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The relationship of neutrophil elastase and proteinase 3 with risk factors, and chronic complications in type 2 diabetes: A Fenofibrate Intervention and Event Lowering in Diabetes (FIELD) sub-study

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Abstract

Introduction: Neutrophil elastase (NE) and proteinase 3 (PR3) are novel inflammation biomarkers. We investigated their associations with chronic complications, determinants of biomarker levels and effects of fenofibrate in patients with type 2 diabetes mellitus (T2DM) from Fenofibrate Intervention and Event Lowering in Diabetes study.

Methods: Plasma NE and PR3 levels were quantified at baseline ($n = 2000$), and relationships with complications over 5-years assessed. Effects of fenofibrate on biomarker levels ($n = 200$) were determined at four follow-up visits.

Results: Higher waist-to-hip ratio, homocysteine and C-reactive protein and lower apoA-II were determinants of higher NE and PR3 levels. Higher NE levels were associated with on-trial stroke and cardiovascular mortality, and higher PR3 levels with on-trial stroke, but associations were not significant after adjustment for confounding factors. Although higher NE and PR3 levels were associated with baseline total microvascular disease, only NE levels were associated with on-trial neuropathy or amputation. These associations were not significant after adjusting for multiple comparisons. NE and PR3 levels did not change with fenofibrate.

Conclusions: In T2DM plasma NE and PR3 levels are associated with vascular risk factors, and total microvascular disease at baseline, but on rigorous analyses were not associated with on-trial complications. Levels were not changed by fenofibrate.

Keywords

Cardiovascular disease, diabetes, microvascular disease, neutrophil elastase, proteinase 3, serine protease

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Key messages

- This is the first study to assess relationships of plasma NE and PR3 levels with vascular risk factors, cardiovascular and microvascular outcomes and of fenofibrate effects.
- Higher waist-to-hip ratio, homocysteine and high-sensitivity C-reactive protein levels and lower apoA-II levels were independently associated with higher levels of both NE and PR3.
- Higher baseline NE was associated with on-trial stroke and cardiovascular mortality, and higher PR3 with on-trial stroke, but associations were not significant after adjustment for confounding factors.
- T2DM adults with prevalent total microvascular disease at baseline had higher baseline NE and PR3 than those without complications, but only baseline NE tended to be related to new neuropathy and amputations over a median 5-year period.
- Fenofibrate treatment did not change plasma NE or PR3 levels.

Introduction

Inflammation is a hallmark feature of obesity, type 2 diabetes mellitus (T2DM), diabetic vascular complications and cardiovascular disease (CVD). Chronic low-grade tissue inflammation is an important cause of systemic insulin resistance and T2DM,[1,2] which is mediated by immune cells such as macrophages, T-cells, B-cells, mast cells and eosinophils. Neutrophils, the most abundant (40%–75%) type of white blood cells, are the first immune cells to respond to inflammation. They secrete several serine proteases, including neutrophil elastase (NE, also known as leucocyte elastase and serine elastase) and proteinase 3 (PR3), both of which are stored in primary granules and are released after neutrophil activation and degranulation.[3,4]

In 2008, a cooperative role for PR3 and NE in vivo in neutrophil activation and non-infectious inflammation was identified. PR3 and NE can enhance neutrophil-dependent inflammation by eliminating the local anti-inflammatory activity of progranulin.[5] They also play a role in mediating vascular endothelial inflammation.[6] NE deletion can greatly increase hepatic and adipose tissue insulin sensitivity in mice with high-fat diet (HFD)-induced obesity.[7] In obese mice and human subjects, there was increased serum NE activity.[8] NE-knockout mice were resistant to HFD-induced bodyweight gain, insulin resistance, inflammation and fatty liver. A NE inhibitor reversed insulin resistance and body weight gain in HFD-fed mice.[8] NE expression is also increased in atherosclerotic plaques where it degrades components of the extracellular matrix, with macrophages being the main source of NE production.[9] In HFD-fed apolipoprotein E (apoE)-knockout mice, NE was detected in mature atherosclerotic plaques, predominantly in the endothelium, alongside interleukin (IL)-1β and promote IL-1β secretion from human coronary endothelial cells.[10] On the other hand, PR3 can induce insulin resistance in the mouse and inhibition of PR3 activity can increase glucose clearance.[11] A recent study has shown elevated plasma NE and PR3 levels in patients with type 2 diabetes.[12] Therefore, both NE and PR3 play a role in linking inflammation to T2DM and its vascular complications.

However, there are few human studies to complement these interesting animal studies. In humans with type 1 diabetes, circulating protein levels and enzymatic activities of NE and PR3 are markedly elevated relative to non-diabetic subjects.[13] In a prospective study of acute myocardial infarction patients, PR3 was a significant predictor of death or heart failure.[14] As yet relationships between circulating levels of NE and PR3 with cardiovascular and microvascular complications in T2DM patients are not known. Also unknown are effects of the peroxisome proliferator-activated receptor (PPAR) α agonist, fenofibrate, which has anti-inflammatory effects and can protect against microvascular and some macrovascular complications in adults with T2D.[15,17–20] In the present study, we investigated whether plasma NE and PR3 levels were associated with vascular risk factors, and with concurrent and/or future cardiovascular and microvascular events in T2DM adults from the Fenofibrate Intervention and Event Lowering in Diabetes (FIELD) study and effects of fenofibrate.

Methods

Study design

The FIELD study was a double-blind placebo-controlled randomised clinical trial to study the effects of long-term lipid-lowering treatment with fenofibrate on adverse cardiovascular and microvascular disease outcomes in 9795 adults with T2DM. The study design, baseline subject characteristics and major findings of the FIELD study have been described previously.[16–21] All patients were aged 50–75 years at baseline and were randomly allocated to once-daily co-micronised fenofibrate 200 mg or matching placebo for a median of 5-years (International Standard Randomised Controlled Trial number ISRCTN64783481).[16] All participants in both placebo and treatment groups were prescribed single-blind fenofibrate therapy during a 6-week active run-in phase before randomisation. The study protocol was approved by national and local ethics committees and all participants gave written informed consent. The study was undertaken in accordance with the Declaration of Helsinki and Good Clinical Practice Guidelines.

Plasma NE and PR3 levels were measured at baseline in a random sub-sample of 2000 participants with stratification

by sex and subsequent fenofibrate/placebo treatment allocation. No significant differences in age, sex, race and treatment allocation were found between the 2000 participants in this biomarker sub-study, and the other 7795 patients (Supplemental Table 1), but included participants had higher body mass index (BMI), lower waist-to-hip ratio, shorter known diabetes duration and lower percentages of having prior CVD and baseline microvascular disease, than those not included. In a subsample of 200 participants, both NE and PR3 levels were also measured at the time of randomisation (after a 16-week run-in period that included the last 6-weeks with fenofibrate), 1 year and 5-years or study close-out to assess the effect of fenofibrate on biomarker levels.

Biomarker measurement

Enzyme-linked immunosorbent assay (ELISA) kits (Antibody and Immunoassay Services, University of Hong Kong, Hong Kong) were used for NE and PR3 measurement in citrate plasma as described previously.[13] Briefly, plasma was diluted 1:100 (v:v) with assay diluent and analysed together with quality controls as per manufacturer's instructions. For NE, the intra- and inter-assay coefficients of variation (CVs) were $<8\%$ and $<17\%$ respectively. For PR3, the intra- and inter-assay CVs were $<7\%$ and $<13\%$ respectively. In a pilot study, plasma NE and PR3 levels were demonstrated to be stable up to eight freeze-thaw cycles with CVs of 9.8% and 3.6% respectively. All samples were analysed masked for subject identity, study treatment allocation and sample order. There were the same numbers of participants in both FIELD treatment groups, and all samples from the same subject were analysed in the same assay plate.

Clinical characteristics and outcome events

The detailed study protocol and measurement methods of clinical characteristics have been described previously.[16,17,22–24] The Chronic Kidney Disease Epidemiology Collaboration algorithm was used to calculate the estimated Glomerular Filtration Rate (eGFR).[25] The homeostasis model assessment estimate of insulin resistance (HOMA-IR) was calculated according to a computer model.[26] High-sensitivity C-reactive protein (hs-CRP) levels were measured using an automated immune-turbidometric assay on a Modular E170 analyser (Roche Diagnostics, Mannheim, Germany).[27]

Details on the primary endpoint, other cardiovascular outcomes and microvascular outcomes of the FIELD trial have been described previously.[16–21,27–29] In this analysis, as specified for all FIELD biomarker analyses, the primary cardiovascular outcome was on-trial total CVD events which was a composite of coronary heart disease (CHD) events, total stroke and other cardiovascular death events plus coronary and carotid revascularisation.[28] The secondary cardiovascular outcomes in this analysis were the individual components of total CVD events, that is, CHD event, total stroke, CVD mortality and coronary and carotid revascularisation. In this study, we also analysed the tertiary outcome of hospital admission for angina pectoris which included unstable angina, other forms of angina pectoris and unspecified angina pectoris with matched codes of I20.0, I20.8 and I20.9 by ICD-10 (International Classification of Diseases, Tenth Revision).[28] At baseline, previous CVD history comprised myocardial infarction, stroke, angina, coronary artery bypass grafting (CABG), percutaneous transluminal coronary angioplasty (PTCA), peripheral vascular disease and revascularisation. For microvascular diseases, the primary outcome in this analysis was total (or composite) microvascular disease, defined as the presence of nephropathy, retinopathy, neuropathy and/or microvascular amputation at baseline (baseline microvascular disease) or which developed during follow-up (on-trial microvascular disease).[29] Secondary outcomes were the four individual components of total microvascular disease, that is, nephropathy, retinopathy, neuropathy and microvascular amputation. In this analysis, progression from normoalbuminuria to microalbuminuria (urinary albumin/creatinine ratio (UACR) $\geqslant 3.5$ to <35 for women and $\geqslant 2.5$ to <25 for men) or macroalbuminuria (UACR $\geqslant 35$ for women and $\geqslant 25$ for men), or from microalbuminuria to macroalbuminuria were also treated as a new on-trial nephropathy event. Among 172 participants included in this analysis, standardised retinal photography was performed and photographs graded with Early Treatment Diabetic Retinopathy Study (ETDRS) criteria at the baseline, 2, 5 years and at study close, and progression of retinopathy was defined as at least a 2-step increase in ETDRS grade after 2-years or more of follow-up.[18]

Statistical analysis

Data are presented as mean (SD), median (interquartile range (IQR)) or number (percentage), where appropriate. Comparison of clinical characteristics between two independent groups was performed by Chi square test for categorical variables. For continuous variables, comparison was performed by *t*-test for normally distributed variables and Wilcoxon rank-sum test for skewed variables.

To identity the determinants of baseline biomarker levels, the association of clinical characteristics with biomarker levels at baseline was assessed using univariable and multivariable linear regression analysis with the ln-transformed levels of the biomarkers modeled as the dependent variable. For variables with skewed distribution, data were analysed after natural log (ln) transformation. All variables with a $p < 0.20$ in univariable analysis were entered into the multivariable model with the final model being selected using a backward elimination procedure until all variables had $p < 0.05$. Multi-collinearity issue was assessed in multivariable linear regression

models using the variance inflation factor. When there was multi-collinearity issue between two variables, selection of the variables was based on the r^2 of the model.

Logistic regression was used to assess the cross-sectional association of NE and PR3 levels with baseline history of cardiovascular disease. Cox proportional hazards regression was used to assess the association of baseline NE and PR3 levels with different new on-trial cardiovascular outcome events. In Model 1, data were adjusted for treatment allocation for new on-trial outcome analysis. In Model 2, data were further adjusted for CVD risk factors, including age, sex, known diabetes duration, prior history of CVD (except for analysis of baseline history of CVD), smoking (never, former and current), BMI, glycosylated haemoglobin (HbA1c), HOMA-IR, systolic blood pressure (BP), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), triglycerides, fibrinogen, plasma creatinine and homocysteine at baseline.[28] Logistic regression was used to assess the cross-sectional association of NE and PR3 levels with baseline microvascular disease. Cox regression analysis was used for new on-trial retinopathy and amputation, while logistic regression was used for new on-trial total microvascular disease, nephropathy, neuropathy and two-step progression of ETDRS grade, as the examinations for these outcomes were performed at 3–4 visits only. In Model 1, data were adjusted for treatment allocation (except for baseline microvascular disease). In Model 2, data were further adjusted for traditional risk factors, including age, sex, known diabetes duration, prior history of CVD, smoking (never, former and current), BMI, HbA1c, HOMA-IR, systolic BP, HDL-C, LDL-C, triglycerides, fibrinogen, plasma creatinine, homocysteine and glucose-lowering medication (diet alone, oral hypoglycaemia agent(s) alone, insulin alone and insulin + oral agent(s)) at baseline.[29] To prevent over-fitting in the regression analysis of some outcome events with small number of cases, data were adjusted for treatment (for new on-trial outcomes only) and the most significant predictors of the outcomes (selected by backward elimination) so that the number of predictor parameters estimated in the regression model fulfilled the 1 in 10 rule (i.e. 1 predictor variable can be fitted for every 10 events). For those outcomes which demonstrated a positive association, a sensitivity analysis was done which further adjusted for hs-CRP levels because of the inflammatory properties of NE and PR3.

In all Cox regression analyses, the proportional hazards assumptions were checked using Schoenfeld residuals and no significant deviation from the assumptions was found for all the outcomes. In this analysis, the principal pre-specified analysis was the association of baseline biomarker levels with different cardiovascular and microvascular outcomes. A two-sided $p < 0.05$ was considered significant for the primary total cardiovascular and total microvascular outcomes; $p < 0.01$ for secondary and tertiary outcomes. A two-sided $p < 0.05$ was considered significant for all other analyses. p Values for treatment and sex interaction were estimated by including the multiplicative interaction term in the regression models in the full sample after adjusting for the main effects of the covariates.

All statistical analyses were performed using SPSS 25 (IBM, Armonk, NY).

Results

Baseline characteristics of the 2000 participants are shown in Table 1. Among them, 50% were allocated to fenofibrate treatment. No significant differences were found in clinical characteristics and plasma NE and PR3 levels at baseline between the two groups (Table 1).

Supplemental Figure 1 show the histograms of the distribution of the biomarker levels among all participants as well as in sex-specific subgroups. Both plasma NE and PR3 levels showed a right skewed distribution. Plasma NE and PR3 levels were moderately strongly correlated ($r = 0.745$, $p < 0.001$).

Table 2 shows the univariable and multivariable analysis for the relationship of clinical characteristics with plasma NE and PR3 levels at baseline. Higher waist-to-hip ratio, HOMA-IR, homocysteine and hs-CRP levels, and lower systolic BP, triglycerides, apoA-II levels and eGFR were significantly associated with higher plasma NE levels in the multivariable analysis ($r^2 = 0.041$). Being female and older, higher waist-to-hip ratio, plasma creatinine and hs-CRP levels, shorter known diabetes duration, use of glucose-lowering medication and lower apoA-II levels were significantly associated with higher plasma PR3 levels ($r^2 = 0.083$).

As shown in Supplemental Table 2, participants with prior history of any CVD at baseline had higher PR3 levels than those without ($p = 0.026$), but the association was not significant after adjusting for confounding variables. As shown in Table 3, there were no significant differences in plasma NE and PR3 levels between participants with and without on-trial total CVD events. However, participants who experienced an on-trial 'total stroke' had higher baseline plasma NE and PR3 levels ($p = 0.032$ and 0.015 respectively), and those with CVD mortality had higher baseline plasma NE levels ($p = 0.043$). All these differences did not meet the more rigorous pre-specified criteria for a 'significant' p-value for secondary cardiovascular outcomes. Neither plasma NE or PR3 levels were significantly associated with any cardiovascular outcome after adjusting for confounding variables (Table 3). No significant interactions with treatment allocation and sex were found in all these analyses.

At baseline, both plasma NE and PR3 levels were higher in subjects with any microvascular disease, especially nephropathy (all $p < 0.001$, Table 4). After adjusting

Table 1. Subject baseline characteristics.

	Placebo ($n = 1000$)	Fenofibrate ($n = 1000$)	p
Age (year)	62.0 (6.8)	62.2 (6.9)	0.566
Male (%)	50 (50.0)	50 (50.0)	1.000
White (%)	931 (93.1)	923 (92.3)	0.492
BMI (kg/m^2)	30.3 (27.2–34.1)	30.3 (27.0–34.3)	0.732
Waist-to-hip ratio	0.92 (0.86–0.98)	0.93 (0.86–0.97)	0.733
Known diabetes duration (year)	5 (2–9)	4 (2–9)	0.326
Prior history of CVD (%)	171 (17.1)	198 (19.8)	0.120
Smoking (%)			
Current	87 (8.7)	77 (7.7)	0.534
Former	501 (50.1)	490 (49.0)	
Never	412 (41.2)	433 (43.3)	
Fasting insulin (mU/L)	12 (8–19)	12 (8–19)	0.527
Fasting glucose (mmol/L)	8.4 (6.9–10.4)	8.4 (6.9–10.3)	0.806
HbA1c (%)	6.8 (6.0–7.8)	6.7 (6.0–7.6)	0.520
HOMA-IR	1.80 (1.21–2.72)	1.83 (1.16–2.70)	0.633
Systolic BP (mmHg)	139 (15)	139 (15)	0.377
Diastolic BP (mmHg)	81 (8)	81 (8)	0.386
Total cholesterol (mmol/L)	5.06 (0.71)	5.06 (0.70)	0.879
HDL-C (mmol/L)	1.10 (0.26)	1.10 (0.26)	0.549
LDL-C (mmol/L)	3.08 (0.65)	3.08 (0.64)	0.970
Triglycerides (mmol/L)	1.75 (1.35–2.35)	1.78 (1.37–2.40)	0.406
ApoA-I (g/L)	1.30 (0.21)	1.30 (0.22)	0.823
ApoA-II (g/L)	0.35 (0.07)	0.35 (0.07)	0.832
ApoB (g/L)	0.97 (0.18)	0.97 (0.18)	0.483
Fibrinogen (g/L)	3.58 (0.72)	3.61 (0.75)	0.411
Plasma creatinine (μmol/L)	74.2 (15.6)	74.5 (16.4)	0.618
eGFR (mL/min/1.73 m^2)	85.7 (14.1)	85.4 (14.8)	0.686
Homocysteine (μmol/L)	9.3 (7.8–11.0)	9.2 (7.6–11.0)	0.493
hs-CRP (mg/L)	3.4 (1.5–6.7)	3.1 (1.6–7.3)	0.969
Baseline glucose-lowering medication (%)			
Diet alone	290 (29.0)	320 (32.0)	0.288
Oral agent alone	612 (61.2)	573 (57.3)	
Insulin alone	45 (4.5)	55 (5.5)	
Insulin + oral agent	53 (5.3)	52 (5.2)	
NE (ng/mL)	70.1 (50.1–105.7)	70.5 (52.3–107.4)	0.324
PR3 (ng/mL)	43.5 (32.1–58.0)	43.5 (31.2–58.1)	0.450

Apo: apolipoprotein; BMI: body mass index; BP: blood pressure; CVD: cardiovascular disease; eGFR: estimated glomerular filtration rate; HbA1c: glycosylated haemoglobin; HDL-C: high-density lipoprotein cholesterol; HOMA-IR: homeostasis model assessment estimate of insulin resistance; hs-CRP: high-sensitivity C-reactive protein; LDL-C: high-density lipoprotein cholesterol; NE: neutrophil elastase; PR3: proteinase 3.
Data are expressed as mean (standard derivation), median (interquartile range) or *n* (%), where appropriate.

for confounding variables, higher baseline plasma NE and PR3 levels were both associated with higher odds of total microvascular disease, nephropathy and neuropathy (all $p < 0.01$, Table 4). No significant interaction with sex was found. In a separate analysis, the association of baseline plasma NE and PR3 levels with total microvascular disease, nephropathy and neuropathy remained significant after further adjustment for hs-CRP (all $p < 0.01$).

Baseline plasma NE and PR3 levels did not differ significantly between participants with and without new on-trial microvascular complications (Table 5). After adjusting for confounding variables, both baseline NE and PR3 levels were not significantly associated with new on-trial microvascular disease (Table 5). Elevated baseline NE levels were associated with new on-trial neuropathy and microvascular amputation ($p = 0.021$ and 0.041), but these associations did not meet the more rigorous pre-specified criteria for a 'significant' p value for secondary microvascular outcomes. No significant interaction with treatment allocation and sex was found (Table 5). In a separate analysis, the association of elevated baseline NE levels with new on-trial neuropathy and microvascular amputation

Table 2. Association of different CVD risk factors with baseline NE and PR3 levels using univariable and multivariable linear regression analysis with ln-transformed biomarker levels as the dependent variable.

Characteristics	NE				PR3			
	Univariable analysis		Multivariable analysis		Univariable analysis		Multivariable analysis	
	% Change (95% CI)	*p*	% Change (95% CI)	*p*	% Change (95% CI)	*p*	% Change (95% CI)	*p*
Age (year)	0.6 (0.1, 1.1)	0.018			0.4 (0.1, 0.8)	0.023	0.6 (0.2, 1.0)	0.004
Male	1.3 (−5.1, 8.1)	0.706			−6.7 (−11.4, −1.8)	0.008	−12.6 (−18.7, −6.1)	<0.001
White	−0.4 (−12.1, 13.0)	0.955			8.4 (−1.8, 19.7)	0.109		
BMI (kg/m^2)	1.0 (0.4, 1.5)	<0.001			1.4 (1.0, 1.9)	<0.001		
Waist-to-hip ratio	78.1 (18.2, 168.3)	0.006	79.5 (15.7, 178.4)	0.009	39.2 (0.7, 92.4)	0.045	106.9 (39.4, 207.1)	<0.001
Known diabetes duration (year)	0.0 (−0.6, 0.5)	0.906			−0.4 (−0.8, 0.1)	0.096	−0.8 (−1.3, −0.3)	0.001
Prior history of CVD	5.7 (−2.8, 15.0)	0.195			6.7 (−0.1, 14.1)	0.054		
Current smoker	12.5 (−0.7, 27.4)	0.064			9.6 (−0.7, 20.9)	0.068		
Former smoker	−0.4 (−6.9, 6.7)	0.918			−1.0 (−6.2, 4.5)	0.717		
Fasting insulin (mU/L)	0.3 (0.0, 0.5)	0.033			0.3 (0.1, 0.5)	<0.001		
Fasting glucose (mmol/L)	1.0 (−0.2, 2.2)	0.111			0.8 (−0.1, 1.8)	0.096		
HbA1c (%)	3.1 (0.6, 5.6)	0.014			2.9 (0.9, 4.8)	0.004		
HOMA-IR	4.2 (1.6, 6.9)	0.001	3.4 (0.6, 6.2)	0.015	5.6 (3.5, 7.7)	<0.001		
Systolic BP (mmHg)	−0.2 (−0.4, 0.0)	0.091	−0.3 (−0.5, −0.1)	0.014	0.0 (−0.2, 0.1)	0.747		
Diastolic BP (mmHg)	−0.1 (−0.5, 0.3)	0.568			0.1 (−0.3, 0.4)	0.748		
Total cholesterol (mmol/L)	−4.4 (−8.8, 0.1)	0.055			−4.4 (−7.9, −0.8)	0.016		
HDL-C (mmol/L)	−15.0 (−25.0, −3.6)	0.011			−11.7 (−20.0, −2.6)	0.013		
LDL-C (mmol/L)	−0.5 (−5.4, 4.7)	0.847			−2.5 (−6.3, 1.4)	0.208		
Triglycerides (mmol/L)	−2.8 (−6.5, 0.9)	0.139	−4.1 (−7.9, −0.1)	0.044	−0.9 (−3.9, 2.1)	0.551		
ApoA-I (g/L)	−17.4 (−29.2, −3.6)	0.015			−13.2 (−23.1, −1.9)	0.023		
ApoA-II (g/L)	−59.8 (−75.2, −35.0)	<0.001	−51.8 (−70.6, −20.8)	0.004	−53.4 (−68.1, −32.0)	<0.001	−43.8 (−61.4, −18.0)	0.003
ApoB (g/L)	−12.3 (−27.0, 5.4)	0.161			−12.7 (−24.5, 0.8)	0.065		
Fibrinogen (g/L)	6.5 (1.8, 11.3)	0.006			12.2 (8.3, 16.1)	<0.001		
Plasma creatinine (μmol/L)	0.3 (0.1, 0.5)	0.001			0.1 (0.0, 0.3)	0.090	0.3 (0.1, 0.5)	0.003
eGFR (mL/min/1.73 m^2)	−0.4 (−0.6, −0.2)	<0.001	−0.4 (−0.6, −0.1)	0.004	−0.3 (−0.5, −0.2)	<0.001		
Homocysteine (μmol/L)	2.3 (1.2, 3.5)	<0.001	1.7 (0.4, 2.9)	0.008	1.4 (0.6, 2.3)	0.001		
hs-CRP (mg/L)	1.4 (0.9, 2.0)	<0.001	1.3 (0.8, 1.9)	<0.001	2.2 (1.8, 2.6)	<0.001	2.1 (1.6, 2.5)	<0.001
Baseline glucose-lowering medication								
Oral agent alone	6.3 (−1.1, 14.4)	0.097			5.9 (0.0, 12.2)	0.049	8.3 (2.2, 14.8)	0.007
Insulin alone	15.4 (−1.4, 35.1)	0.074			13.8 (0.5, 28.8)	0.041	19.9 (5.4, 36.5)	0.006
Insulin + oral agent	29.4 (11.0, 51.0)	0.001			22.2 (8.3, 38.0)	0.001	21.7 (7.2, 38.1)	0.002

Apo: apolipoprotein; BMI: body mass index; BP: blood pressure; CI: confidence interval; CVD: cardiovascular disease; eGFR: estimated glomerular filtration rate; HbA1c: glycosylated haemoglobin; HDL-C: high-density lipoprotein cholesterol; HOMA-IR: homeostasis model assessment estimate of insulin resistance; hs-CRP: high-sensitivity C-reactive protein; LDL-C: high-density lipoprotein cholesterol; NE: neutrophil elastase; PR3: proteinase 3.
The percentage change was estimated by the exponentiation of coefficients from linear regression analysis. For baseline NE levels, fasting insulin, total cholesterol and plasma creatinine were not entered into the multivariable model due to multi-collinearity issues with HOMA-IR, apoB and eGFR. For baseline PR3 levels, fasting insulin, total cholesterol and eGFR were not entered into the multivariable model due to multi-collinearity issues with HOMA-IR, apoB and plasma creatinine. The variance inflation factors of all the predictor variables in the multivariable model are <5.0.

remained similar after further adjusting for hs-CRP levels ($p=0.020$ and 0.045 respectively).

In a sub-sample of 100 subjects from the fenofibrate treatment group and 100 sex-matched subjects from the placebo group, plasma biomarker levels were also measured at additional time-points. Supplemental Table 3 shows the clinical characteristics of these 200 subjects at baseline. As shown in Table 6, both NE and PR3 levels did not change significantly over time in both treatment groups, and fenofibrate treatment did not affect their levels.

Discussion

We believe this is the first study of the relationship of both circulating NE and PR3 levels with traditional risk factors, cardiovascular and microvascular outcomes and of fenofibrate effects in a large-scale, well-designed clinical trial in adults with T2DM. In the present FIELD trial sub-study, significant correlations of these inflammation-related biomarkers with vascular risk factors were identified. Higher baseline NE levels were associated with new on-trial stroke and CVD mortality, and higher PR3 levels with prior history of any CVD at baseline and new on-trial stroke, but not with more stringent criteria. Participants with any of the composite (total) microvascular endpoints or with nephropathy at baseline had higher baseline NE and PR3 levels than those without microvascular disease. Only baseline NE was associated with new on-trial neuropathy and amputation, although the association was no longer significant after adjusting for multiple comparisons. Fenofibrate did not alter NE or PR3 levels.

In the present study, we identified some clinical characteristics as major determinants of plasma NE and PR3 levels in adults with T2DM. For example, higher waist-to-hip ratio, homocysteine levels and hs-CRP levels and

Table 3. Association of baseline plasma NE and PR3 levels with on-trial CVD outcome events over 5 years.

Outcome	Levels (ng/mL)		*p*	Model 1		Model 2	
	No event	Event		HR (95% CI)	*p*	HR (95% CI)	*p*
Primary outcome							
Total CVD events (242 events)							
NE	70.1 (50.7–105.6)	72.7 (52.3–108.5)	0.224	1.01 (0.90–1.14)	0.864	1.01 (0.89–1.15)	0.849
PR3	43.3 (31.5–57.8)	44.7 (32.7–59.4)	0.202	1.05 (0.94–1.17)	0.392	1.06 (0.94–1.19)	0.341
Secondary outcomes							
CHD event (104 events)							
NE	70.3 (51.0–105.7)	70.9 (50.1–112.3)	0.757	0.93 (0.72–1.20)	0.581	0.88 (0.66–1.18)	0.407
PR3	43.3 (31.7–57.7)	47.1 (28.4–62.9)	0.476	0.98 (0.79–1.20)	0.816	0.92 (0.72–1.18)	0.513
Total stroke (62 events)							
NE	70.2 (50.6–105.6)	79.4 (60.6–115.8)	0.032	1.06 (0.88–1.27)	0.541	1.07 (0.90–1.29)	0.439
PR3	43.2 (31.5–57.9)	48.8 (38.9–65.3)	0.015	1.06 (0.88–1.28)	0.560	1.05 (0.86–1.30)	0.624
CVD mortality (50 events)							
NE	70.2 (50.6–105.7)	85.3 (59.0–112.5)	0.043	1.02 (0.79–1.31)	0.877	0.99 (0.75–1.30)	0.949
PR3	43.3 (31.7–57.8)	49.5 (27.1–70.9)	0.196	1.05 (0.86–1.29)	0.613	1.00 (0.78–1.29)	0.995
Coronary and carotid revascularization (126 events)							
NE	70.4 (51.1–106.6)	67.5 (46.6–100.4)	0.312	0.97 (0.79–1.18)	0.738	0.99 (0.81–1.21)	0.903
PR3	43.6 (31.7–58.2)	41.1 (31.4–56.0)	0.420	1.02 (0.86–1.21)	0.826	1.06 (0.91–1.24)	0.452
Hospitalization for angina pectoris (79 events)							
NE	70.3 (51.0–106.6)	66.3 (48.8–99.4)	0.489	0.84 (0.55–1.30)	0.438	0.85 (0.52–1.38)	0.503
PR3	43.5 (31.6–58.3)	42.3 (34.2–51.9)	0.807	0.88 (0.62–1.24)	0.462	0.86 (0.57–1.30)	0.462

CHD: coronary heart disease; CI: confidence interval; CVD: cardiovascular disease; HR: hazards ratio; NE: neutrophil elastase; PR3: proteinase 3. Biomarker levels are expressed as median (interquartile range) and *p*-value was estimated by Wilcoxon rank-sum test. HR was expressed per 1 standard deviation (291.2 ng/mL for NE and 64.87 ng/mL for PR3) increase in biomarker levels. Model 1: Data were adjusted for treatment allocation. Model 2: For total CVD events, data were further adjusted for age, sex, known diabetes duration, prior history of CVD, smoking, BMI, HbA1c, HOMA-IR, systolic BP, HDL-C, LDL-C, triglycerides, fibrinogen, plasma creatinine and homocysteine at baseline. For CHD event, data were further adjusted for age, sex, prior history of CVD, smoking, BMI, HbA1c, systolic BP and HDL-C at baseline. For total stroke, data were further adjusted for age, sex, prior history of CVD, BMI and systolic BP at baseline. For CVD mortality, data were further adjusted for age and BMI at baseline. For coronary and carotid revascularization, data were further adjusted for age, sex, known diabetes duration, prior history of CVD, BMI, HbA1c, systolic BP, HDL-C, LDL-C, triglyceride and homocysteine at baseline. For hospitalization for angina pectoris, data were adjusted for known diabetes duration, prior history of CVD, systolic BP, HDL-C, fibrinogen, plasma creatinine (NE only) and homocysteine (PR3 only) at baseline.

Table 4. Association of NE and PR3 levels with microvascular diseases at baseline.

Baseline microvascular disease	*n*		Levels (ng/mL)		*p*	Model 1		Model 2	
	Without	With	Without	With		OR (95% CI)	*p*	OR (95% CI)	*p*
Total microvascular disease									
NE	1423	577	68.2 (49.6–105.1)	75.8 (56.0–112.7)	<0.001*	1.15 (1.04–1.26)	0.004*	1.18 (1.07–1.30)	0.001*
PR3	1423	577	42.1 (31.1–56.6)	47.3 (33.8–63.8)	<0.001*	1.20 (1.08–1.33)	<0.001*	1.22 (1.10–1.35)	<0.001*
Nephropathy									
NE	1569	421	67.9 (49.3–102.9)	79.9 (57.3–126.2)	<0.001*	1.13 (1.03–1.24)	0.008*	1.17 (1.06–1.30)	0.001*
PR3	1569	421	42.1 (31.1–56.3)	49.2 (35.0–67.9)	<0.001*	1.19 (1.08–1.31)	<0.001*	1.22 (1.10–1.35)	<0.001*
Neuropathy									
NE	1877	119	69.9 (50.6–106.2)	76.7 (59.7–107.5)	0.071	1.19 (1.07–1.32)	0.001*	1.22 (1.09–1.36)	<0.001*
PR3	1877	119	43.0 (31.6–57.8)	48.2 (32.8–61.9)	0.058	1.21 (1.08–1.34)	<0.001*	1.23 (1.10–1.38)	<0.001*
Retinopathy									
NE	1846	154	70.4 (50.7–106.6)	69.0 (51.0–99.5)	0.743	1.04 (0.90–1.20)	0.578	1.05 (0.90–1.22)	0.529
PR3	1846	154	43.3 (31.6–58.2)	46.5 (33.2–56.5)	0.761	1.02 (0.87–1.19)	0.823	1.03 (0.86–1.22)	0.767

CI: confidence interval; OR: odds ratio; NE: neutrophil elastase; PR3: proteinase 3.

Biomarker levels are expressed as median (interquartile range) and *p*-value was estimated by Wilcoxon rank-sum test. OR was expressed per 1 standard deviation (291.2 ng/mL for NE and 64.87 ng/mL for PR3) increase in biomarker levels. There are 10 and 4 patients with missing data on baseline nephropathy and neuropathy, respectively. For amputation, data were not shown because there were only four cases and reliable estimates cannot be estimated. Model 1: Unadjusted model. Model 2: For total microvascular disease and nephropathy, data were adjusted for age, sex, known diabetes duration, prior history of CVD, smoking, BMI, HbA1c, HOMA-IR, systolic BP, HDL-C, LDL-C, triglycerides, fibrinogen, plasma creatinine, homocysteine and glucose-lowering medication at baseline. For neuropathy, data were adjusted for sex, known diabetes duration, prior history of CVD, BMI, systolic BP, triglycerides, fibrinogen, homocysteine and glucose-lowering medication at baseline. For retinopathy, data were adjusted for age, known diabetes duration, prior history of CVD, smoking, HbA1c, HOMA-IR, systolic BP, HDL-C, LDL-C, fibrinogen, homocysteine and glucose-lowering medication at baseline.

**p*-Values which meet the pre-specified criteria for a 'significant' *p*-value for primary and secondary microvascular outcomes.

Table 5. Association of baseline biomarker levels with new on-trial total microvascular diseases.

Outcomes	Levels (ng/mL)		*p*	Model 1		Model 2	
	Without	With		OR/HR (95% CI)	*p*	OR/HR (95% CI)	*p*
New total microvascular disease (*n* = 2000, 524 events)							
NE (per SD 291.2 ng/mL)	70.5 (51.0–105.6)	70.1 (50.2–107.5)	0.775	1.06 (0.97–1.16)	0.223	1.05 (0.95–1.15)	0.343
PR3 (per SD 64.87 ng/mL)	43.3 (31.5–58.2)	44.0 (32.9–57.8)	0.411	1.06 (0.96–1.16)	0.244	1.04 (0.94–1.15)	0.439
New nephropathy (*n* = 1823, 312 events)							
NE (per SD 303.6 ng/mL)	70.1 (50.7–104.7)	69.2 (48.9–107.2)	0.804	1.02 (0.91–1.15)	0.696	0.99 (0.88–1.12)	0.914
PR3 (per SD 67.22 ng/mL)	43.4 (31.6–57.7)	42.4 (31.6–57.7)	0.913	1.03 (0.92–1.15)	0.612	0.99 (0.88–1.13)	0.933
New neuropathy (*n* = 1797, 158 events)							
NE (per SD 264.0 ng/mL)	69.3 (50.4–105.7)	69.5 (51.4–101.2)	0.997	1.12 (1.01–1.25)	0.039	1.15 (1.02–1.29)	0.021
PR3 (per SD 57.7 ng/mL)	43.0 (31.5–57.5)	41.9 (32.5–57.4)	0.833	1.10 (0.97–1.24)	0.141	1.10 (0.97–1.25)	0.143
New retinopathy (*n* = 2000, 94 events)							
NE (per SD 291.2 ng/mL)	70.3 (50.9–105.8)	71.3 (49.8–120.1)	0.501	1.04 (0.88–1.23)	0.640	1.09 (0.91–1.29)	0.360
PR3 (per SD 64.87 ng/mL)	43.1 (31.6–58.0)	48.3 (35.0–58.4)	0.114	1.06 (0.92–1.23)	0.400	1.13 (0.94–1.35)	0.199
Two-step progression of ETDRS grade (*n* = 172, 18 events)							
NE (per SD 542.8 ng/mL)	71.6 (51.2–108.6)	80.2 (39.5–101.0)	0.814	0.30 (0.02–4.64)	0.391		
PR3 (per SD 98.88 ng/mL)	43.3 (31.8–65.1)	47.2 (34.0–54.3)	0.954	0.47 (0.10–2.16)	0.335		
New amputation (*n* = 2000, 43 events)							
NE (per SD 291.2 ng/mL)	70.2 (50.6–105.7)	82.9 (61.4–112.8)	0.029	1.13 (0.98–1.32)	0.096	1.18 (1.01–1.39)	0.041
PR3 (per SD 64.87 ng/mL)	43.3 (31.6–58.1)	51.1 (34.3–57.8)	0.108	1.10 (0.92–1.31)	0.286	1.14 (0.93–1.40)	0.223

CI: confidence interval; HR: hazards ratio; NE: neutrophil elastase; OR: odds ratio; PR3: proteinase 3; SD: standard deviation.
Biomarker levels are expressed as median (interquartile range) and *p*-value was estimated by Wilcoxon rank-sum test. OR or HR was expressed per 1 SD increase in biomarker levels. For new on-trial retinopathy requiring laser treatment and amputation, analysis was done using Cox regression and HR was reported. For new on-trial total microvascular disease, nephropathy and neuropathy, analysis was done using logistic regression and OR was reported. Model 1: Data were adjusted for treatment allocation. Model 2: For total microvascular disease and nephropathy, data were further adjusted for age, sex, known diabetes duration, prior history of CVD, smoking, BMI, HbA1c, HOMA-IR, systolic BP, HDL-C, LDL-C, triglycerides, fibrinogen, plasma creatinine, homocysteine and glucose-lowering medication at baseline. For neuropathy, data were further adjusted for age, sex, known diabetes duration, prior history of CVD, smoking, BMI, HbA1c, systolic BP, LDL-C, triglycerides, plasma creatinine and homocysteine and at baseline. For retinopathy, data were further adjusted for age, known diabetes duration, HbA1c, systolic BP, homocysteine and glucose-lowering medication at baseline. For amputation, data were further adjusted for known diabetes duration, HbA1c and fibrinogen. For two-step progression of ETDRS grade, no further adjustment was performed due to low number of cases.

Table 6. Effect of fenofibrate treatment on the relative change in biomarker levels.

Time-point	Geometric mean (95% CI)		Relative change (95% CI)[a]				Ratio of 5-year change (fenofibrate/placebo)	*p*
	Placebo	Fenofibrate	Placebo	*p*	Fenofibrate	*p*		
PR3 (ng/mL)								
Baseline	45.5 (41.5–49.8)	41.2 (37.0–45.9)	Referent		Referent			
Randomization	43.3 (39.7–47.2)	41.1 (37.7–44.7)	−4.7% (−10.8 to +1.7)	0.147	−0.3% (−7.3 to +7.2)	0.930	1.05 (0.95–1.15)	0.363
Year 1	46.2 (42.4–50.5)	46.3 (41.1–52.2)	+1.7% (−6.0 to +10.1)	0.667	+12.4% (−2.0 to +28.9)	0.093	1.10 (0.94–1.29)	0.212
Year 5 or study close	44.2 (40.4–48.4)	43.0 (37.6–49.2)	−2.8% (−10.4 to +5.4)	0.493	+4.4% (−9.9 to +21.0)	0.565	1.07 (0.91–1.27)	0.403
NE (ng/mL)								
Baseline	72.4 (64.0–82.0)	65.1 (55.5–76.3)	Referent		Referent			
Randomisation	71.4 (64.2–79.4)	67.9 (60.6–76.0)	−1.4% (−10.4 to +8.5)	0.764	+4.3% (−6.7 to +16.6)	0.453	1.06 (0.91–1.23)	0.444
Year 1	75.5 (66.4–85.8)	80.7 (67.6–96.2)	+4.3% (−7.5 to +17.5)	0.493	+24.0% (+1.9 to +50.9)	0.032	1.19 (0.95–1.50)	0.136
Year 5 or study close	70.6 (63.0–79.1)	73.5 (61.9–87.3)	−2.3% (−13.9 to +11.0)	0.721	+13.0% (−8.9 to +40.2)	0.263	1.16 (0.90–1.48)	0.252

CI: confidence interval; NE: neutrophil elastase; PR3: proteinase 3; SD: standard deviation.
Treatment effect was derived from the ratio of the relative changes in the fenofibrate group to that in the placebo group. The relative changes from the baseline to each follow-up visit between treatment groups were compared by *t*-test after ln-transformation. *p*-Values between baseline and each follow-up visit in each treatment group are estimated by the paired *t*-test.
[a]Derived as geometric mean of change (95% CI) from ln-transformed data (i.e. 100 × exp(mean change) − 1]).

lower apoA-II levels are independent predictor of higher levels of both NE and PR3. These are all factors often associated with higher levels of inflammation.[30,31] However, these clinical characteristics only explained about 4.1% and 8.3% of the variations of plasma NE and PR3 levels, respectively. This suggests that there could be some other as yet unidentified major determinants, such as inherited factors. As expected, NE levels are found to correlate strongly with PR3 levels, because they are both serine proteases secreted from neutrophils under inflammatory conditions. Fenofibrate has anti-inflammatory effect through its activation of PPARα signalling pathway, which inhibits the expression of different acute-phase proteins and pro-inflammatory cytokines such as IL-1β, IL-6 and tumor necrosis factor (TNF)-α.[15] In the present study, fenofibrate treatment did not affect plasma NE and PR3 levels study after 6 weeks and up to 5-years follow-up, suggesting that the regulation of NE and PR3 expression is independent of the PPARα signalling pathway.

The clinical trial, Canakinumab Anti-inflammatory Thrombosis Outcomes Study (CANTOS), suggested that reducing inflammation by targeting the IL-1β innate immunity pathway can significantly reduce cardiovascular event rates in the absence of lipid lowering.[32] Therefore, NE and PR3 could be novel targets for reducing inflammation for CVD prevention. They are released by neutrophils at the site of inflammation. PR3 can induce apoptosis through a caspase-like activity on endothelial cells,[33] and release proinflammatory cytokines such as TNF-α, IL-1β and IL-18 from their nascent membrane-bound precursor form.[3] PR3 can also induce insulin resistance in the mouse and inhibition of PR3 activity can increase glucose clearance.[11] On the other hand, NE can promote chemokine and cytokine activation and degradation, cytokine receptor shedding, proteolysis of cytokine binding proteins and activation of different specific cell surface receptors.[3,34] Moreover, recent studies in primary mouse and human hepatocytes have also shown that NE treatment directly leads to insulin receptor substrate (IRS) protein degradation, lower insulin signaling, higher glucose production and cellular insulin resistance.[7] Deletion of NE in diet-induced obese mice can reduce adipose tissue inflammation, improve glucose tolerance and increase insulin sensitivity.[7] Therefore, both PR3 and NE have been suggested as therapeutic targets or biomarkers for inflammatory diseases and related conditions such as obesity,[8] insulin resistance,[7,8] type 1 diabetes,[13] cytoplasmic autoantibody-associated vasculitides[35] and inflammatory vascular disease.[36]

Despite studies showing a potential role of neutrophil serine protease in inflammation, and atherosclerosis,[6–10] there only a few human studies on relationship of circulating NE and PR3 levels with CVD or its risk factors. In a study of 900 patients with acute myocardial infarction (21.3% with a history of diabetes), PR3 was a significant predictor of death or heart failure over a median follow-up period of 347 days, with an additive predictive value over N-terminal pro-B-type natriuretic peptide.[14] Elevation of serum NE activity has also been found in obese subjects compared to the lean controls[8] and this is in keeping with BMI being a determinant of NE (and also PR3) levels in the present study. In the present large study of NE and PR3 levels in T2DM with a median of 5-years follow-up, we found a trend for an association of higher NE with on-trial stroke and CVD mortality and higher PR3 with on-trial stroke, but not with other on-trial CVD events, although these associations lost significance after adjusting for confounding factors, suggesting that some of their risk mediation may be through these adjustment variables (such as BMI).

Higher circulating levels of PR3 and NE were associated with baseline total microvascular disease, especially nephropathy and neuropathy. In fact, neutrophil serine proteinase can regulate changes in glomerular permeability through their proteolytic property.[37] It has been reported that NE expression in renal proximal tubules is increased in mouse model of acute kidney injury, and NE treatment can cause proximal tubule cell injury in cell culture studies.[38] Inhibitors of NE have been also shown to reduce diabetic neuropathy in mouse.[39] However, circulating NE and PR3 levels did not predict new on-trial microvascular events in the present study. Further study is needed to elucidate the role of PR3 and NE in microvascular disease.

As plasma NE and PR3 are elevated in T2DM, our study suggests that NE and PR3 may not be useful as biomarkers for evaluating future cardiovascular and microvascular complications in patients who have already elevated NE and PR3 levels due to the presence of T2DM. Nevertheless, this does not exclude the causal role of chronic inflammation in chronic complications in T2DM. As NE and PR3 play important roles in the production of mature cytokine forms by proteolytic cleavage of their membrane-bound precursor, the present results suggested that upstream inflammatory signaling pathway mediators, instead of these proteolytic cleavage processes, are more likely to be useful potential therapeutic targets for chronic complications.

Study strengths include that the FIELD study is a large, well-designed and conducted trial with very well-characterised subjects with validated data on multiple CVD and microvascular events. These outcome events were pre-specified and adjudicated by a committee masked to study treatment allocation with standardised assessments. The use of more stringent pre-specified criteria for the *p* values for statistical significance of the secondary and tertiary outcomes in this study can help reduce the chance of false positive results due to multiple testing. However, there are some study limitations. The number of cases for some CVD and microvascular events, especially

amputation, were small in this FIELD sub-study ($n = 2000$). Moreover, we only assessed the chronic change in circulating levels of NE and PR3, but not the acute change in their levels and their local tissue-specific expressions, which are difficult to achieve in large numbers and in a trial setting. We also have not measured the circulating levels of α1-antitrypsin, which inhibits the enzymatic activity of serine proteinases, including NE and PR3.[4] Finally, the study results may not be generalisable into healthy people, people without diabetes or people with type 2 diabetes who are dis-similar to those studied in the FIELD trial. Further studies with different study design or subject characteristics, are merited.

In summary, despite the potential roles of NE and PR3 in inflammatory diseases and related conditions such as obesity, insulin resistance and vascular disease, circulating NE and PR3 levels are not independently and robustly associated with cardiovascular and microvascular outcome events in T2DM patients, nor are their circulating levels altered by fenofibrate.

Declaration of conflicting interests

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Supplemental material

Supplemental material for this article is available online.

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