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Association of serum bilirubin with longevity: Evidence from a retrospective longitudinal study and cross-sectional data

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ABSTRACT: Bilirubin is a potent antioxidant and an important anti-inflammatory factor. Therefore, there has been an increasing focus on serum bilirubin as a negative risk factor of cardiovascular mortality in men and an indicator of improved survival in both sexes, but the direct mechanisms of these links and the causes of sex differences are not well understood. Moreover, the evidence from longitudinal studies on effects of bilirubin on longevity is limited. In this study, we retrospectively analyzed two groups of older adults to explore age-dependent changes in serum bilirubin levels and their associations with long-term survival in both sexes. Longitudinal data from 142 individuals (68 men and 74 women) aged 45 to 70 years were compared with cross-sectional data from 225 individuals (113 men and 112 women). The latter group was divided into four categories of survival, i.e. 53, 63, 68, and 76+ based on data on lifespan. ANOVA, *t*-test, and regression analysis were run. The analysis of the longitudinal data showed an increase in serum total bilirubin levels in men ($0.3038e^{0.093x}$, $R^2 = 0.667$) and women ($0.1838e^{0.0187x}$, $R^2 = 0.950$), while the analysis of cross-sectional data revealed a U-shaped pattern of age-related changes in men ($0.001x2 - 0.1263x + 4.4524$, $R^2 = 0.999$) but an inverted U-shaped pattern in women ($0.0006x^2 + 0.072x - 1.6924$, $R^2 = 0.195$). On balance, these results suggest that elevated but normal bilirubin levels might confer a survival advantage in older men but not women. Alternatively, the positive relationship between serum total bilirubin and lifespan was not causal but coincidental. Further studies are needed to elucidate the direct mechanisms of the association between serum bilirubin levels and longevity in elderly people of both sexes.

KEY WORDS: aging, antioxidant, bilirubin, inflammation, longevity, longitudinal study, sex differences

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Introduction

Bilirubin is a potent antioxidant that can help protect the cells against oxidative stress elicited by various types of reactive oxygen species (ROS). Apart from its antioxidant activity, serum bilirubin exerts anti-inflammatory effects on the cells of the vasculature and the mononuclear phagocyte system (MPS), thereby protecting against atherosclerosis (Idelman et al. 2015; Kundur et al. 2015) and lowering the overall risk of cardiovascular disease (CVD). Serum total bilirubin (STB), which represents the equilibrium between several concurrent processes involved in the production and metabolism of this bile pigment, is considered to be a major contributor to the total antioxidant capacity of the plasma (Sedlak et al. 2009). In a healthy adult, approximately 250–350 mg of bilirubin is produced daily in the liver, spleen, and other parts of the MPS, deriving mainly from heme catabolism but also from ineffective erythropoiesis and certain proteins (Rodwell et al. 2015). Thus, any physiologic or pathologic process that increases breakdown of erythrocytes, such as toxins, infections, immune reactions, and genetic defects in erythrocyte plasma membranes, can lead to hyperbilirubinemia (i.e. raised plasma bilirubin levels) and jaundice.

For a long time, there has been an increasing focus on STB as a negative risk factor of cardiovascular mortality and an indicator of improved survival in both sexes, but especially in men (Djousse et al. 2001; Temme et al. 2001; Vitek et al. 2002; Perlstein et al. 2008; Kimm et al. 2009; Lin et al. 2009; Tanaka et al. 2009; Lin et al. 2010; Li et al. 2013; Ong et al. 2014; Kunutsor 2015; Li et al. 2015). According to the oxidation-inflammation theory of aging (De la Fuente and Miquel 2009; Franceschi and Campisi 2014; Chmielewski and Strzelec 2017), elevated serum bilirubin levels that protect against oxidative damage, atherosclerosis, CVD, tissue inflammation, etc. should be associated with improved survival among healthy older individuals, i.e. when hyperbilirubinemia is not caused by any serious disease. However, some studies have shown that elevated STB levels do not confer any survival advantage in older adults (Boland et al. 2014). Long-term analyses of these associations at the population level are extraordinarily rare and such data are scarce. In addition, longitudinal studies of aging usually do not explore the links between total bilirubin and longevity. Therefore, there is a paucity of information on the association between STB and long-term survival in the Polish population.

We have overcome these difficulties by collecting extensive data, both longitudinal and cross-sectional, that include numerous physiological and biochemical parameters from older adults of both sexes who were studied longitudinally for at least 25 years (between the ages of 45 and 70), which means that this could be an introduction to the Polish Longitudinal Study of Aging (PLSA) or at least useful data for meta-analysis (Chmielewski et al. 2015a; 2015b; 2016a; 2016c; 2017). Based on these data, we aim to explore the association between STB and long-term survival in older adults of both sexes from the same population that was studied longitudinally for a very long time.

Materials and methods

Of 3500 patients who resided at the Regional Specialist Hospital in Cibórz, Lubuskie Province, Poland, in the years

1960–2000, longitudinal data were available for 142 individuals aged 45 to 70 years (including 68 men and 74 women) and cross-sectional data were available for 225 individuals (including 113 men and 112 women) who differed in lifespan. Therefore, the latter sample was divided into four categories of survival using both individual (ILS) and mean lifespan (MLS), i.e. 53, 63, 68, and 76+ years of age. The limit of ILS for the first category of survival from the cross-sectional data was 57.5 years of age. This subgroup consisted of 34 individuals, including 22 men and 12 women, and MLS was 53 and 52 years of age, respectively, which means that this category comprised only short-lived subjects. The limit of ILS for the second category of survival was 65 years and this subgroup was made up of 57 individuals, including 27 men and 30 women; MLS was 63 years for both sexes, which means that these subjects lived longer compared to the first subgroup of inmates. The limit of ILS for the third category was 72.5 years and this subgroup consisted of 89 individuals, including 49 men and 40 women; MLS was 67.6 years for men and 68 years for women. The last category of survival (76+) consisted of 45 individuals, including 15 men and 30 women, all aged 76 years and over, which means that this subgroups comprised only long-lived subjects (Chmielewski et al. 2016a).

In the Polish People's Republic, these individuals resided continuously at this institution that provided care for older and chronically ill people from the lowest socioeconomic strata. These inmates and residents lived there until their death or, in the case of long-lived subjects, until the 2000 reform of the healthcare system in Poland. During that time, they underwent routine medical check-ups. Thus, their health status was constantly evaluated by medical staff at this institution. The hospital staff had their consent to take all these measurements as well as to set up the database for medical and scientific purposes. After the 1989 systemic transformation in Poland, many of these residents stayed at this institution until the year 2000, when the system of health care was reformed and reorganized.

The study was carried out in accordance with the principles of the Declaration of Helsinki. The process of exploring the medical records and collecting the data was performed with permission and consent of both local and hospital authorities in the years 2005–2007. For the purpose of this study, we have used only data from those subjects who were physically healthy and who did not take any psychoactive drugs. Hyperbilirubinemic subjects were excluded from the study sample, and data from individuals who had clinically significant liver dysfunctions (e.g. cirrhosis, cancer, and other serious diseases) were not used for the analysis. Noteworthy, ill and treated subjects were thus excluded from the study sample and this was the only selection criterion, which means that the sample is random because no other selection criteria were applied. Further details of the study population and data collection are described elsewhere (Chmielewski et al. 2015a; 2015b; 2016a; 2016c; 2017).

In the Polish Longitudinal Study of Aging (PLSA), age-related changes in numerous anthropometric, physiological, and biochemical characteristics were analyzed. All measurements were performed in accordance with internationally accepted standards and requirements. Blood samples from the median cubital vein were drawn by a nurse. During the 25-year study period, blood tests were

performed from 10 up to 18 times within each five-year period for a very long time, i.e. 25 years in the case of the longitudinal sample. STB levels were estimated in accordance with the modified Malloy-Evelyn procedure for total serum bilirubin assay. On the basis of such frequently repeated measurements, we were able to calculate arithmetic means, standard deviations (SD), standard errors, and other statistics for each five-year interval. Thus, an arithmetic mean for a given age category in both longitudinal and cross-sectional sample was always calculated on the basis of frequently repeated measurements in the studied individuals.

Normality of the data distribution was tested with the K-S test. To determine and compare the rate and patterns of changes with age in the analyzed parameters in the compared groups of subjects as well as derive mathematical formulae describing these changes and differences, analysis of variance (ANOVA), *t*-test, and regression analysis were performed. To find the best approximation of the function, the standard method of least squares was used. A given function of regression was confirmed as the best fitting model only when a coefficient of determination (R^2) reached the highest value and an unknown parameter (β_0) along with a coefficient of regression (β_1) were statistically significant at $p<0.05$. Five types of regression models were tested: (1) linear function, i.e. $\beta_1 x + \beta_0$, (2) logarithmic function, i.e. $\beta_1 \mathrm{Ln}(x) + \beta_0$, (3) polynomial function, i.e. $\beta_1 x^2 + \beta_2 x + \beta_0$, (4) exponential function type I, i.e. $\beta_1 x^a$, and (5) exponential function type II, i.e. $\beta_1 e^{a(x)}$, where x is age (an independent variable), y is a value of an analyzed biochemical characteristic (a dependent variable), β_2 stands for the second coefficient of regression, *a* represents the exponent, and *e* denotes the base of the natural logarithm.

Results

In the two samples, data on STB were normally distributed (K-S test). The statistical characteristics pertinent to longitudinal and cross-section changes with age in STB in the individuals from the PLSA over the period under study are presented in Tables 1 and 2.

The regression analysis of the longitudinal data revealed that in the analyzed group of patients STB levels were positively correlated with age in both sexes (Fig. 1). The best fitting regression model was exponential type II in men ($0.3038e^{0.093x}$, $R^2 = 0.667$) and in women ($0.1838e^{0.0187x}$, $R^2 = 0.950$). No statistically significant differences between STB levels in the consecutive age categories in the analyzed individuals were observed (*t*-test, $p>0.05$). In men, the trough oc-

Table 1. Longitudinal changes with aging in STB levels (in mg/dL) in men and women ($N = 142$) aged 45 to 70 years in the six consecutive age categories

Age	Men ($N = 68$)		Women ($N = 74$)		*t*-test	*p*
	Mean	SD	Mean	SD		
45	0.486	0.445	0.456	0.448	0.40	0.691
50	0.501	0.415	0.437	0.243	1.13	0.259
55	0.460	0.292	0.503	0.334	–0.82	0.416
60	0.499	0.343	0.582	0.570	–1.04	0.301
65	0.583	0.382	0.614	0.536	–0.40	0.692
70	0.593	0.263	0.686	0.343	–1.80	0.074

Table 2. Cross-sectional changes with age in STB levels (in mg/dL) in men and women (N = 225) from four categories of mean lifespan

Age category	Men (N=113)		Women (N=112)		t-test	p
	Mean	SD	Mean	SD		
53	0.582	0.340	0.465	0.238	1.00	0.328
63	0.487	0.264	0.655	0.625	–1.27	0.208
68	0.505	0.442	0.396	0.154	1.47	0.145
76+	0.660	0.909	0.463	0.504	0.87	0.389

curred at the age of 55, while in women the lowest level of STB was observed five years earlier, i.e. at the age of 50. In both sexes, the peak occurred at the age of 70 and a steady increase was observed throughout the period under study (Table 1).

The curve of regression representing cross-sectional changes with age in STB assumed a U-shaped pattern in men but an inverted U-shaped pattern in women (Fig. 2). The best fitting regression model in both sexes was polynomial (for men $0.001x2 – 0.1263x + 4.4524$, $R^2 = 0.999$; for women $–0.0006x^2 + 0.072x – 1.6924$, $R^2 = 0.195$). In men, the lowest level of STB was observed at the age of 63, and in women the trough occurred at the age of 68. The peak in total bilirubin occurred at the age of 76+ in men. Thus, the highest age at death was associated with elevated yet normal bilirubin levels among men ($p<0.05$ for the trend). By contrast, in women the peak occurred at the age of 63, i.e. when in men the trough in STB was noted (Table 2). No significant sex differences in STB levels were found. Starting from the age of 63 years onward, a steady increase in total bilirubin level was observed in men. However, starting from the age of 68 years onward, a gradual increase in total bilirubin level was observed in both sexes. Nevertheless, considering the observed associations between STB levels and mean lifespan in the compared types of data, i.e. longitudinal vs. cross-sectional, as well as on the basis of general tendencies after the age of 68 in the cross-sectional assessment, elevated level of STB seems to be a predictor of longer life expectancy in older men but not necessarily in older women.

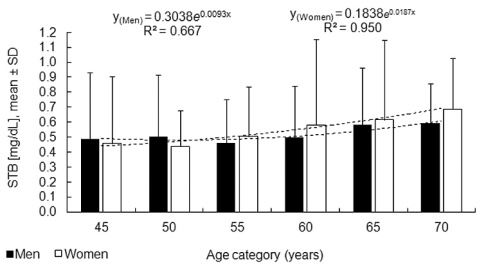

Fig. 1. Longitudinal changes in serum total bilirubin (STB) with aging (bilirubin levels versus age), N =142, in six consecutive age categories, arithmetic means ± standard deviations (SD); models of regression with coefficients of determination (R^2) are shown

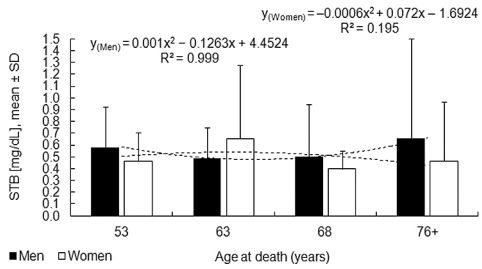

Fig. 2. Cross-sectional changes in serum total bilirubin (STB) with age (bilirubin levels versus age at death), N = 225, in four categories of survival: arithmetic means ± standard deviations (SD); models of regression with coefficients of determination (R^2) are shown

Discussion

The results revealed a statistically significant trend toward higher serum bilirubin levels in men who had the highest age at death, which suggests that elevated but normal bilirubin levels are associated with greater longevity, especially among older men. Interestingly, the highest age at death was not related to elevated bilirubin levels in older women. These findings may have implications for our understanding of the links between serum bilirubin levels, overall health, and long-term survival in older adults, and especially the observed sex differences need further examination.

For many decades, bilirubin has been considered to be only a toxic byproduct of heme catabolism and a yellowish pigment whose significantly increased levels in the blood are associated with poor health and decreased survival. Nevertheless, in numerous studies moderately elevated STB levels have been shown to be linked to increased survival and a decreased risk of cardiovascular events (Djousse et al. 2001; Vitek et al. 2002; Tanaka et al. 2009; Li et al. 2013; Vitek 2012; Oda 2014; Tatami et al. 2014; Agrawal and Sharma 2015; Kundur et al. 2015). Although the characteristics, metabolism, and various effects of this pigment has been of abiding interest to both clinicians and researchers, there is still no general consensus with respect to the association between STB levels and longevity in the elderly (cf. Kapitulnik and Maines 2012; Boland et al. 2014; Kundur et al. 2015).

Moreover, there are several methodological issues surrounding these investigations. First, comparatively little attention has been paid to longitudinal and cross-sectional associations between STB levels and longevity in physically healthy individuals, and there is a paucity of such data. Second, the results of some large and seminal studies on the beneficial effects of elevated serum bilirubin levels on survival in both patients and healthy individuals are mixed (Chen et al. 2008; Perlstein et al. 2008; Boland et al. 2014; Jørgensen et al. 2014; Ong et al. 2014; Li et al. 2015). Further, the predictive value of STB with respect to longevity seems to decrease with age as some prospective longitudinal studies have found that in older adults slightly elevated STB levels do not confer any survival advantage (Boland et al. 2014). Furthermore, other protective effects of bilirubin have been demonstrated mainly *in vitro* or in peripheral tissues, where the well-known effect of neurotoxicity of bilirubin is eliminated. Findings from *in vivo* studies show that higher than normal STB levels are strongly associated with liver-related mortality. On the other hand, a growing body of evidence suggests now that bilirubin is an important antioxidant that has several protective functions and STB levels were reported to be inversely associated with the risk of CVD (Djousse et al. 2001; Vitek et al. 2002; Lin et al. 2006; Vitek 2012; Agrawal and Sharma 2015), arterial calcification and stiffness (Tanaka et al. 2009; Zhang et al. 2012; Li et al. 2013), atherosclerosis (Novotny and Vitek 2003; Oda 2014; 2016; Tatami et al. 2014), inflammation (Erdogan et al. 2006; Vitek and Schwertner 2007; Maruhashi et al. 2012; Wallner et al. 2013), insulin resistance (Lin et al. 2009), and cancer mortality (Temme et al. 2001; Li et al. 2015). Some authors suggest that increased bilirubin levels in healthy subjects are linked to low cancer mortality, especially among older men, probably because the antioxidant activity of this

pigment can help protect against ROS and cancer, which means that measurements of serum bilirubin concentrations might contribute to cancer risk estimation (Temme et al. 2001). Interestingly, some earlier studies have demonstrated that cigarette smoking is associated with both decreased serum bilirubin concentrations and increased risk of coronary heart disease (Schwertner 1998). Moreover, it has been established that patients with Gilbert's syndrome have relatively low presence of CVD, including coronary heart disease (Vitek et al. 2002; Vitek 2012), presumably because hyperbilirubinemia protects against atherosclerosis and myocardial infarction (Boon et al. 2014).

The associations between STB levels and mortality have been investigated in both patients and healthy individuals. However, we know of no studies that have analyzed the links between STB levels and longevity in healthy older individuals studied longitudinally for several decades. The beneficial effects of elevated but normal serum bilirubin levels on long-term survival have been described in the medical literature and the longevity benefits of greater antioxidant capacity of the plasma due to higher STB levels are supported by compelling evidence and a variety of studies. For example, it has been shown that mild to moderately elevated levels of circulating bilirubin are associated with reduced risks of cardiovascular mortality, metabolic syndrome, and type 2 diabetes (Schwertner et al. 1994; Hopkins et al. 1996; Djousse et al. 2001; Ohnaka et al. 2010; Ajja et al. 2011; Choi et al. 2013; Boon et al. 2014; Bossard et al. 2014; Jung et al. 2014; Nano et al. 2016). Moreover, the results of some earlier comparative studies have suggested that smoking is associated with decreased serum bilirubin levels and increased risk of CVD due to lower antioxidant concentrations and higher levels of oxidized blood lipid and lipoprotein concentrations (Schwertner 1998).

Currently, several independent mechanisms through which higher but normal bilirubin levels may reduce the risk of CVD and premature death are recognized. For example, high arterial blood pressure, which is associated with increased oxidative stress (Baradaran et al. 2014), is a strong predictor of increased mortality and shortened life expectancy (Chobanian et al. 2003; Franco et al. 2005). Hypertension is a major risk factor of atherosclerosis, stroke, chronic kidney failure (CKF), heart failure, CHD, and myocardial infarction. Several studies have shown that bilirubin may decrease the level of oxidative stress, protect against atherosclerosis, ameliorate renal hemodyncamics, thereby protecting against hypertension (Stec et al. 2012; Kundur et al. 2015). As regards STB levels in hypertensive patients, some investigations have shown that subjects with non-dipper hypertension had significantly lower STB levels and higher leukocyte count compared to patients with dipper hypertension (Demir et al. 2014). The lack of studies on longitudinal and cross-sectional associations between STB levels and longevity in physically healthy individuals presumably results from the fact that such longitudinal data remain scarce. As regards the links with dyslipidemia, bilirubin inhibits the process of oxidation of low-density lipoproteins (LDL) and reduces the formation of oxidized LDL (Boon et al. 2014; Oda 2014; 2016). Individuals with elevated STB levels tend to have lower levels of total cholesterol, LDL, and triglycerides but

higher levels of HDL, and HDL-cholesterol is an independent negative risk factor for cardiovascular mortality (Bulmer et al. 2013). Serum bilirubin level within the normal range has been shown to be inversely related to the ApoB/ApoA-1 ratio (Wallner et al. 2013), which is considered to be a superior parameter for predicting the risk of cardiovascular events as it reflects the cholesterol transport and has been shown to be strongly related to some CVD risk factors and cardiovascular events such as myocardial infarction and stroke (Walldius et al. 2004; Sniderman et al. 2006; Lima et al. 2007). Furthermore, some authors suggest that elevated serum bilirubin levels in hyperbilirubinemic subjects are associated increased activity of endothelial nitric oxide synthase (eNOS), which stimulates vasodilatation and is a fundamental determinant of cardiovascular homeostasis (Kundur et al. 2015). Bilirubin also suppresses the formation of advanced glycation-end products, inhibits the proliferation and migration of endothelial cells, and decreases platelet hyperactivity through several independent mechanisms (Peyton et al. 2012). Thus, it ameliorates endothelial function, decreases the risk of dyslipidemia and atherosclerosis, and therefore protects against CVD. Recent investigations have also shown that STB affects some important factors involved in the inflammatory response such as the transcription factor NF-κB, IL-2, IL-6, TNF-α, INF-γ, and CRP (Kundur et al. 2015). Other studies suggest that bilirubin inhibits the toll-like receptor 4-mediated up regulation of inducible nitric oxide synthase (iNOS) by preventing activation of HIF-1α through scavenging of Nox-derived ROS (Idelman et al. 2015). Moreover, bilirubin has been shown to suppress INF-β release via a ROS-independent mechanism. Thus, it is clear that bilirubin may exert anti-inflammatory effects on the cells of the vasculature and the MPS, which might confer a longevity advantage because it ameliorates chronic systemic inflammation, a process that contributes to organismal senescence. Furthermore, recent studies show that it scavenges chloramines and inhibits myeloperoxidase-induced protein/lipid oxidation in hyperbilirubinemic serum (Boon et al. 2015). Remarkably, endogenously elevated bilirubin levels protect from myocardial infarction, which could explain why hyperbilirubinemic individuals who are healthy have low cardiovascular mortality (Bakrania et al. 2014; 2017). Moreover, it has been established that an increase in total bilirubin correlates negatively with the development of hyper-LDL cholesterolemia, and this protective effects of higher bilirubin levels can contribute to the beneficial effects on CVD in addition to the well-known role as a potent antioxidant (Wu et al. 1994; 1996; Sedlak et al. 2009; Oda 2014; 2016). Furthermore, the protective role of bilirubin is not confined to these effects as it was earlier demonstrated that bilirubin displays also antiviral activity (Santangelo et al. 2012; Schmidt et al. 2012), improves renal hemodynamics and blood pressure in an animal model of hypertension (Stec et al. 2012), and has some beneficial effects in cardiovascular, inflammatory, and pulmonary diseases (Erdogan et al. 2006; Vitek and Schwertner 2007; Kapitulnik and Maines 2012; Maruhashi et al. 2012; Ryter 2012; Wegiel and Otterbein 2012; Wallner et al. 2013).

In regards to sex differences, our results support previous research suggesting that older men with higher but normal STB levels have a longevity ad-

vantage over those with lower but normal levels. It is well known that men are more susceptible to atherosclerosis and CVD compared to women and they are more likely to develop these conditions at a relatively younger age, whereas women's risk increases significantly following menopause. It is well known that women have other protective mechanisms ensuring their survival in adulthood and making them less dependent on serum bilirubin (e.g. the mechanisms associated with gender gap in longevity such as the genetic and molecular benefits from the homogametic state, higher levels of estrogens, lower levels of testosterone, the cessation of reproduction, etc.). Therefore, the beneficial effects of elevated STB levels on long-term survival are more pronounced and perspicuous in older men since their survival is more dependent on such a single factor compared to women whose longer lifespan is ensured by other adaptive mechanisms linked to the gender gap in longevity, including the molecular mechanisms of ROS production and scavenging, the insulin/IGF-1 signaling pathway, hyperfunction of mTOR, the hormonal milieu, telomere length and attrition, and other processes that are responsible for longer life expectancy in women (for a review, see Rincon et al. 2005; Jiang et al. 2007; Kirkwood 2010; Borysławski and Chmielewski 2012; Chmielewski 2012; 2016; 2017; Regan and Partridge 2013; Chmielewski and Borysławski 2016; Chmielewski et al. 2016b).

This study has certain limitations that should be acknowledged. By design, it cannot prove that elevated bilirubin levels confer a longevity advantage. Based on findings from other studies, we discuss the protective role of higher serum bilirubin levels with regard to CVD, atherosclerosis, hypertension, systemic inflammation, etc., but the present study does not permit causal conclusions. However, it does provide an insight into what might be beneficial in terms of long-term survival and it suggests that there are sex differences that need further examination. It should be remembered that the study sample is a fairly specific group of subjects. They lived for many years at the medical institution that provided care for older and ill people and some of them had mental problems. Therefore, the results should be interpreted with the utmost caution. More research on serum bilirubin level as a negative risk factor for CVD and a predictor of long-term survival in older adults needs to be performed.

Conclusions

The results of the present study are in line with previous investigations and suggest that elevated but normal STB might confer a survival advantage, especially among older men. However, it is also possible that the positive relationship between STB and age at death in the studied population was not causal but coincidental. Further studies are needed to elucidate the direct mechanisms that link higher but normal STB levels to greater longevity in older adults as well as to unravel the mystery of sex differences in the protective function of serum bilirubin against CHD and other cardiovascular events.

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Authors' contributions

PC conceived the study, performed bibliographic search, conducted the statistical analysis, interpreted the results, wrote the manuscript, revised it and was a proofreader. BS helped analyze and interpret the data, provided comments and advice on the overall study and critically revised the article. JC, KC and KB collected the data and supervised the research. All authors take responsibility for integrity of the work, including data analysis.

Conflict of interest

The authors declare that there is no conflict of interests.

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