Complement C3 and Risk of Diabetic Microvascular Disease: A Cohort Study of 95202 Individuals from the General Population
Katrine Laura Rasmussen,1 Børge Grønne Nordestgaard,1 and Sune Fallgaard Nielsen1∗

BACKGROUND: Whether the complement system is involved in the development of diabetic microvascular disease is unknown. We tested the hypothesis that high concentrations of complement C3 are associated with increased risk of diabetic retinopathy, nephropathy, and neuropathy in individuals from the general population.

METHODS: We studied 95202 individuals from the general population with baseline measurements of complement C3, genotyped for rs1065489, rs429608, and rs448260 determining concentrations of complement C3, and enrolled in the Copenhagen General Population Study from 2003 through 2013, following them until April 10, 2013. Rs1065489, rs429608, and rs448260 were identified with genome-wide association scans in 3752 individuals from the Copenhagen City Heart Study.

RESULTS: The cumulative incidence was increased from the lowest tertile to the highest tertile of complement C3 for diabetic retinopathy (log-rank trend, \( P = 1 \times 10^{-10} \)), nephropathy (\( P = 7 \times 10^{-15} \)), and neuropathy (\( P = 5 \times 10^{-16} \)). Multifactorially adjusted hazard ratios for a 1 SD higher concentration of complement C3 were 1.87 (95% CI, 1.61–2.18) for diabetic retinopathy, 1.90 (1.62–2.23) for diabetic nephropathy, and 1.56 (1.29–1.89) for diabetic neuropathy. The multifactorially adjusted hazard ratio for individuals with the highest vs lowest tertile of complement C3 was 3.29 (1.78–6.07) for retinopathy, 2.71 (1.42–5.16) for nephropathy, and 2.40 (1.26–4.54) for neuropathy.

CONCLUSIONS: High baseline concentrations of complement C3 were associated with increased risk of diabetic retinopathy, nephropathy, and neuropathy in individuals from the general population. These epidemiological findings were substantiated by a Mendelian randomization approach, potentially indicating causality.
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Diabetes mellitus is a major health problem leading to high risk of developing microvascular disease complications like retinopathy, nephropathy, and neuropathy, the pathogenesis of which is largely unclear. Besides monitoring glucose and hemoglobin A1c concentrations, combined with routine screening of individuals with diabetes for already-developed microvascular diseases (1, 2), we lack biomarkers to aid clinically in risk prediction of microvascular disease and to understand parallel and independent mechanisms besides glycemic control. Complement C3 is an acute-phase reactant and a central component in activation of the complement system (3, 4), and high complement C3 concentrations are associated with adiposity, insulin resistance, prediabetes, diabetes, and dyslipidemia, and are further recognized to be implicated as a cardiometabolic risk factor (4–9).

Presently, there is some evidence suggesting complement C3 may play a role in the development of microvascular disease (10); for retinopathy, both complement C3d and C5b-9 have been found simultaneously in the choriocapillaris of patients with diabetic retinopathy (11), corroborating the notion that complement activation had occurred in situ, whereas the absence of complement C1q and C4 suggests that complement C5b-9 was generated via the alternative pathway (11, 12). For nephropathy, deposition of complement C3 has been found in glomeruli and glomerular capillaries in animal models (13, 14), and a study in mice showed complement inhibition leading to thrombomodulin-ameliorated albuminuria and glomerular damage (15). Also, glomerular structures
ments of complement C3 from the Copenhagen General Population Study, a prospective study of the Danish general population with first enrollment from 2003 to 2013 and with follow-up examinations ongoing (21–23). Participation rate was 43%.

**MICROVASCULAR DISEASE END POINTS**

Information on diagnoses of diabetic microvascular disease was collected from the national Danish Patient Registry with data on all patient contacts from all clinical hospital departments in Denmark since 1977, including emergency wards and outpatient clinics since 1995. Multiple records of the same diabetic microvascular complication from different hospitals and different departments were combined to include only the first event and the date for this event. Information on diagnoses was registered using World Health Organization International Classification of Diseases, 10th revision (ICD10)\(^2\) codes. In Denmark, a diagnosis of diabetes typically leads to an eye examination by a medical eye specialist, as well as other clinical and biochemical testing. After this first examination at the time of diabetes diagnosis, tests will typically be repeated either every 12 months for individuals without any signs or positive indicators of microvascular disease or every 3 to 6 months otherwise.

Signs of simple or proliferative diabetic retinopathy or diabetic macular edema are recorded as diabetic retinopathy in the Danish Patient Registry (ICD10 codes E10.3, E11.3, E13.3, and H36.0). Abnormal urinary measurements (e.g., albumin/creatinine ratio or 24-h albumin) are diagnosed as diabetic nephropathy (ICD10 codes E10.2, E11.2, and E13.2). Symptoms such as loss of sensory abilities or autonomic symptoms such as erectile dysfunction lead to a diagnosis of diabetic neuropathy (ICD10 codes E10.4, E11.4, and E13.4).

Follow-up began at the time of blood sampling (2003–2013). The national Danish Civil Registration System records all births, immigrations, emigrations, and deaths in Denmark. Follow-up ended at occurrence of an event (n = 158 for retinopathy; n = 132 for nephropathy; n = 115 for neuropathy), death (n = 3960), emigration (n = 230), or on April 10, 2013 (last update of the registries), whichever came first. The composite end point for all microvascular disease was defined as the first event of diabetic retinopathy, nephropathy, and/or neuropathy. Median follow-up period was 4.6 years (range, 0–9 years), with no individuals lost to follow-up. The numbers of person-years were 437,273 for diabetic reti-
nopathy, 437595 for nephropathy, and 437614 for neuropathy.

**BIOCHEMICAL MEASUREMENTS AND GENOTYPING**

Plasma complement C3 was measured turbidimetrically on fresh samples with a Kone autoanalyzer (Konelab, Thermo Fisher Scientific) immediately after sampling using polyclonal antibodies (Complement C3 antiserum 981931, Thermo Scientific). Measurements were blinded to later development of diabetic microvascular disease. The precision of the assay was evaluated daily by internal quality controls: Typical monthly CVs were 3% to 5% at the level of 1.02 g/L. The same calibrator was used for all measurements (SpeciCal 980997, Thermo Fisher Scientific), and performance was stable for analyses of participant samples during the study period. C-reactive protein (CRP) and plasma glucose were measured using standard hospital assays. Complement C3 measurement rate was 99.2%.

Rs1065489 in the gene for complement factor H (CFH)\(^3\), rs429608 in Ski2 like RNA helicase region of the class III gene region of the major histocompatibility complex (SKIV2L), and rs448260 in the gene for complement C3 (C3) were first identified to determine high concentrations of complement C3 by genome-wide association scan in 3752 individuals from the fourth examination of the Copenhagen City Heart Study in 2001 to 2003 using genotype data from the Metabo+ chip, a customized Illumina Infinium iSelect array, designed by the Emerging Risk Factors Consortia targeting cardiometabolic traits (24) (Fig. 1, Discovery). Subsequently, these 3 single-nucleotide polymorphisms were genotyped in 95,202 individuals from the Copenhagen General Population Study using Applied Biosystems Viia 7 system (Life Technologies, a part of Thermo Fisher Scientific) and Taqman-based assays (Fig. 1, Replication). Call rates were $\geq$ 99.9%, and Hardy–Weinberg equilibrium was fulfilled ($P \geq 0.44$) for all 3 individual single-nucleotide polymorphisms.

**OTHER COVARIATES**

Body mass index (BMI) was measured with weight in kilograms divided by measured height in meters squared. All other covariates were self-reported from questionnaire, dichotomized or ordered, and defined in detail in the legend to Table 1.

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\(^3\) Human genes: CFH, complement factor H; SKIV2L, superkiller viralicidic activity 2-like RNA helicase; C3, complement C3.
hours (9 groups of 0 – 1 h to > 8 h) were used to define adjusted logarithmically transformed glucose values deviated from mean log(glucose).

The epidemiological findings were substantiated by Mendelian randomization when plasma complement C3 determining genotypes, combined into a weighted or a simple gene score, were used to predict the risk of microvascular disease based on the epidemiological associations and, subsequently, compared with the observed genetically determined risk of microvascular disease, as done previously (22). Pleiotropy was assessed beforehand by evaluation of the distribution of baseline characteristics in the 3 groups of the weighted gene score. We used Cuzick’s extension of a Wilcoxon rank sum test

Fig. 1. Metabo+ genome-wide association scan of complement C3 in the 2001 to 2003 examination of the Copenhagen City Heart Study, and plasma concentrations of complement C3 for 3 single-nucleotide polymorphisms genotyped in the Copenhagen General Population Study.

Discovery: The Copenhagen City Heart Study is a prospective study of the Danish general population initiated in 1976 to 1978 with follow-up examinations in 1981 to 1983, 1991 to 1994, and 2001 to 2003. The genome-wide association scan was performed for 3752 individuals from the fourth examination in 2001 to 2003 with genotype data from the Metabo+ chip, a customized Illumina Infinium iSelect array, designed by the Emerging Risk Factors Consortia targeting cardiometabolic traits (24). Each participant was genotyped for approximately 200,000 single-nucleotide polymorphisms. Single-nucleotide polymorphisms that were not in Hardy–Weinberg equilibrium or showed evidence of genotyping errors were excluded from further analysis. Significance level is indicated by the horizontal line (−log₁₀(2.5 × 10⁻⁷) = 6.6) corresponding to the Bonferroni corrected P value: P < 0.05/200 000 = 2.5 × 10⁻⁷. Based on the genome-wide association scan, rs1065489 in the gene for complement factor H (CFH), rs429608 in SKIV2L like RNA helicase region of the class III gene region of the major histocompatibility complex (SKIV2L), and rs448260 in the gene for complement C3 (C3) were identified by having the highest F-statistics for each gene to determine high concentrations of complement C3. Individuals were recruited and examined similarly as in the Copenhagen General Population Study but served only as the discovery cohort for the genome-wide association scan, and individuals from the Copenhagen City Heart Study were not included in the main analyses. Replication: Rs1065489 (CFH), rs429608 (SKIV2L), and rs448260 (C3) were subsequently genotyped in 95,202 individuals of the Copenhagen General Population Study and replicated discovery findings. Mean and SDs are given. Cuzick’s extension of a Wilcoxon rank sum test for trend across genotypes is given. Values for R² are 0.7%, 0.3%, and 0.4%, and F-statistics from the trend for the linear regression across genotypes are 697, 305, and 363 for rs1065489, rs429608, and rs448260, respectively.
Table 1. Baseline characteristics of individuals from the Copenhagen General Population Study by tertiles of complement C3 and weighted gene score in 3 groups.*

<table>
<thead>
<tr>
<th>Tertile/group</th>
<th>Epidemiologic</th>
<th>Genetic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>First</td>
<td>Second</td>
</tr>
<tr>
<td>Range of C3, g/L</td>
<td>0.14–1.02 (n = 32 802)</td>
<td>1.03–1.20 (n = 32 141)</td>
</tr>
<tr>
<td>Men (%)</td>
<td>14 268 (43)</td>
<td>15 393 (48)</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>23.9 ± 0.02</td>
<td>25.9 ± 0.02</td>
</tr>
<tr>
<td>Smoking (%)</td>
<td>5487 (17)</td>
<td>5978 (19)</td>
</tr>
<tr>
<td>Hypertension (%)</td>
<td>15 908 (48)</td>
<td>19 591 (61)</td>
</tr>
<tr>
<td>Alcohol consumption (%)</td>
<td>6076 (19)</td>
<td>5828 (18)</td>
</tr>
<tr>
<td>Physical inactivity (%)</td>
<td>13 016 (40)</td>
<td>15 532 (48)</td>
</tr>
<tr>
<td>Postmenopausal (%)b</td>
<td>10 850 (59)</td>
<td>11 678 (70)</td>
</tr>
<tr>
<td>Hormonal replacement therapy (%)P</td>
<td>1906 (10)</td>
<td>1898 (11)</td>
</tr>
<tr>
<td>Lipid-lowering therapy (%)</td>
<td>2240 (7)</td>
<td>3531 (11)</td>
</tr>
<tr>
<td>Education &lt;8 years (%)</td>
<td>2358 (7)</td>
<td>3360 (10)</td>
</tr>
<tr>
<td>Type 1 diabetes (%)</td>
<td>168 (1)</td>
<td>112 (0)</td>
</tr>
<tr>
<td>Type 2 diabetes (%)</td>
<td>401 (7)</td>
<td>792 (2)</td>
</tr>
<tr>
<td>CRP, mg/Lc</td>
<td>0.9 ± 0.02</td>
<td>1.4 ± 0.01</td>
</tr>
<tr>
<td>Glucose, mmol/Lc</td>
<td>5.2 ± 0.006</td>
<td>5.2 ± 0.007</td>
</tr>
</tbody>
</table>

* Data are range, numbers of individuals (%), or mean (± SE) and are from the day of enrollment. Missing data on categorical and continuous covariates (0.3%) were imputed from age, sex, and day of enrollment using multiple imputation procedure; however, if only individuals with complete data were included, results were similar to those reported. Hypertension was use of antihypertensive medication and/or a systolic blood pressure of ≥ 140 mmHg, and/or a diastolic blood pressure of ≥ 90 mmHg. Diabetes mellitus was self-reported or register-reported disease before or on day of enrollment, use of insulin or oral hypoglycemic agents, and/or fasting plasma glucose concentrations of > 11 mmol/L (> 198 mg/dL). Smoking was current smoking. Alcohol consumption was > 1421 units per week for women/ men [1 unit = 12 g of alcohol, equivalent to 1 glass of wine or spirit or 1 beer (33 cl)]. Physical inactivity was ≤ 4 h per week of light physical activity in leisure time.

b Women reported menopausal status and use of hormonal replacement therapy. Lipid-lowering therapy was primarily statins (yes/no), and education was < 8 years of education. To convert glucose values from mmol/L to mg/dL, multiply by 18.0. To convert CRP values from mg/L to nmol/L, multiply by 9.524. P for trend was by nonparametric trend test.

c Except for postmenopausal status and hormonal replacement therapy and diabetes, for which Kruskal–Wallis equality-of-populations rank test was used across 3 groups (premenopausal/postmenopausal without hormonal replacement therapy/postmenopausal with hormonal replacement therapy, and no diabetes/type 1 diabetes/type 2 diabetes) and in women only.

Geometric mean = SE for CRP and glucose.

C3 = plasma complement C3.
for trend, whereas corresponding $R^2$ and $F$-statistics were evaluated by linear regression.

Results

Baseline characteristics of study participants by plasma complement C3 tertiles and by 3 groups of weighted gene score are shown in Table 1. Survival and risk of diabetes in responders and nonresponders are shown in Fig. 1 of the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol64/issue7. During 9 years of follow-up, 158 individuals developed diabetic retinopathy, 132 developed diabetic nephropathy, and 115 developed diabetic neuropathy. We estimated that optimal thresholds for ROC curves of plasma complement C3 at baseline and future risk of individual diabetic microvascular events were 1.24 g/L for retinopathy, 1.12 g/L for nephropathy, and 1.11 g/L for all diabetic microvascular disease (see Fig. 2 in the online Data Supplement). There was a positive association between plasma concentrations of complement C3 and risk for 1, 2, or 3 microvascular complications (see Fig. 6 in the online Data Supplement), as well as when comparing high and low concentrations of complement C3 (see Fig. 7 in the online Data Supplement). Sensitivity analyses showed no major interaction (see Fig. 8 in the online Data Supplement).

PLASMA CONCENTRATIONS OF COMPLEMENT C3 AND RISK OF DIABETIC RETINOPATHY, NEPHROPATHY, AND NEUROPATHY

Risk of retinopathy, nephropathy, and neuropathy increased with increasing plasma complement C3 as evaluated by restricted cubic spline regressions using the optimal threshold concentration of plasma complement C3 for all microvascular disease as reference (1.11 g/L) (Fig. 2).

The cumulative incidence for retinopathy as a function of age increased stepwise from the lowest tertile (complement C3, $\leq 1.02$ g/L) through the middle tertile (C3, 1.03–1.20 g/L) to the highest tertile (C3, $\geq 1.21$ g/L) (log-rank trend: $P = 1 \times 10^{-20}$) (Fig. 3, top). Similar patterns were found for nephropathy (log-rank trend: $P = 7 \times 10^{-15}$) (Fig. 3, middle) and for neuropathy ($P = 5 \times 10^{-19}$) (Fig. 3, bottom).

There was a positive association between plasma concentrations of complement C3 and risk of individual microvascular end points for a Cox regression model multifactorially adjusted for age (time scale), sex, BMI, smoking, hypertension, lipid-lowering therapy, alcohol consumption, physical inactivity, education, postmenopausal status, and hormonal replacement therapy (Fig. 4, left), with further attenuation after adjustment for glucose and baseline diabetes mellitus (Fig. 4, middle), and yet further after adjustment for CRP (Fig. 4, right), evaluated both on the continuous scale and for tertiles of complement C3. For 1 SD increase of complement C3, multifactorially adjusted hazard ratios were 1.87 (95% CI, 1.61–2.18) for retinopathy, 1.90 (1.62–2.23) for nephropathy, and 1.56 (1.29–1.89) for neuropathy (Fig. 4, left). The multifactorially adjusted hazard ratio for individuals with the highest vs lowest tertile of complement C3 was 3.29 (1.78–6.07) for retinopathy, 2.71 (1.42–5.16) for nephropathy, and 2.40 (1.26–4.54) for neuropathy. Results for nephropathy were attenuated by further adjustment for CRP, whereas results for neuropathy were augmented by this adjustment (Fig. 4, middle and right). Results were similar using type 1 and type 2 diabetes status as a time-dependent covariate (see Figs. 4 and 5 in the online Data Supplement). There was also a positive association between plasma concentrations of complement C3 and risk for 1, 2, or 3 microvascular complications (see Fig. 6 in the online Data Supplement), as well as when comparing high and low concentrations of complement C3 (see Fig. 7 in the online Data Supplement). Sensitivity analyses showed no major interaction (see Fig. 8 in the online Data Supplement).

GENE SCORES IN GROUPS, PLASMA CONCENTRATIONS OF COMPLEMENT C3, AND RISK OF DIABETIC MICROVASCULAR DISEASE

Rs1065489 (CFH), rs429608 (SKIV2L), and rs448260 (C3) each independently showed a stepwise per genotype association with plasma complement C3 (all $P \leq 1 \times 10^{-66}$) (Fig. 1). Plasma complement C3 increased with increasing gene score group for both the weighted gene score and the simple gene score in groups ($P \leq 1 \times 10^{-239}$) (Fig. 5, left). A 5% higher plasma complement C3 for the third weighted gene score group vs the first group theoretically predicted a hazard ratio of 1.14 (1.13–1.16) (Fig. 5, middle) for risk of diabetic microvascular disease. The corresponding observed hazard ratio was 1.17 (1.01–1.36) (Fig. 5, right).

Discussion

Our principal finding in 95202 individuals from the general population is that individuals with increased baseline plasma complement C3 had increased risk of diabetic retinopathy, nephropathy, and neuropathy. Further, in Mendelian randomization, a weighted genotype score of rs1065489, rs429608, and rs448260 determining high plasma concentrations of complement C3 was associated with increased risk of microvascular disease, potentially indicating causality. Comparing the highest vs lowest tertile of complement C3, we found risk estimates for all 3 microvascular end points to be 2- to 3-fold, which is considered clinically significant. Thus, plasma complement C3 measurements have the potential to play
Fig. 2. Multifactorially adjusted hazard ratios for diabetic retinopathy, nephropathy, and neuropathy according to plasma concentrations of complement C3 in the Copenhagen General Population Study.

Solid lines are multifactorially adjusted hazard ratios, whereas dashed lines indicate 95% CIs derived from restricted cubic spline regressions with 3 knots and the reference defined as the optimal threshold concentration (1.11 g/L) from ROC curves for all microvascular disease events. Graphs are truncated at the concentrations of 0.89 g/L and 2.01 g/L owing to statistically unstable estimates at extreme low and high concentrations, thus including only 82,910 individuals in these analyses. Cox regression models were adjusted for age (time scale), sex, BMI, smoking, hypertension, lipid-lowering therapy, alcohol consumption, physical inactivity, education, postmenopausal status and hormonal replacement therapy, adjusted glucose, diabetes mellitus, and CRP.
a central role in future risk stratification and screening programs of individuals with prediabetes with respect to development of microvascular complications.

Mechanistically, the complement system may be involved in the pathogenesis of microvascular disease in several ways that work in concert. First, complement activation can promote systemic inflammation and contribute to inflammation-mediated vascular damage (3), as supported by our observation that risk estimates were attenuated by CRP adjustment as a measure of inflammation. Second, complement activation may lead to endothelial dysfunction (30), which precedes and may contribute to microvascular complications (31). Although endothelial function cannot be measured directly in humans (31), punctiform microaneurysms, hard exudates, and cotton wool spots in retinal photos, as well as microalbuminuria, provide figurative examples of increased permeability of the microvasculature. Also, complement C3 has been identified in extracts from atherosclerotic plaques (32, 33) and may even be considered integral to macrovascular disease (34), further strengthening the link to microvascular disease. Third, the complement system interplays with the coagulation system and is activated during blood clotting, suggesting participation of complement proteins in thrombus formation, and the function of endothelial cells and platelets can be modulated by the complement system (30, 35, 36). Interestingly, we previously showed that increased complement C3 was associated with increased risk of deep venous thrombosis and pulmonary embolism (9). Additionally, complement C3 has been suggested to be implicated in hypofibrinolysis in diabetes, with a diabetes-dependent

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**Fig. 3.** Cumulative incidence of diabetic retinopathy, nephropathy, and neuropathy as a function of age and tertiles of plasma concentrations of complement C3 in the Copenhagen General Population Study.

C3, plasma concentrations of complement C3.
incorporation of complement C3 in the clot, possibly via glucose toxicity-induced inflammation (37). Together, this leads to the speculation that complement activation could pose an additional risk factor for further damage, leakage, and microthrombosis of the microvasculature (12). Fourth and finally, the complement system is activated in postprandial lipemia (38). Activated complement C3, or desarginated C3a, is considered a paracrine metabolic factor that stimulates the uptake of glucose and fat storage in human adipose tissue, with the effects being additive and independent of the effects of insulin (34), and has been found significantly increased in postprandial type II diabetes serum compared with postprandial case serum (39). Also, desarginated complement C3a was increased in the cases with macroalbuminuria or proliferative retinopathy (39). If circulating C3 concentrations represent the potential for complement activation, and desarginated C3a has paracrine rather than endocrine functions, then a moderate increase in C3 might represent an increased supply for the augmentation of potent local processes induced by desarginated C3a and other downstream products of C3 activation. Strengths of the study include the large prospective general population design with no losses to follow-up and plasma complement C3 measurements preceding the diagnosis of microvascular disease. Importantly, the associations observed in the present study were present even in those without diabetes or high glucose at baseline. Further, a pivotal strength of this study is the substantiation of the epidemiological findings in a Mendelian randomization design, thus minimizing the risk of reverse causation and thereby substantiating the level of evidence for causality.

Theoretical possible limitations of this study include selection bias and the possibility that responding participants in this study were healthier than nonresponders; however, owing to the recruitment of 95,202 individuals from the general population without knowledge of complement C3 concentration, genetic variation, or disease status, selection bias would be unlikely to explain our findings. On the contrary, this is most likely to result in a more conservative epidemiological association and is further unlikely to disturb the genetic findings, as variation in the single-nucleotide polymorphisms most likely would be randomly distributed in responders and nonresponders. A related potential limitation concerns the

### Table 1: Risk of diabetic retinopathy, nephropathy, and neuropathy as a function of 1 SD increase and tertiles of complement C3.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Hazard ratio (95% CI)</th>
<th>P for trend</th>
<th>Hazard ratio (95% CI)</th>
<th>P for trend</th>
<th>Hazard ratio (95% CI)</th>
<th>P for trend</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retinopathy</td>
<td>1 SD increase in C3</td>
<td>1.87 (1.61–2.18)</td>
<td>1.34 (1.15–1.56)</td>
<td>1.26 (1.06–1.46)</td>
<td>1.00 (reference)</td>
<td>P = 0 x 10^-7</td>
</tr>
<tr>
<td>N, events</td>
<td>Total</td>
<td>32,802</td>
<td>13</td>
<td>32,141</td>
<td>31</td>
<td>30,269</td>
</tr>
<tr>
<td>N, tertiles</td>
<td>1.03–2.02</td>
<td>1.00 (reference)</td>
<td>1.00 (reference)</td>
<td>1.00 (reference)</td>
<td>1.35 (0.70–2.62)</td>
<td>1.46 (0.75–2.84)</td>
</tr>
<tr>
<td>C3 ≥ 2.11</td>
<td>2.71 (1.42–5.12)</td>
<td>1.96 (1.01–3.78)</td>
<td>1.18 (0.59–2.37)</td>
<td>1.37 (0.69–2.71)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nephropathy</td>
<td>1 SD increase in C3</td>
<td>1.90 (1.62–2.23)</td>
<td>1.40 (1.19–1.65)</td>
<td>1.25 (1.03–1.50)</td>
<td>1.00 (reference)</td>
<td>P = 0.001</td>
</tr>
<tr>
<td>N, events</td>
<td>Total</td>
<td>32,802</td>
<td>12</td>
<td>32,141</td>
<td>29</td>
<td>30,269</td>
</tr>
<tr>
<td>N, tertiles</td>
<td>1.03–2.02</td>
<td>1.00 (reference)</td>
<td>1.00 (reference)</td>
<td>1.00 (reference)</td>
<td>1.34 (0.67–2.66)</td>
<td>1.38 (0.70–2.78)</td>
</tr>
<tr>
<td>C3 ≥ 2.11</td>
<td>2.71 (1.42–5.12)</td>
<td>1.96 (1.01–3.78)</td>
<td>1.18 (0.59–2.37)</td>
<td>1.37 (0.69–2.71)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neurology</td>
<td>1 SD increase in C3</td>
<td>1.56 (1.29–1.89)</td>
<td>1.15 (0.95–1.38)</td>
<td>1.23 (1.01–1.51)</td>
<td>1.00 (reference)</td>
<td>P = 0.002</td>
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<tr>
<td>N, events</td>
<td>Total</td>
<td>32,802</td>
<td>13</td>
<td>32,141</td>
<td>36</td>
<td>30,269</td>
</tr>
<tr>
<td>N, tertiles</td>
<td>1.03–2.02</td>
<td>1.00 (reference)</td>
<td>1.00 (reference)</td>
<td>1.00 (reference)</td>
<td>1.40 (0.72–2.72)</td>
<td>1.46 (0.75–2.85)</td>
</tr>
<tr>
<td>C3 ≥ 2.11</td>
<td>2.40 (1.26–4.54)</td>
<td>1.71 (0.89–3.30)</td>
<td>1.59 (0.80–3.11)</td>
<td>1.99 (1.01–3.95)</td>
<td></td>
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</table>

**Fig. 4.** Risk of diabetic retinopathy, nephropathy, and neuropathy as a function of 1 SD increase and tertiles of complement C3.

Hazard ratios were multifactorially adjusted for age (time scale), sex, BMI, smoking, hypertension, lipid-lowering therapy, alcohol consumption, physical inactivity, education, postmenopausal status and hormonal replacement therapy (left); further adjusted for glucose and baseline diabetes mellitus (middle); and yet further adjusted for CRP (right). 1 SD increase in C3 = 0.21 g/L C3, complement C3.
availability and completeness of the diagnostic information; however, the Danish Patient Registry includes all hospital visits and outpatient visits, and in Denmark practically all patients with diabetes enter a rigorous program for monitoring the potential development of microvascular disease. Unregistered events of microvascular disease would only bias our results toward the null hypothesis. In line with this, the diagnosis of diabetic microvascular complications can be preceded by years of prediabetes, undiagnosed diabetes, or overt diabetes (40, 41); in this study, we provide both estimates using diabetes as a fixed covariate and as a time-dependent covariate with similar results, and it could further be argued that the general population approach represents the most direct evaluation of the association between baseline measurement of complement C3 and development of microvascular complications. Although we did not adjust for use of antidiabetic therapy, which might affect results, effect sizes of estimates for all 3 individual microvascular end points were not attenuated in individuals without diabetes at baseline. Also, we were not able to adjust for HbA1c because such measurements were not available; however, as our findings are supported by Mendelian randomization, lifestyle factors such as glucose and HbA1c concentrations are unlikely to disturb our main findings. Still, we did adjust for diabetes status, and glucose was adjusted for time since last meal. Another theoretical potential limitation is that the complement C3 determining alleles used in our study are coupled to the known familial/genetic components to microvascular complication development explaining our genetic findings; however, this is unlikely because they are located on entirely different genes. Another potential limitation is that blood samples were not drawn in the fasting state, which could add to the random measurement error for plasma glucose concentrations. However, the interindividual and intra-individual glucose fluctuations that are associated with time from last meal were considered in the Cox regression model by adjusting the glucose concentration for time since last meal. Another potential limitation is the discovery nature of our study, which ideally should be validated in an independent co-

Fig. 5. Plasma concentrations of complement C3 and corresponding theoretically predicted and observed hazard ratios for diabetic microvascular disease for weighted and simple gene scores in 3 groups.

As the exposure was a genetic instrument, individuals entered into the study at date of birth, thus including 94,982 participants with available genotypes, who developed 1089 events of diabetic microvascular disease, with diabetic microvascular disease being defined as the first event of diabetic retinopathy, diabetic nephropathy, or diabetic neuropathy. Means and SD for plasma complement C3 are given (left). The simple gene score was calculated for each participant using the sum of the number of complement C3 increasing alleles for rs1065489 (CFH), rs429608 (SKIV2L), and rs448260 (C3), whereas the weighted gene score was the sum of weights, with the per allele weights being the β-coefficients for the 3 variants obtained from a linear regression. Both gene scores were subsequently grouped in 3 similarly sized groups (left). Theoretically predicted hazard ratios (middle) were calculated from delta mean concentrations of complement C3 for groups of weighted gene score and for simple gene score and the epidemiological association between plasma complement C3 and risk of microvascular disease from a Cox regression model adjusted for age (time scale) and sex. Observed hazard ratios (right) were adjusted for age (time scale) and sex. Values for $R^2$ are 1% and 1%, and F-statistics from the trend for the linear regression across genotypes are 1085 and 1118 for the weighted and unweighted allele score in the 3 groups, respectively.
hort; however, we are not of a similar large modern general population cohort with measurements of plasma complement C3, the single-nucleotide polymorphisms determining complement C3 concentrations, and diabetic microvascular end points. As a final potential limitation, we studied only white individuals of Danish descent; however, we are not aware of any data to suggest that our results should not be applicable to all humans.

In conclusion, high concentrations of complement C3 were associated with increased risk of diabetic retinopathy, nephropathy, and neuropathy in individuals from the general population. The epidemiological findings were substantiated by a Mendelian randomization approach, potentially indicating causality. Comparing the highest vs lowest tertile of complement C3, we found risk estimates for all 3 microvascular end points to be 2- to 3-fold, which is considered clinically significant. Thus, plasma complement C3 measurements have the potential to play a central role in future risk stratification and screening programs of individuals with prediabetes with respect to development of microvascular complications.

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