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Homocysteine, S-adenosylmethionine and S-adenosylhomocysteine are associated with retinal microvascular abnormalities: the Hoorn Study

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Short title: homocysteine and retinal microangiopathy

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Abstract

Objective To investigate the relationship of homocysteine and homocysteine metabolism components with retinal microvascular disorders in subjects with and without type 2 diabetes.

Methods In this population-based study of 256 participants, aged 60-85 years, we determined total plasma homocysteine, S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH) in plasma and erythrocytes, total folate in serum and erythrocytes, 5-methyltetrahydrofolate (5-MTHF), and vitamin B12 and B6. Participants were ophthalmologically examined by means of indirect funduscopy and 2-field 45° fundus photography, and were graded for retinopathy and retinal sclerotic vessel abnormalities. A computer-assisted method was used to measure retinal vessel diameters.

Results Total plasma homocysteine was inversely associated with retinal arteriolar diameters (standardized beta (95% CI): -0.20 (-0.33;-0.07) or a decrease of 3.78 µm CRAE per 1 SD increase of homocysteine level (= 4.6 µmol/l) ). In addition, SAM/SAH ratio in plasma was inversely associated with retinal sclerotic vessel abnormalities and retinopathy (odds ratios (95% CI): 0.61 (0.39-0.96) and 0.50 (0.30-0.83) per 1 SD, respectively). The associations were independent of age, sex, glucose tolerance status, other homocysteine metabolism components and cardiovascular risk factors.

Conclusions The results of this study support the concept that total plasma homocysteine and a low SAM/SAH ratio in plasma, which may reflect reduced transmethylation reactions, may contribute to the pathogenesis of (retinal) microangiopathy.
Hyperhomocysteinemia has been associated with an increased risk for cardiovascular disease independently of conventional cardiovascular risk factors [1,2]. It remains unclear, however, whether homocysteine itself is a direct cause of atherosclerosis. Other components of homocysteine metabolism, such as S-adenosylmethionine (SAM), S-adenosylhomocysteine (SAH) and folate, have also been suggested to play a role in, and possibly mediate, the relationship with cardiovascular disease [3-6]. Methionine – homocysteine metabolism involves methionine transmethylation, which involves the formation of SAM and SAH, and leads to the production of homocysteine (Figure 1). Homocysteine can be remethylated to methionine, which requires vitamin B12 and folate, or can go through the transsulfuration pathway, which requires vitamin B6. However, the exact extent of contribution of these metabolites to cardiovascular disease risk has not yet been established [7].

Homocysteine is thought to increase cardiovascular risk by inducing endothelial dysfunction, increasing oxidative stress, and inducing thrombophilia and proliferation of smooth muscle cells, which can lead to narrowing of the intravascular lumen [2,7-9]. Experimental studies, mainly in rats, suggest that hyperhomocysteinemia may also play a role in the pathogenesis of renal microvascular disease [10-12]. In humans, however, little is known about the relationship of homocysteine and its metabolites with microangiopathy. High levels of homocysteine were reported to be strongly associated with the development of microalbuminuria [13,14]. Hyperhomocysteinemia is also thought to be a strong risk factor for retinal vascular occlusive disease [15]. For retinopathy, this relationship is not very clear. In the Hoorn study, we previously reported a relationship of hyperhomocysteinemia with retinopathy in type 2 diabetic individuals [16], whereas others did not find any relationship [17].

In light of these considerations, we hypothesized that homocysteine and (or) components of homocysteine metabolism are involved in the pathogenesis of microvascular disease. As the retina offers a unique opportunity to non-invasively analyze the microvasculature, we investigated the relationship of homocysteine and components of homocysteine metabolism with computer-assisted measurements of retinal vessel diameters, retinal sclerotic vessel abnormalities and retinopathy. Because hyperhomocysteinemia appears to be more strongly related to cardiovascular disease in the presence of type 2 diabetes [18], we studied the above-mentioned relationships in a population-based study of subjects with and without type 2 diabetes.
Methods

Study population

For the present study, we used data of the 2000-2001 Hoorn Study follow-up examination. The Hoorn Study is a population-based cohort study of type 2 diabetes and its cardiovascular complications among 2484 Caucasian persons, aged 50-74 years, which started in 1989. Full details have been provided elsewhere [19,20]. Fasting and 2-hour post-load plasma glucose levels after a 75-g oral glucose tolerance test (OGTT) were measured in plasma, and were used for classification into glucose tolerance categories [21]. Subjects who were already known to have diabetes or use glucose-lowering treatment were excluded from the OGTT. At baseline, an age-, sex-, and glucose-tolerance-stratified subsample of 631 participants was extensively studied for reasons of efficiency. In the years 2000-2001, a follow-up examination was carried out among surviving participants who gave their permission to be re-contacted [20]. Of the 631 participants who had an ophthalmological examination at baseline, almost 60% dropped out for the follow-up examination, because of the following reasons: 119 (19%) persons had died, 49 (8%) had moved out of the region, and 207 (33%) did not participate because of mobility or health problems, or lack of motivation. Finally, 256 persons were included in the follow-up ophthalmological examination [22], 70 with normal glucose metabolism (NGM), 69 with impaired glucose metabolism (IGM), and 109 with type 2 diabetes [21]. Written informed consent was obtained from all participants. The Ethical Review Committee of the VU University Medical Center (VUmc) in Amsterdam, the Netherlands, approved the Hoorn Study.

Sample preparation and determination of homocysteine, SAM, SAH, folates and vitamins

All samples were processed within 30 minutes after collection, stored at –80 degrees Celsius and analyzed within two years. Ethylenediaminetetraacetic acid (EDTA)-anticoagulated blood samples were put on ice for the determination of homocysteine, SAM and SAH. For SAM and SAH measurements, samples were immediately deproteinized by the addition of 0.625 ml of a 10% perchloric acid solution to 1 ml plasma and by adding 1 ml of 5% perchloric acid to 1 ml whole blood, followed by mixing [23]. For the determination of total folate, 0.5 mg of ascorbic acid was added to 0.5 ml of serum [23], and for the determination of total folate in erythrocytes, 1 ml of reagent with ascorbic acid, human serum albumin and sodium azide (ACS:180, Chiron Diagnostics) was added to 50 µl of whole blood.
Total plasma homocysteine was determined with an automated fluorescence polarisation immunoassay on an Abbott Imx analyzer (interassay coefficient of variation (CV) $[CV = \text{(standard deviation of the mean difference) / ((square root of 2) x pooled mean)}]$ was 4%) [24]. Tandem mass spectrometry was used to determine SAM and SAH in plasma and whole blood (intra-assay CV were 4% for both determinations, interassay CV were 8% and 6%, respectively) [25]. Erythrocyte concentration for SAM and SAH were calculated as: [whole blood concentration – plasma concentration * (1 - hematocrit)] / hematocrit. Total folate in serum and erythrocytes and serum vitamin B12 were measured with automated chemiluminescence (Chiron Diagnostics ASC: 180® Automated Chemiluminescence Systems). Intra- and interassay CV were 4% and 5% for total folate, respectively. 5-Methyltetrahydrofolate (5-MTHF), the active form of folate, was measured by high-performance liquid chromatography [26], which was also used to measure plasma vitamin B6 [27]. The interassay CV was 7%.

**Ophthalmological examination and retinal vessel diameter measurements**

After mydriasis with 0.5% tropicamide and 2.5% phenylephrine eye drops, the retina was examined by funduscopy and fundus photography, as previously described [22]. In short, fundus photography was performed with a 45º CR5 non-mydriatic retinal camera (Canon Inc., Tokyo, Japan), interfaced to a 3CCD Color Video Camera (Sony Corp., Japan). The quality of each photo was checked immediately on a connected color video monitor (Trinitron, Sony, Tokyo, Japan) and a new photograph was taken if the quality was insufficient. The photographs were digitized, compressed (10:1 JPEG), and stored on a magneto-optical disc using the TEAC MV-300P Viewfile system (TEAC Corp., Tokyo, Japan). Two photographs were made of each eye, one centered on the macula and one centered on the optic disc.

Methods used to measure retinal vessel diameters from digitized photographs followed a standardized protocol, which has been described elsewhere [28]. Briefly, one investigator (M.V.H.) independently measured all arterioles and venules 0.5 to 1 disc diameter from the optic disc margin using a computer-imaging program (Retinal Analysis, Optimate, Madison, Wisconsin), masked to participant identity. The branches of arterioles were also measured if the trunk measures were $\geq 85 \, \mu m$. Computer-assisted measurements of the diameters of arterioles and venules were obtained and combined according to the revised formulas of Parr and Hubbard [28-31], which account for magnification differences and the number of vessels in photographs. Average diameters of arterioles (central retinal arteriolar equivalents (CRAE)) and venules (central retinal venular equivalents (CRVE)) in one eye were assessed, and
combined into an arteriole-to-venule ratio (AVR). An AVR of 1.0 indicates that the diameters of the arterioles are approximately equal to the diameters of the venules, whereas a smaller AVR indicates narrower arterioles or wider venules. For each subject, one photograph centered on the optic disc was used, alternately selected of the left and right eye (ratio: 50%-50%). In the case of insufficient quality, the photograph of the other eye was examined. The intra-observer intersession CV were 5% for CRVE, 8% for CRAE and 9% for AVR.

Both fundus photographs were independently analyzed by two individuals (H.A.L. en A.C.M.) to grade retinal sclerotic vessel abnormalities and retinopathy. In the case of disagreement, the judgment of a third investigator (B.C.P.P.) was taken to be decisive. Retinal sclerotic vessel abnormalities were defined as the presence of venous beading, focal narrowing, arteriovenous crossing changes, “copper” or “silver” wiring, dilated or tortuous retinal veins, or central or branch venular occlusion. Retinopathy was defined as the presence of one or more microaneurysms, hemorrhages, or hard exudates, possibly in combination with areas of neovascularization, fibrous proliferation, pre-retinal or vitreous hemorrhages and/or laser coagulation scars in at least one eye according to the Eurodiab classification [22,32].

Other measurements
Brachial systolic and diastolic blood pressures, glycated hemoglobin, fasting insulin, serum total, high-density and low-density lipoprotein cholesterol, serum triglycerides, serum creatinine, serum albumin, body mass index, waist and hip circumferences, smoking and prior cardiovascular disease (CVD) were determined according to methods described elsewhere [33-35]. Hypertension was defined as diastolic blood pressure >= 90 mmHg, systolic blood pressure >= 140 mmHg and/or use of anti-hypertensive medication [36].

Statistical analyses
Clinical and ophthalmological characteristics, expressed as mean ± SD, percentage, or median (interquartile range) in the case of a skewed distribution, were computed according to tertiles of levels of total plasma homocysteine. Overall group differences in continuous variables were tested by means of analyses of variance, and differences in categorical measures were tested with Pearson’s chi-square test.

Multivariable linear regression analyses were used to calculate the associations of homocysteine and its metabolites with AVR, CRAE, and CRVE. To make the results of linear regression models comparable among different determinants, we report standardized beta values. A standardized beta of 0.1 indicates that if the determinant at issue increases by 1 SD,
the outcome increases by 0.1 SD. Furthermore, we report change of AVR and change in µm CRAE and CRVE. Logistic regression analyses were used to calculate the associations of components of homocysteine metabolism with retinal sclerotic vessel abnormalities and retinopathy (odds ratios were reported per 1 SD). Because all determinants showed linearity with AVR, CRAE, CRVE, retinal sclerotic vessel abnormalities and retinopathy, we used homocysteine and its metabolism components as continuous variables in the models. First, we adjusted the associations for the stratification variables, age, sex and glucose tolerance status. Then, we additionally adjusted the associations for the other components of the homocysteine metabolism, and for other potential confounders, i.e. glycated hemoglobin, systolic and diastolic blood pressure, body mass index, total cholesterol, microalbuminuria, and prior CVD.

Effect-modification by type 2 diabetes was investigated by entering product terms of diabetes yes/no times the predictor variable in the regression models. P-values less than 0.05 were considered statistically significant. All analyses were performed in SPSS 12.0 for Windows 98.

Results
Of the 256 participants, 6 had photographs that could not be graded for AVR and CRAE, and 2 had photographs that could not be graded for CRVE. Furthermore, 4 subjects had missing data of total plasma homocysteine and 68 subjects had missing data of one or more components of the homocysteine metabolism. Subjects with one or more missing variables did not differ substantially from subjects with available data (data not shown), and therefore, were not excluded from further analyses.

Mean (± SD) CRAE (in µm) was 170.6 ± 18.9 (range: 119.9 - 225.1), mean (± SD) CRVE (in µm) was 231.2 ± 27.1 (range: 148.7 - 333.5), and mean (± SD) AVR was 0.75 ± 0.10 (range: 0.49 - 1.31). Of the 256 subjects, 31 had retinal sclerotic vessel abnormalities, and 32 had retinopathy.

Clinical characteristics
Table 1 presents the clinical and ophthalmological characteristics according to tertiles of total plasma homocysteine levels. AVR and CRAE were inversely correlated with levels of homocysteine.
Associations of homocysteine, SAM and SAH with retinal vessel diameter

Figure 2a shows the distribution of homocysteine levels against levels of CRAE for persons with normal glucose metabolism, impaired glucose metabolism and diabetes. Figure 2b shows that, after adjustment for age, sex, and glucose tolerance status, subjects in the highest tertile of homocysteine had an 8 µm lower CRAE than subjects with homocysteine levels in the lowest tertile (p < 0.05). Indeed, when we analyzed homocysteine as a continuous variable, total plasma homocysteine was inversely associated with CRAE and AVR after adjustment for age, sex, and glucose tolerance status (st.ß (95%CI) –0.20 (-0.33;-0.07) and -0.15 (-0.28 ; -0.01), respectively). This implies that CRAE decreases with 3.78 µm per 1 SD increase of homocysteine level (= 4.6 µmol/l) (Table 2). After additional adjustments for SAM-plasma, 5-MTHF, and cardiovascular risk factors, the association of total plasma homocysteine with small retinal arteriolar diameter did not change (Table 3). Furthermore, individual adjustment for other components of the homocysteine metabolism and other cardiovascular risk factors, such as lipid levels or current smoking, also did not affect the results (data not shown).

High SAM-plasma and SAH-plasma were both associated with lower AVR after adjustment for age, sex and glucose tolerance status (Table 2). After adjustment for 5-MTHF, plasma homocysteine, SAM-plasma or SAH-plasma, body mass index, waist-to-hip ratio, waist circumference or microalbuminuria the associations lost statistical significance. After adjustment for total folate in serum and erythrocytes, or vitamins B6 and B12, the association of SAM and SAH in plasma with lower AVR remained statistical significant (data not shown).

Associations of SAM and SAH with retinal sclerotic vessel abnormalities and retinopathy

The SAM/SAH ratio in plasma was lower in subjects with retinal sclerotic vessel abnormalities than in subjects without retinal sclerotic vessel abnormalities (median (interquartile range): 5.6 (4.5-6.5) vs. 6.0 (5.1-7.0), p=0.05). In addition, SAH in plasma was significantly higher and the SAM/SAH ratio in plasma lower, in subjects with retinopathy as compared with subjects without retinopathy (SAH-plasma: 17.6 (15.6-25.6) vs. 15.3 (12.5-20.2) nmol/l, p=0.003; SAM/SAH ratio in plasma: 5.1 (4.2-5.8) vs. 6.0 (5.1-7.0), p<0.001).

After adjustment for age, sex and glucose tolerance status, the SAM/SAH ratio in plasma was inversely and strongly associated with retinal sclerotic vessel abnormalities and retinopathy (ORs (95% CI): 0.61 (0.39-0.96) and 0.50 (0.30-0.83), respectively) (Table 2). Adjustments for other homocysteine metabolism components showed little impact on both associations. Further adjustments for cardiovascular risk factors did not change the results.
substantially, except for prior CVD, which slightly attenuated the association for retinal sclerotic vessel abnormalities (data not shown).

In addition, SAM/SAH ratio in plasma showed a borderline association with microalbuminuria after adjustments for age, sex and glucose tolerance status (OR (95% CI) microalbuminuria (yes/no): 0.81 (0.62-1.06)). The standardized beta for microalbuminuria as a linear variable was –0.153 (p=0.029, SD=28.1) (data not shown).

**Additional analyses**

The presence of type 2 diabetes did not statistically significantly modify the associations of homocysteine metabolism components with AVR, CRAE, CRVE, retinal sclerotic vessel abnormalities or retinopathy (p>0.05). SAM and SAH in erythrocytes, the SAM/SAH ratio in erythrocytes, 5-MTHF, total folate in serum and in erythrocytes, and vitamins B12 and B6 were not associated with AVR, CRAE, CRVE, retinal sclerotic vessel abnormalities and retinopathy (data not shown).

**Discussion**

The present study, which included subjects with and without type 2 diabetes, had two main findings. First, a high total plasma homocysteine was significantly associated with retinal arteriolar narrowing, independently of other components of the homocysteine metabolism or cardiovascular risk factors. Second, a lower SAM/SAH ratio in plasma was independently associated with retinal sclerotic vessel abnormalities and retinopathy. These results did not differ between subjects with and without type 2 diabetes.

The present study is the first to show an independent association between total plasma homocysteine and retinal arteriolar narrowing. This finding is in line with the general concept that homocysteine has vasculotoxic properties. Homocysteine is considered an independent risk factor for atherosclerotic vascular disease and mortality [1,2]. In addition, hyperhomocysteinemia has been associated with retinal vascular occlusive disease, which was recently shown in a meta-analysis [15]. In contrast with our results, one study including 84 recently diagnosed type 2 diabetic individuals and 115 non-diabetic individuals did not find a relationship of plasma homocysteine levels with AVR [37]. This study, however, did not use a computer-assisted method to accurately measure arteriolar and venular diameters, as we did. Further support for an association between hyperhomocysteinemia and microvascular disease derives from studies of renal microvascular disease [11,13,14], which show an independent association of homocysteine with the presence and development of microalbuminuria [13,14].
In addition, Fassbender et al. demonstrated that homocysteine levels were increased among 82 subjects with cerebral microangiopathy, compared with subjects without cerebrovascular disease [38]. Therefore, our and previous studies suggest that hyperhomocysteinemia may not only be a risk factor for macrovascular disease, but may also be associated with microvascular disease in the retina and elsewhere.

Our results showed that subjects with homocysteine levels in the highest tertile had 8 µm smaller retinal arteriolar diameter compared with subjects with homocysteine levels in the lowest tertile. To put this into perspective, higher blood pressure (a known determinant of retinal arteriolar narrowing) was, in our study, associated with decreases of 0.6 and 1.1 µm in retinal arteriolar diameter per 10 mmHg higher systolic and diastolic blood pressure, respectively, which is comparable to previous findings [39,40]. Therefore, these data suggest that homocysteine could be a biologically relevant risk factor for retinal arteriolar narrowing.

The exact mechanism by which homocysteine affects the vascular system is not completely understood. However, homocysteine is thought to exert its toxic effects on the vascular wall by impairing endothelial function, increasing oxidative stress, decreasing availability of nitric oxide, inducing a prothrombotic state, and by inducing proliferation of smooth muscle cells [2,9]. This may cause vasoconstriction, intimal thickening, medial hyperplasia, hyalinization and sclerosis, consequently leading to a smaller arteriolar lumen.

We did not find any association between homocysteine and prevalent retinopathy. Previously, Hoogeveen et al. found a significant relationship of homocysteine with retinopathy in diabetic subjects, but not in non-diabetic subjects in the Hoorn Study population of 1989 [16]. These discordant findings might probably be due to limited power of the present study (N retinopathy = 32), which might also explain the absence of interaction of diabetic status. Moreover, selective mortality of diabetic individuals with hyperhomocysteinemia [41] may have resulted in a ‘healthy survivor effect’, and may have affected our results. On the other hand, data on the association between homocysteine and diabetic retinopathy are not consistent [42,43].

Unlike total plasma homocysteine, a lower SAM/SAH ratio in plasma was strongly associated with retinal sclerotic vessel abnormalities and retinopathy. Previous studies have shown that a low SAM/SAH ratio was associated with end-stage renal failure [44], peripheral arterial occlusive disease [23], and vascular disease (stroke and myocardial infarction) [45]. Our additional results that SAM/SAH ratio in plasma was borderline associated with microalbuminuria support these findings. Indeed, the ratio of SAM and SAH is crucial in the regulation of multiple enzymatic transmethylation reactions [46]. A decrease of this ratio may
result in inhibition of transmethylation reactions, which affects the biosynthesis of proteins, hormones, phospholipids, neurotransmitters, RNA and DNA [47,48], and consequently might lead to inhibition of vascular endothelial cell growth [49] and impairment of endothelial function. In addition, increased SAH, which could be caused by elevated homocysteine concentrations, also potentially acts as an inhibitor of transmethylation reactions [46]. Taken together, we hypothesize that a reduction of transmethylation reactions may be an important factor in the pathogenesis of retinal microvascular disease.

In the present study, we used a detailed measurement of retinal microvessel diameters by a computer-assisted method on fundus photographs made in mydriasis. Moreover, we used the revised formulas of Parr and Hubbard to quantify vessel caliber, by which the vessels were measured independently of image scale and the number of measured vessels [31]. A second advantage of this study is its population-based design, including subjects with and without type 2 diabetes.

This study has also limitations. The results that homocysteine is associated with retinal arteriolar narrowing and SAM/SAH ratio in plasma is only associated with sclerotic vessel abnormalities and retinopathy might be confusing, suggesting different pathophysiological mechanisms. As the complete pathophysiological pathway of the association of homocysteine/methionine metabolism with microangiopathy is still unclear, our results may represent different pathophysiological pathways, however we cannot exclude the possibility that these differences are cause by chance. Due to the cross-sectional design of this study, we cannot interpret the present results as cause-and-effect relationships. In addition, the study population consisted of individuals aged 60 to 85 with a considerable prevalence of cardiovascular risk factors, and we do not know whether the results of this study can be generalized to a younger or healthier population or to other ethnicities. However, an older population has also advantages in studying homocysteine and its components, because homocysteine levels increase with age, and low to low-normal concentrations or deficiencies of folate and vitamin B12 are relatively common in this age group, which provide wider ranges of concentrations to study in the elderly.

In conclusion, this is the first population-based study that shows an independent relationship of total plasma homocysteine with retinal arteriolar narrowing. In addition, the SAM/SAH ratio in plasma was strongly associated with retinal sclerotic vessel abnormalities and retinopathy. This suggests that homocysteine, and, possibly, reduced transmethylation reactions may be involved in the pathogenesis of retinal microvascular disease. Further studies are needed to clarify the microvascular effects of homocysteine-lowering treatment.
with folic acid, which may also increase transmethylation reactions [50], in order to create new treatment modalities for (retinal) microvascular disease.

Acknowledgements

We thank H.A. van Leiden and A.C. Moll for their important contribution to the retinal examination of the participants of the present study. Also, we would like to thank M.K. Ikram and J.R. Vingerling for their help with judging the retinal vessel diameter measurements.
Table 1: Baseline characteristics according to tertiles of homocysteine levels

<table>
<thead>
<tr>
<th></th>
<th>Homocysteine &lt;10.0 µmol/l</th>
<th>Homocysteine 10.0-12.7 µmol/l</th>
<th>Homocysteine &gt;= 12.8 µmol/l</th>
<th>P trend</th>
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<tbody>
<tr>
<td>N</td>
<td>85</td>
<td>83</td>
<td>84</td>
<td></td>
</tr>
<tr>
<td>Age (yrs)</td>
<td>69 ± 6</td>
<td>72 ± 7</td>
<td>74 ± 7</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Gender (% male)</td>
<td>47</td>
<td>49</td>
<td>61</td>
<td>0.076</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>6.3 ± 1.0</td>
<td>6.3 ± 0.9</td>
<td>6.2 ± 0.9</td>
<td>0.262</td>
</tr>
<tr>
<td>Diabetes (%)</td>
<td>41</td>
<td>46</td>
<td>42</td>
<td>0.894</td>
</tr>
<tr>
<td>Hypertension (%)</td>
<td>75</td>
<td>77</td>
<td>82</td>
<td>0.351</td>
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<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>144 ± 19</td>
<td>150 ± 21</td>
<td>148 ± 23</td>
<td>0.181</td>
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<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>85 ± 11</td>
<td>85 ± 12</td>
<td>83 ± 12</td>
<td>0.344</td>
</tr>
<tr>
<td>Use of ACE-inhibitor/ca-antagonist (%)</td>
<td>18.8</td>
<td>21.7</td>
<td>21.4</td>
<td>0.730</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>27.2 ± 4.0</td>
<td>27.2 ± 3.6</td>
<td>27.8 ± 4.5</td>
<td>0.345</td>
</tr>
<tr>
<td>Waist-to-hip ratio</td>
<td>0.94 ± 0.10</td>
<td>0.94 ± 0.09</td>
<td>0.95 ± 0.09</td>
<td>0.306</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>95.6 ± 12.7</td>
<td>95.5 ± 11.4</td>
<td>97.9 ± 10.4</td>
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</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>5.7 ± 1.1</td>
<td>5.9 ± 1.0</td>
<td>5.6 ± 1.0</td>
<td>0.623</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/l)</td>
<td>1.3 ± 0.4</td>
<td>1.5 ± 0.4</td>
<td>1.3 ± 0.4</td>
<td>0.862</td>
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<tr>
<td>LDL cholesterol (mmol/l)</td>
<td>3.5 ± 0.9</td>
<td>3.7 ± 0.9</td>
<td>3.6 ± 0.8</td>
<td>0.861</td>
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<tr>
<td>Triglycerides (mmol/l)</td>
<td>1.5 (1.0-2.0)</td>
<td>1.4 (1.0-1.8)</td>
<td>1.4 (1.1-1.7)</td>
<td>0.186</td>
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<td>Statin therapy (%)</td>
<td>17.6</td>
<td>12.0</td>
<td>10.7</td>
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<td>Microalbuminuria (%)</td>
<td>14</td>
<td>19</td>
<td>21</td>
<td>0.234</td>
</tr>
<tr>
<td>Albumin-to-creatinine ratio</td>
<td>0.7 (0.5-1.3)</td>
<td>0.7 (0.5-1.6)</td>
<td>0.8 (0.5-1.9)</td>
<td>0.037</td>
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<tr>
<td>Current smoking (%)</td>
<td>11</td>
<td>10</td>
<td>16</td>
<td>0.329</td>
</tr>
<tr>
<td>Prior cardiovascular disease (%)</td>
<td>45</td>
<td>59</td>
<td>57</td>
<td>0.261</td>
</tr>
<tr>
<td>AVR</td>
<td>0.76 ± 0.12</td>
<td>0.75 ± 0.09</td>
<td>0.72 ± 0.10</td>
<td>0.024</td>
</tr>
<tr>
<td>CRAE (µm)</td>
<td>173 ± 19</td>
<td>172 ± 18</td>
<td>166 ± 19</td>
<td>0.011</td>
</tr>
<tr>
<td>CRVE (µm)</td>
<td>231 ± 30</td>
<td>232 ± 27</td>
<td>231 ± 25</td>
<td>0.900</td>
</tr>
<tr>
<td>Retinal vessel sclerosis (%)</td>
<td>9</td>
<td>18</td>
<td>17</td>
<td>0.184</td>
</tr>
<tr>
<td>Retinopathy (%)</td>
<td>9</td>
<td>16</td>
<td>13</td>
<td>0.458</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SD, percentage or median (interquartile range) in the case of skewed distribution. P-values were calculated by analyses of variance, or a chi-square test. AVR: arteriole-to-venule ratio; CRAE: central retinal arteriolar equivalent; CRVE: central retinal venular equivalent.
Figure 1: Methionine-homocysteine metabolism

Legend: SAM: S-adenosylmethionine; SAH: S-adenosylhomocysteine; 5-MTHF: 5-methyltetrahydrofolate; B6: vitamin B6; B12: vitamin B12

Legend: SAM: S-adenosylmethionine; SAH: S-adenosylhomocysteine; 5-MTHF: 5-methyltetrahydrofolate; B6: vitamin B6; B12: vitamin B12
Figure 2a: scatterplot of central retinal arteriolar equivalent against homocysteine levels with different symbols assigned to persons with normal glucose metabolism (NGM), impaired glucose metabolism (IGM), and diabetes (DM).

Figure 2b: Age-, sex-, and glucose-tolerance-status-adjusted regression coefficients of tertiles of total plasma homocysteine associated with retinal arteriolar diameter.

Legend: CRAE: central retinal arteriolar equivalent; homocysteine tertiles: 1<10.0 µmol/l; 2=10.0-12.7 µmol/l; 3 >= 12.8 µmol/l

P<0.05
Table 2: Age-, sex-, and glucose-tolerance-status-adjusted associations between homocysteine, components of homocysteine metabolism and retinal vascular abnormalities

<table>
<thead>
<tr>
<th></th>
<th>Standard deviation</th>
<th>AVR</th>
<th>CRAE (18.9 µm)</th>
<th>CRVE (27.1 µm)</th>
<th>RSVA</th>
<th>RP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard</td>
<td>0.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>deviation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Homocysteine</td>
<td>4.6 µmol/l</td>
<td>-0.15 (-0.28 ; -0.01) / -0.02</td>
<td>-0.20 (-0.33 ; -0.07) / -3.78</td>
<td>-0.04 (-0.17 ; 0.09) / -1.03</td>
<td>0.97 (0.65-1.45)</td>
<td>0.98 (0.65-1.47)</td>
</tr>
<tr>
<td>SAM-plasma</td>
<td>28.5 nmol/l</td>
<td>-0.13 (-0.26 ; 0.00) / -0.01</td>
<td>-0.05 (-0.18 ; 0.07) / -0.95</td>
<td>0.10 (-0.02 ; 0.23) / 2.71</td>
<td>0.92 (0.62-1.36)</td>
<td>0.94 (0.64-1.40)</td>
</tr>
<tr>
<td>SAH-plasma</td>
<td>9.2 nmol/l</td>
<td>-0.15 (-0.29 ; -0.01) / -0.02</td>
<td>-0.12 (-0.25 ; 0.01) / -2.27</td>
<td>0.06 (-0.07 ; 0.19) / 1.63</td>
<td>1.17 (0.85-1.62)</td>
<td>1.16 (0.84-1.60)</td>
</tr>
<tr>
<td>SAM/SAH ratio*</td>
<td>1.5</td>
<td>0.05 (-0.10 ; 0.20) / 0.01</td>
<td>0.09 (-0.05 ; 0.23) / 1.70</td>
<td>0.02 (-0.12 ; 0.16) / 0.54</td>
<td>0.61 (0.39-0.96)</td>
<td>0.50 (0.30-0.83)</td>
</tr>
<tr>
<td>5-MTHF</td>
<td>8.9 nmol/l</td>
<td>0.02 (-0.13 ; 0.16) / 0.00</td>
<td>-0.00 (-0.14 ; 0.13) / -0.07</td>
<td>0.00 (-0.14 ; 0.14) / -0.11</td>
<td>0.98 (0.67-1.44)</td>
<td>0.92 (0.59-1.45)</td>
</tr>
<tr>
<td>Vitamin B12</td>
<td>101.1 pmol/l</td>
<td>-0.01 (-0.14 ; 0.13) / -0.00</td>
<td>-0.03 (-0.16 ; 0.09) / -0.57</td>
<td>-0.02 (-0.15 ; 0.12) / -0.55</td>
<td>1.11 (0.74-1.64)</td>
<td>1.01 (0.68-1.49)</td>
</tr>
<tr>
<td>Vitamin B6</td>
<td>37.2 nmol/l</td>
<td>0.03 (-0.10 ; 0.16) / 0.00</td>
<td>-0.06 (-0.18 ; 0.06) / -1.13</td>
<td>-0.10 (-0.22 ; 0.03) / -2.71</td>
<td>1.30 (0.97-1.74)</td>
<td>1.01 (0.70-1.45)</td>
</tr>
</tbody>
</table>

Data are calculated with linear regression analyses (AVR, CRAE, and CRVE), and with logistic regression analyses (RSVC and RP), and are shown as standardized betas (St.ß) (95% confidence interval (CI)) / change in AVR or µm CRAE/CRVE and odds ratios (OR) per 1 SD (95% CI). * SAM/SAH ratio in plasma; SAM: S-adenosylmethionine; SAH: S-adenosylhomocysteine; 5-MTHF: 5-Methyltetrahydrofolate; AVR: arteriole-to-venule diameter; CRAE: central retinal arteriolar equivalent; CRVE: central retinal venular equivalent; RSVC: retinal sclerotic vessel abnormalities; RP: retinopathy.
Table 3 Multivariate associations of homocysteine with the central retinal arteriolar equivalent (CRAE)

<table>
<thead>
<tr>
<th>Model</th>
<th>Added variables</th>
<th>St.ß (95% CI) / change in µm</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Homocysteine, age, sex, glucose tolerance status</td>
<td>-0.20 (-0.33 ; -0.07) / -3.78</td>
</tr>
<tr>
<td>2</td>
<td>As model 1 + SAM-plasma and 5-MTHF</td>
<td>-0.25 (-0.39 ; -0.09) / -4.73</td>
</tr>
<tr>
<td>3</td>
<td>As model 1 + glycated hemoglobin, systolic and diastolic blood pressure, body mass index, total cholesterol, microalbuminuria and prior CVD</td>
<td>-0.22 (-0.35 ; -0.09) / -4.16</td>
</tr>
<tr>
<td>4</td>
<td>As model 2 + glycated hemoglobin, systolic and diastolic blood pressure, body mass index, total cholesterol, microalbuminuria and prior CVD</td>
<td>-0.25 (-0.41 ; -0.09) / -4.73</td>
</tr>
</tbody>
</table>

Data are calculated with linear regression analyses, and are showed as standardized beta (95% confidence interval) and change in µm CRAE. SAM: S-adenosylmethionine; SAH: S-adenosylhomocysteine; 5-MTHF: 5-Methyltetrahydrofolate
References


