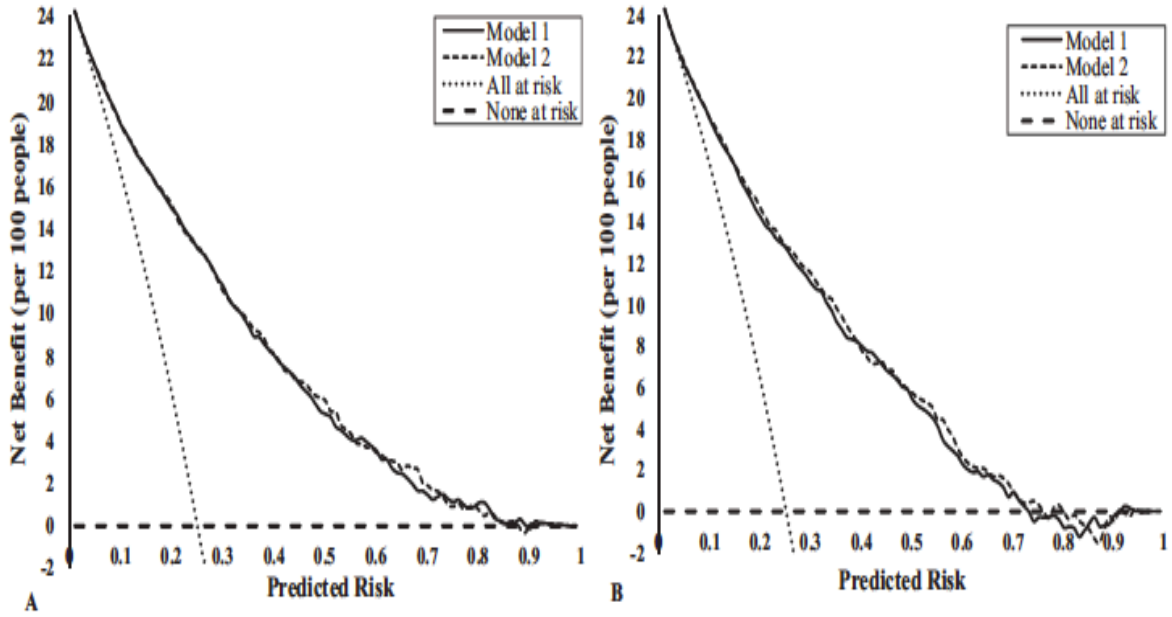
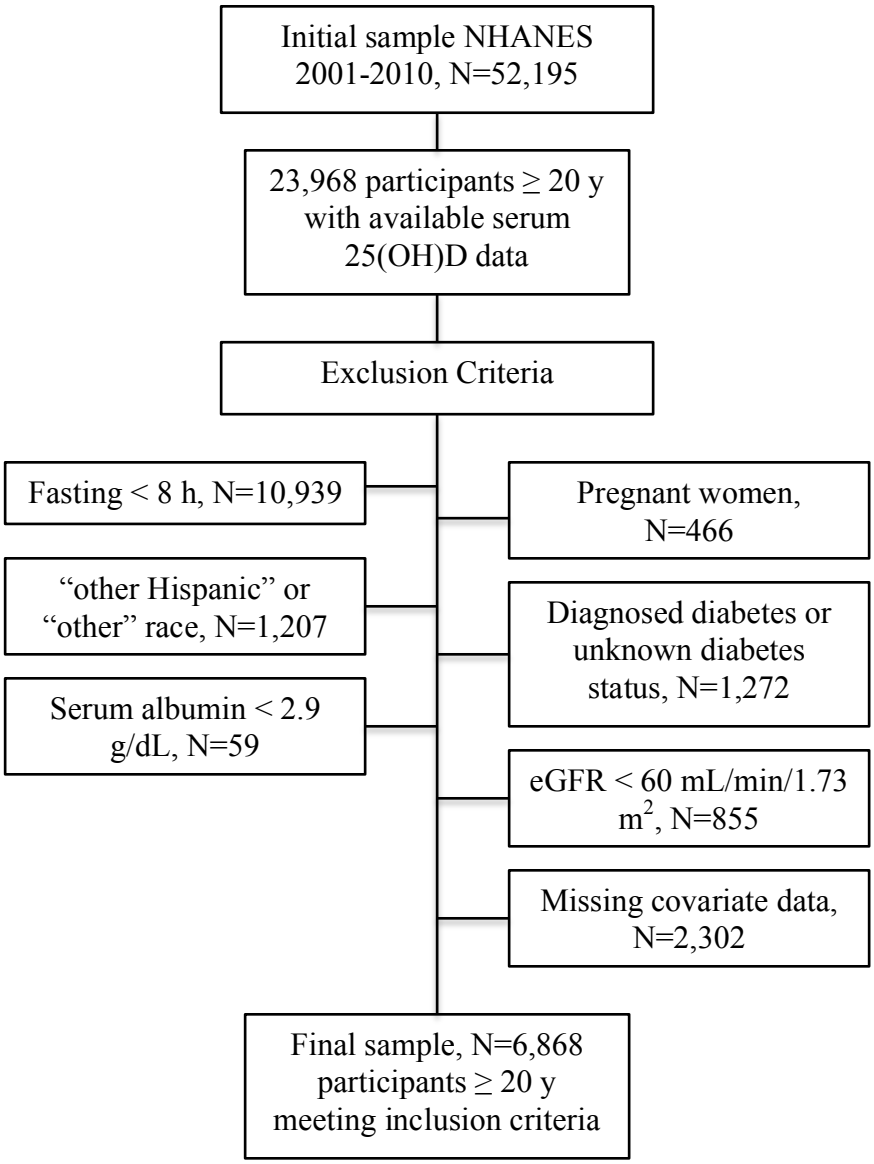


Figure 5. 2 Decision curve analysis.

Decision curve analysis (DCA) for models predicting insulin resistance in the training (A) and testing (B) datasets for Model 1 (solid line) and Model 2 (small dashed line), NHANES 2001–2010. The dotted line indicates the net benefit for assuming ‘‘all at risk”, and the horizontal dashed line assumes ‘‘none at risk”. The y-axis represents the number of true positive cases identified per 100 people using the models. Model 1 included age, sex, ethnicity (Mexican-American; non-Hispanic white; non-Hispanic black), a high fasting plasma glucose level of ≥ 100 mg/dL or medication, abdominal obesity (WC ≥ 102 cm in men or ≥ 88 cm in women), a high triglyceride level of ≥ 250 mg/dL or lipid-modifying medication, a low HDL-cholesterol level of < 40 mg/dL in men or < 50 mg/dL in women or HDL-increasing medication, family history of diabetes, and physical activity (Inactive; 0 MET min./week; Somewhat Active; < 500 MET min/week; Active; ≥ 500 MET min/week). Model 2 included selected risk factors in Model 1 plus serum 25(OH).

Chapter 5: Supplementary data



Flowchart showing the exclusion criteria for sample derivation, NHANES 2001-2010.

***The risk models were further adjusted for hypertension (140/90 mm Hg and above or on medication)**

Table 1. Serum 25(OH)D thresholds in relation to normal glucose and insulin homeostasis in the training and testing datasets overall and by age and racial/ethnic background, NHANES 2001-2010.

		Estimated 25(OH)D thresholds			IOM threshold (50 nmol/L)		ES threshold (75 nmol/L)	
	AUC (95% CI)	25(OH)D (nmol/L)	Sensitivity	Specificity	Sensitivity	Specificity	Sensitivity	Specificity
Training sample (N=4120)								
Overall	0.62 (0.60, 0.64)	60.7	55	62	72	42	31	84
20-39 yrs	0.66 (0.63, 0.69)	56.7	57	64	70	53	29	90
40-59 yrs	0.62 (0.60, 0.65)	60.7	58	60	74	42	33	84
≥ 60 yrs	0.58 (0.54, 0.61)	61.2	57	57	73	32	33	78
MAs	0.63 (0.60, 0.67)	54.4	57	65	66	52	17	96
NH-Whites	0.63 (0.61, 0.66)	68.4	55	63	86	26	44	74
NH-Blacks	0.54 (0.50, 0.58)	40.9	55	58	31	71	5.0	94
Testing sample (N=2748)								
Overall	0.64 (0.62, 0.66)	60.5	56	66	72	49	31	85
20-39 yrs	0.71 (0.68, 0.75)	54.4	63	70	70	59	30	92
40-59 yrs	0.64 (0.60, 0.67)	60.1	55	64	71	50	31	85
≥ 60 yrs	0.57 (0.53, 0.61)	61.2	58	55	75	34	33	75
MAs	0.66 (0.62, 0.70)	53.2	59	65	64	57	18	96
NH-Whites	0.65 (0.62, 0.68)	66.4	60	62	86	31	44	74
NH-Blacks	0.59 (0.54, 0.64)	40.5	52	60	35	75	8.0	95

Abbreviations: AUC, Area Under Curve; HMD, the Health and Medicine Division of the National Academies (formerly known as the Institute of Medicine); ES, the Endocrine society; MAs, Mexican-Americans; NH-Whites, non-Hispanic whites; NH-Blacks, non-Hispanic blacks.

Sensitivity, proportion of true positive cases [true positive/(true positive + false negative)]. Specificity, proportion of true negative cases [true negative/(true negative + false positive)].

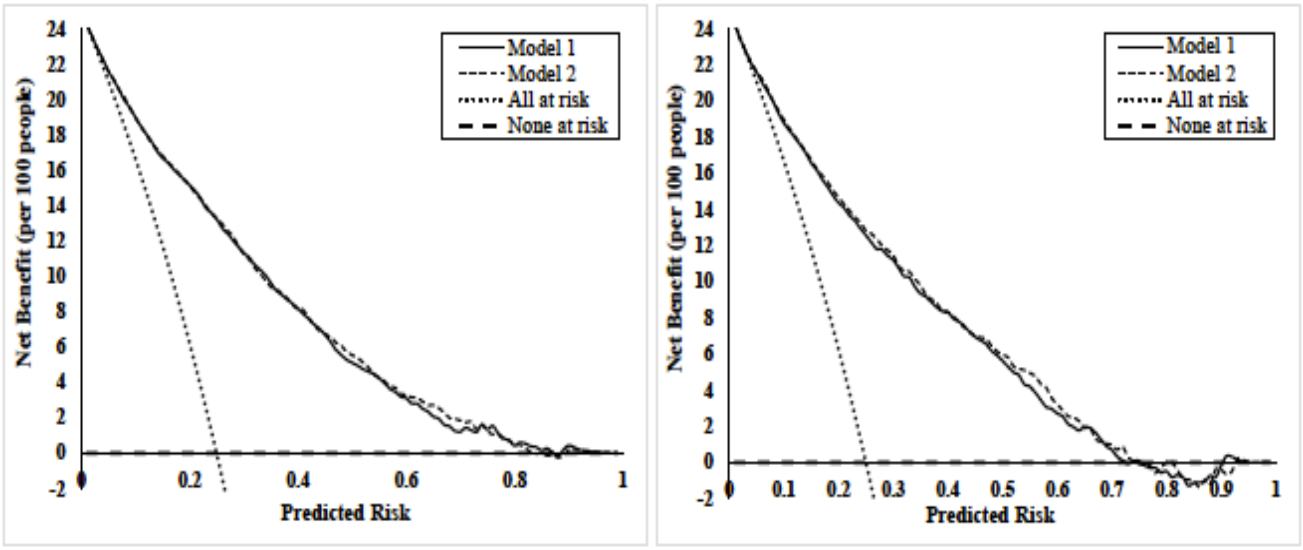


Figure 1. Decision curve analysis (DCA) for models predicting insulin resistance in the training (A) and testing (B) datasets for Model 1 (solid line) and Model 2 (small dashed line), NHANES 2001-2010. The dotted line indicates the net benefit for assuming "all at risk", and the horizontal dashed line assumes "none at risk". The y-axis represents the number of true positive cases identified per 100 people using the models.

Model 1 included age, sex, ethnicity (Mexican-American; non-Hispanic white; non-Hispanic black), a high fasting plasma glucose level of ≥ 100 mg/dL or medication, abdominal obesity (WC ≥ 102 cm in men or ≥ 88 cm in women), a high triglyceride level of ≥ 250 mg/dL or lipid-modifying medication, a low HDL-cholesterol level of < 40 mg/dL in men or < 50 mg/dL in women or HDL-increasing medication, family history of diabetes, physical activity (Inactive; 0 MET minutes/week; Somewhat Active; < 500 MET minutes/week; Active; ≥ 500 MET minutes/week) and hypertension (140/90 mm Hg and above or BP medication).

Model 2 included selected risk factors in Model 1 plus serum 25(OH).

Chapter 6: Study 3- Metabolically Healthy Obesity, Vitamin D, and All-Cause and Cardiometabolic Mortality Risk in NHANES III

Chapter 6 has been previously published:

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Authors' contribution:

All authors have read and approved the final version being submitted with contributions as follows: conception (BA, SMK, CIA), data acquisition (BA), statistical analysis (BA) and interpretation of data (BA, SMK, JLK, CIA), drafting of manuscript (BA), critical review and editing (BA, SMK, JLK, CIA).

6.1 Abstract

Background & Aims: Previous studies assessing the prognosis of metabolically healthy obesity (MHO) have been limited by a lack of a harmonized definition of MHO phenotype. Furthermore, obesity is a risk factor for vitamin D deficiency and low vitamin D status has been associated with a higher risk of mortality; however, few studies have evaluated the joint association between vitamin D, metabolic health phenotype, and mortality risk. Using a harmonized definition, we investigated whether MHO is associated with subsequent all-cause and cardiometabolic mortality, and whether serum 25-hydroxyvitamin D [25(OH)D] modifies these associations.

Methods: This study included participants aged ≥ 20 years from the Third National Health and Nutrition Examination Survey (NHANES III). MHO phenotype was defined as a combination of obesity (≥ 30 kg/m^2) and zero components of metabolic syndrome. Multivariable Cox regression was used to assess the risk of mortality across metabolic phenotypes, and the joint association between metabolic phenotype and 25(OH)D. Fine and Gray regression was performed to account for competing risk events.

Results: Among 11,333 participants, a total of 2,980 deaths (937 cardiometabolic death outcomes) occurred during a median follow-up of 19.1 years. In the absence of any metabolic abnormality, obesity (MHO) was not associated with a higher risk of all-cause (hazard ratio [HR], 0.89 [95% CI, 0.52-1.51]) or cardiometabolic mortality (cause-specific HR, 1.21 [95% CI 0.33-4.46]). Similar results were obtained from competing risk analysis. No significant differences in average 25(OH)D levels were observed between MHO and non-MHO participants; however, there was a significant interaction between metabolic health phenotype and serum 25(OH)D in relation to cardiometabolic mortality such that levels of serum 25(OH)D < 50 nmol/L were associated with increased risk of cardiometabolic mortality, particularly in participants within the normal weight and obesity BMI ranges.

Conclusions: Our results support the hypothesis that MHO phenotype is a benign health condition. Vitamin D deficiency may exacerbate the risk of cardiometabolic death outcomes associated with metabolic dysfunction in participants within the normal weight and obesity BMI ranges. Further research is warranted to validate our findings.

6.2 Introduction

The existence of the metabolically healthy obesity (MHO) has become increasingly recognized [347]; however, the benign health condition of the MHO phenotype has been widely debated and studies have reported conflicting findings [97–103,348–355]. Moreover, the prevalence of MHO phenotype varies significantly across studies, where the prevalence has been estimated to range between 10% and 40% in the adult population [347]. Although study-specific factors such as age, ethnicity, environmental factors and genetics may explain some of these discrepant findings, the lack of a harmonized definition for risk stratification accounts for a large proportion of the reported discrepancy [28]. Most previous studies have included at least one metabolic abnormality [97–101,103,355], yet the type of metabolic abnormality differs across studies and this may affect the long-term risk estimates differently. Therefore, use of a harmonized definition for MHO phenotype, defined as having zero metabolic abnormality [28], is important for establishing whether obesity or metabolic health is a more important predictor of adverse health outcomes.

Identification of novel biomarkers is also needed to have a better understanding of the risk factors that modulate the prognosis of the MHO phenotype. Suboptimal vitamin D status has been associated with increased risk of chronic diseases and mortality [33,356,357]. Obesity is an established risk factor for vitamin D deficiency, where a causal link between obesity and hypovitaminosis D has been reported in a Mendelian randomization study [23], and high adiposity is consistently associated with low serum 25-hydroxyvitamin D levels [25(OH)D, the accepted measure of vitamin D status] in observational studies [301]. Vitamin D is thought to play a key role in preserving the healthy lipid and inflammatory profile of the MHO phenotype [25]; however, conflicting evidence exists in the literature [26,27].

Although previous studies have evaluated the dose-response relationship between vitamin D status and mortality risk [33,261], with some studies reporting a U-shaped or inverse J-shaped association [199,358], lack of standardized serum 25(OH)D data have impeded our understanding of the dose-response relationship between vitamin D status and non-skeletal health outcomes [7,8]. Moreover, previous studies have evaluated the individual contributions of

metabolic health, obesity, and vitamin D status in relation to long-term health outcomes; however, their joint associations have been largely unexplored. Elucidating the interrelation between metabolic health, obesity, and vitamin D may aid in the development of more effective treatment strategies that may ultimately reduce the burden of obesity-related complications.

The purpose of this study was therefore to investigate whether MHO, defined as having zero metabolic abnormality, is associated with subsequent all-cause and cardiometabolic mortality, and to evaluate the joint association of metabolic health phenotype and vitamin D status in relation to mortality risk using data from the Third National Health and Nutrition Examination Survey (NHANES III, 1988-1994).

6.3 Subjects and Methods

6.3.1 Data Source

The NHANES III (1988-1994) is a representative survey of the civilian, non-institutionalized US population conducted by the National Center for Health Statistics (NCHS) of the Center for Disease Control and Prevention. The NCHS has updated mortality data for NHANES III up to December 31, 2011, and the baseline data collected in 1988-1994 was linked to mortality data using a probabilistic record matching with death certificates records obtained from the National Death Index (NDI). Follow-up time was calculated from examination date until date of death or end of study (December 31, 2011). Detailed descriptions of the survey design and mortality matching method are published elsewhere [359,360]. The NCHS institutional board approved NHANES III and all participants provided written informed consent [359].

6.3.2 Outcome Measures

All-cause and cardiometabolic mortality status were obtained from publicly available dataset. For participants classified as “assumed deceased”, death cases were coded according to the Tenth Revision of the International Classification of Diseases (ICD-10) and with underlying cause of death further categorized into ten broad groups: Diseases of heart; malignant neoplasms; chronic lower respiratory diseases; accidents (unintentional injuries); cerebrovascular diseases; Alzheimer’s disease; diabetes mellitus; influenza and pneumonia; nephritis, nephrotic syndrome

and nephrosis; and all other causes (residual). For cardiometabolic death outcomes, we combined deaths classified as diseases of heart, cerebrovascular diseases and diabetes mellitus.

6.3.3 Exposure Measures

Exposure was defined as a combination of body mass index (BMI: kg/m^2) and metabolic health. A harmonized definition of metabolic health was used [28], where 'metabolically healthy' individuals are defined as having zero of the metabolic syndrome (MetS) criteria (excluding waist circumference) [60]. Participants with at least one of the four metabolic risk factors were defined as 'metabolically unhealthy'.

Systolic and diastolic blood pressure (BP) was the average of up to 6 measurements collected under standard conditions [361]. Individuals who self-reported a history of hypertension or use of BP medication were defined as having hypertension. Serum triglyceride, HDL-cholesterol, and fasting plasma glucose (FPG) concentrations were measured using standardized laboratory procedures as reported by NCHS [361]. Participants reporting a history of diabetes or current diabetes medications were considered to have dysglycemia. Similarly, participants taking lipid-modifying medications were considered to have dyslipidemia. Medication use was self-reported by questionnaire during the in-home interview.

BMI (kg/m^2) was categorized into normal weight (18.5 to 24.9), overweight (25 to 29.9), and obesity ($\geq$ 30). Participants were subsequently divided into six metabolic phenotypes: metabolically healthy normal-weight (MHNW), metabolically healthy overweight (MHOW), metabolically healthy obesity (MHO), metabolically unhealthy normal-weight (non-MHNW), metabolically unhealthy overweight (non-MHOW), and metabolically unhealthy obesity (non-MHO).

6.3.4 Covariate Data

Age, sex, ethnicity (Non-Hispanic White, Non-Hispanic Black, Mexican-American or other), smoking status, educational level, and leisure-time physical activity (LTPA) were self-reported by questionnaire during the in-home interview. Smoking status was categorized into current, former or never smokers (cigarettes only). Educational attainment was coded as less than high school, high school diploma, and more than high school. LTPA was assessed by a questionnaire that asked participants about the type and frequency of the following 9 activities: run/jog, swim,

bicycle, aerobics, calisthenics, dancing, yard or garden work, weight lifting, or other physical activity). For each of these 9 activities, a validated metabolic equivalent (MET) score was assigned and using these scores, we estimated the total weekly LTPA MET. Serum creatinine was recalibrated to be traceable to an isotope-derived mass spectroscopy method using the following equation: standardized creatinine (mg/dL)= [0.960 x NHANES III serum creatinine (mg/dL)]- 0.18 [362]. Estimated glomerular filtration rate (eGFR) was calculated using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation [363]. Serum C-reactive protein (CRP) was measured using a low sensitivity method that can detect CRP levels > 0.22 mg/dL [361]. Because most individuals had values below the minimum detectable level, CRP was treated as a categorical variable using clinical cut-off points as previously described [364]: low (< 0.22 mg/dL), moderate (0.22-< 1.0 mg/dL), and high (≥ 1.0 mg/dL). We used standardized serum 25(OH)D values in this study [278]. Season of blood draw was categorized as winter (November to April) and summer (May to October).
Poverty-to-income ratio (PIR), a ratio of total family income to the official poverty threshold, was used to assess the socioeconomic status. The Healthy Eating Index (HEI) scores derived from 24-hour dietary recall was used to assess the overall diet quality of participants [365]. Alcohol consumption was assessed by questionnaire and estimated as number of drinks per month.

6.3.5 Analytical Sample

A total of 18,825 adults aged 20 years or older were interviewed in NHANES III, and 16,573 completed the mobile examination center (MEC) and laboratory examinations. After applying the exclusion criteria, the total analytical sample included 11,333 participants aged ≥ 20 years with no history of CVD and cancer (excluding skin cancer) (**Chapter 6: supplementary data**, **Figure 1**).

6.3.6 Statistical Analysis

Baseline characteristics were analyzed according to metabolic health phenotype using sampling weights to account for differential probability of selection [359]. Differences in continuous and categorical data were assessed using weighted Wald-F and Chi-square tests as appropriate. Survey weighted Cox proportional hazards (PH) regression models were constructed and

adjusted for age, sex, ethnicity, smoking status, education level, LTPA, eGFR, serum CRP and 25(OH)D. For cardiometabolic mortality, we also performed Fine and Gray regression to estimate the cumulative incidence of cardiometabolic death while accounting for competing events (i.e., cancer and other deaths) [366]. SAS macro (%PSHREG) was used to plot the cumulative incidence curves for cardiometabolic mortality [367]. The PH assumption was tested using log-minus-log survival plots and analysis of Schoenfeld residuals, and no violations were observed.

Unweighted Cox PH models with restricted cubic splines (RCS) were used to graphically display the dose-response relations between serum 25(OH)D and all-cause and cardiometabolic mortality in the overall sample [368,369]. Knots in the RCS models were set at the 25^{th}, 50^{th}, and 75^{th} percentiles of serum 25(OH)D, with 50 nmol/L set as the reference level. We examined the non-linear relation between 25(OH)D and cardiometabolic mortality non-parametrically with RCS models; tests for non-linearity used the likelihood ratio test, comparing the model with only the linear term to the model with the linear and the cubic spline terms [369]. On the basis of previous literature, the RCS models were adjusted for age, sex, ethnicity, smoking status, education, LTPA, eGFR, serum CRP and season. The joint association between metabolic phenotype and serum 25(OH)D with mortality (all-cause and cardiometabolic death outcomes) was assessed by a formal test of interaction. Significant interaction terms were further stratified by the effect modification term.

All analyses were conducted with SAS version 9.4 (Cary, NC). P-value < 0.05 was considered significant.

6.3.7 Sensitivity Analyses

Adjustment for Additional Confounders

Due to the large number of missing data for alcohol consumption, income, and diet quality, we adjusted for these covariates in a sensitivity analysis to further account for potential residual confounding. This analysis included 9,744 participants.

Using Waist Circumference to Define Obesity

We used waist circumference (WC) to define abdominal obesity and examine whether metabolically healthy but abdominally obese individuals are at increased risk of mortality. WC (cm) was defined as low (men, ≤ 94; women, ≤ 80), intermediate (men, > 94 and ≤ 102; women, > 80 and ≤ 88), and elevated (men, > 102; women, > 88) [370]. This analysis included 10,988 participants.

6.4 Results

6.4.1 Baseline Characteristics

The total sample included 11,333 adults aged ≥ 20 years with no history of CVD and cancer; 2,980 participants died during a median follow-up of 19.1 years, with 937 cardiometabolic death outcomes.

Table 6.1 shows the weighted characteristics of the study population according to metabolic phenotypes. A total of 367 participants met the MHO definition (3.2% of the total sample). The mean age was 43.6 (42.8, 44.5) years in the overall sample. Mean age was consistently higher among metabolically unhealthy participants compared to their metabolically healthy counterparts. Metabolically healthy participants had lower BMI and mean values for the four metabolic risk factors than their metabolically unhealthy counterparts. Metabolically healthy participants (irrespective of BMI status) also had lower prevalence of current smokers, better kidney function, lower CRP values and were more educated. Overall, mean serum 25(OH)D was negatively associated with BMI and was significantly higher in MHNW (67.6 nmol/L) compared to non-MHNW (63.3 nmol/L), however, no significant differences were observed between metabolically healthy and unhealthy participants within the overweight and obesity BMI ranges (overweight: 61.4 vs. 62.4 nmol/L; obesity: 53.8 vs. 55.5 nmol/L).

6.4.2 Metabolic Health Phenotype and Mortality Risk

Table 6.2 shows the hazard ratios (HRs) for all-cause mortality and cause-specific HRs for cardiometabolic and non-cardiometabolic mortality. MHNW participants were used as reference group (HR=1.0). After adjusting for confounders, we observed a higher risk of all-cause

mortality among metabolically unhealthy participants within the normal-weight and obesity BMI range. MHOW, non-MHOW, and MHO were not associated with risk of all-cause mortality. In relation to cardiometabolic mortality, being metabolically unhealthy irrespective of BMI status was associated with a significantly higher cause-specific hazard for cardiometabolic death outcomes, whereas no significant association was observed in relation to non-cardiometabolic mortality. For MHOW and MHO participants, the cause-specific HRs for cardiometabolic and non-cardiometabolic death outcomes were not significant.

Figure 6.1 shows the cumulative incidence curves (obtained from Fine and Gray model to adjust for competing events) for cardiometabolic mortality stratified by metabolic health phenotype. In the unadjusted cumulative incidence (Figure 6.1A), the risk of cardiometabolic mortality over 23 years of follow-up remained low in metabolically healthy participants irrespective of their BMI status (< 4%), whereas the risk of cardiometabolic mortality was higher in metabolically unhealthy participants, with the highest incidence observed among non-MHNW participants (12.8%). After adjusting for age, sex, ethnicity, smoking status, education, LTPA, eGFR, serum CRP and 25(OH)D (Figure 6.1B), the estimated cumulative incidence of cardiometabolic mortality was lowest for MHO participants followed by MHNW and MHOW participants. Among metabolically unhealthy participants, non-MHO participants had the highest risk of cardiometabolic mortality, and non-MHNW and non-MHOW participants had comparable risk of cardiometabolic death outcomes over 23 years of follow-up.

6.4.3 Standardized Serum 25(OH)D Status and Mortality Risk

Figure 6.2 shows the dose-response relation between 25(OH)D and all-cause and cardiometabolic mortality risk in the overall sample. Serum 25(OH)D levels below 50 nmol/L were significantly and inversely associated with increased risk of all-cause and cardiometabolic mortality, and the minimum relative risk was observed with values near 60-75 nmol/L.
There was a significant interaction between metabolic phenotype and serum 25(OH)D in relation to cardiometabolic mortality (*P* interaction < 0.0001). **Figure 6.3** shows the dose-response relation between serum 25(OH)D and cardiometabolic mortality further stratified by metabolic health phenotype. Within the normal-weight BMI range, there was a linear inverse association between serum 25(OH)D and cardiometabolic mortality. This inverse association was stronger

for non-MHNW participants where there was a clear relative risk reduction in cardiometabolic mortality with increasing serum 25(OH)D levels. For individuals within the overweight BMI range, we observed a non-significant curvilinear relation between 25(OH)D and cardiometabolic mortality. Lastly, for MHO participants, we observed a non-significant inverse association between serum 25(OH)D and cardiometabolic mortality risk. For non-MHO participants, there was a significant inverse association between low 25(OH)D (< 50 nmol/L) and cardiometabolic mortality risk, however this inverse relation attenuated at the higher end of 25(OH)D concentrations (> 50 nmol/L).

6.4.4 Sensitivity Analyses

When we further adjusted our models for income, alcohol consumption, and diet quality in a subset sample of 9,744 participants with complete data, HR estimates were similar to the main analysis (**Chapter 6: supplementary data, Table 1**). When using WC to define obesity, a total of 630 participants were categorized as MHO (5.7% of the total sample), and results were also similar to the main analysis (**Chapter 6: supplementary data, Table 2**).

6.5 Discussion

In a population study of 11,333 U.S. adults with a median follow-up of 19.1 years, we found that in the absence of any metabolic dysfunction, obesity (MHO) was not associated with a higher risk of all-cause and cardiometabolic mortality. We found a significant interaction between metabolic health and serum 25(OH)D in relation to cardiometabolic death outcomes. Specifically, 25(OH)D levels were inversely associated with cardiometabolic mortality in normal-weight individuals irrespective of metabolic health status (linear association) and in non-MHO individuals (non-linear association). For overweight individuals and MHO participants, we did not observe a significant inverse association between serum 25(OH)D and cardiometabolic mortality. Overall, metabolic health was a more important predictor of adverse outcomes in our study and effect modification by 25(OH)D raises the possibility that optimizing vitamin D status may improve the prognosis of cardiometabolic health outcomes, particularly in high-risk groups (i.e., individuals with metabolic dysfunction and vitamin D deficiency).

Our study findings are in agreement with studies showing MHO is not associated with increased risk of all-cause or cardiometabolic mortality [348–354], yet other studies have questioned the benign health condition of MHO [97–103,355]. Most recently, Lassale et al. [103] found that metabolically healthy participants within the overweight and obesity BMI ranges had higher coronary heart disease (CHD) risk than their normal-weight counterparts. However, they classified participants as metabolically healthy if they did not meet the MetS criteria (i.e., ≤ 2 components) [103]. In their sensitivity analysis to assess the contribution of overweight and obesity with zero MetS components, they found that overweight and obesity was not associated with subsequent CHD risk [103]. While they appear inconsistent, their results are, in fact, in agreement with our findings. In addition, when using WC to define abdominal obesity, our results were similar to the main analysis in that metabolically healthy abdominally obese participants were not at increased risk of all-cause or cardiometabolic mortality. Our results are in agreement with a recent study by Doustmohamadian et al. in relation to all-cause mortality [371], however, Keihani et al. reported an increased risk of CVD outcomes among metabolically healthy abdominally obese individuals [372]. Studies that have questioned the benign health status of the metabolically healthy obesity (using either BMI or WC to define obesity) have included a large proportion of individuals with ≤ 2 components of the MetS criteria [97–101,103,355,372]. The differences in estimated risk could be ascribed to the variation in type and severity of the MetS components across different studies, and evidence suggests that cardiometabolic risk increases with the presence of just one metabolic abnormality [373]. Therefore, we argue that use of a strict criterion to define metabolic health is more appropriate for risk stratification [28].

In relation to cardiometabolic mortality, competing risk events are common in epidemiologic research and are particularly relevant to studies with long-term follow-up [366,374]. In our study, the frequency of competing events was ~18% in the overall sample and ~5% among MHO participants. For risk prediction, it is important to account for competing events because standard survival methods overestimate the cumulative incidence of an event of interest [366,374]. Additionally, a higher hazard rate may not necessarily coincide with a higher cumulative incidence because the one-to-one correspondence between the hazard rate and the CIF is lost in the presence of competing risks [366,374]. In our study, we found the results from the cause-

specific HRs for MHO participants were similar to the cumulative incidence estimates because the frequency of competing events was low among MHO participants (i.e, ~ 5%). To our knowledge, previous studies assessing CVD prognosis among MHO participants have not accounted for competing events, and depending on the frequency of competing events in each study cohort, an increase in hazard rate may not necessarily imply an increase in the cumulative incidence of events [374]. Studies assessing the prognosis of the MHO phenotype in relation to cause-specific events should be aware of the potential bias introduced by standard survival methods and should account for competing events to more appropriately estimate the CVD prognosis of MHO participants.

We found evidence of a nonlinear association between 25(OH)D and all-cause and cardiometabolic mortality in the overall sample, with the minimum risk observed with values near 60-75 nmol/L. In our study, 25(OH)D modified the association between metabolic phenotype and cardiometabolic mortality. Effect modification by 25(OH)D was particularly pronounced among metabolically unhealthy normal-weight individuals such that higher 25(OH)D status (> 50 nmol/L) attenuated the increased risk of cardiometabolic mortality, with no evidence of a threshold effect. Higher 25(OH)D status among MHO participants had a non-significant inverse association with cardiometabolic mortality; this non-significance may be attributed to the small sample size (n=367). For non-MHO participants, there was a significant nonlinear inverse relationship between 25(OH)D and cardiometabolic mortality, with the highest risk observed for 25(OH)D < 50 nmol/L. The nonlinear association and the wide confidence intervals for participants within the obesity BMI range may either reflect a threshold effect of vitamin D or may be a function of the insufficient data for high 25(OH)D levels (> 50 nmol/L). For participants within the overweight BMI range, the non-linear association of serum 25(OH)D and cardiometabolic mortality was not significant. These results could reflect a chance finding. Moreover, the non-MHOW subgroup had the highest average age compared to other subgroups, and although we adjusted for age in our models, there may be residual confounding due to older age of non-MHOW participants, thus contributing to the non-significant association.
Vitamin D may mitigate the detrimental effects of metabolic dysfunction on cardiometabolic death outcomes (i.e., diabetes, hypertension, CVD) in several ways. Insulin resistance (IR) is the core metabolic trait associated with type-2 diabetes and several lines of evidence support a role

of vitamin D in glucose homeostasis and insulin sensitivity [3]. A recent meta-analysis of randomized control trials (RCT) showed that vitamin D supplementation (with a minimum dose of 4000 IU/d) significantly improved FPG, HbA1c, and insulin sensitivity in patients with type-2 diabetes [375]. Another recent RCT showed that 2,800 IU/d of vitamin D_3 in vitamin D deficient participants with obesity significantly reduced HbA1c by 3.52 mmol per mol of vitamin D [376]. Vitamin D supplementation has also been shown to be an effective intervention in hypertensive patients; several RCTs have shown a beneficial effect of vitamin D on central BP parameters in hypertensive patients with vitamin D deficiency [222,224]. While other RCTs have found null results in relation to BP parameter (particularly in untargeted populations), a Mendelian randomization study supported a causal role of vitamin D in hypertension [208]. Thus, the effect modification observed in our study could reflect changes in glycemic control and hypertension, particularly in metabolically unhealthy participants within the normal weight and obesity BMI ranges. While vitamin D has been shown to improve glycemic and BP parameters, no clinical trials have been performed specifically to investigate whether vitamin D supplementation can help individuals with metabolic dysfunction to transition in the metabolically healthy state. Although weight-loss through lifestyle modification is recommended for patients with obesity [24], long-term weight-loss maintenance remains challenging, and vitamin D supplementation has been proposed as an adjunct therapy in high-risk individuals [375]. Individuals with obesity have a higher risk of vitamin D deficiency [23] and may require at least 6,000 IU vitamin D_3/d to maintain serum 25(OH)D > 50 nmol/L [377]. Future well-designed RCTs are needed to investigate whether vitamin D supplementation is beneficial for transition and maintenance of metabolic health in targeted populations.

The present study has several limitations. Biomarkers for metabolic health, adiposity, and serum 25(OH)D were measured only once at baseline, therefore we were not able to assess the transition from metabolically healthy to unhealthy states, as well as any changes in 25(OH)D over time. In addition, only 3% of participants were categorized as MHO and consequently there were relatively few deaths among MHO phenotype, and this lead to a relatively wide confidence intervals for the risk estimates; therefore, these estimates should be interpreted with caution. For competing risk analysis, we were unable to further adjust for NHANES survey weights using the Fine and Gray model and it is difficult to obtain a true estimate of the frequency and the

distribution of competing events at the population level. Therefore, our estimate of competing events may not be transferable to other populations with a different distribution of competing events. As with any observational study, the presence of residual confounding related to measurement errors and unmeasured variables (i.e., genetic factors) cannot be eliminated in our study.

In conclusion, our study supports the hypothesis that metabolically healthy obesity is a benign health condition and our results suggest that metabolic health is a more important predictor of adverse events. MHO individuals did not have higher mean 25(OH)D levels compared to non-MHO individuals; however, vitamin D status modified the association between metabolic phenotype and cardiometabolic mortality, particularly among metabolically unhealthy normal-weight and obesity phenotypes. Future large interventional studies are needed to help clarify the joint association between vitamin D and metabolic health.

Table 6. 1 Baseline characteristics of participants aged 20 years and older by metabolic health status: NHANES III survey 1988 to 1994.

	Normal weight (n=4380)		Overweight (n=4022)		Obesity (n=2931)		
	Healthy (n=1939)	Unhealthy (n=2441)	Healthy (n=922)	Unhealthy (n=3100)	Healthy (n=367)	Unhealthy (n=2564)	P-value
Age, y	35.1 (34.2, 36.0)	46.3 (44.6, 47.9)	38.2 (37.0, 39.4)	48.5 (47.4, 49.6)	37.9 (36.4, 39.5)	46.9 (45.9, 47.9)	<.0001
FPG, mmol/L	4.90 (4.87, 4.92)	5.32 (5.27, 5.38)	5.02 (4.99, 5.05)	5.55 (5.49, 5.61)	4.97 (4.89, 5.05)	5.87 (5.75, 5.99)	<.0001
Triglyceride, mmol/L	0.88 (0.86, 0.91)	1.47 (1.42, 1.52)	1.01 (0.98, 1.04)	1.93 (1.85, 2.01)	1.07 (1.01, 1.12)	2.10 (2.02, 2.20)	<.0001
HDL-cholesterol, mmol/L	1.56 (1.54, 1.59)	1.30 (1.27, 1.32)	1.42 (1.39, 1.46)	1.18 (1.15, 1.20)	1.47 (1.41, 1.52)	1.13 (1.11, 1.14)	<.0001
SBP, mmHg	111.1 (110.3, 111.8)	123.4 (121.9, 124.9)	113.9 (112.9, 114.9)	127.2 (125.9, 128.4)	115.1 (113.6, 116.7)	129.5 (128.5, 130.5)	<.0001
DBP, mmHg	69.2 (68.7, 69.8)	73.9 (73.1, 74.7)	71.9 (71.0, 72.8)	77.3 (76.7, 77.8)	72.9 (72.0, 73.8)	79.1 (78.5, 79.8)	<.0001
BMI, kg/m^2	22.1 (21.9, 22.2)	22.5 (22.4, 22.6)	26.9 (26.7, 27.0)	27.2 (27.1, 27.2)	32.9 (32.5, 33.5)	34.8 (34.4, 35.2)	<.0001
Serum 25(OH)D, nmol/L	67.6 (65.7, 69.4)	63.3 (61.7, 64.9)	61.4 (59.2, 63.5)	62.4 (60.9, 63.8)	53.8 (50.2, 57.4)	55.5 (53.7, 57.4)	<.0001
Male, %	41.1 (39.5, 46.8)	45.3 (42.4, 48.2)	56.3 (51.3, 61.4)	60.7 (58.3, 63.0)	37.7 (27.2, 48.1)	43.2 (39.8, 46.7)	<.0001
Ethnicity, %							<.0001
Non-Hispanic Whites	78.7 (74.6, 82.9)	79.1 (75.8, 82.4)	73.8 (69.3, 78.3)	79.2 (76.1, 82.3)	67.2 (59.9, 74.5)	74.3 (71.1, 77.5)	
Non-Hispanic Blacks	8.99 (7.30, 10.7)	7.52 (6.34, 8.69)	12.7 (10.4, 15.1)	8.53 (7.48, 9.58)	20.5 (15.9, 25.1)	12.4 (10.5, 14.4)	
Mexican-Americans	4.28 (3.32, 5.24)	3.98 (3.12, 4.83)	6.60 (5.01, 8.20)	5.89 (4.78, 7.00)	4.61 (3.14, 6.08)	6.28 (5.08, 7.48)	
Other	7.99 (5.03, 10.9)	9.36 (6.59, 12.1)	6.83 (3.83, 9.84)	6.42 (4.07, 8.77)	7.61 (3.25, 12.0)	6.93 (4.82, 9.04)	
Current smokers, %	29.8 (26.7, 32.9)	32.8 (29.8, 35.8)	18.6 (15.3, 22.0)	28.5 (25.6, 31.3)	20.9 (13.9, 28.0)	23.9 (21.3, 26.6)	<.0001
Education, %							<.0001
Less than high school	15.1 (12.8, 17.4)	24.6 (21.8, 27.4)	15.7 (11.9, 19.5)	26.6 (22.9, 30.4)	17.2 (12.4, 22.0)	28.2 (25.4, 30.9)	
High school	31.2 (28.4, 34.0)	32.4 (29.9, 35.0)	33.0 (28.1, 38.0)	34.7 (31.8, 37.6)	43.9 (36.3, 51.4)	37.6 (34.1, 41.2)	
More than high school	53.7 (49.9, 57.4)	42.9 (39.4, 46.4)	51.2 (45.6, 56.9)	38.6 (34.6, 42.6)	38.9 (31.3, 46.5)	34.1 (30.1, 38.2)	
Leisure-time PA, total weekly, MET	30.5 (28.0, 33.0)	27.5 (25.5, 29.6)	30.5 (26.9, 34.2)	24.6 (22.6, 26.7)	20.1 (15.9, 24.4)	21.4 (18.9, 23.9)	<.0001
eGFR, ml/min/1.73 m^2, %							<.0001
≥ 90	85.7 (83.1, 88.3)	69.1 (65.9, 72.4)	80.6 (76.4, 84.8)	63.3 (60.8, 65.8)	84.3 (78.2, 90.3)	64.5 (61.8, 67.2)	
60-< 90	14.0 (11.4, 16.6)	26.3 (23.4, 29.3)	19.2 (15.0, 23.3)	32.3 (29.8, 34.7)	15.3 (9.20, 21.4)	30.1 (27.4, 32.7)	
< 60	0.28 (0.06, 0.50)	4.50 (3.65, 5.34)	0.20 (0.00, 4.44)	4.44 (3.46, 5.42)	0.41 (0.00, 1.23)	5.45 (4.52, 6.37)	

CRP mg/dL, %							<.0001
< 0.22	90.6 (89.0, 92.2)	78.5 (75.3, 81.7)	81.4 (77.6, 85.2)	71.1 (67.0, 75.2)	58.4 (48.7, 68.1)	50.0 (46.0, 54.0)	
0.22-1.0	7.76 (6.04, 9.48)	15.7 (12.7, 18.7)	15.7 (11.8, 19.6)	22.4 (19.4 , 25.5)	29.7 (19.6, 39.7)	36.4 (32.8, 40.1)	
≥ 1.0	1.61 (0.76, 2.47)	5.75 (4.30, 7.19)	2.84 (1.22, 4.45)	6.42 (4.81, 8.03)	11.9 (7.45, 16.4)	13.5 (11.6, 15.4)	

Values are survey-weighted means or frequencies (%), and 95% CIs in parenthesis. P-values are based on Wald-F test and Chi-square tests, which test the independence of means and frequencies, respectively, across metabolic health groups.

Table 6. 2 All-cause, cardiometabolic (CM) and non-CM mortality hazard ratios (HRs) for participants aged 20 years and older according to metabolic health status: NHANES III survey 1988 to 1994 with follow-up through 2011.

N=11,333			All-cause mortality			CM mortality			Non-CM mortality	
	Median follow-up, yr	**All-cause deaths/N**	**HR**	**95% CI**	**CM deaths/N**	**HR**	**95% CI**	**non-CM deaths/N**	**HR**	**95% CI**
MHNW	20.0	179/1939	1.00	-	37/1939	1.00	-	142/1939	1.00	-
MHOW	19.7	87/922	0.85	0.59, 1.22	20/922	0.94	0.38, 2.33	67/922	0.84	0.56, 1.27
MHO	19.6	25/367	0.89	0.52, 1.51	6/367	1.21	0.33, 4.46	19/367	0.84	0.47, 1.50
non-MHNW	18.7	891/2441	**1.38***	**1.12, 1.69**	286/2441	**2.22***	**1.28, 3.82**	605/2441	1.23	0.95, 1.59
non-MHOW	18.7	1055/3100	1.15	0.91, 1.47	345/3100	**1.94***	**1.12, 3.34**	710/3100	1.02	0.77, 1.33
non-MHO	18.7	743/2564	**1.44***	**1.17, 1.76**	243/2564	**2.51***	**1.53, 4.09**	500/2564	1.26	0.99, 1.61

HRs are adjusted for age, sex, ethnicity, smoking status, educational level, leisure-time physical activity, kidney function (eGFR), serum CRP and 25(OH)D. *P-value ≤ 0.05.

MHNW: Metabolically Healthy Normal-Weight

MHOW: Metabolically Healthy Overweight

MHO: Metabolically Healthy Obesity

Non-MHNW: Metabolically Unhealthy Normal Weight

Non-MHOW: Metabolically Unhealthy Overweight

Non-MHO: Metabolically Unhealthy Obesity

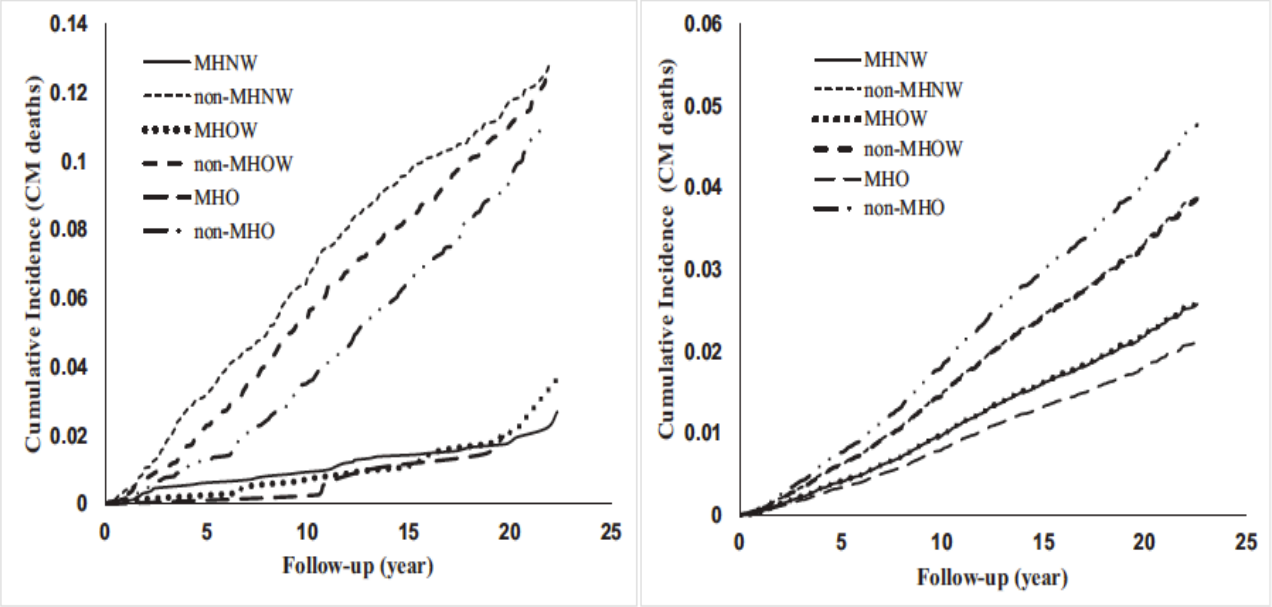

Figure 6-1A **Figure 6-1B**

Figure 6. 1 Cumulative incidence curves for cardiometabolic (CM) death outcomes by metabolic health phenotypes among participants aged 20 years and older: NHANES III survey with follow-up through 2011.

Unadjusted (Figure 6-1A) and adjusted (Figure 6-1B) cumulative incidence curves for cardiometabolic (CM) death outcomes by metabolic health phenotypes among participants aged 20 years and older: NHANES III survey with follow-up through 2011. Cumulative incidence functions (CIFs) were obtained from Fine and Gray model (i.e., adjusting for non-cardiometabolic deaths). CIFs (Figure 1B) were adjusted for age, sex, ethnicity, smoking status, educational level, leisure-time physical activity, kidney function (eGFR), serum CRP and 25(OH)D.

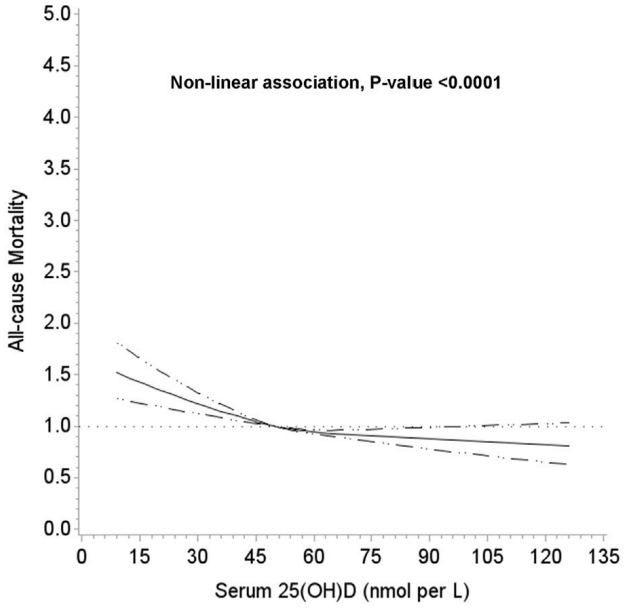

Figure 6-2A

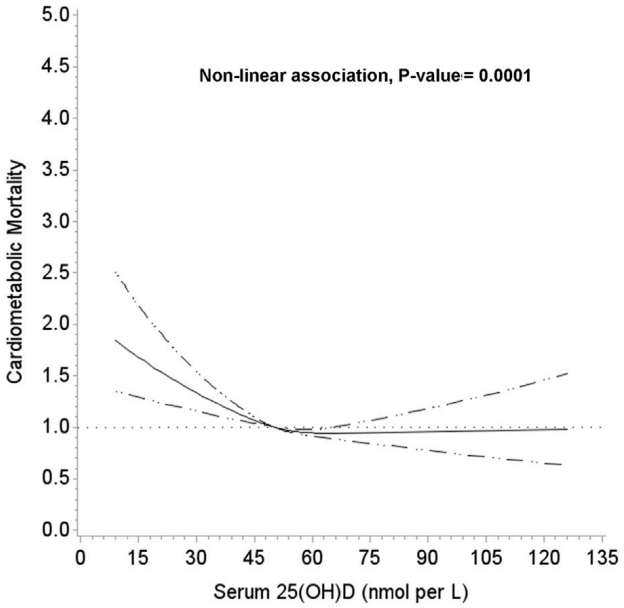

Figure 6-2B

Figure 6. 2 Adjusted cubic spline models showing hazard ratios (HR) for all-cause and cardiometabolic mortality according to serum 25(OH)D concentration in participants aged 20 years and older: NHANES III survey.

Models are adjusted for metabolic health phenotype, age, sex, ethnicity, smoking status, education level, leisure-time PA, kidney function (eGFR), serum CRP, and season of blood draw. The solid line represents HR for all-cause and cardiometabolic mortality and the dashed lines represent the 95% confidence intervals. Knots are at the 25th, 50th, and 75th percentiles for serum 25(OH)D. Reference value for serum 25(OH)D is 50 nmol/L (HR=1.0).

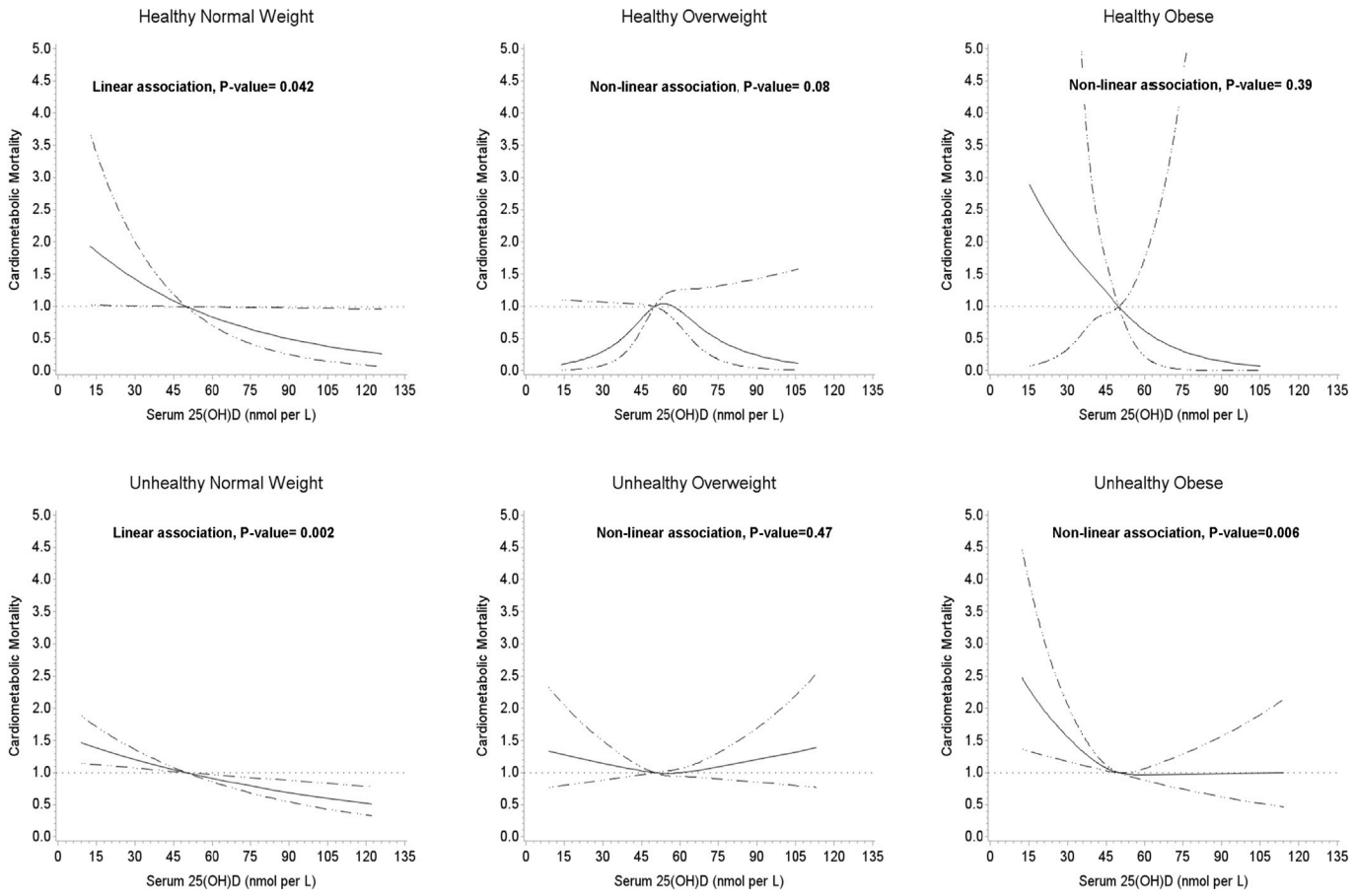

Figure 6. 3 Adjusted cubic spline models showing association between serum 25(OH)D levels and hazard ratios (HRs) for cardiometabolic mortality stratified by metabolic health status among participants aged 20 years and older: NHANES III survey 1988 to 1994.

Models are adjusted for age, sex, ethnicity, smoking, education level, leisure-time PA, kidney function (eGFR), serum CRP, and season of blood draw. The solid line represents HR for cardiometabolic mortality and the dashed lines represent the 95% CIs. Knots are at the 25th, 50th, and 75th percentiles for serum 25(OH)D. Reference value for serum 25(OH)D is 50 nmol/L (HR=1.0).

Chapter 6: Supplementary data

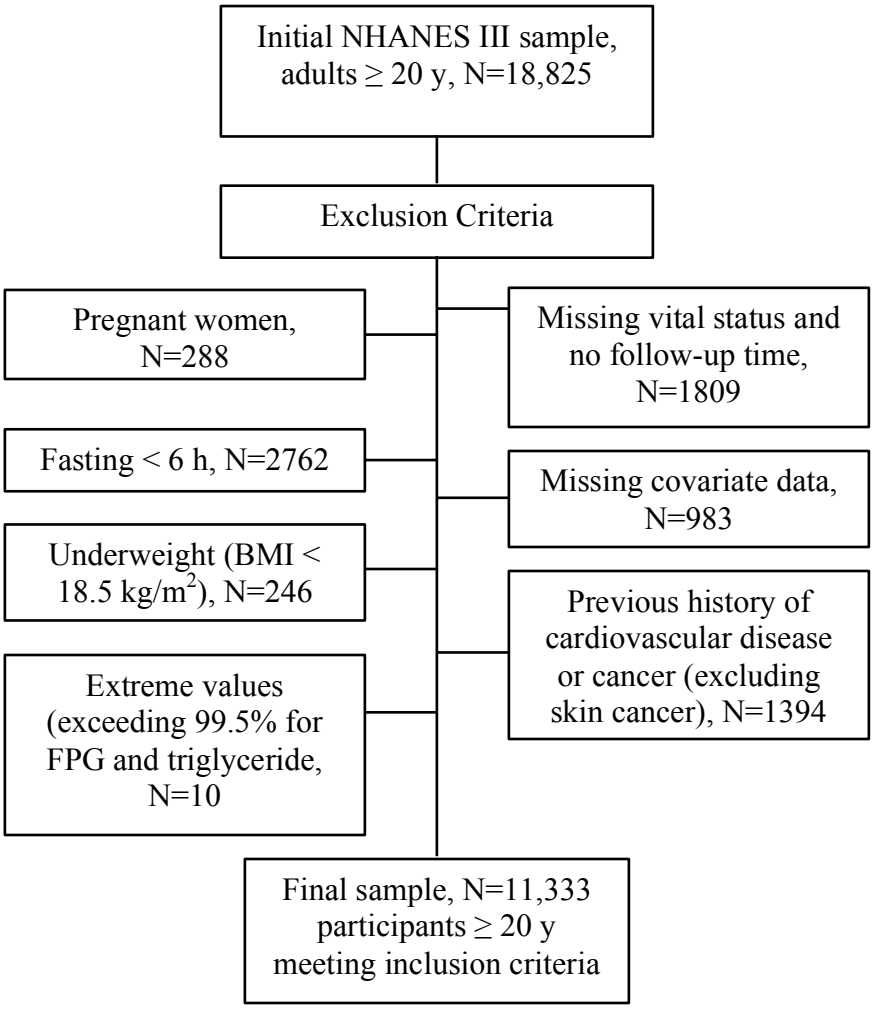

Figure 1: Flowchart showing the exclusion criteria for sample derivation, NHANES III (1988-1994).

Adjustment for additional confounders (income, alcohol consumption, HEI score)

Table 1. All-cause, cardiometabolic (CM) and non-CM mortality hazard ratios (HR) for participants aged 20 years and older according to metabolic health status: NHANES III survey 1988 to 1994 with follow-up through 2011.

N=9,744			All-cause mortality			CM mortality			Non-CM mortality	
	Median follow-up, yr	**All cause deaths/N**	**HR**	**95% CI**	**CM deaths/N**	**HR**	**95% CI**	**non-CM deaths/N**	**HR**	**95% CI**
MHNW	19.9	146/1699	1.00	-	31/1699	1.00	-	115/1699	1.00	-
MHOW	19.7	71/814	0.89	0.58, 1.35	14/814	0.84	0.31, 2.31	57/814	0.91	0.57, 1.44
MHO	19.6	19/317	0.82	0.42, 1.58	4/317	1.21	0.28, 5.21	15/317	0.75	0.34, 1.65
non-MHNW	18.8	717/2084	**1.34***	**1.07, 1.66**	224/2084	**1.90***	**1.11, 3.27**	493/2084	1.24	0.94, 1.63
non-MHOW	18.7	875/2634	1.18	0.90, 1.55	286/2634	**1.82***	**1.03, 3.22**	589/2634	1.06	0.79, 1.44
non-MHO	18.7	619/2196	**1.44***	**1.15, 1.81**	201/2196	**2.46***	**1.48, 4.10**	418/2196	1.26	0.97, 1.64

Hazard ratios are adjusted for age, sex, ethnicity, smoking, education level, leisure-time PA, kidney function (eGFR), serum CRP, serum 25(OH)D, income, alcohol consumption, and diet quality (HEI score). *P-value ≤ 0.05.

MHNW: Metabolically Healthy Normal-Weight

MHOW: Metabolically Healthy Overweight

MHO: Metabolically Healthy Obesity

Non-MHNW: Metabolically Unhealthy Normal Weight

Non-MHOW: Metabolically Unhealthy Overweight

Non-MHO: Metabolically Unhealthy Obesity

Different definition of obesity (using waist circumference (WC) to define obesity)

Table 2. All-cause, cardiometabolic (CM) and non-CM mortality hazard ratios (HRs) for participants aged 20 years and older according to metabolic health status: NHANES III survey 1988 to 1994 with follow-up through 2011.

N=10,988			All-cause mortality			CM mortality			non-CM mortality	
	Median follow-up, yr	**All cause deaths/N**	**HR**	**95% CI**	**CM deaths/N**	**HR**	**95% CI**	**non-CM deaths/N**	**HR**	**95% CI**
Healthy low WC	20.0	147/1917	1.00	-	28/1917	1.00	-	119/1917	1.00	-
Healthy intermediate WC	19.7	60/610	0.83	0.53, 1.30	12/610	0.63	0.22, 1.79	48/610	0.88	0.52, 1.49
Healthy elevated WC	19.6	71/630	1.02	0.70, 1.48	20/630	1.34	0.53, 3.36	51/630	0.99	0.63, 1.55
Unhealthy low WC	19.2	537/2029	**1.38***	**1.14, 1.68**	163/2029	**2.26***	**1.28, 3.99**	374/2029	1.24	0.97, 1.59
Unhealthy intermediate WC	18.8	593/1776	**1.27***	**1.02, 1.58**	189/1776	**2.17***	**1.15, 4.11**	404/1776	1.11	0.85, 1.47
Unhealthy elevated WC	18.5	1422/4026	**1.37***	**1.12, 1.68**	473/4026	**2.28***	**1.21, 4.31**	949/4026	1.23	0.97, 1.56

HRs are adjusted for age, sex, ethnicity, smoking status, educational level, leisure-time physical activity, kidney function (eGFR), serum CRP and 25(OH)D.

*P-value ≤ 0.05. Waist circumference (WC) defined as low (men, ≤ 94 cm; women, ≤ 80 cm), intermediate (men, > 94 and ≤ 102 cm; women, > 80 and ≤ 88 cm), and elevated (men, > 102 cm; women, > 88 cm).

Chapter 7: Study 4- Lifetime risk of cardiometabolic mortality according to vitamin D status of middle and older-aged adults: NHANES III mortality follow-up

Manuscript is under review by the Journal of Steroid Biochemistry and Molecular Biology (submitted May 8, 2018).

Authors' contribution:

All authors have read and approved the final version being submitted with contributions as follows: conception (BA), data acquisition (BA), statistical analysis (BA) and interpretation of data (BA, JLK, CIA), drafting of manuscript (BA), critical review and editing (BA, JLK, CIA).

7.1 Abstract

Background & Objectives: The predictive value of total 25-hydroxyvitamin D level (25(OH)D, a biomarker of vitamin D status) in relation to lifetime risk of cardiometabolic mortality is not known. The purpose of this study was to determine the association between standardized total 25(OH)D levels and lifetime risk for cardiometabolic mortality in middle- to older-aged adults.
Methods: We followed up 7,958 participants in the Third National Health and Nutrition Examination Survey from 1988-1994 until the occurrence of cardiometabolic death or attainment of 95 years of age (median follow-up 17.9 years, 1,371 cardiometabolic-deaths). Lifetime risks to 70 and 95 years of age were estimated according to recommended total 25(OH)D cutoffs by national guidelines, and a combination of total 25(OH)D category and traditional risk factor burden. We also explored variation in lifetime risk estimates by levels of body mass index (BMI).
Results: Participants with total 25(OH)D <30 nmol/L had high lifetime risk for cardiometabolic mortality (≥ 36%). Lifetime risk was highest among participants with total 25(OH)D < 30 nmol/L and with ≥ 2 major traditional risk factors (25% and 43% at 70 and 95 years of age), whereas lifetime risk was lowest among participants with 25(OH)D ≥ 30 nmol/L and low-intermediate traditional risk factors (3% and 28% at 70 and 95 years of age). Lifetime risk estimates were consistent across BMI categories.
Conclusion: A single measurement of vitamin D deficiency (< 30 nmol/L) in middle- to older-aged adults is a strong predictor of high lifetime risk for cardiometabolic mortality, particularly among those with high burden of traditional risk factors.

7.2 Introduction

Cardiovascular disease (CVD) is the leading cause of morbidity and mortality in the United States [378]. The most recent national guidelines suggest that clinicians should consider patients' risk factor burden within the context of lifetime risk to capture the cumulative burden of CVD with advancing age [29]. Several well-established traditional risk factors, including hypertension, diabetes, and hypercholesterolemia, are viewed as target risk factors for treatment and management of CVD outcomes. Although the development of traditional CVD risk factors can be delayed or altogether prevented through healthy lifestyle behaviors, long-term maintenance of a healthy diet, non-smoking, physical activity, and appropriate weight outside of a clinical trial setting is often challenging [110]. Therefore, prevention and management of CVD requires a multifaceted approach and complementary strategies are needed to reduce the cumulative burden of CVD events with advancing age.

Of interest, prior research has demonstrated an independent and inverse association between vitamin D status, as measured by total 25-hydroxyvitamin D (25(OH)D) levels, and cardiometabolic disease risk including CVD mortality [34,379]. Although several studies have shown a significant inverse association, such associations do not necessarily support the clinical usefulness or the predictive value of total 25(OH)D in the context of long-term disease risk prediction at the population level [2,21]. In addition, the relationship between total 25(OH)D and risk of cardiometabolic disease may be confounded by obesity, given that excess adiposity is a risk factor for vitamin D deficiency [23]. Therefore, excess weight is an important factor to consider and it is unknown whether the predictive ability of 25(OH)D in relation to cardiometabolic disease risk is similar in individuals classified as normal-weight, overweight, or obesity [380].

Although most RCTs of vitamin D supplementation to date have failed to show a beneficial role of vitamin D in relation to cardiometabolic health outcomes, prior RCTs have had significant shortcomings [5,6]. Moreover, it is known that RCTs are more difficult to conduct properly and to test the right-dose response in a targeted population for a sufficient time-period [381]. Given these current limitations, lifetime risk analysis provides a unique opportunity to assess the

predictive value of total 25(OH)D in the context of long-term risk prediction (>10 years), which may not be a feasible approach in RCTs.

The objective of this study was to determine the association between total 25(OH)D levels and lifetime risk for cardiometabolic mortality overall, and to further explore variation by levels of body mass index (BMI) in adults ≥ 40 years of age from the Third National Health and Nutrition Examination Survey (NHANES III).

7.3 Methods

7.3.1 Study Sample

The NHANES III (1988-1994) is a representative survey of the civilian, non-institutionalized US population conducted by the National Center for Health Statistics (NCHS) of the Center for Disease Control and Prevention. Detailed description of the survey design and methodology are published elsewhere [359]. The baseline data collected in 1988-1994 was linked to mortality data (up to December 31, 2011) by a probabilistic record matching methodology with death certificate records obtained in the National Death Index (NDI). Detailed description of the matching methodology used to determine mortality status has been published elsewhere [360]. Follow-up time was calculated from clinical examination date until date of death or end of study (December 31, 2011). The NCHS institutional board approved NHANES III and all participants provided written informed consent [359].

A total of 23,258 adults aged 20 years and older were invited to participate in NHANES III, 18,825 were interviewed, and 16,573 completed the mobile examination center (MEC) and laboratory examinations. After applying the exclusion criteria, 7,958 participants had complete information on study parameters. A flowchart for sample derivation is shown in **Figure 1** (**Chapter 7: supplementary data).**

7.3.2 Risk Factor Measurement

Age, sex, and smoking status were self-reported by questionnaire during the in-home interview. Diabetes status was defined as previous diagnosis of diabetes or a FPG ≥ 126 mg/dL or use of

insulin or any diabetes medication. Smoking status was categorized into current, former or never smokers (cigarettes only). BMI was calculated as weight in kilograms divided by height in meters squared (kg/m^2) and categorized as normal weight (18.5-24.9 kg/m^2), overweight (25-29.9 kg/m^2), and obesity (≥ 30 kg/m^2).

Systolic and diastolic BP was the average of up to 6 measurements. Total cholesterol and blood glucose were measured using standardized laboratory procedures as reported by NCHS [361]. We used standardized serum 25(OH)D values in this study [278].

7.3.3 Cardiometabolic Mortality

We used the publicly available dataset to obtain information on cardiometabolic death outcomes. Death cases were coded according to the Tenth Revision of the International Classification of Diseases (ICD-10) and with the underlying cause of death further categorized into ten broad groups: 1) Diseases of heart; 2) Malignant neoplasm; 3) Chronic lower respiratory diseases; 4) Accidents (unintentional injuries); 5) Cerebrovascular diseases; 6) Alzheimer's disease; 7) Diabetes mellitus; 8) Influenza and pneumonia; 9) Nephritis, nephrotic syndrome and nephrosis; and 10) All other causes (residual). For cardiometabolic mortality, we combined primary causes of death classified as diseases of heart, cerebrovascular diseases and diabetes mellitus.

7.3.4 Statistical Analysis

Participant data were stratified according to baseline traditional CVD risk factor (RF) levels using a previously validated algorithm [32]. For traditional CVD RFs, we included measures of BP, total cholesterol, current smoking, presence of diabetes, and medications for hypertension and dyslipidemia. Participants were initially stratified a priori into 5 mutually exclusive risk categories (i.e., all-optimal, ≥ 1 not optimal, ≥ 1 elevated, 1 major, and ≥ 2 major RFs). Due to the small sample size for the "all-optimal" subgroup (n=298), we combined this subgroup with the "≥ 1 not optimal" subgroup and renamed it as "normal risk factors" (see Table 1 footnote for detailed description of RF levels).

For the lifetime risk analysis by vitamin D status, we stratified total 25(OH)D according to national guidelines by Health and Medicine Division (HMD) of the National Academies

(formerly known as Institute of Medicine, IOM) [2] and the Endocrine Society [16]; < 30 nmol/L, 30-< 50 nmol/L, 50-< 75 nmol/L, and ≥ 75 nmol/L.

For the joint effect of traditional risk burden and total 25(OH)D, participants were stratified according to combination of aggregate CVD risk burden and total 25(OH)D levels. First, traditional RF burden was re-categorized into "high-risk" (≥ 2 major RFs) and "low-intermediate risk" (normal RFs, ≥ 1 elevated RFs, and 1 major RFs). We then dichotomized total 25(OH)D status as < 30 nmol/L and ≥ 30 nmol/L. Finally, we created 4 mutually exclusive risk groups based on traditional RFs and total 25(OH)D: 1) low-intermediate risk / < 30 nmol/L; 2) high-risk / < 30 nmol/L; 3) low-intermediate risk/ ≥ 30 nmol/L; and 4) high-risk / ≥ 30 nmol/L.

We used the lifetime risk model, a modified Kaplan-Meier (KM) analysis, as described previously [382]. In brief, lifetime risk model adjusts for competing events (i.e., deaths due to cancer and other non-cardiometabolic death outcomes) by treating them as separate events and adjusts for risk inflation introduced by standard KM analysis [32,382]. Lifetime risk model also uses age as the underlying time-scale where participants contribute information on event status for each age attained during follow-up [382]. As such, each participant contributes person-time from study entry to the occurrence of cardiometabolic mortality, competing event, or the attainment of 95 years of age. We also conducted a sensitivity analysis by excluding participants with baseline CVD (i.e., with history of heart attack, stroke, or congestive heart failure). All analyses were performed with SAS statistical software version 9.4 (Cary, NC).

7.4 Results

7.4.1 Baseline Characteristics

We followed a sample of 7,958 participants 40 years of age or older for a total of 129,294 person-years (median follow-up of 17.9 years); there were 1,371 cardiometabolic deaths and 2,457 deaths due to other causes. Baseline characteristics of the overall sample and by BMI status are shown in **Table 7.1**.

7.4.2 Lifetime Risk Estimates Stratified by Individual Traditional Risk Factors and BMI

Table 7.2 shows the effect of individual traditional RFs on the lifetime risk of cardiometabolic mortality in the overall sample and by BMI status. In the overall sample, increasing BP and total cholesterol were associated with increased lifetime risk; the lifetime risk to 95 years of age was > 30% among participants with BP ≥ 140/ ≥ 90 mm Hg and total cholesterol ≥ 200 mg/dL. The presence of diabetes resulted in the highest lifetime risk for cardiometabolic mortality, at 46% through 95 years of age. Lifetime risk to 70 years of age was almost three times higher among current smokers (11.6%) compared to nonsmoker (4.25%); however lifetime risk to 90 years of age was similar for nonsmokers (26.6%) and smokers (27.6%). Because of sparse data, we were not able to provide lifetime risk estimates through 95 years of age, as few smokers survived past the age of 90. Similar trends were observed for lifetime risk of cardiometabolic mortality stratified by BMI status, however, presence of diabetes among normal-weight participants conferred the highest lifetime risk for cardiometabolic mortality; 34% and 59.4% at 70 and 95 years of age, respectively.

7.4.3 Lifetime Risk Estimates Stratified by Aggregate Risk Factor Burden and BMI

Lifetime risks for cardiometabolic mortality through 95 years of age stratified by aggregate risk factor burden are shown in **Figure 7.1.** In the overall sample, the lifetime risk to 95 years of age for cardiometabolic mortality was highest among those with ≥ 2 major RFs (41.1%) followed by those with only 1 major RF (29.7%), ≥ 1 elevated RF (26.8%), and normal RFs (24.7%). Similar trends were observed for lifetime risk of cardiometabolic mortality stratified by BMI status.

7.4.4 Lifetime Risk Estimates Stratified by 25(OH)D Status and BMI

Lifetime risks for cardiometabolic mortality through 95 years of age stratified by total 25(OH)D levels are shown in **Figure 7.2**. In the overall sample, lifetime risk to 70 years of age was highest for those with total 25(OH)D < 30 nmol/L (11.7%) and lowest for those with 25(OH)D levels between 50-75 nmol/L (4.2%). Lifetime risk to 95 years of age remained high for total 25(OH)D < 30 nmol/L (37.1%). Similar trends were observed for lifetime risk analyses stratified by BMI status; for participants with obesity, we omitted the lifetime risk estimates for total 25(OH) ≥ 75

nmol/L because the sample size was not sufficient to provide a reliable lifetime risk estimate (n=265).

7.4.5 Lifetime Risk Estimates Stratified by Aggregate Risk Factor, 25(OH)D Status, and BMI

Figure 7.3 shows the combined effect of traditional RF burden and total 25(OH)D status in the overall sample and further stratified by BMI status. The lifetime risks were lowest in the low-intermediate risk / ≥ 30 nmol subgroup in the overall sample and across all BMI categories. Conversely, the lifetime risks were highest in the high risk / < 30 nmol/L subgroup. Among participants with high-risk burden (i.e., ≥ 2 major RFs) in the overall sample, total 25(OH)D ≥ 30 nmol/L was associated with lower lifetime risk (i.e., 10% and 3% absolute risk reduction through 70 and 95 years of age, respectively). Among participants with low-intermediate risk burden, total 25(OH)D ≥ 30 nmol/L was also associated with lower lifetime risk (i.e., 3% and 6% absolute risk reduction through 70 and 95 years of age, respectively). Similar trends were observed across all BMI categories.

7.4.6 Sensitivity Analysis

When we excluded participants with a history of CVD (i.e., history of heart attack, stroke or congestive heart failure) at baseline, the lifetime risk estimates were similar to the main analysis (**Chapter 7: supplementary data, Figures 2-4**).

7.5 Discussion

In a population study of 7,958 U.S. adults with 129,294 person-years of follow-up, participants with total 25(OH)D levels < 30 nmol/L had high lifetime risk for cardiomatabolic mortality (≥ 36%), and this finding was consistent across all BMI categories. In relation to traditional risk factor burden, having ≥ 2 major RFs conferred the highest lifetime risk for cardiometabolic mortality (≥ 41%), and this finding was also consistent across all BMI categories. When participants were stratified by a combination of traditional risk factor burden and total 25(OH)D levels, the lifetime risk for cardiometabolic mortality was highest in the ≥ 2 major RFs / < 30 nmol/L subgroup, whereas the lifetime risk was lowest in the low-intermediate risk / ≥ 30 nmol/L subgroup. Lastly, lifetime risks were intermediate for subgroups with low-intermediate

risk / < 30 nmol/L and ≥ 2 major risk factors / ≥ 30 nmol/L. These results remained similar across all BMI categories. Overall, our study findings indicate that a single measurement of total 25(OH)D < 30 nmol/L in middle- to older-aged adults is a strong predictor of high lifetime cardiometabolic mortality risk independent of BMI status, and that the effect of vitamin D deficiency (< 30 nmol/L) is particularly pronounced in individuals with ≥ 2 major traditional CVD risk factors.

In our study, the highest lifetime risk was observed among participants with ≥ 2 major traditional risk factors, followed by those with 1 major traditional risk factor only. Our results are in agreement with previous studies assessing the association between traditional CVD risk factor burden and lifetime risk for CVD [32]. Moreover, using data from the Lifetime Risk Pooling Project, a recent study by Khan et al. found that overweight and obesity was associated with an increased lifetime risk for CVD [92]. However, prior studies have not assessed the potential variation in lifetime risk estimates according to a combination of traditional risk factor burden and BMI status. Therefore, our study extends previous studies by estimating the lifetime risk according to traditional risk factors stratified BMI status; our results indicate that a single measurement of traditional risk factor burden in mid-life is a strong predictor of lifetime risk for cardiometabolic mortality with little to no variation by BMI status. This finding is in agreement with prior studies showing that risk factor burden is a more important driver of future health outcomes than overweight/obesity alone [380].

In relation to vitamin D status, participants with total 25(OH)D < 30 nmol/L had lifetime risk for cardiometabolic mortality that was 12% and 37% at 70 and 95 years of age, respectively. In the overall sample, the lowest lifetime risk was observed among participants with total 25(OH)D levels of 50-< 75 nmol/L (i.e., 4% and 26% at 70 and 95 years of age, respectively); however, after 90 years of age, no marked differences were observed for total 25(OH)D between 30- < 50 nmol/L, 50-< 75 nmol/L and ≥ 75 nmol/L. These findings persisted across all BMI categories; however, for participants with obesity, due to the low number of participants with total 25(OH)D ≥ 75 nmol/L (n=265), we were unable to provide a robust lifetime risk estimate for cardiometaboilc mortality. To our knowledge, no previous study has estimated the absolute risk of cardiometabolic mortality associated with total 25(OH)D levels. However, prior observational

studies have mostly assessed the association between total 25(OH)D levels and CVD mortality using relative risk measures [198,265,383]. Further, meta-analyses of cohort studies have demonstrated a consistent, inverse association between total 25(OH)D and CVD mortality in participants with and without CVD history, which persisted after adjustment for traditional risk factors [34,379,384].

Although prior studies have assessed the association between total 25(OH)D and CVD mortality using relative risk measures, the utility of relative risk is often limited because it requires a standard base group, which may not be directly applicable within a clinical setting [385]. Moreover, the establishment of a standard base group with respect to total 25(OH)D levels has been challenging due to the unavailability of standardized total 25(OH)D data in previous studies [11]. Use of an unstandardized total 25(OH)D data may lead to misclassification of individuals with respect to specific total 25(OH)D cutoffs (i.e., < 30 nmol/L, < 50 nmol/L, < 75 nmol/L) [136]. As such, most studies to date have used a quantile-based approach for classification of vitamin D deficiency (i.e., participants in the lowest quartile of the total 25(OH)D distribution) [34,265,383,386,384]. However, use of a quantile-based approach results in different base groups across studies and the relative risk estimates vary widely depending on the quantile cutoffs for total 25(OH)D levels. Therefore, use of standardized total 25(OH)D data is of utmost importance for the accurate assessment of vitamin D status and for reaching a consensus on a 25(OH)D cut-off that reliably predicts a health outcome of interest [11]. To date, only one recent meta-analysis of eight prospective studies (median follow-up of 10.5 years) from Europe used standardized total 25(OH)D data and the results from this meta-analysis showed that in participants with total 25(OH)D < 30 nmol/L, the rate of CVD mortality was twice that of participants with levels between 75-< 100 nmol/L [adjusted hazard ratio= 2.21 (1.50-3.26)] [379]. While the meta-analysis by Gaksch et al. [379] used a relative risk model to assess the association between standardized 25(OH)D and CVD mortality, our results are in agreement with this previous work [379]. Our study also extends prior work by showing that a single measurement of total 25(OH)D < 30 nmol/L in middle- to old-aged adults is a strong predictor of high lifetime risk of cardiometabolic mortality. Therefore, while the < 30 nmol/L cutoff is currently used by the IOM to signify ‘vitamin D deficiency’ in relation to poor bone health outcomes (i.e., low bone mineral density and risk of osteoporosis in adults) [2], the results from

our study suggest that it may also be important to consider the cardiometabolic health of middle and old-aged adults with vitamin D deficiency.

When we stratified participants according to a combination of traditional risk factor burden and total 25(OH)D status, we found that lifetime risk for cardiometabolic mortality was lowest for participants with low-intermediate risk and total 25(OH)D ≥ 30 nmol/L. In contrast, lifetime risk was highest for participants with ≥ 2 major risk factors and total 25(OH)D < 30 nmol/L. These findings remained consistent across all BMI categories. In a further sensitivity analysis, our results were similar to the main analysis after exclusion of participants with previous history of cardiovascular disease. In the presence of major risk factors/poor health status, it has been hypothesized that vitamin D may act as a resilience factor, meaning that sufficient vitamin D levels may prevent or delay death from a major disease by modulating the immune system [387]. In our study, participants with ≥ 2 major traditional risk factors and total 25(OH)D ≥ 30 nmol/L had lower lifetime risk of cardiometabolic morality compared to those with levels < 30 nmol/L, an observation that is in line with the resilience hypothesis. Further, while we acknowledge that the causal relationship between vitamin D deficiency and cardiometabolic mortality is not fully established, according to Bradford Hill criteria for causality, the evidence to date on the role of vitamin D in relation to cardiometabolic health outcomes satisfies 8 of the 9 criteria for causality (i.e., strength of association, consistency, specificity, temporality, biological gradient, plausibility, coherence, and analogy), except for experiment [388]. To date, RCTs of vitamin D supplementation have had significant shortcomings [5]. While we await the results of on-going RCTs to establish whether vitamin D deficiency is a *risk factor* in the development of cardiometabolic diseases, our study shows that a single measurement of total 25(OH)D < 30 nmol/L in middle- to old-aged adults is a strong *risk marker* of cardiometabolic mortality, particularly among participants with ≥ 2 major traditional CVD risk factors.

The present study has several limitations. First, biomarkers for vitamin D status, cholesterol, blood pressure, smoking and diabetes status were only available at baseline; therefore, we were unable to capture changes in these parameters over the long-term. However, it has been previously shown that tracking total 25(OH)D over the long-term is similar to that seen for cardiovascular parameters such as blood pressure and lipids [389]. It has been also reported that

75% of participants with serum 25(OH)D levels < 30 nmol/L have levels less than 50 nmol/L 14 years later [389]. Therefore, in our study, participants with 25(OH)D < 30 nmol/L were unlikely to have substantial improvements in their vitamin D levels over time. Although we acknowledge this as a limitation in our study, a single measurement of 25(OH)D < 30 nmol/L in middle- to old-aged adults appears to be a strong predictor of high lifetime risk of cardiometabolic mortality; however, the predictive ability of total 25(OH)D for participants with levels ≥ 30 nmol/L is less certain as total 25(OH)D levels may have fluctuated and decreased over time (i.e., due to weight gain with advancing age [390]). Therefore, in individuals with 25(OH)D ≥ 30 nmol/L, repeat measurements of total 25(OH)D is required to track changes in vitamin D status due to weight gain and changes in other lifestyle factors (i.e., sun exposure, diet, supplement use). Moreover, using the lifetime risk model, we were unable to incorporate NHANES survey weights to adjust for complex survey design, meaning that lifetime risk estimates in this study may not be representative of the general U.S. population. Nonetheless, our sample size was fairly large (n=7,958) and our lifetime risk estimates according to traditional risk factors were similar to previous studies using other U.S. cohorts, such as the cardiovascular disease lifetime risk pooling project [32]. Lastly, even though our lifetime risk estimates in a subset of participants without a history of CVD remained similar to the main analysis, the presence of reverse causality cannot be eliminated in our study.

In summary, we assessed the long-term predictive ability of standardized total 25(OH)D in relation to lifetime risk of cardiometabolic mortality in middle- to older-aged adults and found that total 25(OH)D < 30 nmo/L was associated with a high lifetime risk of cardiometabolic mortality, particularly among participants with high traditional risk factor burden. Although the causal relationship between vitamin D and cardiometabolic diseases remains to be established, total 25(OH)D < 30nmol/L consistently predicted high lifetime cardiometabolic mortality risk across all BMI categories. It is unknown whether vitamin D supplementation in individuals with ≥ 2 major traditional CVD risk factors would be beneficial. Therefore, future clinical trials are needed to assess whether vitamin D supplementation as an adjunct therapy in high-risk populations is a cost-effective strategy in the primary prevention of cardiometabolic mortality.

Table 7. 1 Baseline characteristics of participants (adults ≥ 40 years) in NHANES III (1988-1994)

	Overall	Normal Weight (18.5-< 25 kg/m^2)	Overweight (25-< 30 kg/m^2)	Obesity (≥ 30 kg/m^2)
No. of participants	7958	2612	3090	2256
Person-years of follow-up	129 294	40 604	50 150	38 540
Survival time, median (IQR), y	17.9 (10.5-20.2)	17.6 (8.83-20.2)	17.9 (10.4-20.2)	18.1 (12.8-20.2)
Total deaths, No. (%)	3828 (48.1)	1364 (52.2)	1493 (48.3)	971 (43.0)
Cardiometabolic deaths, No. (%)	1371 (17.2)	469 (17.9)	537 (17.4)	365 (16.2)
Age, y	61.1 ± 13.9	62.7 ± 14.7	61.8 ± 13.8	58.3 ± 12.4
BMI, kg/m2	27.7 ± 5.39	22.5 ± 1.68	27.3 ± 1.42	34.4 ± 4.46
Systolic blood pressure, mmHg	133.6 ± 20.1	131.2 ± 21.1	134.5 ± 19.8	135.1 ± 18.9
Diastolic blood pressure, mmHg	76.3 ± 10.4	73.9 ± 10.1	76.8 ± 10.2	78.4 ± 10.5
Total Cholesterol, mg/dL	218.2 ± 44.1	213.3 ± 42.9	220.0 ± 43.5	221.3 ± 45.7
Serum 25(OH)D, nmol/L	54.9 ± 19.9	57.7 ± 21.0	55.7 ± 19.3	50.8 ± 18.6
Current Smokers, No. (%)	1685 (21.2)	708 (27.1)	609 (19.7)	368 (16.3)
Diabetes Mellitus, No. (%)	934 (11.7)	168 (6.43)	356 (11.5)	410 (18.2)
Normal risk factors*	1030 (12.9)	395 (15.1)	395 (12.8)	240 (10.6)
≥ 1 elevated risk factor†	1453 (18.3)	512 (19.6)	561 (18.2)	380 (16.8)
1 major risk factor‡	3302 (41.5)	1128 (43.2)	1254 (40.6)	920 (40.8)
≥ 2 major risk factor‡	2173 (27.3)	577 (22.1)	880 (28.5)	716 (31.7)

*Normal risk factors category was defined as blood pressure < 140/ <90 mm Hg, total cholesterol as < 200 mg/dL, non-smoker, and nondiabetic. †≥ 1 elevated risk factor category was defined as systolic blood pressure 140-159 mm Hg or diastolic blood pressure 90-99 mm Hg, total cholesterol 200-239 mg/dL non-smoker, and nondiabetic. ‡1 major risk factor category was defined as having only 1 of the major risk factors: systolic blood pressure ≥ 160 mm Hg or diastolic blood pressure ≥ 100 mm Hg or treated or total cholesterol ≥ 240 mg/dL or current smoker or diabetic. ≥ 2 major risk factors category was defined as having at least 2 of the major risk factors.

Table 7. 2 Lifetime risk of cardiometabolic mortality according to traditional CVD risk factors overall and stratified by BMI category: NHANES III (1988-1994) with follow-up through 2011

	Overall (n=7958)		Normal Weight (n=2612)		Overweight (n=3090)		Obesity (n=2256)	
	Lifetime risk, %		Lifetime risk, %		Lifetime risk, %		Lifetime risk, %	
	To 70 y	To 95 y	To 70 y	To 95 y	To 70 y	To 95 y	To 70 y	To 95 y
Overall	…	…	5.66	24.8	5.69	26.3	7.74	30.3
SBP or DBP, mm Hg								
< 120 and < 80	2.78	25.8	2.57	23.8	2.18	27.9	4.29	24.7
120-139 or 80-89	4.62	26.7	3.98	23.9	4.67	27.0	5.36	33.1
140-159 or 90-99	7.15	32.9	11.1	35.3	6.32	31.7	6.18	32.2
≥ 160 or ≥ 100 or treated	13.1	38.7	15.8	40.4	12.3	38.2	12.7	38.6
Total cholesterol, mg/dL								
< 180	5.71	28.9	7.62	31.7	3.87	24.3	5.68	28.6
180-199	5.69	29.4	3.84	26.1	6.02	35.7	7.16	23.7
200-239	5.67	32.3	5.73	30.1	5.2	33.2	6.16	33.8
≥ 240	7.91	35.8	5.3	28.0	6.63	34.9	11.3	43.5
Nondiabetic	4.89	30.3	4.33	27.7	4.83	31.1	5.61	32.6
Diabetic	18.3	46.0	34	59.4	12.2	40.6	17.8	43.9
Nonsmoker	4.25	26.6*	3.7	25.4*	3.18	25.4*	5.8	29.4*
Smoker	11.6	27.6*	8.92	23.3*	12.6	28.6*	14.6	35.1*

*Lifetime risk estimated to 90 y due to few current smokers surviving past 90 years.

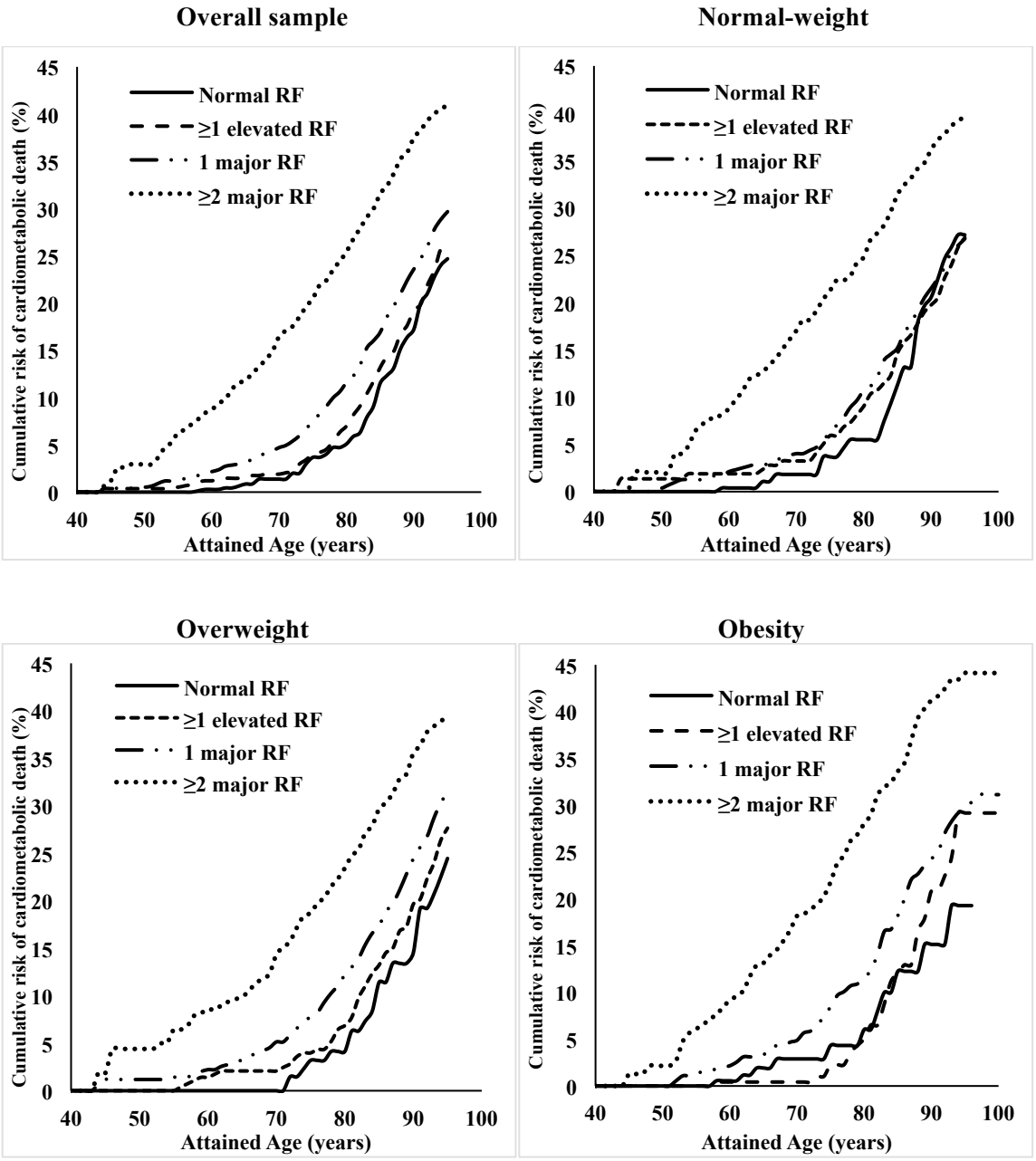

Figure 7. 1 Cumulative incidence of cardiometabolic mortality adjusted for competing risk events according to aggregate risk factor (RF) burden stratified by BMI: NHANES III (1988-1994) with follow-up through 2011.

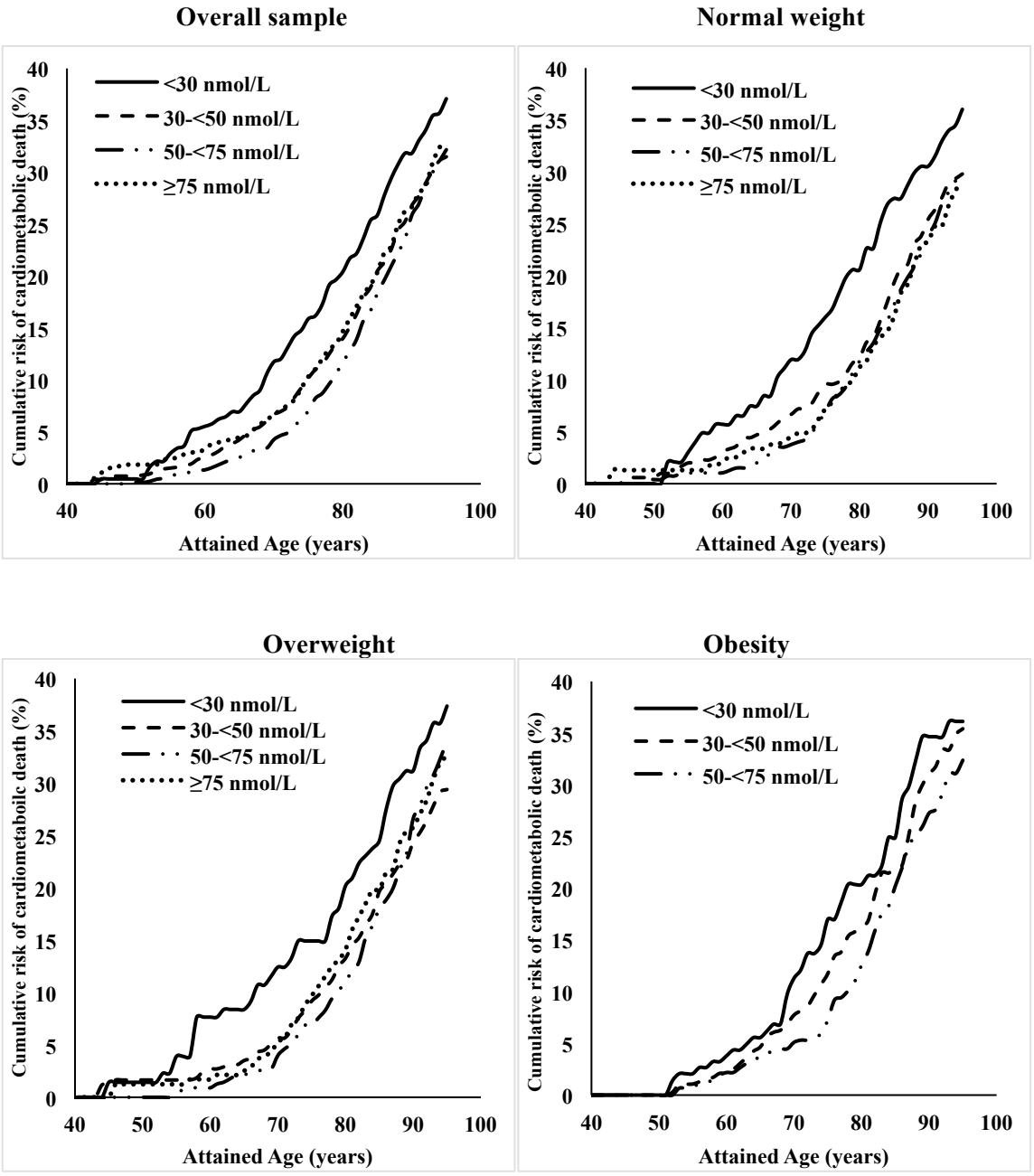

Figure 7. 2 Cumulative incidence of cardiometabolic mortality adjusted for competing risk events according to serum 25(OH)D status stratified by BMI: NHANES III (1988-1994) with follow-up through 2011.

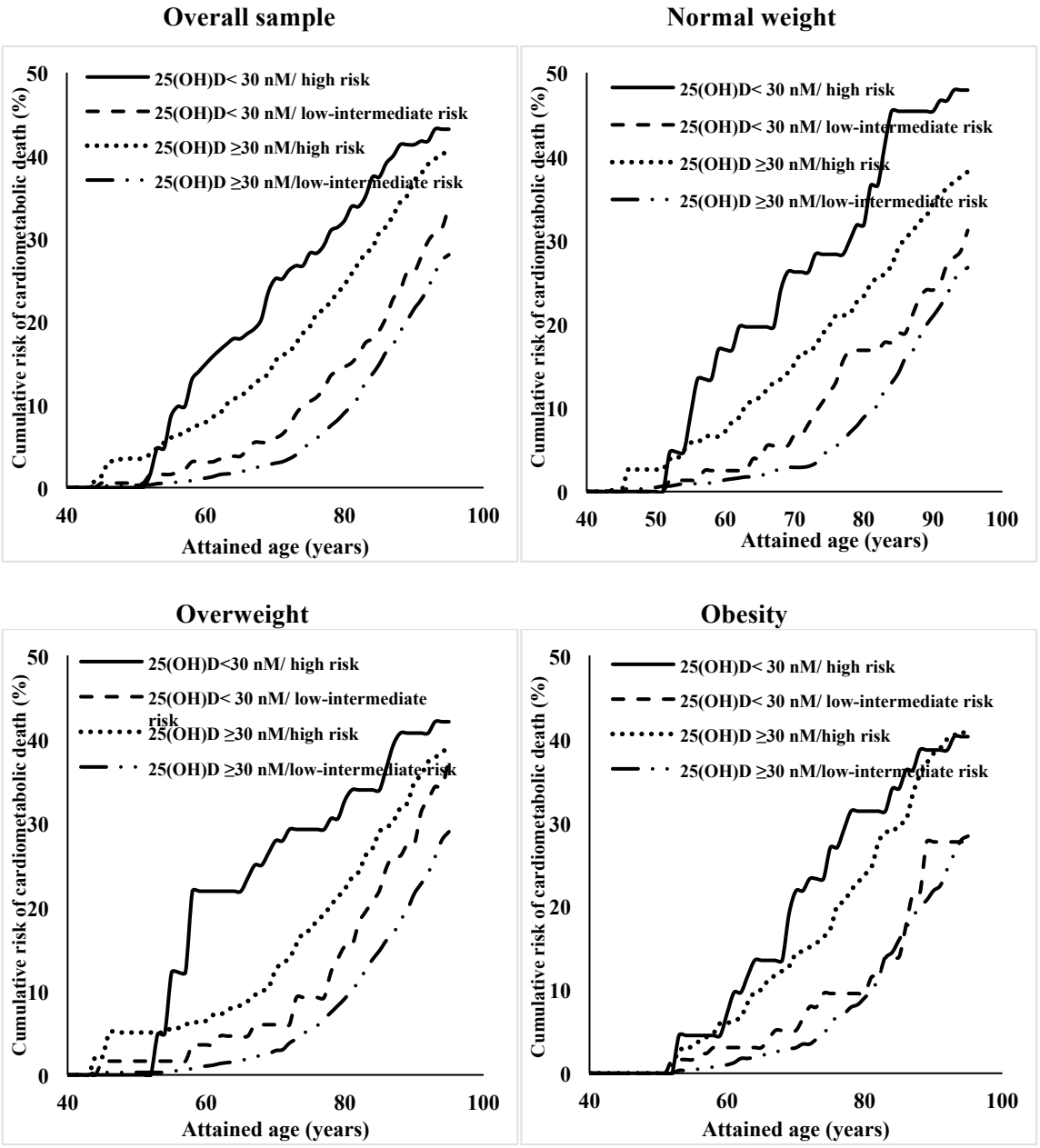

Figure 7. 3 Cumulative risk of cardiometabolic mortality adjusted for competing risk events according to risk burden and serum 25(OH)D status stratified by BMI category.

Chapter 7: Supplementary data

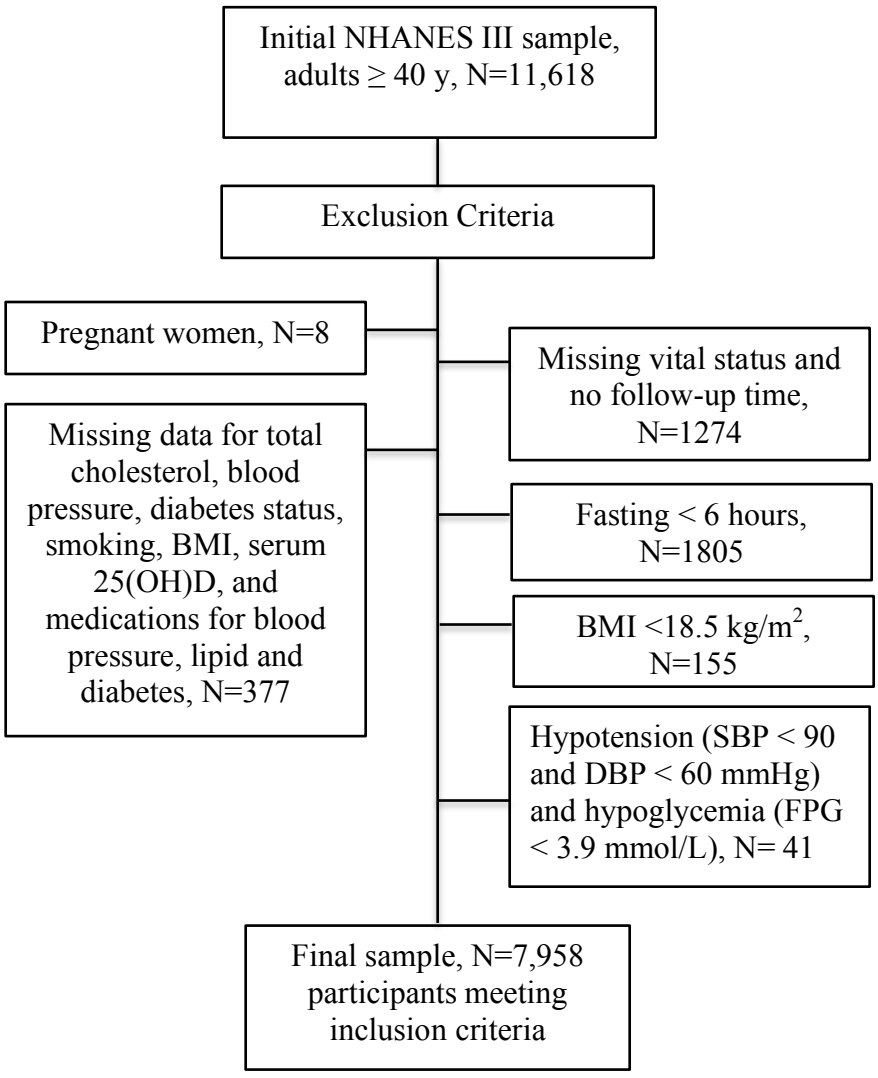

Figure 1: Flow chart showing exclusion criteria.

Sensitivity Analyses: excluding cases of CVD at baseline (n=6896)

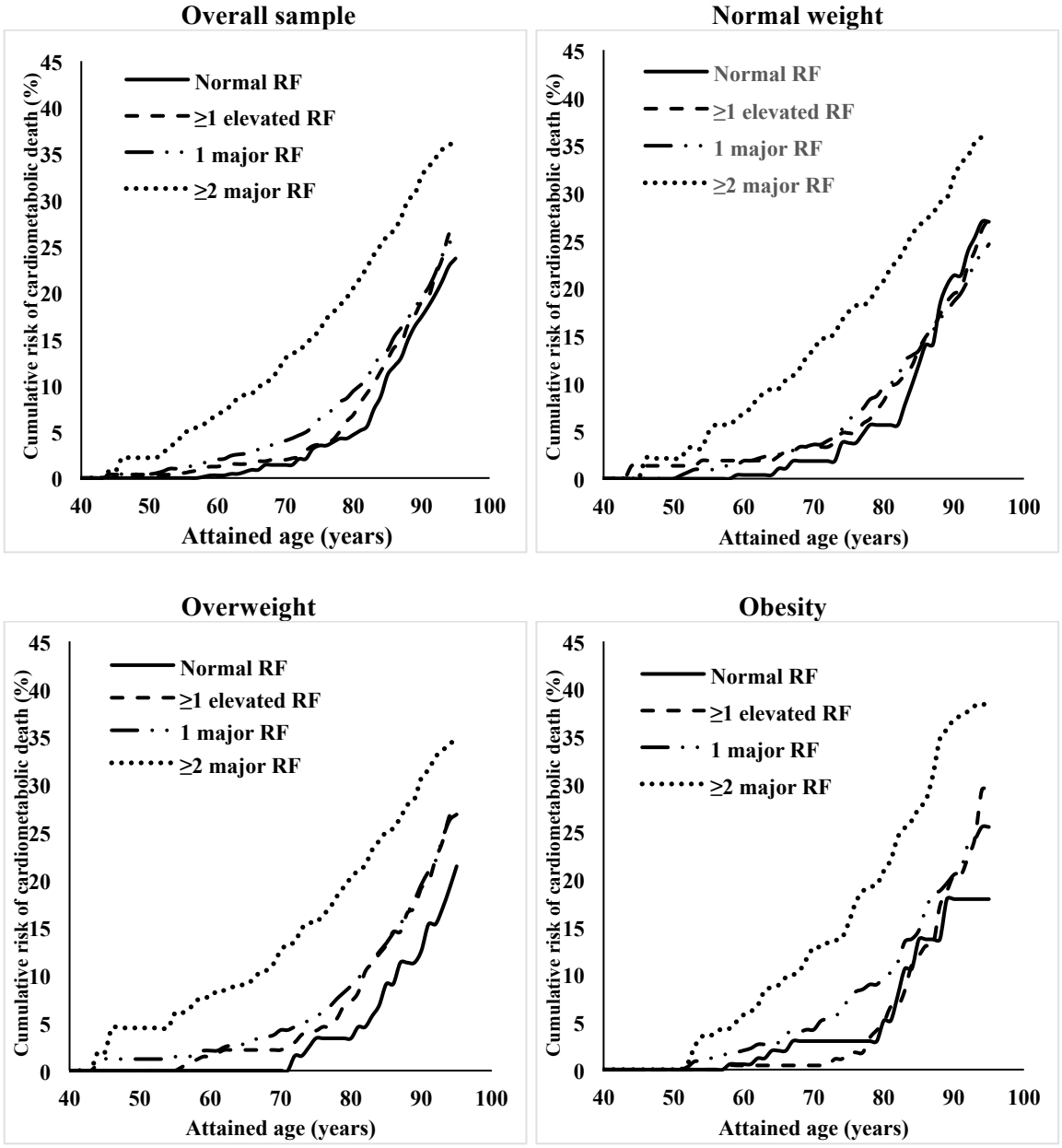

Figure 2: Cumulative incidence of cardiometabolic mortality adjusted for competing risk events according aggregate risk factor (RF) burden stratified by BMI: NHANES III (1988-1994) with follow-up through 2011. Excluded participants with history of CVD at baseline. Total n=6896.

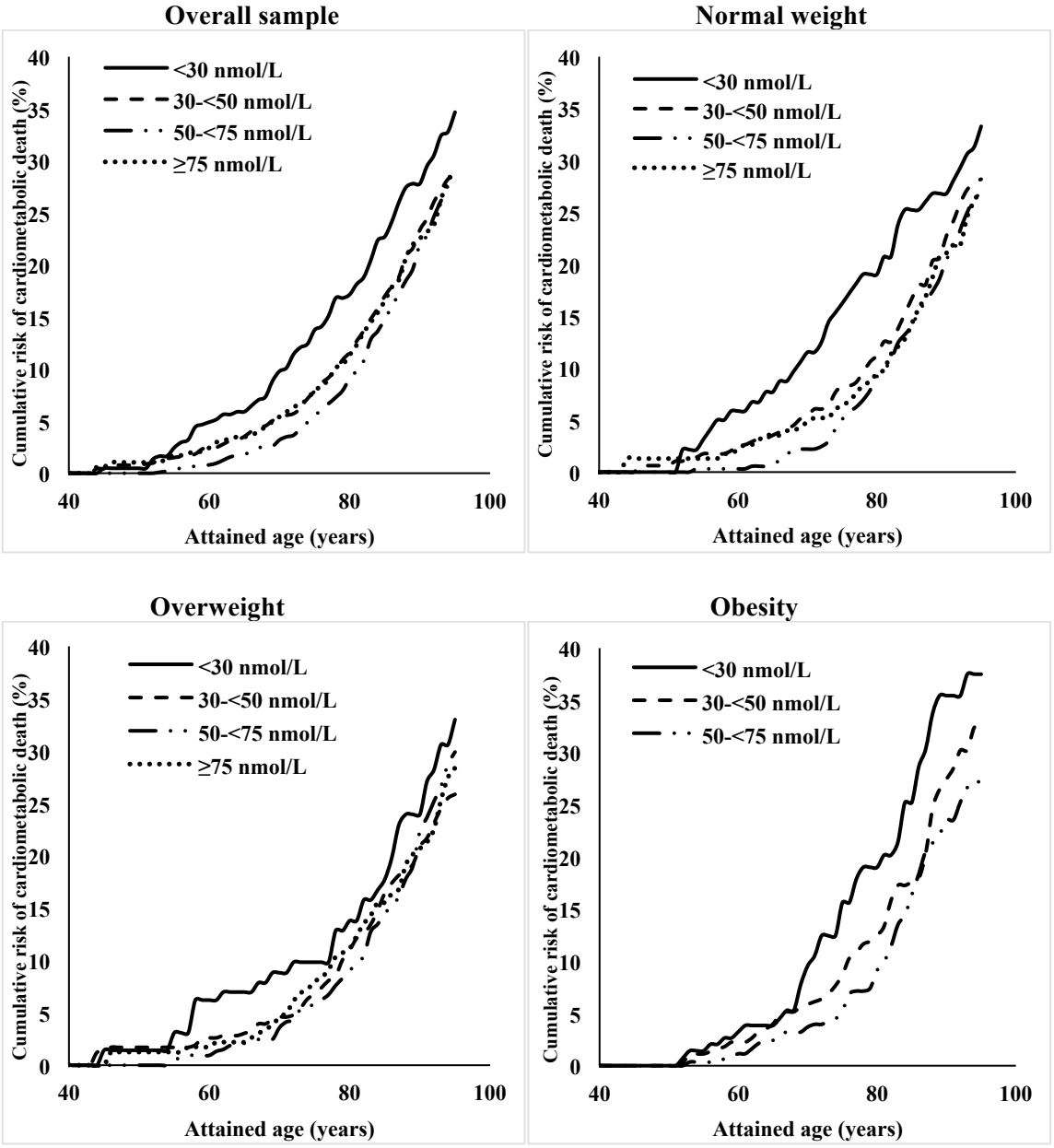

Figure 3: Cumulative incidence of cardiometabolic mortality adjusted for competing risk events according total 25(OH)D status stratified by BMI: NHANES III (1988-1994) with follow-up through 2011. Excluded participants with history of CVD at baseline. Total n=6896.

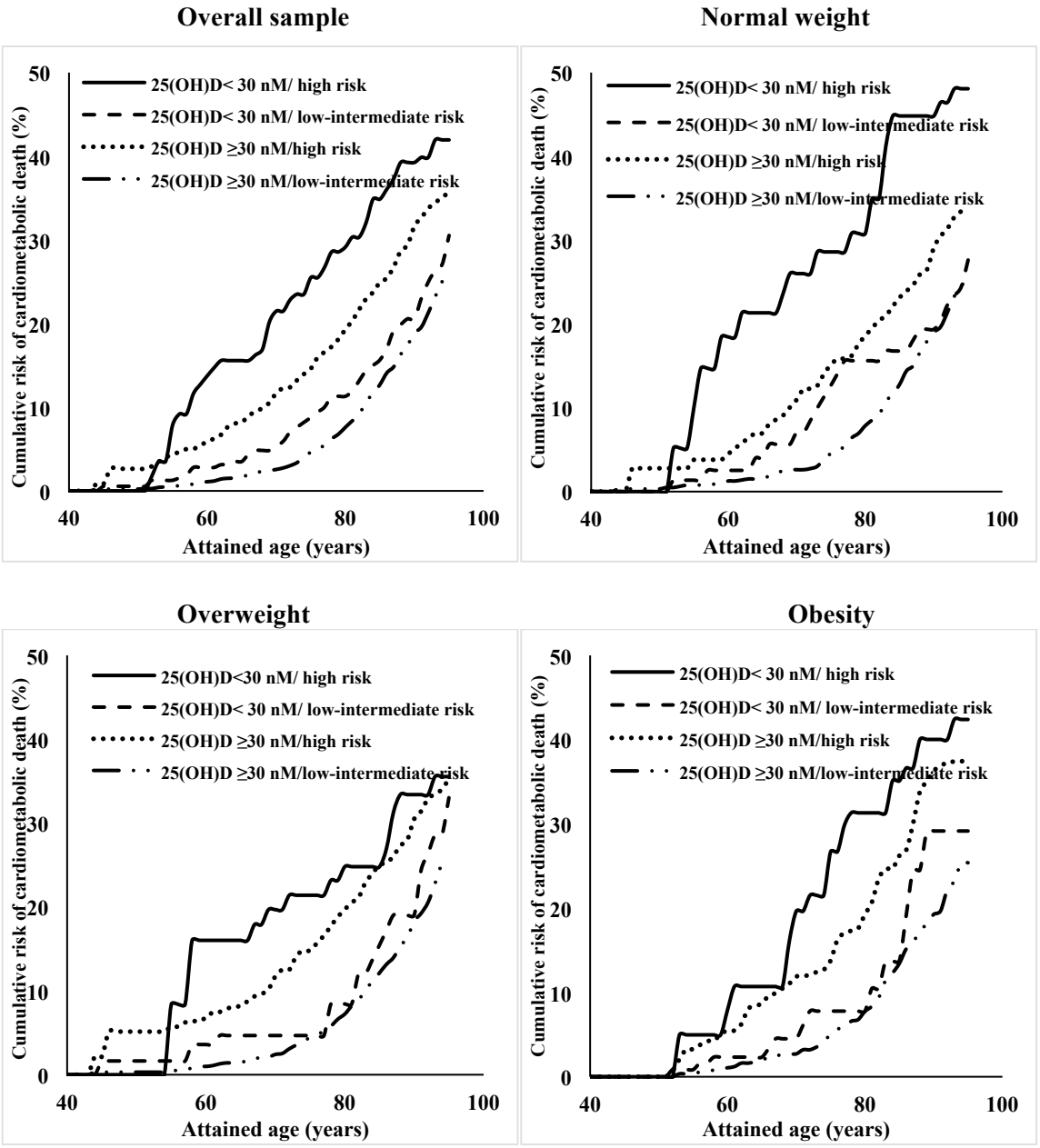

Figure 4: Cumulative risk of cardiometabolic mortality adjusted for competing risk events according to traditional risk burden and serum 25(OH)D status stratified by BMI category: NHANES III (1988-1994) with follow-up through 2011. Excluded participants with history of CVD at baseline. Total n=6896.

Chapter 8: Overall Discussion

8.1 Summary

Interest in the role of vitamin D in relation to cardiometabolic health outcomes has been rapidly growing. The identification of multiple physiological functions of vitamin D beyond its currently recognized role in bone health has brought considerable attention to the non-skeletal function of vitamin D. However, given the limitations of the existing evidence at the time of commencing this thesis work and the different interpretations for optimal vitamin D status, there were several research gaps that needed to be addressed. To date, the vitamin D guidelines by the IOM [2] and the ES [16] have led to substantial disagreement about what defines adequate levels of vitamin D status, as assessed by total 25(OH)D level. Notably, the current recommendations from the IOM and ES lack clarity in that i) unstandardized data for total 25(OH)D was used to assess the relationship between vitamin D status and health outcomes ii) there is no evidence that the currently recommended total 25(OH)D thresholds provide further discrimination of health risks beyond bone health, and iii) the current guidelines do not consider potential variation in health risk among adults from multi-ethnic subpopulations or individuals with overweight or obesity. Therefore, given the existing gaps in the literature, the overall objectives of this thesis were to examine the association between standardized total 25(OH)D and cardiometabolic health outcomes, and to further explore potential variation by ethnicity, metabolic health and BMI. In addition, this thesis also examined the utility of total 25(OH)D as a biomarker of effect by assessing the performance of total 25(OH)D as a screening biomarker for insulin resistance in asymptomatic adults and by evaluating the predictive ability of the recommended 25(OH)D thresholds by the IOM and the ES in relation to lifetime risk of cardiometabolic mortality.

8.1.1 Examination of the Cross-Sectional Association between Standardized Total 25(OH)D and Cardiometabolic risk

The objective for the first study of this thesis was to examine the cross-sectional association between standardized total 25(OH)D and cardiometabolic health outcomes (i.e., HOMA-IR, MetS, and Framingham 10-year CVD risk) in a nationally representative sample of U.S. adults (Chapter 4). Overall, results indicated significant negative associations between standardized

total 25(OH)D levels and HOMA-IR, MetS, and Framingham 10-year CVD risk, and these associations remained significant after adjustment for sociodemographics, season of blood draw, NHANES survey cycle, BMI, smoking status, vitamin D supplement use, physical activity and medications for dyslipidemia and diabetes. In the overall sample, the lowest risks for cardiometabolic health outcomes were observed among individuals in the highest total 25(OH)D quartile (≥ 74.3 nmol/L) compared to those in the lowest quartile (< 43.4 nmol/L). Our results are in line with previous epidemiological studies reporting negative association between total 25(OH)D and cardiometabolic health outcomes including insulin resistance and pre-diabetes [191,269,287], MetS [264,268], and CVD risk [265,288]. In our study, the negative association between standardized total 25(OH)D and cardiometabolic risk remained significant across all ethnic subgroups in NHANES (i.e., NH-whites, NH-blacks, and Mexican Americans), with the lowest risks observed among individuals in the highest 25(OH)D quartile (≥ 74.3 nmol/L) compared to those in the lowest quartile (< 43.4 nmol/L) across all ethnic subgroups. Previous studies investigating the association between vitamin D status and cardiometabolic risk across different ethnic subpopulations have reported ethnic differences in the association of total 25(OH)D with cardiometabolic health outcomes. Specifically, using unstandardized 25(OH)D data from NHANES III, Scragg et al. reported a negative association between total 25(OH)D and type-2 diabetes risk in Mexican-Americans and NH-whites, but not in NH-blacks [12]; this finding was consistent in NHANES 2001-2006 [201]. Using data from NHANES III, Michos et al. also observed a negative association between unstandardized 25(OH)D levels and fatal stroke in whites but not blacks [14]. In another study, Robinson-Cohen et al. reported a significant inverse association between serum 25(OH)D and risk of coronary heart disease in whites and Chinese participants, but not in blacks or Hispanic participants from the Multi-Ethnic Study of Atherosclerosis [13]. In relation to heart failure, Lutsey et al. observed that low serum 25(OH)D was significantly associated with incident heart failure among whites, but not among black participants from the Atherosclerosis Risk in Communities study [15]. In contrast to these studies, we did not find ethnic differences in the association between serum 25(OH)D and cardiometabolic risk using standardized total 25(OH)D data in NHANES 2001-2010 [320]. In relation to the observed ethnic differences between whites and blacks in the literature, it has been hypothesized that blacks exhibit a compensatory mechanism in response to their lower total 25(OH)D levels; specifically, it has been reported that blacks have lower levels of the circulating

vDBP and thus have similar concentrations of the free or bioavailable 25(OH)D relative to whites [15,292]. Therefore, according to this compensatory hypothesis, the lower concentrations of total 25(OH)D observed among blacks may not uniformly reflect a state of vitamin D deficiency. Moreover, it has been hypothesized that the free or bioavailable 25(OH)D may be a better marker of vitamin D status in blacks than measurement of total 25(OH)D levels [391]. However, using a reliable LC-MS/MS method, Henderson et al. [294] reported no significant differences in the circulating vDBP levels between blacks and whites. Hence, the results by Henderson et al. [294] do not support the compensatory hypothesis given that there are no significant differences in the circulating vDBP levels. In line with these results by Henderson et al. [294], the results from our first study also do not support the compensatory hypothesis among blacks given that low total 25(OH)D levels were significantly associated with increased cardiometabolic disease risk in NH-blacks. Use of a standardized data for total 25(OH)D levels likely reduced the misclassification of vitamin D deficiency/sufficiency subcategories in our study. Moreover, unstandardized measurement of 25(OH)D levels in previous studies may have led to null associations among black participants. In studies comparing performance of different 25(OH)D assays, a large variation in total 25(OH)D levels has been reported, where one-in-five to one-in-three participants are misclassified as 'deficient' using a commercial 25(OH)D assays, and the largest discrepancy between two different assays for a single sample has been reported to be 137 nmol/L (190 nmol/L compared to 53 nmol/L) [8,136]. Therefore, the adoption of standardized measurement of total 25(OH)D is important, especially in population-based surveys where multiple survey cycles are generally combined to assess the association between total 25(OH)D and cardiometabolic health outcomes. In summary, the results from our first study were valuable in that we reconfirmed the significant negative association between standardized total 25(OH)D levels and cardiometabolic risk, and our results also suggest that low total 25(OH)D is a risk marker of cardiometabolic risk across all ethnic subgroups, including NH-blacks.

8.1.2 Clinical Utility of Total 25(OH)D Status and Estimation of Optimal 25(OH)D Level

To date, inter-laboratory differences in the measurement of vitamin D status have impeded the development of reliable thresholds for total 25(OH)D level in relation to skeletal and non-skeletal health outcomes. Currently, there is no consensus on the recommended levels for total

25(OH)D in relation to bone health, and additionally, the level for a sufficient total 25(OH)D may differ for non-skeletal health outcomes [18]. Moreover, in their 2012 report, the IOM raised concerns over the utility of total 25(OH)D as a biomarker of effect (i.e., whether level of total 25(OH)D is causally related to and is a reliable predictor of the health outcome of interest) [2]. A large number of studies have evaluated the association between total 25(OH)D levels and cardiometabolic health outcomes, including insulin resistance, MetS, type-2 diabetes, and CVD; however, such associations are not necessarily causal or predictive of the health outcome of interest [2]. To date, the clinical utility of total 25(OH)D as a screening biomarker has not been evaluated. Therefore, using standardized total 25(OH)D data from NHANES (2001-2010), the primary objectives of the second study were to assess the clinical utility of measuring total 25(OH)D along with traditional risk factors in the diagnosis of insulin resistance and to estimate the optimal total 25(OH)D threshold associated with normal glucose and insulin homeostasis in asymptomatic, ethnically-diverse U.S. adults. Overall, this study indicated that measurement of total 25(OH)D along with established risk factors would provide a small incremental improvement in detection of insulin resistance in asymptomatic adults (i.e., an estimated 2 to 4 additional cases per 1,000 people) and the optimal 25(OH)D threshold was estimated to be at least 60 nmol/L. The optimal thresholds varied slightly by 1-5 nmol/L according to age categories (20-39 y, 55-58 nmol/L; 40-59y, 60 nmol/L ; ≥ 60 y, 61 nmol/L); however, the thresholds differed substantially by ethnicity (Mexican-Americans, 54 nmol/L; NH-whites, 68 nmol/L; NH-blacks, 41 nmol/L). To date, relatively few studies have evaluated the utility of total 25(OH)D in the context of CHD risk prediction [332], CVD mortality [333] and all-cause mortality in patients undergoing coronary catheterization [334].While these studies have evaluated the utility of total 25(OH)D in the context of CVD and mortality risk prediction among patients at intermediate-to-high risk, to our knowledge, no previous study has evaluated the utility of total 25(OH)D as a screening biomarker in asymptomatic adults; therefore, it is difficult to make direct comparison with previous studies. Hence, our study was novel in that we assessed the utility of serum 25(OH)D as a screening biomarker in asymptomatic individuals who were presumably at low-to-intermediate risk; however, due to the cross-sectional nature of our data, our results highlight the need for further research into the clinical utility of total 25(OH)D as a screening biomarker using a longitudinal cohort design. Moreover, the optimal level of 25(OH)D in our study was estimated to be ~ 60 nmol/L in the overall sample; therefore, our results

indicated that the optimal total 25(OH)D in relation to normal glucose and insulin homeostasis is slightly above the IOM's recommended threshold of 50 nmol/L for bone health [2]. Furthermore, we also assessed the performance of the recommended cutoffs by the IOM (i.e., 50 nmol/L) and the ES (i.e., 75 nmol/L) in terms of their sensitivity and specificity. Overall, application of the 50 nmol/L cutoff yielded a higher sensitivity, whereas the 75 nmol/L cutoff yielded a higher specificity across all age and ethnic subgroups. For a biomarker to be considered as a reliable screening tool, the following characteristics need to be fulfilled: high specificity, known reference limits, added value on traditional risk factors, and low cost [392]. Therefore, if total 25(OH)D level is intended to be used as a screening biomarker, then the ES cutoff of 75 nmol/L yields the highest specificity across all age and ethnic subpopulations (≥ 73 %), with the highest specificity observed among Mexican-Americans and NH-blacks (i.e., 95%) (see Table 3, Chapter 5). In contrast, the IOM's cutoff of 50 nmol/L yielded the highest sensitivity. However the sensitivity of the 50 nmol/L cutoff performed poorly among NH-blacks (31-35%) and Mexican-Americans (64%) (see Table 3, Chapter 5). Therefore, the observed consistency for the high specificity of the 75 nmol/L cutoff suggests that total 25(OH)D may prove to be a useful screening biomarker in high-risk individuals at risk of developing future type-2 diabetes and CVD. Future studies using standardized measurement of 25(OH)D levels are needed to validate our findings in other populations using a prospective study design.

8.1.3 Vitamin D Status, Metabolically Healthy Obesity and Mortality Risk

The third study in this thesis focused on the prognosis of the MHO phenotype in relation to all-cause and cardiometabolic mortality and whether vitamin D status (as assessed by total 25(OH)D) modified the association between MHO phenotype and mortality risk. Using a harmonized definition of MHO, defined as a BMI ≥ 30 kg/m^2 with zero metabolic abnormality [28], our results from the third study supported the hypothesis that MHO phenotype is a benign health condition in relation to all-cause and cardiometabolic mortality. Previous studies have used a variety of different criteria to define MHO (i.e., absence of MetS or having 1 or 2 MetS components), which have led to inconsistent results [95,97,99–103,349,350,352–354,393]. Given the existence of the substantial differences in the definition of MHO phenotype in the literature, our study was valuable in that we used a harmonized definition of MHO, as recently proposed by Ortega et al. [28]. Secondly, previous studies have reported inconsistent results due to the

different length of follow up (< 10 years vs. > 10 years); a meta-analysis by Kramer et al. [394] showed that MHO was associated with increased risk of all-cause mortality or CVD events only in studies with long-term follow-up (≥ 10 years). In our study, the median follow-up was 19.1 years and our results did not support the hypothesis proposed by Kramer et al [394]; therefore, given that previous studies have used variable follow-up duration, a median follow-up of 19.1 years should be considered another strength of our study. Moreover, in relation to cardiometabolic mortality, we further explored the prognosis of MHO phenotype using Fine and Gray regression to account for competing risk events. The cumulative incidence of cardiometabolic mortality among MHO individuals was similar to metabolically healthy normal-weight individuals, which further supports the hypothesis of benign health condition among MHO individuals. Therefore, our study was novel in that we accounted for competing risk events to assess the long-term prognosis of MHO phenotype in relation to cardiometabolic mortality. To our knowledge, previous studies assessing the long-term cardiometabolic/CVD prognosis of MHO phenotype have not accounted for competing risk events, and depending on the frequency of competing events, the cumulative incidence of CVD events may be overestimated using standard survival methods [366,374]. Therefore, future studies assessing the long-term prognosis of MHO phenotype should explore the potential bias introduced by standard survival techniques that do not account for competing events. In relation to the second objective of the third study, we observed a non-linear association between total 25(OH)D and all-cause and cardiometabolic mortality in the overall sample, with the minimum risk observed with values near 60-75 nmo/L. Studies evaluating vitamin D status in relation to mortality risk have also reported non-linear dose-response relationships [33] with some reporting a U-shaped or inverse J-shaped association [198,358]. In our study, we only observed a non-linear dose-response relationship, where the significant association between total 25(OH)D and mortality risk plateaued with values near 60-75 nmol/L. Moreover, we did not find a significant difference in the average total 25(OH)D levels between participants classified as MHO and non-MHO phenotypes; however, total 25(OH)D modified the association between metabolic health and cardiometabolic mortality risk among participants with BMI in the normal-weight and obesity range. Specifically, among metabolically healthy and unhealthy individuals within the normal-weight BMI range, we observed a significant linear dose-response relationship between total 25(OH)D and cardiometabolic mortality, where cardiometabolic mortality risk declined monotonically with

increasing total 25(OH)D levels with no evidence of a threshold effect. In contrast, we observed a significant non-linear dose response relationship among metabolically *unhealthy* participants within the obesity BMI range, where a threshold effect was observed for total 25(OH)D levels near 50-60 nmol/L. It is well established that individuals with obesity have lower levels of total serum 25(OH)D due to volumetric dilution (i.e., greater fat, muscle and liver mass). Therefore, the non-linear dose response relationship may reflect volumetric dilution in participants with BMI in the obesity range. Among MHO participants, we observed a non-significant inverse dose-response relationship between total 25(OH)D and cardiometabolic mortality. This non-significance could be due to the small sample size of MHO individuals in our study (n=367) and the respective low number of events (n=6). Therefore, we were unable to fully characterize the nature of the dose-response relationship for MHO participants; future studies with more MHO participants are needed to characterize the nature of this dose-response relationship. Further, among participants with BMI in the overweight range, total 25(OH)D did not modify the association between metabolic health and cardiometabolic risk (i.e., metabolically healthy and unhealthy participants with BMI in the overweight range were at low and high risk of cardiometabolic mortality, respectively, irrespective of total 25(OH)D levels). Whether this finding reflects a physiological difference in vitamin D metabolism in individuals with BMI in the overweight category or reflects a chance finding is not known; therefore, additional research is needed to investigate the potential differences in the dose-response relationship according to body weight. Further research into the nature of the dose-response relationship is important because of the potential application of this knowledge in the design of future RCTs of vitamin D supplementation (i.e., a non-linear dose-response relationship suggests a threshold effect and recruitment of participants should be based on this threshold effect to increase the statistical power of an RCT).

8.1.4 Vitamin D Status and Lifetime Risk of Cardiometabolic Mortality

The fourth study focused on the long-term predictive value of total 25(OH)D by assessing whether a single measurement of total 25(OH)D in middle- to old-aged adults is associated with lifetime risk of cardiometabolic mortality, while also accounting for competing risk events. Given that previous studies have primarily used relative risk measures to assess the association between total 25(OH)D and cardiometabolic disease risk, an estimation of the absolute risk

according to total 25(OH)D levels recommended by the IOM and ES would help to clarify the predictive value of these cutoffs. Therefore, the primary objective of the last study was to evaluate i) whether a single measurement of standardized total 25(OH)D in middle- to old-aged adults is associated with lifetime risk of cardiometabolic mortality, and ii) to evaluate the combined effect of total 25(OH)D and traditional CVD risk factors on the remaining lifetime risk of cardiometabolic mortality. We also explored variations in lifetime risk estimates across BMI categories. In a sample of 7,958 participants with 129,294 person-years of follow-up in NHANES III, the lifetime risk of cardiometabolic mortality was highest for participants with ≥ 2 major risk factors. Our results are consistent with previous studies reporting lifetime risk estimates according to traditional risk factor burden [32,395]; however, our study extends prior work by providing estimates for lifetime risks according to BMI status, and our results indicate that risk factor burden is a stronger predictor of long-term mortality risk than BMI status alone. This finding is also in line with our third study where we found that metabolic health is a more important driver of future disease risk than overweight/obesity alone [396]. With respect to total 25(OH)D levels, to our knowledge, our study is the first to estimate the lifetime risk of cardiometabolic mortality according to total 25(OH)D thresholds currently recommended by the IOM [2] and the ES [16]. We found that total 25(OH)D < 30 nmol/L was consistently associated with high lifetime risk of cardiometabolic mortality through 95 years of age (≥ 36%) and results were consistent across all BMI categories. Although we used an absolute risk model, our results are in line with a previous meta-analysis by Gaksch et al. [379] which used standardized total 25(OH)D data to assess the relative risk of CVD mortality over a median follow-up of 10.5 years; compared to participants with total 25(OH)D level of 75-< 100 nmol/L, the rate of CVD mortality was twice as high in participants with levels <30 nmol/L [379]. Therefore, taken together, our results along with the meta-analysis by Gaksch et al. [379] indicate that a single measurement of total 25(OH)D < 30 nmol/L in middle- to old-aged adults is a strong predictor of high lifetime risk of cardiometabolic mortality. Although serum 25(OH)D levels are known to fluctuate with season, it has been shown that a single measurement of total 25(OH)D is reflective of vitamin D status over 14 years, particularly for individuals with total 25(OH)D levels < 30 nmol/L [389]. Therefore, in our study, participants with total 25(OH)D < 30 nmol/L were unlikely to have had a substantial improvement in their total 25(OH)D levels. Moreover, although participants with total 25(OH)D ≥ 30 nmol/L consistently had lower lifetime risks

compared to those with levels < 30 nmol/L, the predictive value of total 25(OH)D levels of 30-< 50 nmol/L, 50-< 75 nmol/L and ≥ 75 nmol/L is less certain, as total 25(OH)D may fluctuate over time due to changes in lifestyle factors (i.e., sun exposure, diet, supplement use) and weight gain with advancing age. Furthermore, we also assessed the lifetime risk of cardiometabolic mortality according to a combination of traditional risk burden and total 25(OH)D. Among participants with high-risk burden (i.e., ≥ 2 major RFs) in the overall sample, total 25(OH)D ≥ 30 nmol/L was associated with lower lifetime risk (i.e., 10% and 3% absolute risk reduction through 70 and 95 years of age, respectively). Among participants with low-intermediate risk burden, total 25(OH)D ≥ 30 nmol/L was also associated with lower lifetime risk (i.e., 3% and 6% absolute risk reduction through 70 and 95 years of age, respectively). Similar trends were observed across all BMI categories. The effect modification by total 25(OH)D observed in this study is consistent with our third study where we found that total 25(OH)D modified the association between metabolic health and cardiometabolic mortality. In the third study, effect modification was dependent on BMI status where total 25(OH)D modified the association between metabolic health and cardiometabolic mortality in individuals with BMI in the normal-weight and obesity range. In contrast, in the fourth study, we found that total 25(OH)D modified the absolute risk estimates independent of BMI status, an effect that could be due to differences in relative versus absolute risk models. In the third study, we used a relative risk model (i.e., restricted cubic spline Cox proportional hazards model) to assess the dose-response relationship between baseline total 25(OH)D and cardiometabolic mortality risk. However, the restricted cubic spline Cox proportional hazards model cannot be extrapolated to assess the cumulative risk burden. Using the relative risk model, a reference group (i.e., those with total 25(OH)D equal to 50 nmol/L) must be chosen; however, baseline 25(OH)D levels may change over time and it is possible that participants with BMI in the overweight range are more susceptible to changes in total 25(OH) over time due to weight gain with advancing age. Therefore, repeat measurement of total 25(OH)D levels is needed to assess the nature of the dose-response relationship in participants with BMI in the overweight range. Although our results from the third and fourth study may seem inconsistent, use of relative versus absolute risk model most likely explains these discrepant findings. Our last study was novel in that it assessed the long-term predictive value of a single measurement of standardized total 25(OH)D on lifetime absolute risk of cardiometabolic mortality in middle- to old-aged adults. In summary, our results indicate that IOM's definition of

vitamin D deficiency (i.e., total 25(OH)D < 30 nmol/L) is a strong predictor of high lifetime risk of cardiometabolic mortality across all BMI categories, whereas the predictive value of vitamin D insufficiency and sufficiency as defined by the IOM and the ES is less certain, as total 25(OH)D levels ≥ 30 nmol/L may fluctuate over time. Therefore, our data emphasize the need for future clinical trials to target individuals with total 25(OH)D < 30 nmol/L, as these individuals have a high lifetime risk of cardiometabolic mortality and that their total 25(OH)D levels are unlikely to substantially improvement over the course of their lifespan (i.e.., from middle age to old age). Moreover, the effect of vitamin D deficiency (< 30 nmol/L) was particularly pronounced in individuals with ≥ 2 major traditional CVD risk factors. It is unknown whether vitamin D supplementation in individuals with ≥ 2 major traditional CVD risk factors would be beneficial. Therefore, future clinical trials are needed to assess whether vitamin D supplementation as an adjunct therapy in high-risk populations is a cost-effective strategy for the primary prevention of cardiometabolic mortality.

8.2 Strengths and Limitations

At the time of commencing this thesis project, there was a growing interest in defining the optimal vitamin D status in relation to multiple health outcomes; however, prior studies have primarily been limited by the unavailability of standardized data for total 25(OH)D levels. In accordance with the VDSP [138], total 25(OH)D data was standardized in NHANES to reduce assay variations in the measurement of total 25(OH)D levels. Therefore, a major strength of this thesis work is the application of standardized total 25(OH)D data from NHANES in the assessment of the association between total 25(OH)D and cardiometabolic health outcomes. The standardization of total 25(OH)D data allowed us to pool multiple cycles of NHANES data from 2001 to 2010, which substantially increased our sample size. Moreover, prior studies have predominantly included populations of White European origin, and in studies that have included ethnic minorities, ethnic-specific analyses were further constrained by the small sample sizes in underrepresented populations, such as U.S. blacks. Therefore, the large sample size obtained via pooling multiple survey cycles increased the precision of our study for stratification by ethnicity. Most importantly, standardization of total 25(OH)D allowed us to estimate the optimal 25(OH)D cutoff associated with normal insulin and glucose metabolism, and we were also able to further explore the potential variation in the optimal 25(OH)D levels by ethnicity. Another strength of

this thesis work is the long-term follow-up in study 3 (median follow-up of 19.1 years) and study 4 (median follow-up of 17.9 years). In the third study, given that prior studies had different length of follow-up (< 10 years versus > 10 years), having a median follow-up of 19.1 years is a strength of our study. Moreover, prior studies with long-term follow-up (> 10 years) have mainly used standard survival methods that did not account for competing risk events. Therefore, in the third study, we used a state of the art methodology to account for competing risk events to assess the long-term prognosis of MHO phenotype in relation to cardiometabolic mortality. Further, prior studies have used less strict criteria to define MHO phenotype; therefore, use of a standardized definition of MHO is another strength of our study. Lastly, previous studies assessing the prospective association of total 25(OH)D levels with cardiometabolic health outcomes also had different length of follow-up and used standard survival methods that did not account for competing deaths. Therefore, the lifetime risk model in the fourth study allowed us to assess the long-term predictive ability of standardized total 25(OH)D in relation to cardiometabolic mortality, while also accounting for competing deaths. To our knowledge, our study is the first to assess the lifetime risk of cardiometabolic mortality according to total 25(OH)D cutoffs currently endorsed by the IOM and the ES.

While this thesis had several strengths, there were several limitations as well. The first two studies in this thesis were cross-sectional, and as with any observational data, unmeasured confounding in our data precludes us from making any causal interpretation. Moreover, in the third and fourth study, although we excluded participants with a previous history of chronic disease to study the prospective association between total 25(OH)D and mortality risk, the presence of reverse causality cannot be eliminated. In the second study, given that the gold-standard measure of insulin resistance (i.e., hyperinsulinemic-euglycemic clamp) is time-consuming, expensive, and not practical for use in large epidemiological studies, we used HOMA-IR as a proxy measure for insulin resistance. While HOMA-IR has been shown to have a good correlation with the gold-standard method [346], it only measures hepatic insulin resistance. In addition, in study 2, while we internally validated our risk model (i.e., by randomly splitting the dataset into training and testing samples with ratio of 3:2, respectively), we were unable to externally validate the risk model using a different population dataset. Moreover, estimation of the optimal level of total 25(OH)D using endemic 25(OH)D data is limited to the

range of total 25(OH)D values in the sample; therefore, estimation of optimal level of 25(OH)D may differ in another population with a different distribution for total 25(OH)D values. In a separate study (unpublished), using data from the Canadian Health Measures Survey (CHMS), the optimal level of total 25(OH)D was similar to study 2 results using NHANES data (see Table 1 in appendix). However, as both NHANES and CHMS datasets represent the North American population, the applicability of these thresholds to other populations is not known. In the third and fourth study, we used BMI as a proxy measure of adiposity. The use of more accurate measures of fat mass (i.e., dual-energy x-ray absorptiometry (DEXA)) would have allowed a better assessment of body fat distribution. However, data from DEXA were not available in NHANES III. Furthermore, with respect to the primary exposure variables in the third and fourth study, measures for total 25(OH)D, BMI and metabolic health parameters (i.e., total cholesterol, triglycerides, HDL-cholesterol, blood pressure, fasting plasma glucose) were only available at baseline; therefore, we were unable to assess any changes in these variables over time. Changes in diet, sun exposure, physical activity, and other health conditions could affect these variables; therefore, repeated measures are required to adequately capture changes in these variables over time. Lastly, the Fine and Gray and the lifetime risk models used in the third and fourth study, respectively, did not incorporate NHANES survey weights. These models have not yet made their way to standard survey packages in SAS and incorporating survey weights would require extensive custom programming. Therefore, our risk estimates may not represent the general U.S. population. Moreover, it is difficult to estimate the frequency and distribution of competing risk events at the population level; therefore, our estimate of competing events may not be transferable to other populations with a different distribution of competing events. To reduce this limitation, obtaining a large sample size via pooling multiple observational studies would increase the robustness and generalizability of the lifetime risk estimates (i.e., this undertaking would be similar to the rationale of the lifetime risk pooling project [397]). Lastly, for the lifetime risk estimates in the fourth study, we were not able to statistically test the differences between lifetime risk estimates according to total 25(OH)D levels and traditional risk factor burden; doing so would also require custom programming of the Practical Incidence Estimator (PIE) macro by Beiser et al [382].

8.3 Implications & Future Directions

Our understanding of the role of vitamin D in human health has evolved substantially over the past decade; however, the role of vitamin D in relation to non-skeletal health outcomes remains an active area of research. Although RCTs of vitamin D supplementation are the gold-standard in establishing a causal relationship, high-quality observational studies will still continue to contribute to our understanding of the role of vitamin D in non-skeletal health outcomes [5]. This thesis utilized secondary data from NHANES to address existing research gaps regarding the association between vitamin D status, as measured by standardized total 25(OH)D concentration, and cardiometabolic health outcomes. In particular, we found that low total 25(OH)D levels were cross-sectionally associated with cardiometabolic disease risk irrespective of ethnic background. It is important to note that previous studies reporting ethnic differences in the association between total 25(OH)D and cardioemtabolic health outcomes have used unstandardized total 25(OH)D data, which has likely contributed to misclassification of individuals and resultant null findings [8]. Therefore, future prospective cohort studies with standardized total 25(OH)D data are needed to validate our findings in a large multi-ethnic cohort.

In recent years, there has also been much controversy over the optimal vitamin D status; however, the unavailability of standardized total 25(OH)D measure has impeded the development of reliable 25(OH)D thresholds. Therefore, using standardized data, the optimal total 25(OH)D level associated with normal glucose and insulin homeostasis was estimated to be 60 nmol/L; however, we also found that the optimal total 25(OH)D level may differ substantially by ethnicity. We also assessed the clinical utility of measuring total 25(OH)D along with traditional risk factors and found that addition of total 25(OH)D incrementally improved the detection of insulin resistance in asymptomatic adults. Moreover, we further assessed the sensitivity and specificity of the total 25(OH)D cutoffs currently endorsed by the IOM and the ES. We found that the 75 nmol/L cutoff endorsed by the ES consistently yielded a higher specificity, whereas the 50 nmol/L yielded a higher sensitivity; however, the 50 nmol/L cutoff performed poorly in NH-blacks and Mexican-Americans. In this context, a ‘rule-in’ strategy with high specificity may be more important to avoid misdiagnosis in asymptomatic adults [21]; therefore, the ≥ 75 nmol/L may be a useful threshold for screening insulin resistance in

asymptomatic adults. Due to the cross-sectional nature of our data, additional longitudinal studies are needed to validate our findings and determine whether the optimal 25(OH)D level varies substantially by ethnicity.

In the third study, we assessed whether MHO is associated with subsequent all-cause and cardiometabolic mortality risk, and whether total 25(OH)D modified these associations. We found that MHO was not associated with increased all-cause or cardiometabolic mortality. However, due to the single measurement of BMI and metabolic health parameters in our study, additional longitudinal studies are needed to track changes in BMI and metabolic health parameters over time to assess the transient nature of the MHO phenotype [24,398], and to further elucidate risk factors that are associated with this transition. Furthermore, in our study, a higher total 25(OH)D level was not significantly related to the MHO phenotype. However, we found that total 25(OH)D modified the association between metabolic health phenotype and cardiometabolic mortality, particularly in high-risk subgroups (i.e., metabolically unhealthy individuals within the normal-weight and obesity BMI ranges). Our results indicate that vitamin D deficiency exacerbates the risk of cardiometabolic mortality in high-risk individuals. Vitamin D supplementation in high-risk individuals may prove to be beneficial [254,375,376,399] and may improve the metabolic milieu of metabolically unhealthy phenotypes; however, to our knowledge, no clinical trial has been performed to investigate whether vitamin D supplementation can help individuals with metabolic dysfunction to transition into a metabolically healthy state. Future intervention studies are needed to answer this question.

In the fourth study, we estimated the absolute risk of cardiometabolic mortality according to traditional risk factor burden, total 25(OH)D, and the combination of these parameters. Specifically, we used the lifetime risk model to assess the long-term predictive value of total 25(OH)D cutoffs currently recommended by the IOM and the ES, and found that total 25(OH)D < 30 nmol/L was consistently associated with high lifetime risk of cardiometabolic mortality across all BMI categories. However, the predictive value of total 25(OH)D ≥ 30 nmol/L is less certain, as total 25(OH)D levels may fluctuate over time due to changes in lifestyle behaviors and weight gain due to advancing age. Therefore, our results indicate that while the IOM's definition of vitamin D deficiency was set in relation to bone health outcomes, the < 30 nmol/L

cutoff is also a strong predictor of high lifetime risk of cardiometabolic mortality. To date, most of the RCTs of vitamin D supplementation have not been targeted towards individuals with vitamin D deficiency (i.e., including individuals with sufficient vitamin D levels dilutes any beneficial effect of vitamin D supplementation and decreases the power of the study to detect statistically significant effects) [6]. Therefore, future RCTs should target individuals with total 25(OH)D levels < 30 nmo/L because these individuals are unlikely to have substantial improvement in their vitamin D levels from middle-age to old-age.

8.4 Summary of Findings in Relation to Global Cardiometabolic Risk Assessment

The overall objective of this thesis was to examine the role of vitamin D in relation to global cardiometabolic risk assessment. Specifically, we evaluated the performance of total 25(OH)D as a novel and emerging risk biomarker in the assessment of global cardiometabolic risk (see **Figure 2.1** in Chapter 2 for conceptual framework). The clinical utility of novel biomarkers as indicators of disease trait remains an active area of research and there is increasing interest in utility of novel biomarkers in the assessment of global cardiometabolic risk. Generally, the overall expectation of a novel biomarker is to improve the ability of a clinician to identify high-risk individuals, thereby enabling them to optimally manage a patient. If a biomarker is used as an indicator of a disease trait, it can either be classified as a *risk factor* (i.e., associated with a disease because it is in the causal pathway leading to the disease) or as a *risk marker* (i.e., associated with a disease but not necessarily in the causal pathway leading to the disease; it may be an indicator of the disease process) [21]. While the causal link between vitamin D and cardiometabolic health outcomes remains an active area of research [400–404], this dissertation showed that a low total 25(OH)D level is a strong risk marker of adverse cardiometabolic health outcomes (i.e., MetS, insulin resistance, CVD risk, and cardiometabolic mortality). When assessing the clinical utility of total 25(OH)D as a risk marker of insulin resistance in asymptomatic adults, addition of total 25(OH)D to traditional risk markers and factors marginally improved detection of insulin resistance in asymptomatic adults (i.e., addition of total 25(OH)D to established risk factors resulted in detection of 2 to 4 extra cases of insulin resistance per 1,000 people screened). We also estimated the optimal level of total 25(OH)D associated with normal glucose and insulin homeostasis; the optimal level was estimated to be at least 60 nmol/L. Further, we assessed the performance of total 25(OH)D cutoffs currently

recommended by the IOM and the ES. We found that the ES cutoff of ≥ 75 nmol/L consistently yielded a higher specificity across all age and ethnic subgroups; therefore, the ≥ 75 nmol/L cutoff may be a useful threshold for screening insulin resistance in asymptomatic adults. On the other hand, the assessment of the long-term predictive value of total 25(OH)D showed that the IOM's cutoff of < 30 nmol/L was consistently associated with high lifetime risk of cardiometabolic mortality. Taken together, results from study 2 and study 4 showed that total 25(OH)D values below 60-75 nmol/L are indicative of suboptimal vitamin D status in relation to glucose and insulin homeostasis, and if total 25(OH)D levels continue to decline over time then total 25(OH)D < 30 nmol/L is a strong risk marker of cardiometabolic mortality. Therefore, while the recommended thresholds by the IOM and the ES were set in relation to bone health outcomes (i.e., bone mineral density, osteoporosis in adults and rickets in children), the demonstrated applicability of these thresholds to cardiometabolic health outcomes in this thesis suggests that the cutoffs for total 25(OH)D levels in relation to cardiometabolic health outcomes are within the range of recommended cutoffs for bone health outcomes.

Due the observational nature of this thesis work, we were unable to assess whether vitamin D deficiency is also a risk factor for adverse cardiometabolic health outcomes. However, based on the evidence to date, evidence on the role of vitamin D in relation to cardiometabolic health outcomes satisfies eight of the Hill criteria for causality (i.e., strength of association, consistency, specificity, temporality, biological gradient, plausibility, coherence, and analogy) [388]. The only criterion for causality between vitamin D and cardiometabolic health that has not been satisfied is experimental evidence [388]. Vitamin D deficiency is known to be causally related poor bone health outcomes [2] and if the results from ongoing large-scale clinical trials [400–404] show that vitamin D deficiency is also causally linked to adverse cardiometabolic health outcomes, then vitamin D supplementation may be an effective treatment strategy for both chronic conditions (i.e., osteoporosis and cardiometabolic disease) given that these conditions share similar risk factors. Moreover, if the causal link between vitamin D and adverse cardiometabolic health outcomes is established, then monitoring total 25(OH)D levels along with traditional risk factors may be useful for identification of high-risk individuals who are likely to benefit the most from vitamin D supplementation. In the case that RCTs fail to show a causal link between vitamin D and cardiometabolic health outcomes, then vitamin D deficiency only

serves as an indicator of the disease process itself (i.e., risk marker). However, given the bidirectional communication between the vasculature and bone [405–407], the role of vitamin D in decreasing the risk of adverse cardiometabolic health may be via its mediating effects on bone health [408]. Emerging evidence suggests that poor bone health is a significant risk factor for cardiovascular disease [407]; therefore, future studies are needed to disentangle the potential mechanisms that link poor bone health to adverse cardiometabolic health outcomes, and whether vitamin D mediates this relationship.

8.5 Conclusion

The results of this dissertation highlight the importance of using standardized measurement of total 25(OH)D levels, particularly in population-level health surveys where multiple survey cycles are generally combined to study the association between total 25(OH)D and cardiometabolic health outcomes. In this dissertation, it was found that low vitamin D status, as assessed by standardized total 25(OH)D levels, was cross-sectionally related to insulin resistance, MetS, and high CVD risk, irrespective of ethnic background. The optimal total 25(OH)D threshold in relation to normal glucose metabolism was estimated to be at least 60 nmol/L. This dissertation also assessed the clinical utility of standardized total 25(OH)D level as a screening biomarker for insulin resistance and found that measurement of total 25(OH)D along with traditional risk factors only marginally improved the detection of insulin resistance in asymptomatic adults. In addition, this dissertation also assessed the long-term prognosis of the MHO phenotype and found that MHO phenotype is not associated with increased all-cause or cardiometabolic mortality. While a higher total 25(OH)D level did not characterize the underlying healthy metabolic profile of the MHO phenotype, low vitamin D status exacerbated the risk of cardiometabolic mortality in metabolically unhealthy participants with BMI in the normal-weight and obesity ranges. Lastly, this dissertation assessed the long-term predictive value of standardized total 25(OH)D and found that total 25(OH)D < 30 nmol/L was a strong predictor of high lifetime risk for cardiometabolic mortality. Taken together, these findings suggest that a low total 25(OH)D level is a strong risk marker of adverse cardiometabolic health outcomes. Results from several ongoing RCTs of vitamin D supplementation will establish whether vitamin D deficiency is a causal risk factor for the development of cardiometabolic diseases [400–404].

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Appendix A: Serum 25(OH)D thresholds in relation to normal glucose and insulin homeostasis, Canadian Health Measures Survey

Table 1: Serum 25(OH)D thresholds overall and by age and race/ethnic background, Canadian Health Measures Survey (cycles 1 & 2 combined)

	Serum 25(OH)D (nmol/L)	AUC (95% C.I.)	Sensitivity (%)	Specificity (%)
Men (N=1457)				
Overall	60.7	0.57 (0.54-0.60)	55	56
20-39 yrs	59.2	0.57 (0.51-0.63)	65	50
40-59 yrs	60.5	0.61 (0.55-0.66)	58	56
≥ 60 yrs	72	0.57 (0.52-0.63)	66	48
Whites	66.5	0.58 (0.54-0.62)	63	50
Non-Whites	48.2	0.54 (0.45-0.63)	54	57
Women (N=1634)				
Overall	63.9	0.59 (0.55-0.62)	56	56
20-39 yrs	62.8	0.60 (0.54-0.65)	60	55
40-59 yrs	62.9	0.61 (0.56-0.66)	61	57
≥ 60 yrs	67.6	0.58 (0.53-0.64)	54	61
Whites	67.1	0.60 (0.57-0.64)	60	55
Non-Whites	n/a*	0.47 (0.39-0.55)	n/a	n/a

Abbreviations: AUC, Area Under Curve.

Sensitivity, proportion of true positive cases [true positive/(true positive + false negative)].

Specificity, proportion of true negative cases [true negative/(true negative + false positive)].

*If AUC <0.50, cutoffs cannot be estimated.