

# Nonlinear Association Between Serum Total Bilirubin and Femoral Bone Mineral Density Among U.S. Adults Aged $\geq 50$ Years: A Cross-Sectional NHANES Study

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## Research Article

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# Abstract

## Background

Oxidative stress contributes to impaired bone remodeling, whereas bilirubin is involved in redox homeostasis. We investigated the association between serum total bilirubin and femoral bone mineral density (BMD) in older adults.

## Methods

We analyzed 8,930 adults aged  $\geq 50$  years from five cycles of the National Health and Nutrition Examination Survey (NHANES). Survey-weighted multivariable linear regression and restricted cubic spline (RCS) models were used to examine associations of serum total bilirubin with femoral neck and total femur BMD. Subgroup and sensitivity analyses were performed.

## Results

In fully adjusted linear models, serum total bilirubin was not significantly associated with femoral neck BMD ( $\beta = 0.0114$  g/cm<sup>2</sup> per 1 mg/dL; 95% CI,  $-0.0028$  to  $0.0256$ ;  $P = 0.114$ ) or total femur BMD ( $\beta = 0.0061$  g/cm<sup>2</sup> per 1 mg/dL; 95% CI,  $-0.0088$  to  $0.0210$ ;  $P = 0.417$ ). However, the highest bilirubin quartile was associated with higher femoral neck BMD compared with the lowest quartile ( $\beta = 0.0118$  g/cm<sup>2</sup>; 95% CI,  $0.0010$ – $0.0226$ ;  $P = 0.033$ ;  $P$  for trend =  $0.025$ ). RCS analyses showed significant nonlinear associations with femoral neck BMD ( $P$ -nonlinearity =  $0.006$ ) and total femur BMD ( $P$ -nonlinearity =  $0.003$ ). No statistically significant interactions were observed. Sensitivity analyses showed generally similar patterns.

## Conclusions

Serum total bilirubin was nonlinearly associated with femoral BMD among adults aged  $\geq 50$  years. These findings suggest that the association may not be adequately characterized by a simple linear model. Prospective studies are needed to clarify its temporal and potential causal relevance.

## Introduction

Reduced bone strength and increased fracture susceptibility are key features of osteoporosis and low bone mass. Bone mineral density (BMD) is widely used to assess skeletal health and fracture risk, particularly at clinically relevant sites such as the hip and femoral neck. Bone remodeling depends on the coordinated activities of osteoblast-mediated bone formation and osteoclast-mediated bone resorption, and disruption of this balance can lead to bone loss. National estimates from the National Health and

Nutrition Examination Survey (NHANES) indicate a substantial burden of osteoporosis and low bone mass among U.S. adults aged  $\geq 50$  years [1].

Oxidative stress is increasingly recognized as an important contributor to abnormal bone remodeling. Excessive production of reactive oxygen species can impair osteoblast survival and differentiation, promote osteoclastogenesis, and disrupt redox-sensitive signaling involved in skeletal homeostasis [2–4]. These observations have stimulated interest in circulating markers of redox balance and endogenous antioxidant mechanisms in relation to skeletal health.

Bilirubin, an end product of heme catabolism and a commonly measured marker of hepatobiliary function, also has antioxidant and redox-modulating properties at physiological concentrations [5]. Observational studies have reported associations between higher circulating bilirubin concentrations and a lower prevalence of osteoporosis or reduced bone loss [6, 7], and an NHANES-based study reported an association between total bilirubin and BMD among adolescents [8]. However, Mendelian randomization studies have not established a causal association of circulating bilirubin with estimated BMD or fracture risk [9]. Moreover, experimental studies in settings of jaundice or excessive bilirubin exposure have suggested potentially adverse effects on osteoblast and osteoclast biology [10, 11].

Collectively, these findings suggest that the association between circulating bilirubin and skeletal health may be concentration-dependent and potentially nonlinear. In addition, analyses combining multiple NHANES cycles require appropriate consideration of the complex survey design and potential differences across survey periods. Therefore, we investigated the associations of serum total bilirubin with femoral neck and total femur BMD among U.S. adults aged  $\geq 50$  years across five NHANES cycles. We further assessed potential nonlinearity using restricted cubic spline models and evaluated the consistency of the findings through prespecified subgroup analyses, liver-related sensitivity analyses, and survey cycle-specific analyses.

## Results

### 2.1 Study population and baseline characteristics

Among 50,463 participants from the five included NHANES cycles, 36,297 participants aged  $< 50$  years, 1,395 without serum total bilirubin data, and 2,270 without valid femoral neck BMD measurements were excluded, yielding a primary analytical cohort of 10,501 participants. After further excluding 1,571 participants with missing covariate data, 8,930 participants constituted the final complete-case sample (Fig. 1).

The final analytical sample included 4,681 men and 4,249 women. The survey-weighted quartile cut points for serum total bilirubin were 0.5, 0.7, and 0.8 mg/dL, corresponding to Q1 ( $\leq 0.5$  mg/dL), Q2 ( $> 0.5$ – $0.7$  mg/dL), Q3 ( $> 0.7$ – $0.8$  mg/dL), and Q4 ( $> 0.8$  mg/dL). Among the 8,930 complete cases, these groups included 3,062, 2,766, 1,067, and 2,035 participants, respectively. Unequal group sizes reflected

ties in bilirubin values at the quartile cut points. Survey-weighted baseline characteristics according to serum total bilirubin quartiles are presented in Table 1.

## 2.2 Associations of serum total bilirubin with femoral BMD

In crude models, serum total bilirubin was positively associated with femoral neck BMD ( $\beta = 0.0440$  g/cm<sup>2</sup> per 1 mg/dL; 95% CI, 0.0283–0.0597;  $P < 0.001$ ) and total femur BMD ( $\beta = 0.0560$  g/cm<sup>2</sup> per 1 mg/dL; 95% CI, 0.0383–0.0737;  $P < 0.001$ ). These associations were attenuated after multivariable adjustment (Table 2).

In fully adjusted Model 3, serum total bilirubin modeled as a continuous linear term was not significantly associated with femoral neck BMD ( $\beta = 0.0114$  g/cm<sup>2</sup> per 1 mg/dL; 95% CI, – 0.0028 to 0.0256;  $P = 0.114$ ) or total femur BMD ( $\beta = 0.0061$  g/cm<sup>2</sup> per 1 mg/dL; 95% CI, – 0.0088 to 0.0210;  $P = 0.417$ ).

When serum total bilirubin was modeled in quartiles, the fully adjusted  $\beta$  coefficients for femoral neck BMD were 0.0084 g/cm<sup>2</sup> (95% CI, – 0.0015 to 0.0183;  $P = 0.096$ ), 0.0126 g/cm<sup>2</sup> (95% CI, – 0.0002 to 0.0254;  $P = 0.053$ ), and 0.0118 g/cm<sup>2</sup> (95% CI, 0.0010–0.0226;  $P = 0.033$ ) for Q2, Q3, and Q4 versus Q1, respectively. The  $P$  for trend was 0.025.

For total femur BMD, the corresponding fully adjusted estimates were 0.0105 g/cm<sup>2</sup> (95% CI, 0.0003–0.0208;  $P = 0.044$ ), 0.0124 g/cm<sup>2</sup> (95% CI, – 0.0018 to 0.0266;  $P = 0.087$ ), and 0.0103 g/cm<sup>2</sup> (95% CI, – 0.0006 to 0.0212;  $P = 0.063$ ) for Q2, Q3, and Q4 versus Q1, respectively. The  $P$  for trend was 0.050.

## 2.3 Nonlinear associations

Restricted cubic spline analyses showed significant overall and nonlinear associations between serum total bilirubin and both femoral BMD outcomes after full adjustment (Fig. 2). For femoral neck BMD, the  $P$  values for the overall association and nonlinearity were 0.004 and 0.006, respectively. For total femur BMD, the corresponding  $P$  values were 0.006 and 0.003. The fitted curves suggested that BMD increased across the lower range of serum total bilirubin and subsequently plateaued, with no evidence of a continued linear increase at higher concentrations.

## 2.4 Subgroup analyses

Subgroup analyses are presented in Fig. 3. For femoral neck BMD, positive within-stratum associations were observed among current smokers ( $\beta = 0.0393$ ; 95% CI, 0.0084–0.0702;  $P = 0.016$ ) and participants with diabetes ( $\beta = 0.0480$ ; 95% CI, 0.0107–0.0854;  $P = 0.015$ ). However, the corresponding interaction tests were not statistically significant ( $P$  for interaction = 0.170 and 0.064, respectively). Interaction tests were also not statistically significant for age ( $P = 0.902$ ), sex ( $P = 0.068$ ), BMI ( $P = 0.879$ ), race/ethnicity ( $P = 0.532$ ), physical activity ( $P = 0.659$ ), or hypertension ( $P = 0.281$ ).

For total femur BMD, a positive within-stratum association was observed among participants with diabetes ( $\beta = 0.0482$ ; 95% CI, 0.0117–0.0846;  $P = 0.012$ ); however, the interaction test was not statistically significant ( $P$  for interaction = 0.057). Interaction tests were also not statistically significant

for age ( $P = 0.610$ ), sex ( $P = 0.127$ ), BMI ( $P = 0.339$ ), race/ethnicity ( $P = 0.409$ ), smoking status ( $P = 0.314$ ), physical activity ( $P = 0.960$ ), or hypertension ( $P = 0.227$ ). Overall, none of the prespecified interaction tests reached statistical significance.

## 2.5 Sensitivity analyses

Sensitivity analyses are summarized in Supplementary Table S2 and Supplementary Figure S1. After excluding participants with serum total bilirubin  $> 1.7$  mg/dL ( $n = 8,864$ ), the continuous linear association was statistically significant for femoral neck BMD ( $\beta = 0.0203$  g/cm<sup>2</sup> per 1 mg/dL; 95% CI, 0.0046–0.0360;  $P = 0.012$ ), whereas the association with total femur BMD did not reach statistical significance ( $\beta = 0.0157$  g/cm<sup>2</sup> per 1 mg/dL; 95% CI, – 0.0002 to 0.0317;  $P = 0.053$ ).

After excluding participants with ALT or AST  $> 40$  U/L or missing aminotransferase values ( $n = 8,152$ ), the corresponding estimates were 0.0102 g/cm<sup>2</sup> per 1 mg/dL (95% CI, – 0.0033 to 0.0237;  $P = 0.135$ ) for femoral neck BMD and 0.0047 g/cm<sup>2</sup> per 1 mg/dL (95% CI, – 0.0100 to 0.0195;  $P = 0.522$ ) for total femur BMD. When both exclusion criteria were applied ( $n = 8,099$ ), serum total bilirubin was positively associated with femoral neck BMD ( $\beta = 0.0171$  g/cm<sup>2</sup> per 1 mg/dL; 95% CI, 0.0010–0.0333;  $P = 0.038$ ), whereas the association with total femur BMD was not statistically significant ( $\beta = 0.0121$  g/cm<sup>2</sup> per 1 mg/dL; 95% CI, – 0.0049 to 0.0291;  $P = 0.158$ ).

RCS sensitivity analyses showed broadly similar exposure–response patterns, although the statistical evidence for overall and nonlinear associations varied across the restricted samples. After excluding participants with serum total bilirubin  $> 1.7$  mg/dL,  $P$ -overall and  $P$ -nonlinearity were 0.005 and 0.033, respectively, for femoral neck BMD and 0.015 and 0.026, respectively, for total femur BMD. After excluding participants with elevated or missing aminotransferase values, the corresponding  $P$  values were 0.027 and 0.019 for femoral neck BMD and 0.025 and 0.010 for total femur BMD. When both exclusion criteria were applied,  $P$ -overall and  $P$ -nonlinearity were 0.032 and 0.052, respectively, for femoral neck BMD and 0.063 and 0.045, respectively, for total femur BMD (Supplementary Table S2 and Supplementary Figure S1).

## 2.6 Survey cycle-specific analyses

Cycle-specific analyses are presented in Supplementary Table S3. For femoral neck BMD, fully adjusted  $\beta$  estimates ranged from – 0.0150 g/cm<sup>2</sup> per 1 mg/dL (95% CI, – 0.0480 to 0.0180) in 2005–2006 to 0.0366 g/cm<sup>2</sup> per 1 mg/dL (95% CI, 0.0071–0.0662) in 2009–2010. The bilirubin-by-cycle interaction did not reach statistical significance ( $P$  for interaction = 0.051). For total femur BMD, there was also no statistically significant bilirubin-by-cycle interaction ( $P$  for interaction = 0.212) [12].

## Methods

### 5.1 Study design and population

NHANES is a continuous, nationally representative survey of the noninstitutionalized U.S. population conducted by the National Center for Health Statistics. It uses a complex, multistage probability sampling design and combines interviews, physical examinations, and laboratory assessments. We combined five survey cycles in which the exposure, femoral DXA outcomes, and required covariates were available: 2005–2006, 2007–2008, 2009–2010, 2013–2014, and 2017–2018. Femoral DXA examinations were performed in NHANES Mobile Examination Centers using standardized protocols and provided measurements including femoral neck and total femur BMD [13]

Across the five included NHANES cycles, 50,463 participants were initially considered. We excluded 36,297 participants aged < 50 years, leaving 14,166 participants aged  $\geq$  50 years. Among these, 1,395 participants without serum total bilirubin data were excluded, leaving 12,771 participants. We then excluded 2,270 participants without valid femoral neck BMD measurements, yielding a primary analytical cohort of 10,501 participants. For the fully adjusted analyses, a complete-case approach was applied to the prespecified covariates; 1,571 participants with missing covariate data were excluded, resulting in a final complete-case sample of 8,930 participants (Fig. 1).

All NHANES protocols were approved by the National Center for Health Statistics Research Ethics Review Board, and written informed consent was obtained from participants [14]. The present study used publicly available, de-identified data.

## 5.2 Serum total bilirubin

Serum total bilirubin was obtained from the NHANES standard biochemistry profile and expressed in mg/dL. In the primary regression analyses, bilirubin was modeled both as a continuous variable (per 1 mg/dL increase) and categorically. Detailed laboratory methods and quality-control procedures for serum total bilirubin measurements are provided in the NHANES laboratory documentation [15].

## 5.3 Femoral bone mineral density

The primary outcomes were femoral neck BMD and total femur BMD, expressed in g/cm<sup>2</sup> and measured by dual-energy X-ray absorptiometry (DXA). NHANES femur DXA documentation identifies DXNKBMD as femoral neck BMD and DXOFBMD as total femur BMD and describes standardized quality-control procedures for the examination [13]. Only participants with valid outcome measurements were included in the corresponding analyses.

## 5.4 Covariates

Covariates were selected a priori based on their potential associations with serum bilirubin, BMD, or both. Demographic covariates included age, sex, and race/ethnicity; socioeconomic covariates included educational attainment and poverty-income ratio (PIR); anthropometric and lifestyle covariates included body mass index (BMI), smoking status, alcohol consumption, and physical activity; clinical covariates included diabetes mellitus and hypertension; and laboratory covariates included serum vitamin D, calcium, phosphorus, and estimated glomerular filtration rate (eGFR).

Smoking status was categorized as never, former, or current. Participants who had smoked fewer than 100 cigarettes during their lifetime were classified as never smokers; those who had smoked at least 100 cigarettes but did not currently smoke were classified as former smokers; and those who had smoked at least 100 cigarettes and currently smoked every day or some days were classified as current smokers.

Alcohol consumption was harmonized across cycles and categorized as yes or no using ALQ101 in 2005–2006, 2007–2008, 2009–2010, and 2013–2014, and ALQ111 in 2017–2018. ALQ101 assessed whether participants had consumed at least 12 alcoholic drinks in any 1 year, whereas ALQ111 assessed whether participants had consumed at least one alcoholic drink in their lifetime, excluding small tastes or sips [16, 17]. Thus, alcohol consumption was harmonized as a binary covariate across cycles while retaining the cycle-specific questionnaire definitions.

Physical activity was harmonized across survey cycles. In 2005–2006, participants reporting either vigorous or moderate activity were classified as active, whereas those reporting neither were classified as inactive. In the later included cycles, participants reporting any vigorous work, moderate work, walking or bicycling for transportation, vigorous recreational activity, or moderate recreational activity were classified as active; those reporting none of these activities were classified as inactive.

Diabetes mellitus was defined according to self-reported physician diagnosis (DIQ010), with responses of yes classified as diabetes and responses of no or borderline classified as no diabetes. Hypertension was defined according to self-reported physician diagnosis (BPQ020), with yes and no responses classified accordingly.

eGFR was calculated using the 2021 race-free CKD-EPI creatinine equation [18]:

$$\text{eGFR} = 142 \times \min(\text{SCr}/\kappa, 1)^{\alpha} \times \max(\text{SCr}/\kappa, 1)^{-1.200} \times 0.9938^{\text{Age}} \times 1.012 \text{ (if female),}$$

where SCr is serum creatinine in mg/dL,  $\kappa$  is 0.7 for women and 0.9 for men, and  $\alpha$  is  $-0.241$  for women and  $-0.302$  for men. Survey cycle was included as an adjustment variable in pooled multivariable models. Cycle-specific source files and variable codes are provided in Supplementary Table S1.

## 5.5 Statistical analysis

All analyses incorporated the NHANES complex survey design, including examination weights, strata, and primary sampling units (PSUs). For pooled analyses, the 2-year examination weight (WTMEC2YR) was divided by five, while cycle-specific stratum and PSU identifiers were retained [19, 20]. For Table 1, continuous variables are presented as survey-weighted means with standard errors (SEs), categorical variables as survey-weighted percentages, and sample sizes as unweighted counts. No between-group hypothesis tests were performed.

Serum total bilirubin was categorized according to survey-weighted quartiles among the 10,501 participants with available bilirubin and BMD data. Because ties could occur at the percentile cut points,

the resulting quartile groups were not required to contain equal numbers of participants.

Survey-weighted linear regression models were fitted to estimate the associations of serum total bilirubin with femoral neck and total femur BMD. The crude model was unadjusted. Model 1 adjusted for survey cycle, age, sex, and race/ethnicity. Model 2 additionally adjusted for education, PIR, BMI, smoking status, alcohol consumption, and physical activity. Model 3 further adjusted for diabetes, hypertension, serum vitamin D, calcium, phosphorus, and eGFR. Regression coefficients ( $\beta$ ) and 95% confidence intervals (CIs) were reported. For quartile analyses, Q1 was the reference category. Linear trends across quartiles were tested by assigning each participant the median bilirubin concentration of the corresponding quartile and modeling this value continuously.

Potential nonlinear associations were evaluated using restricted cubic spline (RCS) models [21] with the same covariate adjustment as Model 3. Boundary knots were placed at 0.3 and 1.2 mg/dL and internal knots at 0.6 and 0.7 mg/dL. A serum total bilirubin concentration of 0.7 mg/dL was used as the reference value. Models included the full exposure range, whereas spline curves were displayed over the range of 0.3–1.2 mg/dL. The overall association was evaluated using a design-based Wald test of the linear and nonlinear spline components jointly. Nonlinearity was assessed using a design-based Wald test of the nonlinear spline components.

Prespecified subgroup analyses were conducted by age, sex, BMI, race/ethnicity, smoking status, physical activity, diabetes, and hypertension. Continuous linear associations were estimated within each subgroup. The stratifying variable was excluded from the corresponding within-stratum model; continuous age and BMI were also excluded from models stratified by these variables. Effect modification was assessed using interaction terms, with interpretation based on P values for interaction rather than differences in statistical significance between individual strata [12]. These interaction tests compared linear slopes and did not assess differences between complete nonlinear exposure-response curves.

Sensitivity analyses were performed after excluding (1) participants with serum total bilirubin > 1.7 mg/dL, (2) participants with alanine aminotransferase (ALT) or aspartate aminotransferase (AST) > 40 U/L or missing aminotransferase values, and (3) participants meeting either exclusion criterion. RCS models were repeated in these restricted samples. We also estimated fully adjusted associations separately within each survey cycle and tested bilirubin-by-cycle interactions in the pooled sample.

Statistical analyses were performed using IBM SPSS Statistics version 26.0 (IBM Corp., Armonk, NY, USA) and R version 4.5.3 (R Foundation for Statistical Computing, Vienna, Austria). The principal R packages used for survey-weighted and spline analyses included *survey* [20], *splines*, *dplyr*, and *ggplot2*. All statistical tests were two-sided, and  $P < 0.05$  was considered statistically significant.

## Discussion

In this nationally representative cross-sectional analysis of 8,930 U.S. adults aged  $\geq 50$  years, serum total bilirubin showed significant nonlinear associations with femoral neck and total femur BMD. After comprehensive adjustment, serum total bilirubin modeled as a continuous linear term was not significantly associated with either BMD outcome. In contrast, quartile analyses showed a positive trend for femoral neck BMD, while RCS analyses provided statistical evidence of nonlinearity at both skeletal sites. Subgroup analyses did not identify statistically significant effect modification, and sensitivity analyses generally showed associations in the same direction, although the strength of statistical evidence varied after liver-related exclusions.

Our findings add nuance to previous observational evidence regarding bilirubin and skeletal health. A longitudinal study of healthy middle-aged men [7] reported less proximal femur bone loss across increasing bilirubin concentrations, while a study of postmenopausal women without potential liver disease reported lower bilirubin concentrations among women with osteoporosis [6]. An NHANES-based study also examined total bilirubin in relation to BMD among adolescents [8]. Collectively, these studies suggest an association between circulating bilirubin and skeletal phenotypes, but they do not establish that higher bilirubin concentrations are uniformly beneficial. Moreover, Mendelian randomization did not support a causal effect of genetically predicted circulating bilirubin on estimated BMD or fracture risk [9]. The nonlinear association observed in the present study may partly explain why studies involving different populations, exposure distributions, skeletal outcomes, and modeling assumptions have yielded apparently inconsistent findings.

A biologically plausible interpretation involves the dual context of bilirubin as a redox-active molecule and a biomarker influenced by hepatobiliary physiology. Oxidative stress can promote osteoblast apoptosis, impair osteogenesis and mineralization, and favor osteoclastogenesis [2–4]. Within physiological ranges, the antioxidant and redox-related properties of bilirubin may therefore be associated with a more favorable skeletal environment [5]. However, these potential effects should not be extrapolated to pathological hyperbilirubinemia. Experimental studies in jaundice-related settings have demonstrated dose-dependent impairment of osteoblast viability, differentiation, mineralization, and osteogenic gene expression [10], while other evidence suggests that bilirubin may increase osteoclast viability and reduce osteoclast apoptosis in advanced liver disease [11]. These observations are compatible with a concentration-dependent relationship rather than an indefinitely monotonic protective effect and are consistent with the nonlinear pattern observed in the present analysis.

The crude positive associations between serum total bilirubin and BMD were substantially attenuated after multivariable adjustment, suggesting that the unadjusted associations were partly explained by measured covariates. Because survey cycle was entered together with demographic and other covariates, this attenuation cannot be attributed to any single adjustment variable. Pooling nonconsecutive NHANES cycles may also introduce temporal heterogeneity related to population composition, laboratory procedures, lifestyle characteristics, and other secular factors. In cycle-specific analyses, bilirubin-by-cycle interactions did not reach statistical significance for either femoral neck BMD ( $P$  for interaction = 0.051) or total femur BMD ( $P$  for interaction = 0.212), although the near-threshold

result for femoral neck BMD suggests that some between-cycle variation cannot be excluded. Accordingly, the pooled estimates should be interpreted in the context of potential residual temporal heterogeneity.

The sensitivity analyses provide additional context for interpreting the main findings. Excluding participants with serum total bilirubin > 1.7 mg/dL strengthened the continuous linear association with femoral neck BMD, whereas excluding participants with elevated or missing aminotransferase values yielded estimates closer to those of the primary analysis. When both exclusion criteria were applied, the association with femoral neck BMD remained positive, whereas the estimate for total femur BMD was not statistically significant. Evidence from the RCS analyses also varied across the restricted samples and was attenuated under the most restrictive exclusions. Thus, although the overall direction and nonlinear pattern were generally maintained, the strength of statistical evidence was partly sensitive to the analytical population. This finding is relevant because serum bilirubin may reflect both physiological redox variation and underlying hepatobiliary abnormalities.

This study has several strengths. First, it included a large sample from NHANES and accounted for the complex survey design by incorporating examination weights, strata, and primary sampling units. Second, serum total bilirubin was evaluated using complementary continuous, categorical, and spline approaches rather than relying solely on a single linear specification, allowing the shape of the exposure–outcome association to be examined more comprehensively. Third, the regression models adjusted for a broad range of demographic, socioeconomic, anthropometric, lifestyle, clinical, and biochemical covariates, including survey cycle. Fourth, prespecified subgroup analyses, liver-related sensitivity analyses, and cycle-specific analyses were used to assess the robustness and potential heterogeneity of the observed associations.

Several limitations should also be acknowledged. First, the cross-sectional design precludes inference regarding temporality or causality, and reverse causation cannot be excluded. Residual or unmeasured confounding remains possible despite extensive covariate adjustment, including potential confounding related to medications, liver disease, reproductive factors, and other characteristics not fully captured in the available data. Second, serum total bilirubin was measured at a single time point and did not distinguish between conjugated and unconjugated bilirubin; therefore, it may not adequately represent long-term bilirubin exposure, within-person variability, or potentially distinct biological effects of bilirubin fractions. Third, 1,571 of the 10,501 participants in the primary analytical cohort (15.0%) were excluded because of missing covariate data. Complete-case analysis may introduce selection bias if missingness is related to bilirubin, BMD, or other participant characteristics, and survey weighting does not necessarily eliminate such bias [22]. Fourth, pooling five nonconsecutive NHANES cycles introduces potential temporal and measurement heterogeneity. In particular, alcohol and physical activity measures differed across cycles despite harmonization, which may have resulted in residual measurement variability. Fifth, multiple subgroup comparisons increase the possibility of chance within-stratum findings, particularly because none of the prespecified interaction tests reached statistical significance. Sixth, the outcomes were DXA-measured BMD rather than incident fractures or clinically diagnosed

osteoporosis; therefore, the findings should not be directly interpreted as evidence of reduced fracture or osteoporosis risk. Finally, the findings pertain to the noninstitutionalized U.S. population represented by the included NHANES cycles and may not be directly generalizable to other populations.

In conclusion, serum total bilirubin was nonlinearly associated with femoral neck and total femur BMD among U.S. adults aged  $\geq 50$  years. The association was not adequately characterized by a simple linear model, and the present cross-sectional findings should be interpreted as evidence of an epidemiological dose–response pattern rather than proof that bilirubin itself protects bone. Longitudinal studies incorporating repeated bilirubin measurements, detailed assessment of liver function and bilirubin fractions, and clinically relevant skeletal outcomes such as incident osteoporosis and fractures are needed to clarify the temporal and potential causal relevance of these findings. Mechanistic studies may further help distinguish potentially favorable redox-associated relationships within physiological bilirubin ranges from adverse skeletal effects associated with pathological hyperbilirubinemia.

## Conclusions

Serum total bilirubin was nonlinearly associated with femoral neck and total femur BMD among U.S. adults aged  $\geq 50$  years. Although fully adjusted linear models did not show statistically significant associations, restricted cubic spline analyses provided evidence of significant nonlinearity at both skeletal sites. These findings suggest that the association between serum total bilirubin and femoral BMD may not be adequately characterized by a simple linear model. Prospective studies are needed to clarify the temporal and potential causal relevance of these findings.

## Declarations

### Ethics Approval and Consent to Participate

The National Center for Health Statistics Research Ethics Review Board approved all National Health and Nutrition Examination Survey (NHANES) protocols, and all participants provided written informed consent. This study was a secondary analysis of publicly available, de-identified data and therefore did not require additional institutional ethics approval.

## Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

## Consent for Publication

Not applicable.

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# Author Contribution

Wenjun Yao contributed to the study conception and design, data acquisition, data analysis and interpretation, and drafting of the manuscript. Ya Sun and Bo Wang contributed to data acquisition, data curation, and statistical analysis. Xingxin Yan and Fei Gao contributed to data interpretation and visualization. Hao Chen contributed to methodology and critical revision of the manuscript. Weiwei Hua contributed to the study conception and design, supervision, interpretation of the results, and critical revision of the manuscript. All authors reviewed and approved the final manuscript.

# Data Availability

The datasets analyzed in this study are publicly available from the National Health and Nutrition Examination Survey (NHANES), conducted by the National Center for Health Statistics, Centers for Disease Control and Prevention, at <https://www.cdc.gov/nchs/nhanes/>

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## Tables

Table 1

Survey-weighted characteristics of the complete-case study population according to serum total bilirubin quartiles.

| Characteristic           | Overall         | Q1<br>( $\leq 0.5$<br>mg/dL) | Q2<br>( $> 0.5-0.7$<br>mg/dL) | Q3<br>( $> 0.7-0.8$<br>mg/dL) | Q4<br>( $> 0.8$<br>mg/dL) |
|--------------------------|-----------------|------------------------------|-------------------------------|-------------------------------|---------------------------|
| Unweighted N             | 8930            | 3062                         | 2766                          | 1067                          | 2035                      |
| Age, years               | 62.88<br>(0.18) | 62.71<br>(0.27)              | 62.84 (0.24)                  | 63.06 (0.35)                  | 63.08<br>(0.28)           |
| Male, %                  | 48.9%           | 35.9%                        | 45.3%                         | 57.2%                         | 68.6%                     |
| <b>Race/ethnicity, %</b> |                 |                              |                               |                               |                           |
| Mexican American         | 4.7%            | 5.3%                         | 4.6%                          | 4.4%                          | 4.3%                      |
| Other Hispanic           | 3.5%            | 5.0%                         | 2.6%                          | 3.5%                          | 2.5%                      |
| Non-Hispanic White       | 77.9%           | 72.6%                        | 78.7%                         | 80.7%                         | 83.3%                     |
| Non-Hispanic Black       | 8.3%            | 10.6%                        | 8.5%                          | 6.4%                          | 5.8%                      |
| Other Race               | 5.5%            | 6.5%                         | 5.6%                          | 5.0%                          | 4.2%                      |
| <b>Education, %</b>      |                 |                              |                               |                               |                           |
| <9th grade               | 5.7%            | 6.1%                         | 5.5%                          | 5.9%                          | 5.2%                      |
| 9-11th grade             | 10.1%           | 9.7%                         | 11.1%                         | 10.1%                         | 9.5%                      |
| High school/GED          | 25.8%           | 28.2%                        | 25.3%                         | 24.2%                         | 23.9%                     |
| Some college/AA          | 29.0%           | 30.2%                        | 30.9%                         | 25.3%                         | 26.3%                     |
| College or above         | 29.4%           | 25.8%                        | 27.2%                         | 34.5%                         | 35.0%                     |
| Poverty-income ratio     | 3.30 (0.04)     | 3.12 (0.06)                  | 3.32 (0.06)                   | 3.42 (0.07)                   | 3.46 (0.06)               |
| BMI, kg/m <sup>2</sup>   | 28.68<br>(0.09) | 29.23<br>(0.20)              | 28.73 (0.13)                  | 28.18 (0.24)                  | 28.06<br>(0.16)           |
| <b>Smoking status, %</b> |                 |                              |                               |                               |                           |
| Never                    | 50.1%           | 50.0%                        | 49.6%                         | 50.9%                         | 50.3%                     |
| Former                   | 34.6%           | 30.6%                        | 35.2%                         | 35.3%                         | 39.4%                     |
| Current                  | 15.3%           | 19.4%                        | 15.2%                         | 13.8%                         | 10.3%                     |
| Alcohol use, %           | 78.4%           | 80.9%                        | 75.0%                         | 75.9%                         | 80.5%                     |
| Physically active, %     | 71.9%           | 69.7%                        | 71.7%                         | 73.0%                         | 74.9%                     |
| Diabetes, %              | 14.5%           | 17.5%                        | 14.8%                         | 12.5%                         | 10.8%                     |

| Characteristic                      | Overall       | Q1<br>( $\leq 0.5$ mg/dL) | Q2<br>( $> 0.5-0.7$ mg/dL) | Q3<br>( $> 0.7-0.8$ mg/dL) | Q4<br>( $> 0.8$ mg/dL) |
|-------------------------------------|---------------|---------------------------|----------------------------|----------------------------|------------------------|
| Hypertension, %                     | 48.7%         | 49.2%                     | 49.9%                      | 46.3%                      | 47.4%                  |
| 25-hydroxyvitamin D, nmol/L         | 73.80 (0.76)  | 76.43 (1.29)              | 72.95 (1.04)               | 73.50 (1.15)               | 71.26 (0.84)           |
| Serum calcium, mg/dL                | 9.44 (0.01)   | 9.39 (0.01)               | 9.46 (0.01)                | 9.46 (0.01)                | 9.46 (0.02)            |
| Serum phosphorus, mg/dL             | 3.74 (0.01)   | 3.78 (0.02)               | 3.77 (0.01)                | 3.76 (0.02)                | 3.65 (0.02)            |
| eGFR, mL/min/1.73 m <sup>2</sup>    | 82.23 (0.34)  | 82.60 (0.50)              | 81.71 (0.51)               | 81.84 (0.73)               | 82.58 (0.57)           |
| Femoral neck BMD, g/cm <sup>2</sup> | 0.763 (0.002) | 0.745 (0.004)             | 0.764 (0.004)              | 0.774 (0.005)              | 0.782 (0.004)          |
| Total femur BMD, g/cm <sup>2</sup>  | 0.925 (0.002) | 0.903 (0.004)             | 0.925 (0.005)              | 0.938 (0.006)              | 0.952 (0.005)          |

Values are survey-weighted means (SEs) for continuous variables and survey-weighted percentages for categorical variables; sample sizes are unweighted counts. Serum total bilirubin quartiles were defined as Q1  $\leq 0.5$  mg/dL, Q2  $> 0.5-0.7$  mg/dL, Q3  $> 0.7-0.8$  mg/dL, and Q4  $> 0.8$  mg/dL. No between-group hypothesis tests were performed. BMD, bone mineral density; BMI, body mass index; eGFR, estimated glomerular filtration rate; PIR, poverty-income ratio; SE, standard error.

Table 2

Associations of serum total bilirubin with femoral neck and total femur BMD. Categorical estimates compare survey-weighted bilirubin quartiles.

| Exposure                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                   | Crude<br>$\beta$ (95% CI); P    | Model 1<br>$\beta$ (95% CI); P  | Model 2<br>$\beta$ (95% CI); P  | Model 3<br>$\beta$ (95% CI); P  |
|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|
| <b>Femoral neck BMD</b>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                    |                                 |                                 |                                 |                                 |
| Continuous bilirubin (per 1 mg/dL)                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                         | 0.0440 (0.0283, 0.0597); <0.001 | 0.0065 (-0.0092, 0.0222); 0.414 | 0.0109 (-0.0032, 0.0251); 0.128 | 0.0114 (-0.0028, 0.0256); 0.114 |
| Survey-weighted bilirubin quartiles                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                        |                                 |                                 |                                 |                                 |
| Q1                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                         | Reference                       | Reference                       | Reference                       | Reference                       |
| Q2                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                         | 0.0190 (0.0092, 0.0288); <0.001 | 0.0080 (-0.0029, 0.0189); 0.146 | 0.0085 (-0.0013, 0.0184); 0.088 | 0.0084 (-0.0015, 0.0183); 0.096 |
| Q3                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                         | 0.0293 (0.0161, 0.0425); <0.001 | 0.0091 (-0.0038, 0.0220); 0.165 | 0.0128 (0.0002, 0.0255); 0.047  | 0.0126 (-0.0002, 0.0254); 0.053 |
| Q4                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                         | 0.0376 (0.0260, 0.0492); <0.001 | 0.0075 (-0.0048, 0.0197); 0.226 | 0.0116 (0.0010, 0.0222); 0.033  | 0.0118 (0.0010, 0.0226); 0.033  |
| P for trend                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                | < 0.001                         | 0.187                           | 0.024                           | 0.025                           |
| <b>Total femur BMD</b>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                     |                                 |                                 |                                 |                                 |
| Continuous bilirubin (per 1 mg/dL)                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                         | 0.0560 (0.0383, 0.0737); <0.001 | 0.0024 (-0.0155, 0.0202); 0.793 | 0.0056 (-0.0094, 0.0205); 0.460 | 0.0061 (-0.0088, 0.0210); 0.417 |
| Survey-weighted bilirubin quartiles                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                        |                                 |                                 |                                 |                                 |
| Q1                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                         | Reference                       | Reference                       | Reference                       | Reference                       |
| Q2                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                         | 0.0223 (0.0099, 0.0348); <0.001 | 0.0110 (-0.0010, 0.0231); 0.073 | 0.0107 (0.0005, 0.0208); 0.040  | 0.0105 (0.0003, 0.0208); 0.044  |
| Q3                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                         | 0.0356 (0.0219, 0.0492); <0.001 | 0.0091 (-0.0053, 0.0235); 0.212 | 0.0125 (-0.0017, 0.0266); 0.082 | 0.0124 (-0.0018, 0.0266); 0.087 |
| <p>Note: <math>\beta</math> coefficients represent differences in BMD (g/cm<sup>2</sup>). Q1 was the reference group. P for trend was calculated by assigning the median serum total bilirubin concentration of each quartile (Q1 = 0.4, Q2 = 0.7, Q3 = 0.8, Q4 = 1.0 mg/dL) and modeling this value as a continuous variable. Crude: unadjusted. Model 1: adjusted for NHANES survey cycle, age, sex, and race/ethnicity. Model 2: additionally adjusted for education, poverty-income ratio, body mass index, smoking status, alcohol consumption, and physical activity. Model 3: additionally adjusted for diabetes, hypertension, serum vitamin D, calcium, phosphorus, and estimated glomerular filtration rate. BMD, bone mineral density; CI, confidence interval; eGFR, estimated glomerular filtration rate.</p> |                                 |                                 |                                 |                                 |

| Exposure                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                   | Crude<br>$\beta$ (95% CI); P    | Model 1<br>$\beta$ (95% CI); P  | Model 2<br>$\beta$ (95% CI); P  | Model 3<br>$\beta$ (95% CI); P  |
|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|
| <b>Femoral neck BMD</b>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                    |                                 |                                 |                                 |                                 |
| Q4                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                         | 0.0489 (0.0354, 0.0625); <0.001 | 0.0067 (-0.0072, 0.0206); 0.337 | 0.0099 (-0.0010, 0.0207); 0.073 | 0.0103 (-0.0006, 0.0212); 0.063 |
| P for trend                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                | < 0.001                         | 0.287                           | 0.056                           | 0.050                           |
| <p>Note: <math>\beta</math> coefficients represent differences in BMD (g/cm<sup>2</sup>). Q1 was the reference group. P for trend was calculated by assigning the median serum total bilirubin concentration of each quartile (Q1 = 0.4, Q2 = 0.7, Q3 = 0.8, Q4 = 1.0 mg/dL) and modeling this value as a continuous variable. Crude: unadjusted. Model 1: adjusted for NHANES survey cycle, age, sex, and race/ethnicity. Model 2: additionally adjusted for education, poverty-income ratio, body mass index, smoking status, alcohol consumption, and physical activity. Model 3: additionally adjusted for diabetes, hypertension, serum vitamin D, calcium, phosphorus, and estimated glomerular filtration rate. BMD, bone mineral density; CI, confidence interval; eGFR, estimated glomerular filtration rate.</p> |                                 |                                 |                                 |                                 |

## Figures

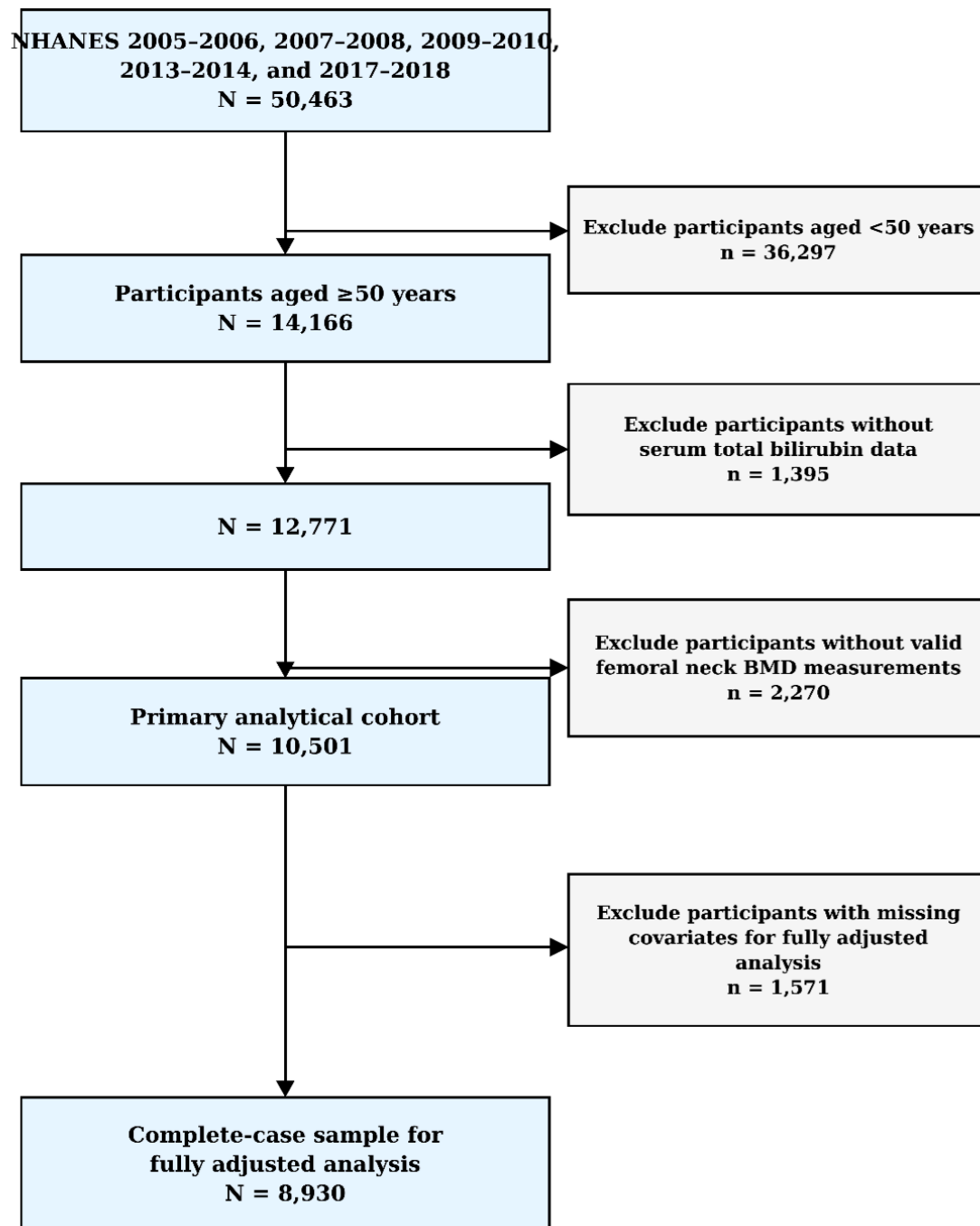


**Figure 1**

**Flowchart of participant selection from NHANES.**

Participants were selected from five NHANES cycles (2005–2006, 2007–2008, 2009–2010, 2013–2014, and 2017–2018). Of 50,463 participants, 8,930 remained in the final complete-case sample after sequential exclusions.

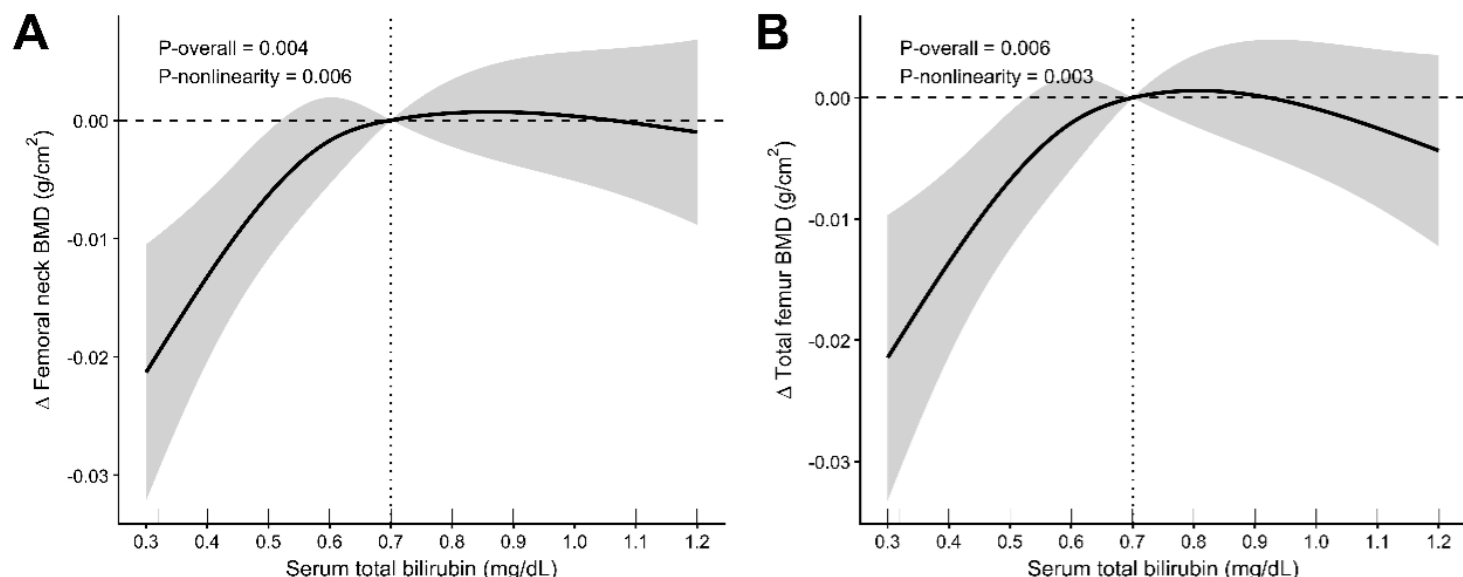


**Figure 2**

## Restricted cubic spline associations between serum total bilirubin and femoral BMD.

Restricted cubic spline associations with (A) femoral neck BMD and (B) total femur BMD. Models were fully adjusted as described in Methods. Solid lines represent estimated differences in BMD relative to 0.7 mg/dL bilirubin, and shaded areas represent 95% CIs. P-overall and P-nonlinearity were obtained from design-based Wald tests.

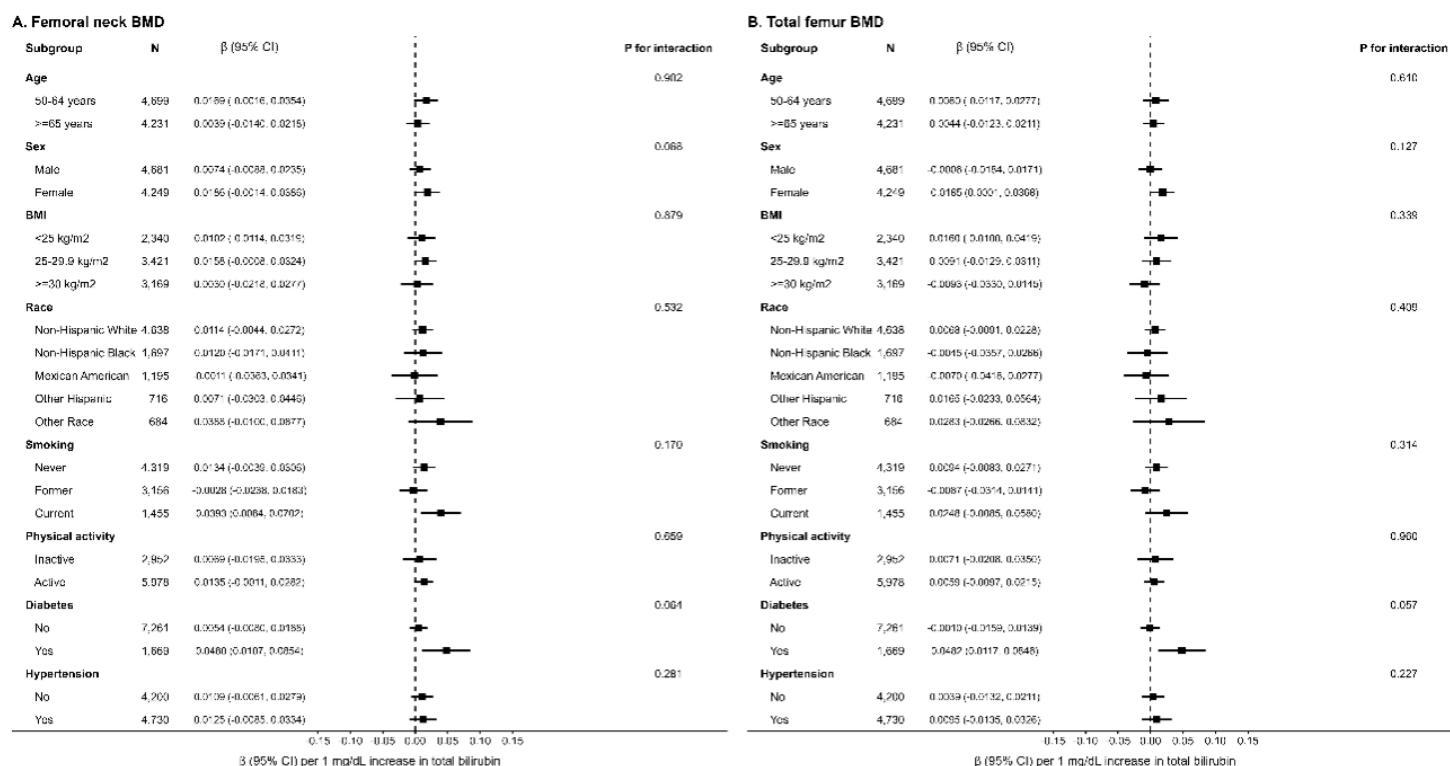


**Figure 3**

### **Subgroup analyses of the associations between serum total bilirubin and femoral BMD.**

Subgroup analyses for (A) femoral neck BMD and (B) total femur BMD.  $\beta$  coefficients and 95% CIs represent differences in BMD per 1-mg/dL increase in serum total bilirubin. Models were fully adjusted as described in Methods, excluding the corresponding stratifying variable. P values for interaction assess differences in linear associations across strata.

## **Supplementary Files**

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