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ARTICLE

Relationship of fibroblast growth factor 21 with baseline and new on-study microvascular disease in the Fenofibrate Intervention and Event Lowering in Diabetes study

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Abstract

Aims/hypothesis Baseline circulating fibroblast growth factor 21 (FGF21) levels can predict total cardiovascular disease events in the Fenofibrate Intervention and Event Lowering in Diabetes (FIELD) study. This paper describes the relationship of baseline FGF21 levels and new on-study microvascular disease in patients with type 2 diabetes from the FIELD study.

Methods Baseline FGF21 levels were measured in plasma by enzyme-linked immunosorbent assay in 9697 study participants. Total microvascular disease was defined as the presence of any nephropathy, retinopathy, neuropathy and/or microvascular amputation. The relationship between FGF21 levels and microvascular disease was assessed by multivariable logistic regression.

Results Higher baseline FGF21 levels were found in patients with baseline total microvascular disease ($p<0.001$). The association remained significant after adjusting for potential confounding factors (OR [95% CI] 1.13 [1.08, 1.19] per SD increase in $\log_e$-transformed FGF21 levels, $p<0.001$). Of 6465 patients without baseline total microvascular disease, 1517 developed new on-study total microvascular disease over 5 years of follow-up. Higher baseline FGF21 levels were associated with a higher risk of new on-study total microvas-

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cular disease after adjusting for potential confounding factors (OR [95% CI] 1.09 [1.02, 1.16] per SD increase in $\log_e$-transformed FGF21 levels, $p=0.01$). Addition of FGF21 levels in a model of new on-study total microvascular disease with established risk factors significantly, but modestly, increased the integrated discrimination improvement and the net reclassification improvement (both $p<0.01$).

Conclusions/interpretation Higher baseline FGF21 levels are seen in patients with type 2 diabetes and established microvascular disease, and predict the future development of new microvascular disease.

Keywords Fenofibrate · Fenofibrate Intervention and Event Lowering in Diabetes (FIELD) study · Fibroblast growth factor 21 · Microvascular disease · Nephropathy

Abbreviations

CVD	Cardiovascular disease
ETDRS	Early Treatment Diabetic Retinopathy Study
FGF21	Fibroblast growth factor 21
FIELD	Fenofibrate Intervention and Event Lowering in Diabetes
IDI	Integrated discrimination improvement
IQR	Interquartile range
NRI	Net reclassification improvement
UACR	Urinary albumin/creatinine ratio

Introduction

Fibroblast growth factor 21 (FGF21) is a novel metabolic regulator that plays an important role in glucose and lipid metabolism [1, 2]. Administration of recombinant FGF21 to mice with hyperglycaemia and insulin resistance decreases circulating glucose, triacylglycerol and fasting insulin levels and it improves glucose clearance during an oral glucose tolerance test without causing mitogenicity, hypoglycaemia or weight gain [1]. Human recombinant FGF21 also increases glucose uptake in both mouse and human adipocytes [1]. FGF21 regulates peroxisome proliferator-activated receptor-γ (PPAR-γ) and the glucose-lowering activity of thiazolidinediones [3, 4]. Despite these beneficial effects of FGF21 in glucose metabolism in animal studies, circulating FGF21 levels are often elevated in obesity, dyslipidaemia, insulin resistance, type 2 diabetes and coronary artery disease [2, 5–7]. The elevation in FGF21 levels may be a compensatory response to underlying metabolic stress or may be due to impaired FGF21 signalling leading to FGF21 resistance [8].

Microvascular diseases, such as nephropathy, neuropathy, retinopathy and amputation, are often found in people with type 2 diabetes. In the Fenofibrate Intervention and Event Lowering in Diabetes (FIELD) study, fenofibrate treatment has been demonstrated to reduce the risk of total cardiovascular disease (CVD) events as well as the risks of albuminuria, laser treatment for diabetic retinopathy and amputation in patients with type 2 diabetes over a follow-up period of 5 years [9–11]. Recently, we have reported that higher FGF21 levels at baseline predict a higher risk of total cardiovascular disease (CVD) events in patients with type 2 diabetes taking part in the FIELD study [12]. Previous studies have demonstrated an association between higher circulating FGF21 levels and diabetic nephropathy and retinopathy [13, 14]. However, these studies are limited by their cross-sectional design and there are no reports on the association of FGF21 levels with other microvascular diseases, such as neuropathy and amputation, in patients with type 2 diabetes. Therefore, in this study, we investigated the relationship between FGF21 levels and microvascular diseases in patients who had taken part in the FIELD trial, using both a cross-sectional and a prospective study design.

Methods

Study design The study design, baseline participant characteristics and major findings of the FIELD study have been described previously [9–11, 15, 16]. Briefly, the FIELD study was a double-blind placebo-controlled randomised trial, involving a total of 9795 patients with type 2 diabetes recruited into the study between February 1998 and November 2000 (International Standard Randomised Controlled Trial no. ISRCTN64783481). All patients were aged 50–75 years and were randomly allocated to receive once-daily co-micronised fenofibrate 200 mg or matching placebo for 5 years. The study was undertaken in accordance with the Declaration of Helsinki and Good Clinical Practice Guidelines and the protocol was approved by national and local ethics committees. All participants gave written informed consent.

Plasma FGF21 measurement Early-morning fasting baseline blood specimens had been obtained in all individuals prior to randomisation in the FIELD study. In the present study, baseline plasma samples were available for FGF21 measurement from 9697 (99.0%) out of 9795 patients from the FIELD study. FGF21 levels were measured using enzyme-linked immunosorbent assay kits (Antibody and Immunoassay Services, University of Hong Kong, Hong Kong) as described previously [7, 12, 17]. Briefly, a 60 μl plasma sample was diluted 1:1 (vol.:vol.) with assay diluent and analysed together with quality controls according to manufacturer's instructions. The intra-assay and inter-assay coefficients of variation were <6%. All samples were analysed masked for patient identity and study treatment allocation.

Microvascular disease The primary outcome in this analysis was total microvascular disease (defined as the presence of any nephropathy, retinopathy, neuropathy and/or microvascular amputation) at baseline (baseline microvascular disease) or which developed during the follow-up (on-study microvascular disease). The secondary outcomes were the four individual components of total microvascular disease (i.e. nephropathy, retinopathy, neuropathy and microvascular amputation). Information on microvascular diseases in the FIELD study has been given elsewhere [9–11, 18].

Non-traumatic amputation was a pre-specified tertiary endpoint of the FIELD trial. All amputations that had occurred during the study follow-up (on-study amputations) were adjudicated by two clinicians (separately masked to treatment allocation) and any discrepancies were resolved by mutual agreement [11]. Pre-study amputations were adjudicated in the same fashion [11]. In the present study, we only included amputations of digits and forefoot without previous or concurrent large-vessel disease (including angioplasty and bypass surgery) in the same limb or evidence of causative embolism. These amputations were classified as minor amputations without known large-vessel disease and were judged to be related to microvascular disease [11].

Any self-reported history of retinopathy was recorded at baseline but retinal photographs were not gathered routinely from all participants in the main study [10]. However, all instances of laser photocoagulation therapy for diabetic retinopathy were recorded routinely at every follow-up visit and supporting documentation was requested for validation [10]. In the present study, retinopathy during follow-up was defined as the need for laser treatment for retinopathy, which was a pre-specified tertiary endpoint of the main FIELD study [9, 10]. Documentation regarding the use of laser treatment was adjudicated, masked to treatment allocation, by at least two ophthalmologists to ascertain the reason for each episode of laser treatment. Participants were excluded from the analysis if their laser treatment was identified as being for treatment of capsular opacity, iridotomy, retinal breaks or for other non-diabetic conditions. For amputation and retinopathy, patients were followed up at 4–6 month intervals for a median period of 5 years. In a substudy of 1012 patients, standardised retinal photography was performed and photographs were graded using Early Treatment Diabetic Retinopathy Study (ETDRS) criteria at baseline, 2 years, 5 years and at study close to determine the progression of retinopathy, which was defined as at least a two-step increase in ETDRS grade after 2 years or more of follow-up [10]. After excluding patients with missing data on ETDRS grade and baseline FGF21 levels, 937 patients were included in this analysis.

Neuropathy was determined if the patient's foot had absent sensation on monofilament testing. Nephropathy was defined as urinary albumin/creatinine ratio (UACR) ≥2.5 mg/mmol for men and ≥3.5 mg/mmol for women (i.e. including both microalbuminuria and macroalbuminuria). For neuropathy and nephropathy, the examination was performed at baseline, 2 years, 5 years and at study close. At each clinic visit, a spot urine sample was collected in addition to blood samples. Fasting patients emptied their bladders on waking and then produced a spot urine specimen in the container provided at the time of their blood collection. Urinary creatinine was measured by the Jaffe reaction using a Hitachi 917 analyser (Roche Diagnostics, Basel, Switzerland). Urine albumin was measured by immunonephelometry on an Array 360 analyser (Beckman, Fullerton, CA, USA). In a separate analysis, the association of baseline FGF21 levels with baseline and new on-study abnormal estimated GFR (<60 ml min^{-1} 1.73 m^{-2}), as well as abnormal estimated GFR at 8 weeks after treatment cessation at study close (washout substudy, n=661 [19]), was assessed. As fenofibrate increased plasma creatinine levels [19], the analysis of new on-study abnormal estimated GFR was limited to the placebo group only.

Other variables of interest The measurement of clinical characteristics and details of the primary endpoint and other outcomes of the FIELD trial have been described previously [9–12, 15–17]. Previous CVD comprised angina, myocardial infarction, coronary artery bypass grafting, percutaneous transluminal coronary angioplasty, stroke, peripheral vascular disease and revascularisation. Estimated GFR was calculated by the four-variable Modification of Diet in Renal Disease formula [20].

Statistical analysis Statistical analysis was performed using SPSS 22 (IBM, Armonk, NY, USA) or STATA 13.0 (StataCorp, College Station, TX, USA). Data are presented as mean (SD) or as a percentage. For variables with skewed distribution, data are presented as median (interquartile range [IQR]) and were analysed after nature $\log_e$-transformation. Comparison of clinical characteristics between two independent groups was performed by χ^2 test for categorical variables and t test for continuous variables.

In this analysis, the principal pre-specified analysis was the association of baseline FGF21 levels with both baseline and new on-study total microvascular disease. When there was a statistically significant association with total microvascular disease, analysis was then performed for its four individual components (i.e. secondary outcomes). For the prospective analysis of new on-study microvascular disease, patients with microvascular disease at baseline were excluded from the analysis. For the cross-sectional association of FGF21 levels with baseline microvascular disease, logistic regression was used to compute OR and 95% CI. For the prospective analysis, Cox regression was used to compute the HR and 95% CI for new on-study retinopathy and amputation, while logistic regression was used for new on-study total microvascular disease, nephropathy and neuropathy, and two-step progression

of ETDRS grade as examinations for these outcomes were performed at three or four visits only. For Cox regression analysis, the proportional hazard assumptions were checked using Schoenfeld residuals, and no significant deviation from the assumptions was found for any of the outcomes. As FGF21 levels were highly skewed, data were analysed after $\log_e$-transformation to achieve a more linear relationship with outcomes.

To investigate the assumption of linearity of the relationship between $\log_e$-transformed FGF21 levels and outcomes, $\log_e$-transformed FGF21 levels were categorised using deciles and plots of $\log_e$ HR/OR (obtained from logistic or Cox models including $\log_e$-transformed FGF21 decile variable) vs the median of the category were constructed. Also, deviation from linearity was tested by fitting models in which both a linear and a categorical version (decile) of the variable-grouped $\log_e$-transformed FGF21 level were included. For this method, the test of the overall effect of the individual categories assesses the significance of a non-linear component [21]. In these tests, data were also adjusted for treatment allocation for on-study diseases. These tests did not indicate a significant deviation from linearity for any of the baseline or on-study microvascular outcomes. Therefore, continuous $\log_e$-transformed levels of baseline FGF21 were used in the main analysis. In a separate analysis, tertiles of FGF21 levels were used and a test of trend was performed by fitting a regression model that included a discrete variable derived using the median FGF21 level of each tertile (as the ranges of FGF21 level within each tertile were not equal). The p values for 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. In all regression models, replacement of plasma creatinine by estimated GFR in adjustment model 2 made little difference to results (data not shown).

The incremental value of the addition of $\log_e$-transformed FGF21 levels in the risk prediction model for new on-study total microvascular disease was assessed by the change in Harrell's C statistic, integrated discrimination improvement (IDI) and net reclassification improvement (NRI) as described previously [22]. The goodness of fit of the models was assessed using the Hosmer–Lemeshow test. As the NRI method is highly sensitive to the chosen cut-points of risk and there are no pre-specified cut-points that can be applied to all different outcomes appropriately, the category-free NRI (NRI>0) approach was used with both 'event NRI' and 'nonevent NRI' calculated [23]. IDI and NRI were analysed using the packages 'PredictABEL' and 'nricens', respectively, in R Project for Statistical Computing version 3.1.1 (http://www.r-project.org/).

To account for the issue of multiple testing, a two-sided $p<0.05$ was pre-specified as significant for the primary outcome of total microvascular disease; $p<0.01$ was considered significant for the secondary outcomes. A two-sided $p<0.05$ was considered significant for all other analyses.

Results

Clinical characteristics according to baseline total microvascular disease Table 1 shows the clinical characteristics of 9697 patients according to FGF21 levels and the presence or absence of baseline total microvascular disease. Patients with baseline total microvascular disease were more likely to be older, men, former or current smokers and taking glucose-lowering medications and to have a prior history of CVD and longer duration of known diabetes than those without baseline total microvascular disease, regardless of the baseline FGF21 levels. Patients with baseline total microvascular disease also had higher HbA_{1c}, BP, fibrinogen and plasma creatinine and homocysteine and lower LDL-cholesterol and HDL-cholesterol levels than those without baseline total microvascular disease regardless of the baseline FGF21 levels. Of the 6465 patients without baseline total microvascular disease, 1517 developed on-study total microvascular disease during follow-up. Patients who developed new on-study total microvascular disease were more likely to be older, men, taking glucose-lowering medications and allocated to the fenofibrate treatment group and to have a history of CVD, longer known diabetes duration, higher systolic BP and higher plasma creatinine and homocysteine levels at baseline than those patients who did not develop new total microvascular disease, regardless of baseline FGF21 levels (see electronic supplementary material [ESM] Table 1).

Association of baseline FGF21 levels with baseline microvascular disease As shown in Table 2, patients with total microvascular disease at baseline had significantly higher FGF21 levels than those without such disease ($p<0.001$). Among the four individual components of total microvascular disease, there was a significant difference in FGF21 levels between patients with and without nephropathy at baseline ($p<0.001$), but not between those with and without neuropathy, retinopathy and amputation at baseline (all $p\geq0.01$). In multivariable regression analysis, a higher baseline $\log_e$-transformed FGF21 level was significantly associated with higher odds of baseline total microvascular disease and nephropathy after adjusting for other confounding factors (both $p<0.001$; Fig. 1 and ESM Table 2). Similar results were obtained when FGF21 tertiles were used in the analysis. No significant association was found for baseline neuropathy, retinopathy and amputation (all $p>0.01$).

Association of baseline FGF21 levels with new on-study microvascular disease As shown in Table 2, patients who developed new on-study total microvascular disease,

Table 1 Characteristics of patients at baseline according to baseline FGF21 levels and baseline total microvascular disease (N=9,697)

Characteristic	Plasma FGF21 tertile 1 (<239.3 pg/ml)		Plasma FGF21 tertile 2 (239.3 to <412.8 pg/ml)		Plasma FGF21 tertile 3 (≥412.8 pg/ml)	
	No total microvascular disease at baseline (n=2,311)	Total microvascular disease at baseline (n=921)	No total microvascular disease at baseline (n=2,168)	Total microvascular disease at baseline (n=1,065)	No total microvascular disease at baseline (n=1,986)	Total microvascular disease at baseline (n=1,246)
Age (years)	61.5±6.7	62.5±6.9***	61.8±6.9	63.2±6.8***	62.1±6.8	63.4±7.1***
Male sex	1526 (66.0)	721 (78.3)***	1278 (59.4)	757 (71.1)***	1022 (51.5)	765 (61.4)***
Smoking						
Never smoker	1000 (43.3)	380 (41.3)	948 (43.7)	355 (33.3)***	786 (39.6)	419 (33.6)***
Ex-smoker	1145 (49.5)	445 (48.3)	1053 (48.6)	596 (56.0)***	969 (48.8)	686 (55.1)***
Current smoker	166 (7.2)	96 (10.4)**	167 (7.7)	114 (10.7)**	231 (11.6)	141 (11.3)
History of CVD	353 (15.3)	227 (24.6)***	378 (17.4)	322 (30.2)***	422 (21.2)	400 (32.1)***
Known diabetes duration (years)	4 (2–9)	9 (3–14)***	4 (2–8)	7 (3–12)***	4 (2–8)	6 (3–11)***
BMI (kg/m^2)	28.3 (25.7–31.6)	29.0 (26.3–32.4)***	29.6 (26.9–33.2)	30.4 (27.5–34.0) **	31.0 (27.8–35.1)	31.4 (28.0–35.6)
HbA_{1c} (%)	6.7 (5.9–7.5)	7.3 (6.4–8.4)***	6.6 (6.0–7.5)	7.2 (6.4–8.3)***	6.8 (6.1–7.6)	7.3 (6.5–8.3)***
HbA_{1c} (mmol/mol)	49.2 (41.0–58.5)	55.7 (46.5–68.3)***	48.6 (41.5–58.5)	55.2 (45.9–67.2)***	50.8 (42.6–59.7)	56.0 (47.0–66.7)***
HOMA-IR (ratio)	1.39 (0.95–2.03)	1.63 (1.06–2.49)	1.71 (1.15–2.52)	1.85 (1.25–2.82)***	2.11 (1.43–3.08)	2.24 (1.54–3.24)*
Systolic BP (mmHg)	137±15	144±16***	139±15	145±16***	139±14	146±16***
Diastolic BP (mmHg)	81±8	83±9***	82±8	83±9***	82±8	84±9***
HDL-cholesterol (mmol/l)	1.14±0.28	1.10±0.24***	1.10±0.25	1.06±0.26***	1.08±0.25	1.04±0.25***
LDL-cholesterol (mmol/l)	3.14±0.62	3.08±0.62*	3.11±0.64	3.06±0.66*	3.00±0.67	2.96±0.69*
Triacylglycerol (mmol/l)	1.49 (1.19–1.94)	1.52 (1.21–1.97)	1.74 (1.38–2.24)	1.81 (1.41–2.42) **	1.96 (1.52–2.61)	2.06 (1.58–2.80)***
Fibrinogen (g/l)	3.54±0.69	3.67±0.73***	3.53±0.75	3.73±0.76***	3.56±0.73	3.65±0.82***
Homocysteine (μmol/l)	9.1 (7.7–10.8)	9.8 (8.0–11.7)***	9.4 (7.9–11.1)	9.8 (8.2–11.9)***	9.6 (7.9–11.7)	10.5 (8.6–13.0)***
Plasma creatinine (μmol/l)	76.3±14.3	79.2±15.1***	76.2±14.9	79.5±17.1***	76.6±15.5	81.2±18.6***
Estimated GFR (ml min^{-1} 1.73 m^{-2})	89.8±17.2	89.3±18.0	88.5±17.8	87.6±19.6	86.0±17.6	83.9±21.0**
Baseline glucose-lowering medication						
Diet alone	758 (32.8)	170 (18.5)***	712 (32.8)	173 (16.2)***	559 (28.1)	212 (17.0)***
Oral agent alone	1279 (55.3)	504 (54.7)	1263 (58.3)	683 (64.1)**	1247 (62.8)	804 (64.5)
Insulin alone	149 (6.4)	112 (12.2)***	91 (4.2)	89 (8.4)***	77 (3.9)	82 (6.6)***
Insulin + oral agent	125 (5.4)	135 (14.7)***	102 (4.7)	120 (11.3)***	103 (5.2)	148 (11.9)***

Data are expressed as mean ± SD or n (%). For skewed variables, data are expressed as median (IQR) and were $\log_e$-transformed before analysis

The p value was estimated by χ^2 test for categorical variables and t test for continuous variables

*p<0.05, **p<0.01 and ***p<0.001 vs patients without total microvascular disease within each FGF21 tertile group

nephropathy, and neuropathy had significantly higher FGF21 levels than those patients who did not develop such complications (all p<0.001). In multivariable regression analysis, higher baseline $\log_e$-transformed FGF21 levels were significantly associated with a higher risk of new on-study total microvascular disease after adjusting for confounding factors (p=0.01; Fig. 1 and ESM Table 3). Among the four individual components of new on-study total microvascular disease, no significant association with any individual microvascular disease was found (all p≥0.01). Similar trends were obtained when FGF21 tertiles were used in the analysis (p=0.03). For all microvascular outcomes, the association did not differ significantly between the placebo group and the fenofibrate treatment groups (p for treatment interaction ≥0.05 for primary outcome and ≥0.01 for secondary outcomes; ESM Table 3). In a separate analysis, of 937 patients with data on ETDRS grade and baseline FGF21 levels, no significant association with at least a two-step increase in ETDRS grade after 2 years or more of follow-up was found (Table 2 and ESM Table 3). Although baseline FGF21 levels were significantly associated with UACR at baseline, 2 years, 5 years or study close (all p<0.001, ESM Table 4), the highest tertile of baseline FGF21 level tended to be associated with the progression of albuminuria from baseline to 2 years (p for trend=0.04), but not from

baseline to 5 years or study close (ESM Table 5). When estimated GFR data were analysed, baseline FGF21 levels showed a significant inverse association with estimated GFR at baseline among all patients ($p<0.001$) and 4 months later among the placebo group ($p=0.01$), but not with the estimated GFR at other follow-up visits in the placebo group or 8 weeks after treatment cessation at study close (ESM Table 6). Higher baseline FGF21 levels were significantly associated with a higher risk of abnormal estimated GFR at baseline ($p<0.001$) and, in the placebo group, new on-study abnormal estimated GFR ($p<0.05$) but not new abnormal estimated GFR at 8 weeks after treatment cessation at study close (ESM Table 7).

Table 2 Baseline FGF21 levels according to baseline and new on-study microvascular diseases

Microvascular disease	*n*	Baseline FGF21 levels (pg/ml)	*p* value
Baseline			
Total microvascular disease			
No	6,465	301.6 (198.8–461.3)	<0.001
Yes	3,232	345.0 (223.3–530.4)	
Nephropathy			
No	7,193	302.3 (198.3–460.7)	<0.001
Yes	2,478	358.7 (230.7–547.6)	
Neuropathy			
No	9,144	314.7 (204.9–481.5)	0.16
Yes	553	333.1 (215.4–492.5)	
Retinopathy			
No	8,724	315.7 (206.0–481.8)	0.51
Yes	804	317.7 (195.8–494.2)	
Amputation			
No	9,679	315.7 (205.3–482.3)	0.67
Yes	18	302.6 (197.2–449.8)	
On-study[a]			
New total microvascular disease			
No	4,948	295.7 (196.9–453.7)	<0.001
Yes	1,517	328.3 (204.8–489.1)	
New nephropathy			
No	5,849	297.9 (196.3–454.9)	<0.001
Yes	1,344	328.4 (207.9–494.6)	
New neuropathy			
No	8,483	312.2 (204.7–478.3)	<0.001
Yes	661	343.2 (217.1-525.8)	
New retinopathy requiring laser treatment			
No	8,537	316.1 (206.7–482.3)	0.11
Yes	187	303.2 (183.3–453.7)	
Two-step progression of ETDRS grade[b]			
No	835	306.8 (205.2–468.5)	0.97
Yes	102	319.9 (205.7–451.1)	
New amputation			
No	9,630	315.4 (205.1–482.0)	0.01
Yes	49	389.7 (280.2–540.8)	

FGF21 levels are expressed as median (IQR) and were $\log_e$-transformed before analysis

[a] For the analysis of new on-study microvascular disease (except two-step progression of ETDRS grade), patients with the specific type of microvascular disease at baseline were excluded

[b] Serial retinal photography was performed in a substudy of patients at baseline, 2 years, 5 years and at study close

p values were estimated by *t* test

Discrimination and reclassification for new on-study microvascular disease For patients without baseline total microvascular disease, the addition of $\log_e$-transformed FGF21 levels to a model adjusted for risk factors (including treatment allocation, age, sex, known diabetes duration, history of CVD, smoking, BMI, HbA_{1c}, HOMA-IR, systolic BP, HDL-cholesterol, LDL-cholesterol, triacylglycerol, fibrinogen, plasma creatinine, homocysteine and glucose-lowering medication at baseline) did not result in a significant increase in the *C* statistic for new on-study total microvascular disease ($p=0.46$, ESM Table 8). However, both IDI and NRI analysis showed that there was a modest but significant improvement for new on-study total microvascular disease (both $p<0.01$; Table 3). ESM Fig. 1 shows the reclassification plot for new on-study total microvascular disease.

Discussion

The present report is the first to describe the association of circulating FGF21 levels with various microvascular outcomes in a large-scale, well-designed clinical trial conducted in patients with type 2 diabetes. The cross-sectional analysis demonstrated a significant association of higher baseline FGF21 levels with total microvascular disease and nephropathy at study entry. Similar results were also found in the prospective analysis, in which higher baseline FGF21 levels were associated with a higher risk of subsequent first-ever microvascular disease during 5 year follow-up after randomisation of the patients into either the placebo or fenofibrate treatment group.

There are only limited studies in the literature that have reported the relationship between circulating FGF21 levels and microvascular disease. In a previous study of 264 patients with type 2 diabetes, the median serum FGF21 levels were 468, 492 and 595 pg/ml in patients with normoalbuminuria, microalbuminuria and macroalbuminuria, respectively, and serum FGF21 levels were independently associated with urinary albumin excretion, suggesting a potential role for FGF21 in diabetic nephropathy [13]. However, this earlier study was limited by its small sample size and cross-sectional study design. Consistent with this previous study, our study also showed a significant association between FGF21 levels and baseline total microvascular disease and nephropathy. We extended this finding by showing a significant association of

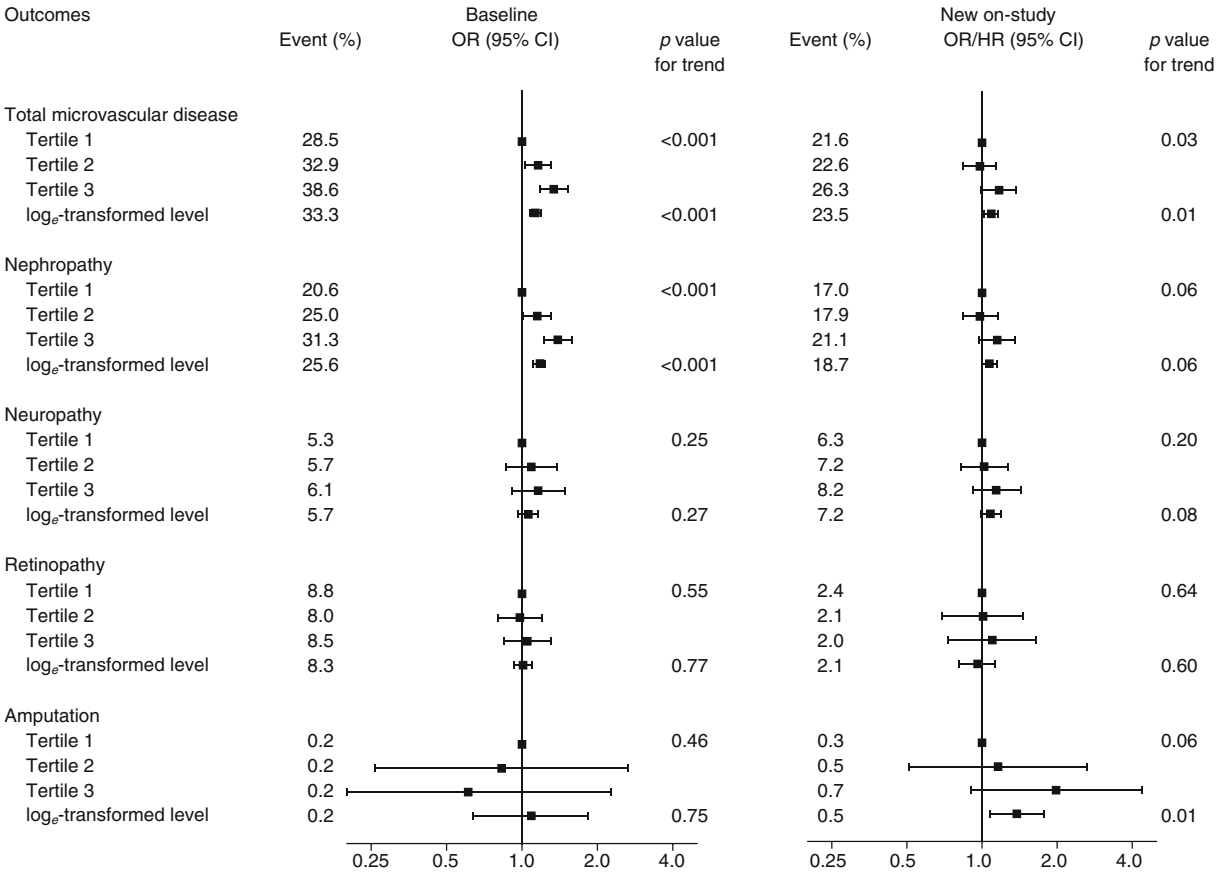

Fig. 1 Association of baseline plasma FGF21 levels with baseline and new on-study microvascular disease. For the analysis of on-study microvascular disease, patients with the specific type of microvascular disease at baseline were excluded. For new on-study retinopathy, data on retinopathy requiring laser treatment are shown. All OR, HR and *p* values were adjusted for treatment allocation (on-study disease only), age, sex, known diabetes duration, history of CVD, smoking (never, former and current), BMI, HbA_{1c}, HOMA-IR, systolic BP, HDL-cholesterol, LDL-cholesterol, triacylglycerol, fibrinogen, plasma creatinine, homocysteine and glucose-lowering medication (diet alone, oral agent alone, insulin alone and insulin + oral agent) at baseline. Error bar indicate 95% CI. For the $\log_e$-transformed FGF21 level, HR and OR are expressed per one SD increase in $\log_e$-transformed level

baseline FGF21 levels with new on-study total microvascular disease among individuals free of any disease at baseline. There is only one other study in the literature that has investigated the relationship of FGF21 levels with retinopathy, a cross-sectional study of 117 Chinese patients with type 2 diabetes reporting that serum FGF21 levels were associated with the severity of diabetic retinopathy [14]. However, in our present study with a larger sample size and 5 year follow-up, we did not observe any significant association of plasma FGF21 levels with baseline or new on-study retinopathy. This discrepancy could be due to the difference in patient characteristics, with higher age, lower BMI, better glycaemic control, lower LDL-cholesterol, lower triacylglycerol, lower HDL-cholesterol, higher BP and lower FGF21 levels in the patients in our study, compared with those in the previous study. To our best knowledge, there are no reports in the literature that have investigated the relationship of FGF21 levels with other microvascular diseases such as neuropathy and amputation.

Moreover, our study is the first to assess the incremental value of the addition of FGF21 levels in a risk prediction model of microvascular disease with established risk factors using *C* statistics, IDI and category-free NRI. The results of these analyses suggest that plasma FGF21 levels could provide incremental information for on-study total microvascular disease and that FGF21 may be a potential biomarker for risk assessment of total microvascular disease, at least in patients with type 2 diabetes. However, interpretation should be made cautiously, as the improvements in IDI and NRI were only modest and the change in *C* statistics was not statistically significant for the outcome. Nevertheless, the small incremental value seen here is typical of models that already contain strong and well-established risk factors [24, 25].

The association of FGF21 levels with nephropathy may be a main contributor to the association between FGF21 levels and both baseline and new on-study total microvascular diseases. FGF21 levels have been reported to correlate positively with plasma creatinine levels and negatively with estimated

Table 3 Assessment of the incremental value of $\log_e$-transformed FGF21 levels for new on-study total microvascular disease using IDI and NRI

Measure	Estimate
IDI	0.001
95% Cl	0.000, 0.002
p value	<0.01
NRI>0 (%)	3.6
95% CI	0.7, 6.6
p value	<0.01
Event NRI[a] (%)	0.5
Non-event NRI[b] (%)	3.2

Comparison was made for the addition of FGF21 ($\log_e$-transformed) to a model containing treatment allocation, age, sex, known diabetes duration, history of CVD, smoking (never, former and current), BMI, HbA_{1c}, HOMA-IR, systolic BP, HDL-cholesterol, LDL-cholesterol, triacylglycerol, fibrinogen, plasma creatinine, homocysteine and glucose-lowering medication (diet alone, oral agent alone, insulin alone and insulin + oral agent) at baseline. Values above zero for the NRI and IDI indicate improved risk prediction and discrimination with the addition of FGF21 to the model

[a] Percentage correctly reclassified among participants who had events

[b] Percentage correctly reclassified among participants who did not have events

GFR in the FIELD study [12, 17] and other studies [13, 26], and be elevated in both chronic and acute renal dysfunction [27]. In a recent study of 1,136 Chinese patients with type 2 diabetes, higher circulating FGF21 levels predicted estimated GFR decline over 4 years, even in a subgroup of 559 patients with normal estimated GFR and normoalbuminuria [28]. Fenofibrate is also known to cause an increase in plasma creatinine [19]. However, in this study, we have demonstrated a significant association of baseline $\log_e$-transformed FGF21 levels with baseline total microvascular disease and nephropathy, as well as new on-study total microvascular disease after adjusting for risk factors including plasma creatinine and treatment allocation. Moreover, the associations did not differ significantly between the fenofibrate and placebo treatment groups with non-significant *p* values for interaction with treatment allocation. We have also demonstrated the significant association of baseline $\log_e$-transformed FGF21 levels with baseline abnormal GFR (<60 ml min^{-1} 1.73 m^{-2}) as well as new on-study abnormal GFR in the placebo group. At present, the underlying mechanism for the independent association of FGF21 levels with microvascular disease and nephropathy is unclear. Lipotoxicity plays an important role in the pathogenesis of diabetic nephropathy [29]. In human clinical trials and animal studies, FGF21 has demonstrated favourable metabolic effects, including lipid-lowering properties [1, 2, 30]. In a recent animal study, FGF21 administration markedly decreased urinary albumin excretion and mesangial expansion and improved renal lipid metabolism and oxidative stress injury in *db/db* mice [31]. In another study, diabetes- and hyperlipidaemia-induced renal damage was enhanced in *Fgf21*-knockout mice, but was prevented by exogenous FGF21 administration [32]. These studies suggest that FGF21 may play a role in diabetic nephropathy through its effect on glucose and lipid metabolism.

Our study has the advantages of making use of the FIELD study, which is well-designed and has good quality control, a large sample size and standardised assessments of different clinical characteristics and outcome events. The availability of data on microvascular disease at both baseline and during follow-up allowed the assessment of FGF21 levels with microvascular disease using both cross-sectional and prospective analysis. The outcome events of retinopathy and amputation were pre-specified in the FIELD study and were adjudicated by a committee masked to study treatment allocation. However, our study also has several limitations. In this analysis, different outcomes were assessed at the same time and thus there may be a chance of false-positive results due to multiple testing. Nevertheless, this limitation was minimised by using more stringent pre-specified criteria for the *p* values for significance (i.e. $p<0.01$) for the secondary outcomes in this study. The analysis of different microvascular outcome events allowed us to gain a comprehensive understanding about the role of FGF21 in microvascular disease in people with type 2 diabetes. Our study was also limited by the small number of cases for some microvascular diseases, especially amputation, and hence power for analyses was substantially lower for some outcomes. As all the patients in the FIELD study had type 2 diabetes at baseline, the findings from this study may not be generalisable to healthy people or other people at high risk but without diabetes. This is particularly important because patients with type 2 diabetes have been reported to have elevated FGF21 levels [2, 7, 14]. Further studies in different clinical settings, or with different participant characteristics, are merited to confirm our findings from the FIELD study.

In summary, higher baseline plasma FGF21 levels are seen in patients with type 2 diabetes and established microvascular disease (especially nephropathy), and predict the future development of new microvascular disease over 5 years. Both confirmatory studies in other clinical settings and mechanistic studies would be useful to better explain the association of circulating FGF21 levels with diabetic microvascular disease.

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Duality of interest Fournier Pharma (now part of Abbott Pharmaceuticals) sponsored the FIELD trial but had no role in data collection or analysis. KLO has received consulting fees and has served as advisory panel member for Pfizer. AJJ has served as an advisory panel member for Medtronic and on the speaker bureau for Abbott, Animaas and Medtronic and has received research support from Eli Lilly, Medtronic, Novo and Sanofi-Aventis. DRS has received research support from Amgen, Amarin, AstraZeneca and MSD and has received consultancy and educational grants from Abbott, Amgen, MSD and Janssen-Cilag. PJB has served as an advisory panel member for Amgen, AstraZeneca, Eli Lilly, Kowa, MSD, Novartis, Roche and Pfizer, has served as a consultant for CSL Behring, Dezima and MSD, has received research support from MSD and Pfizer and has received honoraria for lectures from Amgen, AstraZeneca, Kowa, MSD and Pfizer. ACK has served as an Advisory Board member for Abbott Pharmaceuticals, Amgen and AstraZeneca and has received speaker and/or advisor honoraria and research support from Abbott, Amgen, Astra-Zeneca, Bristol-Myers Squibb, Eli Lilly, Merck, Novartis, Pfizer, Roche Diagnostics and Solvay. All other authors declare that there is no duality of interest associated with their contribution to this manuscript.

Contribution statement KLO wrote the first draft of the manuscript. KLO, RO and ACK designed the study and contributed to the data analysis. KLO, ASJ, AJJ, AX, DRS, PJB, RSS, MRT, KAR and ACK contributed to the acquisition and interpretation of data. KLO, RO and LB contributed to the data analysis. ASJ, KAR and ACK supervised the studies. KLO, AJJ, DRS, PJB, KAR and ACK contributed to the obtaining of funding. All authors reviewed and edited the manuscript and gave final approval of the version to be published. KLO is responsible for the integrity of the work as a whole.

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