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ORIGINAL ARTICLE

Norepinephrine transporter expression is inversely associated with glycaemic indices: a pilot study in metabolically diverse persons with overweight and obesity

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Summary

Objective

The objective of this study was to examine the cross-sectional relationship between the expression of norepinephrine transporter (NET), the protein responsible for neuronal uptake-1, and indices of glycaemia and hyperinsulinaemia, in overweight and obese individuals.

Methods

Thirteen non-medicated, non-smoking subjects, aged 58 ± 1 years (mean ± standard error of the mean), body mass index (BMI) 31.4 ± 1.0 kg m^{-2}, with wide-ranging plasma glucose and haemoglobin A1c (HbA1c, range 5.1% to 6.5%) participated. They underwent forearm vein biopsy to access sympathetic nerves for the quantification of NET by Western blot, oral glucose tolerance test (OGTT), euglycaemic hyperinsulinaemic clamp, echocardiography and assessments of whole-body norepinephrine kinetics and muscle sympathetic nerve activity.

Results

Norepinephrine transporter expression was inversely associated with fasting plasma glucose ($r = -0.62$, $P = 0.02$), glucose area under the curve during OGTT (AUC_{0-120}, $r = -0.65$, $P = 0.02$) and HbA1c ($r = -0.67$, $P = 0.01$), and positively associated with steady-state glucose utilization during euglycaemic clamp ($r = 0.58$, $P = 0.04$). Moreover, NET expression was inversely related to left ventricular posterior wall dimensions ($r = -0.64$, $P = 0.02$) and heart rate ($r = -0.55$, $P = 0.05$). Indices of hyperinsulinaemia were not associated with NET expression. In stepwise linear regression analysis adjusted for age, body mass index and blood pressure, HbA1c was an independent inverse predictor of NET expression, explaining 45% of its variance.

Conclusions

Hyperglycaemia is associated with reduced peripheral NET expression. Further studies are required to identify the direction of causality.

Keywords: Hyperglycaemia, norepinephrine transporter, obesity, sympathetic nervous system.

Background

Rising levels of obesity, the ageing of the population and greater longevity due to better health care have contributed to the emerging worldwide pandemic in type 2 diabetes (T2D)(1). Cardiovascular disease is the leading cause of death in diabetes, and people with diabetes have an earlier onset of cardiovascular events and a worse prognosis compared with their non-diabetic counterparts (2,3). Moreover, even in non-diabetic populations,

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hyperglycaemia confers increased cardiovascular risk and all-cause mortality (3,4). The sympathetic nervous system is an important regulator of both cardiovascular and metabolic function and there is a growing body of scientific evidence showing that T2D and pre-diabetes are characterized by sympathetic neural perturbations at multiple levels (5–7). Interestingly, manifestations of sympathetic activation are observed early on in genetically predisposed individuals. Elevated resting muscle sympathetic nerve activity (MSNA), heightened sympathetic nervous system responses to hyperinsulinaemia, and altered circadian rhythm of autonomic activity have been reported in non-diabetic offspring of T2D patients (8–10). In a recent appraisal of obese individuals with newly-diagnosed T2D, we demonstrated elevated arterial norepinephrine concentration, augmented central sympathetic outflow and reduced whole-body plasma norepinephrine clearance, compared with age-matched and body mass index (BMI)-matched controls with impaired glucose tolerance (IGT)(5). In addition, T2D subjects had lower steady-state ratio of tritiated 3,4-dihydroxyphenylglycol (DHPG, the major intraneuronal metabolite of norepinephrine) to tritiated norepinephrine ([^{3}H]-DHPG : [^{3}H]-NE) – an index of neuronal norepinephrine transporter (NET) mediated re-uptake (uptake-1)(5,11).

Norepinephrine transporter belongs to the monoamine transporter family and functions to translocate norepinephrine from the neuroeffector junction, and to a lesser extent from the general circulation, back into sympathetic nerves via an active transport process (11,12). *In vitro* and *in vivo* experimental studies indicate that hyperglycaemia and/or hyperinsulinaemia may down-regulate NET expression (13–18). Using a rodent model of insulin resistance with sustained hyperglycaemia (8-weeks high-fat diet and moderate streptozotocin treatment) Thackeray *et al.* demonstrated a 17% reduction in cardiac NET expression and an increase in plasma and cardiac norepinephrine content compared with controls (13). Notably, the reduction in NET expression was independent of any change in sympathetic nerve density. In another study, enhanced polyol pathway flux was found to correlate with reduction in regional cardiac NET expression in streptozotocin treated rats and could be prevented by aldose reductase inhibition (16). Similarly, protein kinase C (PKC) activation acutely diminished NET capacity (Vmax), binding density and cell surface protein expression in cultured cells (17), highlighting that alternate glucose disposition pathways may adversely impact on NET functionality. In parallel to these data on the effects of hyperglycaemia, there is experimental evidence that acute or chronic hyperinsulinaemia may also down-regulate NET, in both the central and peripheral nervous systems (15,18).

In the clinical setting, cardiac scintigraphic studies with ^{123}I-metaiodobenzylguanidine (MIBG), a norepinephrine analogue transported into sympathetic nerves by NET, demonstrate decreased uptake and enhanced washout rate in T2D patients compared with controls and associated disturbances in left ventricular (LV) diastolic function (19). Furthermore, ^{123}I-MIBG uptake has been shown to correlate inversely with haemoglobin A1c (HbA1c) and to predict future cardiac and cerebrovascular events within diabetic populations (20,21). However, no studies to date have examined the metabolic determinants of NET protein expression in humans. This is clinically relevant because peripheral impairment of uptake-1 augments the sympathetic neural signal (5,13). Therefore, the aim of the present study was to quantify the expression of NET in individuals with differing levels of glycaemia, encompassing normal glucose tolerance (NGT), IGT and treatment naïve T2D. We chose subcutaneous forearm veins as the tissue source, because of their dense sympathetic innervation and because the biopsy procedure is relatively minor and well tolerated (22). We hypothesized that hyperglycaemia and/or hyperinsulinaemia would be associated with lower NET expression.

Methods

Subjects

Consecutive non-smoking and non-medicated Caucasian subjects ($n = 13$), participating in clinical trial NCT01771042, who consented to forearm vein biopsy were studied. The purpose of trial NCT01771042 is to examine the benefits of weight loss on neuroadrenergic function within different metabolic strata. Subjects were recruited through newspaper advertising and poster displays in primary healthcare centres, on the basis of being overweight or obese and having a stable body weight (±1 kg) in the previous 6 months as ascertained during screening telephone interview. Exclusion criteria comprised history of cardiovascular, cerebrovascular, renal, liver or thyroid diseases and use of drugs known to affect measured parameters (e.g. oral hypoglycaemic, antihypertensive, antidepressant and cholesterol-lowering therapies) or continuous positive airway pressure treatment. All women were post-menopausal, and none were taking hormone replacement therapy. The study was approved by the Alfred Hospital Ethics Committee, and written informed consent was obtained from each participant. The car parking costs of participants were reimbursed, but they received no other monetary payment. Screening tests comprised physical examination,

electrocardiogram and fasting blood biochemistry. Blood pressure was determined by Dinamap monitor (Model 1846SX, Critikon Inc, Tampa, FL, USA) as the average of five supine recordings after 5-min rest.

Clinical investigations

Clinical investigations were performed on three separate mornings within a 2-week period, in a temperature controlled research room (22 °C). Subjects attended at 8:00 h after an overnight fast, having abstained from alcohol and vigorous exercise for 36 h and caffeine for 18 h. Body composition was determined by whole-body dual-energy X-ray absorptiometry (DEXA, GE-LUNAR Prodigy Advance PA + 130510, GE Medical Systems, Lunar, Madison, W1, USA).

Assessments of glucose metabolism

Subjects underwent a standard 75-g OGTT (Glucaid, Fronine PTY, LTD, Taren Point, NSW 2229, Australia) with 30-minutely blood sampling. The Matsuda insulin sensitivity index was calculated as a measure of insulin sensitivity to endogenously produced insulin (23). World Health Organization criteria were used to categorize subjects as NGT, IGT or as having T2D. On a separate day, the euglycaemic hyperinsulinaemic clamp was initiated by an intravenous bolus injection of insulin (9 mU kg^{-1}; Actrapid 100 IU ml^{-1} Novo Nordisk, Gentofte, Denmark), followed by a constant infusion rate of 40 mU m^2 min^{-1}. Arterialized blood glucose was clamped at 5.0 mmol L^{-1} by the variable infusion of 25% glucose (Baxter, Toongabbie, Australia) and measured 5-minutely using an ABL8XX glucose auto-analyzer (Radiometer, Copenhagen, Denmark). The mean glucose infusion rate between 90 and 120 min was used to calculate whole-body glucose utilization (M value, expressed as mg kg fat-free mass min^{-1}) and the M/I value, adjusted for steady-state plasma insulin concentration. Insulin secretion was estimated by insulinogenic index based on insulin and glucose incursions during OGTT: ($insulin_{30}$–$insulin_0$)/($glucose_{30}$–$glucose_0$) and expressed as mU $mmol.^{-1}$

Sympathetic nervous system measurements

Isotope dilution methodology was used to quantify the rate at which norepinephrine released from sympathetic nerve endings enters the central plasma compartment (whole-body norepinephrine 'spillover' rate) and simultaneously, its plasma clearance (norepinephrine clearance rate). After a priming intravenous bolus of 1.90 μCi of 1-[ring-2,5,6-^{3}H]-norepinephrine (PerkinElmer, Waltham, MA, US; specific activity, 12-15 Ci $mmol^{-1}$), a constant infusion was commenced at 0.19 μCi min^{-1}. Steady state brachial arterial blood samples were obtained 30 min after commencement of the infusion. Calculations comprised

$$\text{Norepinephrine spillover } \left(\text{ng min}^{-1}\right) = \frac{\text{plasma norepinephrine } \left(\text{pg ml}^{-1}\right) \times \text{clearance} \left(\text{ml min}^{-1}\right)}{1,000}$$

$$\text{Norepinephrine clearance } \left(\text{L min}^{-1}\right) = \frac{[^3\text{H}] - \text{norepinephrine infusion rate } \left(\text{dpm min}^{-1}\right)}{[^3\text{H}] - \text{norepinephrine plasma concentration } \left(\text{dpm ml}^{-1}\right) \times 1,000}$$

The dynamic rate of neuronal norepinephrine uptake was estimated by the steady state [^{3}H]-DHPG: [^{3}H]-NE ratio (5,11). Efferent sympathetic nervous activity directed to the skeletal muscle vasculature (MSNA) was quantified in the right peroneal nerve using the technique of microneurography, as previously described (5). MSNA was manually analysed and expressed as burst frequency (bursts/minute) and burst incidence (bursts/100 heartbeats), averaged over 15 min.

Norepinephrine transporter expression in forearm vein biopsies

Our group has previously validated a forearm vein biopsy technique to quantify NET protein in healthy subjects and individuals with the postural orthostatic tachycardia syndrome (22). Briefly, a skin incision was performed on the dorsum of the forearm under local anaesthesia to identify a vein with a diameter of 1–2 mm. One centimetre length of the vein was removed after ligation at both ends with absorbable suture material. Three sutures were used to provide adequate closure of the skin. After removal, the vein was immediately frozen in liquid nitrogen and stored at −80 °C until assay.

Western blots were prepared by using lysates from vein biopsies. The tissue samples were homogenized in PRO-Prep protein extraction solution (17081, INtRON Biotechnology, Korea). Proteins were separated by electrophoresis on a 10% acrylamide minigel and transferred onto polyvinylidene fluoride membranes (NEF1002, PerkinElmer Life Sciences Inc, Waltham, MA, USA). Membranes were incubated with antibodies to NET (anti-NET 1:1000, NET17-1, MAb Technologies Inc, Neenah, WI), tyrosine hydroxylase (anti-TH 1:1000, AB152, Merck Millipore, Bayswater, Victoria, Australia), vesicular monoamine transporter-2 (anti-VMAT2 1:500, V9014, Sigma-Aldrich, Castle Hill, New South Wales, Australia) and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1:10,000,

Santa Cruz Biotechnology Inc, Texas, US) as protein loading control. This was followed by secondary antibody incubation with peroxidase-conjugated anti-IgG. Immunoreactive bands were visualized with enhanced chemiluminescence reagents (NEL104, PerkinElmer LAS Inc, Buckinghamshire, UK). Immunoblot densities were analysed with BIO-RAD QUANTITY ONE software and normalized to GAPDH (expressed as a percentage of GAPDH Control).

Cardiovascular assessments

Spontaneous cardiac baroreflex sensitivity was estimated using the sequence method. The slope of the regression line between cardiac interval and intra-arterial systolic blood pressure values was calculated for each validated sequence (when $r > 0.85$). Individual slopes were averaged over a 15 min recording period.

Doppler echocardiography was performed in the left decubitus position using a Vivid 7 ultrasound machine (GE Vingmed, GE Healthcare, USA) with an M4S 1.5–4.0 MHz matrix array probe, according to the guidelines of the American Society of Echocardiography (24) as detailed previously (25). All examinations were performed by an experienced research cardiac technologist (SK) and reported by a cardiologist (CW) who specialized in echocardiography, blinded to the metabolic status of participants.

Laboratory analyses

Plasma glucose and lipid profile were measured on a commercial analytical system (Architect C18000 analyzer, Abbott Laboratories, Illinois, USA). High sensitivity CRP was quantified by immunoturbidimetric assay, insulin by radio-immuno assay (Linco Research, Inc, Missouri, USA), C-peptide by chemiluminescent immunoassay (ADVIA Centaur, Siemens Healthcare Diagnostics, Tarrytown, NY, USA) and non-esterified fatty acids by enzymatic colorimetric method (Wako Pure Chemical Industries, LtD, Osaka, Japan). Plasma norepinephrine was assayed by high-performance liquid chromatography with electrochemical detection, following extraction by alumina adsorption. [^{3}H]-NE was assayed by liquid scintillation chromatography and corrected for loss during extraction using recoveries of internal standard. Intra-assay CVs in our laboratory are 1.3% for norepinephrine and 2.3% for [^{3}H]-NE; inter-assay CVs are 3.8% and 4.5%, respectively.

Statistical analyses

Statistical analysis was performed using SIGMASTAT Version 3.5 (Systat Software Inc, Point Richmond, CA, USA). Data are presented as the mean ± standard error of the mean and the range. Univariate associations between NET expression and metabolic, anthropometric and cardiovascular variables were assessed using Pearson's correlations. Non-parametric data were log-transformed as appropriate. Age, gender, BMI and blood pressure adjusted stepwise linear regression analyses were performed to identify the independent predictors of NET expression in the cohort. Variables with P-values ≤ 0.05 in univariate analyses were entered into the regression model. Sub-group comparisons based on an HbA1c cut-point of 5.7% were made using Student's un-paired *t*-tests and Mann–Whitney rank sum test. This cut-point was selected according to the American Diabetes Association position statement for the stratification of individuals into low (<5.7%) and high (≥5.7%) risk for diabetes (26). Statistical significance was accepted at the $P < 0.05$ (two-tailed) level.

Results

Subjects

Demographic and clinical characteristics of study participants are presented in Table 1. One subject was NGT, nine were IGT and three had treatment naïve T2D. Fasting glucose, 2-h glucose and HbA1c ranged from 4.6 to 8.5 mmol L^{-1}, 6.2 to 19.0 mmol L^{-1} and 5.1% to 6.5%, respectively. One subject had white-coat hypertension, with clinic blood pressures averaging 143/70 mmHg, but normal 24-h ambulatory blood pressure. All other subjects were normotensive as per the European Society of Hypertension definition (27). Based on echocardiographic assessment, one subject had borderline LV hypertrophy, two had mild concentric LV hypertrophy and four had delayed LV diastolic relaxation. Acceptable MSNA recordings were available in 10 of the 13 subjects. We failed to obtain recordings in two IGT and one T2D subject.

Correlates of norepinephrine transporter expression

Immunoblots for NET resulted in consistent protein bands at ~80 kDa (Figure 1). Numerical immunoblot densities for each subject are presented in Table S1 and the whole Western blot in Figure S3. NET expression correlated inversely with fasting plasma glucose ($r = -0.62$, $P = 0.02$), glucose area under the curve during (AUC_{0-120}, $r = -0.65$, $P = 0.02$), HbA1c ($r = -0.67$, $P = 0.01$), homeostasis model assessment-insulin resistance (HOMA-IR) ($r = -0.50$, $P = 0.08$), non-esterified fatty acids ($r = -0.49$, $P = 0.09$) and heart rate ($r = -0.55$, $P = 0.05$), and positively with clamp derived glucose utilization ($r = 0.58$, $P = 0.04$) and insulinogenic index ($r = 0.51$, $P = 0.08$) as illustrated

Table 1 Demographic and clinical characteristics of study participants

Variable	Mean ± SEM	Range
Age (years)	58 ± 1	50–65
Gender (M/F)	5/8	—
Anthropometrics		
Body weight (kg)	92.7 ± 3.3	75.4–112.2
Body mass index (kg m^{-2})	31.4 ± 1.0	27.2–37.6
Waist circumference (cm)	104.3 ± 2.9	91–123
Waist to hip ratio	0.91 ± 0.02	0.81–1.02
Total body fat mass (kg)	37.6 ± 2.3	20.9–53.0
Glucose metabolism		
Fasting glucose (mmol L^{-1})	5.9 ± 0.3	4.6–8.5
Fasting insulin (mU L^{-1})	15.7 ± 1.7	3.0–21.9
Fasting C-peptide (pmol L^{-1})	627 ± 67	352–1155
HbA1c (%)	5.7 ± 0.1	5.1–6.5
HbA1c (mmol mol^{-1})	39 ± 2	32–48
HOMA-IR	4.31 ± 0.55	0.73–8.08
2-h glucose (mmol L^{-1})	9.7 ± 0.9	6.2–19.0
Glucose $AUC_{0–120}$ (mmol · min·L^{-1})	1317 ± 68	1067–1895
Insulin $AUC_{0–120}$ (mU min L^{-1})	8177 ± 971	2961–15036
Insulinogenic index (mU mmol^{-1})	9.2 ± 1.4	1.6–18.7
Matsuda ISI	2.96 ± 0.65	1.31–9.70
M (mg kg FFM min^{-1})	10.7 ± 1.0	4.9–16.0
M/I (mg kg FFM min^{-1}·mU L^{-1}) × 100	9.6 ± 1.1	3.8–16.5
Lipids		
LDL-cholesterol (mmol L^{-1})	3.9 ± 0.3	1.9–5.8
HDL-cholesterol (mmol L^{-1})	1.35 ± 0.06	1.0–1.7
Triglycerides (mmol L^{-1})	1.6 ± 0.2	0.8–3.8
NEFA (mEq L^{-1})	0.49 ± 0.06	0.20–0.79
Clinic blood pressure and heart rate		
Systolic (mmHg)	124 ± 3	106–143
Diastolic (mmHg)	71 ± 2	57–83
Heart rate (bpm)	62 ± 3	46–76

$AUC_{0–120}$, area under the curve during oral glucose tolerance test; FFM, fat-free mass; HOMA-IR, homeostasis model assessment-insulin resistance; ISI, insulin sensitivity index; M, steady-state glucose utilization during euglycaemic hyperinsulinaemic clamp; M/I, M-value adjusted for steady-state plasma insulin concentration; NEFA, non-esterified fatty acids; SEM, standard error of the mean

in Figure 2. NET expression was not associated with measures of hyperinsulinaemia: fasting insulin ($r = -0.33$, $P = 0.28$) and insulin $AUC_{0–120}$ ($r = 0.03$, $P = 0.93$). Exclusion of the three hyposecreting T2D subjects (with insulin $AUC_{0–120}$ 6037 ± 353 mU L min^{-1}) did not alter the associations with fasting insulin ($r = -0.31$, $P = 0.38$) and insulin $AUC_{0–120}$ ($r = -0.03$, $P = 0.93$). Of the echocardiographic variables NET expression was inversely related to LV posterior wall thickness ($r = -0.64$, $P = 0.02$), LV septal wall thickness ($r = -0.50$, $P = 0.08$), but not to LVMI ($r = -0.35$, $P = 0.25$) or Doppler and tissue Doppler variables. NET expression was not associated with age, anthropometric variables or clinic blood pressure and did not differ between genders. The correlation between steady state [^{3}H]-DHPG : [^{3}H]-NE ratio and NET expression was $r = 0.47$, $P = 0.11$. In adjusted stepwise linear regression analysis, HbA1c was found to be an independent predictor of NET ($P = 0.01$), explaining 45% of its variance (Table 2).

Subgroup analyses

Norepinephrine transporter expression was lower by an average of 29% in subjects with HBA1c ≥ 5.7% compared with those with HBA1c < 5.7% ($P = 0.049$, Figure 3A). In addition to higher glycaemic indices and lower insulinogenic index, subjects with HBA1c ≥ 5.7% had lower cardiac baroreflex sensitivity ($P = 0.01$), and tended to have lower whole-body plasma norepinephrine clearance ($P = 0.08$) and increased LV septal wall thickness ($P = 0.07$)(Table 3). Arterial norepinephrine concentration was 47% higher and whole-body norepinephrine spillover rate was 38% higher in the HbA1c ≥ 5.7% group, but these differences were not statistically significant. TH and VMAT2 immunoblots are presented in Supporting Information. TH and VMAT2 expression did not differ between the two sub-groups (Figures 3B and C).

Discussion

This study examined the metabolic correlates of NET expression in a cohort of middle-aged persons who were overweight or obese with wide-ranging glycaemic indices, encompassing NGT, IGT and treatment naïve T2D. The rationale was based on experimental data implicating hyperglycaemia and/or hyperinsulinaemia in the down-regulation of NET (13–18) and clinical data showing inverse associations between plasma glucose level and whole-body plasma norepinephrine clearance in at risk individuals with metabolic syndrome (5). The key finding is that hyperglycaemia, but not hyperinsulinaemia, is inversely associated with NET expression quantified in forearm vein biopsies. Furthermore, after adjustment for age, gender, BMI and blood pressure, HbA1c, representing chronic exposure to basal and postprandial hyperglycaemia, was found to be an independent inverse predictor of NET expression, explaining 45% of its variance.

Norepinephrine transporter plays a pivotal role in the termination of the actions of norepinephrine endogenously released from sympathetic nerve varicosities. It has been estimated that approximately 90% of norepinephrine released by postganglionic sympathetic nerves undergoes immediate neuronal re-uptake, thus only ~10% spills over into the general circulation (11). Neuronal uptake-1 contributes 20% to the overall clearance of norepinephrine from plasma in humans (28); however, in the heart which receives dense sympathetic innervation, regional NET mediated norepinephrine uptake is

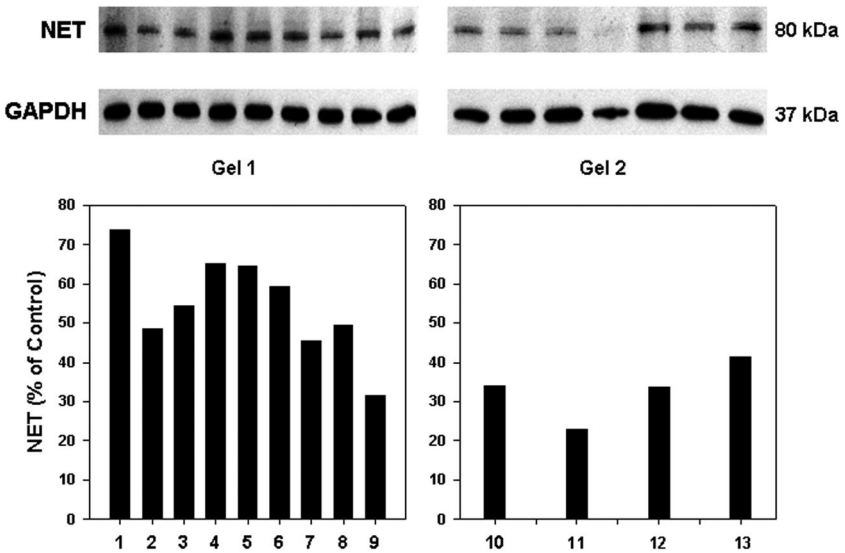

Figure 1 Immunoblots for norepinephrine transporter (NET) and the control house-keeping protein glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Vertical bar graphs show NET expression relative to GAPDH. Subjects 1–6 and subject 11 have HbA1c $< 5.7\%$, whilst subjects 7–10, 12 and 13 have HbA1c $\geq 5.7\%$. Gel 2 immunoblots 2, 4, and 6 represent repeat vein biopsies in subjects 10, 11 and 12 following lifestyle intervention, and do not form part of the present analysis.

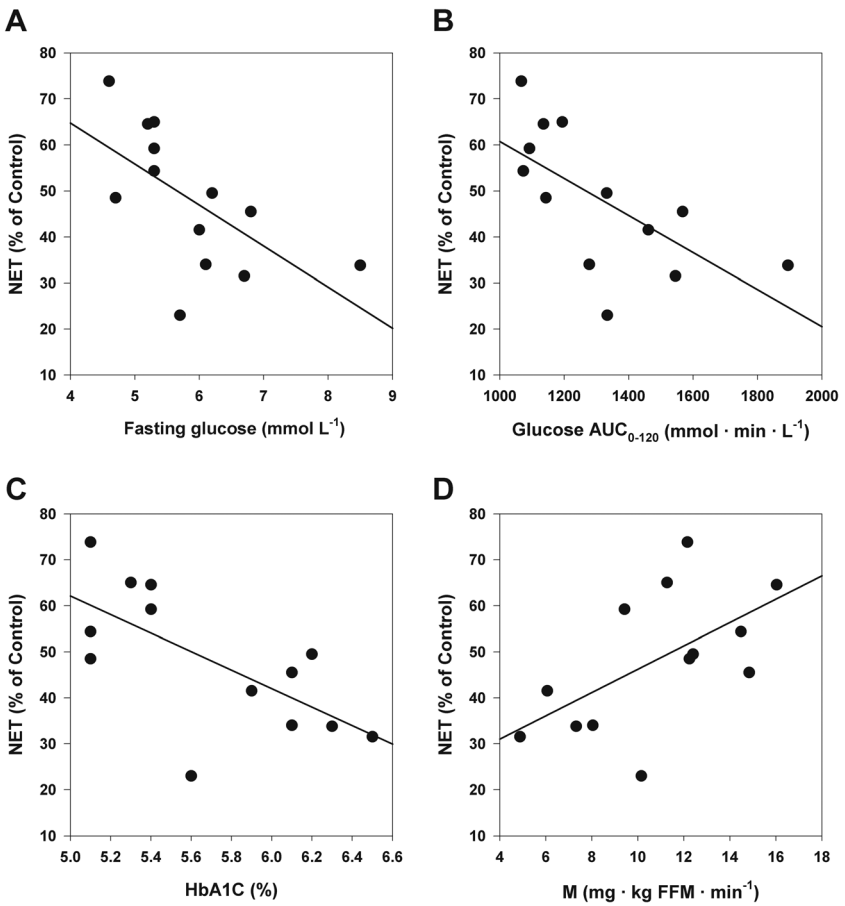

Figure 2 Glycaemic correlates of norepinephrine transporter (NET) expression. **A**. Fasting plasma glucose ($r = {}^{-}0.62$, $P = 0.02$). **B**. Glucose area under the curve (AUC_{0-120}) during OGTT($r = {}^{-}0.65$, $P = 0.02$). **C**. Haemoglobin A1c ($r = {}^{-}0.67$ $P = 0.01$). **D**. Steady state glucose utilization during euglycaemic hyperinsulinaemic clamp (M)($r = 0.58$, $P = 0.04$).

Table 2 Stepwise regression analyses of NET expression (as a % of GAPDH) as the dependent variable ($n = 13$)

Model	Predictor variables	Standardized coefficients	Standard error	R^2	P	β
1	All variables eliminated					
2	HbA1c (%)	−0.67	0.07	0.45	0.012	0.73
3	HbA1c (%)	−0.67	0.07	0.45	0.012	0.73

Model 1 variables entered: age (years), gender, body mass index (kg m^{-2})
Model 2 variables entered: age (years), gender, body mass index (kg m^{-2}), fasting glucose (mmol L^{-1}), glucose AUC_{0-120} during OGTT (mmol L min^{-1}), HbA1c (%) and M (mg kg FFM min^{-1})
Model 3 variables entered: age (years), gender, body mass index (kg m^{-2}), fasting glucose (mmol L^{-1}), glucose AUC_{0-120} during OGTT (mmol L min^{-1}), HbA1c (%) and M (mg kg FFM min^{-1}), clinic mean arterial pressure (mmHg).
Note: β refers to the power of the performed test with $\alpha = 0.05$
NET, norepinephrine transporter.

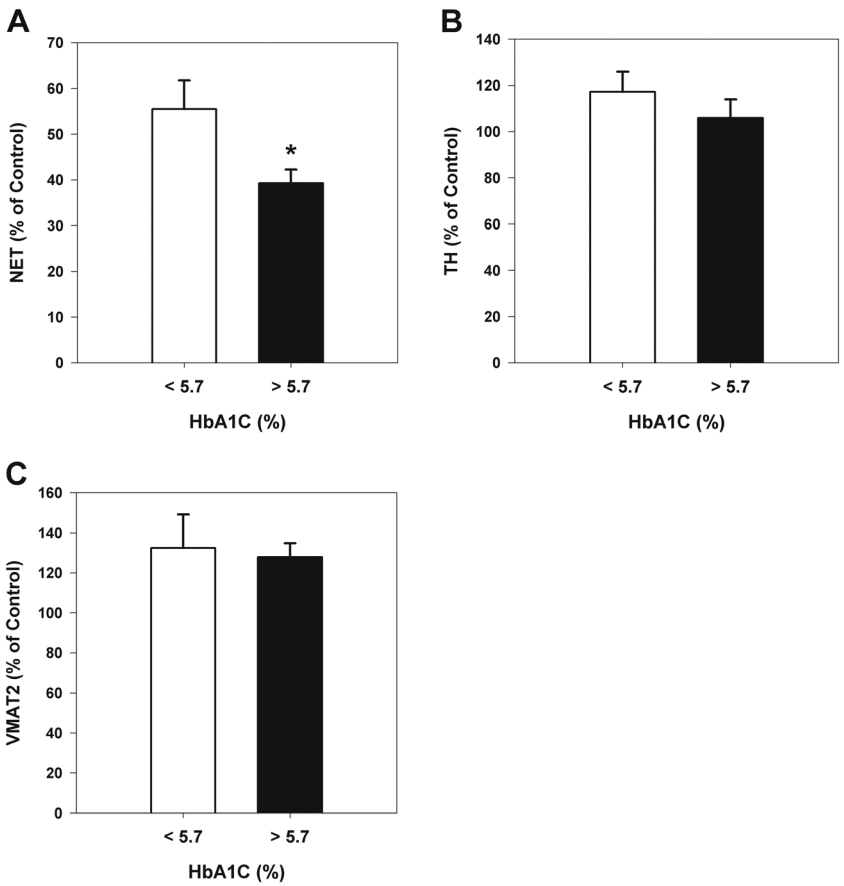

Figure 3 Sympathetic nerve protein expression in different metabolic sub-groups, stratified by haemoglobin A1c using cut-points of $< 5.7\%$ ($n = 7$) and $\geq 5.7\%$ ($n = 6$). **A**. Norepinephrine transporter (NET) expression. **B**. Tyrosine hydroxylase (TH) expression. **C**. Vesicular monoamine transporter-2 (VMAT2) expression. $^{*}P < 0.05$.

much greater, averaging 69% of total cardiac clearance (12). Thus, any perturbation in NET expression or functionality may be of particular relevance to cardiac structure and function (13,19,21). Indeed, in our cohort, NET expression was inversely related to LV posterior thickness, which is in agreement with previous studies showing an association between increased cardiac and whole-body sympathetic activity and LV hypertrophy (25,29). Decreased neuronal uptake-1 has also been linked to the pathogenesis of haemodynamic abnormalities such as arterial hypertension (30) and may predispose to cardiac dysrhythmias. Moreover, an enhanced cardiac washout rate of ^{123}I-MIBG (i.e. decreased NET mediated uptake into cardiac sympathetic nerves) has

Table 3 Comparative clinical data in subjects stratified according to diabetes risk

Variable	HBA1c < 5.7% ($n = 7$)	HBA1c ≥ 5.7% ($n = 6$)	*P*-value
Age (years)	58 ± 2	58 ± 3	1.00
Gender (M/F)	3/4	2/4	1.00
Anthropometrics			
BMI (kg m^{-2})	32.1 ± 1.6	30.5 ± 1.2	0.36
Waist circumference (cm)	105.2 ± 5.0	103.3 ± 2.5	0.53
Waist to hip ratio	0.91 ± 0.03	0.90 ± 0.02	0.73
Total body fat (kg)	38.3 ± 4.3	36.8 ± 1.5	0.76
Android fat (kg)	3.84 ± 0.50	3.77 ± 0.24	0.91
Glucose metabolism			
Fasting glucose (mmol L^{-1})	5.2 ± 0.1	6.7 ± 0.4	0.001
2-h glucose (mmol L^{-1})	8.5 ± 0.5	11.2 ± 1.7	0.14
Glucose $AUC_{0–120}$ (mmol min L^{-1})	1148 ± 35	1513 ± 89	0.002
HbA1c (%)	5.3 ± 0.1	6.2 ± 0.1	<0.001
Fasting insulin (mU L^{-1})	14.0 ± 2.5	17.7 ± 2.2	0.30
Insulin AUC_{0-120} (mU min L^{-1})	7782 ± 1249	8637 ± 1626	0.68
Insulinogenic index (mU mmol^{-1})	12.0 ± 1.6	5.9 ± 1.5	0.02
HOMA-IR	3.44 ± 0.65	5.32 ± 0.77	0.09
Matsuda ISI	3.78 ± 1.12	2.00 ± 0.31	0.18
M/I (mg kg FFM min^{-1} mU L^{-1}) × 100	11.5 ± 1.3	7.4 ± 1.5	0.06
Sympathetic nervous system parameters			
Arterial NE (pg ml^{-1})	309 ± 43	453 ± 89	0.15
NE clearance (L min^{-1})	2.15 ± 0.12	1.86 ± 0.08	0.08
NE spillover (ng min^{-1})	621 ± 76	854 ± 192	0.26
DHPG : NE ratio	4.20 ± 0.77	2.73 ± 0.32	0.13
[^{3}H]-DHPG : [^{3}H]-NE ratio	0.170 ± 0.039	0.119 ± 0.017	0.28
MSNA bursts/minute	38 ± 4	46 ± 5	0.27
MSNA bursts/100 heartbeats	68 ± 4	71 ± 6	0.70
Cardiovascular and echocardiographic parameters			
Systolic blood pressure (mmHg)	126 ± 3	123 ± 6	0.44
Diastolic blood pressure (mmHg)	72 ± 3	70 ± 3	0.48
Heart rate (bpm)	59 ± 3	66 ± 4	0.16
Cardiac BRS (msec mmHg^{-1})	18.0 ± 2.2	10.1 ± 1.2	0.01
LVMI (g m^{-2})	77.1 ± 7.7	92.7 ± 8.7	0.21
Septal wall thickness (mm)	9.9 ± 0.6	11.7 ± 0.9	0.07
Posterior wall thickness (mm)	9.0 ± 0.7	10.0 ± 0.7	0.34
LVEF (%)	66 ± 3	64 ± 3	0.77
Cardiac output (L min^{-1})	5.5 ± 0.1	5.3 ± 0.4	0.69
E/A ratio	1.1 ± 0.0	1.0 ± 0.1	0.46
E wave deceleration time (msec)	181 ± 8	225 ± 35	0.21
Mean E/e' (septal and lateral)	7.9 ± 0.5	8.6 ± 0.7	0.45

BMI, body mass index; BRS, baroreflex sensitivity; DHPG, 3,4-dihydroxyphenylglycol, E/A, ratio of peak early (E) to late (A) transmitral diastolic filling velocities; e', early diastolic annular velocity; HOMA-IR, homeostasis model assessment-insulin resistance; ISI, insulin sensitivity index; LVEF, left ventricular ejection fraction; LVMI, left ventricular mass index; MSNA, muscle sympathetic nerve activity; NE, norepinephrine.

been prospectively linked to cardiovascular and cerebrovascular events in T2D patients without structural heart disease (21).

Experimental studies have highlighted the role of alternate glucose metabolic pathways, specifically the polyol pathway and enhanced PKC activity, in the down-regulation of NET protein expression and functionality under conditions of chronic hyperglycaemia (16,17). The intracellular polyol pathway is normally a minor pathway of glucose metabolism, but its activation in hyperglycaemia causes the accumulation of sorbitol, which in turn leads to decreases in myoinositol and Na^+/K^+-ATPase activity, enhanced consumption of NADPH and oxidative stress (31). Substrate transport

by NET is dependent on an inward sodium gradient, which is maintained by the action of Na^+/K^+-ATPase (32); thus, a reduction in this enzyme would be anticipated to suppress uptake-1. Pharmacological blockade of the polyol pathway with aldose reductase inhibitor protects against streptozotocin-induced reduction in NET expression in rats (16). Vis-a-vis elevation of tissue sorbitol levels by sorbitol dehydrogenase inhibitor exacerbates sympathetic autonomic neuropathy in rats with streptozotocin-induced diabetes but interestingly had no neuropathological effect in non-diabetic rats (33). This observation suggests that sorbitol pathway activity, not absolute levels of sorbitol or fructose, may be most critical in the pathogenesis of diabetic sympathetic neuropathy. NET function is also regulated through the dynamic trafficking of the transporter to and from the plasma membrane. Several studies have demonstrated that PKC through phosphorylation of serine and threonine residues in NET promotes transporter internalization and reduction in surface distribution (17,34). Other proposed mechanisms that may be operational under hyperglycaemic conditions are advanced glycation end-product formation, increased oxidative stress, and altered levels of neurotrophic factors, although their potential contribution to NET expression has yet to be elucidated (35).

There were no significant associations between measures of hyperinsulinaemia (fasting insulin and insulin AUC_{0-120}) and NET expression in the present study. This was also the case after the exclusion of T2D subjects who were insulin hyposecretors from the correlation analyses. However, the findings showed an inverse association between NET expression and insulin resistance (HOMA-IR) and a positive association between NET expression and euglycaemic clamp-derived assessment of whole-body insulin sensitivity. This concurs with a previous report showing lower ^{123}I-MIBG myocardial uptake in T2D subjects with higher visceral fat accumulation and HOMA-IR, compared with matched T2D subjects with lower visceral fat and HOMA-IR (36).

Our study has several limitations. Firstly, the number of subjects studied was small and those with T2D were newly diagnosed and in the early stages of the disease. Therefore, further studies are needed to examine NET expression in persons with longer duration of T2D, the chronology of changes in NET along the diabetes continuum, and its relation to cardiac sympathetic innervation and function. Furthermore, the independent role of hyperinsulinaemia needs to be addressed in cohorts with wide-ranging plasma insulin levels, but normal insulin secretory function, as our findings were likely confounded by reduced insulin secretory capacity with increasing glycaemia. Secondly, formal testing for cardiovascular autonomic neuropathy to ascertain whether differences in NET expression were independent of sympathetic neurodegeneration was not performed, albeit none of the subjects had symptoms of postural hypotension or exercise intolerance at screening. Also, the fact that the expressions of TH, the rate-limiting enzyme in the intraneuronal biosynthesis of norepinephrine, and VMAT2, the enzyme responsible for vesicular storage of norepinephrine, did not differ in the two glycaemic sub-groups, argues against differences in sympathetic fibre density. Thirdly, the cross-sectional nature of the analysis does not permit identification of the direction of causality. It is also feasible, that down-regulation of NET could contribute to elevations in synaptic and plasma norepinephrine levels, which in turn would promote the development of insulin resistance, impaired glucose utilization and alterations in metabolic function (37,38). Fourthly, our protocol did not include a weight-stabilization run-in phase and weight stability was based on self-report. Finally, it merits emphasis that NET was quantified in sympathetically innervated peripheral tissues (forearm veins), thus the results cannot be extrapolated to the effects of glycaemia on NET expression within the central nervous system. Pharmacological studies with NET inhibitors show opposing effects on cardiovascular sympathetic regulation in the brain and periphery, comprising reduced central sympathetic outflow and peripheral blockade of norepinephrine uptake, respectively (32). Thus the clinical ramifications of NET suppression in chronic hyperglycaemia need further exploration.

In summary, this pilot study showed an inverse relationship between glycaemic indices and NET protein expression, and a positive relationship between insulin sensitivity and NET expression, in a metabolically diverse group of persons with overweight or obesity. Excess norepinephrine under conditions of reduced uptake-1, can elicit deleterious effects on cardiovascular regulation, insulin resistance and disease progression (32). It is therefore pertinent that future studies explore the benefits of weight loss and/or pharmacological glucose lowering and insulin sensitization on NET expression, particularly in prediabetes and the early stages of T2D. To this end, 6-months treatment with the nuclear peroxisome proliferator-activated receptor-γ agonist, troglitazone has been shown to increase cardiac uptake of MIBG in non-diabetic patients with essential hypertension (39), whilst pioglitazone increased [^{3}H]-DHPG : [^{3}H]-NE ratio by 83% in hyperinsulinaemic subjects with metabolic syndrome (40).

Conflict of interest statement

N. E. S., L. G., S. J. C, S. E. P., C. I. S., M. T. G., S. K., C. Y. W., N. E., D. K. and E. A. L. have nothing to declare. M. D.

E. and J. A. M. have received research grants and teaching honoraria from Medtronic. J. B. D. is a consultant for Apollo Endosurgical, Bariatric Advantage, and is a member of the Optifast® Medical Advisory Board for Nestle Health, Australia and the Saxenda Advisory Board for Novo Nordisk. G. W. L. has acted as a consultant for Medtronic and has received honoraria from Medtronic, Pfizer and Wyeth Pharmaceuticals for presentations. These organizations played no role in the design, analysis or interpretation of data described here, nor in the preparation, review, or approval of the manuscript.

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Author contributions

N. E. S., M. D. E., J. B. D. and E. A. L. conceived the study and interpreted the data. L. G., S. P. and N. E. performed laboratory analyses. N. E. S., S. C., C. I. S., M. T. G., S. K., C. W., J. M, D. K. and E. A. L. collected clinical data. N. E. S. performed statistical analysis and wrote the manuscript. All authors read and had final approval of the manuscript.

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