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Interleukin-6 is an independent predictor of progressive atherosclerosis in the carotid artery: The Tromsø Study

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ABSTRACT

Background and aims: Novel biomarkers are linked to cardiovascular disease (CVD). The aim of the present study was to investigate the association between 28 blood biomarkers and the formation and progression of carotid plaque.

Methods: In a nested case control study with 703 participants from the population based Tromsø Study, a large biomarker panel was measured in blood obtained at baseline. Carotid ultrasound was assessed both at baseline and at 6 years of follow-up. Four groups were defined: Group 1: no plaque at baseline or at follow-up (reference group); Group 2: novel plaque at follow-up; Group 3: stable plaque at follow-up; Group 4: progression of plaque at follow-up. By multinomial logistic regression analyses, we assessed the risk of being in the different plaque groups with regard to traditional cardiovascular risk factors and levels of biomarkers at baseline.

Results: Adjusted for traditional risk factors, interleukin-6 (IL-6) was an independent predictor of plaque progression (OR 1.44, 95% CI 1.12−1.85 per SD increase in IL-6 level). This result remained significant after inclusion of other novel biomarkers to the model, and when subjects with former CVD were excluded. Neopterin was protective of novel plaque formation (OR 0.73, 95% CI 0.57−0.93). Myeloperoxidase and Caspase-1 were independent predictors of plaque progression, but this effect disappeared when excluding subjects with former CVD.

Conclusions: IL-6 is an independent predictor of plaque progression, suggesting that it may be a marker of progressive atherosclerosis in the general population and that its central role in CVD may be related to promotion of plaque growth.

1. Introduction

Increasing evidence suggests that inflammation plays a pivotal role in the formation, progression and rupture of atherosclerotic plaques [1]. Clinical endpoints such as myocardial infarction (MI), stroke or sudden death may be triggered by an extensive inflammatory reaction at the site of the plaque [2], causing plaque rupture and subsequent thrombosis.

Progression of carotid atherosclerosis evaluated by total plaque area (TPA) [3], plaque volume [4] and degree of stenosis [5] is related to higher risk of vascular events compared to atherosclerosis that remain stable over time. Unstable plaques share some distinctive features, such as a thinner fibrous cap overlying a large necrotic core, a strong intra-plaque inflammatory reaction, a more rapidly progression and an echolucent appearance on ultrasonography [6,7]. Thus, the pathogenesis and subsequent release of certain biomarkers in the bloodstream might differ between stable and unstable plaques. Identification of biomarkers associated with the atherosclerotic process is of interest both for singling out individuals at risk, for understanding the pathophysiological mechanisms involved and subsequently for the development of

Abbreviations: TRFs, traditional cardiovascular risk factors; TPA, total plaque area; FDR, false discovery rates; WBC, white blood cells; DDM, D-dimer; PCT, procalcitonin; MPO, myeloperoxidase; Cu/Zn SOD, copper/zinc superoxide dismutase; BNP, brain natriuretic peptide; CtproAVP, copeptin; MRproADM, midregional proadrenomedullin; MRproANP, midregional proatrial natriuretic peptide.

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preventive therapies [8]. Several markers of inflammation, metabolism, hemodynamic stress, oxidative stress and vascular remodeling circulating in the bloodstream have been associated to atherosclerosis and cardiovascular events in experimental and epidemiological studies [8,9].

The Tromsø study, with its high participation rate and comprehensiveness of clinical examinations including repeated carotid ultrasound assessments, provides a unique opportunity for assessing the association between potential blood based biomarkers and atherosclerosis in a prospective population based setting. The objective of the present study was to identify circulating protein biomarkers associated with formation and progression of carotid plaque. At baseline, we examined 28 novel biomarkers representing different pathophysiological pathways in blood from 703 subjects nested from the large population based Tromsø Study [10]. We studied the association between novel biomarkers and plaque-status at follow-up 6 years later, and assessed whether this association was independent of traditional risk factors (TRFs).

2. Materials and methods

2.1. Subjects

The Tromsø Study is a longitudinal population-based study with repeated health surveys [10]. In the 4th survey in 1994/1995 (baseline), all subjects aged 55–74 years and random 5%–10% samples in other age groups >24 years, were invited to ultrasound scanning of the right carotid artery. Ultrasound assessment was performed in 6727 subjects (76% of the eligible). Subjects who did not consent to medical research (n = 40) were excluded. In the 5th survey in 2000/2001 (follow-up), all subjects who were scanned in 1994 and who were still registered as inhabitants of Tromsø were invited to a second ultrasound examination. 4858 subjects were rescanned at follow-up. Of these, four groups were randomly selected on the basis of carotid ultrasound findings at baseline and follow-up. There were originally 200 subjects in each group, matched on age and sex. A panel of 28 biomarkers was measured in blood obtained at baseline. We excluded 95 subjects due to missing baseline blood samples, and 2 subjects were excluded due to low quality of the ultrasound measurements, leaving 703 subjects to be included in four groups: *1) No plaque group*: Study participants who had no plaque at baseline nor follow-up (n = 126); *2) Novel plaque group*: Participants with no plaque at baseline and novel plaque at follow-up (n = 187); *3) Stable plaque group*: Participants with prevalent plaque at baseline and no increase in total plaque area (TPA) between baseline and follow-up (n = 194); *4) Plaque progression group:* Subjects with plaque at baseline and increase in TPA at follow-up (n = 196). Written informed consent was obtained from each participant included in the study, the study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Regional Committee for Medical Health and Research Ethics.

2.2. Cardiovascular risk factors

Information about smoking, diabetes mellitus, MI, stroke, and use of antihypertensive- and lipid-lowering medication was obtained from self-administered questionnaires. At baseline, standardized measurements of height and weight were taken, non-fasting blood samples for analyses of serum lipids and glucose were collected. Serum total cholesterol was analyzed by an enzymatic colorimetric method using a commercially available kit (CHOD-PAP, Boehringer-Mannheim, Mannheim, Germany). Serum high density lipoprotein (HDL) cholesterol was measured after precipitation of lower-density lipoproteins with heparin and manganese chloride. Determination of glycosylated hemoglobin (HbA1c) in EDTA whole blood was based on an immunoturbidometric assay (UNIMATES, F. Hoffmann-La Roche AG). The HbA1c percent value was calculated from the HbA1c/Hb ratio. Specially trained personnel recorded blood pressure with an automatic device (Dinamap Vital Signs Monitor, Tampa, Fla). Three readings were recorded with 1-min intervals, and the average of the final 2 readings was used in the analyses. Hypertension was defined as systolic blood pressure >140 mmHg and/or diastolic blood pressure >90 mmHg and/or use of antihypertensive medication. Cardiovascular disease (CVD) was defined as previous MI or stroke. Diabetes mellitus was defined as self-reported diabetes and/or regular use of insulin and/or oral antidiabetic medication and/or HbA1c > 6.5.

2.3. Biomarkers

A panel of 28 novel biomarkers that previously have shown promising results on the association with CVD were selected and analyzed in blood obtained at baseline. The selected biomarkers have proposed links to atherosclerosis through different pathophysiological mechanisms: inflammatory markers (C-reactive protein (CRP), fibrinogen, white blood cells (WBC), monocyte count, neopterin, interleukin-6 (IL-6), interleukin-18 (IL-18), soluble intercellular adhesion molecule 1 (ICAM-1), soluble vascular adhesion molecule 1 (VCAM-1), Caspase-1, matrix metallopeptidase 9 (MMP-9), tissue inhibitor of metalloproteinase 1 (TIMP-1), D-dimer (DDM), procalcitonin (PCT), protein S-100); markers of oxidative stress (myeloperoxidase (MPO), copper/zinc superoxide dismutase (Cu/Zn SOD); metabolic markers (adiponectin, leptin, apolipoprotein A1 (ApoA1), apolipoprotein B100 (ApoB100), ApoB100/ApoA1 ratio); markers of hemodynamic stress (brain natriuretic peptide (BNP), copeptin (CtproAVP), midregional proadrenomedullin (MRproADM), midregional proatrial natriuretic peptide (MRproANP); and markers of renal function (creatinine, cystatin-C). The study blood samples underwent no more than three freeze/thaw cycles from time of receipt to protein data production. All samples were kept at 4 °C between sample dilutions, and were otherwise stored at −70 °C until assay production. Fibrinogen, creatinine, and WBC were measured at the Department of Clinical Chemistry, University Hospital of North Norway, Tromsø. Fibrinogen was measured using the PT-Fibrinogen reagent (Instrumentation Laboratory), plasma creatinine was analyzed by a modified Jaffe reaction and WBC counts with automated cell counters by standard techniques. All other biochemical analyses were performed at the Mainz Biomarker Laboratory, Johannes Gutenberg University, Mainz by Biosite stroke panel (protein S-100, DDM, BNP), Biosite MPO panel (MPO), ELISA R&D (IL-6, ICAM-1, VCAM-1, leptin, adiponectin, Caspase-1, MMP-9, TIMP-1), ELISA MBL (IL-18), Bnprospects nephelometry Dade Behring (ApoA1, ApoB100, CRP, cystatin-C) and B.R.A.H.M.S. Cryptor (CtproAVP, MRproADM, MRproANP), ELISA B.R.A.H.M.S. (neopterin), B.R.A.H.M.S. PCT sensitive LIA (PCT) and Ransod test kit, Randox (Cu/Zn SOD). According to manufacturers all inter- and intra-assay coefficients of variation were below 10%, except inter assay coefficients for Adiponectin, IL-18 and PCT which ranged between 10 and 20%.

2.4. Ultrasonography

High-resolution B-mode ultrasonography of the right carotid artery was performed at baseline and follow-up with a duplex scanner (Acuson Xp10 128, ART-upgraded) equipped with a 7.5-MHz linear array transducer and followed the same scanning, reading procedures and reproducibility as published previously

[11,12].

A plaque was defined as a localized protrusion of the vessel wall into the lumen of at least 50% compared with the adjacent intima media thickness (IMT). Six locations of the carotid artery were examined for plaque presence: the far walls and near walls of the common carotid artery, the bifurcation (bulb), and the internal carotid artery. The area of each plaque was outlined manually with automatic calculation of plaque area. In subjects with >1 plaque, the areas of all plaques were summarized to give TPA.

Progression of plaque was defined as an increase in TPA $\geq$1.96 standard deviations (SD) of the mean arithmetic difference between 2 independent measurements performed by 2 independent sonographers i.e. measurement error due to random variation. Based on results from the previous reliability study, the definition of plaque progression was set to an increase in TPA $\geq$7.8 mm^2 [11].

2.5. Statistical analyses

All statistical analyses were performed using SAS software 9.4 (SAS Institute Inc., Cary, NC, USA). Skewed numeric variables were natural log transformed to approximate a normal distribution and geometric means of the baseline levels are presented. Baseline characteristics were reported as mean (standard deviation, SD) or percent within each plaque group. Analyses of variance or Pearson's chi-square test were used to test for differences between groups.

Less than 5% of values were missing for all but 6 biomarkers. There was no more than 11% missing values for any biomarker. Data were assumed to be missing at random, and the FCS command in SAS was used to impute 20 data sets. Rubin's rule was used to combine the results for the imputed data sets. Results from the imputed data sets were compared to complete case analyses and the results did not differ substantially.

To explore biomarker interdependence, we calculated pairwise Spearman correlation coefficients between all variables (biomarkers and continuous TRFs). The resulting correlation matrix was plotted as a heat map (Supplemental Fig. 1).

We used general linear models to assess differences in biomarker levels across plaque groups. Significance level was set to $p < 0.05$. False discovery rates (FDR) were calculated to adjust for multiple comparisons [13]. The FDR was estimated based on the test for main effect for all 28 assessed proteins.

For each biomarker that significantly differed between groups, multinomial logistic regression models were used to assess the association between baseline biomarker level and plaque group, adjusted age and sex and further adjusted for TRFs (hypertension, total cholesterol, HDL cholesterol, current smoking, diabetes mellitus and lipid lowering drugs). “No plaque group” was defined as reference category. Analyses were carried out for the whole study sample, and separately for subjects without former history of CVD to assess the predictive value of biomarkers in a primary intervention setting. Odds ratios (OR) for outcome were reported per 1 SD change in continuous variables or for presence vs. absence of binary variables.

All significant markers in the univariable models and the TRFs were candidates for a final multivariable analysis using a backward selection procedure with a retention p-value of 0.05.

As previous studies have suggested a joint effect of multiple biomarkers in the upper tertile on CVD events, we performed analyses to evaluate the composite measure of the aggregate number of biomarkers in the highest third with respect to plaque progression [14]. We considered the biomarkers which were significantly associated with plaque progression after adjustment for TRFs and used a logistic regression model to estimate OR for being in the plaque progression group versus the no plaque group according to number of biomarkers in the upper tertile [15–17].

3. Results

Baseline characteristics are displayed in Table 1. Mean age was 63.6 years and 66% were men. Prevalence of smoking was higher in all plaque groups compared to the no plaque group, and highest in the plaque progression group. Systolic blood pressure was highest in the novel plaque and the plaque progression groups. Hypertension was more prevalent among subjects with plaque progression than in subjects with no plaque and stable plaques. History of CVD was present in 66 individuals (9.4%). Former CVD was most frequent in the stable plaque and the plaque progression group. The prevalence of diabetes mellitus was U-shaped with highest proportions in the no plaque group and plaque progression group.

A weak to moderate interdependence between several biomarkers was observed (Supplemental Fig. 1). The factors most strongly correlated with age at baseline were cystatin-C, VCAM-1, DDM, BNP and mean systolic blood pressure, with Spearman correlations (r_s) ranging from 0.2 to 0.3. IL-6 was most strongly associated with CRP ($r_s = 0.44$) and fibrinogen ($r_s = 0.31$). Interdependence was also observed between fibrinogen and CRP ($r_s = 0.36$). IL-6 was weakly correlated ($r_s = 0.2–0.3$) with monocyte count, WBC, DDM, ICAM-1, caspase-1 and IL-18. Caspase-1 showed correlations with WBC ($r_s = 0.45$), monocyte count ($r_s = 0.39$), MPO, CRP, ICAM-1 and MMP-9 ($r_s = 0.2–0.3$).

The crude baseline level of 12 biomarkers differed significantly between the four plaque groups (Table 2). These markers were CRP, fibrinogen, WBC, neopterin, DDM, IL-6, caspase-1, ICAM-1, ApoA1, ApoB100, ApoB100/ApoA1 ratio and MPO. Adjustment for multiple comparisons revealed FDR <0.05 for seven biomarkers (fibrinogen, WBC, IL-6, caspase-1, ICAM-1, MPO and ApoB100/ApoA1 ratio). The mean baseline levels of these biomarkers were, except for two, highest in the plaque progression group and lowest in the no plaque group. The exceptions were neopterin and ApoA1. The highest baseline level of neopterin was observed in the no plaque group. The highest level for ApoA1 was observed in the novel plaque group.

Age- and sex-adjusted levels of fibrinogen, Apo B100, ApoB100/ApoA1 ratio, WBC, CRP, MPO, DDM, caspase-1, and IL-6 were significantly associated with plaque progression (Table 3). In addition, an increase in caspase-1 increased the corresponding odds of novel plaque formation, while higher neopterin level decreased the odds for novel plaque formation. The associations between MPO, caspase-1 and IL-6 and plaque progression and between neopterin and novel plaque formation remained significant after adjustment for TRFs (Table 3). When subjects with former CVD were excluded, IL-6 and neopterin remained the only significant biomarkers with OR (95% CI) 1.36 (1.05–1.77) for plaque progression and 0.73 (0.57–0.94) novel plaque formation, respectively.

In the final regression analysis, which included TRFs and the 12 significant biomarkers from the univariable models, IL-6, diabetes, hypertension and smoking remained significant predictors of plaque status at follow-up. One standard deviation increase in IL-6 was associated with OR 1.45 (95% CI 1.14–1.85) for plaque progression (Table 4). The results did not change when subjects with former CVD were excluded.

As shown in Table 5, OR of plaque progression increased with increasing number of biomarkers in the upper tertile (considering IL-6, caspase-1, and MPO). After adjustment for TRFs, individuals with 2 biomarkers in the upper tertile had a 2.2-fold higher odds, and individuals with 3 biomarkers in the upper tertile a 4.4-fold higher odds of plaque progression at follow-up compared to subjects with none of the selected biomarkers in the upper tertile.

Table 1
Baseline characteristics according to plaque groups. The Tromsø Study.

Cardiovascular risk factors at baseline	No plaque (n = 126)	Novel plaque (n = 187)	Stable plaque (n = 194)	Progression of plaque (n = 196)	*p*-value [a]
Age, y	63.1 (6.7)	63.8 (6.4)	63.8 (6.5)	63.7 (6.5)	0.75
Sex, % males	65.1	65.2	66.0	66.3	0.99
BMI, kg/m^2	26.5 (3.2)	26.2 (3.4)	25.90 (3.3)	26.5 (3.5)	0.30
Serum lipids, mmol/L					
Total cholesterol	6.64 (1.09)	6.79 (1.20)	6.81 (1.12)	6.93 (1.34)	0.22
HDL cholesterol	1.49 (0.36)	1.52 (0.49)	1.50 (0.43)	1.41 (0.38)	0.07
Triglycerides	1.82 (1.10)	1.69 (1.03)	1.76 (0.99)	1.87 (1.08)	0.20 [b]
Current smokers, %	14.3	28.3 [d]	29.9 [f]	45.4 [ceg]	0.00
Blood pressure, mmHg					
Systolic	143.2 (19.7)	148.6 (19.5) [d]	147.7 (20.7)	151.2 (21.0) [c]	0.01
Diastolic	83.2 (11.4)	85.9 (10.9)	84.1 (12.1)	86.0 (13.2)	0.10
Selfreported disease/medications,%					
History of MI	2.4	7.0	7.7	11.3 [c]	0.03
History of CVD	3.2	7.0	10.4[f]	14.9 [cg]	0.00
History of DM	6.5	1.1 [d]	2.6 [f]	7.7 [eg]	0.00
Hypertension	50.0	67.4 [d]	63.4 [f]	75.0 [ce]	0.00
Lipid lowering drugs	2.4	1.6	3.1	5.6	0.15
Antihypertensive medications	10.3	11.9	13.4	21.4 [ceg]	0.02

Values are unadjusted means (standard deviations) and percentages.
[a] *p*-value for equality between groups main effect assessed by GLM or X^2; [b] analysis performed on log transformed values; [c] $p < 0.05$ for equality between plaque progression and no plaque; [d] $p < 0.05$ for equality between novel plaque and no plaque; [e] $p < 0.05$ for equality between plaque progression and stable plaque; [f] $p < 0.05$ for equality between stable plaque and no plaque; [g] $p < 0.05$ for equality between plaque progression and novel plaque.

Table 2
Biomarker levels at baseline according to plaque groups. The Tromsø Study.

Biomarkers	No plaque (n = 126)	Novel plaque (n = 187)	Stable plaque (n = 194)	Progression of plaque (n = 196)	*p*-value [b]	FDR
CRP, mg/L[a]	1.25 (1.06–1.48)	1.43 (1.24–1.68)	1.44 (1.22–1.67)	1.76 (1.51–2.04) [c]	**0.04**	0.09
Fibrinogen, g/L[a]	3.18 (3.05–3.32)	3.29 (3.18–3.40)	3.38 (3.28–3.49) [f]	3.51 (3.40–3.63) [cg]	**0.00**	**0.01**
WBC x 10^9/L	6.53 (6.21–6.85)	6.88 (6.61–7.14)	6.87 (6.60–7.14)	7.41 (7.14–7.68) [ceg]	**0.00**	**0.00**
Monocyte x 10^9/L	0.57 (0.54–0.61)	0.61 (0.58–0.63)	0.59 (0.56–0.61)	0.62 (0.60–0.65)	0.07	
IL-6, pg/mL[a]	2.66 (2.41–2.95)	2.84 (2.59–3.12)	2.80 (2.54–3.08)	3.58 (3.31–3.87) [ceg]	**0.00**	**0.00**
IL-18, pg/mL[a]	246.13 (230.25–263.10)	253.49 (239.38–268.43)	252.93 (238.79–267.91)	253.31 (239.34–268.08)	0.91	
Neopterin, nmol/L[a]	7.77 (7.15–8.46)	6.76 (6.32–7.23) [d]	7.19 (6.72–7.70)	7.60 (7.07–8.16)	**0.03**	0.09
Caspase-1, pg/mL[a]	90.34 (83.84–97.33)	99.72 (93.62–106.20) [d]	94.96 (89.51–100.75)	108.90 (102.22–116.01) [ceg]	**0.00**	**0.01**
ICAM-1, ng/mL[a]	246.96 (236.70–257.66)	256.66 (244.95–268.94)	255.68 (245.85–265.92)	278.66 (267.06-290-77) [ceg]	**0.00**	**0.01**
VCAM-1, ng/mL[a]	696.31 (660.02–734.58)	730.10 (700.77–760.65)	731.00 (702.09–761.09)	711.83 (684.18–740.60)	0.39	
Protein S-100, pg/mL[a]	110.17 (103.55–117.23)	108.18 (104.37–112.13)	112.58 (105.95–119.62)	112.56 (106.41–119.07)	0.33	
MMP-9, ng/mL[a]	76.62 (67.20–87.36)	74.27 (66.86–82.51)	82.03 (72.93–92.27)	89.37 (80.20–99.59)	0.10	
DDM, ng/mL[a]	224.63 (201.43–250.50)	260.22 (232.77–290.90)	270.82 (240.52–304.93) [f]	293.21 (258.88–332.08) [c]	**0.02**	0.07
PCT, ng/mL	0.017 (0.015–0.019)	0.016 (0.015–0.018)	0.017 (0.016–0.018)	0.017 (0.015–0.018)	0.75	
Adiponectin, ng/mL[a]	9018.50 (8167.62–9958.03)	8617.55 (7829.00–9485.53)	8545.25 (7809.18–9350.70)	8058.46 (7428.35–8742.03)	0.43	
Leptin, pg/mL[a]	6385.11 (5436.60–7499.12)	5804.73 (5080.80–6631.81)	5978.15 (5321.67–6715.61)	5669.16 (4996.26–6432.69	0.68	
ApoA1 g/L	1.55 (1.50–1.59)	1.59 (1.55–1.64)	1.58 (1.54–1.62)	1.51 (1.48–1.55) [eg]	**0.03**	0.07
ApoB100 g/L	1.14 (1.10–1.18)	1.18 (1.14–1.21)	1.19 (1.16–1.22)	1.23 (1.19–1.27) [c]	**0.02**	0.07
ApoB100/ApoA1	0.76 (0.18)	0.76 (0.20)	0.78 (0.20)	0.83 (0.22) [ceg]	**0.00**	**0.01**
BNP, pg/mL[a]	12.30 (11.35–13.32)	14.03 (12.74–15.45)	13.92 (12.80–15.14)	14.82 (13.29–16.53)	0.12	
CTproAVP, pmol/L[a]	6.82 (6.09–7.64)	6.22 (5.66–6.83)	6.05 (5.55–6.59)	5.89 (5.43–6.40)	0.23	
MRproADM, nmol/L[a]	0.42 (0.40–0.45)	0.42 (0.41–0.44)	0.41 (0.40–0.43)	0.41 (0.39–0.42)	0.39	
MRproANP, pmol/L[a]	62.56 (56.86–68.84)	58.36 (54.69–62.27)	57.87 (54.26–61.71)	58.39 (54.46–62.60)	0.41	
TIMP-1, ng/mL[a]	197.53 (190.14–205.22)	211.08 (202.84–219.66)	205.87 (197.67–214.42)	208.57 (200.65–216.81)	0.18	
MPO, pmol/L [a]	347.36 (302.68–398.63)	407.22 (360.67–459.78)	397.19 (355.82–443.37)	467.80 (420.16–520.84) [c]	**0.00**	**0.02**
Cu/Zn SOD, U/L[a]	5.54 (4.94–6.21)	6.06 (5.51–6.67)	5.80 (5.40–6.24)	6.28 (5.70–6.91)	0.37	
Cystatin-C, mg/L	0.72 (0.70–0.74)	0.73 (0.71–0.75)	0.75 (0.73–0.77)	0.75 (0.73–0.77)	0.20	
Creatinine, μmol/L	81.83 (79.4–84.34)	79.94 (77.48–82.47)	82.22 (79.55–84.97)	79.93 (77.53–82.41)	0.44	

Numbers are unadjusted means (95% confidence intervals).
[a] geometric means, with statistical tests performed on log transformed values; [b] *p*-value for equality between groups main effect assessed by GLM; FDR: false discovery rate. [c] $p < 0.05$ for equality between plaque progression and no plaque; [d] $p < 0.05$ for equality between novel plaque and no plaque; [e] $p < 0.05$ for equality between plaque progression and stable plaque; [f] $p < 0.05$ for equality between stable plaque and no plaque; [g] $p < 0.05$ for equality between plaque progression and novel plaque. Bold: *p*-value or FDR for equality between groups main effect <0.05.

4. Discussion

In a model which included TRFs and 12 biomarkers individually associated with atherosclerosis, IL-6 was the only novel biomarker that remained a significant predictor of plaque progression. To our knowledge, this is the first population based study to demonstrate IL-6 as a predictor of progressive atherosclerotic disease. IL-6 is a master proinflammatory cytokine. It is produced by different cell-types including activated monocytes, macrophages, endothelial cells, adipocytes and Th2-cells. IL-6 production is initiated by infections and raised levels are found in chronic inflammatory conditions which are associated with increased CVD-risk. IL-6

Table 3
Odds ratios of novel plaque, stable plaque and progression of plaque with "no plaque" as reference group, according to baseline levels of biomarkers. The Tromsø Study.

Biomarker		Adjusted for age and sex			Adjusted for age, sex and traditional risk factors		
		Novel plaque *vs.* no plaque	Stable plaque *vs.* no plaque	Progression of plaque *vs.* no plaque	Novel plaque *vs.* no plaque	Stable plaque *vs.* no plaque	Progression of plaque *vs.* no plaque
Fibrinogen [a]	OR(CI)	1.15 (0.90	**1.30 (1.03**	**1.54 (1.21–1.95)**	1.02 (0.79–1.32)	1.15 (0.89–1.49)	1.17 (1.90–1.52)
	p-	–1.45)	**–1.65)**	**0.0004**	0.869	0.275	0.24
	value	0.26	**0.029**				
ApoB100	OR(CI)	1.17 (0.93	1.23 (0.98	**1.46 (1.16–1.84)**	1.28 (0.79–2.05)	1.40 (0.87–2.25)	1.39 (0.86–2.25)
	p-	–1.50)	–1.56)	**0.0015**	0.317	0.1652	0.17
	value	0.191	0.076				
ApoA1	OR(CI)	1.18 (0.93	1.11 (0.88	0.86 (0.67–1.10)	1.49 (0.99–2.25)	1.42 (0.95–2.13)	1.09 (0.72–1.67)
	p-	–1.50)	–1.42)	0.218	0.054	0.088	0.68
	value	0.171	0.354				
ApoB100/ ApoA1	OR(CI)	1.040 (0.82	1.21 (0.88	**1.47 (1.16–1.86)**	1.02 (0.67–1.58)	1.14 (0.74–1.75)	1.33 (0.86–2.05)
	p-	–1.32)	–1.42)	**0.0014**	0.937	0.559	0.20
	value	0.750	0.345				
WBC	OR(CI)	1.27 (0.99	**1.28 (1.01**	**1.71 (1.34–2.18)**	1.13 (0.87–1.48)	1.12 (0.86–1.46)	1.31 (0.99–1.71)
	p-	–1.62)	**–1.63)**	**<0.0001**	0.3495	0.402	0.052
	value	0.052	**0.044**				
CRP [a]	OR(CI)	1.15 (0.91	1.13 (0.90	**1.37 (1.09–1.73)**	1.10 (0.86–1.39)	1.06 (0.83–1.35)	1.14 (0.89–1.47)
	p-	–1.45)	–1.42)	**0.0075**	0.474	0.649	0.295
	value	0.259	0.313				
MPO [a]	OR(CI)	1.23 (0.96	1.24 (0.97	**1.52 (1.20–1.93)**	1.14 (0.87–1.48)	1.14 (0.89–1.48)	**1.29 (1.01–1.66)**
	p-	–1.59)	–1.58)	**0.0005**	0.340	0.302	**0.045**
	value	0.108	0.093				
ICAM-1 [a]	OR(CI)	1.15 (0.91	1.14 (0.90	**1.54 (1.22–1.94)**	1.01 (0.78–1.31)	0.98 (0.76–1.26)	1.14 (0.88–1.49)
	p-	–1.45)	–1.43)	**0.0003**	0.948	0.859	0.328
	value	0.243	0.284				
DDM [a]	OR(CI)	1.21 (0.92	1.31 (1.00	**1.42 (1.09–1.72)**	1.14 (0.86–1.52)	1.24 (0.93–1.64)	1.24 (0.93–1.63)
	p-	–1.59)	–1.72)	**0.009**	0.360	0.140	0.140
	value	0.200	0.052				
Caspase-1 [a]	OR(CI)	**1.29 (1.02**	1.14 (0.91	**1.60 (1.26–2.03)**	1.21 (0.94–1.56)	1.04 (0.81–1.34)	**1.36 (1.05–1.76)**
	p-	**–1.63)**	–1.43)	**0.0001**	0.140	0.742	**0.020**
	value	**0.0337**	0.266				
Neopterin [a]	OR(CI)	**0.73 (0.58**	0.84 (0.67	0.95 (0.76–1.19)	**0.73 (0.57–0.93)**	0.84 (0.66–1.06)	0.95 (0.75–1.21)
	p-	**–0.93)**	–1.06)	0.648	**0.010**	0.149	0.694
	value	**0.010**	0.145				
IL-6 [a]	OR(CI)	1.092 (0.86	1.06 (0.84	**1.60 (1.26–2.03)**	1.06 (0.83–1.35)	1.02 (0.80–1.29)	**1.44 (1.12–1.85)**
	p-	–1.38)	–1.34)	**0.0001**	0.630	0.887	**0.004**
	value	0.466	0.629				

Values are odds ratio per 1 standard deviation increase in biomarker level (95% confidence interval).
Traditional risk factors; sex, age, diabetes, hypertension, smoking, total cholesterol, HDL-cholesterol, and lipid-lowering drugs.
[a] Statistical tests performed on log transformed values. Bold: *p*-value for OR <0.05.

Table 4
Predictors of plaque status at follow-up. The Tromsø Study.

	Novel plaque *vs.* no plaque		Stable plaque *vs.* no plaque		Plaque progression *vs.* no plaque	
	OR (95% CI)	*p*-value	OR (95% CI)	*p*-value	OR (95% CI)	*p*-value
Diabetes	0.37 (0.17–0.81)	0.01	–	ns	–	ns
Hypertension	1.49 (1.17–1.89)	<0.001	1.36 (1.08–1.72)	0.01	1.78 (1.39–2.28)	<0.001
Smoking	1.56 (1.16–2.12)	<0.001	1.62 (1.20–2.18)	<0.001	2.20 (1.64–2.96)	<0.001
IL-6 [a]	–	ns	–	ns	1.45 (1.14–1.85)	<0.001

Values are odds ratio per 1 standard deviation increase in continuous variables (95% confidence interval) or for presence of categorical variables. Variables presented are those selected by backward selection procedure in a model which originally included traditional risk factors (sex, age, diabetes, hypertension, smoking, total cholesterol, HDL-cholesterol, and lipid-lowering drugs) and 12 biomarkers which individually were associated with plaque status at follow-up (Fibrinogen, WBC, ApoB100, ApoA1, ApoB100/ApoA1 ratio, CRP, DDM, MPO, ICAM-1, Caspase-1, Neopterin and IL-6). Age and sex not evaluated due to matched design.
[a] Statistical test performed on log transformed values. ns; not significant (*p*-value > 0.05).

amplifies the inflammatory cascade by stimulating hepatic synthesis of acute phase reactants such as CRP and fibrinogen and is also a pro-coagulant cytokine [18]. IL-6 has a variety of other functions including activation of endothelial cells, activation of the hypothalamic-pituitarity-adrenal axis, promotion of lymphocyte proliferation, differentiation and oxidation of lipoproteins [19]. Through these various effects IL-6 may play a central role in initiation and progression of atherosclerotic plaques [20]. IL-6 is more consistently correlated with CVD than down-stream acute phase reactants such as fibrinogen and CRP [20,21]. The associations between IL-6 and plaque presence [22], plaque size, unstable plaque features including hypodensity and ulceration [22–24] as well as carotid stenosis [25,26], have been documented in previous reports. IL-6 has also been associated with progression of carotid artery stenosis [27] and IMT [28] in high risk populations. In murine experiments, exogenous administrated IL-6 enhanced the development of fatty streaks [29]. Mendelian randomization studies also suggest that IL-6 signaling pathways play a causal role in CVD [30]. Our findings are in line with previous studies underlining the central role of IL-6 in the pathogenesis of atherosclerosis, and suggest an effect through promotion of plaque growth. Further studies should determine if IL-6 also may add incremental value in

Table 5
Odds ratios for plaque progression vs. no plaque at follow-up according to number of biomarkers[a] in the upper tertile at baseline. The Tromsø Study.

	Number of biomarkers [a] in the upper tertile			
	0 (Referent)	1	2	3
% of participants in no plaque group (n = 126)	44.4% (n = 56)	35.7% (n = 45)	16.7 (n = 21)	3.2% (n = 4)
% of participants in plaque progression group (n = 196)	23.0% (n = 45)	39.3% (n = 77)	27.0% (n = 53)	10.7% (n = 21)
Age and sex adjusted				
OR	1	2.19	3.29	7.18
95% CI	–	1.25–3.82	1.69–6.39	2.12–24.3
p-value	–	0.0061	0.0005	0.0015
Fully adjusted				
OR	1	1.84	2.20	4.39
95% CI	–	2.02–3.31	1.08–4.44	1.22–15.7
p-value	–	0.041	0.030	0.023

[a] Biomarkers considered were IL-6, Caspase-1 and MPO which were significantly associated with plaque progression in multivariable adjusted models. Fully adjusted models included sex, age, diabetes, hypertension, smoking, total cholesterol, HDL-cholesterol, and lipid-lowering drugs.

risk estimation tools and serve as a target for preventive therapy. The current available IL-6 antagonist (tocilizumab) has so far shown conflicting results regarding CVD prevention [20,21].

Adjusted for TRFs, MPO and caspase-1 were associated with plaque progression, while neopterin was inversely associated with the risk of novel plaque formation. MPO is an enzyme secreted by activated macrophages and is linked to both oxidative stress and inflammation. MPO may reduce the bioavailability of nitric oxide, resulting in endothelial dysfunction, in particular endothelium dependent vasorelaxation [31]. MPO is involved in the oxidation process of LDL, promoting foam cell formation in the vascular wall [32]. Finally, MPO may play a role in plaque destabilization by activating metalloproteinases, thereby weakening the fibrous cap [31]. Exner et al. found that MPO was significantly associated with progression of carotid artery stenosis and especially in participants with low levels of HDL [31]. Sugiyama et al. found increased numbers of MPO-expressing macrophages in eroded or ruptured plaques [33]. Plaque inflammation detected as high metabolic activity on FDG PET was recently shown to correlate with blood levels of MPO [34]. In a review article from 2009, Schindhelm et al. conclude that a causative role of MPO in initiating CVD is supported by in vitro experiments and pathophysiological observations, indicating that MPO is involved in all stages of atherogenesis from endothelial dysfunction to plaque rupture [35]. Our results support previous studies, suggesting that MPO is a marker of plaque progression, possibly independent of TRFs.

Caspase-1 induces cell death *via* the pyroptotic pathway and is involved in regulation of inflammatory processes by activation of IL-1β through the NLRP3 inflammasome. Recent results from the CANTOS-trial indicate that IL-1β may be a target for CVD prevention [36]. Pyroptosis serves to eject intracellular pathogens, but may also be initiated in macrophages upon engulfment of oxidized lipoproteins in atherosclerosis [37,38]. Autopsy studies have revealed a high prevalence of dead cells in vulnerable and ruptured atherosclerotic plaques [39]. Death of smooth muscle cells leads to attenuation of the fibrous cap, and death of foam cells results in enlargement of the necrotic core; both thin caps and large cores are important determinants of plaque vulnerability [40]. In our study the effect of both caspase-1 and MPO disappeared when subjects with former CVD were excluded, suggesting that these markers may be features of more advanced stages of atherosclerosis related to prevalent CVD.

Surprisingly, we found the highest levels of neopterin in patients without plaque. Neopterin is a product of the catabolism of guanosine triphosphate and is secreted by macrophages upon activation. Neopterin has been suggested as a potential marker for disease activity in patients with cardiovascular disease [41]. Sugioaka et al. have recently shown that s-levels of neopterin correlated with the presence of highly complex carotid and coronary lesions, suggestive of vulnerable plaques in patients with coronary artery disease [42]. The relationship between neopterin and development of atherosclerosis may be complex as our results suggest that high levels of neopterin may be protective against plaque formation in the absence of established atherosclerotic disease. However, as the FDR of neopterin was 0.09 a spurious association between neopterin and plaque formation due to multiple testing may be suspected.

The most widely used serum marker in clinical practice is high sensitive CRP which has shown reproducibility to predict CVD in several large epidemiological studies [43]. In our study, baseline level of CRP was significantly higher in subjects with plaque progression compared to subjects who remained plaque free, but this effect was lost upon adjustment for TRFs. Many of the biomarkers were correlated with each other and with TRFs. This may explain why they did not remain significantly associated with outcome in the multivariable models. The correlation between markers may result from the fact that several markers reflect aspects of the same biological processes and also suggest that inflammatory markers may raise as a response to the presence of TRFs.

Blood-derived biomarkers reflecting progressive subclinical atherosclerosis that can be easily integrated into patient management in a primary care setting are desirable. Several of the examined biomarkers are unspecific markers of inflammation and may be upregulated due to different biological processes. The assessment of multiple markers simultaneously may increase the specificity in regard to atherosclerotic burden and assessment of cardiovascular risk. In our study, evaluation of a multimarker score suggested that the combination of several markers in the upper tertile may increase the risk for plaque progression. These analyses must, however, be considered exploratory and the confidence intervals were wide due to low number of subjects in each category. Future studies should be designed to determine the combination of biomarkers that offers the highest sensitivity and specificity for progressive atherosclerotic disease. It should also be evaluated if implementation of such a multimarker score may add incremental value beyond that obtained from TRFs in CVD risk prediction models.

The majority of former epidemiologic studies have focused on

the association between blood biomarkers and carotid intima media thickness (IMT). However, carotid atherosclerotic plaque is a stronger predictor of cardiovascular events [44,45], and IMT progression did not predict CVD events in large population-based studies [46]. Plaque progression has been found to predict CVD [3–5], can reliably be evaluated in individuals over time [4] and has also been linked to cognitive decline [47,48]. Strengths of the present study are related to its population based, prospective design and large biomarker panel. Our study has several limitations. Serum levels of biomarkers were obtained from frozen plasma samples. This may have influenced the reliability although it should have affected the four groups equally. Biomarkers were measured only once. Substantial day-to day intra-variability may have led to indifferential misclassification, which tends to underestimate true associations. TRFs and biomarkers were assessed at baseline. Therefore, we could not assess the variability of inflammatory markers during the course of the study. Additionally, we cannot rule out the possibility of spurious associations due to sampling or experimental bias. Statistical power to detect associations was reduced due to missing blood samples. Residual confounding is likely to exist and regression to the mean may have affected our outcome categories.

In this nested case control study, baseline interleukin-6 was an independent predictor of plaque progression at 6 years follow-up. This suggests that IL-6 may have a clinical relevant role in the detection of progressive atherosclerotic disease that adds to TRFs. The central role of IL-6 in cardiovascular disease may be related to mechanisms involved in promotion of atherosclerosis. In addition, caspase-1 and MPO were associated with plaque progression after adjustment for TRFs. Elevated levels of multiple biomarkers may increase specificity in the detection of progressive atherosclerosis and future studies aimed at establishing a meaningful molecular signature for prevalent unstable atherosclerosis should be designed.

Conflict of interest

The authors declared they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

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Author contributions

A. Eltoft analyzed and interpreted the data and drafted the manuscript. S.H. Johnsen and E.B. Mathiesen conceived, designed and supervised the research. E.B. Mathiesen handled funding. K.A. Arntzen, S.H. Johnsen and E.B. Mathiesen acquired the data. T. Wilsgaard contributed to data analysis. K.A. Arntzen, E.B. Mathiesen, T. Wilsgaard and S.H. Johnsen made critical revision of the manuscript.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.atherosclerosis.2018.02.005.

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Supplemental figure 1.

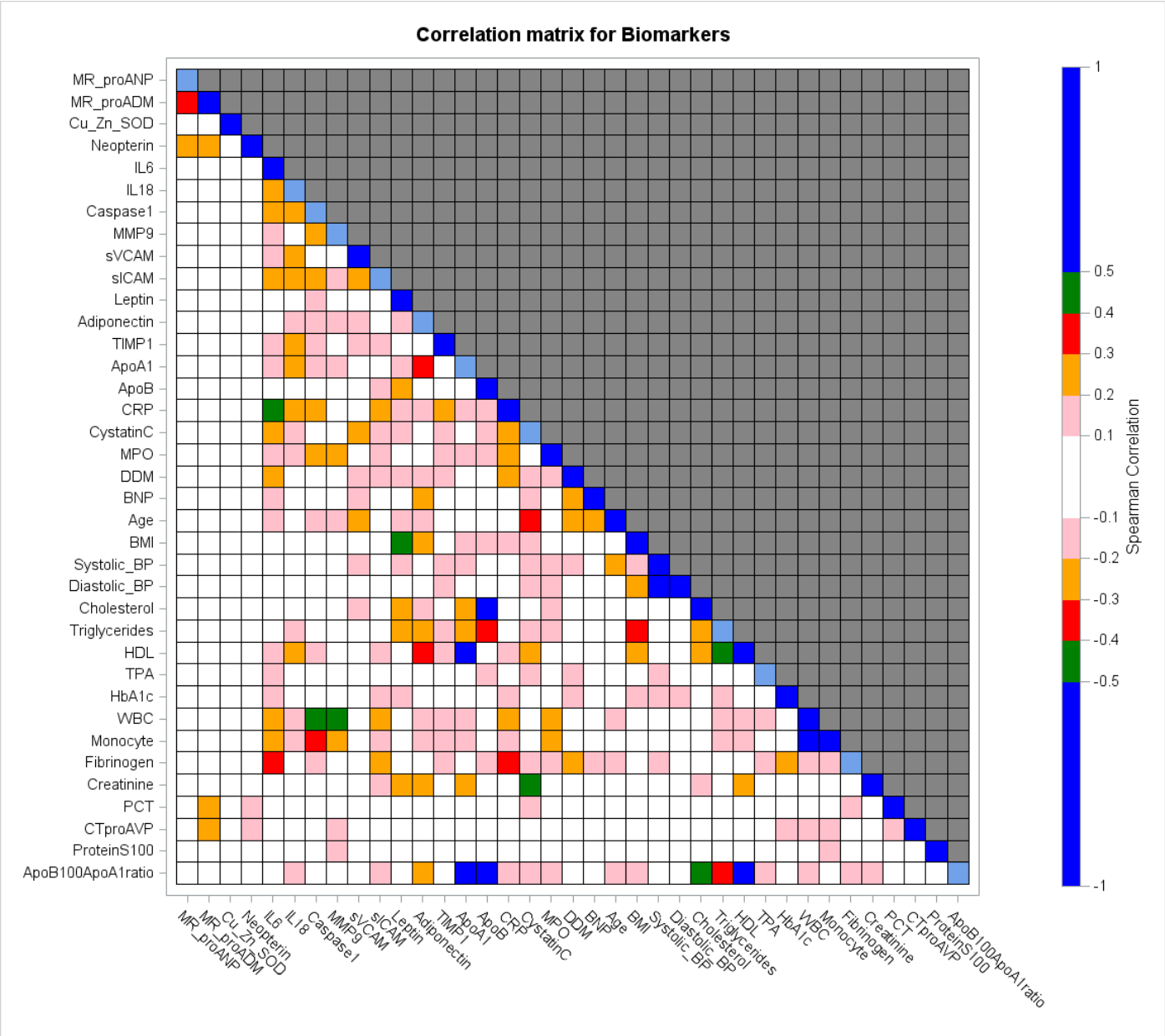

Figure legend.

Pairwise Spearman correlations between biomarkers and continuous traditional risk factors at baseline.

Erratum to "Interleukin-6 is an independent predictor of progressive atherosclerosis in the carotid artery: The Tromsø Study." [Atherosclerosis 271 (April 2018) 1-8]

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The authors realized that an error had occurred in the methods section on page 3, second paragraph (*2.4. Ultrasonography*). The paragraph is now printed correctly below. Furthermore, the years for the 5th survey should read 2001/2002 (page 2, *2.1. Subjects*). The authors would like to apologize for any inconvenience caused.

Progression of plaque was defined as an increase in TPA above the mean absolute difference (2.9 mm^2) between 2 independent measurements performed by 2 independent sonographers, as a measure of the typical magnitude of the measurement error [11]. Stable plaque size was defined as change in TPA of less than ±2.9 mm^2. To reduce risk of misclassification in the plaque progression group, we included subjects with the largest TPA progression.