Clinical Chemistry 64:7
1113–1124 (2018)

Complement C3 and Risk of Diabetic Microvascular Disease: A Cohort Study of 95 202 Individuals from the General Population

Katrine Laura Rasmussen,[1] Børge Grønne Nordestgaard,[1] and Sune Fallgaard Nielsen[1*]

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 95 202 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, $P = 1 \times 10^{-20}$), nephropathy ($P = 7 \times 10^{-15}$), and neuropathy ($P = 5 \times 10^{-10}$). 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.

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

[1] Department of Clinical Biochemistry, Herlev and Gentofte Hospital, Copenhagen University Hospital, Faculty of Health and Medical Sciences, University of Copenhagen, Herlev, Denmark.
* Address correspondence to this author at: Department of Clinical Biochemistry, 54M1, Herlev and Gentofte Hospital, Copenhagen University Hospital, Herlev Ringvej 75, DK-2730 Herlev, Denmark. E-mail sune.fallgaard.nielsen@regionh.dk.

Received January 28, 2018; accepted February 12, 2018.
Previously published online at DOI: 10.1373/clinchem.2018.287581

and medial smooth muscle cells in intrarenal arteries of patients with nephropathy show signs of complement activation *(16, 17)*. For neuropathy, complement C3d and C5b-9 were found in endoneurial microvessels of patients with diabetic neuropathy with an obvious overrepresentation of complement C3 in diabetic vs other chronic neuropathy *(18)*; however, complement C3 deposition is also present in other chronic neuropathies *(18, 19)*. No large-scale study has investigated whether high complement C3 plasma concentrations are associated with high risk of any of these diabetic microvascular complications in the general population.

Large-scale, well-adjusted observational studies can be coupled with Mendelian randomization studies to better address confounding and reverse causation by the use of genetic variants in human populations *(20–22)*. A Mendelian randomization study can be compared with a randomized controlled trial: In the first design, genetic variation that affects the exposure (in this case, plasma concentrations of complement C3) is distributed at random at conception; in the latter design, participants are randomized to control or active treatment (which might not be possible or ethically justifiable). In either design, both measurable and unmeasurable confounders are randomly distributed, thereby overcoming the risk of confounding and reverse causation, which are limitations of the classical epidemiological design.

We tested the hypothesis that high plasma concentrations of complement C3 are associated with increased risk of diabetic retinopathy, nephropathy, and neuropathy in individuals from the general population. Further, by using Mendelian randomization, we tested the hypothesis that there is a causal association between plasma concentrations of complement C3 and risk of microvascular disease. For this purpose, we studied 95 202 individuals from the Copenhagen General Population Study, all with baseline plasma measurements of complement C3 and genotyped for rs1065489, rs429608, and rs448260 determining concentrations of complement C3, and followed them for up to 9 years for development diabetic retinopathy, nephropathy, and neuropathy in a prospective study design.

Materials and Methods

The study was approved by institutional review boards and Danish ethical committees, and was conducted according to the Declaration of Helsinki. Written informed consent was obtained from participants. All participants were white and of Danish descent.

PARTICIPANTS

We included 95 202 individuals with baseline measurements 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-

[2] Nonstandard abbreviations: ICD10, International Classification of Diseases, 10th revision; CRP, C-reactive protein; BMI, body mass index.

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 95202 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 ≥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.

[3] Human genes: *CFH,* complement factor H; *SKIV2L,* superkiller viralicidic activity 2-like RNA helicase; *C3,* complement C3.

STATISTICAL ANALYSES

We used Stata/SE version 13.1. Nonparametric trend tests and Kruskal–Wallis equality-of-populations rank tests were used to evaluate differences across tertiles of complement C3. Plasma glucose and CRP were logarithmically transformed owing to skewed distributions. Missing data on categorical and continuous covariates (0.3%) were imputed from age, sex, and date of examination using multiple imputation with 10 imputations. Multinomial logistic regression was applied for categorical variables and linear regression was applied for continuous variables, and were performed using the "mi impute mlogit" and "mi impute chained (regress)" commands in Stata. We had 80% statistical power at a 2-sided $P < 0.05$ to detect hazard ratios >1.57 for diabetic retinopathy, >1.63 for diabetic nephropathy, and >1.66 for diabetic neuropathy.

ROC curves of plasma complement C3 at baseline and future risk of individual microvascular events were used to estimate the threshold concentration differentiating between groups of high and low complement C3, as well as to define the reference for the restricted cubic splines. Multivariable adjusted restricted cubic splines using 3 knots and Cox regression were used to evaluate the association between plasma complement C3 and development of the 3 individual microvascular disease end points.

Kaplan–Meier curves and log-rank trend tests evaluated cumulative incidence, and Cox proportional hazards regression models estimated hazard ratios for microvascular end points as a function of complement C3 as a continuous exposure, in tertiles, and in high vs low concentrations. For Cox regression models, proportionality of hazards over time was assessed by plotting −ln(−ln[survival]) vs ln(analysis time). There was no sign of nonproportionality. There was no evidence that competing risks changed the estimates to any major extent as examined using Fine–Gray models *(25)*. Regression models were adjusted for known risk factors and markers of lifestyle: age (automatic adjustment as time scale), sex, BMI, hypertension, smoking, alcohol consumption, physical inactivity, menopausal status and hormonal replacement therapy (only women), lipid-lowering therapy, and education. Further analyses were adjusted for diabetes mellitus, glucose adjusted for time since last meal, and CRP. Adjustment for BMI was relevant because adipose tissue produces complement C3, whereas adjustment for CRP performed as acute phase may influence complement C3 concentrations *(26–28)*. Adjustment for diabetes/glucose was relevant, as there is a positive association between glucose and complement C3, as well as an association between diabetes/glucose and development of microvascular complications *(1, 2, 8, 29)*. Residuals from regression of logarithmically transformed glucose and time since last meal in integer

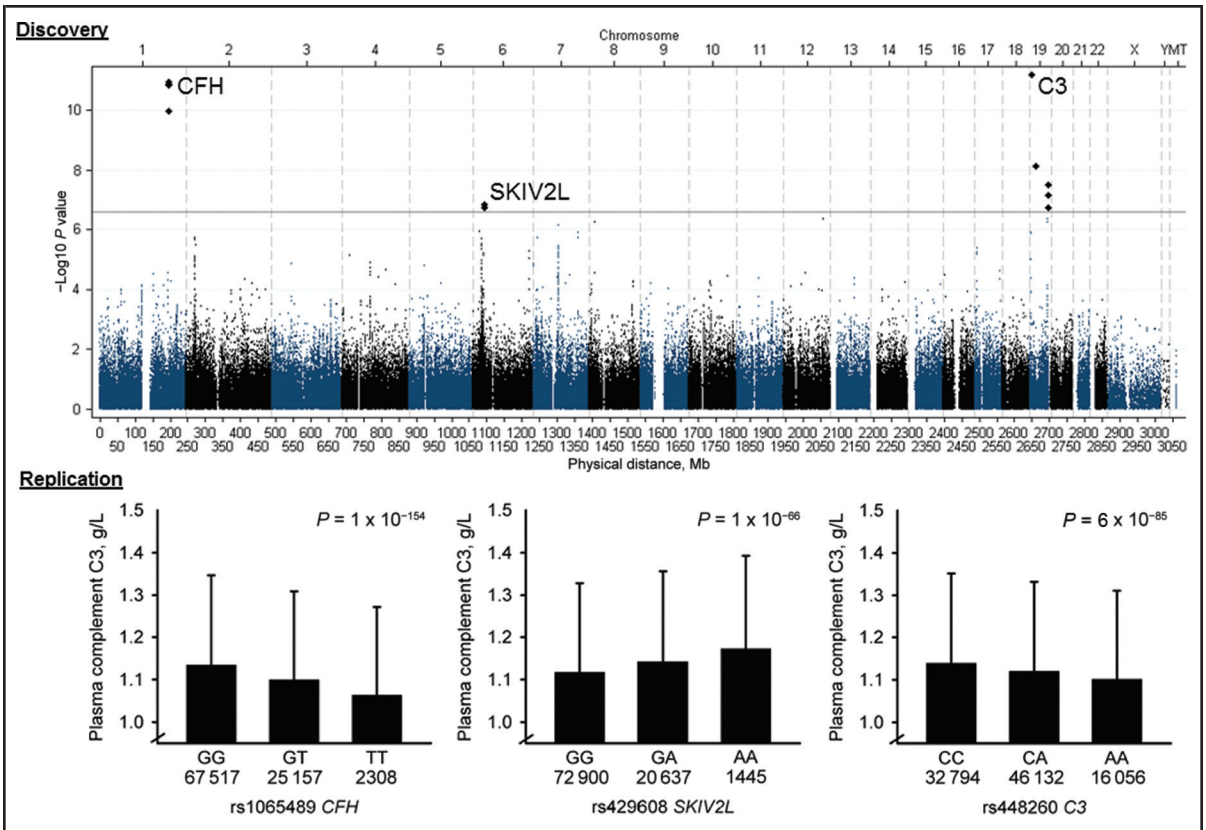

Fig. 1. Metabo+ genome-wide association scan of complement C3 in the 2001 to 2003 examination of the Copenhagen City Heart Study, and plasma concentrations of complement C3 for 3 single-nucleotide polymorphisms genotyped in the Copenhagen General Population Study.

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 ($-\log_{10}(2.5 \times 10^{-7}) = 6.6$) corresponding to the Bonferroni corrected *P* value: $P < 0.05/200\,000 = 2.5 \times 10^{-7}$. Based on the genome-wide association scan, rs1065489 in the gene for complement factor H (*CFH*), 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 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 R^2 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.

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

Table 1. Baseline characteristics of individuals from the Copenhagen General Population Study by tertiles of complement C3 and weighted gene score in 3 groups.[a]

	Epidemiologic				Genetic			
Tertile/group	First	Second	Third	*P* for trend	First	Second	Third	*P* for trend
Range of C3, g/L	0.14–1.02 (n = 32 802)	1.03–1.20 (n = 32 141)	1.21–3.88 (n = 30 259)		0.15–3.88 (n = 33 890)	0.35–2.41 (n = 29 837)	0.14–2.38 (n = 31 255)	
Men (%)	14 268 (43)	15 393 (48)	12 961 (43)	$P = 0.18$	15 215 (45)	13 401 (45)	14 088 (45)	$P = 0.65$
BMI, kg/m^2	23.9 ± 0.02	25.9 ± 0.02	28.8 ± 0.03	$P < 1 \times 10^{-300}$	26.2 ± 0.02	26.2 ± 0.02	26.1 ± 0.02	$P = 0.19$
Smoking (%)	5487 (17)	5978 (19)	5709 (19)	$P = 2 \times 10^{-12}$	6014 (18)	5486 (18)	5646 (18)	$P = 0.27$
Hypertension (%)	15 908 (48)	19 591 (61)	21 730 (72)	$P < 1 \times 10^{-300}$	20 515 (61)	18 020 (60)	18 825 (60)	$P = 0.43$
Alcohol consumption (%)	6076 (19)	5828 (18)	4801 (16)	$P = 4 \times 10^{-18}$	5945 (18)	5309 (18)	5450 (17)	$P = 0.75$
Physical inactivity (%)	13 016 (40)	15 532 (48)	18 286 (60)	$P < 1 \times 10^{-300}$	16 675 (49)	14 731 (49)	15 427 (49)	$P = 0.69$
Postmenopausal (%)[b]	10 850 (59)	11 678 (70)	12 736 (74)		12 546 (67)	11 051 (67)	11 554 (67)	
Hormonal replacement therapy (%)[b]	1906 (10)	1898 (11)	1762 (10)	$P^c = 5 \times 10^{-119}$	2008 (11)	1681 (10)	1847 (11)	$P^c = 0.80$
Lipid-lowering therapy (%)	2240 (7)	3531 (11)	5033 (17)	$P < 1 \times 10^{-300}$	3961 (12)	3524 (12)	3722 (12)	$P = 0.38$
Education <8 years (%)	2358 (7)	3360 (10)	4149 (14)	$P = 6 \times 10^{-159}$	3538 (10)	3165 (11)	3239 (10)	$P = 0.77$
Type 1 diabetes (%)	168 (1)	112 (0)	249 (1)		336 (1)	280 (1)	312 (1)	
Type 2 diabetes (%)	401 (1)	792 (2)	1589 (5)	$P^c = 2 \times 10^{-21}$	1097 (3)	1010 (3)	985 (3)	$P^c = 0.93$
CRP, mg/L[d]	0.9 ± 0.02	1.4 ± 0.01	2.5 ± 0.01	$P < 1 \times 10^{-300}$	1.5 ± 0.02	1.5 ± 0.02	1.4 ± 0.02	$P = 0.12$
Glucose, mmol/L[d]	5.2 ± 0.006	5.2 ± 0.007	5.3 ± 0.007	$P = 9 \times 10^{-127}$	5.3 ± 0.006	5.3 ± 0.007	5.2 ± 0.007	$P = 0.55$

[a] 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 nonfasting plasma glucose concentrations of >11 mmol/L (>198 mg/dL). Smoking was current smoking. Alcohol consumption was >14/21 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 [b] in women only.

[d] 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, 1.31 g/L for nephropathy, 1.12 g/L for neuropathy, 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 CRP and complement C3 (*P* for trend: $P < 1 \times 10^{-300}$) and between plasma glucose and complement C3 (*P* for trend: $P = 3 \times 10^{-142}$) (see Fig. 3 in the online Data Supplement). Correlations of complement C3, age, adjusted glucose, BMI, and CRP are predominantly positive in both men and women (see Table 1 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, ≤1.02 g/L) through the middle tertile (C3, 1.03–1.20 g/L) to the highest tertile (C3, ≥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 ($P = 5 \times 10^{-10}$) (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 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 95 202 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

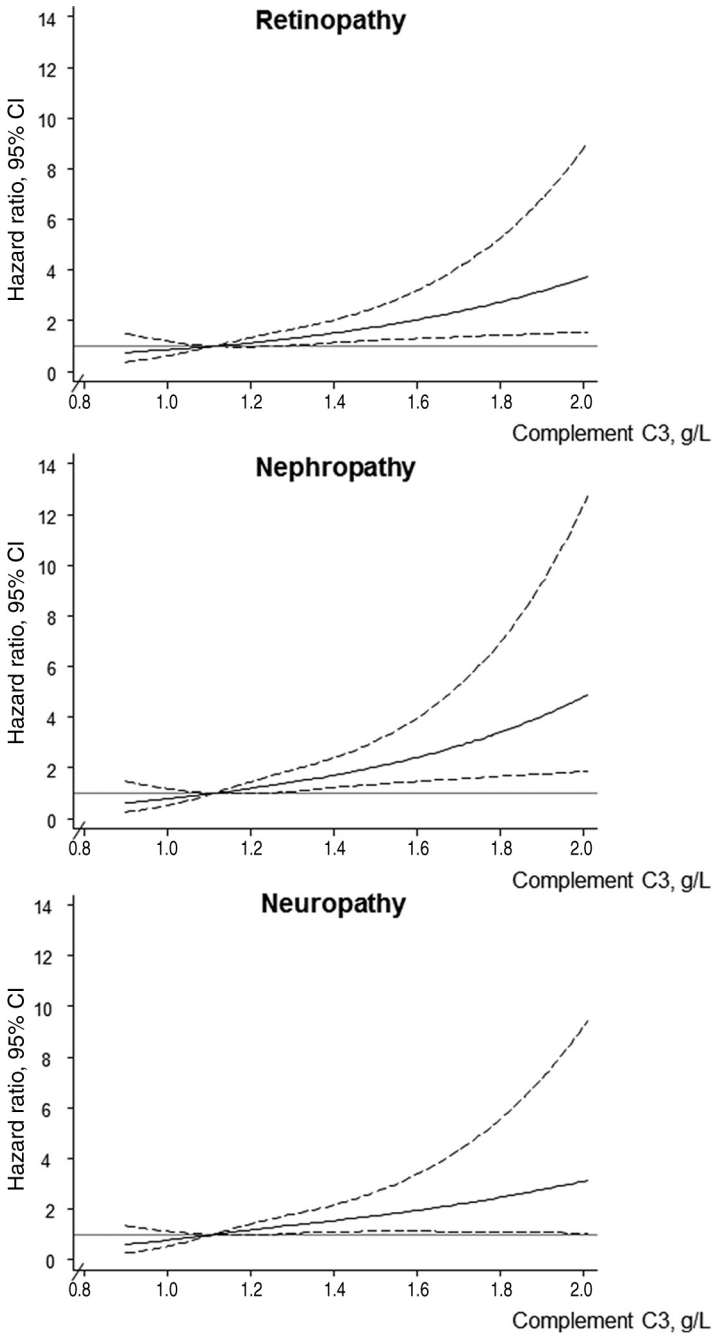

Fig. 2. Multifactorially adjusted hazard ratios for diabetic retinopathy, nephropathy, and neuropathy according to plasma concentrations of complement C3 in the Copenhagen General Population Study.

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.

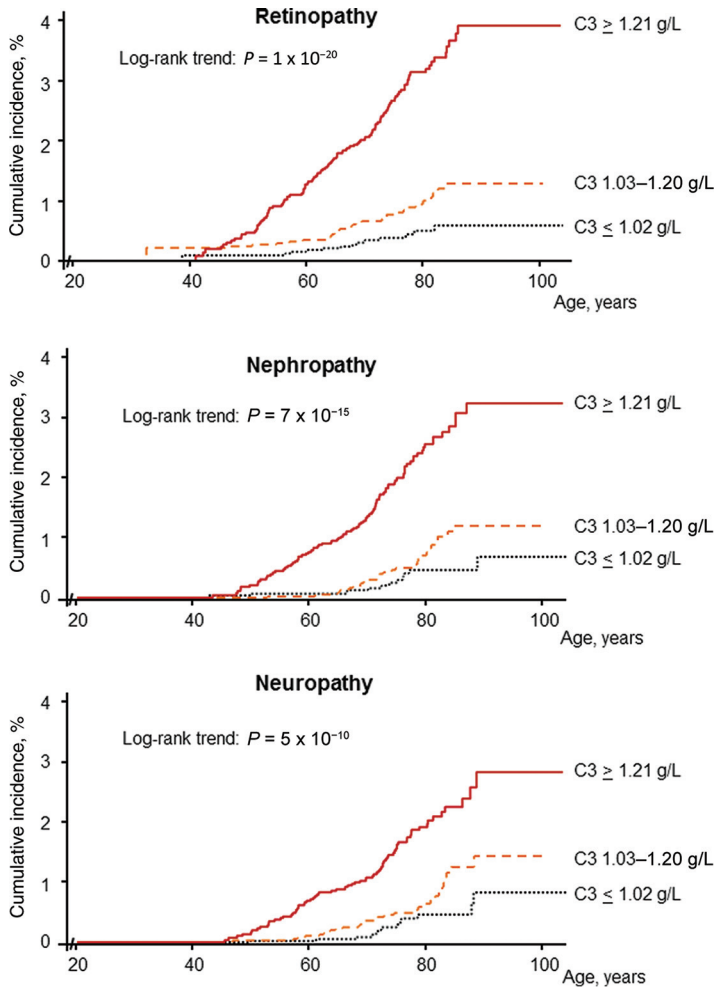

Fig. 3. Cumulative incidence of diabetic retinopathy, nephropathy, and neuropathy as a function of age and tertiles of plasma concentrations of complement C3 in the Copenhagen General Population Study.

C3, plasma concentrations of complement C3.

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

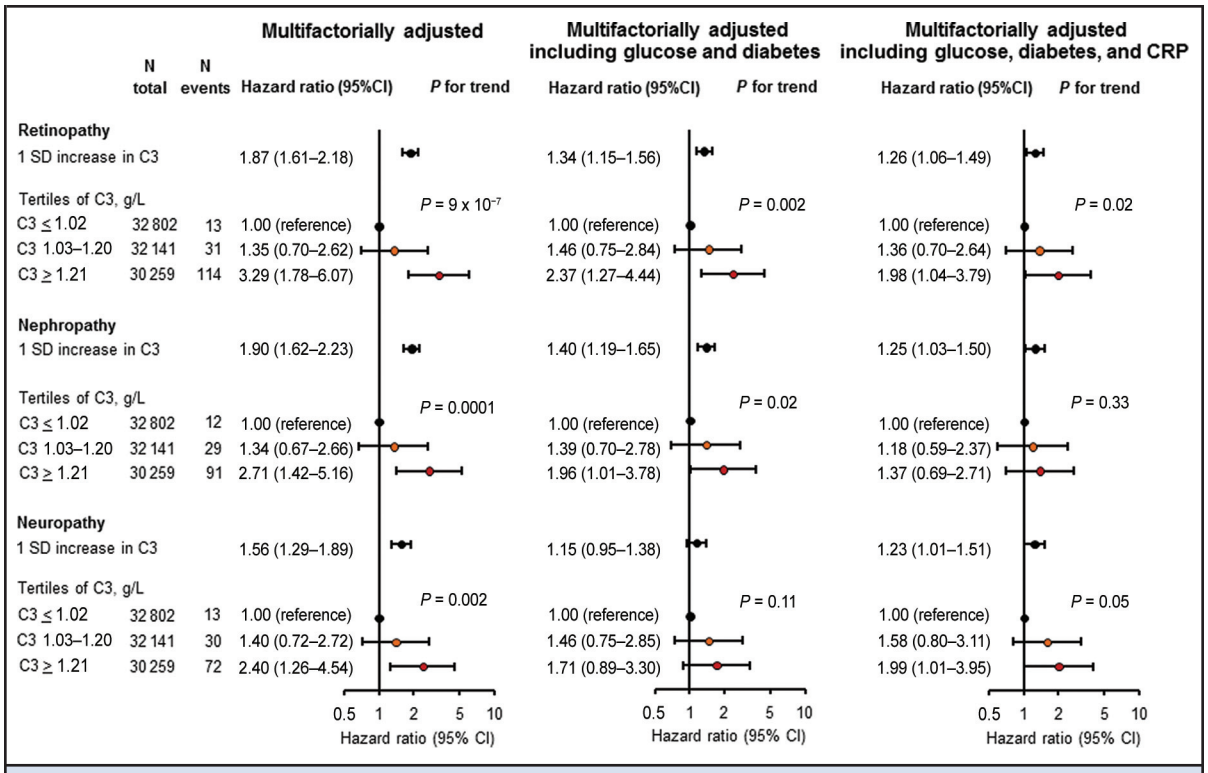

Fig. 4. Risk of diabetic retinopathy, nephropathy, and neuropathy as a function of 1 SD increase and tertiles of complement C3.
Hazard ratios were multifactorially adjusted for age (time scale), sex, BMI, smoking, hypertension, lipid-lowering therapy, alcohol consumption, physical inactivity, education, 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.

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 control and preprandial 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

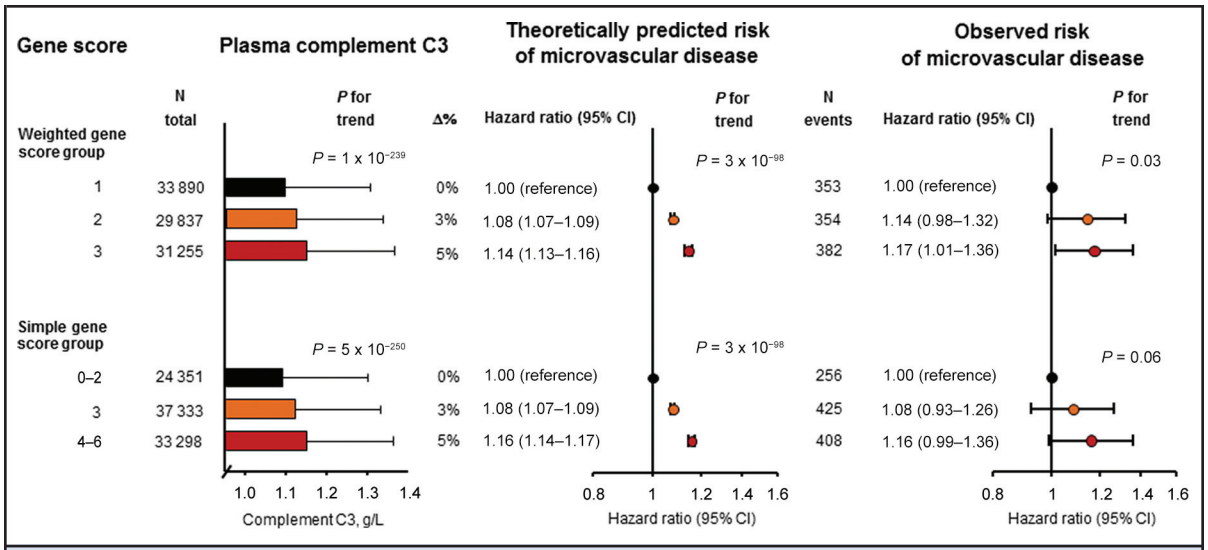

Fig. 5. Plasma concentrations of complement C3 and corresponding theoretically predicted and observed hazard ratios for diabetic microvascular disease for weighted and simple gene scores in 3 groups.

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.

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 intraindividual 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-

hort; however, we are not aware of a similarly 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.

Author Contributions: *All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.*

K.L. Rasmussen: Study concept and design, acquisition of data, statistical analysis, analysis and interpretation of data, drafting of the manuscript, critical revision of the manuscript for important intellectual content, final approval for submission.

B.G. Nordestgaard: Acquisition of data, analysis and interpretation of data, critical revision of the manuscript for important intellectual content, obtained funding, administrative, technical, and material support, study supervision, final approval for submission.

S.F. Nielsen: Study concept and design, acquisition of data, statistical analysis, analysis and interpretation of data, drafting of the manuscript, critical revision of the manuscript for important intellectual content, obtained funding, administrative, technical, and material support, study supervision, final approval for submission, and takes full responsibility for the work as a whole.

Authors' Disclosures or Potential Conflicts of Interest: *Upon manuscript submission, all authors completed the author disclosure form. Disclosures and/or potential conflicts of interest:*

Employment or Leadership: None declared.
Consultant or Advisory Role: None declared.
Stock Ownership: None declared.
Honoraria: None declared.
Research Funding: Department of Clinical Biochemistry, Herlev and Gentofte Hospital, Copenhagen University Hospital.
Expert Testimony: None declared.
Patents: None declared.

Role of Sponsor: The funding organizations played no role in the design of study, choice of enrolled patients, review and interpretation of data, or final approval of manuscript.

References

1. The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. The Diabetes Control and Complications Trial Research Group. N Engl J Med 1993;329:977–86.
2. Intensive blood-glucose control with sulphonylureas or insulin compared with conventional treatment and risk of complications in patients with type 2 diabetes (UKPDS 33). UK Prospective Diabetes Study (UKPDS) Group. Lancet 1998;352:837–53.
3. Markiewski MM, Lambris JD. The role of complement in inflammatory diseases from behind the scenes into the spotlight. Am J Pathol 2007;171:715–27.
4. Hertle E, van Greevenbroek MM, Stehouwer CD. Complement C3: an emerging risk factor in cardiometabolic disease. Diabetologia 2012;55:881–4.
5. Mamane Y, Chung CC, Lavallee G, Morin N, Xu LJ, Huang J et al. The C3a anaphylatoxin receptor is a key mediator of insulin resistance and functions by modulating adipose tissue macrophage infiltration and activation. Diabetes 2009;58:2006–17.
6. Muscari A, Antonelli S, Bianchi G, Cavrini G, Dapporto S, Ligabue A, et al. Serum C3 is a stronger inflammatory marker of insulin resistance than C-reactive protein, leukocyte count, and erythrocyte sedimentation rate: comparison study in an elderly population. Diabetes Care 2007;30:2362–8.
7. Onat A, Can G, Rezvani R, Cianflone K. Complement C3 and cleavage products in cardiometabolic risk. Clin Chim Acta 2011;412:1171–9.
8. Bao X, Xia Y, Zhang Q, Wu HM, Du HM, Liu L, et al. Elevated serum complement C3 levels are related to the development of prediabetes in an adult population: the Tianjin Chronic Low-Grade Systematic Inflammation and Health Cohort Study. Diabet Med 2016;33:446–53.
9. Norgaard I, Nielsen SF, Nordestgaard BG. Complement C3 and high risk of venous thromboembolism: 80517 individuals from the Copenhagen General Population Study. Clin Chem 2016;62:525–34.
10. Ghosh P, Sahoo R, Vaidya A, Chorev M, Halperin JA. Role of complement and complement regulatory proteins in the complications of diabetes. Endocr Rev 2015;36:272–88.
11. Gerl VB, Bohl J, Pitz S, Stoffelns B, Pfeiffer N, Bhakdi S. Extensive deposits of complement C3d and C5b-9 in the choriocapillaris of eyes of patients with diabetic retinopathy. Invest Ophthalmol Vis Sci 2002;43:1104–8.
12. Zhang J, Gerhardinger C, Lorenzi M. Early complement activation and decreased levels of glycosylphosphatidylinositol-anchored complement inhibitors in human and experimental diabetic retinopathy. Diabetes 2002;51:3499–504.
13. Xiao X, Ma B, Dong B, Zhao P, Tai N, Chen L, et al. Cellular and humoral immune responses in the early stages of diabetic nephropathy in NOD mice. J Autoimmun 2009;32:85–93.
14. Ostergaard J, Hansen TK, Thiel S, Flyvbjerg A. Complement activation and diabetic vascular complications. Clin Chim Acta 2005;361:10–9.
15. Wang H, Vinnikov I, Shahzad K, Bock F, Ranjan S, Wolter J, et al. The lectin-like domain of thrombomodulin ameliorates diabetic glomerulopathy via complement inhibition. Thromb Haemost 2012;108:1141–53.
16. Uesugi N, Sakata N, Nangaku M, Abe M, Horiuchi S, Hisano S, Iwasaki H. Possible mechanism for medial smooth muscle cell injury in diabetic nephropathy: glycoxidation-mediated local complement activation. Am J Kidney Dis 2004;44:224–38.
17. Falk RJ, Sisson SP, Dalmasso AP, Kim Y, Michael AF, Vernier RL. Ultrastructural localization of the membrane attack complex of complement in human renal tissues. Am J Kidney Dis 1987;9:121–8.
18. Rosoklija GB, Dwork AJ, Younger DS, Karlikaya G, Latov N, Hays AP. Local activation of the complement system in endoneurial microvessels of diabetic neuropathy. Acta Neuropathol 2000;99:55–62.
19. Graham AR, Johnson PC. Direct immunofluorescence findings in peripheral nerve from patients with diabetic neuropathy. Ann Neurol 1985;17:450–4.
20. Lawlor DA, Harbord RM, Sterne JA, Timpson N, Davey SG. Mendelian randomization: using genes as instruments for making causal inferences in epidemiology. Stat Med 2008;27:1133–63.
21. Kamstrup PR, Tybjaerg-Hansen A, Steffensen R, Nordestgaard BG. Genetically elevated lipoprotein(a) and increased risk of myocardial infarction. JAMA 2009;301:2331–9.
22. Zacho J, Tybjaerg-Hansen A, Jensen JS, Grande P, Sillesen H, Nordestgaard BG. Genetically elevated C-reactive protein and ischemic vascular disease. N Engl J Med 2008;359:1897–908.

23. Frikke-Schmidt R, Nordestgaard BG, Stene MC, Sethi AA, Remaley AT, Schnohr P, et al. Association of loss-of-function mutations in the ABCA1 gene with high-density lipoprotein cholesterol levels and risk of ischemic heart disease. JAMA 2008;299:2524–32.
24. Voight BF, Kang HM, Ding J, Palmer CD, Sidore C, Chines PS, et al. The metabochip, a custom genotyping array for genetic studies of metabolic, cardiovascular, and anthropometric traits. PLoS Genet 2012;8: e1002793.
25. Fine JP, Gray RJ. A proportional hazards model for the subdistribution of a competing risk. J Am Stat Assoc 1999;94:496–509.
26. Gabrielsson BG, Johansson JM, Lonn M, Jernas M, Olbers T, Peltonen M, et al. High expression of complement components in omental adipose tissue in obese men. Obes Res 2003;11:699–708.
27. Choy LN, Rosen BS, Spiegelman BM. Adipsin and an endogenous pathway of complement from adipose cells. J Biol Chem 1992;267:12736–41.
28. Gruys E, Toussaint MJ, Niewold TA, Koopmans SJ. Acute phase reaction and acute phase proteins. J Zhejiang Univ Sci B 2005;6:1045–56.
29. Lauritzen T, Frost-Larsen K, Larsen HW, Deckert T. Effect of 1 year of near-normal blood glucose levels on retinopathy in insulin-dependent diabetics. Lancet 1983;1:200–4.
30. Tedesco F, Fischetti F, Pausa M, Dobrina A, Sim RB, Daha MR. Complement-endothelial cell interactions: pathophysiological implications. Mol Immunol 1999; 36:261–8.
31. Schalkwijk CG, Stehouwer CD. Vascular complications in diabetes mellitus: the role of endothelial dysfunction. Clin Sci (Lond) 2005;109:143–59.
32. Hollander W, Colombo MA, Kirkpatrick B, Paddock J. Soluble proteins in the human atherosclerotic plaque. With spectral reference to immunoglobulins, C3-complement component, alpha 1-antitrypsin and alpha 2-macroglobulin. Atherosclerosis 1979;34:391–405.
33. Pang AS, Katz A, Minta JO. C3 deposition in cholesterol-induced atherosclerosis in rabbits: a possible etiologic role for complement in atherogenesis. J Immunol 1979;123:1117–22.
34. Cianflone K, Xia Z, Chen LY. Critical review of acylation-stimulating protein physiology in humans and rodents. Biochim Biophys Acta 2003;1609:127–43.
35. Sims PJ, Wiedmer T. The response of human platelets to activated components of the complement system. Immunol Today 1991;12:338–42.
36. Oikonomopoulou K, Ricklin D, Ward PA, Lambris JD. Interactions between coagulation and complement–their role in inflammation. Semin Immunopathol 2012;34:151–65.
37. Hess K, Alzahrani SH, Mathai M, Schroeder V, Carter AM, Howell G, et al. A novel mechanism for hypofibrinolysis in diabetes: the role of complement C3. Diabetologia 2012;55:1103–13.
38. Scantlebury T, Maslowska M, Cianflone K. Chylomicron-specific enhancement of acylation stimulating protein and precursor protein C3 production in differentiated human adipocytes. J Biol Chem 1998;273:20903–9.
39. Fujita T, Hemmi S, Kajiwara M, Yabuki M, Fuke Y, Satomura A, Soma M. Complement-mediated chronic inflammation is associated with diabetic microvascular complication. Diabetes Metab Res Rev 2013;29: 220–6.
40. Papanas N, Vinik AI, Ziegler D. Neuropathy in prediabetes: does the clock start ticking early? Nat Rev Endocrinol 2011;7:682–90.
41. Lamparter J, Raum P, Pfeiffer N, Peto T, Hohn R, Elflein H, et al. Prevalence and associations of diabetic retinopathy in a large cohort of prediabetic subjects: the Gutenberg Health Study. J Diabetes Complications 2014;28:482–7.