ORIGINAL PAPER

DOI: http://dx.doi.org/10.15403/jgld-4732

Total Bilirubin Levels in Nonalcoholic Fatty Liver Disease and All-cause and Cause-specific Mortality in US Adults

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Received: 19.11.2022
Accepted: 04.05.2023

ABSTRACT

Background & Aims: Nonalcoholic fatty liver disease (NAFLD) is a chronic progressive illness with a spectrum of disease severity from steatosis to end-stage liver disease. Emerging evidence suggests total serum bilirubin levels are inversely related to the prevalence of metabolic syndrome including NAFLD. We investigated the effects of bilirubin on all-cause and cause-specific mortality stratified by NAFLD status.
Methods: We used the third National Health and Nutrition Examination Survey Cohort (1988-1994) and linked mortality dataset through 2019. Cox-regression models were constructed to assess the association between bilirubin levels categorized by quartile with all-cause and cause-specific mortality.
Results: During the median follow-up of 324 months (n=11,078), higher bilirubin levels were associated with a reduction in risk of all-cause mortality in the multivariable model [hazard ratio (HR): 0.83, 95% confidence interval (CI): 0.71-0.97 for quartile 4 (highest bilirubin levels) vs. quartile 1 (lowest bilirubin levels), p for trend=0.033]. Higher bilirubin levels were associated with a lower risk for all-cause mortality in individuals with NAFLD (HR=0.68, 95%CI: 0.55-0.86 for quartile 4, p for trend=0.010); however, this protective association with higher bilirubin levels was not noted in those without NAFLD. Higher bilirubin levels were associated with a lower risk for cardiovascular and cancer-related mortality in individuals with NAFLD.
Conclusions: In this large national representative sample of American adults, higher bilirubin levels in NAFLD were associated with a lower risk of all-cause mortality, which may be derived from a lower risk of cardiovascular/cancer-related mortality.

Key words: bilirubin – NAFLD – hepatic steatosis – death – NHANES.

Abbreviations: CI: confidence interval; HR: hazard ratio; NAFLD: nonalcoholic liver disease; NASH: nonalcoholic steatohepatitis.

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) has a broad spectrum of disease severity, and it is characterized by progressive hepatic injury with accompanying inflammatory changes. Presentations range from nonalcoholic fatty liver in the form of simple steatosis to nonalcoholic steatohepatitis (NASH) with evidence of lobular inflammatory damage and ballooning hepatocellular injury, to ultimately end-stage liver disease [1]. The global impact of NAFLD is underscored by its rising prevalence, which is estimated to be as high as 25.2% worldwide [2]. NASH cirrhosis accounts for significant healthcare resource utilization, with expenditures stemming largely from hospitalizations and liver transplantation [2]. Liver transplantation shoulders a significant portion of healthcare burden and economic costs [3]. Given NAFLD is the second most common indication for liver transplantation in the United States [2], we anticipate these costs will continue to rise. In the absence of effective therapies for NAFLD [4], identifying prognostic indicators for disease severity and outcome will guide clinical decision-making and help target efforts to prevent and treat modifiable risk factors.

Emerging evidence suggests total serum bilirubin levels are inversely related to the prevalence of metabolic syndrome and its associated metabolic abnormalities, including diabetes mellitus [5, 6], and cardiovascular disease. However, the topic of bilirubin's beneficial vs. harmful effects is still inconclusive [7-13]. While evidence describing the mechanistic links

between elevated bilirubin and metabolic diseases continues to evolve, more recently under scrutiny is the impact of bilirubin in patients with NAFLD [8, 14]. Bilirubin is thought to exert anti-inflammatory properties by neutralizing free radicals and preventing the oxidation of intracellular lipids [7, 15-17]. This cascade is thought to exert protective effects against metabolic syndrome and associated diseases and be responsible for this inverse association seen in several retrospective and prospective designs [15-20]. An experimental study using mice models showed that free radical-scavenging anti-oxidants reduce the amount of adipose accumulation in the liver [18]. Through these mechanisms, higher bilirubin levels are thought to garner health benefits and improve outcomes for individuals with NAFLD.

Given the substantial variation in mortality appreciated across distinct subgroups affected by NAFLD, there is an increasing need for evidence to establish prognostically relevant clinical characteristics associated with poor outcomes among individuals with NAFLD. Employing data from the National Health and Nutrition Examination Survey (NHANES), we aimed to establish the distinct effects of bilirubin on all-cause and cause-specific mortality based on the presence or absence of NAFLD.

METHODS

Study Population

This population-based prospective cohort study analyzed the third NHANES dataset (1988-1994), which includes demographic and anthropometric data, as well as clinical information, including laboratory and hepatic ultrasound data. The third NHANES data was obtained using a clustered, multistage, stratified probability sampling design with the goal of being a representative sample of the United States' general population.

There were 14,797 adult participants (ages 20-74) who underwent an examination at a mobile examination center. Of those, we excluded 2,074 participants with iron overload (transferrin saturation ≥ 50%), viral hepatitis (positive serum hepatitis C antibody and/or positive serum hepatitis B surface antigen), and/or significant alcohol consumption, defined as > 21 or > 14 drinks per week for men and women, respectively. We also excluded participants with missing data for hepatic ultrasonography, total bilirubin, body mass index, aminotransferases, viral hepatitis serology, and mortality outcome (n=1,645). The final sample used for analysis was 11,078 (Fig. 1).

The NHANES survey was approved by the review board of the National Center for Health Statistics. All of the participants gave written informed consent, and our study received an exemption from the institutional review board as the data we used was de-identified.

Clinical and Laboratory Evaluations

The methods for this study have been previously described [19, 20]. Diabetes was defined as either physician diagnosed, HbA1c ≥ 6.5%, fasting glucose ≥ 126 mg/dL, and/or treatment with a hypoglycemic agent or insulin. Hypertension was defined as systolic blood pressure ≥ 140 mmHg or diastolic blood pressure ≥ 90 mmHg and/or previous use of antihypertensive medication. Current smokers were defined as ongoing smokers among individuals who had smoked at least ≥ 100 cigarettes over the previous 5 years. Alcohol consumption was defined based on the amount and frequency of alcohol use as previously described [21]. Sedentary lifestyle was determined by participants answering no to all physical activity questions in the previous month: jogging/running, bicycling, swimming, aerobics, other dancing, calisthenics, garden/yard work, weight lifting, or other sports [4].

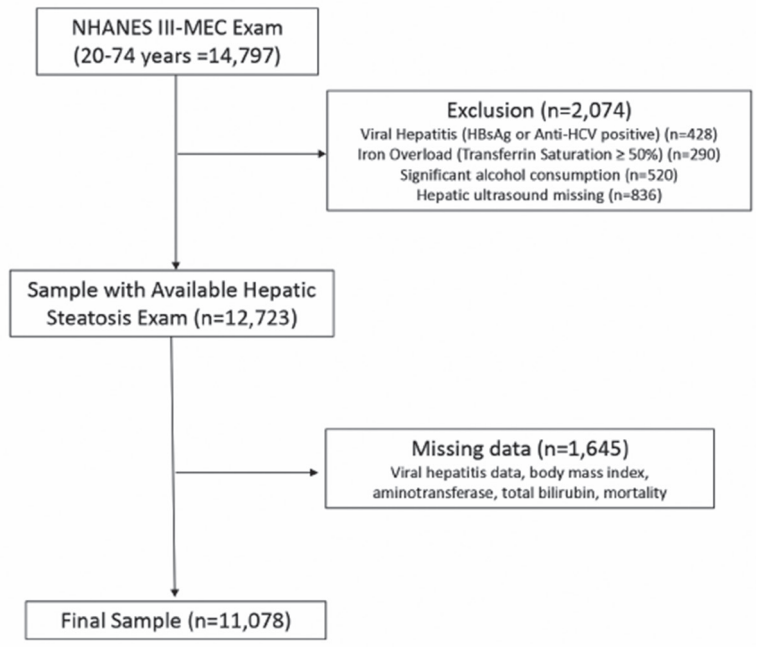

Fig. 1. Flow diagram of participants in the study.

Definition of NAFLD

Detecting hepatic steatosis undergoing ultrasound evaluation at a mobile examination center has been previously described [20]. In short, three radiologists evaluated hepatic ultrasounds using 5 criteria: parenchymal brightness, liver to kidney contrast, deep beam attenuation, bright vessel walls, and gallbladder wall definition [22]. Based on these criteria, the findings were reported as normal, mild, moderate, or severe hepatic steatosis. We then defined NAFLD for our study as the presence of mild to severe steatosis in the absence of other chronic liver disease or other causes for steatosis, such as significant alcohol consumption [23].

Serum Bilirubin Level

Total bilirubin levels in participating adults at mobile examination center were measured and recorded within the third NHANES. Total bilirubin levels were measured using 2,5-dichlorophenyldiazonium tetrafluoroborate in a strongly acidic medium [23]. The intensity of the azobilirubin color was determined photometrically by a Hitachi 737 automated autoanalyzer (Boehringer Mannheim Diagnostics) [23]. As total bilirubin levels were categorized into quartiles based on the weighted sample distribution (quartile 1: < 0.4 mg/dL, quartile 2: ≥ 0.4 and < 0.5 mg/dL, quartile 3: ≥ 0.5 and < 0.7 mg/dL, quartile 4, ≥ 0.7 mg/dL), weighted sample numbers for each quartile are evenly distributed in our analyses. We used the lowest quartile of total bilirubin level as a reference group.

All-cause and Cause-specific Mortality

There was a passive mortality follow-up for all adult participants in the third NHANES up to December 31st, 2019. The National Death index records were used for probabilistic matching to determine mortality status and cause of death for participants from 1988 to 2019. Information for the cause of death in the Underlying Cause of Death 113 code (UCOD_113) was categorized based on the International Classification of Diseases, 9th Revision (ICD-9) for deaths prior to 1998 and ICD-10 for deaths from 1999 and 2019. We then identified all-cause mortality and cause-specific mortalities for cardiovascular disease (UCOD_113: 55-64,70) and cancer (UCOD_113: 19-43). Liver-related mortality in the NHANES III was not able to retrieve as it is restricted public release due to small numbers.

Statistical Analysis

Data was reconstituted using appropriate sampling weights to control for the complex sampling design used by the third NHANES to ensure an unbiased representation of the US general population. The weighted mean ± standard errors for continuous variables and frequencies with 95% confidence intervals (CI) of categorical variables were provided. The statistical significance of differences between bilirubin status was examined using the chi-square test and linear regression as appropriate. Cox-proportion hazard regression models were constructed to estimate the independent association between total bilirubin levels categorized by quartile and all-cause, as well as cause-specific mortality. Models were run within multiple cohorts, including the total population, as well as separated by NAFLD status. The trend of hazards was analyzed to assess whether an increase in the quartile was associated with an increase or decrease in the risk of mortality. Sensitivity analyses were conducted to evaluate the impact of total bilirubin based on the NAFLD disease severity, categorized into mild and moderate/severe hepatic steatosis, on all-cause and cause-specific mortalities. Analyses were performed using STATA 17.1 (StataCorp, College Station, TX) utilizing Taylor series linearization.

RESULTS

A total of 11,078 participants were eligible for inclusion in the final analysis (mean age 42.4 years, men: 48.4%). Out of these, 4,050 individuals met the criteria for NAFLD. Demographic and clinical characteristics are presented based on the bilirubin level quartiles (Table I). According to the bilirubin levels quartiles, there were noticeable differences in the demographic and clinical characteristics. Stratification by bilirubin level showed that younger males were more represented in higher bilirubin quartiles. Body mass index, current smokers, presence of sedentary lifestyle, and blood levels with high-density lipoprotein cholesterol exhibited decreasing frequencies with rising bilirubin levels, while hypertension and blood levels of total cholesterol and hemoglobin A1c showed the lowest in the quartile 4. In contrast, aminotransferase had a linear relation with bilirubin levels.

The median follow-up was 324 months (IQR: 299, 345 months). Throughout the follow-up period, 3,869 deaths were recorded and classified as all-cause mortality. A total of 1,299 cardiovascular disease-related and 936 cancer-related deaths were observed. Results of Cox-regression analyses using the lowest bilirubin level (quartile 1) as the reference category can be seen in Table II. Higher levels of total bilirubin were associated with progressively lower hazards of all-cause mortality in age, sex, and race/ethnicity-adjusted and multivariable models. In the age, gender, race/ethnicity-adjusted model, compared to the reference group with the lowest quartile, those with quartile 3 and quartile 4 were 26% and 27% lower risk for all-cause mortality [hazards ratio (HR): 0.74, 95%CI: 0.64-0.85 for quartile 3 and HR: 0.73, 95%CI: 0.63-0.84 for quartile 4 comparing with quartile 1]. An additional multivariable model taking into account body mass index, smoking status, diabetes, hypertension, alanine aminotransferase, total cholesterol, high-density lipoprotein cholesterol, and sedentary lifestyle produced similar findings. When we classified cohorts by NAFLD status, higher levels of total bilirubin were associated with a lower risk for all-cause mortality in those with NAFLD (adjusted HR=0.68, 95%CI: 0.55-0.86 for quartile 4, p for trend = 0.010). This association was not preserved in populations without NAFLD. In terms of cardiovascular disease-related mortality (Table III), the bilirubin levels were associated with lower cardiovascular mortality in the total population (HR=0.75, 95%CI: 0.59-0.96 for quartile 3); however, trends are not statistically significant (p for trend = 0.180). The inverse association between total bilirubin and cardiovascular mortality was noted in individuals with NAFLD (HR=0.54, 95%CI: 0.37-0.80 for quartile 2 and HR=0.56, 95%CI: 0.38-0.83 for quartile 4).

Table I. Baseline characteristics of the study population based on total bilirubin status

	Quartile 1	Quartile 2	Quartile 3	Quartile 4	p
Age (years)	43.8 ± 0.6	43.8 ± 0.4	43.3 ± 0.6	41.3 ± 0.4	<0.001
Male (%)	15.2 (13.4, 17.2)	31.5 (29.0, 34.2)	49.6 (46.8, 52.4)	68.2 (66.2, 70.2)	<0.001
Body mass index (kg/m^2)	27.4 ± 0.4	26.9 ± 0.2	26.8 ± 0.2	26.1 ± 0.2	<0.001
Waist circumference (cm)	91.1 ± 0.6	91.6 ± 0.5	92.6 ± 0.4	91.7 ± 0.4	0.355
Hypertension (%)	23.4 (20.4, 26.7)	21.8 (19.3, 24.5)	22.0 (20.2, 23.9)	18.2 (15.9, 20.8)	0.008
Diabetes (%)	6.6 (5.3, 8.3)	6.3 (5.1, 7.8)	5.6 (4.4, 7.1)	6.6 (5.6, 7.8)	0.476
Ethnicity (%)					<0.001
Non-Hispanic white	67.8 (63.2, 72.1)	73.1 (69.9, 76.1)	78.8 (76.0, 81.3)	78.7 (75.6, 81.4)	
Non-Hispanic black	17.5 (15.2, 20.0)	12.9 (11.0, 15.0)	8.7 (7.5, 10.1)	7.6 (6.5, 8.8)	
Mexican American	6.0 (4.9, 7.3)	5.5 (4.6, 6.5)	5.2 (4.3, 6.3)	5.2 (4.3, 6.3)	
Others	8.7 (6.0, 12.5)	8.6 (6.1, 11.9)	7.3 (5.9, 9.0)	8.5 (6.5, 11.2)	
Smoking (%)					<0.001
Never	43.7 (40.1, 47.5)	42.7 (39.8, 45.8)	45.1 (42.1, 48.2)	50.9 (48.1, 53.7)	
Current smoker	34.3 (31.0, 37.8)	34.2 (31.2, 37.3)	28.6 (25.7, 31.6)	20.1 (17.8, 22.8)	
Ex-smoker	21.9 (19.0, 25.2)	23.1 (20.5, 25.9)	26.3 (24.1, 28.7)	28.9 (26.8, 31.2)	
Total cholesterol (mg/dL)	205.6 ± 1.3	204.8 ± 1.0	205.9 ± 1.4	199.8 ± 1.3	0.002
HDL-cholesterol (mg/dL)	52.3 ± 0.6	50.8 ± 0.5	50.0 ± 0.4	48.5 ± 0.6	<0.001
Fasting glucose (mg/dL)	96.7 ± 0.9	97.2 ± 0.8	98.3 ± 0.8	99.3 ± 0.9	0.015
Hemoglobin A1c (%)	5.4 ± 0.03	5.4 ± 0.03	5.4 ± 0.03	5.3 ± 0.03	<0.001
Alanine aminotransferase (IU/L)	14.1 ± 0.6	15.6 ± 0.4	18.0 ± 0.4	19.4 ± 0.6	<0.001
Aspartate aminotransferase (IU/L)	18.7 ± 0.3	19.8 ± 0.2	21.0 ± 0.3	22.3 ± 0.2	<0.001
Sedentary lifestyle (%)	29.0 (24.9, 33.5)	24.4 (21.6, 27.4)	17.5 (15.3, 20.1)	15.9 (13.6, 18.5)	<0.001
Total bilirubin (mg/dL)	0.27 ± 0.005	0.4 ± 0.001	0.55 ± 0.001	0.94 ± 0.01	<0.001
NAFLD (%)	34.5(29.7, 39.6)	32.7(29.2, 36.3)	35.1 (32.8-37.5)	33.4 (29.3, 37.7)	0.653

Data are shown as the weighted mean or frequency ± standard errors or 95% confidence intervals as appropriate. Range of quartile 1: < 0.4 ng/dL; quartile 2: ≥ 0.4 and < 0.5 mg/dL, quartile 3: ≥ 0.5 and < 0.7 mg/dL, quartile 4: ≥ 0.7 mg/dL. HDL: high-density lipoprotein; NAFLD: nonalcoholic fatty liver disease.

Additionally, there was also no significant association between bilirubin levels and cardiovascular mortality in the absence of NAFLD. Regarding cancer-related mortality (Table IV), there was a significant inverse association between bilirubin levels and cancer-related mortality (HR=0.70, 95%CI: 0.50-0.97 for quartile 4, p for trend = 0.025) in the total population and individuals with NAFLD (HR=0.58, 95%CI: 0.36-0.94 for quartile 4, p for trend = 0.016).

To investigate the robustness of our results, we performed sensitivity analyses based on the severity of NAFLD (Table V). Increasing bilirubin levels in mild NAFLD did not demonstrate significant dose-response association with all-cause (p for trend = 0.205), cardiovascular (p for trend = 0.821), and cancer-related mortality (p for trend = 0.171). In contrast, the bilirubin levels among individuals with moderate-severe NAFLD were associated with lower all-cause and cause-specific mortality. Those in quartile 4 were 32% (HR=0.68, 95%CI: 0.55-0.84), 47% (HR=0.53, 95%CI: 0.31-0.90), and 46% (HR=0.54, 95%CI: 0.31-0.96) lower risk for all-cause, cardiovascular, and cancer-related mortality, respectively. An inverse dose-response association was noted among individuals with moderate-severe NAFLD (p for trends < 0.05 for all).

DISCUSSION

In this prospective, nationally representative, population-based study, we found a strong inverse relationship between increasing bilirubin levels in NAFLD and all-cause mortality. This association may in part be derived from the distinct mortality benefit gained in those with higher total bilirubin in the cardiovascular disease and cancer-related mortality subgroups. These findings suggest that higher bilirubin levels in NAFLD are closely associated with a beneficial reduction in all-cause, cardiovascular disease, and cancer-related mortality independent of known metabolic risk factors. The protective effect of bilirubin is further magnified among individuals with moderate-severe NAFLD. To date, this is the most comprehensive study to explore these questions, providing considerable insights relevant to the care of patients with NAFLD. Importantly, these findings suggest bilirubin as a potential biomarker for high-risk patients with NAFLD.

Findings from this work are consistent with previous studies demonstrating the protective effects gained from elevated bilirubin across various metabolic conditions [6]. Employing similar data from NHANES, Cheriyath et al. [6] showed an inverse association between bilirubin levels and diabetes, reporting that a 26% reduction in the prevalence of diabetes was observed in individuals with elevated serum bilirubin. Similar findings resonate in the study by Perlstein et al. [9], who evaluated the association between total serum bilirubin and stroke. They found a 9% reduction in the odds of having a stroke with a 0.1mg/dL incremental increase in bilirubin levels [9].

Table II. Association between total bilirubin status and all-cause mortality based on NAFLD status

	Age, gender, race/ethnicity-adjusted model		Multivariable model	
	HR (95%CI)	p	HR (95%CI)	p
Total population				
Quartile 1	1	<0.001*	1	0.033*
Quartile 2	0.84 (0.72-1.00)	0.045	0.84 (0.72-0.99)	0.040
Quartile 3	0.74 (0.64-0.85)	<0.001	0.80 (0.69-0.92)	0.003
Quartile 4	0.73 (0.63-0.84)	<0.001	0.83 (0.71-0.97)	0.021
Total bilirubin (per 1-SD)	0.95 (0.90-1.00)	0.042	1.00 (0.95-1.06)	0.968
No NAFLD				
Quartile 1	1	0.026	1	0.669*
Quartile 2	0.85 (0.67-1.08)	0.186	0.90 (0.72-1.13)	0.346
Quartile 3	0.79 (0.62-1.00)	0.053	0.89 (0.70-1.12)	0.300
Quartile 4	0.77 (0.62-0.97)	0.028	0.93 (0.72-1.20)	0.577
Total bilirubin (per 1-SD)	0.97 (0.90-1.04)	0.395	1.04 (0.95-1.13)	0.378
NAFLD				
Quartile 1	1	<0.001*	1	0.010*
Quartile 2	0.81 (0.64-1.03)	0.082	0.75 (0.60-0.94)	0.013
Quartile 3	0.65 (0.51-0.82)	0.001	0.68 (0.53-0.87)	0.003
Quartile 4	0.65 (0.52-0.82)	<0.001	0.68 (0.55-0.86)	0.001
Total bilirubin (per 1-SD)	0.91 (0.84-0.98)	0.018	0.94 (0.88-1.01)	0.099

The multivariable model was adjusted for age, sex, race/ethnicity, body mass index, smoking status, diabetes, hypertension, alanine aminotransferase, total cholesterol, high-density lipoprotein cholesterol, and sedentary lifestyle. *P-value for the test of trend of hazards. HR: hazard ratio; CI: confidence interval; SD: standard deviation; NAFLD: nonalcoholic fatty liver disease.

Table III. Association between total bilirubin status and cardiovascular disease-related mortality based on NAFLD status

	Age, sex, race/ethnicity-adjusted model		Multivariable model	
	HR (95% CI)	p	HR (95%CI)	p
Total population				
Quartile 1	1	0.027*	1	0.180*
Quartile 2	0.81 (0.63-1.03)	0.085	0.78 (0.60-1.01)	0.060
Quartile 3	0.71 (0.56-0.91)	0.007	0.75 (0.59-0.96)	0.022
Quartile 4	0.72 (0.56-0.92)	0.010	0.80 (0.62-1.02)	0.066
Total bilirubin (per 1-SD)	0.96 (0.86-1.08)	0.496	1.01 (0.91-1.12)	0.887
No NAFLD				
Quartile 1	1	0.315*	1	0.799*
Quartile 2	0.93 (0.69-1.26)	0.636	0.96 (0.73-1.25)	0.745
Quartile 3	0.71 (0.48-1.03)	0.073	0.79 (0.56-1.12)	0.188
Quartile 4	0.85 (0.57-1.26)	0.401	0.98 (0.66-1.45)	0.920
Total bilirubin (per 1-SD)	1.01 (0.88-1.17)	0.839	1.07 (0.92-1.24)	0.393
NAFLD				
Quartile 1	1	0.031*	1	0.098*
Quartile 2	0.61 (0.43-0.88)	0.009	0.54 (0.37-0.80)	0.003
Quartile 3	0.67 (0.47-0.97)	0.034	0.68 (0.44-1.04)	0.074
Quartile 4	0.53 (0.36-0.80)	0.003	0.56 (0.38-0.83)	0.005
Total bilirubin (per 1-SD)	0.86 (0.70-1.05)	0.136	0.91 (0.76-1.09)	0.293

The multivariable model was adjusted for age, sex, race/ethnicity, body mass index, smoking status, diabetes, hypertension, alanine aminotransferase, total cholesterol, high-density lipoprotein cholesterol, and sedentary lifestyle. *P-value for the test of trend of hazards. For abbreviations see Table II.

Table IV. Association between total bilirubin status and cancer-related mortality based on NAFLD status

	Age, gender, race/ ethnicity-adjusted model		Multivariable model	
	HR (95% CI)	p	HR (95%CI)	p
Total population				
Quartile 1	1	<0.001*	1	0.025*
Quartile 2	0.80 (0.60-1.06)	0.121	0.80 (0.59-1.08)	0.141
Quartile 3	0.59 (0.43-0.83)	0.003	0.66 (0.47-0.94)	0.022
Quartile 4	0.58 (0.43-0.78)	0.001	0.70 (0.50-0.97)	0.031
Total bilirubin (per 1-SD)	0.84 (0.76-0.94)	0.002	0.91 (0.82-1.02)	0.094
No NAFLD				
Quartile 1	1	0.030*	1	0.351*
Quartile 2	0.77 (0.49-1.21)	0.248	0.83 (0.52-1.34)	0.446
Quartile 3	0.67 (0.41-1.11)	0.115	0.78 (0.47-1.27)	0.307
Quartile 4	0.59 (0.37-0.94)	0.028	0.79 (0.48-1.30)	0.346
Total bilirubin (per 1-SD)	0.82 (0.69-0.97)	0.020	0.93 (0.78-1.10)	0.397
NAFLD				
Quartile 1	1	0.007*	1	0.016*
Quartile 2	0.82 (0.54-1.25)	0.353	0.79 (0.50-1.24)	0.293
Quartile 3	0.48 (0.30-0.78)	0.004	0.52 (0.31-0.85)	0.011
Quartile 4	0.56 (0.35-0.89)	0.017	0.58 (0.36-0.94)	0.028
Total bilirubin (per 1-SD)	0.88 (0.72-1.07)	0.192	0.89 (0.73-1.07)	0.213

The multivariable model was adjusted for age, sex, race/ethnicity, body mass index, smoking status, diabetes, hypertension, alanine aminotransferase, total cholesterol, high-density lipoprotein cholesterol, and sedentary lifestyle. *P-value for the test of trend of hazards. For abbreviations see Table II.

Furthermore, increased bilirubin levels were associated with improved overall stroke outcomes, including decreased long-term complications such as memory dysfunction or decreased mobility [9]. The protective effects exerted by bilirubin are also appreciated in cardiovascular disease, as demonstrated by a multivariate analysis of 877 men with angiographically confirmed coronary artery disease [10]. Consistent with previous studies, an inverse dose-response relationship was noted between serum bilirubin levels and prevalence of coronary artery disease [11]. In fact, incremental increases in the odds of severe coronary artery disease, as high as 47%, were accompanied by bilirubin level reductions of 50% [11], indicating that higher bilirubin may function as a protective biomarker against coronary artery disease as well.

The mortality benefit gained from bilirubin in patients with metabolic syndrome is hypothesized to derive from its well-recognized cytoprotective properties [15-17, 24, 25]. While no prior studies have evaluated whether bilirubin exerts a mortality benefit in individuals with NAFLD, it is known that there is an inverse relationship between NAFLD prevalence across varying bilirubin levels [8, 14]. This may be explained by similar anti-inflammatory mechanisms and neutralization of free radicals preventing the oxidation of intracellular lipids [7, 15, 16, 26] observed in other cardiometabolic diseases, including coronary artery disease [11] and diabetes [5, 6]. The antioxidative and cytoprotective properties of bilirubin have a well-recognized role in neutralizing oxidative stress, a common pathway associated with the development of NAFLD [15-17, 24, 25, 27]. Interestingly, our study did not show a mortality benefit consistently in the non-NAFLD population. We hypothesize that the cytoprotective and antioxidant properties of bilirubin might be more protective against mortality in more metabolically advanced or increased inflammatory state such as severe NAFLD. Future prospective studies are required to confirm a more prominent association between total bilirubin and mortality in NAFLD and explore the pathophysiologic mechanism by which bilirubin exerts such protective effects. The current body of evidence is clear and would suggest that serum bilirubin levels could be used as a biomarker to classify patients at high risk for increased mortality in NAFLD.

Utilizing the NHANES database affords a large nationally representative sample with high-quality measurements, including anthropometric measures, medical questionnaires, as well as laboratory tests, including ultrasonographic diagnosed NAFLD. Results from this work may be generalizable to much of the Western population that shares similar social- and lifestyle-related behavioral patterns with those of the United States. Additionally, because we have the opportunity to get a more recent update for mortality up to 2019, extended follow-up (median 27 years) allowed sufficient time for longitudinal capture of outcomes, enabling greater accuracy in establishing the directional association between bilirubin levels and mortality. There are of course limitations to this study. First, because the third NHANES was performed in 1988-1994, the prevalence of metabolic syndrome, obesity, and NAFLD may be much lower than the current period, However, there is no database including ultrasonography diagnosed NAFLD and mortality data over 25 years of follow-up, which is suitable for studying for the association between total bilirubin and mortality based on the NAFLD status. Second, because the

Table V. Association between total bilirubin status and all-cause and cause-specific mortality based on severity of NAFLD status

	Age, gender, race/ ethnicity-adjusted model		Multivariable model	
	HR (95% CI)	p	HR (95% CI)	p
All-cause mortality				
Mild NAFLD				
Quartile 1	1	0.021*	1	0.205*
Quartile 2	0.75 (0.48-1.19)	0.222	0.68 (0.42-1.10)	0.117
Quartile 3	0.68 (0.45-1.03)	0.071	0.72 (0.48-1.08)	0.109
Quartile 4	0.61 (0.40-0.95)	0.030	0.70 (0.44-1.09)	0.111
Total bilirubin (per 1-SD)	0.89 (0.76-1.05)	0.162	0.96 (0.82-1.13)	0.610
Moderate-severe NAFLD				
Quartile 1	1	0.001*	1	0.004*
Quartile 2	0.83 (0.63-1.10)	0.194	0.78 (0.58-1.06)	0.112
Quartile 3	0.63 (0.50-0.79)	<0.001	0.67 (0.52-0.85)	0.002
Quartile 4	0.66 (0.54-0.82)	<0.001	0.68 (0.55-0.84)	0.001
Total bilirubin (per 1-SD)	0.91 (0.83-1.00)	0.041	0.94 (0.86-1.02)	0.142
Cardiovascular mortality				
Mild NAFLD				
Quartile 1	1	0.510*	1	0.821*
Quartile 2	0.39 (0.17-0.89)	0.026	0.33 (0.16-0.66)	0.003
Quartile 3	0.68 (0.28-1.70)	0.405	0.70 (0.32-1.55)	0.376
Quartile 4	0.55 (0.20-1.50)	0.222	0.64 (0.29-1.38)	0.246
Total bilirubin (per 1-SD)	0.96 (0.69-1.35)	0.831	1.07 (0.80-1.43)	0.645
Moderate-severe NAFLD				
Quartile 1	1	0.009*	1	0.025*
Quartile 2	0.74 (0.38-1.43)	0.364	0.67 (0.34-1.31)	0.239
Quartile 3	0.68 (0.41-1.12)	0.127	0.69 (0.39-1.20)	0.184
Quartile 4	0.53 (0.32-0.88)	0.015	0.53 (0.31-0.90)	0.021
Total bilirubin (per 1-SD)	0.80 (0.65-1.00)	0.048	0.84 (0.68-1.03)	0.085
Cancer mortality				
Mild NAFLD				
Quartile 1	1	0.091*	1	0.171*
Quartile 2	0.87 (0.40-1.89)	0.724	0.80 (0.33-1.94)	0.621
Quartile 3	0.59 (0.26-1.33)	0.200	0.60 (0.26-1.38)	0.226
Quartile 4	0.56 (0.25-1.25)	0.151	0.63 (0.29-1.38)	0.244
Total bilirubin (per 1-SD)	0.82 (0.59-1.16)	0.254	0.87 (0.64-1.17)	0.348
Moderate-severe NAFLD				
Quartile 1	1	0.022*	1	0.031*
Quartile 2	0.78 (0.47-1.29)	0.319	0.76 (0.45-1.27)	0.285
Quartile 3	0.41 (0.23-0.73)	0.004	0.44 (0.24-0.81)	0.009
Quartile 4	0.55 (0.32-0.93)	0.027	0.54 (0.31-0.96)	0.037
Total bilirubin (per 1-SD)	0.92 (0.73-1.16)	0.468	0.90 (0.72-1.17)	0.439

The multivariable model was adjusted for age, sex, race/ethnicity, body mass index, smoking status, diabetes, hypertension, alanine aminotransferase, total cholesterol, high-density lipoprotein cholesterol, and sedentary lifestyle. *P-value for the test of trend of hazards. For abbreviations see Table II.

third NHANES data does not provide serial abdominal ultrasonography and bilirubin data, we are unable to assess longitudinal changes in NAFLD status or bilirubin status in this study and were also not able to access information on direct and indirect bilirubin or the gold standard diagnostic tool, liver biopsy. Third, while we have attempted to adjust known confounders to determine an independent association between total bilirubin and mortality, unknown residual confounders may have impacted our results. Finally, we were unable to investigate the association between total bilirubin and liver-

related mortality in NAFLD because liver-related mortality in the NHANES III was not released publicly due to the small numbers of deaths.

Previous well-known papers have discussed bilirubin as a negative prognosticator, which appears to be paradoxical to our, and similar studies, findings. There are a multitude of reasons for these differences with the most likely hypotheses being study design, population characteristics, and disease type evaluated. Our study used a retrospective observational approach. Given this, it is likely that confounders could not all be accounted for, and that bias could not be fully removed. Similarly, there are recent retrospective studies, such as the 2021 study by Lopez-Velazquez et al. [28] that showed that bilirubin has a negative predictive value on mortality, results which may have also suffered from observational design flaws.

Several studies looking at the negative effects of bilirubin have not used the same population as was used in our study. Even within our own study, the protective effect of bilirubin were not observed in all population groups assessed, and was most often seen in the NAFLD as opposed to the non NAFLD group. The exact reason for this we can only hypothesize as we have above. Other studies that have found bilirubin to be a negative prognosticator such the famous Child-Pugh and MELD studies, which are widely accepted in clinical practice for prognostication, may have found differing results in NAFLD patients. Zero percent of patients included in the Child-Pugh or King's College non acetaminophen criteria validation studies [29, 30] and only 2.4% of patients included in the MELD validation study [31] had a NAFLD diagnosis. It is thus likely that our findings may not have been generalizable to these populations which in part explains the paradoxical findings. Elaborating on population differences, it is important to note that over 90% of our study population had a normal range bilirubin level. The fourth quartile was bilirubin levels ≥ 0.7 mg/dL, with many patients falling into the normal range. Given that other validation studies (MELD, Child-Pugh and King's College) look at grossly elevated bilirubin levels [29-31], and our study looked mainly at levels within and only just above normal, perhaps there is a therapeutic window in which bilirubin is most, and potentially least, beneficial to mortality. Future studies with further quartile separation may be helpful in bringing to light these differences.

Furthermore, elevated bilirubin in our study, as well as in many recent studies showing the inverse relationship between metabolic syndrome severity and bilirubin, may be related to the cause and type of bilirubin elevation. This was likely not consistent across all studies evaluating the relationship. To elaborate, bilirubin elevation can be due to physiologic baseline differences, hemolysis, impaired conjugation or uptake, or impaired clearance [32]. Given clinical studies have not, to our knowledge, consistently elucidated the causes of bilirubin elevation in their population, it is possible that these differences may be relevant to determining whether or not the oxidative effects of bilirubin outweigh the potentially negative causes of its elevation.

Further confirmation of bilirubin's potentially protective effect needs to be considered through re-assessment, in randomized control trials, alternative database, or large observational studies.

CONCLUSIONS

Given the absence of effective therapies for NAFLD, the identification of prognostic indicators of disease progression and outcome can help target efforts toward preventing and treating modifiable risk factors. We found that higher bilirubin levels exert a protective benefit for both all-cause and cause-specific mortality for the total population, which is amplified in individuals with NAFLD, especially in moderate to severe NAFLD. Although it is still unclear how we might be able to employ these results in the clinical setting, we believe its prognostic utility may help inform further risk stratification to distinguish which subgroups of patients may benefit from aggressive risk factor surveillance and lifestyle modification.

Conflicts of interest: None to declare.

Authors' contribution: Conceived and designed the studyK.M.. B.B.D., L.N., A.A., and D.K. interpreted the data and drafted the manuscript. A.A. and D.K. analyzed the data and revised critically the manuscript. A.A. and D.K. supervised the study. All the authors approved the final version of the manuscript.

REFERENCES

1. Puri P, Sanyal AJ. Nonalcoholic fatty liver disease: Definitions, risk factors, and workup. Clin Liver Dis (Hoboken) 2012;1:99–103. doi:10.1002/cld.81
2. Younossi ZM, Koenig AB, Abdelatif D, Fazel Y, Henry L, Wymer M. Global epidemiology of nonalcoholic fatty liver disease-Meta-analytic assessment of prevalence, incidence, and outcomes. Hepatology 2016;64:73–84. doi:10.1002/hep.28431
3. Bentley TS, Phillips SJ. 2017 U.S. organ and tissue transplant cost estimates and discussion. Milliman Research Report 2017. Available from: https://costprojections.com/wp-content/uploads/2019/03/milliman.2017.pdf
4. Kim D, Wijarnpreecha K, Sandhu KK, Cholankeril G, Ahmed A. Sarcopenia in nonalcoholic fatty liver disease and all-cause and cause-specific mortality in the United States. Liver Int 2021;41:1832–1840. doi:10.1111/liv.14852
5. Lin LY, Kuo HK, Hwang JJ, et al. Serum bilirubin is inversely associated with insulin resistance and metabolic syndrome among children and adolescents. Atherosclerosis 2009;203:563–568. doi:10.1016/j.atherosclerosis.2008.07.021
6. Cheriyath P, Gorrepati VS, Peters I, et al. High Total Bilirubin as a Protective Factor for Diabetes Mellitus: An Analysis of NHANES Data From 1999 - 2006. J Clin Med Res 2010;2:201-206. doi:10.4021/jocmr425w
7. Lin JP, O'Donnell CJ, Schwaiger JP, et al. Association Between the UGT1A1*28 Allele, Bilirubin Levels, and Coronary Heart Disease in the Framingham Heart Study. Circulation. 2006;114:1476-1481. doi:10.1161/CIRCULATIONAHA.106.633206
8. Kwak MS, Kim D, Chung GE, et al. Serum bilirubin levels are inversely associated with nonalcoholic fatty liver disease. Clin Mol Hepatol 2012;18:383–390. doi:10.3350/cmh.2012.18.4.383
9. Perlstein TS, Pande RL, Creager MA, Weuve J, Beckman JA. Serum total bilirubin level, prevalent stroke, and stroke outcomes:

NHANES 1999-2004. Am J Med 2008;121:781-788.e1. doi:10.1016/j.amjmed.2008.03.045
10. Kang SJ, Kim D, Park HE, et al. Elevated serum bilirubin levels are inversely associated with coronary artery atherosclerosis. Atherosclerosis 2013;230:242–248. doi:10.1016/j.atherosclerosis.2013.06.021
11. Schwertner HA, Jackson WG, Tolan G. Association of low serum concentration of bilirubin with increased risk of coronary artery disease. Clin Chem 1994;40:18–23.
12. Perlstein TS, Pande RL, Beckman JA, Creager MA. Serum total bilirubin level and prevalent lower-extremity peripheral arterial disease: National Health and Nutrition Examination Survey (NHANES) 1999 to 2004. Arterioscler Thromb Vasc Biol 2008;28:166–172. doi:10.1161/ATVBAHA.107.153262
13. Krijgsman B, Papadakis JA, Ganotakis ES, Mikhailidis DP, Hamilton G. The effect of peripheral vascular disease on the serum levels of natural anti-oxidants: Bilirubin and albumin. Int Angiol 2002;21:44–52.
14. Jang BK. Elevated serum bilirubin levels are inversely associated with nonalcoholic fatty liver disease. Clin Mol Hepatol 2012;18:357–359. doi:10.3350/cmh.2012.18.4.357
15. Stocker R, Yamamoto Y, McDonagh AF, Glazer AN, Ames BN. Bilirubin is an antioxidant of possible physiological importance. Science 1987;235:1043–1046. doi:10.1126/science.3029864
16. Barañano DE, Rao M, Ferris CD, Snyder SH. Biliverdin reductase: a major physiologic cytoprotectant. Proc Natl Acad Sci U S A 2002;99:16093–16098. doi:10.1073/pnas.252626999
17. Oliveira CPMS, Da Costa Gayotto LC, Tatai C, et al. Oxidative stress in the pathogenesis of nonalcoholic fatty liver disease, in rats fed with a choline-deficient diet. J Cell Mol Med 2002;6:399–406. doi:10.1111/j.1582-4934.2002.tb00518.x
18. Terao K, Niki E. Damage to biological tissues induced by radical initiator 2,2'-azobis(2-amidinopropane) dihydrochloride and its inhibition by chain-breaking antioxidants. J Free Radic Biol Med 1986;2:193–201. doi:10.1016/s0748-5514(86)80070-8
19. Kim D, Kim W, Kwak MS, Chung GE, Yim JY, Ahmed A. Inverse association of marijuana use with nonalcoholic fatty liver disease among adults in the United States. PLoS One 2017;12:e0186702. doi:10.1371/journal.pone.0186702
20. Kim D, Kim WR, Kim HJ, Therneau TM. Association between noninvasive fibrosis markers and mortality among adults with nonalcoholic fatty liver disease in the United States. Hepatology 2013;57:1357–1365. doi:10.1002/hep.26156
21. Ruhl CE, Everhart JE. Joint effects of body weight and alcohol on elevated serum alanine aminotransferase in the United States population. Clin Gastroenterol Hepatol 2005;3:1260–1268. doi:10.1016/s1542-3565(05)00743-3
22. Center for Health Statistics N. Hepatic Steatosis Ultrasound Images Assessment National Health and Nutrition Examination Survey (NHANES) III Hepatic Steatosis Ultrasound Images Assessment Procedures Manual. 2010. Available from: https://www.cdc.gov/nchs/data/nhanes/nhanes3/Hepatic_Steatosis_Ultrasound_Procedures_Manual.pdf
23. Plan and operation of the Third National Health and Nutrition Examination Survey, 1988-94. Series 1: programs and collection procedures. Vital Heal Stat 1 1994;32:1–407.
24. Sumida Y, Nakashima T, Yoh T, et al. Serum thioredoxin levels as a predictor of steatohepatitis in patients with nonalcoholic fatty liver disease. J Hepatol 2003;38:32–38. doi:10.1016/s0168-8278(02)00331-8
25. Seki S, Kitada T, Sakaguchi H. Clinicopathological significance of oxidative cellular damage in non-alcoholic fatty liver diseases. Hepatol Res 2005;33:132–134. doi:10.1016/j.hepres.2005.09.020
26. Frei B, Stocker R, Ames BN. Antioxidant defenses and lipid peroxidation in human blood plasma. Proc Natl Acad Sci U S A 1988;85:9748–9752. doi:10.1073/pnas.85.24.9748
27. Tian J, Zhong R, Liu C, et al. Association between bilirubin and risk of Non-Alcoholic Fatty Liver Disease based on a prospective cohort study. Sci Rep 2016;6:31006. doi:10.1038/srep31006
28. López-Velázquez JA, Chávez-Tapia NC, Ponciano-Rodríguez G, et al. Bilirubin alone as a biomarker for short-term mortality in acute-on-chronic liver failure: an important prognostic indicator. Ann Hepatol 2013;13:98-104. doi:10.1016/S1665-2681(19)30910-X
29. Pugh RN, Murray-Lyon IM, Dawson JL, Pietroni MC, Williams R.. Transection of the oesophagus for bleeding oesophageal varices. Br J Surg 1973;60:646-649. doi:10.1002/bjs.1800600817
30. O'Grady JG, Alexander GJ, Hayllar KM, Williams R. Early indicators of prognosis in fulminant hepatic failure. Gastroenterology 1989;97:439-445. doi:10.1016/0016-5085(89)90081-4
31. Wiesner R, Edwards E, Freeman R, et al. Model for end-stage liver disease (MELD) and allocation of donor livers. Gastroenterology 2003;124:91-96. doi:10.1053/gast.2003.50016
32. Guerra Ruiz AR, Crespo J, López Martínez RM, et al. Measurement and clinical usefulness of bilirubin in liver disease. Adv Lab Med 2021;2:352-372. doi:10.1515/almed-2021-0047