Genetic Stabilization of Transthyretin, Cerebrovascular Disease, and Life Expectancy

Louise S. Hornstrup, Ruth Frikke-Schmidt, Børge G. Nordestgaard, Anne Tybjærg-Hansen

Objective—Transthyretin can cause amyloidosis attributable to destabilization of transthyretin tetramers in plasma. We tested the hypothesis that genetic stabilization of transthyretin associates with reduced risk of vascular disease and increased life expectancy.

Approach and Results—we included 68,602 participants from 2 prospective studies of the general population. We genotyped for 2 stabilizing genetic variants in the transthyretin gene (TTR), R104H and T119M, and determined the association of genotypes with plasma levels of transthyretin, measures of thyroid function, risk of vascular disease, and life expectancy. During a mean follow-up of 32 years, 10,636 participants developed vascular disease. We identified 321 heterozygotes for T119M (frequency, 0.47%); R104H was not detected. First, mean plasma transthyretin and thyroxine levels were increased by 17% (26 μg/mL) and 20% (19 nmol/L), respectively, in heterozygotes versus noncarriers (P≈0.007 and P<0.0001), demonstrating functionality of this variant in the general population. Second, corresponding hazard ratios were 0.70 (95% confidence interval, 0.51–0.97) for all vascular diseases, 0.85 (0.59–1.23) for cardiovascular disease, 0.45 (0.25–0.81) for cerebrovascular disease, 0.47 (0.25–0.88) for ischemic cerebrovascular disease, and 0.31 (0.04–2.22) for hemorrhagic stroke. The cumulative incidence of cerebrovascular disease as a function of age was decreased in heterozygotes versus noncarriers (P=0.005). Third, median age at death from all causes, from vascular and cerebrovascular diseases, and after diagnosis of vascular disease, and median age at diagnosis of vascular disease, was increased by 5 to 10 years in heterozygotes versus noncarriers (P=0.002–0.05).

Conclusions—These results are compatible with an association between genetic stabilization of transthyretin and decreased risk of cerebrovascular disease, and with increased life expectancy in the general population.

Key Words: amyloid ▪ amyloid angiopathy ▪ cerebrovascular disease/stroke ▪ genetic epidemiology ▪ vascular disease

Transthyretin is one of many proteins that can cause the degenerative disease amyloidosis, a term for a group of diseases characterized by extracellular deposition of pathological insoluble fibrillar proteins in organs and tissues. Transthyretin is primarily synthesized in the liver, but is also produced in the choroid plexus. Although the main function is to transport retinol-binding protein, transthyretin also transports 15% and 80% of thyroxine in blood and cerebrospinal fluid, respectively. In both plasma and cerebrospinal fluid, transthyretin circulates as a tetramer comprising 4 identical subunits. Destabilization of these tetramers leads to monomer misfolding and amyloidogenesis, a process that can be prevented by ligands, such as thyroxine, and by drugs that stabilize transthyretin tetramers.

In humans, both wild-type transthyretin tetramers and tetramers comprising mutant and wild-type subunits can cause amyloidosis. Senile systemic amyloidosis is a common aging phenomenon caused by extracellular deposition of wild-type transthyretin in many organs and in the walls of small arteries, but with clinical manifestations mainly from the heart attributable to restrictive cardiomyopathy. The hereditary forms of transthyretin amyloidosis are presumably rare and caused by mutations in the transthyretin gene (TTR) that make the tetrameric protein more unstable and prone to misfold into fibrils. The hereditary forms of transthyretin amyloidosis are typically of earlier onset and more severe than in senile systemic amyloidosis. Clinically, these patients may present with polyneuropathy, cardiomyopathy, or cerebral amyloid angiopathy characterized by deposition of amyloid in cerebral blood vessels, and clinically by both hemorrhagic and ischemic lesions. Both senile systemic amyloidosis and hereditary forms may have deposition of amyloid in small arteries.
Although most mutations in TTR increase the amyloidogenic potential of the protein by destabilizing the tetramers, 2 variants, R104H (rs121918095) and T119M (rs28933981), have been shown to stabilize both wild-type transthyretin tetramers and tetramers comprising mutant and wild-type subunits, and thereby to prevent amyloidogenesis in vitro.14–17 These 2 mutations were first identified in compound heterozygotes, carrying one of these variants together with a known disease-causing mutation in Portuguese (T119M) and Japanese (R104H) families, respectively, and were reported to have milder disease than heterozygotes for the disease-causing mutation alone.14,18 Finally, some family studies have suggested increased thyroxine binding or increased levels of thyroxine or transthyretin in carriers of these 2 stabilizing variants.14,18–21

Whether any of these variants are common in the general population, whether they associate with increased thyroxine and transthyretin levels as markers of increased transthyretin tetramer stabilization, and whether they associate with reduced risk of vascular disease and increased life expectancy in the general population is currently unknown. We tested these hypotheses by genotyping for R104H and T119M in 2 similar studies of the Danish general population, the Copenhagen General Population Study and the Copenhagen City Heart Study, totalling 68,602 participants, of which 10,636 participants developed vascular disease.

### Materials and Methods

Materials and Methods are available in the online-only Supplement.

### Results

#### Characteristics

Genotyping the Copenhagen City Heart Study and the Copenhagen General Population Study identified 68,281 TTR T119M noncarriers (genotype CC) and 321 TTR T119M heterozygotes (genotype CT; frequency, 0.47%; Table). Genotype distribution did not deviate from the Hardy–Weinberg equilibrium (P=0.54). R104H, a stabilizing genetic variant previously identified in Asians,18,22 was not detected. Neither risk factors for ischemic vascular disease (Table) nor measures of glucose metabolism, renal function, liver function, or inflammation (Table I in the online-only Data Supplement) differed by T119M genotype, confirming that the effect of genotype on risk of vascular disease was not confounded by risk factors for vascular disease, or other markers of disease, including inflammatory disease. Comparing continuous traits as a function of genotype using parametric t tests did not markedly change these results (P values, 0.62–0.86 for Table and 0.05–0.99 for Table I in the online-only Data Supplement).

#### TTR T119M Genotype and Plasma Transthyretin

Plasma levels of transthyretin were measured in a total of 1650 participants in the Copenhagen City Heart Study: in all heterozygotes with plasma available (n=35) and in a random sample of noncarriers (n=1615). As previously reported,23 plasma transthyretin levels decreased as a function of age (in 10-year age groups) in both women and men (mean decrease in women and men, respectively, 50 µg/mL and 47 µg/mL; P values for trend <0.0001 from 20 to 80+ years). Mean plasma levels of transthyretin were 16 µg/mL lower in women than in men (Figure I in the online-only Data Supplement; P<0.0001). Results were similar when using parametric tests (P values <0.0001 by linear regression analysis).

T119M heterozygotes had a 17% (26 µg/mL) increase in plasma levels of transthyretin compared with noncarriers. Mean transthyretin levels were 183 µg/mL and 157 µg/mL, respectively (Figure 1A; P=0.007). When matching 1:7 by age (in 10-year age groups) and by sex to account for the effect of age and sex on transthyretin levels (Figure I in the online-only Data Supplement), the corresponding increase was 20% (31 µg/mL; Figure 1B; P=0.003). Results were similar

<table>
<thead>
<tr>
<th>TTR T119M Genotype</th>
<th>No. of subjects (%)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Noncarriers (CC)</td>
<td>68,281 (99.5)</td>
<td></td>
</tr>
<tr>
<td>Heterozygotes (CT)</td>
<td>321 (0.47)</td>
<td></td>
</tr>
<tr>
<td>Age, y</td>
<td>58 (47–67)</td>
<td>0.71</td>
</tr>
<tr>
<td>Sex (F/M)</td>
<td>37,805/30,476</td>
<td>0.25</td>
</tr>
<tr>
<td>Total cholesterol, mmol/L</td>
<td>5.6 (4.9–6.4)</td>
<td>0.88</td>
</tr>
<tr>
<td>Triglycerides, mmol/L</td>
<td>1.4 (1.0–2.1)</td>
<td>0.41</td>
</tr>
<tr>
<td>LDL cholesterol, mmol/L</td>
<td>3.2 (2.6–3.9)</td>
<td>0.92</td>
</tr>
<tr>
<td>HDL cholesterol, mmol/L</td>
<td>1.6 (1.3–1.9)</td>
<td>0.65</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>26 (23–28)</td>
<td>0.63</td>
</tr>
<tr>
<td>Hypertension, %</td>
<td>53</td>
<td>0.81</td>
</tr>
<tr>
<td>Diabetes mellitus, %</td>
<td>4</td>
<td>0.60</td>
</tr>
<tr>
<td>Smoking, %</td>
<td>61</td>
<td>0.69</td>
</tr>
<tr>
<td>Physical activity, %</td>
<td>45</td>
<td>0.71</td>
</tr>
<tr>
<td>Lipid-lowering therapy, %</td>
<td>8</td>
<td>0.54</td>
</tr>
</tbody>
</table>

Values are median (interquartile range) or percentage. Diabetes mellitus was self-reported disease, use of insulin, use of oral hypoglycemic drugs, or nonfasting plasma glucose ≥11 mmol/L. Smoking was active and former smokers. Hypertension was systolic blood pressure ≥140 mm Hg, diastolic blood pressure ≥90 mm Hg, or use of antihypertensive medication. Physical activity was ≥2 hours per week of light physical activity in leisure time. Lipid-lowering therapy was mainly statins. P values by Mann–Whitney U test or Pearson χ² test. HDL indicates high-density lipoprotein; and LDL, low-density lipoprotein.

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after adjusting for age and sex by linear regression analysis (β-coefficient=30, corresponding to a mean increase in trans-
thyretin of 30 μg/mL in heterozygotes versus noncarriers; 
P<0.001, data not shown).

**TTR T119M Genotype, Plasma Thyroxine and Other Measures of Thyroid Function**

*TTR* T119M genotype associated with a 20% (19 nmol/L) increase in mean plasma levels of total thyroxine in heterozy-
gotes versus noncarriers. Mean total thyroxine levels were 114 
nmol/L and 95 nmol/L, respectively (Figure 2; *P*<0.0001). In 
contrast, genotype did not associate with other measures of 
thyroid function, that is, thyroid stimulating hormone, triiod-
thyronine or triiodothyronine uptake test, an indirect measure 
of the amount of thyroxine bound to thyroid-binding globu-
lin, (Figure 2; *P*=0.56–0.63), or with plasma levels of albu-
min (Table I in the online-only Data Supplement; *P*=0.89). In 
plasma, thyroid-binding globulin normally binds 70% to 80% 
of thyroxine, transthyretin binds 10% to 15%, and the rest is 
bound to albumin. Hence, these data suggest that T119M 
heterozygotes have increased total thyroxine levels attributable 
to an increased binding of thyroxine to transthyretin, but 
normal thyroid function. Results were similar after adjusting 
for age and sex by linear regression analysis (β-coefficient=18, 
corresponding to a mean increase in thyroxine of 18 nmol/L 
in heterozygotes versus noncarriers, *P*<0.0001; remaining 
*P* values, 0.57–0.73; data not shown).

**TTR T119M Genotype and Risk of Vascular and Cerebrovascular Disease**

During a mean follow-up of 32 years (range, 0–34 years), the 
multifactorially adjusted (adjusted for age, sex, total cholesterol, 
triglycerides, low-density lipoprotein cholesterol, high-density 
lipoprotein cholesterol, body mass index, hypertension, diabetes 
mellitus, smoking, physical activity, lipid-lowering therapy, 
high-sensitivity C-reactive protein, and fibrinogen) hazard 
ratios for *TTR* heterozygotes versus noncarriers were 0.70 
(95% confidence interval, 0.51–0.97; *P*=0.03) for all vascular 
disease, 0.85 (0.59–1.23; *P*=0.39) for cardiovascular disease, 
0.45 (0.25–0.81; *P*=0.008) for cerebrovascular disease, 0.47 
(0.25–0.88; *P*=0.02) for ischemic cerebrovascular disease, and 
0.31 (0.04–2.22; *P*=0.24) for hemorrhagic stroke (Figure 3). 
There were no interactions between genotype and study cohort 
(Copenhagen City Heart Study and Copenhagen General 
Population Study) on risk of any of the end points mentioned 
avove, indicating a similar effect of genotype on risk in both 
cohorts (Figure II in the online-only Data Supplement).

The cumulative incidence of cerebrovascular disease was 
reduced in T119M heterozygotes versus noncarriers (Figure 4; 
log rank, *P*=0.005). At the age of 75 years, the cumulative 
icidence of cerebrovascular disease was 5% in T119M heter-
ozygotes and 12% in noncarriers (Figure 4).

**TTR T119M Genotype and Life Expectancy**

In T119M heterozygotes versus noncarriers, median age at 
death was increased for all causes of death by 5 years (*P*=0.04), 
for death from vascular disease by 7 years (*P*=0.01), and for 
death from cerebrovascular disease by 10 years (*P*=0.03); age 
at death from nonvascular disease did not differ by genotype 
(*P*=0.61; Figure 5, left and middle). Furthermore, median age 
at death after diagnosis of vascular disease was increased by 7 
years in heterozygotes versus noncarriers (*P*=0.002; Figure 5, 
right). Finally, median age at diagnosis of all vascular diseases 
and cardiovascular disease was increased by 7 and 8 years, 
respectively (Figure III in the online-only Data Supplement; 
*P*=0.05 and *P*=0.02), while the data suggested a similar trend 
for median age at diagnosis of cerebrovascular disease (*P*=0.59). 
Results were similar, but slightly attenuated, after adjusting for 
sex by linear regression analysis (*P* values for age at death, 0.01– 
0.62; *P* values for age at disease, 0.06–0.75; data not shown).

**Discussion**

Our results were obtained in 2 similar prospective studies of 
the general population comprising 68 602 participants, including 
321 heterozygotes for *TTR* T119M and 10 636 individuals 
with incident vascular disease.

The principal findings of this study are the following: (1) 
One in 200 individuals (0.47%) in a white general population 
is heterozygous for *TTR* T119M, a variant known to stabilize 
transthyretin tetramers and to increase binding of thyroxine in 
vitro; (2) T119M is associated with increases in transthyretin 
and total thyroxine levels in heterozygotes versus noncarriers,
providing direct evidence for the stabilizing function of this variant in the general population in vivo; (3) Genetic stabilization of transthyretin tetramers in T119M heterozygotes versus noncarriers is associated with reduced risk of vascular disease, mainly cerebrovascular disease; (4) T119M heterozygotes have increased life expectancy overall and in those who die from vascular and cerebrovascular disease, increased life expectancy after diagnosis of vascular disease and increased age at diagnosis of vascular disease compared with noncarriers. These novel findings need to be confirmed in independent

<table>
<thead>
<tr>
<th>Genotype</th>
<th>No. of Subjects</th>
<th>No. of Events</th>
<th>Adjusted for age and sex</th>
<th>P-value</th>
<th>Adjusted multifactorially</th>
<th>Hazard Ratio (95% CI)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>All vascular disease</td>
<td>CC 68209</td>
<td>10599</td>
<td>319</td>
<td>0.04</td>
<td>1.0</td>
<td>0.70 (0.51-0.97)</td>
<td>0.03</td>
</tr>
<tr>
<td>Cardiovascular disease</td>
<td>CC 68229</td>
<td>7064</td>
<td>320</td>
<td>0.44</td>
<td>1.0</td>
<td>0.85 (0.59-1.23)</td>
<td>0.39</td>
</tr>
<tr>
<td>Cerebrovascular disease</td>
<td>CC 68261</td>
<td>4872</td>
<td>320</td>
<td>0.008</td>
<td>1.0</td>
<td>0.45 (0.25-0.81)</td>
<td>0.008</td>
</tr>
<tr>
<td>Ischemic cerebrovascular disease</td>
<td>CC 68278</td>
<td>4161</td>
<td>321</td>
<td>0.02</td>
<td>1.0</td>
<td>0.47 (0.25-0.88)</td>
<td>0.02</td>
</tr>
<tr>
<td>Hemorrhagic stroke</td>
<td>CC 68279</td>
<td>650</td>
<td>321</td>
<td>0.24</td>
<td>1.0</td>
<td>0.31 (0.04-2.22)</td>
<td>0.24</td>
</tr>
</tbody>
</table>

Figure 2. Plasma levels of thyroxine, thyroid stimulating hormone, triiodothyronine, and triiodothyronine uptake test in T119M heterozygotes vs noncarriers. Values are mean±standard error of the mean. CC indicates noncarriers; CT, T119M heterozygotes; and mIU/L, milli international units per liter. P values by Mann–Whitney U test.

Figure 3. Risk of all vascular disease, cardiovascular disease, cerebrovascular disease, ischemic cerebrovascular disease, and hemorrhagic stroke as a function of T119M genotype. CC indicates noncarriers; CI, confidence interval; and CT, T119M heterozygotes.
studies, but may suggest that drugs, such as tafamidis,\textsuperscript{4,5,25} which kinetically stabilize transthyretin, may have similar beneficial effects in the general population.

The stabilizing effect of the T119M variant in vitro has been convincingly shown by subunit exchange, where an increasing number of T119M subunits in the tetrameric structure associated strongly with decreased fibril formation and increased tetramer stability.\textsuperscript{16,17} The stabilizing property of the T119M variant is suggested to explain why compound heterozygotes for T119M and disease-causing mutations in \textit{TTR} are protected from disease.\textsuperscript{14,15} However, the frequency and impact of T119M in the general population has not been determined previously. Thus far, only a few studies with a limited number of carriers have examined the effect of heterozygosity for T119M on plasma levels of transthyretin\textsuperscript{20,21,26,27}; most suggest that carriers may have increased levels of transthyretin. In the largest study to date, we show that T119M heterozygotes have a 17\% (26 μg/mL) increase in transthyretin levels compared with noncarriers. These findings are in contrast to the more unstable, pathogenic \textit{TTR}-variants, where carriers typically display lower transthyretin levels than normal.\textsuperscript{12} Therefore, the higher transthyretin levels indicate a protein less likely to dissociate and aggregate into fibrils.

It has been shown both by extensive physicochemical studies in vitro and by ex vivo studies of sera from T119M heterozygotes\textsuperscript{14,19,20} that increased binding affinity for thyroxine reflects the stabilizing effect of T119M on the transthyretin tetramer. Furthermore, adding thyroxine, a natural ligand for transthyretin, has been shown to stabilize wild-type transthyretin and inhibit fibril formation in vitro.\textsuperscript{28} Therefore, increased plasma level of total thyroxine in heterozygotes is also a marker for the increased transthyretin tetramer stability attributable to T119M. We found that carriers had a 20\% (19 nmol/L) increase in total thyroxine levels compared with noncarriers, but normal thyroid function, indicating that the increased thyroxine level was attributable to increased binding to transthyretin. Thus, our study demonstrates that T119M is functional and stabilizes transthyretin in the general population in vivo.

In senile systemic amyloidosis, amyloid fibrils are derived from normal wild-type transthyretin.\textsuperscript{6,9} Postmortem studies have shown that senile systemic amyloidosis affects \approx 25\% of individuals >80 years; however, the impact of this condition in the general population is not known.\textsuperscript{9,10} Although cardiomyopathy may be the most severe manifestation of senile systemic amyloidosis, this condition has been associated with an increased risk of myocardial infarction in autopsy studies,\textsuperscript{9,10} and possibly an increased risk of cardioembolic stroke.\textsuperscript{29,30} Furthermore, vascular deposits of transthyretin amyloid have been found systemically in cases with senile systemic amyloidosis,\textsuperscript{7,8} indicating that the vascular integrity may be compromised in other organs as well, including the brain. Therefore, we propose that the protective effect on vascular disease associated with the stabilizing variant T119M may be attributable to a reduced deposition of wild-type transthyretin in the wall of small arteries. In addition, in the present study, heterozygotes had increased life expectancy overall and
among those who died from vascular and cerebrovascular disease, increased life expectancy after diagnosis of vascular disease and increased age at diagnosis of vascular disease compared with noncarriers. These results support that T119M may act through an effect on the vascular wall.

The sporadic form of cerebral amyloid angiopathy with deposition of Aβ, the protein also implicated in Alzheimer disease, is a common condition affecting ≈40% to 50% of individuals aged ≥60 years, and leading to an increased risk of both ischemic and hemorrhagic cerebral events. Several studies have shown that transthyretin can interact with Aβ and possibly inhibit Aβ amyloid deposition.32–34 Thus, another potential explanation for the apparent protective effect of T119M on cerebrovascular disease could be that T119M stabilizes transthyretin in the cerebrospinal fluid and, by binding Aβ, protects against sporadic cerebral amyloid angiopathy.

Other studies have pointed to a general role for transthyretin in neurobiology and repair. More specifically, transthyretin β-bilizes transthyretin in the cerebrospinal fluid and, by binding potential explanation for the apparent protective effect of T119M on cerebrovascular disease. Thus, our data could suggest that T119M protects against amyloid deposition in vitro and in animal studies. Positive results from human trials have also been reported. Therefore, one can speculate that the beneficial effects observed with high or moderately high dietary intake of these compounds may be partly through their stabilizing effect on transthyretin.

Iodinated transthyretin derivatives are candidate drugs for transthyretin amyloidosis, showing improved stability as compared with transthyretin. Thyroxine binding stabilizes transthyretin through 4 iodine substituents, and there are additional binding sites for thyroxine left unbound on transthyretin. Hence, total plasma iodine levels may correlate with transthyretin amyloid deposition in vitro and in animal studies. Positive results from human trials have also been reported. Therefore, one can speculate that the beneficial effects observed with high or moderately high dietary intake of these compounds may be partly through their stabilizing effect on transthyretin.

References
Transthyretin can cause amyloidosis attributable to destabilization of transthyretin tetramers in plasma. We tested the hypothesis that genetic stabilization of transthyretin associates with reduced risk of vascular disease and increased life expectancy. Our novel findings include the following: (1) One in 200 individuals (0.47%) is heterozygous for TTR T119M, a variant known to stabilize transthyretin tetramers; (2) T119M is associated with increases in transthyretin and total thyroxine levels in heterozygotes versus noncarriers, providing direct evidence for the stabilizing function of this variant in vivo; (3) T119M heterozygotes have reduced risk of vascular disease, mainly cerebrovascular disease; (4) T119M heterozygotes have increased life expectancy overall and after diagnosis of vascular disease. These results have potential clinical implications, because they suggest that current drugs, which inhibit the amyloid cascade by mimicking the stabilizing effect of T119M on transthyretin, may slow the progression of vascular deposits leading to cerebrovascular disease in the general population.
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Material and Methods

Study cohorts
Studies were approved by institutional review boards and Danish ethical committees, and conducted according to the Declaration of Helsinki. Written informed consent was obtained from all participants. All participants were white and of Danish descent, and were randomly selected to reflect the adult Danish population aged 20 to 80+ years.

Participants
We included participants in two similar prospective studies of the Danish general population: The Copenhagen General Population Study and The Copenhagen City Heart Study. Combining these studies yielded a total of 68,602 participants, of whom 10,636 developed vascular disease.

The Copenhagen General Population Study
This is a prospective study of the Danish general population initiated in 2003 with ongoing enrollment. Data were obtained from a questionnaire, a physical examination, and from blood samples including DNA extraction. We included the first 58,206 participants from this study in the present analysis. Of these; 7,714 developed vascular disease (4,895 developed cardiovascular, that is ischemic heart disease, and 3,687 developed cerebrovascular disease, that is ischemic cerebrovascular disease or hemorrhagic stroke).

The Copenhagen City Heart Study
This prospective study of the Danish general population was initiated in 1976-78 with follow-up examinations in 1981-83, 1991-94, and 2001-03. Participants were recruited and examined exactly as in the Copenhagen General Population Study. We included 10,396 participants who gave blood for DNA analysis at the 1991-94 or 2001-03 examinations. Of these, 2,922 developed vascular disease (2,198 developed cardiovascular disease and 1,196 developed cerebrovascular disease). Follow-up time for vascular disease for each participant in either study began at the establishment of the National Danish Patient Registry (January 1, 1977) or on the participant’s birthday, whichever came later. For all endpoints, follow-up ended at the date of death, occurrence of event, emigration, or on May 10th 2011 (last update of registry), whichever came first. Mean follow-up was 32 years, and was 100% complete, i.e. none were lost to follow-up.

Endpoints
Vascular disease was cardiovascular disease or cerebrovascular disease as defined in the following. Information on diagnoses of cardiovascular disease (World Health Organization; International Classification of Disease, 8th edition codes 410-14; 10th edition codes I20-I25) was collected and verified by reviewing all hospital admissions and diagnoses entered in the National Danish Patient Registry, all causes of death entered in the National Danish Causes of Death Registry, and medical records from hospitals and general practitioners. Cardiovascular disease was fatal or non-fatal myocardial infarction or characteristic symptoms of angina pectoris, including revascularisation procedures; death from other causes lead to censoring. A diagnosis of myocardial infarction followed the changing
definitions over time and required a typical rise and fall of biochemical markers (troponin or CK-MB), with later changes as indicated.

Information on diagnoses of cerebrovascular disease, including ischemic cerebrovascular disease, that is transitory ischemic attacks, amaurosis fugax and ischemic stroke, and hemorrhagic stroke (WHO; International Classification of Diseases, 8th edition, 431-438; 10th edition, I60-I69, G45) were collected by reviewing all hospital admissions and diagnoses entered in the National Danish Patient Registry and the National Danish Causes of Death Registry.

Laboratory analyses
Plasma levels of transthyretin were measured in a total of 1,650 participants in the Copenhagen City Heart Study: In all heterozygotes with plasma available (n=35) and in a random sample of non-carriers (n=1,615), using a human transthyretin double antibody sandwich enzyme-linked immunosorbent assay, according to the manufacturer’s recommendations (ICL E-80PRE, Dunn Labortechnik GmbH, Asbach, Germany). All samples were run in duplicate.

Plasma levels of thyroid stimulating hormone, total thyroxine, total triiodothyronine and triiodothyronine uptake-test was available on 62,776, 10,091, 4,603 and 4,590 participants, respectively. Triiodothyronine uptake, an indirect measure of the amount of thyroxine bound to thyroid binding globulin, was expressed as thyroid hormone-binding ratio, in which the percent thyroid hormone uptake is divided by a percent uptake from a reference serum. Thyroid function tests were measured using solid phase, competitive chemiluminescent enzyme immunoassays (Siemens Healthcare Diagnostics, Erlangen, Germany).

Plasma total cholesterol, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, triglycerides, glucose, creatinine, albumin, alanine aminotransferase, gamma glutamyltransferase, coagulation factors II-VII-X, pancreatic amylase, C-reactive protein and fibrinogen were measured using standard hospital assays (Boehringer Mannheim GmbH, Mannheim, Germany; Konelab, Helsinki, Finland; DAKO, Glostrup, Denmark; Dade Behring, Deerfield, IL, USA, or Thermo Fisher Scientific, Waltham, MA, USA). Glomerular filtration rate was estimated.

Other covariates
Body mass index was measured weight in kilograms divided by measured height in meters squared. Diabetes mellitus, smoking, hypertension, physical activity, and lipid-lowering therapy were dichotomized and defined as diabetes (self-reported disease, use of insulin, use of oral hypoglycemic drugs and/or nonfasting plasma glucose >11mmol/L), smoking (active and former smoker), hypertension (systolic blood pressure ≥140 mmHg, diastolic blood pressure ≥90 mmHg and/or daily use of antihypertensive drugs), physical activity (four hours or more per week of light physical activity in leisure time versus less than four hours), and lipid-lowering therapy (yes/no).

Genotyping
Genotyping of the only two variants previously reported to increase transthyretin stability in vitro R104H c.371 G>A (rs121918095, chromosome 18, position 29,178,565) and T119M c.416 C>T (rs28933981, chromosome 18, position 29,178,610), was by Taqman based assays on an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems Inc., Foster City, CA, USA). T119M heterozygotes were verified by running the
Taqman assay twice; concordance between the two rounds of TaqMan was 100%. In addition, 10% of all heterozygotes were verified by sequencing. R104H, a variant previously exclusively identified in Asians, was not detected.

**Statistical analyses**

Data were analyzed using STATA/S.E. version 12.0 (Stata Corp., College Station TX, USA). Chi-square test evaluated Hardy-Weinberg equilibrium. The Mann-Whitney U test and Pearson’s $\chi^2$ test were used to compare continuous and categorical values, respectively, as a function of TTR genotype or sex. Plasma transthyretin levels as a function of age in 10-year age groups in women and men separately were by Cuzick’s extension of a Wilcoxon rank-sum test.

First, to test whether TTR genotype associated with increased plasma levels of transthyretin and thyroxine, and with other measures of thyroid function, heterozygotes were compared with non-carriers. For transthyretin, heterozygotes were also matched 1:7 by age (in 10 year age groups) and by sex, to account for the effect of age and sex on transthyretin levels (Supplementary Figure I). Furthermore, linear regression analyses adjusted for age and sex evaluated the effect of genotype on transthyretin levels and measures of thyroid function, and linear regression analyses adjusted for sex evaluated age at death from all endpoints and age at event.

Second, to test whether genetically elevated transthyretin levels associated with decreased risk of vascular disease, we tested for association between TTR genotype and vascular disease in the CCHS and the CGPS combined to obtain maximal power. Because genotype is constant throughout life, and hence imperious to reverse causation, risk of vascular disease as a function of genotype was analyzed from 1977 to 2011. Individuals with events before entry were excluded in prospective analyses.

Cox proportional hazard regression models with age as time scale and left truncation (delayed entry) were used to estimate hazard ratios. Models were adjusted for age and sex, or multifactorially for age, sex, total cholesterol, triglycerides, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, body mass index, hypertension, diabetes, smoking, physical activity, lipid-lowering therapy, high-sensitivity C-reactive protein and fibrinogen. Proportionality of hazards over time was assessed by plotting $-\ln(-\ln[\text{survival}])$ versus ln(analysis time). There was no suspicion of nonparallel lines. Kaplan Meier curves and log-rank tests were used to estimate the cumulative incidence of cerebrovascular disease as a function of age and TTR genotype. To test whether the effect of genotype in predicting disease endpoints was similar in the two studies of the general population, the Copenhagen City Heart Study and the Copenhagen General Population Study, bivariate interaction terms between genotype and cohort were included in the models, and tested statistically by likelihood ratio tests.

Third, to test whether TTR genotype was associated with life expectancy, that is, age at death, or age at death after diagnosis of vascular disease, or with age at diagnosis of vascular disease, cardiovascular disease or cerebrovascular disease, we determined differences in age between heterozygotes and non-carriers for these endpoints. Sensitivity analyses evaluated whether association with age at death was due primarily to vascular, cerebrovascular or non-vascular causes of death, by restricting analyses to participants with register-based (National Danish Causes of Death Registry) vascular, cerebrovascular or non-vascular causes of death.
References


Supplemental Material

Supplementary Table I, page 2.

Supplementary Figure I, page 3. Plasma levels of transthyretin as a function of age (in 10-year age groups) and by sex (women in red and men in blue). Values are median and interquartile range. Tests for trend (transthyretin as a function of age in women and men separately) by Cuzick’s extension of a Wilcoxon rank-sum test.

Supplementary Figure II, page 4. Risk of all vascular disease, cardiovascular disease, cerebrovascular disease, ischemic cerebrovascular disease and hemorrhagic stroke as a function of T119M genotype stratified by cohort. CCHS= the Copenhagen City Heart Study; CGPS= the Copenhagen General Population Study; CC=non-carriers; CT=T119M heterozygotes. P-values for interaction between genotype and cohort on risk of endpoint by likelihood ratio tests.

Supplementary Figure III, page 5. Age at diagnosis of vascular disease, cardiovascular disease and cerebrovascular disease in T119M heterozygotes versus non-carriers from the general population. Values are median and upper interquartile range. CC=non-carriers; CT=T119M heterozygotes. P-values by Mann-Whitney U-test.
Supplementary Table I.

Biochemical characteristics of subjects heterozygous for the T119M variant in *TTR* versus non-carriers in the Copenhagen City Heart Study and the Copenhagen General Population Study combined.

<table>
<thead>
<tr>
<th><em>TTR T119M</em> Genotype</th>
<th>Non-carriers (CC)</th>
<th>Heterozygotes (CT)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of subjects (%)</td>
<td>68,281 (99.5)</td>
<td>321 (0.47)</td>
<td></td>
</tr>
<tr>
<td><strong>Glucose metabolism</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>5.1 (4.7-5.7)</td>
<td>5.2 (4.8-6.0)</td>
<td>0.20</td>
</tr>
<tr>
<td>Hemoglobin A1c (%)</td>
<td>5.8 (5.6-6.2)</td>
<td>5.8 (5.5-6.2)</td>
<td>0.80</td>
</tr>
<tr>
<td><strong>Renal function</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Creatinine (μmol/L)</td>
<td>84.0 (76.0-94.0)</td>
<td>84.0 (76.0-94.0)</td>
<td>0.83</td>
</tr>
<tr>
<td>Estimated GFR (mL/min per 1.73 m²)</td>
<td>76.4 (66.0-87.2)</td>
<td>77.2 (64.5-87.2)</td>
<td>0.95</td>
</tr>
<tr>
<td><strong>Liver function</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Albumin (μmol/L)</td>
<td>590.0 (544.0-630.0)</td>
<td>591.0 (533.0-635.0)</td>
<td>0.89</td>
</tr>
<tr>
<td>Alanine aminotransferase (U/L)</td>
<td>19.0 (14.0-27.0)</td>
<td>19.0 (15.0-26.0)</td>
<td>0.66</td>
</tr>
<tr>
<td>Gamma glutamyltransferase (U/L)</td>
<td>28.6 (21.0-43.0)</td>
<td>28.0 (21.0-40.0)</td>
<td>0.21</td>
</tr>
<tr>
<td>Factor II-VII-X (%)</td>
<td>100.0 (86.0-115.0)</td>
<td>102.0 (89.0-115.0)</td>
<td>0.12</td>
</tr>
<tr>
<td>Pancreatic amylase (U/L)</td>
<td>33.0 (26.8-41.0)</td>
<td>33.0 (26.0-41.0)</td>
<td>0.83</td>
</tr>
<tr>
<td><strong>Inflammatory markers</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High-sensitivity C-reactive protein (mg/L)</td>
<td>1.5 (1.1-2.6)</td>
<td>1.5 (1.1-2.5)</td>
<td>0.74</td>
</tr>
<tr>
<td>Fibrinogen (g/L)</td>
<td>3.6 (3.1-4.3)</td>
<td>3.6 (3.1-4.3)</td>
<td>0.98</td>
</tr>
</tbody>
</table>

Values are median (interquartile range). Numbers vary slightly according to availability of data. Hemoglobin A1c was available in the Copenhagen City Heart Study in 5,737 participants. P-values by Mann Whitney U test. GFR= glomerular filtration rate.
Supplementary Figure I.

**Women, P for trend <0.0001, as a function of age**

**Men, P for trend <0.0001, as a function of age**
Supplementary Figure II.

### All vascular disease

<table>
<thead>
<tr>
<th>Genotype</th>
<th>No. of Subjects</th>
<th>No. of Events</th>
<th>Adjusted for age and sex</th>
<th>P for Interaction</th>
<th>Adjusted multifactorially</th>
<th>Hazard Ratio (95% CI)</th>
<th>P for Interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCHS</td>
<td>CC 10,281</td>
<td>2,913</td>
<td></td>
<td></td>
<td></td>
<td>1.0</td>
<td></td>
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<tr>
<td></td>
<td>CT 41</td>
<td>9</td>
<td></td>
<td></td>
<td></td>
<td>0.60 (0.31-1.16)</td>
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</tr>
<tr>
<td>CGPS</td>
<td>CC 57,928</td>
<td>7,686</td>
<td></td>
<td>0.90</td>
<td></td>
<td>1.0</td>
<td></td>
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<tr>
<td></td>
<td>CT 278</td>
<td>28</td>
<td></td>
<td></td>
<td></td>
<td>0.76 (0.53-1.11)</td>
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### Cardiovascular disease

<table>
<thead>
<tr>
<th>Genotype</th>
<th>No. of Subjects</th>
<th>No. of Events</th>
<th>Adjusted for age and sex</th>
<th>P for Interaction</th>
<th>Adjusted multifactorially</th>
<th>Hazard Ratio (95% CI)</th>
<th>P for Interaction</th>
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<td></td>
<td>CT 42</td>
<td>9</td>
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<td></td>
<td></td>
<td>0.88 (0.46-1.70)</td>
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<td>CGPS</td>
<td>CC 57,928</td>
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<td>0.76</td>
<td></td>
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<tr>
<td></td>
<td>CT 278</td>
<td>20</td>
<td></td>
<td></td>
<td></td>
<td>0.89 (0.57-1.38)</td>
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### Cerebrovascular disease

<table>
<thead>
<tr>
<th>Genotype</th>
<th>No. of Subjects</th>
<th>No. of Events</th>
<th>Adjusted for age and sex</th>
<th>P for Interaction</th>
<th>Adjusted multifactorially</th>
<th>Hazard Ratio (95% CI)</th>
<th>P for Interaction</th>
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<tbody>
<tr>
<td>CCHS</td>
<td>CC 10,333</td>
<td>1,194</td>
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<td>0.73</td>
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<td></td>
<td>CT 42</td>
<td>2</td>
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<td>0.29 (0.07-1.14)</td>
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<tr>
<td>CGPS</td>
<td>CC 57,928</td>
<td>3,678</td>
<td></td>
<td>0.73</td>
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<td>CT 278</td>
<td>9</td>
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<td></td>
<td></td>
<td>0.51 (0.27-0.98)</td>
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### Ischemic cerebrovascular disease

<table>
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<th>Genotype</th>
<th>No. of Subjects</th>
<th>No. of Events</th>
<th>Adjusted for age and sex</th>
<th>P for Interaction</th>
<th>Adjusted multifactorially</th>
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<th>P for Interaction</th>
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<tr>
<td>CCHS</td>
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<td></td>
<td>CT 43</td>
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<td>0.32 (0.08-1.28)</td>
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<tr>
<td>CGPS</td>
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<tr>
<td></td>
<td>CT 278</td>
<td>8</td>
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<td></td>
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<td>0.54 (0.27-1.08)</td>
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### Hemorrhagic stroke

<table>
<thead>
<tr>
<th>Genotype</th>
<th>No. of Subjects</th>
<th>No. of Events</th>
<th>Adjusted for age and sex</th>
<th>P for Interaction</th>
<th>Adjusted multifactorially</th>
<th>Hazard Ratio (95% CI)</th>
<th>P for Interaction</th>
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<tbody>
<tr>
<td>CCHS</td>
<td>CC 10,351</td>
<td>147</td>
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<td>1.0</td>
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<tr>
<td></td>
<td>CT 43</td>
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<td></td>
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<tr>
<td>CGPS</td>
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<td>503</td>
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<td>1.0</td>
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<td>0.42 (0.06-3.00)</td>
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<tr>
<td></td>
<td>CT 278</td>
<td>1</td>
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</tbody>
</table>
Supplementary Figure III.

The figure shows the age at diagnosis (in years) for different groups with different conditions:

1. **Vascular disease**
   - CC: n=10,671
   - CT: n=39
   - P=0.05

2. **Cardiovascular disease**
   - CC: n=7,116
   - CT: n=30
   - P=0.02

3. **Cerebrovascular disease**
   - CC: n=4,892
   - CT: n=12
   - P=0.59

The bars represent the age distribution with error bars indicating variability.