Complement factors D and C3 cross-sectionally associate with arterial stiffness, but not independently of metabolic risk factors

Original Article

Complement factors D and C3 cross-sectionally associate with arterial stiffness, but not independently of metabolic risk factors: The Maastricht Study

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Background: Arterial stiffness predicts cardiovascular outcomes. The complement system, particularly the alternative complement pathway, has been implicated in cardiovascular diseases. We herein investigated the associations of factor D, the rate-limiting protease of the alternative pathway, and C3, the central complement component, with arterial stiffness.

Methods: In 3019 population-based participants (51.9% men, 60.1 ± 8.2 years, 27.7% type 2 diabetes [T2D], oversampled]), we measured carotid-femoral pulse wave velocity (cfPWV), carotid distensibility coefficient (DC) and carotid Young's elastic modulus (YEM), and plasma concentrations of factors D and C3. We conducted multiple linear regression to investigate the association of factors D and C3 (main independent variables, standardized) with cfPWV (primary outcome) and DC and YEM (secondary outcomes), adjusted for potential confounders.

Results: Per SD higher factors D and C3, cfPWV was 0.41 m/s [95% confidence interval: 0.34; 0.49] and 0.33 m/s [0.25; 0.41] greater, respectively. These associations were substantially attenuated when adjusted for age, sex, education, mean arterial pressure, and heart rate (0.08 m/s [0.02; 0.15] and 0.11 m/s [0.05; 0.18], respectively), and were not significant when additionally adjusted for T2D, waist circumference and additional cardiovascular risk factors (0.06 m/s [−0.01; 0.13] and 0.01 m/s [−0.06; 0.09], respectively). Results were comparable for carotid YEM and DC. In persons with T2D, but not in those without, the association between factors D and cfPWV was significant in the fully adjusted model (0.14 m/s, [0.01; 0.27], $P = 0.038$, $P_{interaction} < 0.05$).

Conclusion: The strong association of plasma factors D and C3 with arterial stiffness in this population-based cohort was not independent of T2D and other metabolic risk factors. Our data suggest that a possible causal pathway starting from alternative complement activation may via hypertension and T2D contribute to greater arterial stiffness.

Keywords: arterial stiffness, C3, carotid DC, carotid Young's elastic modulus, carotid-femoral pulse wave velocity, complement factor D

Abbreviations: BMI, body mass index; carotid DC, carotid distensibility coefficient; carotid YEM, carotid Young's elastic modulus; cfPWV, carotid-femoral pulse wave velocity; CVD, cardiovascular diseases; DHD, Dutch Healthy Diet; eGFR, glomerular filtration rate; HDL, high-density lipoprotein; HR, heart rate; MAP, mean arterial pressure; T2D, type 2 diabetes

INTRODUCTION

Arterial stiffening is one of the key processes in the development of cardiovascular diseases (CVD). Greater arterial stiffness may contribute to a higher risk CVD via an increase in systolic blood pressure but the association of arterial stiffness with CVD is, at least in part, independent of blood pressure [1]. Arterial stiffness is higher in obese individuals of all ages than in their non-obese peers [2]. Arterial stiffness is, among others, determined by properties of elastin and collagen in the arterial wall [3]. It is also affected by changes in endothelial cell signaling and vascular smooth muscle cell tone [4].

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The complement system, an intricate protein network that is part of the innate immune system, has been suggested as a potential modulator of arterial stiffness. Complement factors D and C3 comprise major components of the alternative pathway of complement activation which has consistently been implicated in CVD in humans [5]. Complement factor D is the rate-limiting serine-protease in activation of the alternative complement pathway. Complement activation results in the activation of C3, the central complement component.

Factor D, also known as adipsin, is produced in high amounts in adipocytes as well as monocytes/macrophages of adipose tissue [6]. It is also expressed in the aortic endothelium and its expression is higher in endothelial cells derived from diabetic mice [7]. Notably, in a mouse model of vascular calcification, factor D was identified as the major elastase involved in elastin fragmentation and subsequent elastocalcinosis [8]. In line with the above, we previously showed that higher factor D concentration was associated with worse endothelial dysfunction [9]. We also showed that a greater plasma factor D concentration was significantly associated with incident cardiovascular events after adjustment for age, sex and glucose metabolism status, although the association was strongly attenuated upon full adjustment for potential confounders [9]. Factor D was also identified as a biomarker for poor prognosis in patients with coronary artery disease [10]. The Women's Health Initiative observational study cohort confirmed factor D as a risk marker for coronary heart disease (CHD), but not for stroke [11] while in another large cohort consisting only of men, factor D was positively associated with the risk of developing stroke [12] but not with risk of CHD [13].

C3 is mainly synthesized in the liver, but is also produced by, for example, the vascular endothelium [14] and adipocytes, including perivascular adipocytes [15,16]. In humans, circulating C3 is strongly associated with adiposity [17,18], is longitudinally associated with cardiovascular disease [5] and was identified as a risk factor for myocardial infarction [19]. C3 was also identified as a risk factor for hypertension, at least in men [20], and recently an association between complement C3 and carotid-femoral pulse wave velocity (cfPWV) was proposed in a large population-based cohort [21], which awaits confirmation in a large independent study. C3 binds to collagen and elastin fibers within the adventitia and may, thereby, contribute to vascular stiffening [22]. C3 is also produced by aortic smooth muscle cells of spontaneously hypertensive rats and contributes to vascular smooth muscle cell proliferation and extracellular matrix synthesis [23].

Taken together, experimental data suggest that factors D and C3 may be associated with changes in the vascular wall that are functionally involved in processes related to large artery function [8,22] but human data to support this are limited for complement C3 and, to the best of our knowledge, absent for factor D. In our current study we addressed, in a large human cohort enriched for individuals with type 2 diabetes (T2D), the cross-sectional associations of plasma factors D and C3 with aortic stiffness defined as cfPWV, and carotid stiffness defined as carotid distensibility coefficient (DC) and carotid Young's elastic modulus (YEM).

RESEARCH DESIGN AND METHODS

Study population and design

We used data from The Maastricht study, an observational prospective population-based cohort study. The rationale and methodology have been described previously [24]. In brief, the study focuses on the etiology, pathophysiology, complications and comorbidities of T2D and is characterized by an extensive phenotyping approach. Eligible for participation were all individuals aged between 40 and 75 years and living in the southern part of the Netherlands. Participants were recruited through mass media campaigns and from the municipal registries and the regional Diabetes Patient Registry via mailings. Recruitment was stratified according to known T2D status, with an oversampling of individuals with T2D, for reasons of efficiency. A flow-chart of the inclusion of study participants in the current analyses is presented in Fig. 1. The present report includes cross-sectional data from the first 7689 participants, who completed the baseline survey between November 2010 and December 2017. The examinations of each participant were performed within a time window of three months. The study has been approved by the institutional medical ethical committee (NL31329.068.10) and the Minister of Health, Welfare and Sports of the Netherlands (Permit 131088-105234-PG). All participants gave written informed consent.

Factors D and C3 measurement

After overnight fasting, venous blood samples were collected in ethylenediaminetetraacetic acid (EDTA) on ice. Blood was immediately centrifuged and plasma samples were stored at −80°C until use. Complement factor D was measured in EDTA plasma using an R&D duoset kit assay, as described before [9]. The inter-assay variation was 4.6%. Complement C3 was measured in EDTA plasma using an MSD R-plex Human Complement C3 Antibody Set. The assay was performed according to the manufacturer's instruction except for the use of a 1:20 000 instead of 1:300 000 dilution, which resulted in better stability of the measurements. The inter-assay variation was 8.9%.

Arterial stiffness measurements

All measurements were done by trained vascular technicians unaware of the participants' clinical or diabetes mellitus status, in a dark, quiet temperature-controlled room (21–23°C), as described previously [25]. Participants were asked to refrain from smoking and drinking coffee, tea or alcoholic beverages 3 h before the measurements. Participants were allowed to have a light meal (breakfast or lunch). All measurements were performed in supine position after 10 min of rest. Talking or sleeping was not allowed during the examination. During the vascular measurements (≈45 min), brachial systolic, diastolic, and mean arterial pressure (MAP) were determined every 5 min with an oscillometric device (Accutorr Plus, Datascope Inc, Montvale, New Jersey, USA). The mean MAP and heart rate (HR) during these measurements were used in the statistical analysis. A 3-lead ECG was recorded continuously during the measurements to facilitate automatic signal processing.

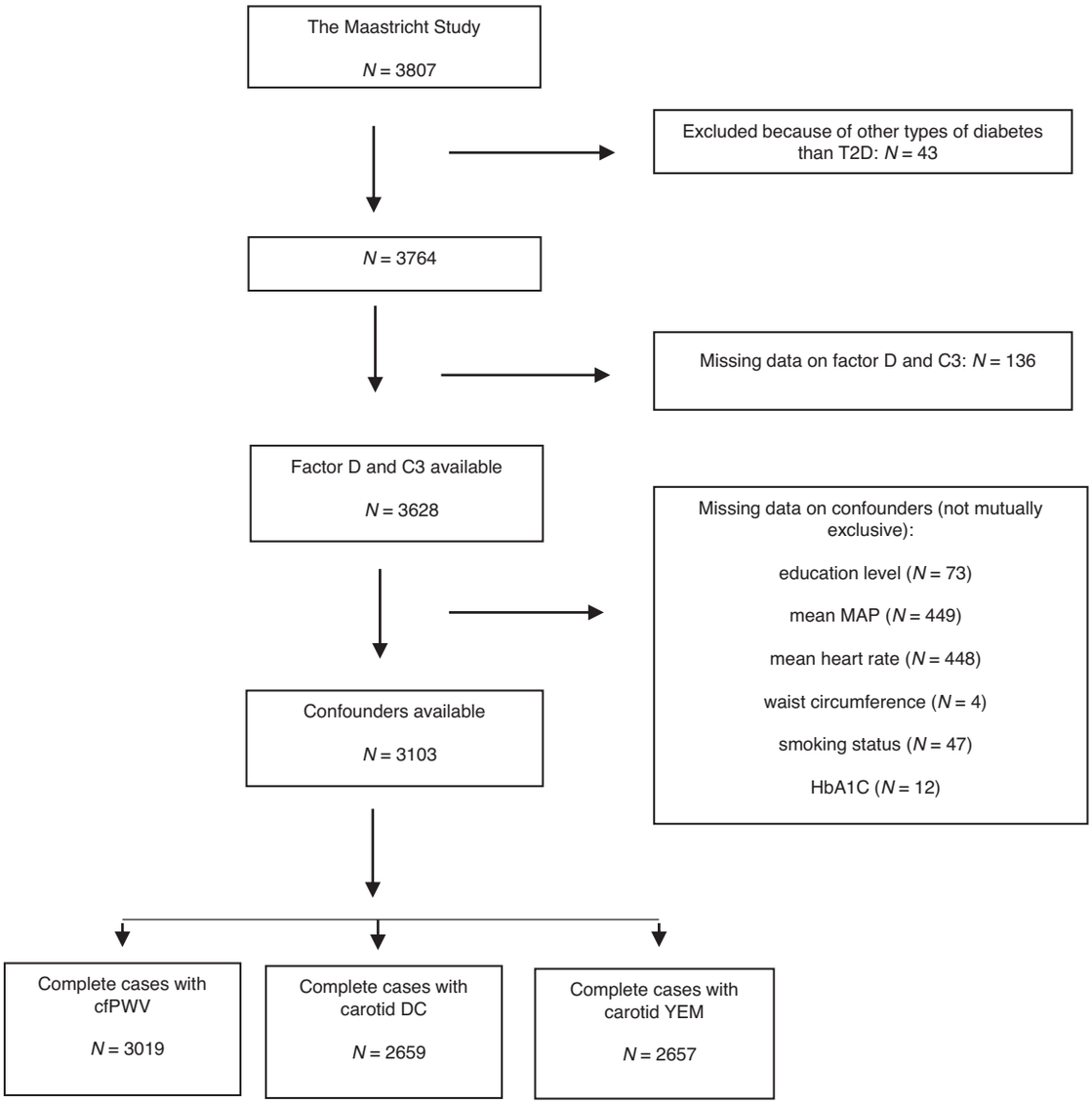

FIGURE 1 Derivation of final study population. cfPWV, carotid-femoral pulse wave velocity; DC, distensibility coefficient; HbA1C, hemoglobin A1c (glycated hemoglobin); MAP, mean artery pressure; YEM, Young's elastic modulus.

Carotid-femoral pulse wave velocity

cfPWV was determined according to guidelines with the use of applanation tonometry (SphygmoCor, Atcor Medical, Sydney, Australia). Pressure waveforms were determined at the right common arteries and right common femoral arteries. Difference in the time of pulse arrival from the R-wave of the ECG between the two sites (transit time) was determined with the intersecting tangents algorithm. The pulse wave travel distance was calculated as 80% of the direct straight distance (measured with an infantometer) between the two arterial sites. The median of 3 consecutive cfPWV (defined as traveled distance/transit time) recordings was used in the analyses.

Local carotid arterial properties

Diameter and distension of the left common carotid artery were measured with an ultrasound scanner equipped with a 7.5-MHz linear probe (MyLab 70, Esaote Europe B.V., Maastricht, the Netherlands). Exactly as reported before [25], during the ultrasound measurements a B-mode image on the basis of 19 M-lines was captured on screen and an online echo-tracking algorithm showed real-time anterior and posterior arterial wall displacements. The M-mode recordings were comprised of 19 simultaneous recordings at a frame rate of 498 Hz. The distance between the M-line recording positions was 0.96 mm; therefore, a total segment of 18.24 mm of each artery was covered by the scan plane. For offline processing, the radiofrequency signal was fed into a dedicated PC-based acquisition system with a sampling frequency of 50 MHz. Data processing was conducted in MatLab. The distension waveforms were acquired from the radio frequency data with the use of a wall track algorithm. The median diameter and distension of two measurements were used in the analyses. Local arterial

elastic properties were quantified by calculating the following indices:

Distensibility coefficient (DC)

$$DC = (2\Delta D * D + \Delta D^2)/(PP * IAD^2)\ [\text{in } 10^3\ kPa^{-1}]$$

Young's elastic modulus (YEM)

$$YEM = D/(IMT * DC)\ [\text{in } 10^3\ kPa]$$

where ΔD is distension; D, arterial diameter; and PP, brachial pulse pressure (calculated as systolic BP minus diastolic BP); and IMT, intima-media thickness. DC represents arterial distensibility and YEM represents the stiffness of the arterial wall material at operating pressure.

Other characteristics of participants

Covariates were measured as reported before [24]. In short, smoking (never, former or current smoker), alcohol consumption (none, low (women ≤7 and men ≤14 glasses/week,) and high (women >7 and men >14 glasses/week)), physical activity (hours/week) and education status (low, medium, high) were obtained through web-based questionnaires. Use of lipid-modifying and antihypertensive medication was collected by means of an interview. Weight and height were measured to calculate body mass index (BMI, kg/m^2) and waist circumference was measured midway between the lower rib margin and the iliac crest. T2D was diagnosed according to World Health Organization criteria, using a 75 g oral glucose tolerance test [26]. Serum total cholesterol, high-density lipoprotein (HDL) cholesterol, and triglycerides were measured using an automatic analyzer (Beckman Synchron LX20, Beckman Coulter Inc.). MAP was calculated as 1/3 systolic blood pressure + 2/3 diastolic blood pressure. Twenty-four-hour blood pressure was measured using an ambulatory device (WatchBP O3, Microlife, Switzerland). Readings were taken each 15 min between 8:00 and 23:00 h and each 30 min from 23:00 to 8:00 h. A validated food frequency questionnaire [27] was administered and used to derive the Dutch Healthy Diet (DHD)15-index that contains 15 components representing the 15 food-based Dutch dietary guidelines of 2015 [28]. Since the FFQ did not distinguish between filtered and unfiltered coffee, the DHD score in the present study ranged from 0 to 140. Kidney function can be affected by arterial stiffness [29], and therefore, the glomerular filtration rate (eGFR) was included as descriptive variable, estimated by means of CKD-EPI equation based on the combination of serum creatinine and serum cystatin C [24].

Statistical analyses

All analyses were performed using IBM SPSS Statistics 27 for windows (version 27; IBM Corp, Somers, New York, USA). A two-tailed P value <0.05 was considered significant. Normality of variable distribution was tested. Normal distributed variables are presented as mean ± SD. Categorical variables are presented as proportions (%). Characteristics were compared across quartiles of factors D and C3, using an ANOVA for continuous variables, or X^2-test in case of categorical variables.

Multiple linear regression was used to investigate the associations of plasma concentrations of factors D and C3 (main independent variables) with cfPWV (primary outcome), and carotid DC and YEM (secondary outcomes). Regression models were as follows: model 1 was the crude association; model 2 was additionally adjusted for age (years) and sex (male or female), education status (medium high, each yes/no); model 3 was additionally adjusted for MAP (mmHg) and HR (bpm); model 4 was additionally adjusted for T2D (as yes/no, because of oversampling of T2D); model 5 was additionally adjusted for waist circumference (in cm); model 6 was additionally adjusted for smoking habits (current or former smoker, each yes/no), lipid-modifying and/or antihypertensive medication (each yes/no), total/HDL cholesterol ratio triglycerides (mmol/l) and HbA1c (mmol/mol).

Note models 4 and 5 may be overadjusted for the association of factor D and arterial stiffness, because factor D [30,31] has been implicated in β-cell preservation, strongly expressed in adipose tissue, and additionally implicated in adipocyte differentiation and progression of obesity [32]. Models 3 and 4 may be overadjusted for the association of C3 and arterial stiffness, because complement C3 has been implicated in development of hypertension [20], and T2D [33].

We tested for interaction with T2D because of the oversampling of T2D in the Maastricht study and for interaction with sex to explore potential differences in these relationships between men and women, by including interaction terms of interest, that is, factor D*T2D, factor D*sex, C3*T2D, C3*sex, respectively, plus T2D or sex*all confounders included in the model [34].

To assess the robustness of the findings in the main analyses, we performed several sensitivity analyses. First, associations between factor D or C3 and arterial stiffness were re-evaluated using income level and occupation status substituted for education status. Second, associations between factor D or C3 and arterial stiffness were re-evaluated using MAP calculated from 24-h blood pressure measurements substituted for MAP at the time of ultrasound measurement. Third, associations between complement factor D or C3 and arterial stiffness were re-evaluated using BMI instead of waist circumference. Fourth, associations between complement factor D or C3 and arterial stiffness were re-evaluated by additionally adjusting for physical activity (hours/week), and for DHD15 (score). This reduced the number of participants in the analyses because of a relatively large number of missing in these variables. We also substituted energy intake (kJ/day) and alcohol consumption (low alcohol consumption and high alcohol consumption, each yes/no) for DHD15 (score).

RESULTS

General characteristics of the study population

Factor D concentration was 0.92 ± 0.23 mg/l (range 0.33–2.32), C3 concentration was 1.15 ± 0.23 g/l (range 0.45–2.50). The correlation between factors D and C3 was $r = 0.123$, $P < 0.001$ ($n = 3019$). General characteristics of the study population across quartiles of factors D and C3 are presented in Table 1. Participants in the higher quartiles of both factors D and C3 were older, which was more

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TABLE 1. General characteristics of the study population based on quartiles of factors D and C3 concentration

	Quartiles of plasma factor D concentration					Quartiles of plasma factor C3 concentration				
	1st quartile N = 755 0.33–0.78 mg/l	2rd quartile N = 755 0.78–0.90 mg/l	3rd quartile N = 755 0.90–1.04 mg/l	4th quartile N = 754 1.04–2.32 mg/l	*P*-value[a]	1st quartile N = 757 0.45- 0.99 g/l	2nd quartile N = 767 0.99–1.13 g/l	3rd quartile N = 751 1.13–1.28 g/l	4th quartile N = 744 1.29- 2.50 g/l	*P*-value[a]
Demographics										
Age (years)	56.8 ± 7.9	58.9 ± 8.1	60.8 ± 8.0	63.8 ± 7.1	<0.001	58.7 ± 8.3	60.5 ± 8.0	60.7 ± 7.9	60.4 ± 8.5	<0.001
Sex (%men)	40.5	50.2	56.3	60.7	<0.001	46.8	54.0	55.8	51.2	0.003
Medium/high Education status (%)	31.4/39.5	28.2/41.9	27.8/38.4	25.3/35.1	<0.001	25.0/52.0	30.9/39.4	27.6/33.8	29.3/29.4	<0.001
lifestyle										
BMI (kg/m^2)[b]	25.4 ± 3.8	26.5 ± 4.1	27.2 ± 4.1	28.7 ± 4.8	<0.001	24.6 ± 3.2	26.2 ± 3.7	27.5 ± 3.9	29.7 ± 4.8	<0.001
Waist circumference (cm)	90.3 ± 12.5	94.0 ± 12.7	96.7 ± 12.6	101.5 ± 13.1	<0.001	88.2 ± 11.1	93.5 ± 11.9	97.7 ± 12.0	103.3 ± 13.6	<0.001
Physical activity (h/week)[b]	15.4 ± 8.3	14.6 ± 8.0	13.8 ± 8.0	12.9 ± 7.8	<0.001	15.2 ± 7.9	14.7 ± 8.0	13.9 ± 8.4	12.7 ± 7.8	<0.001
Former smokers/current smokers (%)	49.9/14.2	51.9/13.2	49.9/13.8	59.3/12.2	0.005	52.6/12.4	49.4/15.6	53.8/13.4	55.4/11.8	0.195
Energy (kcal)[b]	2181 ± 611	2137 ± 601	2212 ± 602	2160 ± 602	0.113	2185 ± 591	2199 ± 591	2179 ± 607	2126 ± 628	0.118
Low/high alcohol consumption (%)	53.4/29.1	54.7/28.2	55.9/27.3	58.9/20.1	0.001	53.6/34.4	56.8/30.2	60.1/20.9	52.4/19.0	<0.001
DHD15-sum[b]	83.7 ± 15.4	84.2 ± 14.4	82.9 ± 14.6	83.0 ± 14.3	0.266	85.4 ± 14.5	84.0 ± 14.5	83.0 ± 14.8	81.3 ± 14.7	<0.001
Biological/clinical										
Fasting plasma glucose (mmol/l)[b]	5.8 ± 1.6	5.9 ± 1.6	6.1 ± 1.7	6.3 ± 1.6	<0.001	5.5 ± 1.2	5.8 ± 1.2	6.2 ± 1.5	6.7 ± 2.1	<0.001
HbA1c (mmol/mol)	39.5 ± 9.3	39.9 ± 9.2	41.2 ± 10	42.5 ± 9.8	<0.001	37.7 ± 7.1	39.0 ± 7.7	41.7 ± 9.2	44.8 ± 12.4	<0.001
Systolic blood pressure (mmHg)[b]	131.9 ± 17.3	133.9 ± 17.8	136.6 ± 18.0	137.2 ± 18.3	<0.001	129.9 ± 17.7	133.9 ± 17.4	137.3 ± 17.6	138.2 ± 17.8	<0.001
Diastolic blood pressure (mmHg)[b]	74.9 ± 10.0	75.8 ± 9.5	77.2 ± 10.0	76.2 ± 9.9	<0.001	73.8 ± 9.8	75.6 ± 9.8	77.0 ± 9.6	77.7 ± 9.9	<0.001
Mean of MAP (mmHg)	95.8 ± 10.6	95.9 ± 9.6	97.7 ± 10.3	97.5 ± 10.5	<0.001	95.3 ± 10.6	96.3 ± 10.2	97.6 ± 10.4	97.8 ± 9.8	<0.001
Heart rate-mean (bpm)	63.0 ± 9.5	62.4 ± 9.2	62.8 ± 9.4	62.9 ± 9.6	0.676	60.6 ± 8.5	61.6 ± 8.9	63.1 ± 9.1	65.7 ± 10.3	<0.001
Total/HDL cholesterol	3.5 ± 1.1	3.6 ± 1.2	3.7 ± 1.1	3.9 ± 1.2	<0.001	3.4 ± 1.1	3.7 ± 1.1	3.7 ± 1.1	3.9 ± 1.3	<0.001
Triglycerides (mmol/l)	1.3 ± 0.9	1.3 ± 0.8	1.4 ± 0.8	1.6 ± 0.9	<0.001	1.1 ± 0.6	1.3 ± 0.7	1.5 ± 0.9	1.8 ± 1.0	<0.001
eGFR (ml/min per 1.73m^2	99.0 ± 10.8	92.5 ± 10.1	86.6 ± 11.0	74.8 ± 14.2	<0.001	90.3 ± 13.6	87.8 ± 14.2	88.2 ± 14.5	85.9 ± 16.1	<0.001
Cardiovascular disease (%)	11.6	15.1	18.8	23.0	<0.001	13.1	13.0	19.3	23.2	<0.001
prediabetes/T2D (%)	12.8/22.3	13.2/23.6	15.5/27.9	17.4/36.9	<0.001	10.8/11.1	15.9/19.8	14.8/32.8	17.5/47.4	<0.001
Medication use										
Use of glucose-lowering medication (%)	16.7	18.1	21.3	29.7	<0.001	8.9	15.5	24.6	37.2	<0.001
Ues of antihypertensive medication (%)	27.2	30.9	40.0	58.4	<0.001	24.4	35.3	42.2	54.7	<0.001
Use of lipid-modifying medication (%)	29.7	30.5	38.1	47.5	<0.001	19.4	30.9	44.5	51.3	<0.001
Outcome variables										
cfPWV (m/s)	8.6 ± 1.9	8.9 ± 2.1	9.2 ± 2.1	9.6 ± 2.4	<0.001	8.5 ± 1.9	9.0 ± 2.2	9.2 ± 2.2	9.5 ± 2.4	<0.001
Carotid DC (10^{-3} kPa)[b]	15.4 ± 5.4	14.7 ± 5.3	14.0 ± 4.9	13.1 ± 4.6	<0.001	15.2 ± 5.3	14.3 ± 5.0	13.7 ± 4.9	13.9 ± 5.1	<0.001
Carotid YEM (10^3 kPa)[b]	0.69 ± 0.41	0.72 ± 0.32	0.76 ± 0.35	0.83 ± 0.40	<0.001	0.70 ± 0.36	0.74 ± 0.33	0.79 ± 0.39	0.78 ± 0.41	<0.001

Data are presented as mean ± SD (continuous variables) or proportion (%, categorical variables).
BMI, body mass index; cfPWV, carotid-femoral pulse wave velocity; DC, distensibility coefficient; DHD-15, Dutch Health Diet index; eGFR, estimated glomerular filtration rate; HbA1c, hemoglobin A1c (glycated hemoglobin); HDL, high-density lipoprotein; MAP, mean artery pressure; YEM, Young's elastic modulus.
[a]*P*-values were obtained by ANOVA or Pearson chi-square.
[b]BMI, *n* = 3018; physical activity, *n* = 2669; energy intake, *n* = 2850; DHD15-sum, *n* = 2850; fasting plasma glucose, *n* = 3017; diastolic blood pressure, *n* = 3017; systolic blood pressure, *n* = 3017, eGFR, *n* = 2701; carotid DC, n = 2659; carotid YEM, *n* = 2657.

TABLE 2. Associations of factor D and factor C3 with aortic stiffness measurements

	cfPWV (m/s)[a]					
	Factor D[b]			C3[b]		
	β	[95% CI]	*P*-value	β	[95% CI]	*P*-value
Model 1	**0.413**	**[0.337; 0.490]**	**<0.001**	**0.331**	**[0.254; 0.407]**	**<0.001**
Model 2	0.074	[0.000; 0.147]	0.051	**0.247**	**[0.177; 0.317]**	**<0.001**
Model 3	**0.083**	**[0.017; 0.150]**	**0.014**	**0.113**	**[0.048; 0.177]**	**0.001**
Model 4	0.062	[−0.003; 0.127]	0.062	0.028	[−0.037; 0.094]	0.395
Model 5	0.064	[−0.003; 0.132]	0.062	0.029	[−0.041; 0.099]	0.419
Model 6	0.058	[−0.010; 0.126]	0.097	0.014	[−0.058; 0.085]	0.707

cfPWV, carotid-femoral pulse wave velocity; CI, confidence interval.
[a]$n = 3019$.
[b]Factors D and C3 are standardized.
Bold typefont represents statistically significant data.
Model 1: Crude association.
Model 2: adjusted for age, sex, education status.
Model 3: additionally adjusted for mean arterial pressure, mean heart rate.
Model 4: additionally adjusted for yes/no T2D.
Model 5: additionally adjusted for waist circumference.

TABLE 3. Associations of factor D with aortic stiffness measurements stratified on T2D

	cfPWV (m/s)					
	Participants with T2D[a]			Participants without T2D[a]		
	β factor D[b]	[95% CI]	*P*-value	β factor D[b]	[95% CI]	*P*-value
Model 1	**0.310**	**[0.168; 0.452]**	**<0.001**	**0.322**	**[0.236; 0.408]**	**<0.001**
Model 2	0.058	[−0.081; 0.196]	0.414	0.027	[−0.058; 0.111]	0.536
Model 3	0.120	[−0.007; 0.246]	0.064	0.019	[−0.056; 0.095]	0.615
Model 4	**0.136**	**[0.006; 0.267]**	**0.041**	0.007	[−0.072; 0.085]	0.869
Model 5	**0.139**	**[0.008; 0.271]**	**0.038**	−0.004	[−0.083; 0.075]	0.916

cfPWV, carotid-femoral pulse wave velocity; CI, confidence interval.
[a]Persons with T2D; $n = 835$, persons without T2D; $n = 2184$.
[b]Factor D is standardized.
Bold typefont represents statistically significant data.
Model 1: Crude association.
Model 2: adjusted for age, sex, education status.
Model 3: additionally adjusted for mean arterial pressure, mean heart rate.
Model 4: additionally adjusted for waist circumference.
Model 5: additionally adjusted for smoking habits, lipid-modifying and/or antihypertensive medication, Total/high-density lipoprotein cholesterol ratio, triglycerides and HbA1c.

pronounced for factor D. Those with higher factor D were more often men, while this was less so for C3. Both were strongly and positively associated with measures of obesity (BMI and waist). The higher prevalence of prediabetes and T2D in those with higher factors D and C3 was more pronounced for C3. The prevalence of CVD was comparable for factors D and C3, and was higher in the higher quartiles. Higher C3 was more prominently than higher factor D characterized by higher fasting triglycerides and glucose, which was also reflected in the use of medication. With respect to the primary and secondary outcomes of this study, those with higher factors D and C3 had higher cfPWV, lower carotid DC and higher carotid YEM, all consistent with worse arterial stiffness.

Associations of factors D and C3 with carotid-femoral pulse wave velocity

The associations of factors D and C3 with cfPWV are presented in Table 2. Factor D was positively associated with cfPWV (per SD higher factor D, cfPWV was 0.413 m/s greater, 95% confidence interval (CI) [0.337; 0.490], model 1). After adjustment for age, sex, education status, MAP and HR, the association was attenuated to 0.083 m/s ([0.017; 0.150], model 3) and after additional adjustment for T2D it was no longer significant (0.062 m/s, [−0.003; 0.127], model 4). C3 was also positively associated with cfPWV (0.331 m/s, [0.254; 0.407], model 1). After adjustment for age, sex, education status, MAP and HR, the association was attenuated to 0.113 [0.048; 0.177] (model 3). After additional adjustment for T2D, the association was no longer significant (0.028 m/s, [−0.037; 0.094], model 4). Note that the associations in model 4 may to some extent be overadjusted because of reasons indicated in the methods.

Next, we tested whether the associations of factors D and C3 with cfPWV differed between participants with or without T2D or between men and women. T2D, but not sex, modified the association of factor D, but not C3, with cfPWV. In the fully adjusted models, $P_{\text{interaction}}$ with T2D was 0.04 and 0.83 and $P_{\text{interaction}}$ with sex was 0.22 and 0.87, respectively, for factors D and C3. Subsequent stratified analyses on T2D (Table 3) showed that the crude associations between factors D and cfPWV did not differ between participants with (0.310 m/s [0.168; 0.452]) and without T2D (0.322 m/s [0.263; 0.408]). This association was fully

attenuated in the nondiabetic participants (−0.004 m/s [−0.083; 0.075]), but remained positive and significant in those with T2D (0.139 m/s [0.008; 0.271]).

In subsequent sensitivity analyses, exchanging income level and occupation status for education status, 24-h MAP for MAP at the time of ultrasound measurement, BMI for waist circumference, or additionally adjusting for physical activity and DHD15, with subsequently substituting energy intake and alcohol consumption for DHD15 did not materially alter the results (data not shown).

Associations of factors D and C3 with carotid distensibility coefficient and carotid Young's elastic modulus

Factor D was inversely associated with carotid DC (per SD higher factor D, the β for carotid DC was $-0.832{\cdot}10^{-3}$/kPa [−1.027; −0.638], Table 4, model 1), indicating that a higher concentration of factor D was associated with stiffer arteries. After adjustment for age, sex and education status, this association was no longer significant -0.090×10^{-3}/kPa, [−0.277; 0.096], model 2). C3 was also inversely associated with carotid DC (-0.547×10^{-3}/kPa [−0.742; −0.353], Table 4, model 1). After adjustment for age, sex, education status, MAP, HR, and T2D, the association attenuated to -0.176×10^{-3}/kPa [−0.344; −0.008], model 4) and in the fully adjusted model it was no longer significant (-0.023×10^{-3}/kPa [−0.207; 0.161], model 6).

Factor D was positively associated with carotid YEM (per SD higher factor D, carotid YEM was higher 0.049×10^3 kPa [0.035 0.063], Table 4, model 1), but after adjustment for age this association was not significant (0.011×10^3 kPa [−0.003 0.026], model 2). C3 was positively associated with carotid YEM (per SD higher C3, carotid YEM was higher 0.034×10^3 kPa [0.020; 0.049], model 1), which was attenuated after adjustment for age, sex, education status, MAP (0.017×10^3 kPa, 95% CI [0.004; 0.030], model 3) and no longer significant after additional adjustment for T2D (0.007×10^3 kPa [−0.006; 0.021], model 4). Neither T2D nor sex modified the association of factor D or C3 with carotid DC or carotid YEM ($P_{\text{interaction}}$ ranging from 0.29 to 1.0).

In subsequent sensitivity analyses, exchanging income level and occupation status for education status respectively, 24-h MAP for MAP at the time of ultrasound measurement, BMI for waist circumference, or additionally adjusting for physical activity and DHD15, with subsequently substituting energy intake and alcohol consumption for DHD15 did not materially alter the results (data not shown).

DISCUSSION

In a large population-based cohort, we examined cross-sectional associations of the alternative complement factors D and C3 with arterial stiffness. The main finding of this study was that higher concentrations of factors D and C3 were associated with greater aortic and carotid stiffness, as represented by cfPWV, carotid DC and carotid YEM, but not independently of age, sex, education status, HR, MAP, and presence of T2D.

The positive associations of factors D and C3 with adiposity were as expected, given their strong expression in adipose tissue, and consistent with previous reports [6,17]. The higher concentration of factor D in older individuals was shown before [9], although in a small group of healthy Caucasians an inverse association was reported [35]. Notably, when we restricted our analysis to individuals without T2D and CVD, the associations remained positive and significant (data not shown). The slightly higher concentration of C3 with increasing age is not consistently confirmed by existing literature [36] and may be related to higher prevalence of T2D in the older individuals. The higher concentration of factor D in men is consistent with our previous observation in an independent cohort [9] but not always confirmed [35]. Again, when we restricted our analyses to the participants without T2D and CVD, this sex-difference remained (data not shown). Factors D and C3 were both associated with higher MAP. This is corroborated by our own observations on systolic and diastolic blood pressure in an independent cohort [9,37], as well as by reports that C3 is associated with development of hypertension [20] and that a polymorphism in *CFD* is associated with pulmonary hypertension in patients with systemic

TABLE 4. Associations of factor D and factor C3 with carotid stiffness measurements

	Carotid DC (10^{-3}/kPa)[a]						Carotid YEM (10^3 kPa)[a]					
	Factor D[b]			C3[b]			Factor D[b]			C3[b]		
	β	[95% CI]	*P*-value	β	[95% CI]	*P*-value	β	[95% CI]	*P*-value	β	[95% CI]	*P*-value
Model 1	**−0.832**	**−1.027; −0.638**	**<0.001**	**−0.547**	**[−0.742; −0.353]**	**<0.001**	**0.049**	**0.035; 0.063**	**<0.001**	**0.034**	**[0.020; 0.049]**	**<0.001**
Model 2	−0.090	−0.277; 0.096	0.342	**−0.432**	**[−0.607; −0.256]**	**<0.001**	**0.011**	−0.003;0.026	0.123	**0.028**	**[0.014; 0.041]**	**<0.001**
Model 3	−0.118	−0.288; 0.052	0.173	**−0.268**	**[−0.429; −0.107]**	**0.001**	**0.013**	0.000;0.027	0.058	**0.017**	**[0.004; 0.030]**	**0.010**
Model 4	−0.093	−0.263; 0.076	0.281	**−0.176**	**[−0.344; −0.008]**	**0.040**	**0.011**	−0.003;0.025	0.120	0.007	[−0.006; 0.021]	0.279
Model 5	−0.030	−0.206; 0.145	0.736	−0.097	[−0.278; 0.084]	0.293	0.005	−0.009;0.020	0.452	−0.001	[−0.015; 0.014]	0.909
Model 6	−0.004	−0.180; 0.173	0.968	−0.023	[−0.207; 0.161]	0.804	0.003	−0.011;0.018	0.657	−0.002	[−0.017; 0.013]	0.776

Carotid DC, carotid distensibility coefficient; CI, confidence interval; YEM, Young's elastic modulus.
[a]Carotid DC, $n = 2659$, carotid YEM, $n = 2657$.
[b]Factors D and C3 are standardized.
Bold typefont represents statistically significant data.
Model 1: Crude association.
Model 2: adjusted for age, sex, education status.
Model 3: additionally adjusted for mean arterial pressure.
Model 4: additionally adjusted for yes/no T2D.
Model 5: additionally adjusted for waist circumference.
Model 6: additionally adjusted for smoking habits, lipid-modifying and/or antihypertensive medication, Total/high-density lipoprotein cholesterol ratio, triglycerides and HbA1c.

sclerosis [38]. The association of C3, but not factor D, with HR has, to the best of our knowledge, not been reported before.

Arterial stiffness is, among others, determined by properties of the extracellular matrix such as collagen and elastin, and by vascular endothelial and smooth muscle cell function [3,4,39]. Factor D may contribute to arterial stiffness via cleavage of elastin fibers [8], while C3 may bind to collagen and elastin fibers within adventitia [22]. Factors D and C3 are implicated in worse endothelial dysfunction and low-grade inflammation via alternative pathway activation [9]. Complement activation results in generation of anaphylatoxins which are potent soluble mediators of inflammation [40], and induces membrane disruption in target cells [41], hence activating endothelial and smooth muscle cells [40,41]. Low-grade inflammation results in functional stiffening of large arteries through impairment of endothelial function [42,43], proliferation of smooth muscle cells [3], and increased synthesis of extracellular matrix proteins [3].

Approximately 80% of the crude association between factors D and cfPWV was explained by age, sex and education status and this was largely attributable to age. Little to no additional effect of MAP and HR was observed. Age-induced arterial wall remodeling contributes to arterial stiffness [44]. This is generally attributed to 'wear and tear' but the proposed function of factor D as a locally produced elastase [18] combined with strong relationship between age and factor D (Pearson's correlation 0.328, $P < 0.001$) is striking. In contrast, age, sex and education status explained only 25% of the association between C3 and cfPWV, while strong additional attenuation was attributable to MAP and HR. A role for complement activation in hypertension-related vascular dysfunction has been proposed [45], and complement C3 has been implicated in development of hypertension [20]. Our current observation corroborates a potential contribution of C3 to cfPWV via the induction of hypertension.

Upon adjustment for T2D, the associations of factors D and C3 with cfPWV were further attenuated and nonsignificant. A previous study reported a positive association between C3 and cfPWV [21], but did not adjust for presence of diabetes at the time of cfPWW measurements, and the findings reported are hence in line with our current results. Factor D [30,31] and C3 [46] have both been implicated in β-cell preservation. At the same time, C3 has been implicated in obesity-associated insulin resistance, and we [47] and others [33] identified C3 as an independent risk factor for T2D. As such, adjustment for T2D may to some extent represent overadjustment, as the relationships between these complement factors and arterial stiffness may partly result from their effects on glucose metabolism and insulin resistance.

Upon full adjustment neither factor D nor C3 remained statistically significantly associated with greater carotid stiffness, as represented by carotid DC and carotid YEM. This does not corroborate the idea that carotid stiffness may act as mediator of the previously-reported association between factor D and stroke [12]. The association of factor D with cfPWV differed somewhat according to T2D status, as indicated by a significant interaction term, and in the fully adjusted models in stratified analyses the association was only significant in those with T2D, not in those without T2D. Interaction with T2D was only observed for aortic (cfPWV), not for carotid (YEM or DC) stiffness, and only for factor D, not for C3. This result should therefore be interpreted with caution, as it may represent a chance finding.

Strengths of our study include the large population-based cohort and the extensive phenotyping, which allowed thorough adjustment for potential confounders. Moreover, this is, for as far as we are aware, the first large human study in which both factor D and arterial stiffness are available. This study also has limitations. First, factors D and C3 have various sites of production including adipose tissue, perivascular fat, immune cells, β-cells, and for C3 also liver. Plasma measurements represent the integral of different cellular sources. Moreover, factors D and C3 exert multiple biological functions. Some of their effects place some of the potential confounders we included in our analyses, at least partly, in the causal path towards arterial stiffness. Indeed, as noted above, factors D and C3 have implicated in in β-cell preservation, while C3 has been particularly implicated in the development of hypertension, and development of insulin resistance and T2D. This may have introduced overadjustment. Also, factors D and C3 were measured systemically, while their production and effects in the vessel wall may be partly local. Moreover, our cross-sectional design hampers causal inference. Lastly, our study only focused on Caucasian individuals aged 40–75 years, which prohibits extension of the findings to other ethnicities and ages.

In conclusion, in this population-based study the association of factor D with arterial stiffness is for a large part explained by age, while the association of C3 with arterial stiffness is primarily explained by HR and MAP. A small part of the observed associations might be attributed to a causal path leading from alternative complement activation via hypertension and T2D to arterial stiffness.

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Availability of data and materials: The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflicts of interest

There are no conflicts of interest.

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