MATERIALS AND METHODS

Study design and participants

The NHS is an ongoing prospective cohort study of 121,700 female registered nurses aged 30 to 55 years who were enrolled in 1976 and have been continuously followed through biennial questionnaires. Two blood collections, 10 years apart, have been completed in the NHS. In 1989-1990, all NHS participants who had completed the 1988 questionnaire and agreed to provide blood samples were eligible for the first blood collection, and a total of 32,826 women (aged 43-69 years) provided blood samples. In 2000-2001, 18,717 women (aged 53-79 years) who participated in the first collection provided a second blood sample. The samples were sent via an overnight courier, and the majority (95%) of the samples arrived within 24 hours of phlebotomy. Upon arrival, samples were centrifuged and aliquoted into cryotubes as plasma, buffy coat, and erythrocyte fractions, which were then stored in liquid nitrogen freezers at –130 ºC or colder until analysis.

Nested case-control study design

Participants who provided blood samples and who were free of diagnosed cancer and cardiovascular diseases at the time of blood draw were eligible for the current nested case-control study. We prospectively identified incident CHD cases and selected 1~2 controls for each case using risk-set sampling from participants who remained free of CHD events at the time of case diagnosis through the end of follow-up (1990-1998 for the first blood collection and 2000-2010 for the second blood collection). Cases and controls were matched on age at blood draw (±1 year), smoking status (never, past, and current), fasting status at blood draw (fasting for 10 hours or not), and time of blood draw. After excluding participants with missing plasma biomarker values, the analysis of 25OHD and PTH included a total of 382 cases of nonfatal myocardial infarction (MI) and fatal CHD, 575 controls; the analysis of VDBP included 396 cases and 398 controls.

The study protocol was approved by the institutional review board of Brigham and Women's Hospital and the Human Subjects Committee Review Board of Harvard T.H. Chan School of Public Health. Informed consent was obtained from the study participants.

Ascertainment of CHD

On the baseline and all biennial follow-up questionnaires, participants were queried about the occurrence of physician-diagnosed CHD events and other diseases. Permission to access medical records of those who reported having a nonfatal MI was requested. Exposure-blinded study physicians reviewed all medical records and confirmed the self-reports of nonfatal MI using the World Health Organization criteria, which require typical symptoms plus either electrocardiographic abnormality or elevated cardiac enzyme levels[1]. Deaths were identified by reports from next of kin, postal authorities, or by searching the National Death Index. Fatal CHD was identified if CHD was listed as the cause of death on death certificates, hospital records, or autopsy reports, and these cases were confirmed if there was a previous report of CHD and no other more

plausible cause of death. Unconfirmed CHD deaths were excluded from the present study. Total CHD was defined as nonfatal MI and fatal CHD cases.

Measurement of plasma levels of 25OHD, VDBP, PTH, and other biomarkers

Circulating 25OHD and PTH levels were measured at two time points in 2011 and 2015, and VDBP levels were entirely measured in 2015. Samples of the case-control pairs were shipped in the same batch and analyzed in the same run. Within each batch, samples of each pair were assayed by the same technician in a random sequence under identical conditions to minimize systematic biases. Plasma levels of 25OHD were assayed by using an enzyme immunoassay from Immunodiagnostic Systems Inc. (Fountain Hills, AZ). VDBP was measured by enzyme-linked monoclonal antibody assay from R & D Systems (Minneapolis, MN). Intact PTH was measured by electrochemiluminescence immunoassay on the Roche E Modular system (Roche Diagnostics, Indianapolis, IN). Albumin was measured by a colorimetric assay (Roche Diagnostics, Indianapolis, IN). Plasma levels of total cholesterol, high-density lipoprotein (HDL) cholesterol, and triglycerides were measured with a Hitachi 911 analyzer using reagents and calibrators from Roche Diagnostics (Indianapolis, IN). Low-density lipoprotein (LDL) cholesterol levels were determined using a homogeneous direct method from Genzyme (Cambridge, MA). High-sensitivity C-reactive protein was measured using a latex-enhanced immunoturbidimetric assay from Denka Seiken (Tokyo, Japan) on a Hitachi 911 system. Creatinine was measured by a modified Jaffe method. Hemoglobin A1c was measured by turbidimetric immunoinhibition using packed red cells (Roche Diagnostics).

Coefficients of variation (CVs) of the assays were assessed by repeatedly analyzing quality-control samples. The average intra-assay CV was 3.2% for 25OHD, 6.7% for VDBP, and 3.4% for PTH. The CVs were generally <10% for other biomarker assays.

Calculation of bioavailable 25OHD

Bioavailable 25OHD is defined as 25OHD that is either bound to albumin or free (i.e., not bound to VDBP). We used validated equations to determine bioavailable 25OHD[2], with previously estimated binding affinity constants for 25OHD with VDBP and albumin[3]. It was demonstrated that the calculated levels of bioavailable 25OHD were highly correlated with levels directly measured by centrifugal ultrafiltration[3]. Details on the calculation of bioavailable 25OHD levels are presented in **Supplemental material**.

Assessment of covariates

Information on the occurrence of major chronic diseases, medical history, lifestyle, body weight and height, family history of MI, menopausal status, and postmenopausal hormone use was collected at baseline and updated biennially using follow-up questionnaires. Body mass index (BMI) was calculated as weight in kilograms divided by the square of height in meters (kg/m^2). Physical activity was assessed as metabolic equivalents per week (MET-hr/wk) using the duration of moderate or vigorous forms of exercise multiplied by the intensity of the activity[4]. Usual dietary habits were assessed by means of validated semiquantitative food-frequency questionnaires every 2-4 years, which inquired

about usual intake over the previous year. The Alternative Healthy Eating Index (AHEI) score was calculated to measure the overall diet quality[5].
Covariates assessed at the time of blood draw (1990 for the first collection and 2000 for the second collection) were used in the current analysis.

Statistical analysis

Owing to batch-to-batch variation over time, we recalibrated 25OHD and PTH levels from all labs to have a comparable distribution to an average batch according to Rosner et al.'s method[6]. In brief, we assumed that all batches combined represented an average batch. We then regressed levels of 25OHD or PTH on predictors including case-control status, age at blood draw and postmenopausal status and hormone use, as well as indicator variables for each batch. Within each batch, 25OHD and PTH levels were recalibrated by adding the resulting value of the coefficients for that batch minus the average of the batch coefficients. Therefore, these recalibrated levels accounted for the variability between batches independent of varying distributions of predictors between batches.

Spearman partial correlation coefficients were used to evaluate the interrelations between biomarkers of interest including total and bioavailable 25OHD, VDBP, and PTH among controls. The study population was categorized into quartiles based on the distributions of biomarkers among controls to assess their associations with CHD risk. In the current analysis we presented results from unconditional logistic regression because it preserved more power especially for testing interactions[7] and conditional logistic regression yielded largely similar results. We adjusted for matching factors in the crude model. In the multivariate model, we further adjusted for BMI, physical activity, alcohol intake, postmenopausal status and hormone use, aspirin use, parental history of MI before age 65 years, and AHEI score. We further evaluated factors that could be potential confounders or mediators of the effects of vitamin D-related biomarkers, including history of hypertension or diabetes, HDL cholesterol, LDL cholesterol, C-reactive protein, and creatinine. To evaluate the independence of the biomarkers, we mutually adjusted for the other biomarkers of interest as well. Linear trend was assessed by assigning participants the median value in their quartiles and evaluating this as a continuous variable. We also evaluated the linear relationship by modeling the associations for each standard deviation (SD) increment of biomarker levels. We created joint categories of levels of these biomarkers to examine the joint effects. Potential effect modification was assessed by multiplicative interaction terms, with statistical significance determined by Wald's test.

All P values were 2-sided, and 95% confidence intervals (CIs) were calculated for odds ratios (ORs). Data were analyzed with the Statistical Analysis Systems software package, version 9.1 (SAS Institute, Inc., Cary, NC, US).

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