Materials and Methods

Study population

The HPFS is a prospective cohort study of 51,529 U.S. male health professionals aged 40 to 75 years at initial enrollment in 1986. Since 1986, lifestyle information, medical history, and disease diagnosis have been updated every two years by validated questionnaires.[1] Upon request, a total of 18,159 men provided blood samples during the period of 1993-1994, which was our study baseline. The details of the HPFS cohort data collection have been described elsewhere.[1-3] Of the men providing blood samples, 1000 participants who had a confirmed diagnosis of diabetes at study baseline (1986) or during follow up through 1998 were selected and comprised the study population for the present analysis.[3,4] After excluding participants (n=36) with type 1 diabetes at baseline or participants (n=14) without data for FABP4, RBP4, or HMW adiponectin, 950 diabetic men were included in our final analysis. We prospectively followed these individuals and documented deaths through 2015. The study was approved by the institutional review boards at Brigham and Women's Hospital and Harvard T.H. Chan School of Public Health, and all participants provided written informed consent.

Confirmation of T2D diagnosis

A validated supplementary questionnaire regarding symptoms, diagnostic tests, and hypoglycemic therapy was mailed to participants who reported having diabetes on any biennial questionnaire. Prior to the release of the American Diabetes Association criteria in 1997, the National Diabetes Data Group criteria were used to diagnose diabetes: (1) unequivocal elevation of glucose levels (fasting levels ≥7.8 mmol/l, blood glucose ≥11.1 mmol/l during an oral glucose tolerance testing, or random blood glucose ≥11.1 mmol/l) together with one or more diabetes-related symptoms (weight loss, excessive thirst, polyuria, or hunger); (2) elevated glucose levels on more than one occasion in the absence of symptoms; or (3) treatment with anti-diabetic drugs (insulin or oral hypoglycemic medication).[5] For participants with T2D identified after 1998, the cutoff point used for fasting plasma glucose concentrations was lowered to 7.0 mmol/l according to the American Diabetes Association criteria.[6] In a previous HPFS validation study, 57 of 59 (97%) questionnaire-confirmed self-reported T2D cases were re-confirmed by the review of medical records.[7]

Ascertainment of cause of death

Death was reported by the next of kin or the postal authorities or by searching the National Death Index. In a previous validation study, we demonstrated that at least 98% of deaths were identified using these methods.[8] Deaths due to CVD were defined as International Classification of Diseases (ICD)-8 codes of 390-458 or 795. Fatal CVD were identified if these conditions were listed as the cause of death in autopsy reports, hospital records, or death certificates. Fatal CVD was then confirmed by reviewing hospital records or autopsy reports if CVD was listed as the underlying cause of death and if evidence of previous CVD was available from medical records. Using similar protocol, we also confirmed deaths due to cancer (ICD-8 codes 140-207) and other causes occurred in HPFS.

Sample collection and laboratory measurements

Interested HPFS participants were sent a blood collection kit containing instructions and the necessary supplies. The participants made arrangements for the blood to be drawn and then sent the samples back by overnight courier. The vast majority (97%) of samples arrived in our laboratory within 24 hours of blood draw. Upon arrival, whole blood samples were centrifuged and aliquoted into cryotubes as plasma, buffy coat, and red blood cells. The cryotubes were stored in the vapor phase of liquid nitrogen freezers with a temperature ≤ -130°C. We also requested information on the date and the time of the blood sample draw, and the time elapsed since the preceding meal to define fasting status (<8 versus ≥8 hours). Plasma FABP4 levels were measured using an ELISA kit from Caymen Chemical (Ann Arbor, MI). The average intra-assay coefficient of variation (CV) was 6.7%. Plasma RBP4 was measured by an ELISA assay from R & D Systems (Minneapolis, MN). The average intra-assay CV was 6.0%. Plasma concentration of HMW adiponectin was measured using ELISA kits from ALPCO Diagnostics Inc. (Salem, NH). The average intra-assay CV was 8.3%. In the current study, we also tested the potential impact of sample collection conditions on the stability of these adipokines. We collected blood samples from local volunteers and then made three splits for each sample: one split was immediately processed and put in a freezer for storage; another split was kept chilled as whole blood with a cool pack and processed 24 hours after collection; the third split was kept chilled and processed 48 hours after collection. Interclass correlation coefficients (ICCs) of biomarkers between these split samples (0-24 hours, 0-48 hours, and 24-48 hours) were

calculated. The levels of the adipokines were all stable with an ICC of 0.94 observed for FABP4, 0.92 for RBP4, and 0.94 for HMW adiponectin. In addition, pilot studies conducted among HPFS participants have demonstrated very good reproducibility for levels of total and HMW adiponectin and FABP4 in blood samples collected one year apart: the ICCs were 0.85, 0.91, and 0.89, respectively.[9,10] Another study also showed good reproducibility of RBP4 measurements over a 4-month period (ICC 0.77).[11] In additon, high-sensitivity C-reactive protein (hsCRP), hemoglobin A1C (HbA1c), triacylglycerol (TG), total cholesterol (TC), high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol, and plasma creatinine levels were also measured. The methodology of these assays was described elsewhere.[12,13]

Assessment of covariates

In the HPFS questionnaires, we obtained information on body weight, physical activity, cigarette smoking, alcohol consumption, medical history (including use of aspirin and cholesterol-lowering medication), family history of MI, history of hypertension, hypercholesterolemia, angina, CABG, MI, stroke, and diabetes. Body mass index (BMI) was calculated as weight in kilograms divided by the square of height in meters (kg/m^2). Dietary information was obtained using a validated semi-quantitative food frequency questionnaire (FFQ) every four years. The Alternate Healthy Eating Index (aHEI) was calculated as an overall measure of diet quality using the FFQ data.[14] Diabetes duration was defined as the period from the diagnosis of T2D until blood draw. Estimated glomerular filtration rate (eGFR) was derived from the 4-variable Modification of Diet equation.[15] In our study, blood samples were provided by most participants in 1993-1994. Therefore, demographics, lifestyle, medical history, and other variables estimated by 1992 follow-up questionnaire were used as baseline covariates.

Single nucleotide polymorphism (SNP) selection in Mendelian randomization analysis

Based on findings from previous genome-wide association studies or analyses using candidate gene approach, we constructed unweighted genetic risk scores by summing up the number of risk alleles (coding as 0, 1, 2) and used the scores as instrumental variables. For instrumental variable analysis (n=781), we selected 19 SNPs for FABP4 (rs16909187, rs16909192, rs10808846, rs7018409, rs2290201, rs6992708, rs3824088, rs7835371, rs1054135, rs2305319, rs8192688, rs2290201, rs2200477, rs1843560, rs7017115, rs1486004, rs10808846, rs10958025,

rs7018409),17-19 7 SNPs for RBP4 (rs10882272, rs108822723, rs17108993, rs3758538, rs3758539, rs34571439, and rs7091052),[16-18] 7 SNPs for RBP4 (rs10882272, rs108822723, rs17108993, rs3758538, rs3758539, rs34571439, and rs7091052),[19-22] and 21 SNPs for HMW adiponectin (rs3774261, rs1648707, rs1342387, rs12733285, rs822354, rs1426810, rs266717, rs6810075, rs16861194, rs17300539, rs266729, rs822394, rs17366568, rs1501299, rs17366743, rs6773957, rs1063538, rs1063539, rs6444175, rs7615090, rs2241766).[23-27] After applying filtering criteria of linkage disequilibrium (LD) correlation >0.8 and minor allele frequency ≥5%, we finally included 7 SNPs for FABP4 (rs3824088_G, rs7835371_A, rs1054135_T, rs6992708_C, rs8192688_A, rs1843560_C, rs10808846_T), 3 SNPs for RBP4 (rs10882272_C, rs17108993_G, and rs3758538_G), and 19 SNPs for HMW adiponectin (rs1342387_T, rs12733285_T, rs822354_A, rs1426810_G, rs266717_T, rs6810075_C, rs16861194_G, rs17300539_A, rs266729_G, rs822394_A, rs17366568_A, rs1501299_T, rs3774262_A, rs17366743_C, rs6773957_A, rs1063538_T, rs1063539_C, rs6444175_A, rs7615090_G) in the Mendelian randomization analysis.

Statistical methods

The comparisons between patients who died of CVD and the remaining patients were examined by using the Student's *t* test for normally distributed variables, the Wilcoxon rank-sum test for skewed variables, or the *Chi*-Square test for categorical variables. To explore the relationship among FABP4, RBP4, HMW adiponectin, and other CVD risk factors, we calculated Spearman partial correlation coefficients (r_s) and adjusted for age at blood draw, BMI at age 21, physical activity, smoking status (never smoked, <10 pack-years, 10-24 pack-years, 25-44 pack-years, 45-64 pack-years, 65+ pack-years, missing), alcohol consumption (non-drinker, <5.0 g/day, 5.0-9.9 g/day, 10.0-14.9 g/day, 15.0-29.9 g/day, ≥30.0 g/day, missing), diabetes duration, aHEI score, family history of MI, use of aspirin or cholesterol-lowering medication (yes, no), baseline history of hypertension, high cholesterol, angina, CABG, MI, and stroke. The study population was categorized into tertiles according to the distribution of FABP4, RBP4, and HMW adiponectin levels. Person-time was calculated for each participant from baseline examination to the occurrence of death or the date of return of the last questionnaire. Cox proportional hazards regression was used to examine the associations between adipokines (FABP4, RBP4, and HMW adiponectin) and CVD mortality. In our study, with a type I error (alpha) of 0.05 (two-sided), we

had 80% power to detect an HR of 1.60 comparing extreme tertiles. In the multivariate analysis, we controlled for the aforementioned covariates as well as TG (mg/dL), HDL (mg/dL), LDL (mg/dL), and hsCRP (mg/L). The median value of each category of adipokines was modeled as a continuous variable to test for linear trends. To minimize residual confounding, additional stratified analyses were conducted by age (<65 yr, ≥65 yr), BMI at age 21 (<25 kg/m2, ≥25 kg/m^2), and current smoking status (no, yes). Likelihood ratio tests were performed to examine potential interactions. We also conducted several sensitivity analyses to examine the robustness of the associations of interest. First, FABP4, RBP4, and HMW adiponectin were further mutually adjusted for in the models. Second, we restricted our analysis among T2D patients without CVD at baseline. In Mendelian randomization analysis, we used the two-stage control function instrumental variable approach with genetic risk scores as instrumental variables and assumed an additive model.[28,29] First, we fitted a linear regression model to predict adipokines from instrumental variables. Next, we fitted a logistic regression model with adipokines as predictors and CVD mortality as the outcome using robust standard errors and adjusted for the residuals from the first step.[29,30] The coefficients for adipokines from the second-stage model were the causal estimates for adipokines. In Mendelian randomization studies, the instrumental variable was considered sufficiently strong if the first-stage F-statistic exceeded 10.[29] Two-sided *P* values <0.05 were considered as statistically significant. All analyses were performed using SAS 9.4 (SAS Institute, Inc., Cary, North Carolina).

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