Endothelin-1 predicts incident diabetic peripheral neuropathy in Type 2 Diabetes: a cohort study

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Abstract

Introduction: Diabetic peripheral neuropathy (DPN) is a common microvascular complication in patients with type 2 diabetes (T2D). Apart from hyperglycemia, few modifiable risk factors have been identified. Endothelin-1 is a potent vasoconstrictor peptide, implicated in the causal pathway of microangiopathy. We investigated whether baseline plasma endothelin-1 and other metabolic and vascular risk factors predicted the incidence of DPN.

Design: This is a 3-year observational, cohort study.

Methods: In patients with T2D ($n = 2057$), anthropometric data, fasting blood, and urine were collected for biochemistry and urine albumin/creatinine measurements. Forearm cutaneous endothelial reactivity was assessed by iontophoresis and laser Doppler flowmetry/imaging. Measurements were repeated on follow-up. Incident DPN was considered present if an abnormal finding in monofilament (<8 of 10 points) or neurothesiometer testing was ≥ 25 volts on either foot at 3-year follow-up, but normal at baseline. Plasma endothelin-1 was assessed by ELISA.

Results: At baseline, mean age of patients was 57.4 ± 10.8 years old and prevalence of DPN was 10.8%. Of the 1767 patients without DPN, 1250 patients returned for follow-up assessment ((2.9 ± 0.7) years), with a 10.7% incidence of DPN. Patients with incident DPN had significantly higher baseline endothelin-1 (1.43 (1.19 – 1.73) vs 1.30 (1.06 – 1.63)) pg/mL, $P < 0.0001$. Multivariable Cox proportional hazards ratio showed a 1-s.d. increase in log endothelin-1 (adjusted HR: 4.345 (1.451 – 13.009), $P = 0.009$), systolic blood pressure (per 10-unit) (adjusted HR: 1.107 (1.001 – 1.223), $P = 0.047$) and diabetes duration (adjusted HR: 1.025 (1.004 – 1.047), $P = 0.017$) predicted incident DPN, after adjustment for glycemic control, eGFR, albuminuria, peripheral arterial disease and retinopathy status.

Conclusion: Higher baseline endothelin-1, blood pressure and diabetes duration were significant and independent predictors for incident DPN. Validation of our findings in independent cohorts and molecular mechanistic studies will help better our understanding on the role of endothelin-1 in DPN.

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Introduction

Prevalence of type 2 diabetes mellitus (T2D) is increasing globally and has been identified as the ninth major cause of reduced life expectancy (1). Diabetic peripheral neuropathy (DPN) is one of the common microvascular complications

in patients with T2D, resulting in peripheral nerve dysfunction, accompanied with typical characteristics of pain and numbness (2, 3). DPN is one of the leading causes of lower limb amputations and morbidity in the

world. Aggressive glucose-lowering has been shown to be effective in delaying onset of DPN for type 1 diabetes but less so in T2D (4), probably arising from differences in their aetiology, where T2D is characterized by defects in insulin resistance, β -cell failure and pathophysiologic abnormalities in multiple organs (5). Comparing patients with intensified multifactorial intervention and those on conventional care, the STENO-2 study showed significant relative risk reductions for autonomic neuropathy, nephropathy and retinopathy (6), but no changes in the progression of DPN was observed.

Patients with DPN have microvascular dysfunction, as evidenced by reduced endothelial-dependent and endothelial-independent vasodilation (7). Impairment of endothelial function, characterized by reduced bioactivity of nitric oxide (NO), is an early finding in patients with insulin resistance (8) and T2D (9). Early alterations in microcirculation have been identified in pre-diabetic states, suggesting that endothelial dysfunction occurs early in the diabetes pathway (10). We showed in a previous cross-sectional study that reduced endothelial-dependent vasodilation and apolipoprotein C3 were significantly associated with DPN (11).

Endothelin-1 is a potent vasoconstrictor and pro-inflammatory peptide produced from various cell types, including the endothelial (12), neuronal (13) and renal tubular cells (14). Endothelin-1 acts on endothelin-1 receptors, ET_A and ET_B receptors, on adjacent endothelial or smooth-muscle cells, producing either vasoconstrictive or vasodilative effects, respectively. A large body of evidence has implicated endothelin-1 to be in the causal pathway of diabetic microangiopathy. Besides the negative effects on NO availability, insulin-resistant states such as T2D (15), obesity (16), essential hypertension (17) and coronary artery disease (18) have been shown to be associated with elevated plasma levels of endothelin-1.

While higher plasma endothelin-1 has been reported in patients with DPN compared to those without, these studies were conducted in a cross-sectional manner and causality of endothelin-1 to DPN is unknown. We hypothesize that in patients with T2D, higher baseline endothelin-1 predicts incident DPN. The aim of the study was to determine if baseline clinical parameters and plasma endothelin-1 are predictive of incident DPN.

Methods

This is a cohort study, where patients were recruited from August 2011 to March 2014 (SMART2D: Singapore

study of MAcro-angiopathy and micro-vascular Reactivity in Type 2 Diabetes) and followed-up after 3 years, as described previously (19, 20). We consecutively enrolled adults between 21 and 90 years of age, with T2D seen in our institution's Diabetes Centre and a community medical centre in Singapore. Patients were re-contacted through phone calls or mails. All procedures, performed at baseline, were repeated at follow-up. This study was approved by our institutional Ethics Review Board (NHG Domain Specific Review Board), and all participants signed informed consent at both visits.

Anthropometric data, fasting blood and urine were collected for blood biochemistry, urine albumin and creatinine measurements (Roche, Diagnostics) at both baseline and follow-up in the same routine laboratory. All blood samples were centrifuged within 1 h after collection and kept at 4°C during this period. Thereafter, they were stored at -80°C in aliquots and used without additional freeze-thaw cycles. Patients were advised not to take their morning medications until fasting blood was obtained. Estimated GFR (eGFR) was calculated using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation (21). Blood pressure (BP) and heart rate (Omron, Japan) were measured at the clinic and the average of three most consistent readings was taken. Baseline plasma endothelin-1 and insulin were measured by ELISA (RnD systems, USA and Mercodia, Sweden, respectively). Plasma samples were measured in duplicates with intra-assay CV of 2.02% and 2.83% and inter-assay CV of 8.00% and 7.40% for endothelin-1 and insulin, respectively. The homeostasis model assessment (HOMA)-2 of insulin resistance (HOMA2-IR) was calculated using an excel-based HOMA index of insulin resistance (HOMA2-IR) obtained from (<https://www.dtu.ox.ac.uk/homacalculator/>) (22). Assessment for neuropathy was by a neurothesiometer (Horwell Scientific, Yorkshire, UK) for vibration and with a 10-g monofilament for light touch. DPN was present if an abnormal finding in monofilament (inability to detect more than 2 of 10 points on either foot) or neurothesiometer testing of ≥ 25 volts on either foot was detected. The outcome in this study is incident DPN. Baseline and follow-up foot examinations were performed by the same team of research nurses ($n=4$) who received standardized training and accreditation (11).

Vascular measurements

Microvascular endothelial function assessments were performed in temperature controlled rooms by laser

Doppler flowmetry/imaging (Lisca PIM 3.0, Lisca Development AB, Linköping, Sweden) to measure cutaneous perfusion, accompanied by iontophoresis of acetylcholine or sodium nitroprusside to assess endothelium-dependent and endothelium-independent vasodilation, respectively, as described previously (23). Vasodilation was presented as percentage change from baseline flowmetry. Similarly, the same team performed both baseline and follow-up measurements.

Statistical analyses

All analyses were performed using SPSS version 22 (SPSS Inc) and STATA SE 14. Results for continuous variables with normal distribution were presented in mean $\pm$ S.D.; medians and interquartile ranges were presented for continuous but non-normally distributed variables. Binary variables were presented as absolute numbers or in percentages. Binary and continuous variables with normal distribution were analyzed using Chi-square and Student's *t*-test or ANOVA, respectively, while non-parametric tests were used for quantitative variables not normally distributed. Bonferroni correction was used for multiple testing. Bivariate associations of log-transformed endothelin-1 with clinical variables were analyzed using Pearson analyses. Associations of independent variables with endothelin-1 were analyzed using multivariable generalized linear regression. Only variables significant in Pearson's correlation (baseline age, BMI, HDL-C, triglyceride, systolic blood pressure, eGFR, urine ACR, retinopathy, endothelium-dependent, endothelium-independent vasodilation and metformin usage) or known confounders (gender and ethnicity) were added into the multivariable model. Multivariable Cox proportional hazards model was used to estimate the coefficient (β) and hazards ratio (HR) for incident DPN and 95% CI for each risk factor assessed at baseline, after adjustment. Age, gender, ethnicity, metabolic derangements (diabetes duration, baseline HbA1c and systolic BP), which were significant in the univariate analysis, baseline eGFR, urinary ACR, PAD, and retinopathy status, the main diabetic complications, were included as covariates in the Cox model. Proportionality assumptions were tested using Schoenfeld residuals. Skewed data such as triglycerides, urinary ACR, endothelial-dependent and endothelial-independent vasodilation were analyzed after logarithmic transformation. All statistical tests were two-sided with a level of significance being <0.05 .

Results

A total of 2057 patients with T2D were recruited at baseline. Prevalence of DPN was 10.8% in the cross-sectional cohort, as reported previously (11). Of 1767 patients without DPN at baseline (August 2011–March 2014), 1250 patients returned for a follow-up assessment within a mean of (2.9 ± 0.7) years. One hundred and thirty-four (10.7%) patients developed incident DPN. Characteristics of patients with no DPN at baseline, comparing those with incident DPN and those who remained free of DPN, are shown in Table 1 and follow-up characteristics of the cohort are shown in Supplementary Table 1 (see section on [supplementary materials](#) given at the end of this article). Prevalence of patients, who were lost to follow-up, was not statistically significantly different between patients with and without DPN, at baseline (Supplementary Fig. 1). Patients, with incident DPN, were significantly older at baseline, had longer duration of T2D, had poorer glycemic control with higher baseline fasting glucose and HbA1c, Systolic BP (SBP), heart rate, poorer eGFR, higher urine ACR, poorer endothelium-independent vasodilation and higher Endothelin-1 (1.30 ($1.06 - 1.63$) vs 1.43 ($1.19 - 1.73$) pg/mL, $P < 0.0001$). At baseline, a higher proportion of patients with incident DPN were on insulin and aspirin (Table 1).

Bivariate analyses of plasma endothelin-1 is shown in Table 2 (left column). Women had lower plasma endothelin-1 (1.35 ($1.08 - 1.67$)) pg/mL levels, compared to men (1.38 ($1.11 - 1.70$)) pg/mL ($P = 0.07$). Malays had significantly higher endothelin-1 (1.45 ($1.18 - 1.77$)) pg/mL compared to Chinese (1.33 ($1.07 - 1.66$)) pg/mL, Indians (1.35 ($1.11 - 1.67$)) pg/mL and other ethnicities (1.27 ($1.06 - 1.66$)) pg/mL ($P < 0.0001$). Patients with proliferative retinopathy and non-proliferative retinopathy had significantly higher endothelin-1 than those without retinopathy (1.44 ($1.19 - 1.78$) vs 1.42 ($1.15 - 1.69$) vs 1.32 ($1.07 - 1.65$)) pg/mL, $P < 0.0001$, respectively. Patients on metformin had significantly lower endothelin-1 (Supplementary Table 3). In the multivariable linear regression model (Table 2, right), age, Malay ethnicity, BMI, log-triglycerides and eGFR remained significant determinants of log-transformed endothelin-1, after adjusting for gender, HDL-C, SBP, urine ACR, retinopathy, endothelium-dependent, independent vasodilation and metformin usage. When endothelium-dependent and independent vasodilation were entered into the model separately, endothelium-dependent vasodilation but not endothelium-independent vasodilation was

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Table 1 Baseline characteristics of patients with and without incident DPN ($n = 1250$).

Baseline characteristics	No DPN on follow-up ($n = 1116$)	Incident DPN ($n = 134$)	P-value
Age (years)	56.3 $\pm$ 10.4	59.6 $\pm$ 9.1	<0.0001
Duration of diabetes (years)	10.1 $\pm$ 8.2	13.9 $\pm$ 9.8	<0.0001
Gender (% Males)	48.9	58.2	0.025
Ethnicity (%)			0.971
Chinese	55.5	54.5	
Malay	18.2	17.9	
Indian	22.6	23.1	
Others	3.7	4.5	
Height (cm)	1.61 $\pm$ 0.09	1.63 $\pm$ 0.09	0.016
BMI (kg/m ²)	27.6 $\pm$ 5.1	27.4 $\pm$ 5.6	0.714
Waist circumference (cm)	96.5 $\pm$ 15.0	95.8 $\pm$ 14.9	0.684
Total Cholesterol (mmol/L)	4.42 $\pm$ 1.01	4.48 $\pm$ 1.11	0.586
HDL-C (mmol/L)	1.31 $\pm$ 0.36	1.25 $\pm$ 0.32	0.057
LDL-C (mmol/L)	2.74 $\pm$ 0.79	2.80 $\pm$ 0.98	0.429
Triglyceride (mmol/L) [^]	1.37 (1.02–1.93)	1.46 (1.06–2.19)	0.094
Fasting glucose (mmol/L)	7.94 $\pm$ 2.53	8.48 $\pm$ 2.75	0.017
HbA1c (%)	7.67 $\pm$ 1.27	7.98 $\pm$ 1.43	0.031
Fasting Insulin (μ U/mL) [^]	7.68 (4.58–12.5)	8.00 (4.2–14.1)	0.704
HOMA2-IR [^]	1.15 (0.64–1.90)	1.16 (0.81–2.12)	0.123
Endothelin-1 (pg/mL) [^]	1.30 (1.06–1.63)	1.43 (1.19–1.73)	<0.0001
Systolic BP (mmHg)	135.2 $\pm$ 17.1	142.6 $\pm$ 20.7	<0.0001
Diastolic BP (mmHg)	77.5 $\pm$ 9.4	78.1 $\pm$ 10.0	0.492
Heart Rate (beats/min)	70.1 $\pm$ 10.7	72.3 $\pm$ 11.7	0.025
eGFR (mL/min/1.73 m ²)	90.6 $\pm$ 23.1	75.8 $\pm$ 30.4	<0.0001
Urine ACR (mg/g)	17.0 (5.0–63.5)	41.0 (10.0–221.0)	<0.0001
Endothelium-independent vasodilation (%) [^]	71 (41–110)	54 (29–91)	0.002
Endothelium-dependent vasodilation (%) [^]	110 (52–178)	89 (50–151)	0.083
Prevalence of Peripheral Arterial Disease (%)	10.2	17.3	0.003
Prevalence of Retinopathy (%)			<0.0001
Non-proliferative retinopathy	15.9	20.5	
Proliferative retinopathy	6.2	18.1	
% on Medications			
Metformin	84.4	73.1	0.001
Insulin	22.3	41.8	<0.0001
ACEI	34.6	39.6	0.149
ARB	24.7	30.3	0.100
Statins	80.0	78.2	0.267
Fibrates	10.6	8.2	0.241
Aspirin	19.6	27.6	0.021
Smoking History (%)			0.143
Non-Smoker	85.8	79.5	
Ex-Smoker	6.2	9.8	
Current Smoker	8.1	10.6	

[^]Log transformed before analysis.

statistically significant for log-transformed Endothelin-1 (Supplementary Table 2).

In the multivariable Cox regression (Table 3), baseline log-transformed endothelin-1 ($\beta = 1.469$, $SE = 0.559$, $P = 0.009$) SBP ($\beta = 0.101$, $SE = 0.051$, $P = 0.047$) and duration of diabetes ($\beta = 0.025$, $SE = 0.011$, $P = 0.017$) remained significant predictors of incident DPN, after adjustment for age, gender, ethnicity, HbA1c, eGFR, urine ACR, PAD and retinopathy status at baseline. Baseline endothelin-1 and SBP remained as significant predictors, even after

inclusion of BP medication usage (ACE inhibitors and angiotensin receptor blockers) (Supplementary Table 4).

Discussion

In this observational longitudinal cohort study, with 3-year follow-up, we show that elevated baseline endothelin-1, baseline SBP and longer duration of diabetes predicted incident DPN in a relatively large cohort of patients with

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Table 2 Univariable and multivariable association of plasma endothelin-1 (log transformed) with anthropometric and biochemical parameters ($n = 1250$).

Baseline characteristics	Pearson correlation	P-value	Adjusted odds ratio (95% CI)	P-value
Age (years)	0.152	<0.0001	1.002 (1.001–1.002)	<0.0001
Duration of Diabetes (years)	0.028	0.244		
Gender [#]	0.043	0.07	1.016 (1.000–1.032)	0.053
Ethnicity [†]	0.020	0.396		
Malay			1.027 (1.008–1.047)	0.006
Indian			1.008 (0.990–1.026)	0.399
Others			0.992 (0.957–1.029)	0.682
BMI (kg/m ²)	0.151	<0.0001	1.005 (1.003–1.006)	<0.0001
Waist circumference (cm)	0.130	<0.0001		
Total Cholesterol (mmol/L)	0.047	0.049		
HDL-C (mmol/L)	−0.067	0.005	1.006 (0.982–1.030)	0.650
LDL-C (mmol/L)	0.059	0.223		
Triglyceride (mmol/L) [^]	0.114	<0.0001	1.054 (1.016–1.093)	0.005
Fasting glucose (mmol/L)	0.013	0.589		
HbA1c (%)	−0.005	0.832		
Fasting Insulin [^]	0.007	0.763		
HOMA-2IR [^]	0.002	0.936		
Systolic BP (mmHg)	0.099	<0.0001	1.001 (0.997–1.005)	0.713
Diastolic BP (mmHg)	−0.004	0.865		
eGFR (mL/min/1.73 m ²)	−0.24	<0.0001	0.993 (0.990–0.997)	<0.0001
Urine ACR (mg/g) [^]	0.138	<0.0001	1.003 (0.994–1.012)	0.486
PAD ^{##}	−0.026	0.276		
Retinopathy [*]	0.099	<0.0001		
Non-proliferative			1.015 (0.996–1.034)	0.126
Proliferative			1.018 (0.991–1.046)	0.188
Endothelium-independent vasodilation (%)	−0.082	0.001	0.995 (0.974–1.015)	0.607
Endothelium-dependent vasodilation (%)	−0.069	0.004	0.985 (0.968–1.002)	0.091
Metformin (Usage vs non-usage) ^{**}	−0.104	<0.0001	0.982 (0.964–1.001)	0.064

Pearson's correlation is shown on the left and multivariate association, adjusted for potential confounders are shown on the right. Only variables significant in Pearson's correlation were entered into the multivariate analysis. *P*-values are in bold to indicate statistical significance after adjustment.

[#]Gender was coded as a dummy variable where 1 = female and 2 = male. [†]Ethnicity was coded as a dummy variable where 1 = Chinese, 2 = Malay, 3 = Indian and 4 = Others. ^{##}PAD was coded as dummy variable where 0 = no PAD and 1 = PAD. ^{*}Retinopathy was coded as dummy variable where 0 = no Retinopathy, 1 = non-proliferative retinopathy and 2 = proliferative retinopathy. ^{**}Metformin was coded as a dummy variable where 0 = non-usage and 1 = usage. [^]Log-transformed before analysis.

T2D. These findings suggest a potential pathogenic link between endothelin-1 and the development of clinical DPN. To our knowledge, while high endothelin-1 has been shown to predict incident coronary heart disease (24) and all-cause death in general population (25), this is the first report of elevated baseline endothelin-1 predicting incident DPN in a cohort of patients with T2D.

This study is the first to investigate baseline endothelin-1 with incident DPN using a prospective design. We hypothesize that ET1 may contribute to incident DPN through impact on several pathophysiological mechanisms. Elevated endothelin-1 has been found in both chronic and acute inflammatory demyelinating polyneuropathy compared to healthy controls (26). Most evidence suggests that actions attributed to endothelin-1 are likely to be secondary to reduced blood flow and ischemia of nerve tissue (27, 28). Apart from its vasoconstrictive actions, endothelin-1 possesses pro-inflammatory effects

in vascular walls. It has been reported that endothelin-1 activates macrophages, releasing mediators like TNF- α (29), IL-6 (30), IL-8 (31) and reactive oxidative species (ROS) (32). Endothelin-1 stimulates the production of ROS in arterial smooth muscle cells, endothelial cells and isolated arteries, demonstrating a putative mechanism for the detrimental effects of endothelin-1 on peripheral nerves (33, 34). Raised endothelin-1, at baseline, may directly potentiate the demyelination of peripheral nerves in patients with incident DPN. Direct neuronal actions of endothelin-1 are less well-studied compared to its vasomotor effects. In experimental diabetic models, endothelin receptor antagonism, with bosentan, led to a significant increase in sciatic nerve conduction through improvements in sciatic nutritive endoneurial blood flow (35). Novel therapies of ET_A antagonists, such as Bosentan, led to improvements in microvascular endothelium-dependent vasodilatation in patients with diabetes and microangiopathy (36),

Table 3 Multivariable Cox regression analysis predicting incident diabetic peripheral neuropathy.

Variables	Hazards ratio (95% CI)	P-value
Model		
Endothelin-1 (pg/mL) ^Δ	4.345 (1.451–13.009)	0.009
Age (years)	1.013 (0.992–1.035)	0.214
Gender [#]	1.283 (0.886–1.860)	0.187
Ethnicity (Reference: Non-Malay)		
Malay	0.902 (0.552–1.476)	0.681
Duration of Diabetes (years)	1.025 (1.004–1.047)	0.017
HbA1c (%)	1.148 (0.988–1.333)	0.071
SBP (10-unit) (mmHg)	1.107 (1.001–1.223)	0.047
eGFR (mL/min/1.73 m ²)	0.996 (0.989–1.003)	0.217
Urine ACR (mg/g) ^Δ	1.171 (0.929–1.476)	0.182
PAD vs no PAD	1.448 (0.892–2.350)	0.135
Retinopathy vs no Retinopathy	1.152 (0.776–1.709)	0.483

Only parameters significant in Table 1 or known confounding factors were added sequentially into the Models. Age, gender, ethnicity and metabolic derangements which were significant in the univariate analysis (diabetes duration, baseline HbA1c and systolic BP), baseline eGFR, urinary ACR, PAD and retinopathy status were included as covariates. Schoenfeld residual test: $P = 0.2090$.

^ΔLog-transformed before analysis. [#]Gender was coded as a dummy variable where 1 = female and 2 = male.

suggesting that higher endothelin-1 could contribute to poorer nerve conduction and DPN. On-going trial efforts, like SONAR (37), may provide better insights and possibilities of the endothelin receptor antagonism in improving blood pressure, reducing the incidence of DPN and diabetic ulcers.

Our study showed that elevated baseline blood pressure predicted incident DPN. Previous cross-sectional studies suggest a relationship between hypertension and the presence of neuropathy (38). A prospective cohort study identified hypertension as a vascular risk factor predicting the incidence of DPN in patients with Type 1 Diabetes (39). While the blood pressure lowering medication, angiotensin-converting enzyme inhibitors (ACEI), improved motor and sensory nerve conduction velocity in patients with diabetes (40), no differences were found in intensive and moderate blood pressure therapy on the progression of DPN in patients with T2D (41). In animal models of impaired glucose tolerance and hypertension, although neuropathy development was attributed to hyperglycemia, a combination of diabetes and hypertension led to worsening of sensory nerve function and development of myelin thinning and protein loss at late stages. These phenotypes are postulated to be associated with metalloproteinase dysregulation in Schwann cell (42). These findings are in line with our data that elevated blood pressure in

patients with T2D accelerates DPN development. In our cohort, approximately 60% of patients were on ACEI and/or angiotensin-receptor blocker (ARB) at baseline. Future studies on blood pressure trajectories might shed light on whether early blood-pressure lowering may delay onset of DPN.

Duration of diabetes was an independent predictor of incident DPN in our cohort, in line with data reported by others (43). In the univariate analyses, although HbA1c predicted incident DPN, it was attenuated in the fully adjusted model. This further supports the notion that duration of diabetes, rather than glycemic status, is a critical determinant in DPN pathogenesis. Indeed, while ACCORD showed that intensive glucose-lowering reduced incident DPN (44), Steno-2 (45) and Veterans Affairs Diabetes Trial (VADT) (46) did not show a difference in development of DPN between groups with and without intensive glucose control. Having said so, we would concede that HbA1c observed at baseline may not be sufficient to capture global glycemic burden of the participants and may therefore explain the null association with incident DPN.

In our cohort, we showed that at baseline, older age, Malay ethnicity, higher BMI, elevated triglycerides and poorer eGFR were independently associated with endothelin-1, after adjustment for various risk factors. Endothelin-1 is a well-known marker for vascular endothelial dysfunction/inflammation, which has been reported to reduce availability of nitric oxide (47), in addition to its direct vasoconstriction effects. Therefore, a high plasma circulating endothelin-1, at baseline, could have been reflective of poorer endothelial function and increased the incidence of DPN, within a 3-year follow-up. Significant association of endothelin-1 with obesity (BMI and waist circumference) and triglycerides have been well-reported (48, 49). Impairment of glucose uptake by endothelin-1 (50) may reduce storage of glucose-derived lipids. Although speculative, these mechanisms might lead to dyslipidemia as a result of dysfunctional adipocytes in an insulin resistant state. Congruent to this observation, intralipid infusion to increase triglyceride levels led to significant increase in arterial endothelin-1 in healthy volunteers (51). We showed an independent association of lower eGFR with baseline endothelin-1. The relationship between eGFR and endothelin-1 is not unexpected, as others have shown that higher baseline endothelin-1 predicted incident chronic kidney disease (CKD) (52). One possible mechanism which may explain the relationship of higher endothelin-1 and lower eGFR is the disruption of actin cytoskeleton in podocytes by

endothelin-1, leading to increased permeability of the glomerular filtration barrier (53). Endothelin-1 may also induce endoplasmic reticulum stress and apoptosis in renal tubular cells, resulting in kidney injury (54). Significant association between Malay ethnicity and plasma endothelin-1 has never been reported before. While we previously showed that different genotypes of endothelin-1 may contribute to differences in plasma C-terminal pro-endothelin-1, conferring susceptibility to diabetic nephropathy, the study was conducted in a Chinese cohort (55). Large genotyping efforts, lifestyle and dietary habits will be required to decipher if plasma endothelin-1 is influenced by ethnicity. Future studies focused on genotyping of endothelin-1, lifestyle or dietary habits will be required to explore if plasma Endothelin-1 is influenced by ethnicity. While it is likely that endothelin-1 is a good predictor of incident DPN, its large variability in our multi-ethnic population may limit its use as a prognostic marker.

The pathogenesis of DPN in patients with T2D is complex. Whether elevated endothelin-1 is directly causal in the pathophysiological pathway of DPN or a surrogate indicator as a result of other diabetic complications at baseline is unknown. Recent publications support the prevailing hypothesis that small fiber nerve damage (affecting unmyelinated nerve (A δ and C) fibers) precedes large fiber damage (myelinated (A α and A β) in DPN (56, 57). Small fiber neuropathy usually affects nociceptive and autonomic systems, leading to microvascular dysfunction and impaired NO. In our study, small fiber neuropathy was not specifically assessed at baseline, hence we are not able to exclude the possibility that the presence of small fiber neuropathy led to an increase in endothelin-1 at baseline, which predicted incident DPN. We observed a marginally elevated heart rate, at baseline, in patients with incident DPN, which might reflect autonomic dysfunction, though this is highly speculative. Patients with incident DPN had multiple complications of T2D at baseline. Raised endothelin-1 at baseline could be a consequence of compensatory response to retinopathy (58), increased arterial stiffness (59), endothelial dysfunction (11) or reduced renal clearance. Increased plasma endothelin-1 has been previously linked to increased risk of microvascular and macrovascular complications such as microalbuminuria (60), PAD (61), while its expression in patients with diabetic proliferative retinopathy remains controversial (62, 63, 64). These complications may collectively contribute to incident DPN, rather than endothelin-1, *per se*. Nonetheless, endothelin-1 remains an interesting early plasma biomarker, which may predict incident DPN.

Strengths of the study include the strict enrolment criteria, longitudinal design and the ability to perform detailed, careful and standardized assessment in a relatively large number of T2D patients. A second advantage is the ability to assess prognostic value of plasma endothelin-1 in a relatively large cohort of multi-ethnic patients with T2D. While we attempted to standardize blood collection after an overnight fast, we acknowledge that the limitation that we have only analyzed endothelin-1 in a T2D cohort at a single time point and our findings are not generalizable to other populations. *In vivo* human glucose tolerance tests showed either a reduction (65) or no change of endothelin-1 (66), while fasting states in animal models led to raised plasma endothelin-1 (67). Given the possible influence on fasting changes of plasma endothelin-1, measuring at a single time point would not reflect the dynamic post-prandial changes. Additionally, we did not evaluate small-fibre neuropathy in our cohort, hence may underestimate the prevalence and incidence of neuropathy in our cohort. On entry, patients in our cohort had a diabetes duration of 10.0 years (4.0–16.0), hence our results cannot be applied to diabetes population at different stages. Due to the study protocol, time-to-event was estimated when patient was followed-up for the study; hence, the true incident date of onset may be not precise. A longer follow-up period, in future, will allow elucidation of other risk factors for incident DPN. Another limitation is that our study design of selecting patients from a non-randomized diabetes cohort does not allow us to preclude residual confounding effects.

In conclusion, this study demonstrated that baseline elevated baseline endothelin-1, SBP and longer duration of diabetes predicted incident DPN. Elucidating risk for DPN is important, given that some of these factors are potentially modifiable. The ability to recognize and stratify these patients, who may benefit from treatment, before the development of clinical symptoms will be beneficial.

Supplementary materials

This is linked to the online version of the paper at <https://doi.org/10.1530/EJE-19-0523>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this study.

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