Original Article

Association of circulating total bilirubin level with ischemic stroke: a systemic review and meta-analysis of observational evidence

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Background: Circulating total bilirubin is a biomarker of ischemic stroke and may serve as a potential prognostic factor. It is imperative to systemically evaluate the correlation between circulating total bilirubin and risk for stroke. This systematic review and meta-analysis investigated the relationship between total serum bilirubin and risk for stroke.

Methods: Studies published before 30 June 2017 were searched in four databases (PubMed, EMBASE, Web of Science and Cochrane Central). Additional studies were searched by reviewing references and contacting authors. Cohort, cross-sectional and case-control studies in adults that examined the association between serum total bilirubin and stroke were included irrespective of language and date of publication. The primary outcome of this study was ischemic stroke, and the secondary outcome was stroke. Abstract and full-text were reviewed by two independent reviewers, and disagreement was resolved by consulting a third reviewer. Data were extracted by two independent reviewers using a pre-designed data collection form.

Results: Eleven observational studies (5 prospective and 6 cross-sectional studies) involving 131,450 subjects were included for analysis. In four studies with 83,380 subjects, the relationship between circulating total bilirubin and ischemic stroke was investigated, ischemic stroke was found in 2,496 patients, and the total odds ratio (OR) of the highest bilirubin and the lowest bilirubin for the occurrence of ischemic stroke was 0.66 (95% CI: 0.58–0.74). Eleven studies with 131,450 subjects explored the correlation between bilirubin and stroke, stroke was reported in 5,060 patients, and the total OR of the highest bilirubin and the lowest bilirubin for the occurrence of stroke was 0.73 (95% CI: 0.68–0.79). A stratified analysis based on the gender showed that the total bilirubin level in males correlated with ischemic stroke or stroke, which was not noted in females.

Conclusions: The available studies support an inverse association between circulating total bilirubin and risk for ischemic stroke and stroke in males. Prospective studies with large sample size are needed to establish the role of circulating bilirubin in the prevention of stroke.

Keywords: Bilirubin; ischemic stroke; systemic review; meta-analysis

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Introduction

Stroke accounts for about 1/3 of causes of death worldwide (1). Of great importance, stroke has been the leading cause of death in China in recent years (2). Although much progress has been made in identifying risk factors for stroke, little is known about factors that modulate stroke risk. Serum bilirubin is a normal metabolite of the heme. Traditionally, bilirubin is regarded as a toxic metabolite. In clinical practice, serum bilirubin has been used a marker of liver and hematopoietic diseases. In recent years, a variety of studies have indicated that bilirubin may exert anti-oxidative, anti-inflammatory and neuroprotective effects (3). Some studies have revealed that circulating total bilirubin is negatively related to the risk for ischemic stroke (4-6). Perlstein *et al.* conducted a cross-sectional study on 13,214 adult participants in whom 453 reported a history of stroke; their results showed an increment of total bilirubin by 1.71 μmol/L could reduce the incidence of ischemic stroke by 9% (7). However, there is still controversy on this issue. Kurzepa *et al.* found serum total bilirubin had a weak relationship with the risk for ischemic stroke (8). Kunutsor *et al.* investigated the correlation of circulating total bilirubin with the risk of incident cardiovascular disease in 12 prospective studies involving 9,378 subjects by meta-analysis, but the stroke was not used as an endpoint (9). Li *et al.* investigated the serum total bilirubin in a Chinese population with acute stroke as an endpoint, but only case-control studies were included (10).

Considering that serum total bilirubin is an important biomarker of stroke and has a potential value in the prediction of outcome of stroke, it is imperative to systemically evaluate the correlation between circulating total bilirubin and risk for stroke. This systemic review and meta-analysis was conducted to investigate the relationship between circulating total bilirubin and risk for ischemic stroke.

Methods

Literature search

This study was registered at PROSPERO [https://www.crd.york.ac.uk /PROSPERO/(CRD42017075988)] and reported in accordance with the PRISMA (11) and MOOSE (12) guidelines. PubMed, EMBASE, Web of Science and Cochrane Central were searched for the relevant studies published before 30 June 2017. Studies that examined the association between circulating bilirubin level and stroke in adults (≥18 years) were included for further analysis. Terms related to bilirubin (such as "hyperbilirubin") were combined with key terms related to the outcome (such as "stroke"); no language restriction was applied. The exact search strategy and rationale are shown in the Supplementary file 1.

Study selection and inclusion criteria

Cohort, case-control and cross-sectional studies that examined the association between blood bilirubin level (total bilirubin, direct or indirect bilirubin levels) and stroke were included for further analysis. Two reviewers (P Zhong and D Wu) independently reviewed the title, abstract and full-text, and then input data into a data extraction form. A third reviewer (X Wang) approved the studies selected. When the data were missing, the principal investigators were contacted for further information. If the principal investigator could not provide the missing data, the study was excluded. Finally, the full texts of included studies were retrieved. The primary outcome of this study was ischemic stroke, and the secondary outcome was stroke.

Data extraction

Two investigators independently extract the data and filled them in the form above. Following information was recorded: the first author, publication date, study design, study name, geographical location, population source, time of baseline survey, sample population, sample source (serum/plasma), sample nature (fresh or frozen and storage temperature), assay type, case definition, sample size, sex, mean age at baseline, number of outcome events, summary statistics (using a standardized abstraction form) and degree of adjustment for potential confounders (*Table S1*).

Adjustments were classified as: "+" when risk estimates were adjusted for age and sex; "++" when it was further adjusted for potential risk factors [such as blood pressure, body mass index (BMI)], history of diabetes mellitus, smoking, drinking, excise status and medication); and "+++" when it was further adjusted for inflammatory markers [such as C-reactive protein (CRP)], liver enzymes and blood lipids. The estimates reported with the greatest degree of adjustment were also recorded. If risk estimates were not available, the authors were contacted for further information (*Table S1*).

Quality assessment

Study quality was evaluated using the Newcastle-Ottawa

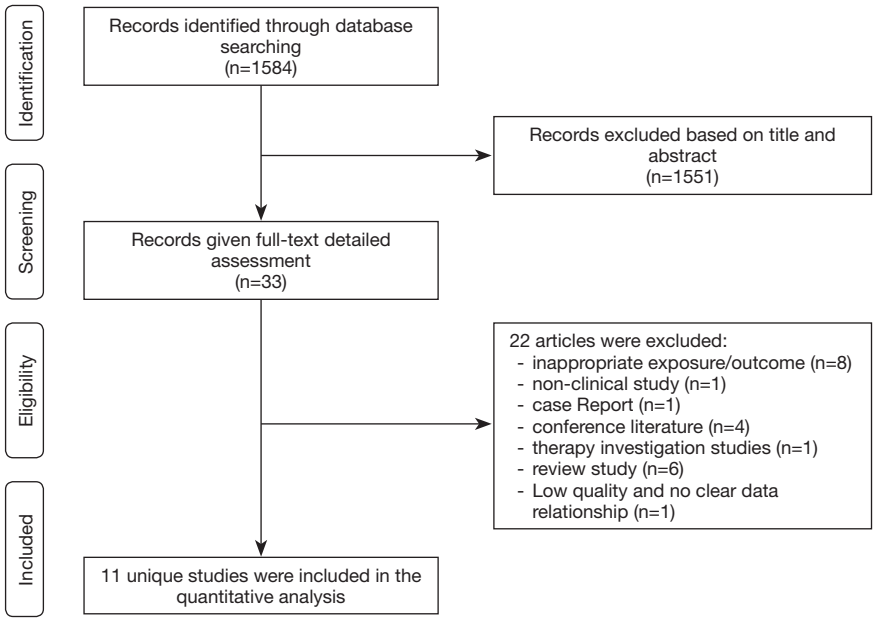

Figure 1 Flow chart of the study collection for the present review and meta-analysis.

Scale (NOS) for cohort studies and case-control studies (13). The quality of studies was determined according to the selection criteria for participants, comparability of cases and controls, and exposure and outcome assessments. For cross-sectional studies, quality was assessed using the NOS modified for cross-sectional studies (14). Over all, a score ≥5 indicated adequate quality for inclusion in the present review (*Tables S2*,*S3*).

Statistical analysis

The effect of bilirubin levels on the risk for stroke was evaluated on the basis of included studies. Odds ratio (OR) was used for the evaluation of the relationship between bilirubin levels and stroke. Because different results were reported in the studies [OR per standard deviation (SD) and OR per quartile], the results were first transformed into OR between upper tertile to lower tertile (15,16), and then the differences observed between groups were expressed as OR with corresponding 95% confidence interval (CI). Individual adjusted OR and 95% CI were extracted or calculated first, and then log OR and its corresponding standard error were estimated for pooling. The I^2 statistic and χ^2 test were employed to assess the variability across studies attributable to heterogeneity beyond chance. A P value greater than 0.10 in the χ^2 test was interpreted as low-level heterogeneity (17). A pooled effect was calculated with a fixed-effects model when there was no statistically significant heterogeneity; otherwise, a random effects model was employed. Subgroup was used to determine the robustness across different groups. Publication bias was evaluated by Egger test and funnel plot. Trim and fill method was used for adjustment once bias was present (18). A value of two-sided P value less than 0.05 was considered statistically significant. Statistical analysis was conducted with the Review Manager (version 5.1 for Windows, Cochrane Collaboration, Oxford, UK, 2011) and STATA 11.0 (Stata Corporation, Lakeway, Texas, USA).

Results

A total of 1,584 studies were identified after initial searching. After reviewing the title and abstract, the full texts of 33 studies were obtained, in which 11 studies were included for further analysis and 22 studies were excluded because they did not meet the inclusion criteria (*Figure 1*). In 11 studies, there were five prospective studies (4,9,19-21) [1 with Mendelian randomization (MR) (16)] and six cross-sectional studies (5,6,22-25). Of these studies, there were 131,450 subjects, including 58,168 females (44.3%). The general features of included studies are shown in *Table 1*. In the included studies, the serum bilirubin was detected in a fasting condition (*Table S1*).

Table 1 General features of included studies

First author and year of publication	Name of study or source of participants	Location of study	Year(s) of study	Age (years) (range or mean ± SD)	Type of study/ follow-up if prospective	Diagnosis	Total no. of participants	Male (%)	No. of cases	Risk factors adjusted	Definition outcome	Study quality
Ekblom *et al.*, 2010	NSHDSC	Sweden	1986–2002	25–74	Prospective/4.9 years	IS	693	55.0	231	Age, BMI, systolic blood pressure, smoking, apolipoprotein B/A1, diabetes and hsCRP	Northern Sweden WHO MONICA Project	7
Kimm *et al.*, 2009	RHE	Korean	1994–2007	30–89	Prospective/14 years	IS	78,724	60.3	1,189	Age, smoking (nonsmoker, ex-smoker, current smoker), alcohol (yes or no), exercise (yes or no), ALT, GGT, total cholesterol, type 2 diabetes, and hypertension	ICD-10	9
						HS	78,724	55.8	473	Age, smoking (nonsmoker, ex-smoker, current smoker), alcohol (yes or no), exercise (yes or no), ALT, GGT, total cholesterol, type 2 diabetes, and hypertension	ICD-10	9
						AS	78,724	57.9	1,964	Age, smoking (nonsmoker, ex-smoker, current smoker), alcohol (yes or no), exercise (yes or no), ALT, GGT, total cholesterol, type 2 diabetes, and hypertension	ICD-10	9
Kunutsor *et al.*, 2015	PREVEND	Netherlands	1997–2009	28–75	Prospective/9.3 years	AS	7,222	48.5	159	Age and sex, smoking status, history of diabetes, systolic blood pressure, total cholesterol, high-density lipoprotein cholesterol, and BMI, alcohol consumption, glucose, and triglycerides, γ-glutamyl transferase and alanine aminotransferase	ICD-9	9
Lee *et al.*, 2017	RHE	Korea	2004–2016	>18	Prospective/7.0 years	AS	5,599	66.9	806	Age, sex	ICD-10	9
Li *et al.*, 2014	RHE	China	2009–2010	30–69	Cross-sectional	SCI	2,856	63.9	343	Age, sex, BMI, smoking status, alcohol consumption, systolic blood pressure, DBP, FPG, TC, triglyceride, high-density lipoprotein cholesterol, LDL-C, aspartate aminotransferase, alanine aminotransferase, γ-glutamyl transpeptidase, eGFR, DM, hypertension, and current use of statins, aspirin, angiotensin-converting enzyme inhibitor/angiotensin II receptor antagonist calcium channel blocker, and hypoglycemic drugs	a	8

Table 1 *(continued)*

Table 1 *(continued)*

First author and year of publication	Name of study or source of participants	Location of study	Year(s) of study	Age (years) (range or mean ± SD)	Type of study/ follow-up if prospective	Diagnosis	Total no. of participants	Male (%)	No. of cases	Risk factors adjusted	Definition outcome	Study quality
Mahabadi *et al.*, 2014	HNRs	Ruhr	2000–2003	45–75	Prospective/9.1years	AS	3,553	44.0	95	Age, gender, BMI, systolic blood pressure, LDL, HDL, antihypertensive medication, lipid-lowering medication, diabetes, smoking status, and CAC score	b	9
Oda and Kawai, 2012	RHE	Japan	2008–2010	>18	Cross-sectional	AS	5,444	62.0	67	Age, aspartate aminotransferase, alanine aminotransferase, gamma glutamyl transferase, current smoking, physical activity, and daily drinking	NS	6
Perlstein *et al.*, 2008	NHANES	United States	1999–2004	≥20	Cross-sectional	AS	13,214	48.1	335	Age, sex, race/ethnicity, smoking, hypertension, total to HDL cholesterol ratio, and diabetes	Questionnaire	7
Zhou *et al.*, 2014	Screening	China	2010–2012	>18	Cross-sectional	SIVD	1,098	45.7	733	Age, sex and vascular risk factors	c	8
Jørgensen *et al.*, 2014	SCOUT	16 countries	2003–2009	≥55	Cross-sectional	NFS	9,742	57.4	221	Sex, age and sibutramine/placebo	d	8
Ren *et al.*, 2016	Screening	China	2013	18–80	Cross-sectional	AS	6,713	56.6	106	Age, duration of diabetes, sex, body mass index, systolic blood pressure, diastolic blood pressure, glycated hemoglobin, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, triglyceride, self-monitoring, log-transformed urinary albumin to creatinine ratio, and drug use and complications	NS	6

a: (I) a focal hyperintense lesion (≥3 mm) in the T2 and FLAIR and hypointense lesion in the T1 image; (II) the absence of signs and symptoms that could be explained by lesions observed by MRI; and (III) no history of clinical stroke. Periventricular white matter lesions were distinguished from SCI based on the high signal intensity on FLAIR2; b: focal neurological deficits over a period of >24 h of presumed cerebrovascular origin; c: subcortical white matter hyperintensity or lacunar infarct on T2-weighted images, and/or fluid attenuated inversion recovery (FLAIR) images and without any acute stroke appearances in diffusion weighted imaging (dWi); d: the presence of acute focal neurological deficit thought to be of vascular origin with symptoms and/or signs lasting more than 24 h. NSHDSC, the Northern Sweden Health and Disease Study; RHE, routine health examination; NS, not stated; N/A, non-available; BMI, body mass index; ALT, alanine transaminase, AST, aspartate aminotransferase; SCOUT, Sibutramine Cardiovascular Outcomes trial; ICD-10, the International Classification of Diseases 10th Revision codes; PREVEND, Prevention of Renal and Vascular End-stage Disease; ICD-9, the International Classification of Diseases, Ninth Revision codes; IS, ischemic stroke; HS, hemorrhagic stroke; AS, all stroke; SCI, silent cerebral infarction; HNRs, the Heinz Nixdorf Recall study; NHANES, the National Health and Nutrition Examination Survey; SIVD, subcortical ischemic vascular disease; NFS, non-fatal stroke.

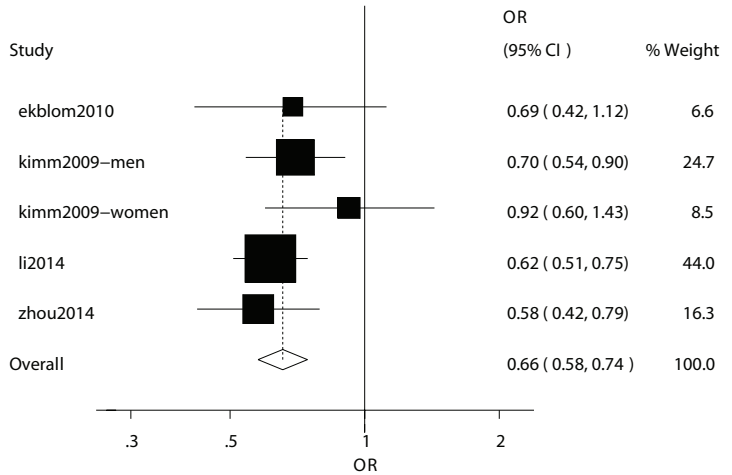

Figure 2 Studies on the association of serum bilirubin level with ischemic stroke.

Correlation between bilirubin level and ischemic stroke

Four studies (4,5,19,25) investigated the correlation between bilirubin level and ischemic stroke, including two cross-sectional studies (5,25) and two prospective studies (4,19) with involvement of 83,380 subjects. In these studies, ischemic stroke was found in 2,496 patients from Korea, China and Sweden, and the median age was 56.56 years. There was no heterogeneity among four studies (I^2=0.0%, P=0.451), and thus fixed effects model was used for further analysis. In four studies, the total OR of the highest bilirubin level and the lowest bilirubin level for ischemic stroke was 0.66 (95% CI: 0.58–0.74) (*Figure 2*).

In two prospective studies (4,19), adjustment was done for the potential risk factors and the total OR of the ratio of highest bilirubin level to lowest bilirubin level for ischemic stroke was 0.74 (95% CI: 0.61–0.91), showing significant difference. In the two cross-sectional studies (5,25), after adjustment for potential risk factors, the total OR of the ratio of highest bilirubin level to lowest bilirubin level for ischemic stroke was 0.61 (95% CI: 0.51–0.71), showing significant difference. There was no heterogeneity in the prospective studies (I^2=0.0%, P=0.540) and cross-sectional studies (I^2=0.0%, P=0.740).

Stratified analysis was done according to the gender. The OR was 0.72 for males (95% CI: 0.57–0.91) with significant difference and 0.78 for females (95% CI: 0.53–1.16) without significant difference. In addition, significant difference was not observed between patients aged ≥60 years and those younger than 60 years; subgroup analysis of studies with involvement of ≥2,000 subjects and those with no more than 2,000 subjects also showed no significant difference (*Table S4*).

Correlation of bilirubin level with stroke

In 11 studies, the correlation between bilirubin level and stroke was investigated (4-7,9,19-22,24,25). There were six cross-sectional studies (5-7,22,24,25) and five prospective studies (4,9,19-21) with involvement of 131,450 subjects. In these studies, stroke was reported in 5,060 patients, and the subjects in these studies were from China, Japan, Korea, Sweden, Netherlands, Germany, and USA. There was heterogeneity among 11 studies (I^2=51.4%, P=0.016), and thus random effects model was used (*Figure 3*).

In six cross-sectional studies (5,6,22-25), the total OR of the ratio of highest bilirubin level to lowest bilirubin level for stroke was 0.65 (95% CI: 0.54–0.79) after adjustment for potential risk factors, showing significant difference. In addition, in five prospective studies (4,9,19-21), the total RR of the ratio of highest bilirubin level to lowest bilirubin level for stroke was 0.72 (95% CI: 0.56–0.92) after adjustment for potential risk factors, showing significant difference. Heterogeneity was noted in six cross-sectional studies (I^2=59.3%, P=0.022) and prospective studies (I^2=48.0%, P=0.087).

Stratified analysis based on the sex showed the OR was 0.75 (95% CI: 0.62–0.90) for males and 0.57 (95% CI: 0.28–1.17) for females, showing no marked differences (*Table S5*). In addition, significant difference was not observed between patients aged ≥60 years and those younger than

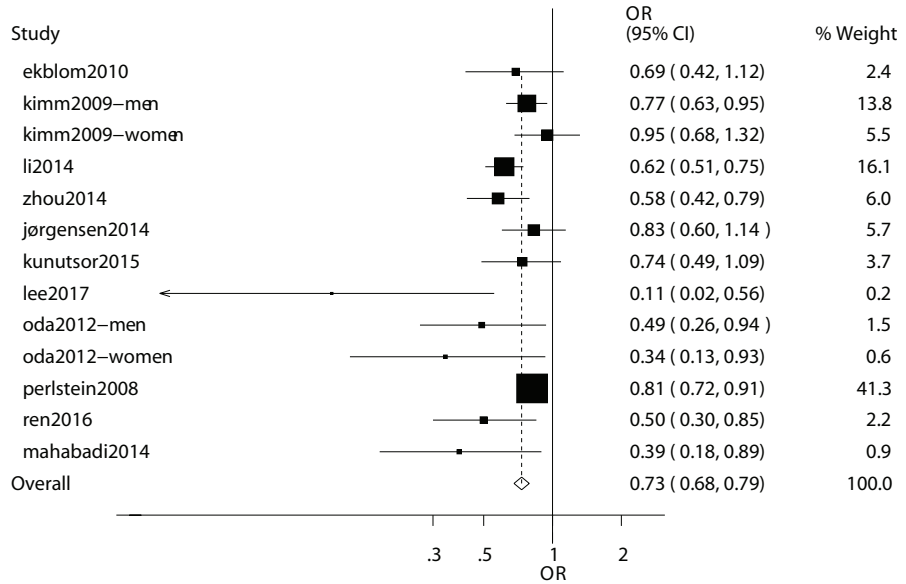

Figure 3 Studies on the association of serum bilirubin level with stroke.

60 years; subgroup analysis of studies with involvement of ≥2,000 subjects and those with no more than 2,000 subjects also showed no significant difference (*Table S5*).

Assessment and management of risk for bias

Results of the Egger test for ischemic stroke (P=0.317) suggested that any publication bias across included studies was unlikely (*Figure S1*). Results of the Egger test for stroke (P=0.011) suggested that any publication bias across included studies was likely (*Figure S1*). Trim and fill method was used for the adjustment. After adjustment, the total OR of ratio of highest bilirubin level to lowest bilirubin level for stroke/ischemic stroke was 0.638 (95% CI: 0.565–0.721; P≤0.0001), showing significant difference.

Discussion

This meta-analysis was conducted to systemically investigate the relationship between circulating total bilirubin level and risk for ischemic stroke and stroke on the basis of available observational studies. The cross-sectional studies and prospective studies indicated that the serum bilirubin level was negatively related to the risk for ischemic stroke and stroke after complete adjustment.

The negative relationship between circulating total bilirubin level and risk for ischemic stroke/stroke was consistent with previously reported. Studies have revealed that bilirubin level may serve as a predictor of some vascular events such as coronary heart disease (26), peripheral artery disease (23), amputation of diabetes mellitus patients (27), diabetic nephropathy (28) and overall mortality (29). Gilbert's syndrome (GS) is a relatively common condition, inducing a benign, non-hemolytic, and unconjugated hyperbilirubinemia. GS patients often present mildly elevated plasma anti-oxidative capacity due to the elevation of unconjugated bilirubin (UCB) and reductions in the thiols and glutathione. Interestingly, the incidence of cardiovascular disease and risk for death from cardiovascular diseases are remarkably reduced in GS patients (30), which might be explained by anti-oxidative capability of bilirubin according to available findings. Baranano *et al.* (31) proposed a mechanism for the cytoprotective action of bilirubin based on an amplification cycle whereby the bile pigment, in the presence of albumin, is itself oxidized to biliverdin by reactive oxygen species (ROS) and, then, recycled by biliverdin reductase back to bilirubin. This hypothesis was challenged by Maghzal *et al.* (32) and McDonagh (33) who demonstrated that the oxidation of both bilirubin and albumin-bound bilirubin by peroxyl radicals or hydrogen peroxide largely degrades bilirubin and generates only negligible amounts of biliverdin.

Stroke can be divided into ischemic stroke and hemorrhagic stroke. The present meta-analysis showed

circulating total bilirubin level was negatively related to the incidence of stroke. In the included studies, only one focused on the relationship between total bilirubin level and hemorrhagic stroke, while results showed no significant relationship between them. Among all included studies, only one study focused on relationship between total bilirubin level and hemorrhagic stroke, while results showed no significant relationship between them (34). Studies on the correlation between bilirubin and hemorrhagic stroke mainly focus on the relationship of bilirubin level with the prognosis of hemorrhagic stroke. Dohi *et al.* (35) found the serum bilirubin increased significantly in the early phase of cerebral hemorrhage and subarachnoid hemorrhage and they speculated that serum bilirubin concentration might serve as a useful marker of oxidative stress in hemorrhagic stroke patients. However, there is evidence showing that a large amount of unbound bilirubin is produced after cerebral hemorrhage, which may cause serious damage to the brain and induce brain edema (36), leading to the deterioration of cerebral hemorrhage. Among included studies, clinical types of ischemic and hemorrhagic stroke were not classified in six studies. The pathophysiology of ischemic stroke is different from that of hemorrhagic stroke, and thus it is necessary to investigate the correlation between serum bilirubin level and stroke of different types. Moreover, ischemic stroke can also be divided into different clinical subtypes. Lin *et al.* investigated 628 patients with ischemic stroke according to the clinical characteristics and imaging findings (37). In this study, etiological grouping was done according to the Trial of Org 10172 in Acute Stroke Treatment (TOAST), and their results showed the increase in the serum bilirubin level was an independent predictor of cardiogenic brain embolism. In the included studies, Li *et al.* conducted a cross-sectional study with involvement of 2,865 subjects. Their results showed the total bilirubin level in the silent cerebral infarction (SCI) group was significantly lower than in the non-SCI group, and multivariate analysis indicated high total bilirubin level was independently related to the reduced risk for SCI (OR 0.925; 95% CI: 0.897–0.954; P<0.001). This indicates that total bilirubin level can be used as a new biomarker of asymptomatic cerebral infarction (5). More studies are needed to confirm the relationship between serum total bilirubin level and risk for stroke of different types.

Our meta-analysis showed the relationship between serum total bilirubin level and incidence of ischemic stroke/stroke was statistically significant in males, but not in females. Kimm *et al.* investigated 78,724 healthy subjects in a prospective study with the median duration of follow-up of 9.4 years. Their results showed patients with high serum bilirubin level had a reduced incidence of ischemic stroke as compared to those with low serum bilirubin level, and the negative relationship between serum bilirubin level and ischemic stroke was only noted in males, not in females (4). In the whole population, the serum bilirubin level in males was slightly higher than in females (23). The difference in serum bilirubin between females and males might be ascribed to the differences in serum estrogen, iron storage in males, higher heme oxygenase in males and lifestyle [drinking, smoking and supplement of anti-oxidants (such as vitamin C)] between them (38,39). Clinical and experimental studies indicated significant differences of liver function between different genders when circulation was poor (39). Under circulation stress status, male sex hormone could impact liver function, while female sex hormone could protect liver function (39). Thus, it is necessary to investigate the relationship of bilirubin with stroke in females and males.

The negative relationship between serum bilirubin and incidence of ischemic stroke may be explained by the anti-oxidative capability of the bilirubin (3), bilirubin induced inhibition of low-density lipoprotein (LDL) oxidation (40), anti-atherosclerotic capability of bilirubin and vascular structure and reactive pathways reported in recent years (41). Serum bilirubin has been confirmed to be a major contributor to total antioxidant capacity of the plasma (42). There is evidence showing that serum bilirubin can reduce the transport and generation of LDL, increase the transport of cholesterol from the blood vessels to the plasma, promote the lipolysis and bile clearance and serve as a physiological lipid antagonist affecting lipid metabolism (43). Animal studies have confirmed bilirubin may phosphorylated ERK1/2 via nNOS/NO/cGMP pathway, exerting neuroprotective effects.

Our study showed not only cross-sectional studies but also prospective studies indicated serum total bilirubin level was negatively related to the incidence of ischemic stroke, however they could not confirm the causal relationship: low serum bilirubin may be present before or after the ischemic stroke. MR uses genetic variants randomly allocated according to the Mendel's second law without any preconception (44,45). MR refers to the use of genetic variants to develop causal inferences from observational data, if the variant genotype is associated with the phenotype and the variant genotype associated with the risk of interest through the phenotype. MR experiments are also warranted to investigate the potential causal implications of bilirubin in the stroke outcomes. Lee *et al.* suggested no causal relationship between a common genetic variant

(rs6742078) of the *UGT1A1* gene, robustly associated with increased circulating total bilirubin level, and risk for stroke in a Korean population, using the MR method (20). However, there are considerable ethnic differences in the genetic association of bilirubin levels (46). Besides, there is the potential existence of pleiotropy. Nevertheless, these results should be interpreted with caution, given the large sample size required for MR analysis and the plausibility of the instrumental variable assumption (47).

A variety of studies have investigated the relationship between serum bilirubin level and risk for cardiovascular diseases or conditions including atherosclerosis (48), hypertension (49), diabetes mellitus (50), metabolic syndrome (51), smoking (27) and excise (52). In patients with appropriate increase in the bilirubin level, bilirubin level may serve as an anti-thrombotic agent to inhibit platelet activation, reducing mortality (30). Heme-1 inducer, *UGT1A1* gene antagonist, and drugs that can reduce liver glucuronidation activity and hepatocyte uptake may induce the mild to moderate increase in the bilirubin level, which may be employed as a promising strategy for the improvement of cardiovascular outcomes (53-56). This means that it is necessary to conduct more prospective studies with large sample size to confirm unresolved issues.

One of advantages in this study was the inclusion of both cross-sectional studies and prospective studies. In the prospective studies, the sample size was large (≥2,000) and the duration of follow-up was relatively long (max: 14 years) in 4 studies except for small sample size (<2,000) and short duration of follow-up (4.9 years) in two nested case-control studies. In addition, not only Asian population but also European population were studied in the studies included. Most included studies adjusted the potential risk factors, and the methodological assessment was satisfactory. In our study, publication bias was noted in studies on the relationship between serum bilirubin level and risk for stroke, but Trim and Fill method was used for adjustment. After adjustment, the results remained unchanged, suggesting that results of meta-analysis were reliable.

Of course, there were limitations in this study. First, the included studies could not confirm the causal relationship between bilirubin level and ischemic stroke/stroke. The subgroup analysis has its own limitations, and meta-regression analysis is infeasible to evaluate the relationship of bilirubin level with different variables of ischemic strokes. In addition, the number of studies on the relationship between bilirubin level and hemorrhagic stroke is still small, which limits the elucidation of relationship between bilirubin level and hemorrhagic stroke. Moreover, few studies focused on the direct bilirubin and/or indirect bilirubin, which limits the elucidation of relationship of stroke with bilirubin of different types.

Taken together, the cross-sectional studies and prospective studies included in this analysis support the negative relationship between serum bilirubin level and risk for ischemic stroke/stroke. The biological basis of this relationship is required to be confirmed in more studies with new methods (such as metabolomics) (53). In addition, more prospective studies with large sample size are needed to confirm the negative relationship between serum bilirubin level and risk for ischemic stroke.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Supplementary

Supplementary file 1

Search strategy

The following search strategy, using a combination of controlled vocabulary (MeSH) and free text terms, was used for MEDLINE (PubMed), and was modified for the other databases searched:

1. "stroke"[MeSH Terms]
2. "cerebral infarction"[MeSH Terms]
3. "brain ischemia"[MeSH Terms]
4. stroke* OR (brain infarct*) OR (cerebral infarct*) OR (brain ischemia) OR (brain ischaemia) OR (cerebral ischemia) OR (cerebral ischaemia) OR (cerebrovascular accident*) OR cva OR (cerebrovascular disorder*) OR (cerebrovascular disease*) OR (brain embol*) OR (brain thromb*) OR (cerebral embol*) OR (cerebral thromb*) OR (intracerebral embol*) OR (intracerebral thromb*) OR (intracranial embol*) OR (intracranial thromb*) OR apoplexy OR ictus
5. "cerebrovascular disorders"[MeSH Terms]
6. "cerebral hemorrhage"[MeSH Terms]
7. "intracranial hemorrhages"[MeSH Terms]
8. (brain haemorrhage*) OR (brain hemorrhage*) OR (cerebral haemorrhage*) OR (cerebral hemorrhage*) OR (intracerebral haemorrhage*) OR (intracerebral hemorrhage*) OR (intracranial haemorrhage*) OR (intracranial hemorrhage*)
9. 1 OR 2 OR 3 OR 4 OR 5 OR 6 OR 7 OR 8
10. (bilirubin[mh] OR (bilirubin*[tiab]))
11. 9 and 10
12. limit 11 to human
13. limit 11 to English

Table S1 Characteristics of included studies

First author, year of publication	Name of study or source of participants	Year of sample collection	Sample source	Fasting samples	Sample state before analysis, storage, temperature (℃) if frozen	Assay method	Assay source (manufacturer)
Ekblom *et al.*, 2010 (19)	NSHDSC	1986–2002	Plasma	Yes	–80 ℃	PCR reactions	A Vitros 5.1 automated analyzer (Ortho-Clinical Diagnostics Inc., Rochester, NY, USA)
Kimm *et al.*, 2009 (4)	RHE	1994–2007	Serum	Yes	Fresh	Automated biochemical assay	Hitachi-7600 analyzer (Hitachi Ltd., Tokyo, Japan)
Kunutsor *et al.*, 2015 (9)	PREVEND	1997–2009	Plasma	Yes	Frozen, –80 ℃	Colorimetric method	(Merck MEGA, Darmstadt, Germany)
Lee *et al.*, 2017 (20)	RHE	2004–2016	Serum	NS	NS	Automated biochemical assay	Hitachi-7600 analyzer (Hitachi Ltd., Tokyo, Japan)
Li *et al.*, 2014 (5)	RHE	2009–2010	Serum	Yes	NS	Modular analytics	Biochemical analyzer (Roche, Mannheim, German)
Mahabadi *et al.*, 2014 (21)	HNRs	2000–2003	Blood	NS	NS	Vanadate oxidation method	An Advia 2400 system (Advia Clinical, Chemistry Analyzer Siemens HealthCare Diagnostics, Eschborn, Germany)
Oda and Kawai, 2012 (6)	RHE	2008–2010	Plasma	Yes	NS	Routine laboratory methods	NS
Perlstein *et al.*, 2008 (7)	NHANES	1999–2004	Serum	NS	NS	Automated biochemical assay	Beckman Synchron LX20 (Beckman Coulter Inc., Fullerton, Calif., USA)
Zhou *et al.*, 2014 (25)	Screening	2010–2012	Serum	Yes	NS	Vanadate oxidase method	Modular dpp ayl-5-001 autoanalyzer (Roche, Switzerland)
Jørgensen *et al.*, 2014 (22)	SCOUT	2003–2009	Serum	Yes	NS	NS	(Abbott Laboratories)
Ren *et al.*, 2016 (24)	Screening	During the 2013	Serum	Yes	NS	NS	NS

NSHDS, the Northern Sweden Health and Disease Study; RHE, routine health examination; NS, not stated; PREVEND, Prevention of Renal and Vascular End-stage Disease; HNRs, the Heinz Nixdorf Recall study; NHANES, the National Health and Nutrition Examination Survey; SCOUT, Sibutramine Cardiovascular Outcomes trial.

Table S2 Quality evaluation of included studies. Quality scoring of prospective cohort studies using Newcastle-Ottawa Scale (maximum score of 9)

References	Selection				Comparability of cohorts on the basis of the design or analysis	Outcome			Overall quality
	Representativeness of the exposed cohort	Selection of the non-exposed cohort	Ascertainment of exposure	Demonstration that outcome of interest was not present at start of study		Assessment of outcome	Was follow-up long enough for outcomes to occur	Adequacy of follow-up of cohorts	
Ekblom *et al.*, 2010 (19)	1	1	1	0	2	1	1	0	7
Kimm *et al.*, 2009 (4)	1	1	1	1	2	1	1	1	9
Kunutsor *et al.*, 2015 (9)	1	1	1	1	2	1	1	1	9
Lee *et al.*, 2017 (20)	1	1	1	1	2	1	1	1	9
Mahabadi *et al.*, 2014 (21)	1	1	1	1	2	1	1	1	9

Table S3 Quality evaluation of included studies. Quality scoring of cross-sectional studies using Modified Newcastle-Ottawa Scale (maximum score of 8)

References	Selection				Comparability on the basis of the design or analysis	Exposure			Overall quality
	Representativeness of the cases	Non-respondents	Adequate definition of exposure	Ascertainment of exposure		The study used a precise definition of outcome and valid and reliable method (individually for each relevant outcome)	Assessment of outcome	Statistical test	
Li *et al.*, 2014 (5)	1	1	1	1	2	Unscored	1	1	8
Oda and Kawai, 2012 (6)	0	1	1	1	2	Unscored	0	1	6
Perlstein *et al.*, 2008 (7)	1	1	1	1	2	Unscored	0	1	7
Zhou *et al.*, 2014 (25)	1	1	1	1	2	Unscored	1	1	8
Jørgensen *et al.*, 2014 (22)	1	1	1	1	2	Unscored	1	1	8
Ren *et al.*, 2016 (24)	0	1	1	1	2	Unscored	0	1	6

Table S4 Subgroup analysis of relationship between total bilirubin level and ischemic stroke based on the highest bilirubin level and the lowest bilirubin level in tri-sectional quantiles

Subgroups	High versus low analyses				
	No. of studies	Odd ratio (95% CI)	I^2 (%)	P for heterogeneity	P for test
Study design					
Prospective cohort	2	0.74 (0.61 to 0.91)	0	0.540	0.004
Cross-sectional	2	0.61 (0.51 to 0.71)	0	0.740	<0.001
Gender					
Male	2	0.72 (0.57 to 0.91)	0	0.613	0.006
Female	2	0.78 (0.53 to 1.16)	61.6	0.107	0221
Age, years					
<60	3	0.67 (0.58 to 0.77)	61.6	0.401	<0.001
≥60	1	–	–	–	–
Sample size					
<2,000	2	0.61 (0.47 to 0.79)	0	0.551	<0.001
≥2,000	2	0.67 (0.58 to 0.78)	31.7	0.231	<0.001

Table S5 Subgroup analysis of total bilirubin level and stroke, based on the highest bilirubin level and the lowest bilirubin level in tri-sectional quantiles

Subgroups	High versus low analyses				
	No. of studies	Odd ratio (95% CI)	I^2 (%)	P for heterogeneity	P for test
Study design					
Prospective cohort	5	0.72 (0.56 to 0.92)	48.0	0.087	0.010
Cross-sectional	6	0.65 (0.54 to 0.79)	59.3	0.022	<0.001
Gender					
Male	3	0.75 (0.62 to 0.90)	0	0.385	0.002
Female	3	0.57 (0.28 to 1.17)	67.3	0.047	0.267
Age, years					
<60	9	0.68 (0.58 to 0.80)	54.4	0.015	<0.001
≥60	2	0.69 (0.55 to 0.86)	59.2	0.117	0.001
Sample size					
<2,000	2	0.61 (0.47 to 0.79)	0	0.551	<0.001
≥2,000	9	0.70 (0.60 to 0.81)	55.2	0.014	<0.001

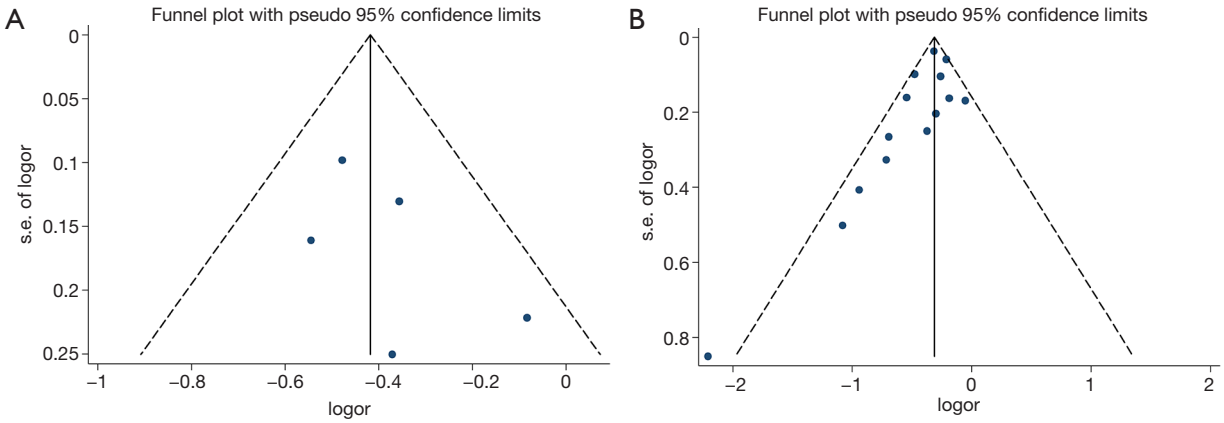

Figure S1 Funnel plot of publication bias. (A) Egger's test for ischemic stroke studies; (B) Egger's test for stroke studies.