SUPPLEMENTAL MATERIAL

Interleukin-6 predicts carotid plaque severity, vulnerability, and progression

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1. SUPPLEMENTAL METHODS

a) Study design and participants

The 5888 participants were randomly sampled from Medicare eligibility lists in four communities: Forsyth (North Carolina), Sacramento (California), Washington (Maryland), and Pittsburgh (Pennsylvania).

b) Clinical and laboratory assessment

Information on prescription medication was collected directly from prescription bottles, and use of nonprescription drugs was ascertained by questionnaire. Collection of blood samples at baseline was performed via venipuncture after a 12-hour fast. Multiple aliquots of plasma and serum were prepared and frozen at -70 ˚C at Field Centers, then shipped weekly on dry ice to the Central Blood Analysis Laboratory. Fasting serum chemistry analyses were performed on the Kodak Ektachem 700 Analyzer (Eastman Kodak Corp., Rochester, NY, USA) and included creatinine, uric acid, C-reactive protein (CRP), and glucose. The plasma lipid profile was obtained on an Olympus Demand system (Olympus Corp., Lake Success, NY, USA) and included total cholesterol, triglycerides, high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) derived using Friedewald equation. Plasma IL-6 levels were measured by enzyme-linked immunosorbent assay (ELISA, High Sensitivity Quantikine kit, R&D Systems, Minneapolis, MN, USA).

Hypertension was defined as blood pressure > 140/90 mmHg or medical history of hypertension or ongoing antihypertensive treatment. Diabetes mellitus was defined as fasting blood glucose > 7 mmol/L or history of diabetes mellitus or ongoing treatment with insulin or oral antidiabetic drugs. Dyslipidemia was defined by at least one of the followings: LDL-C > 100 mg/dL (2.6 mmol/L), HDL-C < 50 mg/dL (1.26 mmol/L), triglycerides > 150 mg/dL (1.7 mmol/L), total cholesterol > 200 mg/dL (5.2 mmol/L), treatment with lipid-lowering drugs. Hyperuricemia was defined as uric acid > 7 mg/dL or ongoing treatment with uric acid-lowering drugs (uricosurics or xanthine oxidase inhibitors).

c) Carotid ultrasound assessment

Sonographers performing the 5-year carotid ultrasound examination were blinded to baseline images. Two-dimensional gray scale imaging was used to detect focal plaques. Pulsed wave, continuous wave, and color Doppler images were also obtained. All images were stored on optical disc and transferred to the CHS Ultrasound Reading Center for centralized reading and interpretation (Ultrasound Reading center, New England Medical Center, Boston MA). Two readings were obtained for the 5-year carotid ultrasound images: the first was blinded to baseline images and the second was not. The latter was used in this study since it reflects real-world practice. Periodic duplicate studies were carried out to assess the intra- and inter-observer agreement between Field Center and Reading Center technicians.

Plaque severity or grade of stenosis was scored 0 to 5 for each of the right and left internal carotid arteries with 0 corresponding to a normal carotid, 1 to 1-24% stenosis, 2 to 25-49% stenosis, 3 to 50-74% stenosis, 4 to 75-99% stenosis, and 5 to an occluded carotid artery. Plaque irregularity was scored 0 for smooth plaque, 1 for mildly irregular (height variations < 0.4 mm), 2 for markedly irregular (height variations of 0.4 - 2.0 mm), and 3 for ulcerated plaques (discrete depression of > 2 mm). Plaque echogenicity was coded 0 for absence of plaque (normal carotid), 1 for hypoechoic or echolucent plaque (echogenicity similar to or lower than that of the vessel lumen), and 2, 3, or 4 for isoechoic, hyperechoic, or calcified plaques.

Mild, moderate, and severe carotid stenosis were defined as 1-49%, 50-74%, and 75-100% stenosis. Plaque vulnerability at baseline was defined as the presence of a markedly irregular plaque, an ulcerated plaque or an echolucent plaque on at least one carotid artery. Plaque progression at 5 years was defined as an increase by one point or more on the plaque severity score for at least one carotid artery.

d) Statistical analyses

- Categorical variables were summarized as frequency and percentage. Continuous variables were summarized as mean (95% CI) or median (IQR) as appropriate. The distribution of continuous variables was assessed by visual inspection of histograms and quantile-quantile plots and by performing skewness and Shapiro-Wilk tests.
- Comparisons between included and excluded participants (missing IL-6 or ultrasound data) were performed using Student t test or Mann-Whitney U test for continuous variables and chi-squared or Fisher exact tests for categorical variables, as appropriate.
- The relationship of log IL-6 with age and biomarkers of cardiovascular disease such as log creatinine, log CRP, uric acid, LDL-C, and cystatin-based glomerular filtration rate was assessed using Pearson correlation test.
- The following independent variables were considered during the modelling process based on available evidence of association with atherosclerosis: age (years), sex, race, atrial fibrillation, hypertension, diabetes, dyslipidemia, smoking status, alcohol consumption (drinks of beer, wine, or liquor per week), history of stroke or transient ischemic attack (TIA), history of coronary heart disease (myocardial infarction, angina pectoris, coronary angiography or coronary bypass surgery), history of peripheral artery disease (claudication, lower extremity angioplasty, leg bypass surgery), body mass index, baseline cystatin-based glomerular filtration rate, log CRP, hyperuricemia, and treatment with anti-inflammatory (steroids or non-steroidal) or antiplatelet drugs. Lipid levels and treatment with statins or uric acid levels and treatment with uric acid-lowering drugs were not considered because they were already included in the definition of dyslipidemia and hyperuricemia.
- Comparison of the relative contribution of each independent variable to the regression models was based on standardized coefficients and odds ratios. The overall significance of multivariable regression models was assessed by the Fisher test for the percent explained variance (linear regression) and the Chi-squared test for the log likelihood ratio (logistic regression). The model performance was assessed by the percent explained variance (R^2); the model calibration by computing the calibration-in-the-large index (CITL), calculating the proportion of observations correctly classified, and inspecting calibration plots; the model discrimination by computing the area under the receiver operating characteristic curve (AUC). Detection of multicollinearity and influential observations was based on variance inflation factor >10 and Cook distance >1. Statistical assumptions governing multivariable linear and logistic regression modelling were verified for all models reported.
- In the sensitivity analyses, we refitted all regression models after excluding patients with history of cardiovascular disease (coronary heart disease, peripheral artery disease, stroke, or transient ischemic attack) and after replacing dyslipidemia by LDL-C and adjusting for treatment with statins.
- To define a candidate clinical cut-off for plasma IL-6, we computed the mean value of log IL-6 in patients with a >50% predicted probability (greater than chance) of plaque progression using the optimism-adjusted multivariable logistic regression model. Then, we derived the corresponding plasma concentration of IL-6 in pg/mL by applying the exponential function and rounding up to the nearest multiple of 0.5. We dichotomized baseline plasma IL-6 levels using the derived cut-off to identify participants with high IL-6 levels at baseline. We then repeated all logistic regression analyses to verify if a high IL-6 level at baseline was independently associated with plaque severity, vulnerability, and progression. We also checked if the performance, calibration, discrimination, and stability of the logistic regression models would be significantly affected. We performed a Wald test for equality of AUC to compare the diagnostic performance of the multivariable logistic regression model to that of IL-6 as a standalone biomarker predictor of plaque progression.
- The CASCO is an international research consortium bringing together investigators from across the world to accelerate the resolution of current and future challenges regarding the diagnosis, assessment, and management of carotid atherosclerosis for optimal stroke prevention.

e) Data Sharing

This manuscript was prepared using CHS data obtained from the National Heart, Lung, and Blood Institute (NHLBI) following an application through its Biologic Specimen and Data Repository Information Coordinating Center (BioLINCC). The content of the manuscript does not necessarily reflect the opinions or views of the CHS or NHLBI. The terms of the Research Materials Distribution Agreement explicitly prohibit the release or distribution of research material in any form to any third party unless required by NHLBI policies and approved by the ad hoc regulatory authorities.

2. SUPPLEMENTAL RESULTS

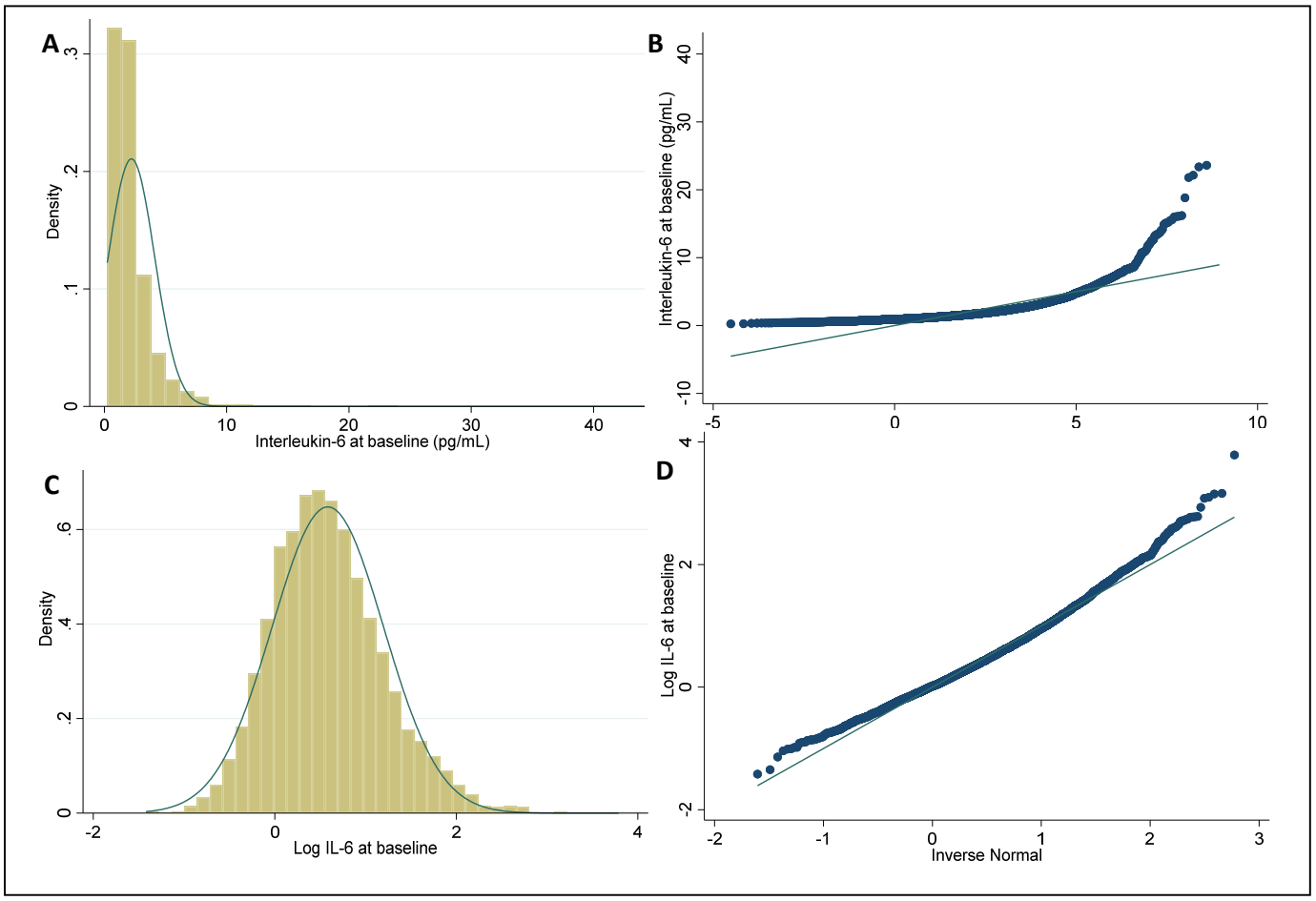
Excluded participants were older, less often women or blacks, and had higher prevalence of diabetes, smoking, coronary or peripheral artery disease. They also had poorer kidney function and higher levels of uric acid and inflammatory markers. The distribution of baseline IL-6, CRP, uric acid, cystatin-based GFR, and LDL-C is shown in.

The prevalence of mild, moderate, and severe stenosis was 72%, 3%, and 0.7%. The baseline carotid ultrasound examination was reported as normal in 24.3%. There were 1267 (29.2%) participants with vulnerable carotid plaque at baseline and 1474 (34.0%) diagnosed with plaque progression at 5 years. Participants with plaque progression at 5 years were less likely to have an ipsilateral vulnerable carotid plaque on the baseline carotid ultrasound examination (16.4 % versus 36.0%, $p < 1.0 \times 10^{-38}$). The characteristics of patients with or without carotid plaque progression are presented in Supplementary Table S2.

Median (IQR) plasma IL-6 level at baseline was 1.4 (1.0-2.2) in participants without carotid plaque and 1.7 (1.2-2.5), 1.6 (1.2-2.8), and 2.3 (1.5-2.7) in participants with mild, moderate, and severe carotid stenosis. Plasma IL6 levels had a moderate positive correlation with log CRP ($r=0.5$, 4.1×10^{-283}) and a moderate negative correlation with cystatin derived GFR ($r = -0.3$, 8.2×10^{-76}) (Supplementary Table S3 and Supplementary Figure S5).

3. SUPPLEMENTAL FIGURES AND FIGURE LEGENDS

Figure S1: Distribution of IL-6 before and after logarithmic transformation



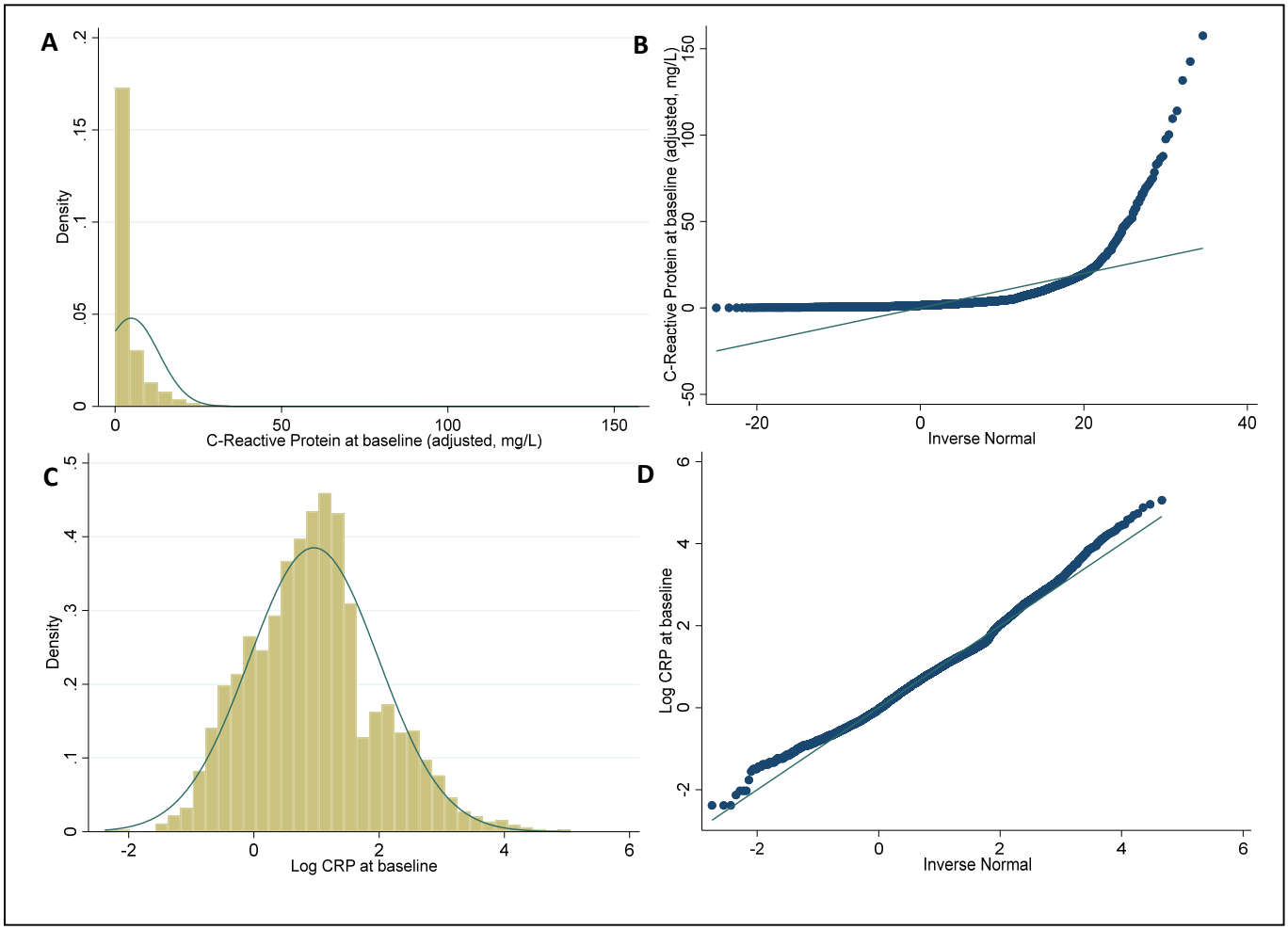
A: Histogram of the distribution of IL-6 levels with overlayed normal density curve

B: Quantiles of IL-6 levels plotted against quantiles of the normal distribution

C: Histogram of the distribution of log IL-6 levels with overlayed normal density curve

D: Quantiles of log IL-6 levels plotted against quantiles of the normal distribution

Figure S2: Distribution of CRP before and after logarithmic transformation



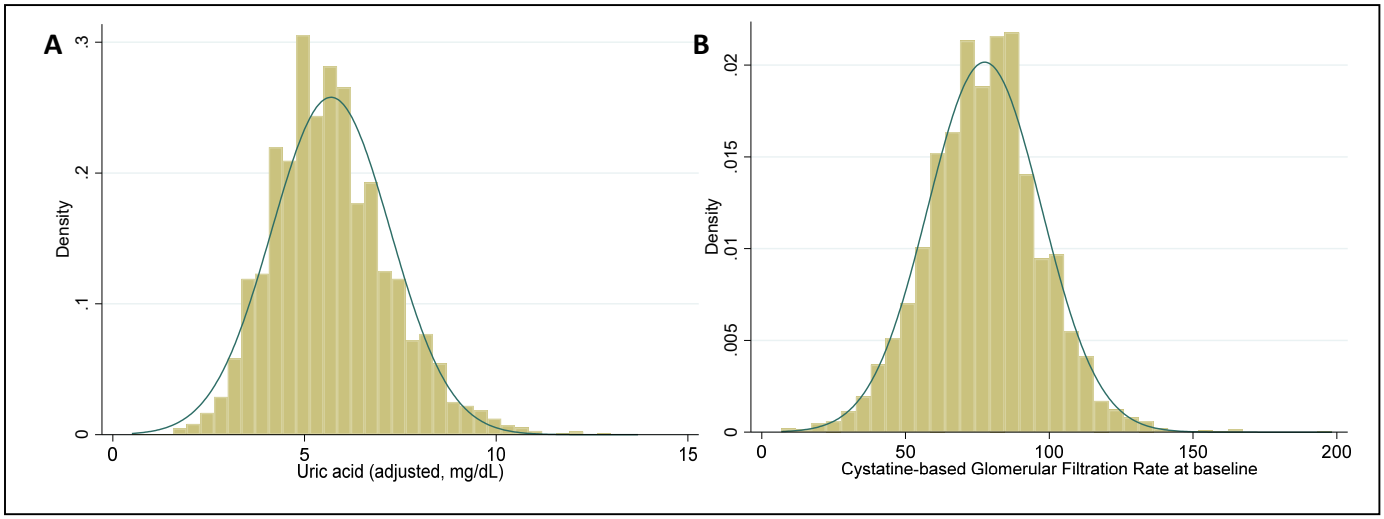
A: Histogram of the distribution of adjusted CRP levels with overlayed normal density curve (adjusted means corrected for instrument drift).

B: Quantiles of CRP levels plotted against quantiles of the normal distribution

C: Histogram of the distribution of log CRP levels with overlayed normal density curve

D: Quantiles of log CRP levels plotted against quantiles of the normal distribution

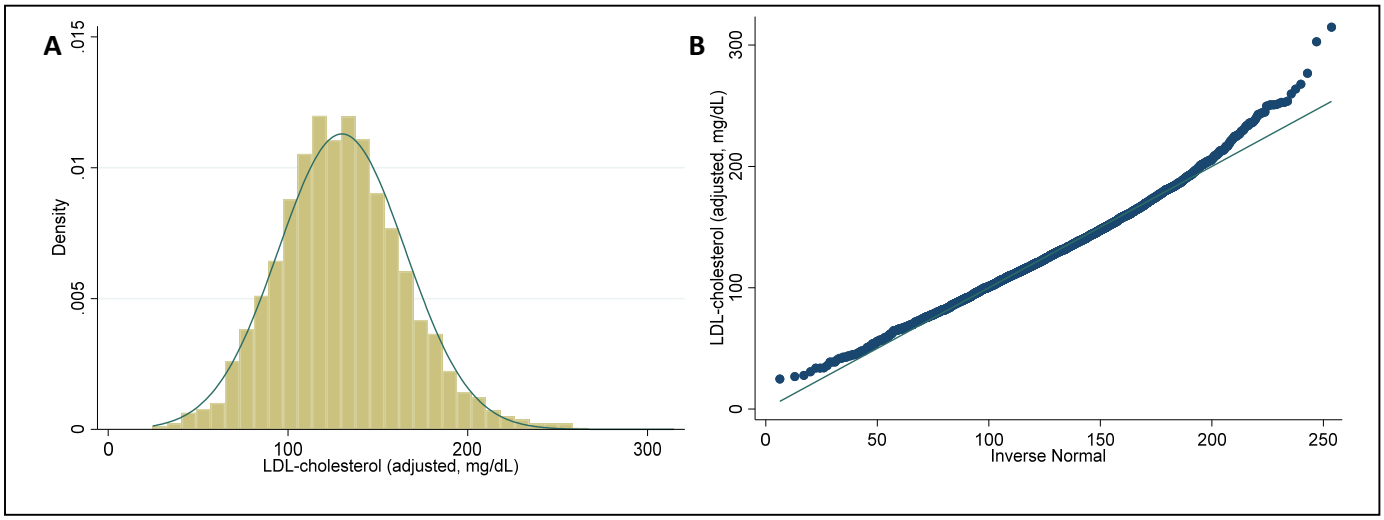
Figure S3: Distribution of uric acid levels and cystatin-based glomerular filtration rate



A: Histogram of the distribution of uric acid levels with overlayed normal density curve (adjusted means corrected for instrument drift).

B: Histogram of the distribution of cystatin-based glomerular filtration rate with overlayed normal density curve.

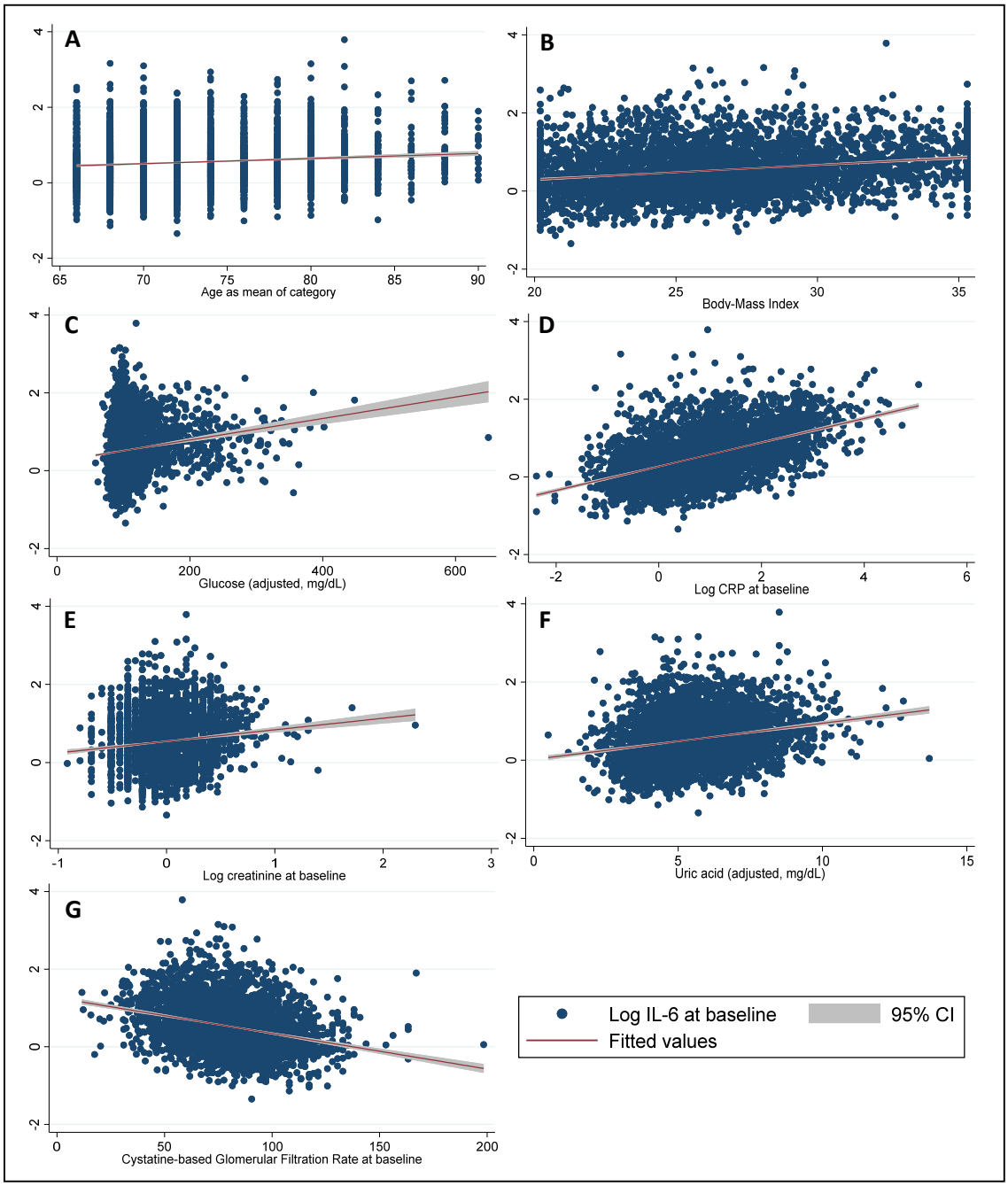
Figure S4: Distribution of LDL-cholesterol levels



A: Histogram of the distribution of LDL-cholesterol levels with overlayed normal density curve (adjusted means corrected for instrument drift).

B: Quantiles of levels plotted against quantiles of the normal distribution.

Figure S5: Scatter plots showing the relationship between log IL-6 and various quantitative parameters



Note: The related Pearson correlation coefficients are reported in Table S2.

Figure S6: Violin plots showing the relationship of IL-6 with cardiovascular risk factors

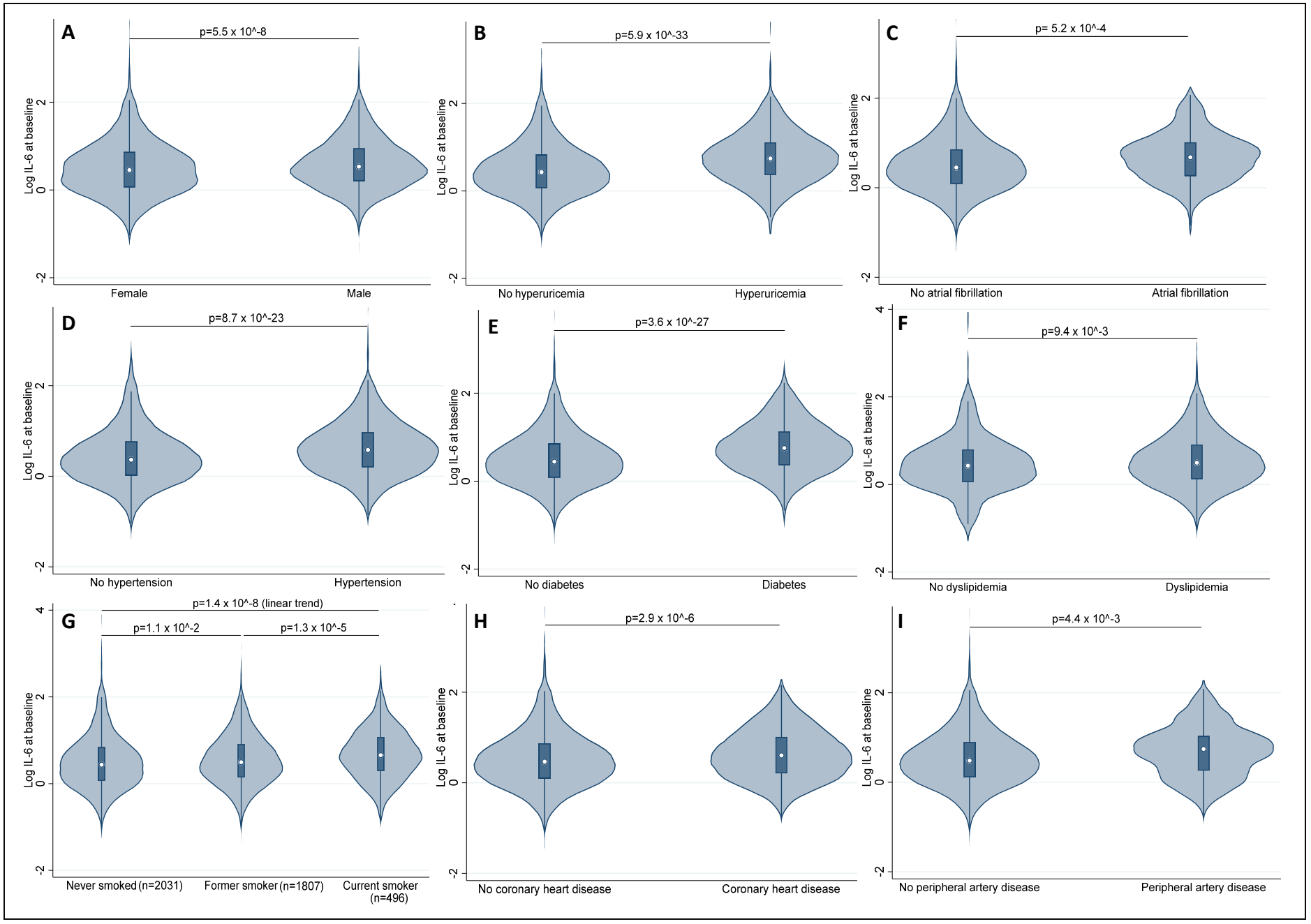
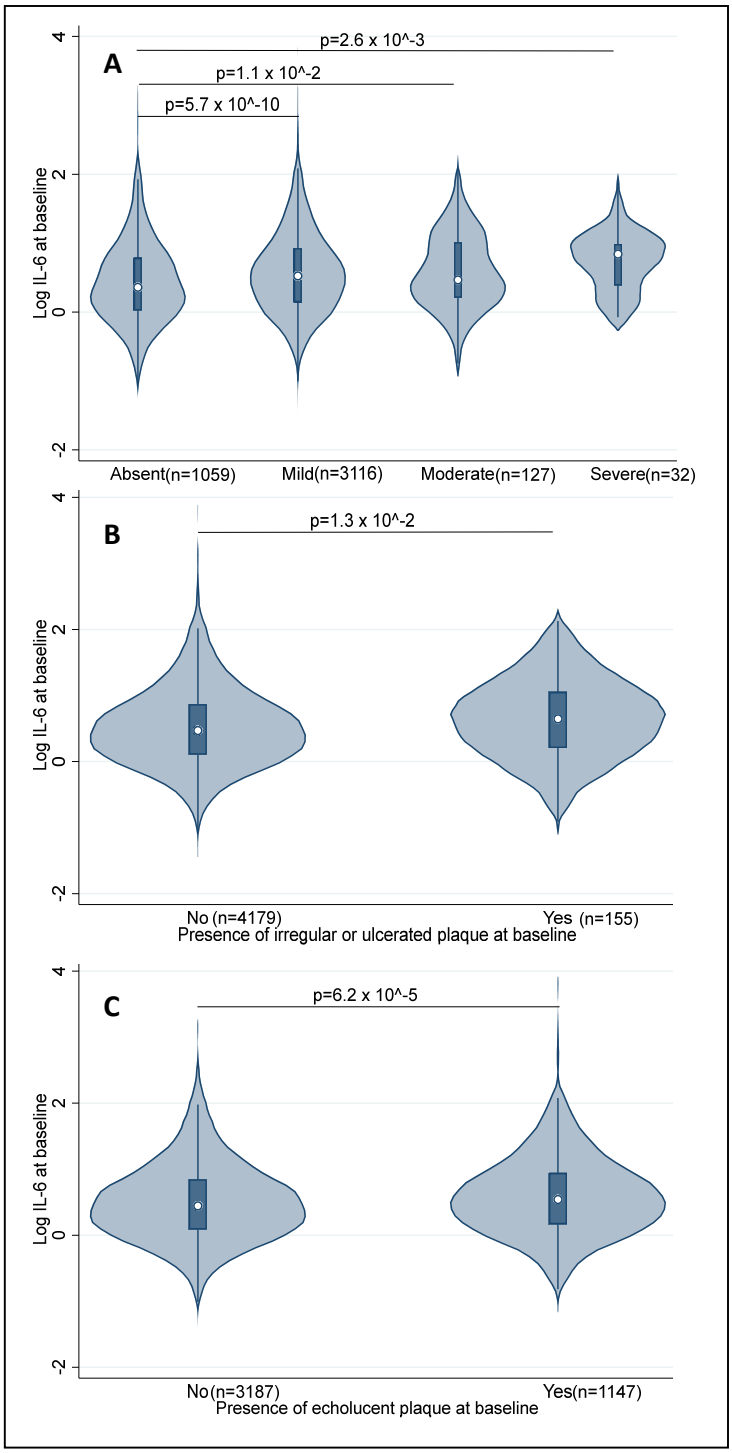

A
p=5.5 x 10^-8
Log IL-6 at baseline
-2
0
2
Female
Male
B
p=5.9 x 10^-33
Log IL-6 at baseline
-2
0
2
No hyperuricemia
Hyperuricemia
C
p= 5.2 x 10^-4
Log IL-6 at baseline
-2
0
2
No atrial fibrillation
Atrial fibrillation
D
p=8.7 x 10^-23
Log IL-6 at baseline
-2
0
2
No hypertension
Hypertension
E
p=3.6 x 10^-27
Log IL-6 at baseline
-2
0
2
No diabetes
Diabetes
F
p=9.4 x 10^-3
Log IL-6 at baseline
-2
0
2
4
No dyslipidemia
Dyslipidemia
G
p=1.4 x 10^-8 (linear trend)
p=1.1 x 10^-2
p=1.3 x 10^-5
Log IL-6 at baseline
-2
0
2
4
Never smoked (n=2031)
Former smoker (n=1807)
Current smoker (n=496)
H
p=2.9 x 10^-6
Log IL-6 at baseline
-2
0
2
No coronary heart disease
Coronary heart disease
I
p=4.4 x 10^-3
Log IL-6 at baseline
-2
0
2
No peripheral artery disease
Peripheral artery disease

Figure S7: Violin plots showing the relationship of IL-6 with the carotid plaque severity and vulnerability at baseline

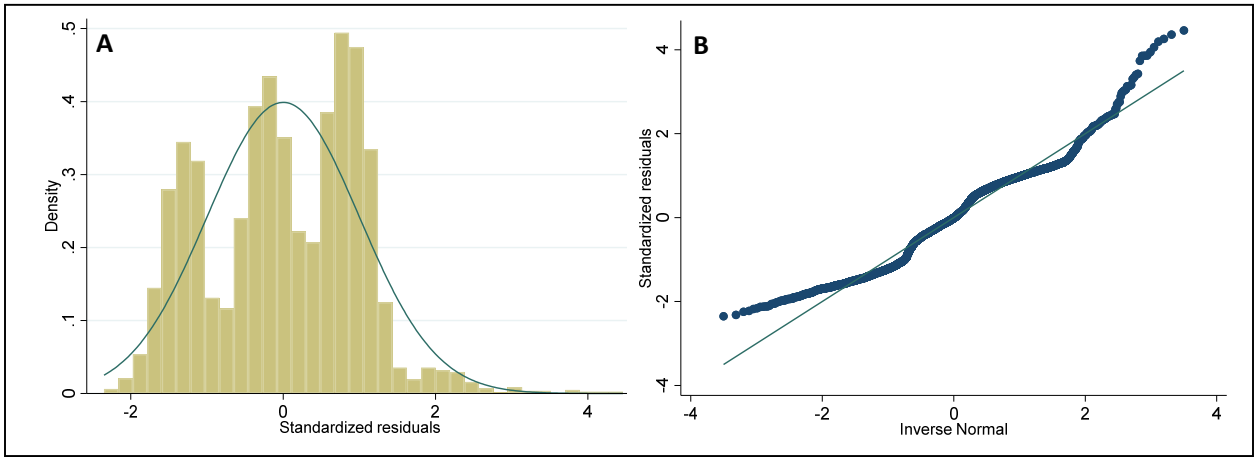


A: Distribution of log IL-6 across categories of stenosis severity. All p-values are derived from unadjusted two-sample Student t tests.

B: Comparison of mean log IL-6 in patients with versus without markedly irregular or ulcerated carotid plaques. The p-value is derived from an unadjusted two-sample Student t test.

C: Comparison of mean log IL-6 in patients with versus without echolucent carotid plaques. The p-value is derived from an unadjusted two-sample Student t test.

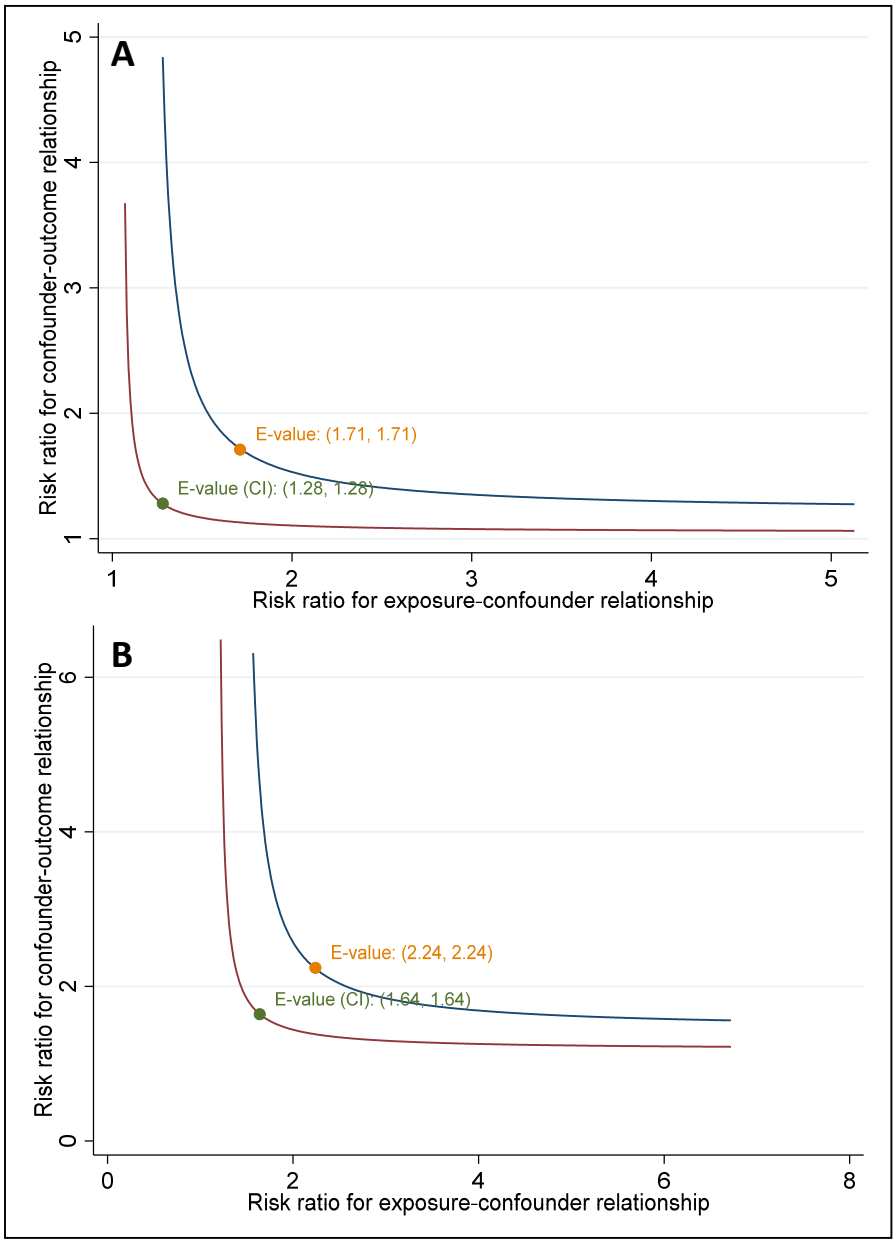
Figure S8: Distribution of standardized residuals for the multivariable regression of carotid stenosis score over log IL-6



A: Histogram of the distribution of standardized residuals with overlayed normal density curve

B: Quantile of standardized residuals plotted against quantiles of the normal distribution

Figure S9: Curves of the sensitivity analysis for unobserved confounders with E-value highlighted



A: curve depicting the range of joint relationships (log IL6-confounder and confounder-plaque vulnerability) that may explain away the estimated effect and its confidence interval for the multivariable logistic regression model to predict plaque vulnerability.
B: curve depicting the range of joint relationships (log IL6-confounder and confounder-plaque vulnerability) that may explain away the estimated effect and its confidence interval for the multivariable logistic regression model to predict plaque progression.

4. SUPPLEMENTAL TABLES AND SUPPORTING INFORMATION

Table S1: Variables extracted from the Cardiovascular Health Study database

Information needed	**Specification**	**Code**
Identification	NA	IDNO
Demographics	Age	AGE2
	Sex	GEND01
	Race	RACE01
Cardiovascular risk factors		
Hypertension	History of hypertension	BPHI, HYPER
	Blood pressure values	SUPSYS16, SUPDIA16,
	Antihypertensive drugs	A2A06, A2AD06, ACE06, ACED06, ALPHA06, ALPHAD06, AMLOD06, NIFIR06, NIFSR06, ANYACE06, ANYDIUR06, ANYVASO06, ANYBETA06, BETA06, BETAD06, CCB06, CCBIR06, CCBSR06, CCBT06, DIHIR06, DIHSR06, DIURET06, DLTIR06, DLTSR06, VERIR06, VERSR06, HCTZ06, HCTZK06, KSPR06, LOOP06, VASO06, VASOD06, HTNMED06,
Body mass index	NA	BMI
Diabetes mellitus	History of diabetes	DIABADA
	Fasting blood glucose	GLU44
	Antidiabetic drugs	INSUL12, AGDI06, BGND06, DPP4I06, OHGA06, SLF106, SLF206, THZD06, INSLN06, INS44
Dyslipidemia	Lipid profile	CHOLADJ, TRIG44, HDL44, LDLADJ
	Lipid lowering drugs	LIPID06, MLPD06, NIAC06, STTN06, FIBR06,
Smoking	NA	PRESSM, EVERSM, SMKAMT, SMOKE
Alcohol consumption	NA	ALCOH
Atrial fibrillation	NA	AFIB
Coronary heart disease	NA	CHD, BPSSUR, CORART, CHDBLMOD, ANBLMOD, MIBLMOD,
Peripheral artery disease	NA	ABTLEG, EXTART, CLBLMOD
History of stroke or TIA	NA	STRKBASE, TIABASE, STBLMOD, TIBLMOD,
Kidney function	NA	MDRD44CLB, CYSGFRBL
Other medications		
Antithrombotic drugs	NA	ADPI06, ASA06, HPRNS06, WARF06,
Anti-inflammatory drugs	NA	NSAID06, OSTRD06,
Uricosurics	NA	URICOS06
Xanthine oxidase inhibitors	NA	XOI06
Biomarkers		
Interleukin-6	NA	IL6BL
C-Reactive Protein	NA	CRPBLADJ
Uric acid	NA	URIC44

Creatinine	NA	CRE44
Carotid ultrasound data		
At baseline	Percent stenosis	PSTEN155, PSTEN255
	Plaque irregularity	LSRFC155, LSRFC255
	Plaque echogenicity	LDENS155, LDENS255
At 5 years	Percent stenosis	PSTEN141, PSTEN241
	Plaque irregularity	LSRFC141, LSRFC241
	Plaque echogenicity	LDENS141, LDENS241

NA means not applicable.

Table S2: Baseline clinical characteristics of the participants

Characteristics	Participants included (n = 4334)	Participants excluded (n = 1554)	p
Age (years, mean ± SD)	72.7 ± 5.1	75.2 ± 6.4	3.2×10^{-53}
Women	2553 (58.9)	776 (49.9)	1.1×10^{-4}
Blacks	744 (17.2)	157 (10.1)	3.2×10^{-11}
Body mass index (kg/m^2, mean ± SD)	26.6 ± 4.1	26.4 ± 4.2	0.04
Atrial fibrillation	160 (3.7)	67 (4.3)	0.28
Hypertension	2543 (58.7)	952 (61.3)	0.08
Diabetes mellitus	646 (14.9)	302 (19.4)	3.1×10^{-5}
Dyslipidemia	3967 (91.5)	1280 (82.4)	2.5×10^{-23}
Current smoker	496 (11.4)	202 (13.0)	0.10
Alcohol consumption (drinks per week, median with IQR)	0.02 (0-1.3)	0 (0-1)	3.3×10^{-5}
Hyperuricemia	860 (19.8)	373 (24.0)	5.5×10^{-4}
Coronary heart disease	761 (17.6)	376 (24.2)	1.3×10^{-8}
Peripheral artery disease	127 (2.9)	86 (5.5)	2.4×10^{-6}
Prior stroke or TIA	227 (5.2)	120 (7.7)	3.6×10^{-4}
Treatment with statins	102 (2.4)	25 (1.6)	0.08
Treatment with antiplatelet drugs	142 (3.3)	52 (3.3)	0.90
Treatment with uric acid-lowering drugs*	118 (2.7)	51 (3.3)	0.26
Treatment with anti-inflammatory drugs†	571 (13.2)	260 (16.8)	1.4×10^{-5}
Cystatin-based GFR (ml/min, mean ± SD)	79.5 ± 19.1	71.0 ± 20.6	4.1×10^{-39}
Interleukin-6 (pg/mL, median with IQR)	1.6 (1.1 – 2.5)	2.1 (1.4 – 3.3)	1.0×10^{-27}
C-reactive protein (mg/L, median with IQR)	2.4 (1.2 – 4.2)	3.0 (1.5 – 6.8)	2.1×10^{-16}
Uric acid (mg/dL, mean ± SD)	5.6 ± 1.5	5.9 ± 1.6	4.7×10^{-7}

CRP: C-Reactive Protein; GFR: Glomerular Filtration Rate; TIA: Transient Ischemic Attack.

* Uric acid-lowering drugs refer to xanthine oxidase inhibitors and uricosurics.

† Anti-inflammatory drugs refer to steroids and non-steroidal anti-inflammatory drugs.

Note: Comparisons between included and excluded participants (missing IL-6 or ultrasound data) were performed using the Student t test (age, body mass index, cystatin-based GFR, uric acid) or the Mann-Whitney U test (alcohol consumption, CRP, interleukin-6) for continuous variables and the chi-squared test for categorical variables. The p-values are not adjusted.

Table S3: Baseline clinical characteristics of participants with carotid plaque progression at 5 years

Characteristics	Carotid plaque progression at 5 years		p
	No (n = 2860)	Yes (n = 1474)	
Age (years, mean ± SD)	72.8 ± 5.2	72.5 ± 5.0	0.18
Women	1689 (59.1)	864 (58.6)	0.80
Blacks	670 (23.4)	74 (5.0)	2.5×10^{-52}
Body mass index (kg/m^2, mean ± SD)	26.7 ± 4.2	26.5 ± 3.8	0.03
Atrial fibrillation	112 (3.9)	48 (3.3)	0.28
Hypertension	1695 (59.3)	848 (57.5)	0.27
Diabetes mellitus	427 (14.9)	219 (14.9)	0.94
Dyslipidemia	2594 (90.7)	1373 (93.1)	0.01
Current smoker	336 (11.7)	160 (10.9)	0.38
Alcohol consumption (drinks per week, median with IQR)	0.02 (0 – 1.3)	0.02 (0 – 1.5)	0.10
Hyperuricemia	570 (19.9)	290 (19.7)	0.84
Coronary heart disease	500 (17.5)	261 (17.7)	0.85
Peripheral artery disease	88 (3.1)	39 (2.7)	0.43
Prior stroke or TIA	163 (5.7)	64 (4.3)	0.06
Treatment with statins	77 (2.7)	25 (1.7)	0.04
Treatment with antiplatelet drugs	108 (3.8)	34 (2.3)	0.01
Treatment with uric acid-lowering drugs*	82 (2.9)	36 (2.4)	0.42
Treatment with anti-inflammatory drugs†	376 (13.1)	195 (13.2)	0.93
Cystatin-based GFR (ml/min, mean ± SD)	79.1 ± 19.4	80.3 ± 18.6	0.07
Interleukin-6 (pg/mL, median with IQR)	1.6 (1.1 - 2.4)	1.6 (1.2 – 2.5)	0.12
C-reactive protein (mg/L, median with IQR)	2.4 (1.2 – 4.3)	2.4 (1.3 – 4.1)	0.79
Uric acid (mg/dL, mean ± SD)	5.7 ± 1.5	5.6 ± 1.5	0.19

CRP: C-Reactive Protein; GFR: Glomerular Filtration Rate; TIA: Transient Ischemic Attack.

* Uric acid-lowering drugs refer to xanthine oxidase inhibitors and uricosurics.

† Anti-inflammatory drugs refer to steroids and non-steroidal anti-inflammatory drugs.

Note: Comparisons between participants with versus without plaque progression were performed using the Student t test (age, body mass index, cystatin-based GFR, uric acid) or the Mann-Whitney U test (alcohol consumption, CRP, interleukin-6) for continuous variables and the chi-squared test for categorical variables. The p-values are not adjusted.

Table S4: Correlation of log IL-6 with various quantitative parameters

Variable	Pearson correlation coefficient	P value
Age (years)	0.11	5.06×10^{-14}
Body mass index (kg/m^2)	0.25	6.4×10^{-63}
Fasting blood glucose (mg/dL)	0.16	7.7×10^{-27}
Log CRP	0.51	4.1×10^{-283}
Log creatinine	0.13	1.22×10^{-17}
Uric acid (mg/dL)	0.23	2.4×10^{-53}
Cystatin-based GFR (ml/min)	-0.30	8.2×10^{-76}

CRP = C-Reactive Protein

GFR = Glomerular filtration rate

Note: The related scatter plots are provided in Supplementary Figure S5.

Table S5: Multivariable linear regression model for the association of IL-6 with carotid plaque severity at baseline after excluding patients with history of cardiovascular disease

Independent variables	β1*	95% CI	p-value	β2†
Log IL-6	0.08	0.03 – 0.14	4.5×10^{-3}	0.06
Age	0.03	0.02 – 0.03	2.3×10^{-12}	0.13
Male	0.11	0.04 – 0.19	1.5×10^{-3}	0.09
Black (African American)	-0.19	(-0.28) – (-0.10)	3.7×10^{-5}	-0.08
Hypertension	0.13	0.06 – 0.20	2.5×10^{-4}	0.11
Diabetes mellitus	0.11	0.01 – 0.22	3.6×10^{-2}	0.03
Dyslipidemia	0.21	0.10 – 0.33	5.0×10^{-4}	0.08
Current smoker	0.32	0.22 – 0.43	4.0×10^{-9}	0.12
Hyperuricemia	0.12	0.02 – 0.22	1.3×10^{-2}	0.04
Intercept	-1.08	(-1.61) – (-0.56)	5.2×10^{-5}	NA

IL-6: interleukin-6; NA: not applicable

* Non-standardized coefficients (linked to change in stenosis severity score per 1 unit increase)

† Standardized coefficients (linked to change in stenosis severity score per 1 standard deviation increase)

Body mass index (p=0.84), alcohol consumption, treatment with antiplatelet drugs, cystatin-based glomerular filtration rate, atrial fibrillation, log C-reactive protein, and treatment with anti-inflammatory drugs were consecutively removed from the model automatically due to coefficients with p-value >0.05.

Fisher F test for significance of the model: F = 19.4, df = 9, $p = 9.4 \times 10^{-32}$. Maximum Cook distance = 0.02. Maximum variance inflation factor = 1.14.

The p values for significance of the regression coefficients were determined by a t-test.

Table S6: Multivariable linear regression model for the association of IL-6 with carotid plaque severity at baseline using LDL-C and statin therapy as independent variables

Independent variables	β1*	95% CI	p-value	β2†
Log IL-6	0.09	0.04 – 0.14	8.8×10^{-4}	0.06
Age	0.02	0.02 – 0.03	9.5×10^{-4}	0.13
Male	0.14	0.07 – 0.20	3.6×10^{-5}	0.09
Black (African American)	-0.22	(-0.30) – (-0.14)	1.1×10^{-7}	-0.08
Hypertension	0.16	0.10 – 0.23	1.1×10^{-6}	0.09
Diabetes mellitus	0.13	0.03 – 0.22	6.6×10^{-3}	0.03
LDL-C	0.002	0.002 – 0.003	6.1×10^{-8}	0.10
Current smoker	0.32	0.22 – 0.42	2.7×10^{-10}	0.12
Hyperuricemia	0.11	0.02 – 0.19	1.2×10^{-2}	0.03
Coronary heart disease	0.14	0.05 – 0.23	2.7×10^{-3}	0.05
Peripheral artery disease	0.31	0.11 – 0.51	2.7×10^{-3}	0.06
Prior stroke or TIA	0.17	0.02 – 0.32	2.4×10^{-2}	0.05
Treatment with statins	0.31	0.10 – 0.52	3.6×10^{-3}	0.04
Intercept	-1.15	(-1.63) – (-0.67)	2.8×10^{-6}	-0.98

IL-6: interleukin-6; CRP: C-reactive protein; LDL-C: low-density lipoprotein cholesterol; NA: not applicable; TIA: transient ischemic attack.

* Non-standardized coefficients (linked to change in stenosis severity score per 1 unit increase)

† Standardized coefficients (linked to change in stenosis severity score per 1 standard deviation increase)

Treatment with antiplatelet drugs (p=0.86), atrial fibrillation, cystatin-based glomerular filtration rate, alcohol consumption, body mass index, log C-Reactive Protein, and treatment with anti-inflammatory drugs (p=0.06) were consecutively removed from the model automatically due to coefficients with p-value >0.05.

Fisher F test for significance of the model: F = 23.3, df = 13, $p = 5.1 \times 10^{-54}$. Maximum Cook distance = 0.02. Maximum variance inflation factor = 1.15.

The p values for significance of the regression coefficients were determined by a t-test.

Note: The counterintuitive association between statin treatment and plaque severity could be explained by two hypotheses. First, statins are typically prescribed to patients with abnormal lipid profile and could reasonably be considered as a surrogate of dyslipidemia. Second, the prescription of statins is often motivated by the discovery of a severe carotid stenosis or a vulnerable plaque.

Table S7: Multivariable logistic regression model for the association of IL-6 with carotid plaque vulnerability at baseline after excluding patients with history of cardiovascular disease

Independent variables	OR1 (95% CI) *	p-value	OR2†	OR3 (95% CI) ‡
Log IL-6	1.14 (0.97 – 1.34)	0.11	1.08	1.12 (0.97 – 1.29)
Body mass index	1.03 (1.00 – 1.05)	0.04	1.11	1.02 (1.00 – 1.05)
Dyslipidemia	1.52 (1.06 – 2.18)	0.02	1.13	1.44 (1.05 – 1.97)
Hyperuricemia	1.43 (1.12 – 1.84)	0.01	1.14	1.37 (1.10 – 1.70)
Intercept (baseline odds)	0.14 (0.07 – 0.29)	3.8×10^{-8}	NA	0.22 (0.11 – 0.44)

IL-6: interleukin-6; NA: not applicable.

* Non-standardized odds ratio (linked to change in odds per 1 unit increase)

† Standardized odds ratio (linked to change in odds per 1 standard deviation increase)

‡ Optimism-adjusted odds ratio using the heuristic shrinkage method.

Log C-reactive protein (p=0.93), treatment with antiplatelet drugs, diabetes mellitus, alcohol consumption, race, hypertension, cystatin-based glomerular filtration rate, treatment with anti-inflammatory drugs, atrial fibrillation, age, smoking status, and sex (p=0.11) were consecutively removed from the model automatically due to coefficients with p-value >0.05. Log IL-6 was forced into the model.

Likelihood ratio chi-squared test for significance of the model: $\chi^2 = 30.5$, df = 4, $p = 3.9 \times 10^{-6}$. Area under the Receiver Operating Characteristic curve (AUC) = 0.57. Count R^2 = 67%. Proportion of patients correctly classified = 66%. Maximum Cook distance = 0.03. Maximum variance inflation factor = 1.10.

The p values for significance of the odds ratios were determined by a Wald test.

Note: The exclusion of 979 patients with cardiovascular disease led to a reduction in statistical power which probably explains why the odds ratio for the association between IL-6 and carotid plaque vulnerability is not significant in this analysis.

Table S8: Multivariable logistic regression model for the association of IL-6 with carotid plaque vulnerability at baseline using LDL-C and statin therapy as independent variables

Independent variables	OR1 (95% CI) *	p-value	OR2†	OR3 (95% CI) ‡
Log IL-6	1.22 (1.06 – 1.41)	5.2×10^{-3}	1.12	1.20 (1.06 – 1.36)
Male	1.20 (1.01 – 1.44)	4.0×10^{-2}	1.10	1.18 (1.01 – 1.38)
Treatment with statins	2.22 (1.17 – 4.21)	1.5×10^{-2}	1.10	2.04 (1.15 – 3.64)
Hyperuricemia	1.45 (1.16 – 1.81)	1.1×10^{-3}	1.15	1.39 (1.14 – 1.69)
Intercept (baseline odds)	0.39 (0.34 – 0.46)	6.9×10^{-49}	NA	0.44 (0.39 – 0.53)

IL-6: interleukin-6; NA: not applicable.

* Non-standardized odds ratio (linked to change in odds per 1 unit increase)

† Standardized odds ratio (linked to change in odds per 1 standard deviation increase)

‡ Optimism-adjusted odds ratio using the heuristic shrinkage method.

Coronary heart disease (p=0.93), diabetes mellitus, log C-reactive protein, race, history of stroke or transient ischemic attack, cystatin-based glomerular filtration rate, alcohol consumption, atrial fibrillation, treatment with anti-inflammatory drugs, hypertension, peripheral artery disease, treatment with antiplatelet drugs, age, LDL-C, body mass index, and smoking status (p=0.08) were consecutively removed from the model automatically due to coefficients with p-value >0.05.

Likelihood ratio chi-squared test for significance of the model: $\chi^2 = 37.5$, df = 4, $p = 1.4 \times 10^{-7}$. Area under the Receiver Operating Characteristic curve (AUC) = 0.57. Count R^2 = 67%. Proportion of patients correctly classified = 66%. Maximum Cook distance = 0.05. Maximum variance inflation factor = 1.06.

The p values for significance of the odds ratios were determined by a Wald test.

Note: The counterintuitive association between statin treatment and plaque vulnerability could be explained by two hypotheses. First, statins are typically prescribed to patients with abnormal lipid profile and could reasonably be considered as a surrogate of dyslipidemia. Second, the prescription of statins is often motivated by the discovery of a severe carotid stenosis or a vulnerable plaque.

Table S9: Multivariable logistic regression model for the association of IL-6 with carotid plaque progression at 5 years after excluding patients with history of cardiovascular disease

Independent variables	OR1 (95% CI) *	p-value	OR2†	OR3 (95% CI) ‡
Log IL-6	1.31 (1.11 – 1.56)	1.9×10^{-3}	1.18	1.31 (1.10 – 1.55)
Current smoker	1.57 (1.11 – 2.22)	1.0×10^{-2}	1.15	1.56 (1.11 – 2.20)
Dyslipidemia	2.17 (1.51 – 3.13)	3.7×10^{-5}	1.24	2.14 (1.49 – 3.08)
Hypertension	1.44 (1.17 – 1.77)	6.2×10^{-4}	1.20	1.43 (1.17 – 1.75)
Male	1.34 (1.08 – 1.66)	8.6×10^{-3}	1.15	1.33 (1.08 – 1.65)
Age (years)	1.04 (1.01 – 1.06)	1.8×10^{-3}	1.18	1.04 (1.01 – 1.06)
Vulnerability at baseline (ipsilateral)	0.69 (0.53 – 0.90)	5.4×10^{-3}	0.85	0.54 (0.90 – 1.56)
Stenosis score at baseline (ipsilateral)	0.26 (0.22 – 0.30)	7.8×10^{-69}	0.30	0.26 (0.23 – 0.30)
Intercept (baseline odds)	0.05 (0.01 – 0.26)	4.2×10^{-4}	NA	0.19 (0.04 – 1.01)

IL-6: interleukin-6; NA: not applicable.

* Non-standardized odds ratio (linked to change in odds per 1 unit increase)

† Standardized odds ratio (linked to change in odds per 1 standard deviation increase)

‡ Optimism-adjusted odds ratio using the heuristic shrinkage method.

The first interaction term (Log IL-6 # ipsilateral vulnerability at baseline, p = 0.70), treatment with anti-inflammatory drugs, log C-reactive protein, atrial fibrillation, body mass index, race, the second interaction term (Log IL-6 # ipsilateral baseline stenosis score), alcohol consumption, treatment with antiplatelet drugs, diabetes mellitus, hyperuricemia, and cystatin-based glomerular filtration rate (p=0.05) were consecutively removed from the model automatically due to coefficients with p-value >0.05.

Likelihood ratio chi-squared test for significance of the model: $\chi^2 = 537.9$, df = 8, $p = 5.2 \times 10^{-111}$. Area under the Receiver Operating Characteristic curve (AUC) = 0.79. Count $R^2 = 71\%$. Proportion of patients correctly classified = 72.6%. Maximum Cook distance = 0.07. Maximum variance inflation factor = 1.20.

The p values for significance of the odds ratios were determined by a Wald test.

Table S10: Multivariable logistic regression model for the association of IL-6 with carotid plaque progression at 5 years using LDL-C and statin therapy as independent variables

Independent variables	OR1 (95% CI) *	p-value	OR2†	OR3 (95% CI) ‡
Log IL-6	1.44 (1.23 – 1.69)	8.8×10^{-6}	1.25	1.43 (1.22 – 1.68)
Current smoker	1.61 (1.17 – 2.20)	3.1×10^{-3}	1.16	1.60 (1.17 – 2.18)
LDL-C	1.01 (1.00 – 1.01)	6.5×10^{-6}	1.25	1.01 (1.00 – 1.01)
Diabetes mellitus	1.54 (1.15 – 2.08)	3.9×10^{-3}	1.15	1.54 (1.15 – 2.05)
Hypertension	1.38 (1.14 – 1.68)	1.2×10^{-3}	1.17	1.38 (1.13 – 1.67)
Coronary heart disease	1.34 (1.00 – 1.78)	5.0×10^{-2}	1.10	1.33 (1.00 – 1.77)
Male	1.36 (1.11 – 1.66)	2.6×10^{-3}	1.16	1.35 (1.11 – 1.65)
Age (years)	1.03 (1.01 – 1.05)	3.1×10^{-3}	1.16	1.03 (1.01 – 1.05)
Vulnerability at baseline (ipsilateral)	0.77 (0.61 – 0.97)	2.7×10^{-2}	0.89	0.77 (0.62 – 0.97)
Stenosis score at baseline (ipsilateral)	0.24 (0.21 – 0.28)	4.3×10^{-91}	0.27	0.25 (0.21 – 0.28)
Intercept (baseline odds)	0.06 (0.01 – 0.29)	3.9×10^{-4}	NA	0.05 (0.01 – 0.23)

IL-6: interleukin-6; NA: not applicable.

* Non-standardized odds ratio (linked to change in odds per 1 unit increase)

† Standardized odds ratio (linked to change in odds per 1 standard deviation increase)

‡ Optimism-adjusted odds ratio using the heuristic shrinkage method.

Atrial fibrillation (p = 0.97), the first interaction term (Log IL-6 # ipsilateral vulnerability at baseline), treatment with anti-inflammatory drugs, race, treatment with statins, history of stroke or transient ischemic attack, alcohol consumption, body mass index, treatment with antiplatelet drugs, log C-reactive protein,the second interaction term (Log IL-6 # ipsilateral baseline stenosis score), peripheral artery disease, hyperuricemia, and cystatin-based glomerular filtration rate (p=0.07) were consecutively removed from the model automatically due to coefficients with p-value >0.05.

Likelihood ratio chi-squared test for significance of the model: $\chi^2 = 691.2$, df = 10, $p = 4.8 \times 10^{-142}$. Area under the Receiver Operating Characteristic curve (AUC) = 0.80. Count $R^2 = 72\%$. Proportion of patients correctly classified = 73%. Maximum Cook distance = 0.06. Maximum variance inflation factor = 1.06.

The p values for significance of the odds ratios were determined by a Wald test.

Table S11: Clinical characteristics of patients with low versus high plasma IL-6 levels at baseline

Characteristics	Low IL-6 levels (n = 2750)	High IL-6 levels (n = 1584)	p
Age (years, mean ± SD)	72.4 ± 5.0	73.2 ± 5.3	5.8×10^{-7}
Women	1670 (60.7)	883 (55.7)	1.3×10^{-3}
Blacks	423 (15.4)	321 (20.3)	4.0×10^{-5}
Body mass index (kg/m^2, mean ± SD)	26.0 ± 3.7	27.8 ± 4.4	1.2×10^{-44}
Atrial fibrillation	82 (3.0)	78 (4.9)	2.8×10^{-4}
Hypertension	1478 (53.7)	1065 (67.2)	3.3×10^{-18}
Diabetes mellitus	295 (10.7)	351 (22.2)	2.2×10^{-24}
Dyslipidemia	2490 (90.5)	1470 (92.8)	6.9×10^{-3}
Current smoker	260 (9.5)	236 (14.9)	6.4×10^{-8}
Alcohol consumption (drinks per week, median with IQR)	0.02 (0.00 – 1.5)	0 (0.00-1.04)	2.2×10^{-3}
Hyperuricemia	406 (14.8)	454 (28.7)	2.2×10^{-28}
Coronary heart disease	429 (15.6)	332 (21.0)	7.9×10^{-6}
Peripheral artery disease	58 (2.1)	69 (4.4)	2.4×10^{-5}
Prior stroke or TIA	116 (4.2)	111 (7.0)	7.2×10^{-5}
Treatment with statins	60 (2.2)	42 (2.7)	3.3×10^{-1}
Treatment with antiplatelet drugs	77 (2.8)	65 (4.1)	2.1×10^{-2}
Treatment with uric acid-lowering drugs*	57 (2.1)	61 (3.9)	5.5×10^{-4}
Treatment with anti-inflammatory drugs†	360 (13.1)	211 (13.3)	8.5×10^{-1}
Cystatin-based GFR (ml/min , mean ± SD)	82.9 ± 18.1	73.4 ± 19.3	3.3×10^{-52}
C-reactive protein (mg/L, median with IQR)	1.8 (0.9 – 3.1)	4.0 (2.3 – 8.7)	2.0×10^{-177}
Uric acid (mg/dL, mean ± SD)	5.4 ± 1.4	6.0 ± 1.6	1.8×10^{-37}
Stenosis severity score (mean ± SD)	1.2 ± 0.9	1.3 ± (0.9)	1.5×10^{-9}
Presence of severe stenosis at baseline	11 (0.4)	21 (1.3)	6 x 10-4
Presence of vulnerable plaque at baseline	760 (27.6)	507 (32.0)	2.3 x 10-3
Plaque progression at 5 years	926 (33.7)	548 (34.6)	5.4×10^{-1}

CRP: C-reactive protein; GFR: glomerular filtration rate; TIA: transient ischemic attack.

* Uric acid-lowering drugs refer to xanthine oxidase inhibitors and uricosurics.

† Anti-inflammatory drugs refer to steroids and non-steroidal anti-inflammatory drugs.

Note: Comparisons between participants with high versus low IL-6 levels were performed using the Student t test (age, body mass index, cystatin-based GFR, uric acid, stenosis severity score) or the Mann-Whitney U test (alcohol consumption, CRP, interleukin-6) for continuous variables and the chi-squared test for categorical variables. The p-values are not adjusted.

Table S12: Multivariable logistic regression model for the association of high IL-6 levels with carotid plaque vulnerability

Independent variables	OR1 (95% CI) *	p-value	OR2†	OR3 (95% CI) ‡
High IL-6 level at baseline	1.21 (1.02 – 1.45)	3.6×10^{-2}	1.10	1.19 (1.01 – 1.39)
Male	1.22 (1.03 – 1.46)	2.3×10^{-2}	1.10	1.19 (1.02 – 1.39)
Dyslipidemia	1.56 (1.11 – 2.17)	9.4×10^{-3}	1.13	1.48 (1.10 – 1.98)
Hyperuricemia	1.38 (1.11 – 1.72)	3.6×10^{-3}	1.13	1.33 (1.09 – 1.61)
Intercept (baseline odds)	0.28 (0.20 – 0.38)	7.6×10^{-15}	NA	0.35 (0.25 – 0.48)

IL-6: interleukin-6; NA: not applicable.

* Non-standardized odds ratio (linked to change in the odds per 1 unit increase)

† Standardized odds ratio (linked to change in the odds per 1 standard deviation increase)

‡ Optimism-adjusted odds ratio using the heuristic shrinkage method.

Coronary heart disease (p=0.93), diabetes mellitus, race, history of stroke or transient ischemic attack, cystatin-based glomerular filtration rate, atrial fibrillation, alcohol consumption, treatment with anti-inflammatory drugs, hypertension, peripheral artery disease, log C-reactive protein, age, treatment with antiplatelet drugs, body mass index, and smoking status (p=0.08) were consecutively removed from the model automatically due to coefficients with p-value >0.05.

Likelihood ratio chi-squared test for significance of the model: $\chi^2 = 34.5$, df = 4, $p = 5.8 \times 10^{-7}$. Area under the Receiver Operating Characteristic curve (AUC) = 0.56. Count R^2 = 67%. Proportion of patients correctly classified = 66%. Maximum Cook distance = 2.2. Maximum variance inflation factor = 1.09.

The p values for significance of the odds ratios were determined by a Wald test.

Table S13: Multivariable logistic regression model for the association of high IL-6 levels with carotid plaque progression

Independent variables	OR1 (95% CI) *	p-value	OR2†	OR3 (95% CI) ‡
High IL-6 level at baseline	1.25 (1.01 – 1.57)	4.7×10^{-2}	1.11	1.25 (1.01 – 1.55)
Current smoker	1.66 (1.21 – 2.27)	1.6×10^{-3}	1.17	1.64 (1.21 – 2.24)
Dyslipidemia	2.29 (1.62 – 3.25)	3.2×10^{-6}	1.15	2.26 (1.60 – 3.19)
Diabetes mellitus	1.48 (1.11 – 1.97)	8.2×10^{-3}	1.14	1.47 (1.10 – 1.95)
Hypertension	1.38 (1.13 – 1.67)	1.3×10^{-3}	1.17	1.37 (1.13 – 1.66)
Coronary heart disease	1.35 (1.02 – 1.80)	3.7×10^{-2}	1.11	1.34 (1.02 – 1.78)
Male	1.34 (1.10 – 1.63)	3.8×10^{-3}	1.15	1.33 (1.10 – 1.62)
Age (years)	1.03 (1.01 – 1.05)	2.2×10^{-3}	1.16	1.03 (1.01 – 1.05)
Vulnerability at baseline	0.77 (0.61 – 0.97)	2.6×10^{-2}	0.89	0.77 (0.62 – 0.97)
Stenosis score at baseline	0.24 (0.21 – 0.28)	1.7×10^{-92}	0.27	0.25 (0.22 – 0.28)
Intercept (baseline odds)	0.06 (0.01 – 0.28)	3.0×10^{-4}	NA	0.07 (0.01 – 0.33)

IL-6: interleukin-6; NA: not applicable.

* Non-standardized odds ratio (linked to change in the odds per 1 unit increase)

† Standardized odds ratio (linked to change in the odds per 1 standard deviation increase)

‡ Optimism-adjusted odds ratio using the heuristic shrinkage method.

Atrial fibrillation (p=0.88), treatment with anti-inflammatory drugs, history of stroke or transient ischemic attack, alcohol consumption, body mass index, race, treatment with antiplatelet drugs, peripheral artery disease, hyperuricemia, and cystatin-based glomerular filtration rate (p=0.11) were consecutively removed from the model automatically due to coefficients with p-value >0.05.

Likelihood ratio chi-squared test for significance of the model: $\chi^2 = 705.84$, df = 10, $p = 3.5 \times 10^{-145}$. Area under the Receiver Operating Characteristic curve (AUC) = 0.80. Count $R^2 = 72.4\%$. Proportion of patients correctly classified = 73%. Maximum Cook distance = 0.07. Maximum variance inflation factor = 1.04.

The p values for significance of the odds ratios were determined by a Wald test.

Table S14: Performance of IL-6 versus multivariable prediction modelling for prediction of plaque progression at 5 years.

	IL-6 ≥ 2 pg/mL	**Multivariable model with dichotomized IL-6***
Sensitivity (%)	37.2	62.4
Specificity (%)	63.8	79.8
Positive Predictive Value (%)	34.6	66.7
Negative Predictive Value (%)	66.3	76.7
Positive Likelihood Ratio	1.0	3.1
Negative Likelihood Ratio	1.0	0.5
Accuracy (%)	57.7	73.0
AUC	0.50 (0.49 – 0.52)	0.80 (0.78 – 0.81)
Wald test for equality of AUC	$\chi^2 = 739.3$, df = 1, p=8.5 x 10^{-163}	

AUC: area under the receiver operating characteristic curve, IL-6: interleukin-6
* Model presented in Supplementary Table S11 above.

Note: This comparison highlights a common pitfall in biomarker research which consists of attempting to use biomarkers as standalone predictors of multifactorial diseases with inherently complex pathobiology. For such conditions, the definition of thresholds should preferably be done only to facilitate the interpretability of prediction models and the design of clinical scores. Moreover, optimal thresholds should ideally be defined based on predicted probabilities derived from optimism-adjusted multivariable models since the performance of biomarkers is modulated by the characteristics of the study population.