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Obstructive Sleep Apnea and Diabetic Neuropathy: a Novel Association in Patients with Type 2 Diabetes

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15.9 Sleep Disordered Breathing: Outcomes

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At Glance Commentary:

Scientific Knowledge on the Subject

Obstructive sleep apnea (OSA) is known to be very common in patients with type 2 diabetes (T2DM). However the consequences of OSA complicating T2DM are unclear, particularly in regard to diabetes-related micro and macro vascular complications.

What This Study Adds to the Field

Our study found that OSA is independently associated with diabetic peripheral neuropathy (DPN) despite adjustment for a wide range of possible confounders. We also found that the severity of DPN correlated with the degree of OSA and the severity of nocturnal hypoxemia. In addition, we identified potential mechanisms linking OSA and DPN including increased nitrosative stress and impaired microvascular blood flow regulation. Our results therefore indicate that OSA complicating T2DM may aggravate and amplify glucose toxicity which has significant implications for tissues susceptible to diabetes complications.

Abstract

Rationale: Diabetic peripheral neuropathy is common and causes significant morbidity. Obstructive sleep apnea (OSA) is also common in patients with type 2 diabetes. Since OSA is associated with inflammation and oxidative stress, we hypothesized that OSA is associated with peripheral neuropathy in type 2 diabetes.

Methods: A cross-sectional study of adults with type 2 diabetes recruited randomly from the diabetes clinic of two UK hospitals**.**

Measurements: Peripheral neuropathy was diagnosed using the Michigan Neuropathy Screening Instrument. OSA (apnea-hypopnea index ≥5 events/hour) was assessed using home-based, multi-channel respiratory monitoring. Serum nitrotyrosine was measured by ELISA, lipid peroxide by spectrophotometer and microvascular function by Laser Speckle Contrast Imaging.

Results: 234 patients (aged 57(12) years) were analysed.

OSA prevalence was 65% (median apnea-hypopnea index 7.2, range 0-93); 40% of which were moderate to severe. Neuropathy prevalence was higher in patients with OSA than those without (60% vs. 27%, p<0.001).

After adjustment for possible confounders, OSA remained independently associated with diabetic neuropathy (OR 2.82, 95% CI 1.44-5.52, p=0.0034).

Nitrotyrosine and lipid peroxide levels (n=102, 74 with OSA) were higher in OSA and correlated with hypoxemia severity. Cutaneous microvascular function (n=71, 47 with OSA) was impaired in OSA.

Conclusions: We describe a novel independent association between diabetic peripheral neuropathy and OSA. We identified increased nitrosative/oxidative stress and impaired microvascular regulation as potential mechanisms. Prospective and interventional studies are needed to assess the impact of OSA and its treatment on peripheral neuropathy development and progression in patients with type 2 diabetes.

Abstract word count 245.

Key words: obstructive sleep apnea, diabetic neuropathy, peripheral neuropathy, type 2 diabetes, intermittent hypoxia, nitrotyrosine, nitrosative stress, microvascular function, endothelial function, laser speckle contrast imaging.

Introduction

Diabetic peripheral neuropathy (DPN) is common and results in great morbidity, mortality and significant economic burden(1). Known DPN risk factors include increasing age and the duration and degree of the antecedent hyperglycemia(2,3) as well as hypertension, dyslipidemia, and obesity(4,5). Putative mechanisms for DPN include increased oxidative/nitrosative stress, advanced glycation end-product formation, activation of the hexosamine and polyol pathways and perturbations of protein kinase C , resulting in direct cellular damage and functional and/or structural defects involving the extra-cellular matrix and/or microvasculature(6,7). Despite our improved understanding of the pathogenesis of DPN, disease-modifying treatments are still lacking (with the exception of improved glycemia)(4,6). Hence, improved understanding of DPN pathogenesis is important in order to identify new treatments(6).

Obstructive sleep apnea (OSA) is a common disorder that is highly prevalent in patients with type 2 diabetes (T2DM) (8,9). It is characterized by upper airway instability during sleep, resulting in markedly reduced (hypopnea) or absent (apnea) airflow(10). These apnea/hypopnea episodes are usually accompanied with cyclical oxygen desaturations and cyclical changes in blood pressure (BP) and heart rate(10). . Since OSA is associated with many of the pathophysiological deficits that are found in diabetes(11-13) , it seems reasonable to speculate that OSA could play an important role in the development or progression of DPN. The primary aim of this study was therefore to explore the interrelationships of OSA and DPN in subjects

with T2DM. A secondary aim was to explore the potential pathophysiological mechanisms.

Methods:

We conducted an observational cross-sectional study in adults with T2DM. Patients with respiratory disease (including pre-diagnosed OSA), end-stage renal disease or non-diabetic neuropathy (<1%) were excluded. Patients were recruited casually from the out-patient diabetes departments of two UK hospitals. Patients were approached in the waiting area before they have seen the clinicians and without any prior knowledge of their medical condition. We avoided any reference to snoring during the recruitment process. Consent was obtained and ethnicity was determined in accordance with the UK decennial census by the study participants. The project was approved by the Warwickshire Research Ethics Committee (REC number 08/H1211/145).

DPN was assessed using the Michigan Neuropathy Screening Instrument (MNSI) (Figure E1)(14-18). DPN was diagnosed if the MNSI examination score was >2 and/or MNSI questionnaire score was ≥ 7(17,19). Foot insensitivity to a 10-g monofilament (applied to 10 foot locations) was defined as < eight correct responses(19).

OSA was assessed by a single overnight home-based cardio-respiratory sleep study using a portable multi-channel device (Alice PDX, Philips Respironics) and scored in accordance with the American Academy of Sleep Medicine guidelines(20). An apnea hypopnea index (AHI) ≥ 5 events/hour was consistent with the diagnosis of OSA(21).

All patients were approached and serum nitrotyrosine and plasma lipid peroxide were assessed in duplicate in all subjects who agreed. 3-nitrotyrosine was measured by ELISA (Oxiselect^{TM}, Cell Biolabs Inc., San Diego, CA, USA) and lipid peroxide by spectrophotometry.

Microvascular assessment was performed on a casually chosen representative patient subset using Laser speckle contrast imaging (Moor Instruments Ltd, Devon, UK)(22,23).

All assessments in the study were blinded.

Data analysis was performed using SPSS 15.0 software (SPSS Inc, Chicago, USA). Data are presented as mean (SD) or median (IQR). Independent continuous variables were compared using the Student's t-test or the Mann-Whitney test. Categorical variables were compared using the Chi-squared test. Correlations between continuous variables were performed using the Pearson or Spearman tests. Differences between independent groups were assessed by analysis of variance (ANOVA). Analysis of covariance (ANCOVA), was used to assess the impact of covariates on the differences between several independent groups. To assess whether OSA status, OSA severity or hypoxemia measures are independent predictors of DPN, multiple logistic regression (forced entry method) was used. Multiple linear (forced entry method) was used to assess independent predictors of continuous variables. Variables included in the regression models were based on known outcome-related risk factors and/or variables that differed between patients with and without OSA. In order to further explore the impact of baseline differences on the associations observed, a sub-group of 70 patients with and 70 without OSA were group matched

for a variety of risk factors. A p value < 0.05 was considered significant unless stated otherwise.

For detailed methodology and details on model building, please see the online supplement.

Results:

We recruited 266 patients; 32 were excluded, leaving 234 patients for analysis (Figure 1). Of these 234 patients, 58% were men, 55% White Caucasians and 45% South Asians.

OSA and DPN prevalence

The overall prevalence of DPN was 48%. The overall prevalence of OSA was 65%. Of the 151 patients with OSA, 60% had mild (AHI 5 to <15), 23% had moderate (AHI 15 to <30) and 17% had severe (AHI ≥30) OSA.

OSA and clinical characteristics in T2DM

Patients with OSA (OSA+) were older, had longer diabetes duration, higher systolic BP, and obesity measures and were sleepier compared to those without OSA (OSA-) (Table 1).

The relationship between OSA and DPN

The overall DPN prevalence was higher in OSA+ compared to OSA- patients (60% vs. 27%, p<0.001). This relationship between OSA and DPN was present irrespective of ethnicity (online supplement, Figure E2).

The relationship between OSA and clinical signs and symptoms of DPN

The overall foot insensitivity prevalence was 37%. Foot insensitivity was higher in OSA+ compared to OSA- patients (50% vs. 15%, p<0.001, respectively). OSA+ patients had more abnormalities on all aspects of the neurological examination (Table 2).

Based on the MNSI questionnaire, OSA+ patients had a higher prevalence of skin hypersensitivity (33% vs. 13, p=0.001). A previous history of "open sore on the foot" was also more common in OSA+ patients (27 vs. 7%, p<0.001); consistent with findings using the monofilament. The rest of the questionnaire components were not significantly different between OSA+ and OSA- patients (data not shown).

A multivariate analysis of the relationship between OSA, its severity and DPN

In order to assess whether the relationship between OSA and DPN is secondary to or independent of the differences observed in baseline characteristics, logistic regression (forced entry method) was used (Table 3). Despite some attenuation by adiposity measures, OSA remained independently associated with DPN (OR 2.82, 95% CI 1.44-5.52, p=0.003) after adjustment for main possible confounders (model 1, Table 3). Further adjustment by inserting other possible confounders into the model did not affect the relationship between OSA and DPN and did not improve the model R^2 (models 4 and 5, Table 3). Replacing BMI with waist circumference or waist/hip ratio in the model 1 did not change the significant relationship between OSA and DPN (models 2 and 3, Table 3). Other independent associations with DPN

included waist circumference (OR 1.03, 95% CI 1.004-1.05, p=0.02), , insulin use (OR 2.59, 95% CI 1.34-5.01, p=0.005) and diabetes duration (OR 1.06, 95% CI 1.01-1.11, p=0.01). Inserting OSA as a 3-category (no OSA, mild OSA and moderate to severe OSA) rather than a dichotomous (no OSA and OSA) variable into model 1, demonstrated that both mild (OR 3.04, 95% CI 1.48-6.25, p=0.002) and moderate to severe OSA (OR 2.43, 95% CI 1.06-5.57, p=0.04) were independently associated with DPN. Furthermore, models that included AHI quartiles and nadir nocturnal oxygen saturation instead of OSA found that these variables were also independently associated DPN (see the online supplement).

A multivariate analysis of the relationship between OSA, its severity and the clinical signs of DPN

Using the monofilament test to detect the "at risk foot" as an outcome, OSA remained independently associated with foot insensitivity (OR 3.97, 95% CI 1.80-8.74, p=0.001, Nagelkerke R Square 0.34) after adjustment as in model 1 in Table 3. Similar to DPN, both mild (OR 4.93, 95% CI 2.10-11.57, p<0.001) and moderate to severe (OR 2.83, 95% CI 1.13-7.13, p=0.03) OSA and AHI quartiles were independently associated with the "at risk foot" after adjustment (see online supplement).

In addition, OSA and AHI quartiles were independently associated with reduced/absent ankle reflexes and vibration perception (see the online supplement).

The relationship of OSA severity and DPN severity

DPN severity (MNSI examination score categories: <2, 2- <4 and ≥4) correlated significantly with OSA severity and nocturnal hypoxemia severity, independently of

age, obesity, diabetes duration, gender and eGFR in the case of AHI (Table 4). There was also a significant trend of higher DPN prevalence in patients with lower nocturnal nadir oxygen saturation (61% vs. 52% vs. 41% vs. 38% for nadir oxygen saturation quartiles <77% vs. 77-<83% vs. 83%-<87% and ≥87% respectively, p=0.02 for the trend). There was however no significant increase in DPN prevalence (as binary variable) between patients with mild (60%), moderate (57%) and severe (62%) OSA.

OSA and DPN: A matched-group analysis

The above findings indicate that OSA is independently associated with DPN after adjusting for the differences observed between patients with and without OSA. However we felt that minimising these differences by matching for as many DPN risk factors as possible would be advantageous to further test this relationship. We were able to group match 140 (70 with and 70 without OSA) patients for BMI and diabetes duration amongst others (For detailed characterisation, see the online supplement, Table E2). DPN prevalence remained higher in the OSA+ group (53% vs. 24%, p=0.001, OSA+ vs. OSA- respectively). The prevalence of the "at risk foot" based on the monofilament examination was also higher in the OSA+ group (43% vs. 13%, p<0.001). After adjustment as in model 4 in Table 3, OSA remained independently associated with DPN (OR 3.92, 95% CI 1.54-9.96, p=0.004, Nagelkerke R Square 0.31) and the at risk foot (based on monofilament perception) (OR 5.56, 95%CI 1.82-16.97, p=0.003, Nagelkerke R Square 0.37).

The relationship of OSA and nitrosative and oxidative stress

In order to explore possible mechanisms that underlie the relationship between OSA and DPN, serum nitrotyrosine and plasma lipid peroxide levels were measured in a cohort of 102 patients (29 without and 73 with OSA; for patients characteristics, see the online supplement Tables E3 and E4). Nitrotyrosine levels were higher in patients with DPN compared to those without [25.6 nM (17.7-35.8) vs. 19.5 nM (11.5 -29.6), p=0.01] and in OSA+ patients compared to OSA- patients [23.5 nM (16.7-36.1) vs. 15.5 nM (11.5-24.3), p=0.007]. There was a stepwise increase in nitrotyrosine abundance between patients without OSA (n=29) and patients with mild (n=45) and moderate to severe OSA (n=28) (P < 0.001 for the trend using ANOVA) (Figure 2), with significant differences between moderate to severe OSA and mild OSA (p=0.04) and patients without OSA (p<0.001). The difference between moderate to severe OSA and no OSA remained significant after adjusting for age, BMI and diabetes duration (p=0.011).

Serum nitrotyrosine levels correlated with OSA severity and nocturnal hypoxemia measures [AHI (r=0.38, p<0.001), time spent with oxygen saturations <80% (r=0.23, p=0.02), ODI (r=0.35, p<0.001) and nadir nocturnal oxygen saturations (r=-0.21, p=0.03)].

Using multiple linear regression, and after adjusting for age at diabetes diagnosis, gender, ethnicity, diabetes duration, BMI, HbA1c, and mean arterial pressure , , OSA (AHI≥10) (B=0.19, p=0.005), OSA (AHI ≥15) (B=0.17, p=0.01), AHI (B=0.24, p=0.001), ODI (B=0.24, p=0.002), and nadir nocturnal oxygen saturations (B= -0.30, p=0.003)

were all independently associated with nitrotyrosine levels. OSA (AHI ≥5) (B=0.12, p=0.11) was not associated with nitrotyrosine levels after adjustment..

Lipid peroxide levels were higher in patients with DPN [21.1 (3.9-42.5) µM/ml] compared to those free from DPN [12.2 (2.9-24.6) µM/ml, p=0.01] and in those with OSA [18.4 (8.3-37.4) µM/ml] compared to those without OSA [7.9 (0.8-22.8) µM/ml, p=0.01] which remained significant after adjusting for age, BMI and diabetes duration (p=0.02) (see the online supplement).

The relationship of OSA and microvascular blood flow regulation

Microvascular assessment was performed in 71 patients (47 with OSA; 28 mild, 11 moderate, 8 severe). For patients characteristics see online supplement (Tables E5 and E6). Patients with OSA had lower basal microvascular flux and lower acetylcholine and sodium nitroprusside induced flux, while heating induced flux was not different between the groups . After adjustment for mean arterial pressure and maximal vasodilatation, basal, acetylcholine and sodium nitroprusside induced flux remained significantly lower in OSA+ patients (Table 5). After adjustment for ethnicity, gender, age at diabetes diagnosis, diabetes duration, BMI, OSA, AHI and nocturnal hypoxemia measures remained independently associated with basal and sodium nitroprusside-induced microvascular function (Tables 5 and E7).

Discussion:

To our knowledge this is the first report identifying a novel independent association between OSA and DPN in patients with T2DM. Different markers of OSA severity correlated with the DPN severity and DPN prevalence increased with worsening

hypoxemia. The OSA prevalence in our sample is consistent with other studies in subjects with T2DM(8,9). The DPN prevalence in our cohort is also similar to previous studies(14,24).

As expected, demographic and metabolic factors differed between patients with and without OSA. Nevertheless, although these differences contributed to the observed relationship between OSA and DPN, OSA remained independently associated with DPN even after adjustment for these possible confounders. Furthermore, OSA remained independently associated with DPN when the groups were matched for obesity and several other DPN risk factors.

Our data also show that OSA is independently associated with the "at risk foot" (based on the 10g monofilament test). Interestingly, the association between OSA and the monofilament test seemed stronger than that with the MNSI. This difference may reflect the different modalities assessed by the 10g monofilament test (which tests for advanced foot insensitivity sufficient to result in ulceration) and the MNSI (a test for DPN) (see online supplement for further information). It is worth noting that all patients with foot ulceration in our sample also experienced OSA and that a previous "sore on the foot" is more common in OSA patients. This provides clinical confirmation of an independent association between OSA and the inability to feel a 10g monofilament.

Potential mechanisms that may link OSA and DPN

There are several possible explanations for a relationship between OSA and DPN (Figure 3). OSA has been shown to increase advanced glycation end-products production(12) and has been associated with altered protein kinase C signaling(25),

which plays an important role in cellular response to hypoxia(26). OSA is associated with decreased endothelial nitric oxide synthase and increased endothelin-1 levels(27). OSA is also associated with hypercoagulability (increased plasminogen activator inhibitor-1)(28) and inflammation(11). The repetitive episodes of re-oxygenation following hypoxemia in OSA patients simulate ischemia–reperfusion injury which results in the generation of reactive oxygen species(11,29). Furthermore, OSA has been recently identified as a "missed" cause in patients with idiopathic peripheral neuropathy(30,31). The role and importance of hypoxemia is supported by our finding of a correlation between DPN severity and measures of nocturnal hypoxemia as well as the increasing prevalence of DPN with worsening intermittent hypoxemia.

In addition, patients with chronic obstructive pulmonary disease (who have sustained hypoxemia) are also known to be at increased risk of peripheral neuropathy(32).

Mild OSA is associated with increased prevalence of DPN

Mild and moderate to severe OSA, AHI and nadir nocturnal oxygen saturations were all found to be independently associated with the presence of DPN. In addition, the severity of DPN was found to be associated with OSA severity (as judged by AHI and nocturnal hypoxemia measures). However, there was no increase in DPN (as a binary variable) prevalence between patients with mild and those with moderate and severe OSA. The significant increase of DPN in patients with mild OSA could reflect the relatively long diabetes duration of these subjects which could amplify the impact of mild OSA/intermittent hypoxemia in vulnerable tissues. Thus assessing

patients with shorter diabetes duration might yield different results. The lack of a further increase in DPN prevalence in patients with AHI ≥ 15 could reflect the small number of patients in that category, the relative insensitivity of the MNSI (compared to nerve electrophysiology) to stage DPN severity, or perhaps a threshold effect of hypoxemia. These issues will need to be explored in larger numbers of patients using a spectrum of quantitative measurements to stage DPN severity.

Sleepiness in patients with T2DM and OSA

An intriguing finding is that our population was not excessively sleepy as assessed by the Epworth Sleepiness Score; even in patients with OSA the median score was less than what considered suggestive of hypersomnolence. This suggests that sleepiness *per se* cannot be used to case identify OSA in patients with T2DM.

Nitrosative and oxidative stress as a potential link between OSA and DPN

The higher serum nitrotyrosine and lipid peroxide levels in our patients with DPN is consistent with reports in experimental DPN implicating nitrosative and oxidative stress in DPN pathogenesis(33) by mechanisms including reducing nerve perfusion and impairing vascular reactivity of epineurial arterioles(34,35). Nitrosative stress also affects all cell types in the peripheral nervous system including endothelial and Schwann cells of the peripheral nerve, neurons, astrocytes and oligodendrocytes of the spinal cord, and neurons and glial cells of dorsal root ganglia(36). Nitrosative stress is associated with the development of thermal hyper- and hypoalgesia, mechanical hypoalgesia, tactile allodynia, and small sensory nerve fiber degeneration(35). More recently the inhibition of nitrosative stress has been shown

to result in improvement of experimental neuropathy in diabetic rodent models(37). To our knowledge, this is the first report of an association of OSA with oxidative/nitrosative stress in patients with T2DM. The significant correlation between serum nitrotyrosine and nocturnal/during sleep hypoxemia measures, suggests that nitrosative stress is a potential mechanistic link between OSA and DPN. Another report in patients **without** diabetes showed that endothelial expression of nitrotyrosine correlated with AHI despite adjustment for age and adiposity(27).

OSA and its association with microvascular function In parallel, microvascular/endothelial dysfunction have been implicated in the pathogenesis of diabetes-related microvascular complications, including DPN(38,39). Our data are therefore consistent with a role for impaired microvascular blood flow regulation as a link between OSA with DPN. Patients with OSA had lower blood flow at baseline and following stimulation with acetylcholine and sodium nitroprusside. These differences (with exception of acetycholine) persisted even when adjusted for maximal vasodilatation, mean arterial pressure, ethnicity, gender, age at diabetes diagnosis, diabetes duration and BMI. Our data also show that AHI, ODI and nadir oxygen saturations are also independently associated with microvascular blood flow regulation abnormalities in patients with T2DM (Table E7 in the online supplement). The impaired response to sodium nitroprusside might in part be due to impaired response to nitric oxide secondary to oxidative stress(40).

The impact of OSA on microvascular blood flow regulation in patients with T2DM has not previously been reported. Previously, impaired brachial artery flow mediated dilatation was identified in obese nondiabetic OSA patients(41). The same report

assessed forearm skin microcirculation using laser Doppler flowmetry and also found that OSA was associated with lower baseline blood flow compared to subjects without OSA. However their findings differed from those reported herein in that the response to acetylcholine and sodium nitroprusside was not impaired by OSA(41). These findings suggest that when OSA is complicated by diabetes there are additional deficits of vasoactive agent metabolism or action, which is thought to mediate the pathogenetic effects of oxidative/nitrosative stress in the development of DPN(42).

Potential clinical implications

The data reported herein provide a rationale for further prospective and interventional studies to assess the impact of OSA and its treatment on DPN development and progression in patients with T2DM. To date, trials examining the impact of continuous positive airway pressure (CPAP) in patients with T2DM have mainly focused on metabolic indices. However, the impact of CPAP on diabetes complications is unknown. While CPAP had a beneficial impact on glycemic indices in some studies(43); others did not show a benefit (44). The association between mild OSA and DPN in this report if confirmed may also have implications for the threshold for OSA treatment, since some authorities only offer CPAP treatment in moderate to severe OSA.

Study limitations

The main limitation of our study is its cross-sectional nature, and the lack of an interventional arm, hence causation cannot be proven. The findings of our observational study, however, should provide the basis for conducting prospective

observational and interventional studies in patients with T2DM. We have used home-based portable multi-channel respiratory devices rather than in-patient overnight polysomnography. However, this approach is well established and validated(45). The MNSI is not the "gold standard" for diagnosing or staging DPN but it has been validated against nerve conduction studies(15,46) and has been used widely in landmark studies(14,16,17,19). We chose to use the MNSI (in concert with the 10g monofilament) since it offers the advantage of consisting of robust, meaningful, clinically detectable end-points.

Conclusions

We have identified a novel association between DPN and OSA in patients with T2DM. In addition we identified some novel potential mechanistic links, including elevated nitrosative stress in patients with OSA and T2DM that correlated with OSA severity and also abnormal microvascular blood flow regulation in these patients. Prospective studies are required to determine the role of OSA and intermittent hypoxemia in the development and progression of DPN in patients with "early" and advanced diabetes as well as the impact of CPAP treatment on DPN.

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Figure Legends

Figure 1: The Consort diagram for our study.

Figure 2: The relationship between OSA and serum nitrotyrosine levels in patients with type 2 diabetes without OSA (n=29) and with mild (n=45) and moderate to severe OSA (n=28). P value for the trend p < 0.001. p= 0.04 for mild vs. moderate to severe OSA. P<0.001 for normal vs. moderate to severe OSA. Normal: patients with type 2 diabetes but without OSA.

Figure 3: The postulated mechanisms linking OSA to DPN (and microvascular complications). HTN: hypertension; ROS: reactive oxygen species; RNS: reactive nitrogen species PKC: protein kinase C; AGE: advance glycation end-products. For more details please refer to the text.

Table 1: Participant characteristics in relation to OSA status. Data presented as median (IQR) or mean (SD). Categorical variables presented as number (% of OSA status). GFR: Glomerular Filtration Rate, TIA: Transient Ischaemic Attack, PVD: Peripheral Vascular Disease. Analysis performed using the Chi-square test for categorical variables, the independent t test for normally distributed variables and the Mann-Whitney U test for non-normally distributed variables.

	OSA- (n=83)	OSA+ (n=151)	P value
Male	34 (41%)	101 (67%)	< 0.001
Caucasians	32 (39%)	97 (64%)	<0.001
Age (years)	54.7 (11.9)	58.5 (11.3)	0.02
Diabetes Duration (years)	9 (5-15)	11 (7-17)	0.02
Body Mass Index (kg/m^2)	30.2 (27.3-35.0)	34.4 (30.9-39.5)	< 0.001
Waist circumference (cm)	105.5 (96.0-115.0)	116.0 (107.5-125.5)	< 0.001
Hip (cm)	106.0 (98.0-117.0)	114.0 (105.0-125.0)	< 0.001
Waist hip ratio	0.97 (0.93-1.02)	1.01 (0.96-1.05)	0.002
Neck circumference (cm)	38.0 (36.5-41.3)	43.0 (39.0-46.0)	< 0.001
Height (cm)	163.5 (8.3)	167.8 (10.0)	0.001
Systolic blood pressure	125.5 (115.0-135.5.0)	130.0 (123.5-140.0)	0.002
Diastolic blood pressure	78.50 (71.0-85.00)	78.00 (71.00-84.50)	0.88
HbA1c (%)	7.7 (7.0-8.7)	8.3 (7.3-9.3)	0.05
Total cholesterol (mmol/L)	3.7 (3.4-4.5)	3.7 (3.3-4.3)	0.57
Triglycerides (mmol/L)	1.5 (1.0-2.1)	1.8 (1.3-2.5)	0.03
HDL (mmol/L)	1.2 (0.9-1.4)	1.1 (0.9-1.2)	0.02
Estimated GFR (ml/min/1.73	92.92 (25.16)	82.41 (26.41)	0.003

TSH	1.6 (1.0-2.2)	1.7 (1.2-2.4)	0.32
Epworth sleepiness score	5.0 (2.0-12.0)	8.0 (4.0-13.0)	0.003
Smoking (current or ex-	32 (39%)	62 (41%)	0.71
Alcohol (drinks alcohol)	12 (15%)	12 (35%)	0.001
Oral anti-diabetes treatment	81 (98%)	137 (91%)	0.05
Insulin	34 (41%)	91 (60%)	0.005
Insulin dose (units)	61 (35-88)	80 (56-118)	0.007
ACE inhibitors	40 (48%)	69 (46%)	0.71
Anti-hypertensive agents	61 (74%)	129 (85%)	0.03
Lipid lowering treatment	71 (86%)	125 (83%)	0.58
Stroke or TIA	60 (7%)	18 (12%)	0.28
Ischemic heart disease	14 (17%)	33 (22%)	0.40
PVD	1 (1%)	10 (7%)	0.06
Albuminurea	20 (24%)	65 (43%)	0.007
Sight threatening retinopathy	17 (21%)	72 (48%)	< 0.001

Table 2: The relationship between OSA status and aspects of foot exam using the MNSI.

Data presented as n (% of abnormal test in the particular OSA group). This was a uni-variate analysis performed using the Chi-square test. The Bonferroni correction was applied and P < 0.01 was considered significant.

	OSA- (n=83)	OSA+ (n=151)	P values
Inspection	34 (41%)	100 (67%)	<0.001
Ulcers	0 (0%)	8 (5%)	0.03
Ankle reflexes	25 (30%)	87 (58%)	<0.001
Vibration	19 (23%)	90 (60%)	<0.001
10g monofilament	12 (15%)	75 (50%)	<0.001

Table 3: Assessing the impact of possible confounders on the association between OSA and DPN (based on the MNSI) using different logistic regression models (forced entry method). The odds ratios (OR) reported are the odds for having DPN in OSA+ compared to OSA- patients. All patients (n=234) were included in all models. Models 1, 2 and 3 include only the main possible confounders for the relationship between OSA and DPN, while models 4 and 5 are adjusted for all variables in our database.

Model	Nagelkerke R Square	Odds ratio	95% confidence interval	P value
Unadjusted: OSA	0.13	4.09	2.28-7.35	<0.001
Model 1	0.25	2.82	1.44-5.52	0.003
Model 2	0.26	2.76	1.41-5.40	0.003
Model 3	0.25	3.33	1.72-6.47	<0.001
Model 4	0.28	2.77	1.36-5.62	0.005
Model 5	0.29	2.72	1.34-5.55	0.006

Model 1: OSA + ethnicity + gender + age at diabetes diagnosis + diabetes duration + eGFR + insulin use + BMI + mean arterial pressure + HbA1c and Alcohol intake (units/week).

Model 2: as model 1 but replacing BMI with waist circumference

Model 3: as model 1 but replacing BMI with waist hip ratio

Model 4: OSA + age at diabetes diagnosis + ethnicity + gender + diabetes duration + BMI + alcohol intake + HbA1c+ insulin use + mean arterial pressure + eGFR + PVD + smoking + total cholesterol + triglycerides + HDL + oral anti-diabetes treatment + anti-hypertensive agents + lipid lowering therapy + anti-platelets + recruitment site

Model 5: As for model 4 but BMI and waist circumference included both in the model

Table 4: The relationship between DPN severity based on the MNSIe score and OSA and nocturnal hypoxemia severity using the Kruskal-Wallis H test. Data presented as median (IQR). Adjusted p values are adjusted for gender, age, BMI, diabetes duration and eGFR. Adjusted p values were calculated using ANCOVA. Interaction between gender and MNSIe categories was not significant in any the analysis performed. Data in the adjusted analysis presented as mean (95% confidence interval). For patients characteristics by MNSIe group, please refer to Table E1 in the online supplement. *These parameters are used as scale variables.

MNSIe	AHI*	ODI*	Time spent with Oxygen saturations < 90%*	Nadir nocturnal Oxygen saturations*
Univariate analysis				
Group 1: < 2 (n=90)	4.7 (1.6-12.2)	4.7 (1.7-13.6)	0.9 (0.1-4.9)	83.5 (79.0-89.0)
Group 2: 2-< 4 (n=100)	7.2 (2.4-16.0)	6.5 (2.7-13.1)	1.2 (0.1-5.6)	83.0 (78.0-88.0)
Group 3: ≥ 4 (n=44)	8.9 (6.8-27.0)	9.8 (6.0-26.8)	2.2 (0.2-9.6)	80.0 (71.5-84.8)
P value for the trend	< 0.001	< 0.001	0.174	0.004
Adjusted analysis				
Group 1	5.5 (4.4-6.9)	5.6 (4.4-6.9)	2.5 (1.7-3.4)	84.0 (82.2-85.4)
Group 2	6.3 (5.1-7.8)	5.9 (4.7-7.2)	2.3 (1.7-3.2)	83.8 (82.3-85.3)
Group 3	11.0 (7.4-16.2)	9.5 (6.4-14.0)	2.7 (1.4-4.9)	80.9 (77.0-84.1)
p value after adjustment	0.02	0.08	0.89	0.26

Table 5: Assessment of microvascular blood flow and endothelial function in with type 2 diabetes with and without OSA. Data presented as median (IQR) or ratios. Blood flux was measured in arbitrary perfusion units (APU). Conductance is calculated by dividing flux by the mean arterial pressure. Analysis was performed using the Mann-Whitney U test. Adjusted p values were calculated using linear regression. For patients characteristics please refer to Tables E5 and E6 in the online supplement. For the results of the adjusted analysis using forced entry method please refer to Table E7 in the online supplement.

	OSA- (n=24)	OSA+ (n=47)	P value- unadjusted	P value- following adjustment
Conductance				
Baseline	0.40 (0.28-0.48)	0.20 (0.16-0.31)	< 0.001	< 0.001
Heating	1.82 (1.43-2.03)	1.66 (1.28-2.07)	0.37	0.42
Ach	1.43 (1.09-1.83)	1.07 (0.75-1.29)	0.002	0.1
SNP	1.61 (1.15-2.14)	1.16 (0.62-1.41)	0.001	< 0.001
Flux in relation to maximum vasodilatation				
Baseline	0.22 (0.16-0.29)	0.14 (0.10-0.17)	< 0.001	0.002
Ach	0.81 (0.670.902)	0.63 (0.430.77)	0.005	0.26
SNP	0.93 (0.77-1.13)	0.57 (0.41-0.89)	< 0.001	0.001

Figure 1: The study Consort diagram

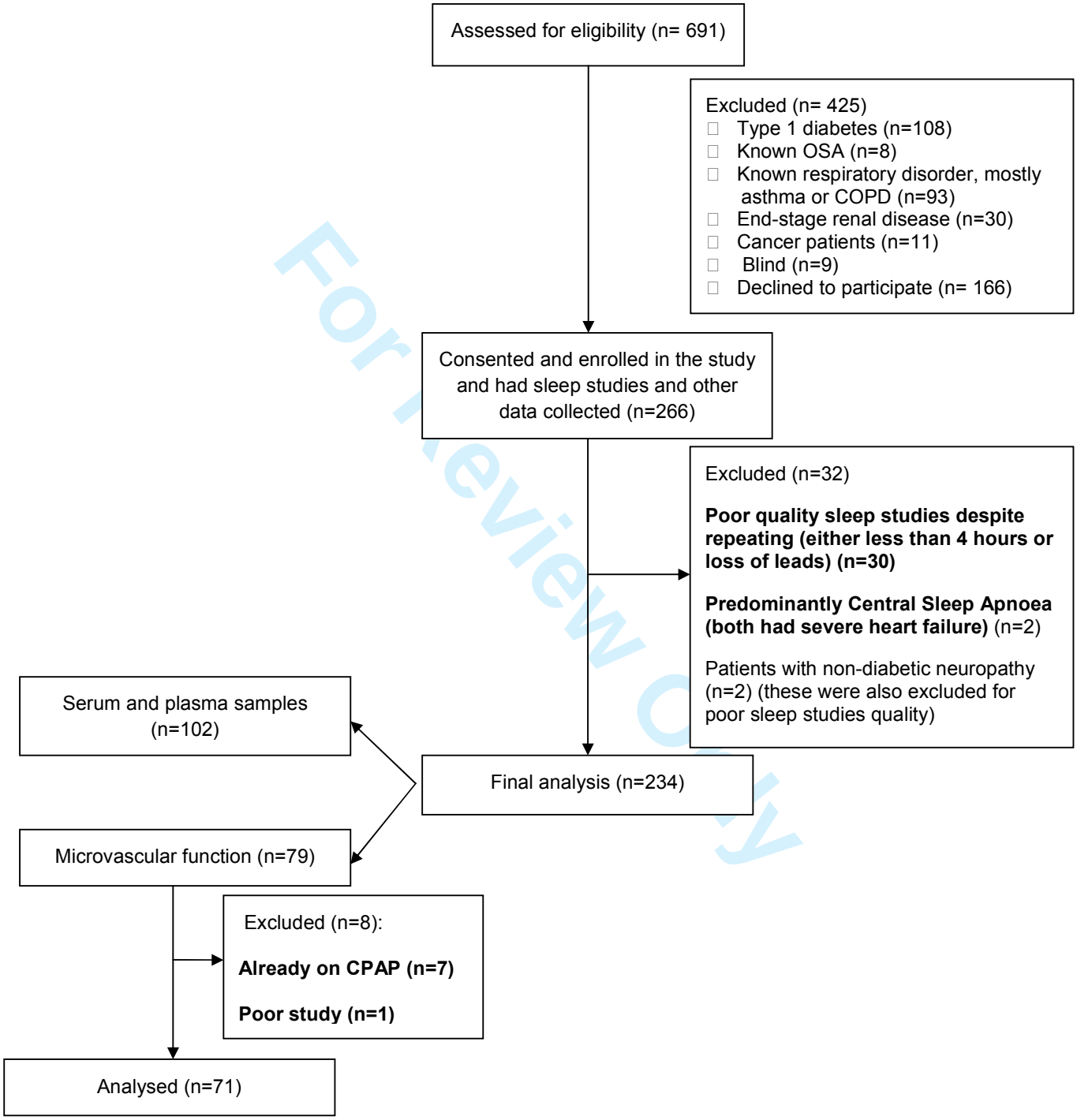


Figure 2: The relationship between OSA and serum nitrotyrosine levels in patients with type 2 diabetes without OSA (n=29) and with mild (n=45) and moderate to severe OSA (n=28, 14 moderate and 14 severe).Analysis was performed using ANOVA. P value for the trend p < 0.001. p= 0.04 for mild vs. moderate to severe OSA. P<0.001 for normal vs. moderate to severe OSA. Normal: patients with type 2 diabetes but without OSA.

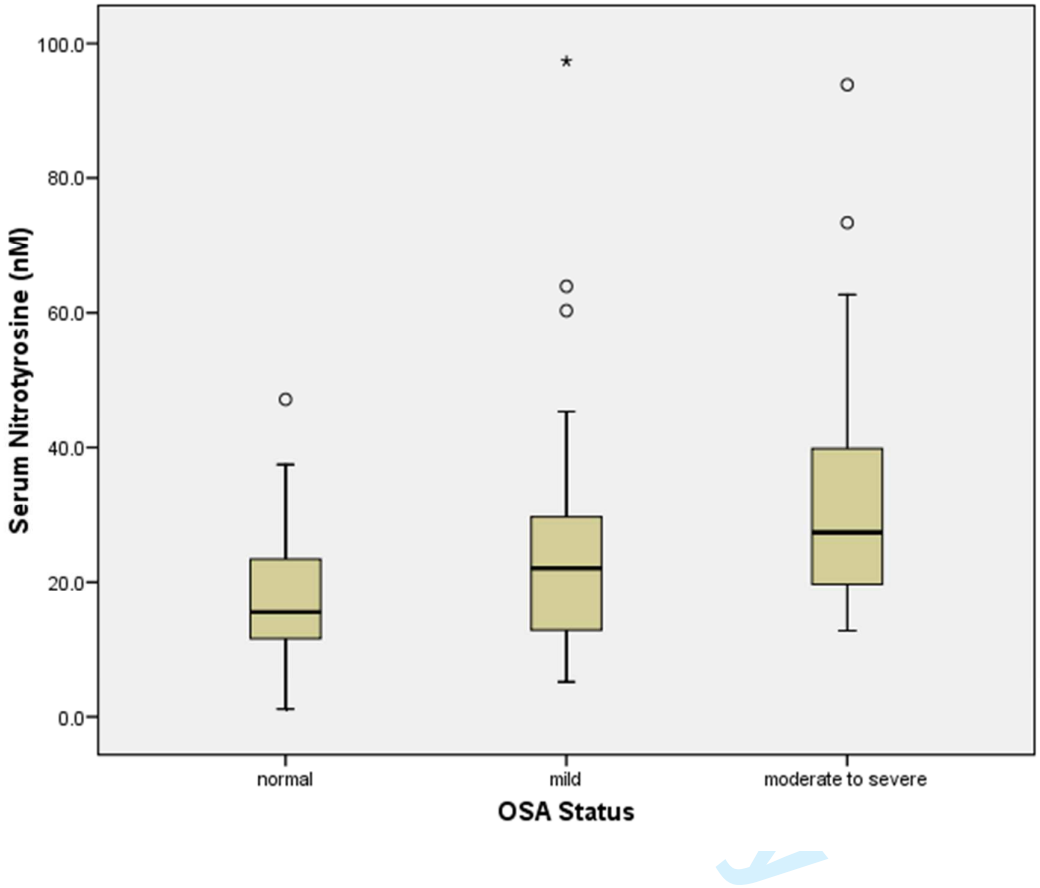
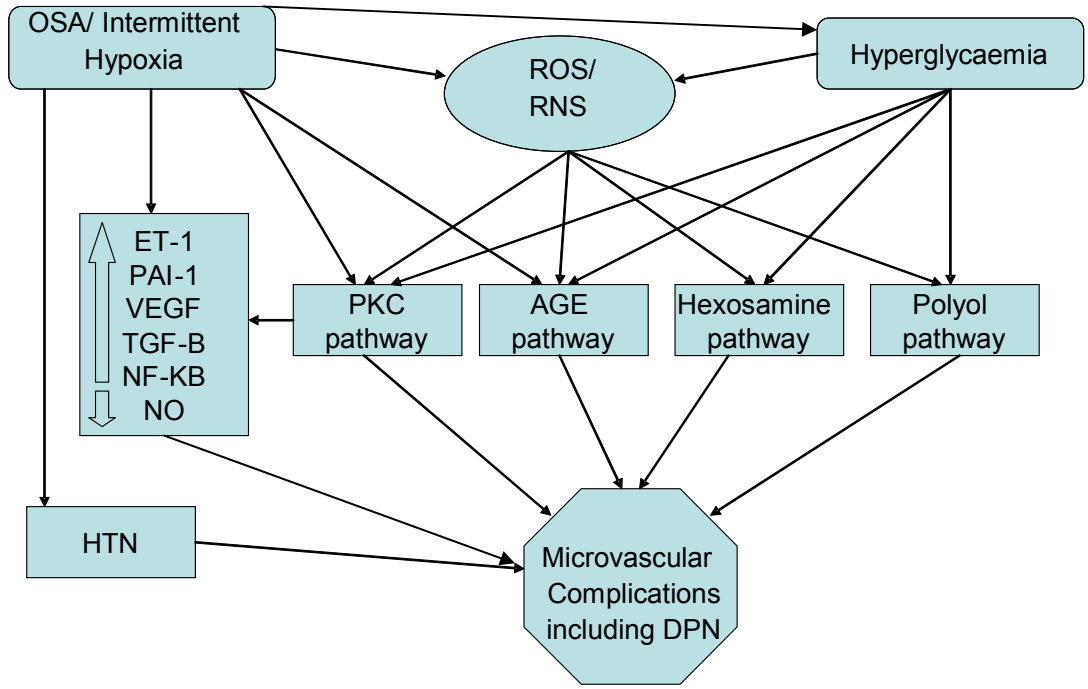


Figure 3: The postulated mechanisms linking OSA to DPN (and microvascular complications). HTN: hypertension; ROS: reactive oxygen species; RNS: reactive nitrogen species PKC: protein kinase C; AGE: advance glycation end-products. For more details please refer to the text.



Obstructive Sleep Apnea and Diabetic Neuropathy: a Novel Association in Patients with Type 2 Diabetes

Abd A Tahrani, Asad Ali, Neil T Raymond, Safia Begum, Kiran Dubb, Shanaz Mughal, Biju Jose, Milan K Piya, Anthony H Barnett, Martin J Stevens .

Online Supplement

Methods

Data collected

Data collected included demographics, anthropometrics, metabolic indices and renal function (estimated glomerular filtration rate (eGFR) using the MDRD equation). Sleep assessment included the use of sleep diaries and the Epworth sleepiness score (ESS).

The Michigan Neuropathy Screening Instrument (MNSI)

The MNSI is a validated, 2-component tool designed to facilitate the early diagnosis of DPN and has been used in several land mark epidemiological studies (1-5). The questionnaire component (MNSIq) comprises 15 questions seeking to characterize sensory disturbance, but also peripheral vascular disease and general asthenia **(Figure E1)**(1). The examination component (MNSIe) comprises a limited foot inspection to identify deformity, skin abnormalities, and ulceration, coupled with an assessment vibratory perception at the great toe (measured using a 128 Hz tuning fork) and ankle tendon reflexes **(Figure E1)**(1).

In the MNSIq, responses of “yes” to items 1-3, 5-6, 8-9, 11-12, 14-15 are each counted as one point. A “no” response on items 7 and 13 counts as 1 point. Item #4 is a measure of impaired circulation and item #10 is a measure of general aesthenia and are not included in scoring.

The MNSIe is scored as indicated in **Figure E1**. Foot Inspection includes looking for evidence of excessively dry skin, callous formation, fissures, frank ulceration, oedema or deformities. Deformities include flat feet, hammer toes, overlapping toes, halux valgus, joint subluxation, prominent metatarsal heads, medial convexity (Charcot foot), edema and amputation. Having any abnormalities on inspection is scored as 1, while 0 is given for normal feet. Vibration sensation and ankle reflexes are scored as 0 for absent, 0.5 for reduced and 1 for absent.

When validated against nerve conduction studies, MNSIe score > 2 had a sensitivity and specificity of at least 80% and 75% respectively (1,6,7). The MNSI also has been reported to have a high inter- and intra observer reproducibility (88.8% and 95% respectively) (6).

We used the MNSI in our study because it has been used in several landmark studies, It is easy to use and can be used to examine a large number of patients with little cost and little time. MNSI has also been validated against nerve conduction studies, and was shown to have a high inter- and intra observer reproducibility. In addition, we chose to use the MNSI (in concert with the 10g monofilament) since they offer the advantage of consisting of robust, meaningful, clinically detectable end-points, rather than just electrical neurophysiology.

Figure E1: The Michigan Neuropathy Screening Instrument.

Patient Version

MICHIGAN NEUROPATHY SCREENING INSTRUMENT

A. History (To be completed by the person with diabetes)

Please take a few minutes to answer the following questions about the feeling in your legs and feet. Check yes or no based on how you usually feel. Thank you.

1.	Are you legs and/or feet numb?	☐ Yes	☐ No
2.	Do you ever have any burning pain in your legs and/or feet?	☐ Yes	☐ No
3.	Are your feet too sensitive to touch?	☐ Yes	☐ No
4.	Do you get muscle cramps in your legs and/or feet?	☐ Yes	☐ No
5.	Do you ever have any prickling feelings in your legs or feet?	☐ Yes	☐ No
6.	Does it hurt when the bed covers touch your skin?	☐ Yes	☐ No
7.	When you get into the tub or shower, are you able to tell the hot water from the cold water?	☐ Yes	☐ No
8.	Have you ever had an open sore on your foot?	☐ Yes	☐ No
9.	Has your doctor ever told you that you have diabetic neuropathy?	☐ Yes	☐ No
10.	Do you feel weak all over most of the time?	☐ Yes	☐ No
11.	Are your symptoms worse at night?	☐ Yes	☐ No
12.	Do your legs hurt when you walk?	☐ Yes	☐ No
13.	Are you able to sense your feet when you walk?	☐ Yes	☐ No
14.	Is the skin on your feet so dry that it cracks open?	☐ Yes	☐ No
15.	Have you ever had an amputation?	☐ Yes	☐ No

Total: _____

MICHIGAN NEUROPATHY SCREENING INSTRUMENT

B. Physical Assessment (To be completed by health professional)

1. Appearance of Feet

	Right	**Left**
a. Normal	☐ 0 Yes ☐ 1 No	Normal ☐ 0 Yes ☐ 1 No
b. If no, check all that apply:		If no, check all that apply:
Deformities	☐	Deformities ☐
Dry skin, callus	☐	Dry skin, callus ☐
Infection	☐	Infection ☐
Fissure	☐	Fissure ☐
Other	☐	Other ☐
specify:	_____	specify: _____

	Right			**Left**		
2. Ulceration	Absent ☐ 0		Present ☐ 1	Absent ☐ 0		Present ☐ 1
3. Ankle Reflexes	Present ☐ 0	Present/ Reinforcement ☐ 0.5	Absent ☐ 1	Present ☐ 0	Present/ Reinforcement ☐ 0.5	Absent ☐ 1
4. Vibration perception at great toe	Present ☐ 0	Decreased ☐ 0.5	Absent ☐ 1	Present ☐ 0	Decreased ☐ 0.5	Absent ☐ 1

The monofilament test

We have also used the perception to a 10-g monofilament (applied to 10 positions, the tip of each toe, under 3 metatarsal heads, the plantar surface of the foot and the dorsal space between the first and second toe) as a test for foot insensitivity; an abnormal monofilament test was defined as < 8 correct responses (8). The sensitivity and specificity of the monofilament test to predict amputations or foot ulceration were 62% and 92% respectively (8). Monofilament insensitivity was found to be an independent predictor of foot ulceration and amputations (9-11).

OSA diagnosis

OSA was assessed by a single overnight home-based cardio-respiratory sleep study using a portable multi-channel device (Alice PDX, Philips Respironics) and scored in accordance with the American Academy of Sleep Medicine guidelines(12). Sleep studies with <4 hours of adequate recordings were repeated and excluded if the quality remained poor. Patients with predominantly central sleep apnea (CSA) were excluded (two patients). All sleep studies were double scored. An apnea hypopnea index (AHI) ≥ 5 events/hour was consistent with the diagnosis of OSA(13). OSA severity was assessed based on the AHI, oxygen desaturation index (ODI, the number of oxygen desaturations of ≥ 4% per hour), the time spent with oxygen saturations < 90% and <80% and the nadir oxygen levels during sleep.

Lipid peroxide

Samples were analysed for lipid peroxides using a modification of a method by el-Saadani et al (14).The principle of this assay is based on the ability of lipid peroxides to convert iodide to iodine, which can be then measured using a spectrophotometer.

Make up the reagent mix containing the following: Potassium Phosphate (0.2M, pH 6.2), Potassium Iodide (0.12M), Sodium Azide (0.15µm), Triton X (2g/ml), Alkylbenzyldimethylammonium Chloride (0.1g/ml), Ammonium Molybdate (10µM).

Add 200ul of sample/blank to a cuvette and 2000ul of reagent mix, incubated in the dark for 30 minutes at 25°C and read the cuvette at 365nm in a spectrophotometer.

The concentration of lipid peroxides was calculated using the Beer-Lambert Law using the extinction coefficient for iodine of 24600. All samples and blanks were analysed in duplicate.

Microvascular/endothelial assessment

Microvascular and endothelial assessment was performed on a casually chosen representative patient subset using Laser speckle contrast imaging (Moor Instruments Ltd, Devon, UK)(15). Microvascular blood flow (measured in arbitrary perfusion units (APU) or flux) was assessed at the left mid thigh level under standardized conditions(16). Imaging was performed over 20 minutes. Blood flow was measured at baseline and following heating to 44^0C and following the iontophoresis of 1% acetylcholine (Ach) and 2% sodium nitroprusside (SNP) (5 pulses over 5 minutes). Data are presented as absolute values, conductance (measured as flux divided by mean arterial pressure(MAP) in order to account for differences in BP), and as percentage of maximal dilatation flow as recommended by previous reports(16).

All microvascular function tests were performed between 10am and 4pm in the same room in our research centre. Room temperature was maintained at 22-24 C. The area of interest was exposed and left to adapt to room temperature for 15-20 minutes before starting the test. We have used the middle of the left thigh in all patients to keep consistency. All studies were performed by the same person following the same protocol.

Laser Speckle Contrast Imaging (LSCI) has been reported to have a mean day-to-day coefficient of variation (CV) of 8%, and intra-class correlation coefficients (ICC) of 0.76 (17). We have examined 4 subjects using the LSCI twice one week apart to assess the reliability and reproducibility of our protocol. The ICC for the baseline, heating, acetylcholine and sodium nitroprusside measurements was 0.9, 0.7, 0.9, and 0.8. The CV for the baseline, heating, acetylcholine and sodium nitroprusside measurements were of 7%, 6%, 14%, and 10% respectively. The ICC for the ratios of baseline, acetycholine and sodium nitroprusside responses to maximum vasodilatation were 0.7, 1.0 and 0.9. The CV for the ratios was 10%, 8% and 11% respectively. These results suggest a robust performance of our protocol which is in line of what is reported in the literature.

Statistical methods

Data analysis was performed using SPSS 15.0 software (SPSS Inc, Chicago, USA). Data are presented as mean (SD) or median (IQR) depending on data distribution. Normality testing was performed using histograms and the Shapiro-Wilk test. Independent continuous variables were compared using the Student's t-test or the Mann-Whitney test. Categorical variables were compared using the Chi-square test. The Bonferroni correction was applied to define statistical significance when comparing the components of the MNSIe and MNSIq between patients with and without OSA. Correlations between continuous variables were performed using the Pearson or Spearman tests. Differences between independent groups were assessed by analysis of variance (ANOVA) with post-hoc analysis. Analysis of covariance (ANCOVA), was used to assess the impact of covariates on the differences between several independent groups. To assess whether OSA status is an independent predictor of DPN, multiple logistic regression (forced entry method) was used. To assess the relation between OSA severity and DPN, AHI quartiles and nocturnal hypoxemia measures were used in the logistic regression models. To assess the independent predictors of continuous variables, multiple linear regression (forced entry method) was used. Non-normally distributed data was normalized by log or square root transformation. Variables included in the regression models were based on known outcome-related risk factors and/or variables that differed between patients with and without OSA. In order to further explore the impact of baseline differences on the associations observed, a sub-group of 70 patients with and 70 without OSA were group matched for a variety of risk factors. A p value < 0.05 was considered significant unless stated otherwise.

Multicollinearity and model checking:

We assessed multicollinearity in both multiple linear and logistic regression models using simple correlations between variables plus the tolerance values, and the condition indices. No tolerance values were < 0.1 and no variables had strong correlations ($r > 0.8$). Condition indices > 30 were taken to indicate multicollinearity problems with variances proportions > 0.5 to indicate the

variables involved. The results of these procedures were to identify evidence of collinearity, but sequentially removing variables involved had limited impact on models estimates for the main exposure.

To investigate and deal with collinearity, where condition indices >30, variables with variances proportions > 0.5 were removed individually and sequentially until no variances proportions > 0.5 remained. Overall, whilst collinearity problems were observed for a number of variables in most models, the impact on estimates for the main exposure variable (OSA) were minimal. For example, in the fully adjusted model (outcome: DPN; Predictors: OSA, age, ethnicity, gender, height, diabetes duration, BMI, alcohol intake, HbA1c, insulin use, blood pressure, eGFR, peripheral vascular disease, smoking, total cholesterol, triglycerides, HDL, oral anti-diabetes treatment, individual anti-hypertensive agents, lipid lowering therapy, anti-platelets and recruitment site) there was significant collinearity with 12 condition indicies > 30. After sequentially removing variables from the model and leaving only Ethnicity, Gender, Age, diabetes duration, OSA, eGFR, Insulin use, and BMI there was only one condition index above 30 and all the rest were below 15. This removal of variables from the model changed the odds ratios for having DPN in patients with OSA from 2.95 (95% CI 1.44-6.05) to 2.83 (95% CI 1.46-5.52). This suggests that the collinearity had had limited impact on models estimates for the main exposure (OSA).

Final models presented thus include variables based on the known outcome-related risk factors and/or possible confounders and/or variables that differed between patients with and without OSA , regardless of the presence of collinearity.

In multiple linear regression models, the residuals were examined. In all the models presented, residuals followed a normal distribution with uniform variance and there was no relationship between the residual and the predictor of interest (OSA, apnea hypopnea index or hypoxemia measures depending on the model).

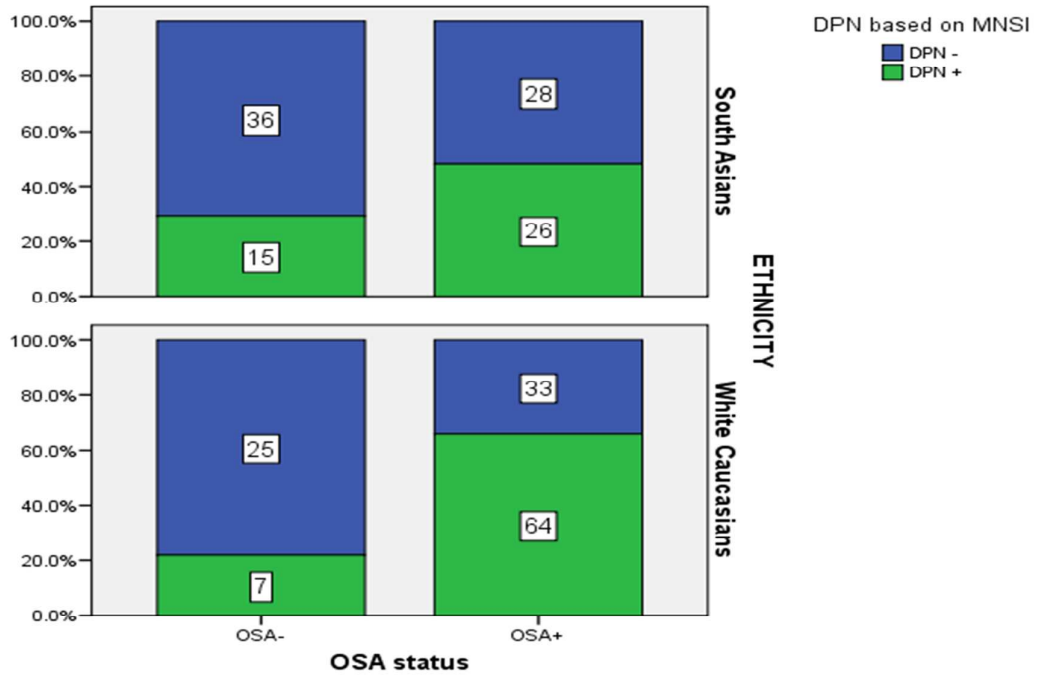
Results

The relationship between OSA and DPN by ethnicity subgroups

The overall prevalence of DPN was significantly higher in OSA+ compared to OSA- patients (60% vs. 27%, p<0.001, respectively). This relationship between OSA and DPN was present irrespective of ethnicity. The prevalence of DPN was higher in patients with OSA whether they were White Caucasians (66% vs. 22%, p<0.001) or South Asians (48% vs. 29%, p=0.049) (**Figure E2**).

The overall prevalence of foot insensitivity was 37%. Foot insensitivity was significantly higher in OSA+ compared to OSA- patients (50% vs. 15%, p<0.001, respectively). The prevalence of an abnormal monofilament test was also more common in patients with OSA whether they were White Caucasians (57% vs. 16%, p<0.001) or South Asians (37% vs. 14%, p=0.006).

Figure E2: The relationship between OSA and DP in ethnicity subgroups.



A multivariate analysis of the relationship between OSA, its severity and DPN:

To assess the relationship between OSA and DPN we used logistic regression models using the forced entry method. The results of the main analysis can be found in Table 3 in the main manuscript.

In order to assess whether any of the OSA metrics or nocturnal hypoxemia parameters are associated with DPN, we have repeated the logistic regression (as in model 1 in Table 3) after removing OSA and inserting AHI (as quartiles), ODI (as quartiles), time spent with oxygen saturation < 80% (as binary variable) and nadir nocturnal oxygen saturations separately into separate models using the forced entry method.

Using AHI quartile 1 (AHI<2.90) as the reference point showed that quartile 2 (AHI 2.90 to 7.59) (OR 2.56, 95% CI 1.10-6.04, p=0.03), quartile 3 (AHI 7.60-16.09) (OR 3.84, 95%CI 1.58-9.33, p=0.003) and quartile 4 (AHI ≥16.01) (OR 3.08, 95%CI 1.20-7.86, p=0.02) were independently associated with DPN. Nadir nocturnal oxygen saturation (OR 0.97, 95% CI 0.93-1.00, p=0.05) was of borderline significance. ODI quartile 3 (ODI 6.65-14.39) was independently associated with DPN when considering quartile 1 (ODI < 2.70) as the reference point (OR 3.03, 95% CI 1.27-7.25, p=0.01). Time spent with oxygen saturation <80% was not independently associated with DPN (OR 1.86, 95%CI 0.93-3.70, p=0.08).

AHI quartiles where also independently associated with abnormal 10g monofilament sensation (p=0.04); with quartile 1 as the reference, quartile 2 (OR 3.22, 95%CI 1.17-8.85, p=0.02), quartile 3 (OR 4.38, 95%CI 1.55-12.36, p=0.005), and quartile 4 (OR 3.16, 95%CI 1.10-9.05, p=0.03) were all independently associated with the “at risk foot”. Only ODI quartile 3 was independently associated with abnormal 10g monofilament perception (OR 3.27, 95% CI 1.23-8.67, p=0.02) when quartile 1 was taken as the reference point. Nadir nocturnal oxygen saturation and time spent with oxygen saturations < 80% were not independent predictors after adjustment.

The relationship between OSA and clinical signs of DPN

To assess the relationship between OSA and the clinical findings on foot examination (MNSIe components), we repeated the logistic regression models as performed in model 1 in **Table 3** , but changed the outcome measure to the aspect of clinical examination of interest.

Using the forced entry method, OSA was independently associated with reduced/absent ankle jerk reflex (OR 2.64, 95% CI 1.35-5.16, p=0.005). OSA was also independently associated with reduced/absent vibration sensation (OR 3.18, 95% CI 1.59-6.39, p=0.001). OSA was not independently associated with having an abnormality on foot inspection (OR 1.82, 95% CI 0.96-3.43, p=0.07). This is in addition to the relationship we described between OSA and the 10g monofilament test, which suggest that OSA is independently associated with different aspects of foot examination. In addition, with quartile 1 being the reference, AHI quartiles were independent predictors of reduced/absent ankle jerk reflex (p=0.03); quartile 2 (OR 2.33, 95%CI 1.01-5.38, p=0.047), quartile 3 (OR 1.94, 95%CI 0.82-4.58, p=0.13), quartile 4 (OR 4.10, 95%CI 1.62-10.31, p=0.003). AHI quartiles were also independent predictors of reduced/absent vibrations sensation (p=0.02); quartile 2 (OR 2.64, 95%CI 1.07-6.50, p=0.04), quartile 3 (OR 4.56, 95%CI 1.78-11.66, p=0.002) and quartile 4 (OR 2.65, 95%CI 1.01-6.94, p=0.048). AHI quartiles were not independently associated with abnormal foot inspection.

Clinical characteristics of patients according to the MNSIe groups

This is referring to **Table 4** in the main manuscript

Table E1: Participants characteristics in relation to MNSIe categories. Data presented as median (IQR) or mean(SD). GFR: Glomerular Filtration Rate.

	Group 1: < 2 (n=90)	Group 2: 2 - < 4 (n=100)	Group 3: ≥ 4 (n=44)	**P value**
Male	51%	54%	80%	0.005
Age (years)	55.0(12.9)	56.7±10.2	62.7±10.5	0.001
Diabetes Duration (years)	10.0 (6.0-12.0)	11.0 (6.0-16.0)	17.0 (11.0-24.7)	< 0.001
Body Mass Index (kg/m^2)	31.6 (27.9-36.4)	33.8 (30.0-38.3)	34.1 (29.2-40.5)	0.032
Alcohol (drinks alcohol)	23%	30.0%	31.8%	0.475
eGFR	90.1(27.1)	87.4±24.4	75.1±26.8	0.009

Clinical characteristics of matched sub-group

Table E2: The characteristics of patients in the matched subgroup in relation to OSA status. Data presented as median (IQR) or mean (SD). GFR: Glomerular Filtration Rate, PVD: Peripheral Vascular Disease. Variables that are matched are highlighted in red. The main aim for this subgroup is to match for BMI and diabetes duration.

	OSA- (n=70)	OSA+ (n=70)	P value
Male	47%	71%	0.003
Caucasians	31%	60%	0.001
Age (years)	55.1(12.2)	59.8(10.2)	0.02
Diabetes Duration (years)	10.0 (6.0-15.0)	10.0 (6.0-15.0)	0.88
Body Mass Index (kg/m^2)	30.0 (26.9-33.9)	31.3 (27.8-33.8)	0.39
Height (cm)	163.6(8.5)	167.7(9.1)	0.006
Systolic blood pressure (mmHg)	125.5 (115.4-136.0)	129.5 (121.5-137.1)	0.08
Diastolic blood pressure (mmHg)	78.5 (70.1-85.6)	78.0 (73.1-82.1)	0.88
HbA1c (%)	7.7 (7.1-8.7)	8.0 (7.0-9.1)	0.68

Total cholesterol (mmol/L)	3.7 (3.3-4.4)	3.6 (3.1-4.2)	0.32
Triglycerides (mmol/L)	1.6 (1.1-2.3)	1.7 (1.2-2.4)	0.76
HDL (mmol/L)	1.2 (1.0-1.4)	1.1 (0.9-1.2)	0.01
Estimated GFR (ml/min/1.73 m^2)	90.3(23.2)	84.7(24.1)	0.16
TSH	1.7 (1.1-2.2)	1.6 (1.0 vs. 2.1)	0.68
Epworth sleepiness score	5 (1-11)	8 (3-13)	0.03
Smoking (current or ex-smoker)	41%	41%	1.0
Alcohol (drinks alcohol)	11%	33%	0.002
Oral anti-diabetes treatment	97%	93%	0.25
Insulin	44%	54%	0.24
Lipid lowering therapy	84%	79%	0.39
Anti-hypertensives	73%	80%	0.32
PVD	1%	4%	0.31

Clinical characteristics of patients undergoing serum nitrotyrosine assessment

Table E3: The characteristics of patients who had undergone serum nitrotyrosine assessment in relation to OSA status. Data presented as median (IQR) or mean (SD). GFR: Glomerular Filtration Rate.

	OSA- (n=29)	OSA+ (n=73)	P value
Male	35%	66%	0.004
Caucasians	55%	67%	0.26
Age (years)	54.8(12.1)	59.0(11.0)	0.10
Diabetes Duration (years)	10.0 (4.5-12.0)	12.0 (6.0-20.0)	0.08
Body Mass Index (kg/m^2)	31.0 (28.1-35.2)	34.6 (31.1-39.8)	0.006
Systolic blood pressure (mmHg)	127.0 (120.5-137.5)	131.5 (123.7-140.5)	0.12

Diastolic blood pressure (mmHg)	78.3(8.9)	77.2(10.1)	0.63
HbA1c (%)	7.4 (6.8-8.5)	8.0 (7.1-9.1)	0.20
Total cholesterol (mmol/L)	3.8 (3.2-4.3)	3.7 (3.2-4.3)	0.77
Triglycerides (mmol/L)	1.6 (1.2-2.3)	1.8 (1.3-2.5)	0.66
HDL (mmol/L)	1.1 (0.9-1.5)	1.1 (0.9-1.2)	0.395
Estimated GFR (ml/min/1.73 m^2)	89.7(24.7)	82.2(27.6)	0.20
Epworth sleepiness score	7.0 (4.0-14.0)	8.0 (3.2-11.7)	1.0
Smoking (current or ex-smoker)	45%	41%	0.73
Alcohol (drinks alcohol)	17%	38%	0.04
Oral anti-diabetes treatment	97%	95%	0.67
Insulin	45%	59%	0.20
Lipid lowering therapy	83%	82.%	0.95
Anti-hypertensives	69%	89%	0.01

Table E4: Comparison of the characteristics of patients who had serum nitrotyrosine measured and those who did not. Data presented as median (IQR) or mean (SD). GFR: Glomerular Filtration Rate.

	Nitrotyrosine not measured (n=132)	Nitrotyrosine measured (n=102)	P value
Male	58%	57%	0.82
Caucasians	49%	64%	0.02
Age (years)	56.7 (11.8)	57.8 (11.4)	0.50
Diabetes Duration (years)	11.0 (6.0-16.0)	11.0 (6.0-16.5)	0.89
Body Mass Index (kg/m^2)	33.7 (7.9)	35.3 (8.8)	0.15
Waist circumference (cm)	112.6 (16.7)	115.2 (16.3)	0.24
Waist hip ratio	1.0 (0.1)	0.9 (0.1)	0.46

Neck circumference (cm)	41.1 (4.7)	41.8 (4.8)	0.25
Systolic blood pressure (mmHg)	128.9 (17.7)	131.7 (16.2)	0.22
Diastolic blood pressure (mmHg)	78.2 (10.4)	77.5 (9.8)	0.64
HbA1c (%)	8.4 (1.5)	8.1 (1.3)	0.07
Total cholesterol (mmol/L)	4.0 (1.1)	3.9 (0.9)	0.69
Triglycerides (mmol/L)	2.0 (1.3)	1.9 (1.1)	0.56
HDL (mmol/L)	1.1 (0.3)	1.1 (0.3)	0.75
Estimated GFR (ml/min/1.73 m^2)	87.5 (26.0)	84.3 (26.9)	0.36
Epworth sleepiness score	7.0 (3.0-13.0)	8.0 (4.0-12.5)	0.95
Smoking (current or ex-smoker)	39%	42%	0.59
Alcohol (drinks alcohol)	24%	32%	0.17
Oral anti-diabetes treatment	92%	95%	0.30
Insulin	52%	55%	0.69
Lipid lowering therapy	85%	82%	0.61
Anti-hypertensives	80%	83%	0.46
Anti-platelets	69%	64%	0.40

Results of lipid peroxide studies

Lipid peroxide levels were higher in patients with DPN (21.14 (3.86-42.48) vs. 12.20 (2.90-24.55), p=0.014). OSA was associated with higher lipid peroxide levels (µM/ml) compared to patients without OSA [18.39 (8.33-37.40) vs. 7.93 (0.81-22.76), p=0.014] which remained significant after adjusting for age, BMI and diabetes duration (p=0.02). Lipid peroxide levels correlated with ODI (r=0.225, p=0.025), time spent with oxygen saturations < 90% (r=0.263, p=0.008), time spent with oxygen saturations < 80% (r=0.229, p=0.022), and nadir nocturnal oxygen saturations (r= -0.236, p=0.019). The correlation with AHI was borderline (r= 0.188, p=0.062). After adjustment for age, BMI

and diabetes duration the correlations between lipid peroxide levels and ODI (r=0.221, p=0.031) and nadir nocturnal oxygen saturations (r=-0.220, p=0.032) remained significant**.** After adjustment for age, gender, ethnicity, diabetes duration, BMI, HbA1c, BMI and mean arterial pressure using multiple linear regression there was no independent association between OSA, OSA metrics or hypoxemia measures and lipid peroxide levels. Only HbA1c was independently associated with lipid peroxide (B=0.61, p=0.001).

Clinical characteristics of patients who had undergone microvascular assessment

Table E5: The characteristics of patients who had undergone microvascular assessment in relation to OSA status. Data presented as median (IQR) or mean (SD). GFR: Glomerular Filtration Rate.

	OSA- (n=24)	OSA+ (n=47)	P value
Male	38%	68%	0.01
Caucasians	33%	64%	0.02
Age (years)	56.0(10.1)	60.6(11.3)	0.09
Diabetes Duration (years)	10.0 (7.2-15.7)	14.0 (6.0-20.0)	0.27
Body Mass Index (kg/m^2)	30.7 (28.0-35.8)	34.4 (31.1-36.6)	0.06
Systolic blood pressure (mmHg)	125.5 (112.5-132.6)	132.0 (121.5-139.0)	0.08
Diastolic blood pressure (mmHg)	77.6(9.106)	76.9(9.5)	0.24
HbA1c (%)	7.4 (6.6-8.4)	7.6 (7.1-8.6)	0.38
Total cholesterol (mmol/L)	3.7 (3.3-4.4)	3.7 (3.1-4.1)	0.28
Triglycerides (mmol/L)	1.6 (1.0-2.7)	1.7 (1.2-2.2)	0.78
HDL (mmol/L)	1.1 (0.9-1.5)	1.1 (0.9-1.3)	0.45
Estimated GFR (ml/min/1.73 m^2)	86.1(25.1)	78.8(24.7)	0.78
Epworth sleepiness score	7.0 (3.2-13.5)	8.5 (3.0-12.2)	0.66
Smoking (current or ex-smoker)	46%	34%	0.33

Alcohol (drinks alcohol)	13%	36%	0.04
Oral anti-diabetes treatment	96%	89%	0.35
Insulin	46%	53%	0.56
Lipid lowering therapy	88%	87%	0.98
Anti-hypertensive agents	75%	89%	0.11

Table E6: Comparison of the characteristics of patients who had Laser Speckle Contrast Imaging/microvascular regulation assessed and those who did not. Data presented as median (IQR) or mean (SD). GFR: Glomerular Filtration Rate.

	Microvascular assessment not preformed (n=156)	Microvascular assessment preformed (n=71)	P value
Male	58%	58%	0.93
Caucasians	55%	54%	0.89
Age (years)	56.1 (11.9)	59.1 (11.0)	0.08
Diabetes Duration (years)	10.0 (6.0-15.8)	11.5 (7.0-20.0)	0.08
Body Mass Index (kg/m^2)	33.8 (7.3)	34.6 (9.2)	0.51
Waist circumference (cm)	113.8 (17.0)	112.2 (14.8)	0.49
Waist hip ratio	1.0 (0.1)	1.0 (0.1)	0.15
Neck circumference (cm)	41.6 (4.9)	40.8 (4.3)	0.24
Systolic blood pressure (mmHg)	130.0 (16.6)	130.1 (18.7)	0.97
Diastolic blood pressure (mmHg)	78.1 (10.5)	77.2 (9.3)	0.51
HbA1c (%)	8.4 (1.4)	8.0 (1.4)	0.02
Total cholesterol (mmol/L)	3.9 (1.0)	3.8 (1.0)	0.39
Triglycerides (mmol/L)	2.1 (1.3)	1.9 (1.2)	0.34

HDL (mmol/L)	1.1 (0.3)	1.2 (0.33)	0.48
Estimated GFR (ml/min/1.73 m^2)	88.2 (27.1)	81.2 (24.9)	0.07
Epworth sleepiness score	7.0 (4.0-12.8)	8.0 (3.0-12.2)	0.77
Smoking (current or ex-smoker)	39.7%	38.0%	0.81
Alcohol (drinks alcohol)	27.6%	28.2%	0.93
Oral anti-diabetes treatment	93.6%	91.5%	0.58
Insulin	55.1%	50.7%	0.54
Lipid lowering therapy	81.4%	87.3%	0.27
Anti-hypertensives	78.8%	84.5%	0.32
Anti-platelets	64.7%	70.4%	0.40

The adjusted analysis for the relationship between microvascular function and OSA metrics:

Table E7: The adjusted analysis of the impact of OSA on microvascular blood flow and endothelial function in patients with type 2 diabetes. Data presented as B and p value. The analysis was performed using blood flow as the outcome and ethnicity, gender, age at diabetes diagnosis, diabetes duration, BMI and OSA (and its metrics) as the independent predictors. Blood flux was measured in arbitrary perfusion units (APU). Conductance is calculated by dividing flux by the mean arterial pressure. Multiple linear regression, the *forced entry method* was used to conduct this analysis. Ach: Acetylcholine, SNP: Sodium nitroprusside.

	OSA	AHI	ODI	Nadir nocturnal oxygen saturation
Conductance				
Baseline	B=-0.22, p <0.001	B= -0.21, p= 0.001	B= -0.14, p= 0.03	B= 0.12, p= 0.34
Heating	B= -0.04, p= 0.42	B= -0.05, p= 0.34	B=-0.03, p=0.49	B= 0.04, p= 0.66
Ach	B= -0.1, p= 0.10	B= -0.05, p= 0.46	B= -0.02, p= 0.80	B= -0.06, p= 0.65
SNP	B= -0.25, p <0.001	B= -0.21, p= 0.005	B= -0.20, p= 0.006	B= 0.29, p= 0.047
Flux in relation to maximum vasodilatation				
Baseline	B= -0.19, p= 0.002	B= -0.16, p= 0.008	B= -0.11, p= 0.09	B= 0.08, p= 0.51
Ach	B= -0.06, p= 0.26	B= -0.002, p= 0.97	B= 0.02, p= 0.78	B= -0.10, p= 0.40
SNP	B= -0.21, p= 0.001	B= -0.16, p= 0.01	B= -0.17, p= 0.009	B= 0.25, p= 0.05

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