

# Plasma total bilirubin levels predict amputation events in type 2 diabetes mellitus: the Fenofibrate Intervention and Event Lowering in Diabetes (FIELD) study

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## Abstract

**Aims/hypothesis** Bilirubin has antioxidant and anti-inflammatory activities. Previous studies demonstrated that higher bilirubin levels were associated with reduced prevalence of peripheral arterial disease (PAD). However, the relationship between bilirubin and lower-limb amputation, a consequence of PAD, is currently unknown. We hypothesised that, in patients with type 2 diabetes, bilirubin concentrations may inversely associate with lower-limb amputation.

**Methods** The relationship between baseline plasma total bilirubin levels and amputation events was analysed in 9,795 type 2 diabetic patients from the Fenofibrate Intervention and Event Lowering in Diabetes (FIELD) study. The analysis plan was pre-specified. Lower-limb amputation was adjudicated blinded to treatment allocation. Relevant clinical and biochemical data were available for analyses. Amputation was a pre-specified tertiary endpoint.

**Results** Bilirubin concentrations were significantly inversely associated with lower-limb amputation, with a greater than

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threefold risk gradient across levels. Individuals with lower bilirubin concentrations had a higher risk for first amputation (HR 1.38 per 5  $\mu\text{mol/l}$  decrease in bilirubin concentration, 95% CI 1.07, 1.79,  $p=0.013$ ). The same association persisted after adjustment for baseline variables, including age, height, smoking status,  $\gamma$ -glutamyltransferase level,  $\text{HbA}_{1c}$ , trial treatment allocation (placebo vs fenofibrate), as well as previous PAD, non-PAD cardiovascular disease, amputation or diabetic skin ulcer, neuropathy, nephropathy and diabetic retinopathy (HR 1.38 per 5  $\mu\text{mol/l}$  decrease in bilirubin concentration, 95% CI 1.05, 1.81,  $p=0.019$ ).

**Conclusions/interpretation** Our results identify a significant inverse relationship between bilirubin levels and total lower-limb amputation, driven by major amputation. Our data raise the hypothesis that bilirubin may protect against amputation in type 2 diabetes.

**Keywords** Amputation · Bilirubin · Fenofibrate Intervention and Event Lowering in Diabetes (FIELD) study · Peripheral arterial disease · Type 2 diabetes mellitus

### Abbreviations

CVD	Cardiovascular disease
FIELD	Fenofibrate Intervention and Event Lowering in Diabetes
GGT	$\gamma$ -Glutamyltransferase
HMOX-1	Haem oxygenase-1
IRR	Incidence rate ratio
PAD	Peripheral arterial disease
UGT1A1	Uridine diphosphate glucuronosyltransferase-1 family polypeptide A1

### Introduction

Non-traumatic lower-limb amputations are often the end-stage clinical events in intractable limb ischaemia. Despite modern therapy, peripheral arterial disease (PAD) remains a major burden on the healthcare system, with at least one amputation due to diabetes occurring every 30 s worldwide, up to eight million patients in the USA being devastated by immobility and significant morbidity, and an annual cost exceeding US\$1.6 billion in 2001 [1, 2]. Furthermore, attention to classic vascular risk factors has failed to substantially reduce the risk of amputation [3], highlighting the need to find novel predictors and biomarkers for PAD and amputation events that may help to identify new therapeutic targets [4–6].

Bilirubin, a product of haem degradation, may confer vascular protective effects [7, 8], and is therefore a possible candidate biomarker for predicting amputation events. Experimental studies have reported that bilirubin possesses potent antioxidant and anti-inflammatory properties *in vitro*

and *in vivo* [9–14]. Given that atherosclerosis and ischaemia are characterised by a state of heightened inflammation and oxidation [15, 16], it is conceivable that bilirubin may confer beneficial effects through these known activities. Consistent with this notion, case–control studies have reported that individuals with elevated bilirubin levels caused by Gilbert’s syndrome have a decreased incidence of atherosclerotic disease compared with normal controls [12, 14]. Also, previous studies reported an inverse relationship between bilirubin levels and PAD prevalence [17–20]. However, the association between bilirubin and the hard clinical endpoint of amputation events has not been reported, to our knowledge. We therefore hypothesised that plasma bilirubin concentrations may inversely associate with lower-limb amputation. We studied this association in a longitudinal cohort of individuals with type 2 diabetes, as these patients have an eightfold higher amputation risk compared with non-diabetic patients [21].

### Methods

**Study participants** The relationship between baseline plasma total bilirubin levels and non-traumatic amputation was analysed in 9,795 type 2 diabetic patients from the FIELD (Fenofibrate Intervention and Event Lowering in Diabetes) study in a subsidiary analysis. In brief, patients in the FIELD study were randomised to either fenofibrate or placebo treatment between February 1998 and November 2000, and were followed up for a median duration of 5 years [22]. All patients were aged 50–75 years and had a diagnosis of type 2 diabetes according to WHO criteria [23]. Individuals with renal impairment, chronic liver disease, symptomatic gallbladder disease, or those who had experienced a cardiovascular event within the 3 months before recruitment were excluded. Non-traumatic amputation was a pre-specified tertiary endpoint. All amputations that occurred during study follow-up (on-study amputations) were adjudicated blinded to treatment allocation by two clinicians separately, and any discrepancies were resolved by mutual agreement. Pre-study amputations were adjudicated in the same fashion. Major amputations were defined as those above the ankle and minor amputations as those below the ankle [3]. All patients provided written informed consent. This study had ethics committee approval in accordance with the Declaration of Helsinki and Good Clinical Practice Guidelines. The original trial was registered with the International Standard Randomised Controlled Trial Number (ISRCTN) 64783481.

**Laboratory measurements** Early-morning fasting baseline blood specimens were obtained in all individuals prior to study randomisation. Plasma (EDTA) levels of total

bilirubin, HbA<sub>1c</sub> and  $\gamma$ -glutamyltransferase (GGT) were determined using an automated analyser (Hitachi 917, Roche Diagnostics, Basel, Switzerland). Total bilirubin was measured as the plasma concentration expressed in  $\mu\text{mol/l}$  using the Diazo method [24].

**Baseline patient variables** A medical history was obtained to determine whether the patient had been diagnosed with or experienced any of: claudication or PAD; prior amputation; diabetic skin ulcer; diabetic retinopathy; neuropathy; or cardiovascular disease (CVD). Age was categorised into <65 years or  $\geq 65$  years. Previous PAD was present if the patient was diagnosed with or experienced claudication or PAD, and/or previous peripheral revascularisation. Prior non-PAD CVD was defined as any history of CHD or stroke. Neuropathy was present if the patient was diagnosed with or experienced diabetic neuropathy, and/or the patient's foot had absent sensation on monofilament testing. Nephropathy was defined by the presence of albuminuria as previously described [3].

**Statistical analyses** All analyses were performed on an intention-to-treat basis. Baseline characteristics were analysed with  $\chi^2$  tests for categorical variables,  $t$  tests for continuous variables or, if the distribution of the data was non-normal, the Wilcoxon rank-sum test. Cox proportional hazards regression was used to compute HRs and 95% CIs to assess the relationship between total bilirubin levels and time to first amputation. As there were 190 amputations occurring in 115 patients, a multiple-event analysis was performed using Poisson regression modelling for all amputation events and adjusting for months of observation and overdispersion, using the Pearson method. The computed HR (or incidence rate ratio [IRR] for the Pearson method) for the bilirubin effect was expressed as the increased risk per 5  $\mu\text{mol/l}$  decrease in bilirubin ( $\approx 1$  SD, a statistically relevant difference in bilirubin concentrations).

A multivariable analysis was also performed adjusting for the following baseline covariates: age; height; smoking status; HbA<sub>1c</sub>; history of previous PAD; amputation or diabetic skin ulcer; neuropathy; nephropathy; diabetic retinopathy; and trial treatment allocation (placebo vs fenofibrate). These covariates (which were determined by use of backwards selection and then confirmed by exhaustive search methods) were selected as they have been reported to be significant predictors of first amputation in the FIELD study [3]. Other prior CVD (non-PAD) was also included as a covariate, as this is a well-recognised risk factor for PAD and amputation [25]. Liver function test variables (alanine transaminase and GGT) and alcohol intake were also considered as covariates, but GGT was the only significant predictor of amputation and was therefore retained as a covariate in the multivariable model. The possibility of

over-fitting because of the large number of potential predictors assessed and the small number of events was examined by calculating the heuristic shrinkage factor. The shrinkage factor of 0.92 indicates that the degree of over-fitting was negligible. A test of interaction was conducted between bilirubin level and treatment allocation. The cumulative risk curves of time to first amputation across five ordered groups of bilirubin level (categorised according to 5  $\mu\text{mol/l}$  [ $\approx 1$ SD] increments in bilirubin concentration, i.e. 0–5, 6–10, 11–15, 16–20 and  $\geq 21$   $\mu\text{mol/l}$ ) was calculated using the Kaplan–Meier method, and the  $p$  value computed using the logrank test of trend. The cumulative rate of amputation across the five ordered groups of bilirubin levels was analysed using the Cochran–Armitage trend test (after testing for linearity). The test for deviation from linearity was conducted by fitting a model including both a linear and a categorical version of the variable-grouped bilirubin level. For this method, the test of the overall effect of the individual categories assesses the significance of a non-linear component [26]. These tests were performed for both a logistic regression (Cochran–Armitage trend test) and a Cox model (logrank trend test) and indicated no deviation from linearity.

Post hoc analyses were also undertaken according to quintile of bilirubin concentration. A test of trend was performed by fitting a Cox model including a discrete variable derived using the median bilirubin level of each quintile (as the ranges of bilirubin level within each quintile were not equal); the level of significance was based on the score test provided by the model. Deviation from linearity was tested using the method described above. A Cox model was also fitted that included the bilirubin quintile group as a categorical variable, both unadjusted and adjusted for the variables mentioned above. The lowest bilirubin quintile was used as the reference category. Analyses were also performed separately for major and minor amputations, sex and smoking status, but in each case, there were insufficient events in at least one subgroup to perform adjusted analyses.

The stability of bilirubin level over time was assessed by calculation of the reliability ratio in a subset of 923 individuals, with repeat measures at year 1. The reliability ratio indicates the proportion of overall variation which is true variation as opposed to true plus random variation (i.e. it gives the ratio of the variance of true to observed bilirubin concentration). Hypotheses and the substudy plan were specified prior to data analyses. The substudy analysis plan stipulated the use of the same statistical methods as in the earlier FIELD amputation study [3], and that the five ordered groups of bilirubin were categorised according to  $\approx 1$  SD increments in bilirubin concentration. As only five patients, none of whom had undergone amputation, had any missing data, no statistical adjustment was made for

**Table 1** Baseline characteristics according to bilirubin levels (5 µmol/l increments) and sex

Variable	Men ( <i>n</i> =6,045)					Women ( <i>n</i> =3,750)					<i>p</i> value <sup>a</sup>
	Bilirubin (µmol/l)					Bilirubin (µmol/l)					
	1–5	6–10	11–15	16–20	≥21	1–5	6–10	11–15	16–20	≥21	
Age ≥65 years	162 (37)	1360 (41)	744 (43)	187 (44)	118 (45)	205 (32)	925 (39)	185 (39)	40 (38)	27 (52)	0.003
Height (cm, median)	173	175	175	175	176	161	161	162	162	160	0.142 <sup>b</sup>
Hypertension	247 (56)	1716 (52)	907 (53)	207 (48)	137 (52)	403 (63)	1521 (64)	301 (63)	69 (65)	34 (65)	0.689
Hypercholesterolaemia	106 (24)	770 (23)	305 (18)	67 (16)	38 (14)	229 (36)	901 (38)	156 (33)	21 (20)	11 (21)	0.001
Smoker	96 (22)	383 (12)	120 (7)	29 (7)	10 (4)	69 (11)	184 (8)	28 (6)	3 (3)	0 (0)	0.000
HbA <sub>1c</sub> >7% (53 mmol/mol)	241 (55)	1514 (46)	727 (42)	156 (36)	93 (35)	277 (43)	1,042 (44)	221 (46)	42 (40)	20 (38)	0.962
Nephropathy	173 (39)	966 (29)	456 (26)	115 (27)	65 (25)	140 (22)	459 (19)	98 (21)	23 (22)	9 (17)	0.594
Previous PAD	55 (13)	298 (9)	118 (7)	30 (7)	23 (9)	53 (8)	147 (6)	33 (7)	5 (5)	2 (4)	0.118
Previous non-PAD CVD	110 (25)	596 (18)	293 (17)	80 (19)	52 (20)	105 (16)	320 (13)	72 (15)	12 (11)	9 (17)	0.489
Prior amputation or diabetic skin ulcer	24 (5)	120 (4)	41 (2)	9 (2)	8 (3)	20 (3)	76 (3)	13 (3)	2 (2)	2 (4)	0.672
Diabetic neuropathy	114 (26)	606 (18)	310 (18)	76 (18)	46 (17)	91 (14)	353 (15)	72 (15)	15 (14)	5 (10)	0.811
Diabetic retinopathy	50 (11)	295 (9)	150 (9)	32 (7)	28 (11)	46 (7)	172 (7)	34 (7)	6 (6)	0 (0)	0.211
Fenofibrate treatment	230 (52)	1,595 (49)	882 (51)	219 (51)	144 (54)	332 (52)	1,167 (49)	247 (52)	54 (51)	24 (46)	0.775

Results are expressed as *n* (%)

To convert µmol/l to mg/dl, divide by 17.1

<sup>a</sup> Differences between categories were analysed using the Cochran–Armitage trend test

<sup>b</sup> Linear regression of height vs bilirubin group variable

**Table 2** Baseline characteristics according to bilirubin levels (quintiles) and sex

Variable	Men ( <i>n</i> =6,045)					Women ( <i>n</i> =3,750)					<i>p</i> value <sup>a</sup>
	Bilirubin (μmol/l)					Bilirubin (μmol/l)					
	1–6	7–8	9–10	11–12	≥13	1–6	7–8	9–10	11–12	≥13	
Age ≥65 years	363 (37)	578 (41)	581 (43)	402 (41)	647 (45)	431 (34)	433 (39)	266 (42)	117 (40)	135 (39)	0.008
Height (cm, median)	174	174	175	175	175	161	161	161	162	161	0.054 <sup>b</sup>
Hypertension	541 (56)	715 (51)	707 (52)	504 (51)	747 (52)	797 (63)	698 (63)	429 (68)	180 (62)	224 (65)	0.211
Hypercholesterolaemia	245 (25)	349 (25)	282 (21)	184 (19)	226 (16)	481 (38)	410 (37)	239 (38)	102 (35)	86 (25)	0.000
Smoker	194 (20)	175 (13)	110 (8)	75 (8)	84 (6)	123 (10)	93 (8)	37 (6)	17 (6)	14 (4)	0.000
HbA <sub>1c</sub> >7% (53 mmol/mol)	507 (52)	671 (48)	577 (43)	428 (43)	548 (38)	567 (45)	477 (43)	275 (44)	129 (44)	154 (45)	0.963
Nephropathy	345 (35)	413 (30)	381 (28)	260 (26)	376 (26)	271 (21)	215 (19)	113 (18)	61 (21)	69 (20)	0.445
Previous PAD	107 (11)	134 (10)	112 (8)	69 (7)	102 (7)	95 (7)	71 (6)	34 (5)	21 (7)	19 (6)	0.151
Previous non-PAD CVD	213 (22)	247 (18)	246 (18)	164 (17)	261 (18)	199 (16)	148 (13)	78 (12)	49 (17)	44 (13)	0.210
Prior amputation or diabetic skin ulcer	46 (5)	57 (4)	41 (3)	21 (2)	37 (3)	42 (3)	35 (3)	19 (3)	8 (3)	9 (3)	0.455
Diabetic neuropathy	215 (22)	262 (19)	243 (18)	179 (18)	253 (18)	182 (14)	177 (16)	85 (14)	43 (15)	49 (14)	0.787
Diabetic retinopathy	102 (10)	129 (9)	114 (8)	84 (9)	126 (9)	91 (7)	76 (7)	51 (8)	25 (9)	15 (4)	0.318
Fenofibrate treatment	472 (49)	715 (51)	638 (47)	507 (51)	738 (52)	627 (49)	554 (50)	318 (51)	155 (53)	170 (49)	0.637

Results are expressed as *n* (%)

To convert μmol/l to mg/dl, divide by 17.1

<sup>a</sup> Differences between categories were analysed using the test of trend using the median bilirubin level of each quintile

<sup>b</sup> Linear regression of height vs bilirubin (median bilirubin level of each quintile)

**Table 3** Association between baseline plasma total bilirubin concentration and risk of first amputation event, and total amputation events

Variable	Risk of amputation (HR per 5 μmol/l decrease in bilirubin, 95% CI)	p value
First amputation event		
Unadjusted	1.38 (1.07, 1.79)	0.013
Adjusted <sup>a</sup>	1.38 (1.05, 1.81)	0.019
Total amputation events <sup>b</sup>		
Unadjusted	1.38 (1.02, 1.86)	0.035
Adjusted <sup>a</sup>	1.37 (1.08, 1.73)	0.009

To convert μmol/l to mg/dl, divide by 17.1

<sup>a</sup> Adjusted for the following baseline patient variables: age, height, smoking status, GGT, HbA<sub>1c</sub>, and history of previous PAD, non-PAD CVD, amputation or diabetic skin ulcer, neuropathy, nephropathy and diabetic retinopathy, as well as trial treatment allocation (placebo vs fenofibrate)

<sup>b</sup> Poisson method: the IRR, analogous to the HR, is shown

this. A two-sided p value <0.05 was considered to indicate statistical significance.

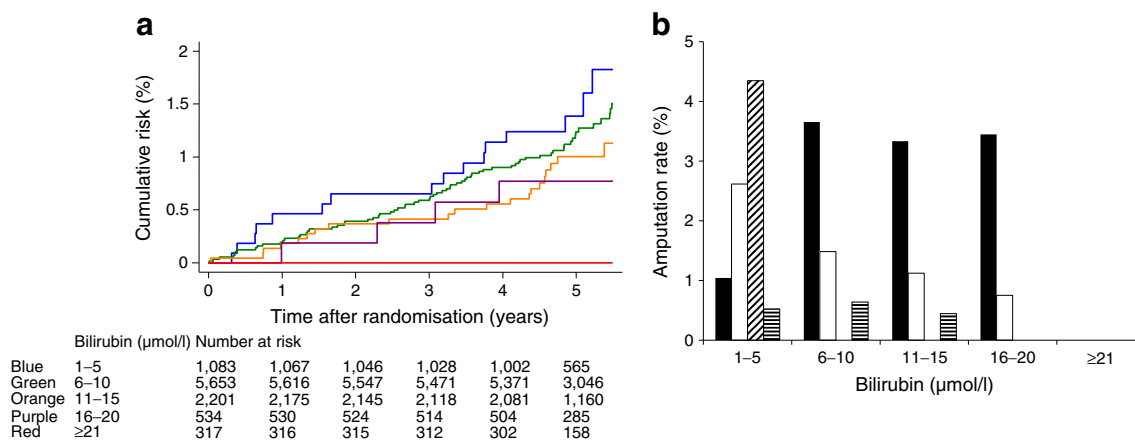
**Results**

The FIELD study enrolled 9,795 individuals, with only 22 being lost to follow-up and nine withdrawing from the study. The mean age of the participants was 62±7 years, with a median diabetes duration of 5 years (interquartile range 2–10 years). There were 190 amputations occurring in 115 patients, with 93 out of 6,045 (1.5%) men and 22 out of 3,750 (0.6%) women suffering from ≥1 amputation. A total of 35 patients experienced major amputations only, 65 minor amputations only, and 15 one or more of both categories. Baseline patient characteristics according to bilirubin

level, sex, and amputation classification are shown in Tables 1 and 2 and electronic supplementary material (ESM) Table 1, respectively. Bilirubin levels were well balanced by study treatment group. The mean baseline plasma total bilirubin concentration was 9.7 μmol/l (median 9.0, SD 4.6, range 1–70 μmol/l). In a subset of 923 patients, bilirubin concentration measured 1 year apart did not change significantly, with a mean difference of only 0.3 μmol/l between measurements and a reliability ratio of 0.73.

*Association between baseline plasma total bilirubin concentrations and amputation events* Baseline plasma total bilirubin concentrations were significantly inversely associated with lower-limb amputation rate (HR 1.38 per 5 μmol/l decrease in bilirubin concentration, 95% CI 1.07, 1.79, p=0.013; Table 3). The same association persisted after adjustment for baseline covariates including age, height, smoking status, GGT, HbA<sub>1c</sub>, trial treatment allocation (placebo vs fenofibrate), and previous PAD, non-PAD CVD, amputation or diabetic skin ulcer, neuropathy, nephropathy and diabetic retinopathy (HR 1.38 per 5 μmol/l decrease in bilirubin concentration, 95% CI 1.05, 1.81, p=0.019). There was also no interaction between bilirubin concentration and treatment allocation (p=0.74). Likewise, the multiple-event analysis revealed a similar relationship (IRR 1.38 per 5 μmol/l decrease in bilirubin concentration, 95% CI 1.02, 1.86, p=0.035). This association also persisted after adjustment for baseline covariates (IRR 1.37 per 5 μmol/l decrease in bilirubin concentration, 95% CI 1.08, 1.73, p=0.009). Patients with only one amputation and multiple (≥2) amputation events had similar bilirubin concentrations (median 8 μmol/l for both, p=0.29).

When the bilirubin levels were categorised into 5 μmol/l (≈1 SD) increments in concentration, the cumulative risk of



**Fig. 1** Amputation risk according to plasma total bilirubin concentration (5 μmol/l increments). (a) Cumulative risk curves (% events over time) to first amputation event. Logrank test of trend, p=0.025. (b) Cumulative 5 year amputation rates by sex and smoking status

classification. Black bars, male smokers; white bars, male non-smokers; diagonally hatched bars, female smokers; horizontally hatched bars, female non-smokers. To convert μmol/l to mg/dl, divide by 17.1

**Table 4** Association between baseline plasma total bilirubin concentration (according to quintiles) and risk of first amputation event

Bilirubin ( $\mu\text{mol/l}$ )	Risk of first amputation (HR, 95% CI)	<i>p</i> value
Unadjusted analysis		
$\leq 6$	1 (reference)	–
7–8	0.76 (0.47, 1.24)	0.269
9–10	0.71 (0.42, 1.21)	0.206
11–12	0.71 (0.38, 1.32)	0.284
$\geq 13$	0.51 (0.27, 0.94)	0.031
Adjusted analysis <sup>a</sup>		
$\leq 6$	1 (reference)	–
7–8	0.74 (0.45, 1.22)	0.244
9–10	0.67 (0.39, 1.16)	0.158
11–12	0.76 (0.39, 1.45)	0.400
$\geq 13$	0.50 (0.27, 0.95)	0.035

To convert  $\mu\text{mol/l}$  to  $\text{mg/dl}$ , divide by 17.1

<sup>a</sup> Adjusted for the following baseline patient variables: age, height, smoking status, GGT,  $\text{HbA}_{1c}$ , and history of previous PAD, non-PAD CVD, amputation or diabetic skin ulcer, neuropathy, nephropathy and diabetic retinopathy, as well as trial treatment allocation (placebo vs fenofibrate)

amputation over the study duration was highest in patients with the lowest (0–5  $\mu\text{mol/l}$ ) bilirubin concentrations, and lowest (with no amputation events) in patients with the highest bilirubin ( $\geq 21 \mu\text{mol/l}$ ) concentrations ( $p=0.025$ ; Fig. 1a). Figure 1b shows the cumulative rate of amputation by sex and smoking status. If the patients with bilirubin  $\geq 21 \mu\text{mol/l}$  were excluded from the analysis, the relationship was no longer significant ( $p=0.11$ ).

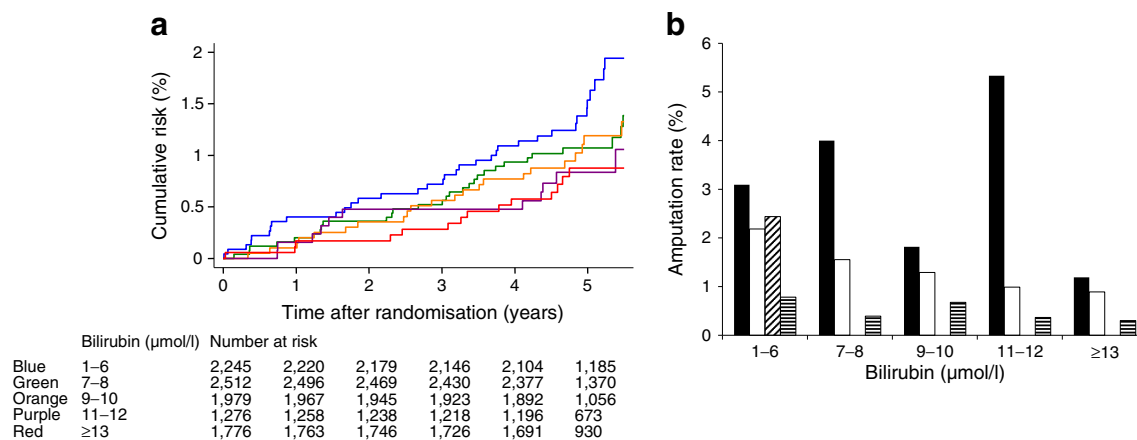
Analyses according to bilirubin quintiles also demonstrated a similar inverse association between bilirubin and amputation. A bilirubin level  $<6 \mu\text{mol/l}$  carries a significantly greater

(twofold higher) risk than that for individuals with bilirubin  $>12 \mu\text{mol/l}$  (HR 0.51 for highest quintile, 95% CI 0.27, 0.94,  $p=0.031$ ; Table 4 and Fig. 2a). This association also persisted after adjusting for baseline covariates (HR 0.50, 95% CI 0.27, 0.95,  $p=0.035$ ). Figure 2b shows the cumulative rate of amputation by sex, smoking status and bilirubin quintile.

Interestingly, the inverse association between bilirubin concentration and amputation event was separately significant for major (HR 1.83 per 5  $\mu\text{mol/l}$  decrease in bilirubin concentration, 95% CI 1.17, 2.87,  $p=0.009$ ), but not minor (HR 1.22 per 5  $\mu\text{mol/l}$  decrease in bilirubin concentration, 95% CI 0.92, 1.61,  $p=0.18$ ) amputation (ESM Fig. 1). Analyses according to bilirubin quintile also revealed a similar inverse association (ESM Table 2 and ESM Fig. 1).

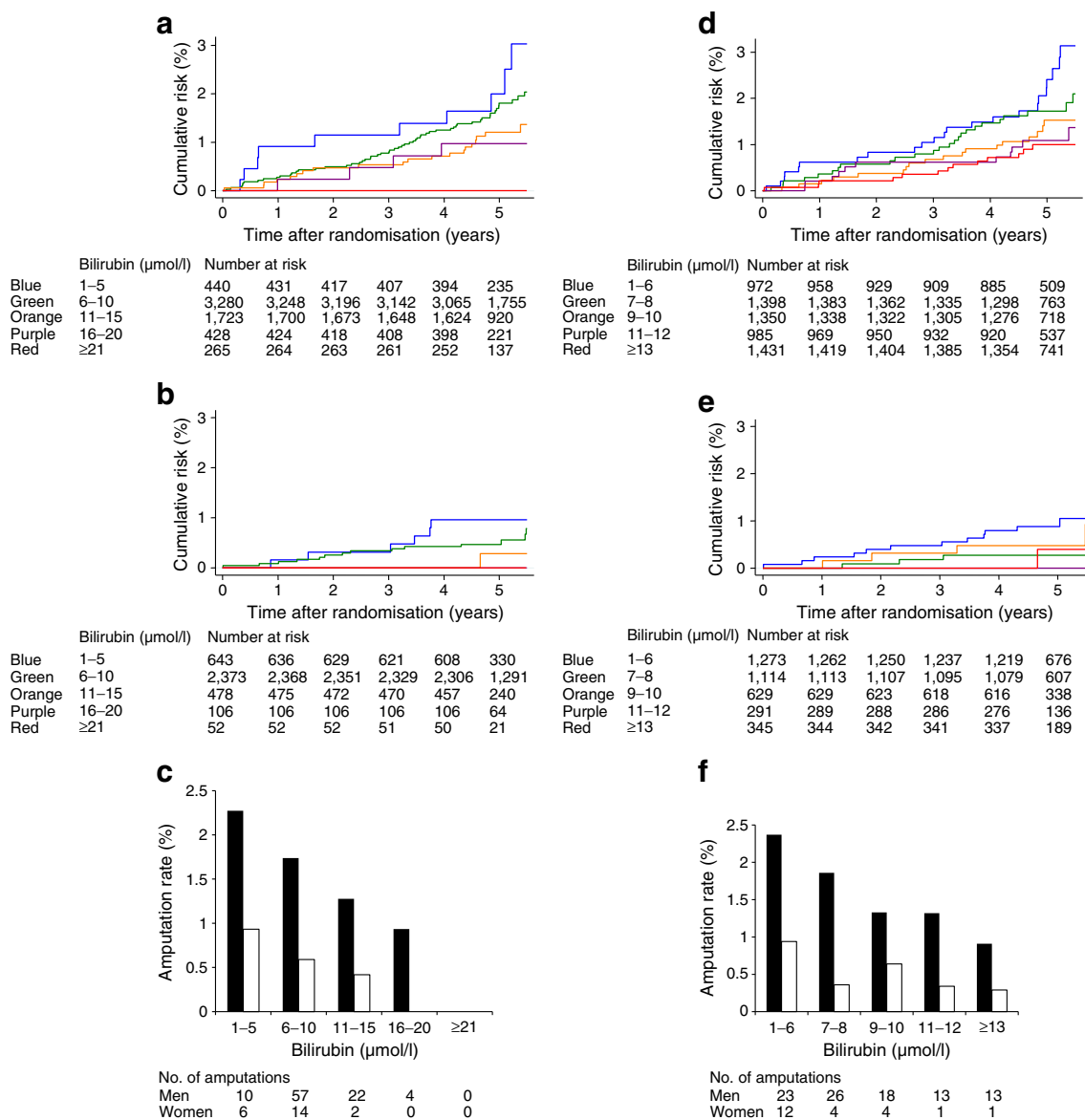
**Analysis according to sex** Consistent with previous studies [4, 5, 19, 27, 28], our study showed that women had lower median bilirubin concentrations than men (8 vs 10  $\mu\text{mol/l}$ ). We found that the inverse relationship between bilirubin concentrations and risk of first amputation was highly significant for men (HR 1.60 per 5  $\mu\text{mol/l}$  decrease in bilirubin concentration, 95% CI 1.19, 2.14,  $p=0.0017$ ), but not women (HR 1.90 per 5  $\mu\text{mol/l}$  decrease in bilirubin concentration, 95% CI 0.84, 4.33,  $p=0.126$ ;  $p$  for interaction by sex = 0.71; Fig. 3a–c). Analyses according to bilirubin quintile also demonstrated a similar inverse association for men (HR 0.38 for highest quintile, 95% CI 0.19, 0.75,  $p=0.005$ ; Table 5 and Fig. 3d–f), but not women. The amputation rate among individuals  $\geq 65$  years old was higher than in younger individuals of both sexes, with cumulative first amputation rates of 2.1% vs 1.1% for men and 1.1% vs 0.3% for women, respectively.

**Analysis according to smoking status** Consistent with previous studies [19, 29], our study showed that current



**Fig. 2** Amputation risk according to plasma total bilirubin concentration (quintiles). (a) Cumulative risk curves (% events over time) to first amputation event. Logrank test of trend (median method),  $p=0.031$ . (b) Cumulative 5 year amputation rates by sex and smoking status

classification. Black bars, male smokers; white bars, male non-smokers; diagonally hatched bars, female smokers; horizontally hatched bars, female non-smokers. To convert  $\mu\text{mol/l}$  to  $\text{mg/dl}$ , divide by 17.1



**Fig. 3** Association by sex classification. **(a, b)** Cumulative risk curves (% events over time) to first amputation event according to baseline plasma total bilirubin concentration (5 μmol/l increments) in **(a)** men, and **(b)** women. Logrank test of trend,  $p=0.0045$  for men and  $p=0.125$  for women. **(c)** Cumulative 5 year amputation rates according to baseline plasma total bilirubin concentration (5 μmol/l increments) in men and women. Cochran–Armitage trend test,  $p=0.0041$  for men and  $p=0.131$  for women. Black bars, men; white bars, women. **(d, e)**

Cumulative risk curves (% events over time) to first amputation event according to baseline plasma total bilirubin concentration (quintiles) in **(d)** men, and **(e)** women. Logrank test of trend (median method),  $p=0.0024$  for men and  $p=0.136$  for women. **(f)** Cumulative 5 year amputation rates according to baseline plasma total bilirubin concentration (quintiles) in men and women. Cochran–Armitage trend test (median method),  $p=0.0023$  for men and  $p=0.136$  for women. Black bars, men; white bars, women. To convert μmol/l to mg/dl, divide by 17.1

smokers had lower median bilirubin levels compared with non-smokers (8 vs 9 μmol/l). We found that the inverse relationship between bilirubin concentration and risk of first amputation was significant for non-smokers (HR 1.34 per 5 μmol/l decrease in bilirubin concentration, 95% CI 1.01, 1.77,  $p=0.04$ ), but not for smokers (HR 1.24 per 5 μmol/l decrease in bilirubin concentration, 95% CI 0.66, 2.33,  $p=0.513$ ;  $p$  for interaction by smoking status=0.81; Fig. 4a–c). The same analyses according to bilirubin quintile were not

statistically significant for either non-smokers or smokers, although there was a trend towards an inverse association in non-smokers (Table 6 and Fig. 4d–f).

**Discussion**

Our results identify a significant inverse relationship between baseline plasma total bilirubin levels and lower-limb



**Table 5** Association between baseline plasma total bilirubin concentration (quintiles) and risk of first amputation event, by sex

Bilirubin ( $\mu\text{mol/l}$ )	Risk of first amputation (HR, 95% CI)	<i>p</i> value
<b>Men</b>		
$\leq 6$	1 (reference)	–
7–8	0.78 (0.44, 1.36)	0.377
9–10	0.56 (0.30, 1.03)	0.062
11–12	0.56 (0.28, 1.10)	0.092
$\geq 13$	0.38 (0.19, 0.75)	0.005
<b>Women</b>		
$\leq 6$	1 (reference)	–
7–8	0.37 (0.12, 1.16)	0.089
9–10	0.67 (0.22, 2.07)	0.483
11–12	0.37 (0.05, 2.83)	0.337
$\geq 13$	0.30 (0.04, 2.34)	0.253

To convert  $\mu\text{mol/l}$  to  $\text{mg/dl}$ , divide by 17.1

amputation in patients with type 2 diabetes. This association was observed between bilirubin and total amputations, as well as separately for major but not minor amputations. The relationship with total (major plus minor) amputation remains robust even after adjusting for numerous other classic vascular and known amputation risk factors, liver function tests (GGT) and study treatment allocation, suggesting that it is likely to be independent of these other factors. Our data are therefore consistent with the hypothesis that bilirubin may be a useful biomarker for amputation in patients with type 2 diabetes. Potentially, this association is of major clinical importance, as the natural range of bilirubin levels seen here covers a striking, greater than threefold, risk gradient in amputation rates over 5 years.

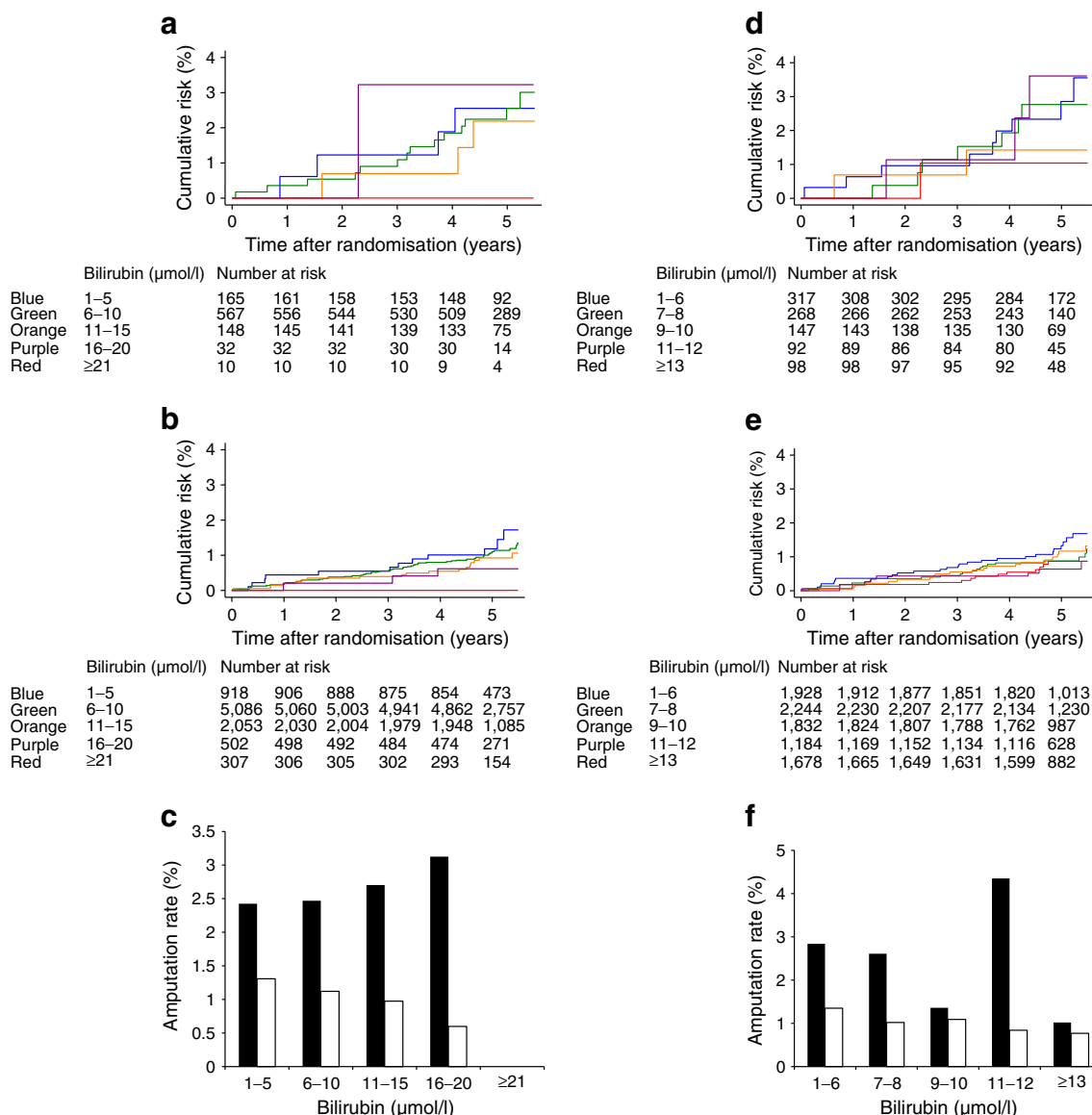
Previous studies focusing on the relationship between bilirubin and PAD also reported a similar inverse association. The largest of these studies analysed 7,075 individuals from the National Health and Nutrition Examination Survey, and reported that increased bilirubin levels were associated with a reduced prevalence of PAD [19]. However, hard clinical endpoints were not explored in that and other similar smaller case-control studies [17–20]. Our study therefore for the first time extends these findings to amputation events. This inverse association is further supported by studies showing a similar relationship between bilirubin levels and CHD, ischaemic stroke, and surrogate markers of atherosclerosis including endothelial dysfunction and carotid intima-media thickness [12, 14, 29–42]. A number of prospective studies found a U- or reversed J-shaped relationship rather than an inverse relationship between bilirubin levels and CHD [4, 5, 27, 28, 30, 33, 34, 37]. The differences between our study and these earlier studies are that they concentrated on CHD and mainly enrolled men, although whether these differences could plausibly explain the differing relationships reported is unclear.

Nonetheless, our results, together with the previously published studies, support the possibility that bilirubin may be protective against atherosclerotic disease.

It is well established that atherosclerosis and its consequence, tissue ischaemia, are characterised by a state of heightened inflammation and oxidative stress [15, 16]. Studies have shown that bilirubin possesses antioxidant and anti-inflammatory activities in vitro [9, 11]. These experimental studies are supported by human studies reporting that individuals with elevated bilirubin levels due to Gilbert's syndrome have higher antioxidant capacity and lower proinflammatory markers compared with control individuals [13, 14, 42]. Moreover, in a rat model of vascular injury, hyperbilirubinaemic Gunn rats had reduced neointima formation after balloon injury compared with control wild-type animals, suggesting that bilirubin itself may prevent the development of intimal hyperplasia [43]. How relevant these findings are, with much higher bilirubin levels in Gunn rats compared with humans, is still debated. The potential anti-atherogenic properties of bilirubin could also be consistent with our findings that the inverse association with amputation events was significant for major (which may more accurately reflect large-vessel atherosclerotic disease) but not minor amputation.

Our results are consistent with the previous study by Perlstein et al [19], showing a significant inverse relationship between bilirubin levels and amputation in men but not women. The lack of significant association in women may simply reflect the lower amputation rates in women compared with men, and it should be noted that there was no significant interaction by sex, so we were unable to conclude that the relationship between bilirubin and amputation risk truly differs by sex. Similarly (in contrast to the study by Perlstein et al), although we found a significant inverse relationship in non-smokers (when analysed according to 5  $\mu\text{mol/l}$  bilirubin increments), but not smokers, again there was no significant interaction by smoking status, and only 9% of the cohort were current smokers. An alternative explanation for this disparate result may be that our study cohort exclusively comprised patients with type 2 diabetes, whereby the adverse combination of hyperglycaemia and smoking might negate any antioxidative benefits that bilirubin might otherwise confer in amputation prevention.

Plasma bilirubin concentrations are determined by the relative activities of the enzymes that form and remove bilirubin. Therefore, a better understanding of the pathways that regulate bilirubin concentrations may lead to new therapeutic targets for the treatment of atherosclerotic disease [7]. This is of particular importance to PAD, in which progressive disease leads to amputation at rates that have not changed significantly over the last three decades despite advances in vascular-targeted therapies [2]. The enzymes



**Fig. 4** Association according to smoking status. **(a, b)** Cumulative risk curves (% events over time) to first amputation event according to baseline plasma total bilirubin concentration (5  $\mu\text{mol/l}$  increments) in **(a)** smokers, and **(b)** non-smokers. Logrank test of trend,  $p=0.96$  for smokers and  $p=0.034$  for non-smokers. **(c)** Cumulative 5 year amputation rates according to baseline plasma total bilirubin concentration trend test,  $p=0.98$  for smokers and  $p=0.032$  for non-smokers. Black bars, smokers; white bars, non-smokers. **(d, e)** Cumulative risk curves (% events over time) to first amputation event according to baseline

plasma total bilirubin concentration (quintiles) in **(d)** smokers, and **(e)** non-smokers. Logrank test of trend (median method),  $p=0.58$  for smokers and  $p=0.095$  for non-smokers. **(f)** Cumulative 5 year amputation rates according to baseline plasma total bilirubin concentration (quintiles) in smokers and non-smokers. Cochran–Armitage trend test,  $p=0.56$  for smokers and  $p=0.09$  for non-smokers. Black bars, smokers; white bars, non-smokers. To convert  $\mu\text{mol/l}$  to  $\text{mg/dl}$ , divide by 17.1

most widely studied to date include haem oxygenase-1 (HMOX-1) and uridine diphosphate glucuronosyltransferase-1 family polypeptide A1 (UGT1A1) [6]. HMOX-1 catalyses the rate-limiting step of haem degradation to carbon monoxide, ferrous iron and biliverdin [44]. Biliverdin is then rapidly reduced to bilirubin via biliverdin reductase. Although initially known for its metabolic role in haem catabolism, it is now well recognised that HMOX-1 may confer vascular protective effects, and this may be mediated, in part, via its haem

catabolism by-products, carbon monoxide and bilirubin [7, 8]. Supporting this, Kawamura et al reported that both HMOX-1 induction and the addition of bilirubin (but not carbon monoxide) conferred anti-inflammatory effects on human endothelial cells in vitro, suggesting that the anti-inflammatory properties of HMOX-1 may be mediated through the action of bilirubin [9].

In humans, it is thought that the length of the (GT)<sub>n</sub> repeat is associated with variation in the *HMOX1* gene

**Table 6** Association between baseline plasma total bilirubin concentration (quintiles) and risk of first amputation event, by smoking status

Bilirubin ( $\mu\text{mol/l}$ )	Risk of first amputation (HR, 95% CI)	<i>p</i> value
Smoker		
$\leq 6$	1 (reference)	–
7–8	0.92 (0.34, 2.48)	0.875
9–10	0.50 (0.11, 2.31)	0.375
11–12	1.58 (0.49, 5.14)	0.445
$\geq 13$	0.36 (0.05, 2.82)	0.328
Non-smoker		
$\leq 6$	1 (reference)	–
7–8	0.75 (0.43, 1.32)	0.319
9–10	0.80 (0.45, 1.43)	0.455
11–12	0.63 (0.30, 1.31)	0.217
$\geq 13$	0.57 (0.30, 1.12)	0.103

To convert  $\mu\text{mol/l}$  to  $\text{mg/dl}$ , divide by 17.1

activity, with shorter  $(GT)_n$  repeats having higher *HMOX1* transcriptional activity and expression compared with longer  $(GT)_n$  repeats [8]. Studies exploring the association between *HMOX1* polymorphism, bilirubin levels and vascular events have yielded conflicting results [6, 38, 45, 46]. One such study reported that longer  $(GT)_n$  repeats (and hence lower *HMOX1* activity) were associated with lower bilirubin levels and higher risk of CHD [38]. After adjusting for bilirubin, the effect of *HMOX1* polymorphism on risk of CHD was no longer present, suggesting that the effect of *HMOX1* polymorphism might be mediated through its influence on bilirubin levels [38]. However, other studies have not found such an association between *HMOX1* polymorphism and bilirubin [45, 46]. Likewise, when comparing FIELD participants who developed on-study amputation with those who did not, there was no significant difference in the presence of the L allele (i.e. longer  $[GT]_n$  repeats) between these two groups of patients (see the ESM Methods, ESM Figs 2 and 3 and ESM Tables 3–6). These results suggest that carbon monoxide, (i.e. an *HMOX1*-derived product other than biliverdin/bilirubin), may not be responsible for the decrease in amputation rate associated with high bilirubin levels.

It is increasingly thought that hepatic *UGT1A1*, rather than *HMOX1*, plays a greater role in regulating bilirubin levels [6]. The link between *UGT1A1* polymorphism, bilirubin and vascular disease was first noted with the low CHD prevalence in patients with Gilbert's syndrome, a hereditary unconjugated hyperbilirubinaemia secondary to *UGT1A1* deficiency [14]. Subsequently, a prospective study involving 1,780 individuals from the Framingham Heart Study Offspring cohort found that *UGT1A1* polymorphism resulting in higher bilirubin levels was associated with lower risk of cardiovascular events [36]. Again, however, some other

smaller studies have failed to show the same association between *UGT1A1* polymorphism and CVD [18, 34, 47]. It is possible that these smaller studies lack the statistical power to detect this association. Nevertheless, clear associations between *UGT1A1* and bilirubin have been demonstrated in most studies, suggesting that *UGT1A1* may be a more important factor than *HMOX1* in regulation of bilirubin levels [6]. In our patient cohort, it is likely that the 317 (out of 9,795) patients with bilirubin concentration  $>20 \mu\text{mol/l}$  have Gilbert's syndrome, which is consistent with the estimated prevalence of 3–5% in the general population, although we do not have the results of *UGT1A1* polymorphism for confirmation. Therefore, future studies exploring the link between *UGT1A1* polymorphism and amputation in the FIELD study cohort will also be important to confirm the results from these previous studies.

There are several limitations of this study. First, bilirubin was, in most cases, only measured once at baseline. It is possible that bilirubin measurements vary significantly for an individual over time. However, random measurement error may result in an underestimation of a true association. Furthermore, in a subset of patients who had their bilirubin remeasured after 1 year, mean bilirubin concentrations had not changed significantly and the individual's levels over 1 year correlated strongly. Second, our study cohort included only patients with type 2 diabetes, and our findings cannot necessarily be generalised to the non-diabetic population. Third, bilirubin levels are inversely associated with glucose levels [48, 49], thereby potentially confounding the results from our study exclusively comprising patients with type 2 diabetes. However, reassuringly, the inverse relationship between bilirubin level and amputation survives adjustment for glycaemic control, and fasting glucose levels were measured at all time points. Fourth, our study does not provide a proven mechanism for the observed association between bilirubin and amputation. Last, the association reported here is not necessarily causal, and could be a false-positive result arising from multiple comparisons, as the FIELD study is exploring risk factors for CHD in diabetes as well as for each of the microvascular complications of diabetes. Therefore, it is possible that the association of bilirubin with amputations is overestimated, and that bilirubin may be a marker rather than an agent directly protective against amputation. Randomised studies raising bilirubin would be better suited to establishing the causal link. This last point is relevant as uric acid, although originally proposed as an antioxidant, is now generally accepted as a biomarker for lifestyle factors rather than a directly protective antioxidant [5, 50].

Despite these limitations, the strengths of the current study include its large sample size, longitudinal nature and the very well-characterised nature of the patient cohort. In

fact, the inverse association between bilirubin level and amputation persisted despite adjusting for a wide range of known risk factors for diabetic complications and amputation (though this did not negate the striking effects of fenofibrate treatment itself to reduce amputation events even after bilirubin adjustment [HR 0.64, 95% CI 0.44, 0.94,  $p=0.02$ ]).

In summary, our study reports for the first time a significant association between higher bilirubin levels and reduced risk of non-traumatic lower-limb amputation in individuals with type 2 diabetes. Furthermore, this association is not explained by other risk factors for amputation in the FIELD study. Our data raise the hypothesis that bilirubin may be a useful biomarker for, and may protect against, amputation in type 2 diabetes. Ultimately, a better understanding of the genes regulating bilirubin level and the potentially protective mechanisms may result in new therapeutic targets for the treatment of PAD and the prevention of amputation in the future.

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**Contribution statement** KHC, LSH, MV, AH, RS, MKCN and ACK were responsible for the conception and design of the study. KHC, RO'C, DRS, LSH, KR, MW, MKCN and ACK acquired the data; KHC, RO'C, DRS, KR, MWD, BY, MKCN and ACK analysed the data. KHC, RO'C, DRS, KR, MWD, MV, AH, BY, RS, MKCN and ACK interpreted the data. KHC, RO'C, LSH, RS, MKCN and ACK were responsible for drafting the article. All authors revised the manuscript and approved the final version.

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