Decreased Serum Bilirubin Is Associated With Silent Cerebral Infarction

Rui-Yan Li, Zhi-Gang Cao, Ji-Rong Zhang, Ying Li, Rui-Tao Wang

**Objective**—The presence of silent cerebral infarction (SCI) increases the risk of transient ischemia attack, symptomatic stroke, cardiovascular disease, and dementia. Total bilirubin (TB) levels were demonstrated to be decreased in carotid intima–media thickness, cardiovascular disease, stroke, and peripheral arterial disease. However, little information is available concerning the correlation between TB and SCI.

**Approach and Results**—A cross-sectional study was conducted to evaluate the association between TB and SCI in 2865 subjects (1831 men and 1034 women) undergoing medical checkup. The participants with SCI had lower TB levels than those without SCI. The subjects with a low TB had a higher prevalence of SCI. Moreover, partial correlation showed that TB levels were tightly correlated with brachial-ankle pulse wave velocity after adjusting for confounding covariates ($r=-0.149$; $P<0.001$). Multivariate logistic regression analysis revealed that higher TB was associated with a lower risk of SCI (odds ratio, 0.925; 95% confidence interval, 0.897–0.954; $P<0.001$).

**Conclusions**—TB is a novel biochemical indicator for SCI regardless of classical cardiovascular risk factors. Early measurement of TB may be useful to assess the risk of SCI. (Arterioscler Thromb Vasc Biol. 2014;34:946-951.)

**Key Words:** atherosclerosis $\bullet$ bilirubin $\bullet$ cerebral infarction

**Results**

Clinical and laboratory data of participants with and without SCI are shown in Table 1. Of the 2865 participants enrolled, 1831 (63.91%) were men and 1034 (36.09%) were women. Median (interquartile range) of total bilirubin (TB) concentration was 10.5 (7.8–13.8; range, 2.1–33.4) μmol/L in the whole cohort of individuals. Three hundred forty-three participants (11.97%; 274 men and 69 women) presented SCI. The patients with SCI were older and had higher body mass index (BMI), systolic blood pressure, diastolic blood pressure (DBP), total cholesterol (TC), triglyceride, low-density lipoprotein cholesterol (LDL-C), fasting plasma glucose (FPG), and brachial-ankle pulse wave velocity (baPWV) levels and reduced TB, indirect bilirubin, direct bilirubin, estimated glomerular filtration rate (eGFR), and high-density lipoprotein cholesterol levels compared with the subjects without SCI. However, the levels of aspartate aminotransferase, alanine aminotransferase, γ-glutamyl transpeptidase, and current use of statins and calcium channel blocker drugs in the 2 groups had no difference. Male sex, smoking, alcohol consumption, type 2 diabetes mellitus (DM), hypertension, and current use of angiotensin-converting enzyme inhibitor/angiotensin II receptor antagonist, aspirin, and hypoglycemic drugs had a higher prevalence in SCI group.

The demographic and biochemical characteristics of the study population according to TB quartiles are shown in Table 2. Mean age, BMI, systolic blood pressure, DBP,
Reduced TB Is Associated With SCI

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FPG, triglyceride, LDL-C, FPG, aspartate aminotransferase, alanine aminotransferase, \( \gamma \)-glutamyl transpeptidase, and baPWV decreased gradually as TB increased. Also, the percentage of alcohol consumption, DM and hypertension, and current use of statins, angiotensin-converting enzyme inhibitor/angiotensin II receptor antagonist, and hypoglycemic drugs reduced as TB quartiles increased. However, eGFR, high-density lipoprotein cholesterol, and the percentage of male sex and smoker increased as TB quartiles increased. TC and the percentage of use of calcium channel blocker and aspirin had no difference as TB quartiles increased.

The partial correlation between baPWV and TB levels was calculated. BaPWV statistically correlated with TB levels after adjustment for age, sex, BMI, smoking status, alcohol consumption, systolic blood pressure, DBP, FPG, TC, triglyceride, high-density lipoprotein cholesterol, LDL-C, aspartate aminotransferase, alanine aminotransferase, \( \gamma \)-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 \( r=-0.149; P<0.001 \).

The prevalence of SCI was calculated by the quartiles of serum TB levels (Figure). The prevalence rate of SCI in Q1, Q2, Q3, and Q4 was 21.33\% (157/736), 11.57\% (82/709), 9.36\% (66/705), and 5.39\% (38/715), respectively. The results indicated that the prevalence of SCI decreased as TB quartiles increased \( P<0.001 \).

Stepwise multiple regression analysis was performed to evaluate risk factors for SCI using logistic regression model in Table 3. Twenty-one variables, including age, sex, BMI, smoking status, alcohol consumption, systolic blood pressure, DBP, TC, triglyceride, high-density lipoprotein cholesterol, LDL-C, FPG, aspartate aminotransferase, baPWV, TB, eGFR, DM, hypertension, and the use of angiotensin-converting enzyme inhibitor/angiotensin II receptor antagonist, aspirin, and hypoglycemic drugs, were entered into the original equation. The risk factors found to be significantly associated with SCI in the logistic regression analysis were sex, BMI, smoking status, DBP, LDL-C, FPG, eGFR, TB, DM, and baPWV. Notably, TB was found to be a newly observed independent risk factor for SCI (odds ratio, 0.925; 95\% confidence interval, 0.897–0.954; \( P<0.001 \)).

### Table 1. Baseline Characteristics of the Analyzed Participants According to SCI Status

<table>
<thead>
<tr>
<th>Variables</th>
<th>With SCI</th>
<th>Without SCI</th>
<th>( P ) Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>343</td>
<td>2522</td>
<td></td>
</tr>
<tr>
<td>Age, y</td>
<td>54.3 (9.4)</td>
<td>49.8 (9.9)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Range</td>
<td>35–68</td>
<td>30–69</td>
<td></td>
</tr>
<tr>
<td>Sex (male, %)</td>
<td>274 (79.9)</td>
<td>1557 (61.7)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>25.9 (2.7)</td>
<td>24.7 (3.2)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Smoker (%)</td>
<td>188 (54.8)</td>
<td>934 (37.0)</td>
<td>0.007</td>
</tr>
<tr>
<td>Alcohol consumption</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never drinker (%)</td>
<td>40 (11.7)</td>
<td>511 (20.3)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Light drinker (%)</td>
<td>196 (57.1)</td>
<td>1361 (54.0)</td>
<td>0.268</td>
</tr>
<tr>
<td>Moderate drinker (%)</td>
<td>67 (19.5)</td>
<td>469 (18.6)</td>
<td>0.676</td>
</tr>
<tr>
<td>Heavy drinker (%)</td>
<td>40 (11.7)</td>
<td>181 (7.2)</td>
<td>0.003</td>
</tr>
<tr>
<td>SBP, mm Hg</td>
<td>135.9 (12.3)</td>
<td>128.8 (12.3)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>DBP, mm Hg</td>
<td>78.8 (8.0)</td>
<td>74.4 (7.9)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>TC, mmol/L</td>
<td>5.25 (4.73–5.74)</td>
<td>5.12 (4.53–5.64)</td>
<td>0.009</td>
</tr>
<tr>
<td>TG, mmol/L</td>
<td>1.98 (1.40–2.58)</td>
<td>1.77 (1.24–2.41)</td>
<td>0.001</td>
</tr>
<tr>
<td>HDL-C, mmol/L</td>
<td>1.42 (1.21–1.62)</td>
<td>1.52 (1.31–1.68)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LDL-C, mmol/L</td>
<td>2.78 (0.77)</td>
<td>2.64 (0.79)</td>
<td>0.004</td>
</tr>
<tr>
<td>FPG, mmol/L</td>
<td>6.10 (5.56–7.05)</td>
<td>5.60 (5.19–6.08)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>AST, U/L</td>
<td>22.0 (18.0–31.0)</td>
<td>21.0 (18.0–28.0)</td>
<td>0.078</td>
</tr>
<tr>
<td>ALT, U/L</td>
<td>19.0 (15.0–30.0)</td>
<td>19.0 (15.0–33.0)</td>
<td>0.431</td>
</tr>
<tr>
<td>GGT, U/L</td>
<td>28.0 (15.0–45.0)</td>
<td>28.0 (15.0–45.0)</td>
<td>0.649</td>
</tr>
<tr>
<td>baPWV, cm/s</td>
<td>1441.3 (156.3)</td>
<td>1277.9 (148.5)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>TB, μmol/L</td>
<td>8.3 (6.8–12.4)</td>
<td>10.7 (8.0–14.1)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>DB, μmol/L</td>
<td>3.4 (2.7–5.2)</td>
<td>3.9 (2.7–5.4)</td>
<td>0.001</td>
</tr>
<tr>
<td>IB, μmol/L</td>
<td>5.0 (3.9–7.1)</td>
<td>6.6 (4.9–9.4)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>eGFR, mL/min per 1.73 m²</td>
<td>70.2 (16.7)</td>
<td>78.5 (15.2)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Hypertension (%)</td>
<td>172 (50.1)</td>
<td>584 (23.0)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>DM (%)</td>
<td>100 (29.2)</td>
<td>199 (7.9)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Medication</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Statin (%)</td>
<td>10 (2.9)</td>
<td>70 (28.8)</td>
<td>0.883</td>
</tr>
<tr>
<td>ACEIs/ARBs (%)</td>
<td>31 (9.0)</td>
<td>147 (5.8)</td>
<td>0.021</td>
</tr>
<tr>
<td>CCBs (%)</td>
<td>25 (7.3)</td>
<td>144 (5.7)</td>
<td>0.244</td>
</tr>
<tr>
<td>Hypoglycemic agents (%)</td>
<td>36 (10.5)</td>
<td>97 (3.8)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Aspirin (%)</td>
<td>53 (15.1)</td>
<td>244 (9.7)</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Values are shown as mean (SD) or median (interquartile range) or percentage. \( P \) value was calculated using the Student’s \( t \) test or Mann–Whitney \( U \) test or \( \chi^2 \) test. ACEI indicates angiotensin-converting enzyme inhibitor; ALT, alanine aminotransferase; ARB, angiotensin II receptor antagonist; AST, aspartate aminotransferase; baPWV, brachial-ankle pulse wave velocity; BMI, body mass index; CCB, calcium channel blocker; DBP, diastolic blood pressure; DB, direct bilirubin; DM, type 2 diabetes mellitus; eGFR, estimated glomerular filtration rate; FPG, fasting plasma glucose; GGT, \( \gamma \)-glutamyl transpeptidase; HDL-C, high-density lipoprotein cholesterol; IB, indirect bilirubin; LDL-C, low-density lipoprotein cholesterol; SBP, systolic blood pressure; SCI, silent cerebral infarction; TB, total bilirubin; TC, total cholesterol; and TG, triglyceride.

**Discussion**

In this cross-sectional study, we showed that the subjects with a low TB had a higher prevalence of SCI. Moreover,
partial correlation found that TB levels were tightly correlated with baPWV after adjusting for covariates. Logistic regression analysis revealed a significant association of TB levels with the risk of SCI after adjustment for conventional vascular risk factors.

SCI has high prevalence in the elderly population. Some studies have demonstrated that SCI is linked to cognitive impairment, depression, and increased risk for stroke.1–3 The majority of SCIs reflect penetrating artery disease and have shared pathogenesis with lacunar infarcts. Recent research has reported that endothelial dysfunction is involved in lacunar infarction and SCI.10

Serum bilirubin (a heme catabolic product) is among the most powerful endogenous antioxidative substances and is positively correlated with the total antioxidant capacity in Gilbert syndrome.11 A lower prevalence of cardiovascular disease is found in Gilbert disease.12 However, TB exerts a toxic effect in certain situations. Gut microflora greatly affects intravascular metabolism of bilirubin.13 On the contrary, l-carnitine in red meat is metabolized by gut microbiota and generates proatherogenic metabolites.14,15 Recent studies showed that the metabolites are associated with an elevated risk for cardiovascular disease.16 In addition, vitamin B12 deficiency, which increases bilirubin levels, adds to the risk of stroke by increasing levels of homocysteine.17 In contrast, higher plasma homocysteine level and decreased TB level are found in patients with diabetic retinopathy.18 The apparent paradox between toxic and protective effects stresses again the double-edged sword character of bilirubin.

### Table 2. Clinical and Biochemical Characteristics of Subjects According to Total Bilirubin Quartiles

<table>
<thead>
<tr>
<th>Quartiles of Total Bilirubin</th>
<th>Q1 (n=736)</th>
<th>Q2 (n=709)</th>
<th>Q3 (n=705)</th>
<th>Q4 (n=715)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>736</td>
<td>709</td>
<td>705</td>
<td>715</td>
<td></td>
</tr>
<tr>
<td>Age, y</td>
<td>52.9 (9.1)</td>
<td>51.0 (9.4)</td>
<td>49.3 (10.6)</td>
<td>48.1 (10.1)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Sex (male, %)</td>
<td>361 (49.0)</td>
<td>393 (55.4)</td>
<td>483 (68.5)</td>
<td>594 (83.1)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>25.2 (3.2)</td>
<td>25.2 (2.9)</td>
<td>24.6 (3.2)</td>
<td>24.4 (3.3)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Smoker (%)</td>
<td>225 (30.6)</td>
<td>250 (35.3)</td>
<td>276 (39.1)</td>
<td>371 (51.9)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

**Alcohol consumption**

- **Never drinker (%)**: 189 (25.7) | 376 (53.0) | 117 (16.6) | 54 (7.6) | <0.001
- **Light drinker (%)**: 162 (22.0) | 387 (54.6) | 113 (16.0) | 47 (6.6) | <0.001
- **Moderate drinker (%)**: 116 (15.8) | 395 (55.7) | 139 (19.7) | 55 (7.7) | <0.001
- **Heavy drinker (%)**: 84 (11.4) | 399 (56.3) | 167 (23.7) | 65 (9.1) | <0.001

**SBP, mmHg**

- Q1: 133.3 (12.4) | Q2: 129.3 (12.9) | Q3: 127.2 (12.4) | Q4: 128.7 (11.6) | <0.001

**DBP, mmHg**

- Q1: 75.6 (8.0) | Q2: 75.4 (8.0) | Q3: 75.2 (7.9) | Q4: 73.4 (8.2) | <0.001

**FPG, mmol/L**

- Q1: 5.87 (5.45–6.38) | Q2: 5.61 (5.10–6.13) | Q3: 5.59 (5.16–6.06) | Q4: 5.59 (5.22–6.06) | <0.001

**TC, mmol/L**

- Q1: 5.09 (4.64–5.58) | Q2: 5.24 (4.52–5.76) | Q3: 5.09 (4.54–5.64) | Q4: 5.14 (4.52–5.64) | 0.151

**TG, mmol/L**

- Q1: 1.93 (1.43–2.51) | Q2: 1.80 (1.08–2.35) | Q3: 1.69 (1.31–2.40) | Q4: 1.73 (1.26–2.45) | <0.001

**HDL-C, mmol/L**

- Q1: 1.44 (1.17–1.64) | Q2: 1.49 (1.31–1.66) | Q3: 1.49 (1.30–1.75) | Q4: 1.54 (1.35–1.71) | <0.001

**LDL-C, mmol/L**

- Q1: 2.73 (0.83) | Q2: 2.60 (0.76) | Q3: 2.60 (0.66) | Q4: 2.72 (0.89) | 0.001

**AST, U/L**

- Q1: 23.0 (19.0–28.0) | Q2: 21.0 (17.5–28.0) | Q3: 21.0 (17.0–28.0) | Q4: 21.0 (18.0–28.0) | <0.001

**ALT, U/L**

- Q1: 21.0 (15.0–34.0) | Q2: 19.0 (15.0–30.0) | Q3: 19.0 (15.0–34.0) | Q4: 20.0 (15.0–33.0) | 0.001

**GGT, U/L**

- Q1: 31.0 (15.0–51.0) | Q2: 29.0 (16.0–50.0) | Q3: 26.0 (14.0–44.0) | Q4: 23.0 (13.0–42.0) | <0.001

**baPWV, cm/s**

- Q1: 1342.9 (158.6) | Q2: 1321.2 (159.7) | Q3: 1279.2 (145.3) | Q4: 1245.0 (152.0) | <0.001

**eGFR, mL/min per 1.73 m²**

- Q1: 74.7 (16.3) | Q2: 80.0 (16.7) | Q3: 78.9 (13.6) | Q4: 76.5 (15.2) | <0.001

**Hypertension (%)**

- Q1: 276 (37.5) | Q2: 165 (23.3) | Q3: 157 (22.3) | Q4: 158 (22.1) | <0.001

**DM (%)**

- Q1: 160 (21.7) | Q2: 48 (6.8) | Q3: 56 (8.2) | Q4: 33 (4.6) | <0.001

**Medication**

- **Statin (%)**: 18 (2.4) | 29 (4.1) | 12 (1.7) | 21 (2.9) | 0.049
- **ACEIs/ARBs (%)**: 57 (7.7) | 29 (4.1) | 47 (6.7) | 45 (6.3) | 0.034
- **CCBs (%)**: 49 (6.7) | 37 (5.2) | 43 (6.1) | 40 (5.6) | 0.677
- **Hypoglycemic agents (%)**: 75 (10.2) | 21 (3.0) | 24 (3.4) | 13 (1.8) | <0.001
- **Aspirin (%)**: 90 (12.2) | 68 (9.6) | 71 (10.1) | 68 (9.5) | 0.280

Data are expressed as mean (SD) or median (interquartile range) or percentage. P value was calculated using 1-way ANOVA test or Kruskal–Wallis or χ² test. ACEI indicates angiotensin-converting enzyme inhibitor; ALT, alanine aminotransferase; ARB, angiotensin II receptor antagonist; AST, aspartate aminotransferase; baPWV, brachial-ankle pulse wave velocity; BMI, body mass index; CCB, calcium channel blocker; DBP, diastolic blood pressure; DM, type 2 diabetes mellitus; eGFR, estimated glomerular filtration rate; FPG, fasting plasma glucose; GGT, gamma-glutamyl transpeptidase; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; SBP, systolic blood pressure; TC, total cholesterol; and TG, triglyceride.
jugated hyperbilirubinemia exerts a protective role in the as well as elevated total mortality.21–24 Second, mild unconjugated hyperbilirubinemia and protect human vascular endothelial cells against complement-mediated injury.20 In addition, our previous studies have demonstrated that unconjugated bilirubin inhibits complement activation via interfering with the interaction between C1 component of complement and immunoglobulins.19 Moreover, heme oxygenase 1 and heme degradation products could attenuate complement-mediated acute inflammation and protect human vascular endothelial cells against complement-mediated injury.20 In addition, our previous studies have observed that serum bilirubin has a tight correlation with baPWV. Furthermore, baPWV has been used as an index of subclinical atherosclerosis. Previous studies documented that increased baPWV is associated with metabolic syndrome, cardiovascular diseases, stroke, and renal disease, as well as elevated total mortality.21–24 Second, mild unconjugated hyperbilirubinemia exerts a protective role in the development of atherosclerosis. Low-grade chronic inflammation induces vascular damage. Some inflammatory markers, such as C-reactive protein and interleukin-6, are reported to have a positive correlation with SCI.25,26 Also, diminished TB is linked with diabetes mellitus, cardiovascular disease, peripheral artery disease, and stroke, which all are relevant to chronic inflammation.27,28

Our results revealed that dyslipidemia is associated with SCI. The results are in line with those of other groups.29,30 Furthermore, LDL-C subfraction LDL-C3 has been found in association with SCI in subjects with hypertension.31 These findings are consistent with the idea that TB may play a protective role in the atherosclerotic process by preventing formation of oxidized LDL-C.8,32 A study reported that there was a higher prevalence of SCI in women than in men, whereas this higher prevalence was not found in the younger cohorts.33–35 These conflicting data may be attributable to the differences in age and race.36 In addition, a report has confirmed that there were significant racial differences in TB levels in different populations.37 However, our study showed that sex was an important factor associated with SCI. Estrogen levels may be responsible for sex disparity. uridine 5‘-diphospho-glucuronosyltransferase 1A1–mediated glucuronidation was an essential step for bilirubin elimination.38 Estrogen could influence the production of bilirubin by inhibiting the uridine 5‘-diphospho-glucuronosyltransferase 1A1–catalyzed bilirubin glucuronidation.39,40 In addition, a recent study confirmed that there was an association between the uridine 5‘-diphospho-glucuronosyltransferase 1A1 and TC and LDL-C levels in women with different hormonal status.41 Similarly, a negative correlation between TB and blood pressure was only found in women.42 Further research is warranted to clarify the sex difference of the effects of bilirubin.

The interpretation of this study has some limitations. First, the study was cross-sectional, and it is difficult to study the direction of causality. A prospective study is needed to clarify this point. Second, we could not get any information on waist circumference, insulin resistance, and C-reactive protein, which are important risk factors. Third, the study is lacking information about the genetic contributions to hyperbilirubinemia. Recent studies indicated that different genetic background plays a key role in controlling serum bilirubin.43

Conclusions

In summary, our study showed that TB is a novel biochemical indicator for SCI regardless of classical cardiovascular risk factors. Early measurement of TB may be useful to assess the risk of SCI.

Acknowledgments

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Disclosures

None.

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Bilirubin is an effective antioxidant molecule. The presence of silent cerebral infarction predicts transient ischemia attack, clinically overt stroke, cardiovascular disease, and dementia. Our study showed that total bilirubin is a novel biochemical indicator for silent cerebral infarction regardless of classical cardiovascular risk factors. Moreover, total bilirubin levels are tightly correlated with brachial-ankle pulse wave velocity after adjusting for confounding covariates. Early measurement of total bilirubin may be useful to assess the risk of stroke.
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Materials and Methods

Study population

We studied 3355 subjects who visited International Physical Examination and Healthy Center, Harbin, China, from January 2009 through December 2010 and who underwent MRI of the brain as part of their routine health check. 357 subjects who met the exclusion criteria were excluded. Another 112 were excluded for having missing data for baPWV. 21 participants with total bilirubin > 34.2 μmol/L were excluded due to a high possibility of Gilbert's syndrome. Thus, the final study population for the present analysis consisted of 2865 participants. We obtained informed consent from all subjects. The study protocol was approved by the Ethics Committee of the Second Hospital of Harbin Medical University, China.

Clinical examination

All the subjects underwent a clinical investigation including medical history, smoking status, physical examinations, laboratory tests, and an MRI scan of the brain. Alcohol consumption was categorized as never, light (≤ 6 drinks/week), moderate (7-14 drinks/week), heavy (≥ 15 drinks/week). Blood pressure was determined using a mercury-gravity sphygmomanometer in a sitting position after a 15-min rest. Body weight was measured in light clothing, without shoes, to the nearest 0.5 kg. Height was measured to the nearest 0.5 cm. Body mass index (BMI) was calculated as weight (kg) divided by height squared (m²).

Biochemical measurements

Fasting venous blood samples were drawn in the morning after an 8-hour fast. The values included total serum bilirubin (TB) concentrations, direct bilirubin (DB), indirect bilirubin (IB), aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyl transpeptidase (GGT), total serum cholesterol (TC), triglyceride (TG), high density lipoprotein cholesterol (HDL), low density lipoprotein cholesterol (LDL) and fasting plasma glucose (FPG). The assays were performed at the Laboratory of Analytical Biochemistry at the Second Hospital of Harbin Medical University, Harbin, using a biochemical analyzer (MODULAR ANALYTICS, Roche, Mannheim, German) using standard methods.

Measurement of baPWV

Brachial-ankle pulse wave velocity (baPWV) was measured using an automatic
device (model MB3000, M&B Electronic Instruments, Beijing, China). The baPWV was automatically calculated according to the formula (L/PTT). L was the difference between the length from heart to ankle and the length from heart to brachium. PTT was the pulse transit time between the brachial and tibial arterial waveforms. Mean right and left baPWV value was calculated during analysis. All measurements were conducted by a single examiner who was blinded to the clinical data. The method was validated in a previous report.¹

Cerebral MRI

Brain MRI examination was performed on a 1.5-T magnetic resonance system (Achieva 1.5T, Philips, Best, The Netherlands). The scan protocol consisted of T2-weighted spin echo (repetition time [TR]: 4,800 ms, echo time [TE]: 100 ms), T1-weighted spin echo (TR: 520 ms, TE: 14 ms), gradient-echo T2* -weighted images (T2*WI; TR = 670 ms, TE = 25 ms), and fluid-attenuated inversion recovery (FLAIR; TR: 8,500 ms, TE: 120 ms, inversion time: 2,000 ms) imaging in axial planes at 5-mm-thick slices with an interslice gap of 1.5 mm. The criteria for SCI were as follows: (1) a focal hyperintense lesion (≥ 3mm) in the T2 and FLAIR and hypointense lesion in the T1 image; (2) the absence of signs and symptoms that could be explained by lesions observed by MRI; and (3) no history of clinical stroke. Periventricular white matter lesions were distinguished from SCI based on the high signal intensity on FLAIR². Dilated perivascular spaces were differentiated from SCI based on their locations (along perforating or medullary arteries, often bilaterally symmetrical, usually in the lower third of the basal ganglia) and by absence of gliosis.³ The diagnoses were made by 2 independent experienced radiologists blinded to subject history and clinical status. The kappa value of agreement for SCI was 0.84. A consensus on inconsistent readings was reached through discussions.

Diagnosis and Exclusion criteria

Diagnosis of type 2 diabetes (DM) was based on American Diabetes Association criteria such as fasting plasma glucose ≥ 7.0 mmol/L, current treatment with a hypoglycemic agent, or casual glucose ≥ 11.1 mmol/L. For the controls or the patients with impaired fasting glucose, DM was diagnosed if a 2-hr post-glucose level after a 75-g oral glucose tolerance test ≥ 11.1 mmol/L. Hypertension was diagnosed if systolic blood pressure ≥ 140 mmHg and/or diastolic pressure ≥ 90 mmHg, or as antihypertensive treatment. Two readings were taken, with a 5-minute interval
between measurements. The mean of the two readings was recorded. The Modification of Diet in Renal Disease (MDRD) equation was used to estimate glomerular filtration rate (eGFR). MDRD equation was: 

$$eGFR=186.3 \times (SCr)^{-1.154} \times (age)^{-0.203} \times 0.742 \text{ if female}.$$ 

Subjects meeting any of the following criteria were excluded: cancer, alcoholism, viral hepatitis, other hemolytic, autoimmune, drug-induced liver diseases associated with hyperbilirubinemia, elevated serum alkaline phosphatase and a positive anti-mitochondrial antibody to rule out primary biliary cirrhosis, transient ischemic attacks, stroke and coronary heart disease.

**Statistical analysis**

The data were expressed as means with standard deviations or medians with interquartile ranges for continuous variables or percentage for categorical variables. Kolmogorov–Smirnov test was used to assess the normality of the continuous variables. Clinical and laboratory data were presented according to SCI status or TB quartiles. For comparisons between the two groups, we used the student's t-test for normally distributed data and Mann-Whitney's U test for non-parametric data. For the comparison of proportions, we used chi-square test. The TB quartiles were quartile 1 (Q1) (≤ 7.8 μmol/L), quartile 2 (Q2) (7.9-10.5 μmol/L), quartile 3 (Q3) (10.6-13.8 μmol/L), quartile 4 (Q4) (≥ 13.9 μmol/L). Group comparisons were conducted using one-way ANOVA test for normally distributed data, Kruskal-Wallis test for non-parametric data and chi-square test for categorical data. Correlations between TB and baPWV were tested by partial correlation after adjusting for other confounding covariates. TB, TC, TG, HDL, AST, ALT, GGT and FPG were logarithmically transformed prior to analysis. Stepwise multiple regression analysis (Backward: Wald; Entry: 0.05, Removal: 0.10) was used to evaluate the risk factors for SCI. Risks were reported as odds ratios (ORs) with 95% confidence interval (CI). Any potential
confounding factors that had at least a modest \((p < 0.20)\) relation to the outcome variable was included in the later multivariate model. Entered into the model were: age, sex, BMI, smoking status, alcohol consumption, SBP, DBP, TC, TG, HDL, LDL, FPG, AST, baPWV, TB, eGFR, DM, hypertension, and the use of drugs. The variables sex, smoking status, DM, hypertension, and the use of drugs were categorized as 0 (absent) or 1 (present) and the variables age, BMI, SBP, DBP, TC, TG, HDL, LDL, FPG, AST, baPWV, eGFR, and TB were included as continuous variables. \(P < 0.05\) (2-tailed test) was considered statistically significant. The SPSS statistical software package version 17.0 (SPSS Inc., Chicago, IL, USA) was used for all statistical analyses.

References