Microvascular complications in patients with type 2 diabetes: the impact of ethnicity, sleep and oxidative stress

by

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Abstract

Background: Diabetes-related microvascular complications are associated with significant morbidity, mortality and economic burden. Effective treatments for microvascular complications, apart from improved metabolic and blood pressure control, are lacking. Hence, improved understanding of the pathogenesis of these complications is needed to develop new treatments.

Obstructive sleep apnoea (OSA) is very common in type 2 diabetes (T2DM) and has been shown to stimulate the same harmful pathways as hyperglycaemia, particularly those that are involved in the pathogenesis of microvascular complications. Hence, it is plausible that OSA is associated with microvascular complications in patients withT2DM.

Aims: To explore the interrelationships between OSA and microvascular complications in patients with T2DM and the possible mechanisms behind such a relationship.

Methods: A cross-sectional study of South Asians and White Europeans with T2DM were randomly recruited from the outpatients of two secondary care diabetes clinics in the UK. Patients were extensively characterised including assessments for OSA and microvascular complications.

Results: Patients (n=234) were included in the analysis. OSA prevalence was 64.5%. OSA patients had worse metabolic profile compared to patients without OSA. The prevalence of all microvascular complications (except cardiac autonomic neuropathy) was higher in patients with OSA compared to patients without. After adjustment for a wide range of confounders, OSA remained independently associated with microvascular complications. OSA and hypoxaemia severity correlated with the severity of complications. Based on blood samples and skin biopsies collected during the study, patients with OSA had increased oxidative and nitrosative stress and impaired microvascular regulation compared with patients without OSA. Furthermore, ethnic differences in OSA accounted for some of the ethnic differences in microvascular complications.

Conclusion: I have identified a novel association between OSA and microvascular complications in patients with T2DM, with increased nitrosative stress and oxidative stress and impaired microvascular regulation as possible mechanisms. Further prospective observational and interventional studies are needed to assess the impact of OSA and its treatment on the development and progression of microvascular complications.

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Table of Contents

Table of Tables

Table of Figures

Abbreviations list

AASM: American Academy of Sleep Medicine

ACEi: Angiotensin converting enzyme inhibitors

Ach: acetylcholine

ACR: albumin creatinine ratio

AGE: advanced glycation end-product

AHI: apnea-hypopnea Index

AR: Aldose reductase

BMI: body mass index

BP: blood pressure

CAN: cardiac autonomic neuropathy

CKD: chronic kidney disease

CPAP: continuous positive airway pressure

CSA: central sleep apnoea

CSMO: clinically significant macular oedema

CV: coefficient of variation

CVD: cardiovascular disease

DAG: diacylglycerol

DAN: diabetic autonomic neuropathy

DCCT: Diabetes Complications and Control Trial

DD: disc diameter

DIP: Distal interphalangeal

DN: Diabetic neuropathy

DPN: diabetic peripheral neuropathy

DPP-4: dipeptidyl peptidase-4

DR: Diabetic retinopathy

DRG: dorsal root ganglia

EDS: excessive daytime sleepiness

eGFR: estimated glomerular filtration rate

ESRD: end-stage renal disease

ET-1: endothelin-1

FRF: the Fundamental Respiratory Frequency

GAFT: glutamine:fructose-6 phosphate amidotransferase

GLP-1: glucagon-like peptide-1

HF: high frequency

HRV: heart rate variability

ICC: intra-class correlation coefficients

IENFD: intra epidermal nerve fibre density

IGF-1: insulin-like-growth factod-1

IGT: impaired glucose tolerance

IQR: Intra quartile range

IR: Insulin resistance

LF: low frequency

Lfa ot LF: low-frequency

LOX-1: lectin-like oxidized LDL-1

LSCI: Laser Speckle Contrast Imaging

MAD: Mandibular advancement devices

MAP: mean arterial pressure

MAPKs: Mitogen activated protein kinases

MNSI: Michigan neuropathy screening instrument

MNSIe: Michigan neuropathy screening instrument-examination

MNSIq: Michigan neuropathy screening instrument-questionnaire

NAFLD: Non-alcoholic fatty liver disease

NASH: Non-alcoholic steatohepatitis

NEFAS: non-esterified fatty acids

NO: nitric oxide

NVD: neovascularisation of optic disc

NVE: neovascularisation elsewhere

OCT: optical coherence tomography

ODI: oxygen desaturation index

OS: oxidative stress

OSA: Obstructive sleep apnoea

oxLDL: Oxidised LDL

PAI-1: plasminogen activator inhibitor-1

PARP: poly(ADP-ribose) polymerase

PKC: protein kinase C

pNN50: percentage of differences between adjacent normal sinus intervals that are greater than 50 ms

PVD: peripheral vascular disease

RAAS: renin–angiotensin–aldosterone

RAGE: receptor of AGE

RCS: reactive chlorine species

RDI: respiratory disturbance index

REM: Rapid Eye Movement

RERA: respiratory effort–related arousal

Rfa: respiratory frequency

rmsSD: square root of the mean of the squares of differences between adjacent normal sinus intervals

RNS: reactive nitrogen species

ROS: reactive oxygen species

SA: South Asians

SDH: sorbitol dehydrogenase

sdNN: standard deviation of normal sinus intervals

SNCV: sural nerve conduction velocity

SNP: Sodium Nitroprusside

SOD: superoxide dismutase

STDR: sight threatening diabetic retinopathy

SWS: slow wave sleep

T2DM: Type 2 diabetes

TCAC: tricarboxylic acid cycle

TIA: transient ischaemic attack

TNF-α: tumour necrosis factor-α

TSP: total spectral power

UCPs: uncoupling proteins

UKPDS: United Kingdom Prospective diabetes Study

VCAM-1: vascular cell adhesion molecule-1

VEGF: vascular endothelial growth factor

VLF: very low frequency

WE: White Europeans

WHO: World Health Organisation

OR: Odds ratio

CI: confidence interval

SD: standard deviation

ANOVA: analysis of variance

ANCOVA: analysis of covariance

1. Chapter one: Introduction

1.1. Diabetes Mellitus

Type 2 diabetes mellitus (T2DM) is a global epidemic with an estimated worldwide prevalence of 6.4% (285 million) in 2010 that is forecast to rise to 7.7% (438 million) in 2030 (1). In addition 344 million people have impaired glucose tolerance (IGT) that is forecast to increase to 472 million by 2030 (1).

The World Health Organisation (WHO) defines diabetes mellitus (DM) as: "a metabolic disorder of multiple aetiology characterized by chronic hyperglycaemia with disturbances of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action, or both. The effects of DM include long-term damage, dysfunction and failure of various organs such as retinopathy with potential blindness, nephropathy that may lead to renal failure, and/or neuropathy with risk of foot ulcers, amputation, Charcot joints, and features of autonomic dysfunction, including sexual dysfunction. People with diabetes are at increased risk of cardiovascular, peripheral vascular and cerebrovascular disease." This definition highlights the complexity of this disorder and that vascular complications are an essential part of this complex disorder.

The health, social, and economic burden of T2DM is significant; patients with diabetes have a reduced life expectancy by 10-15 years (2) and the cost of diabetes was estimated to be 12% of the world's health expenditure in 2010 (1;3;4). The financial impact of DM on the NHS is also remarkable (2;5;6). Because of its increasing prevalence, T2DM (which accounts for 90% of all diabetes) presents a massive challenge to healthcare systems around the world.

T2DM is a complex endocrine and metabolic disorder. The interaction between multiple genetic and environmental factors gives rise to a heterogeneous and progressive condition with variable degrees of insulin resistance (IR) and pancreatic β-cell dysfunction **(Figure 1.1.)** (7). Overweight and obesity are major contributors to the development of IR and IGT (7-9). When β-cells are no longer able to secrete sufficient insulin to overcome IR, IGT progresses to T2DM (7;9). IR usually emerges many

years before the onset of T2DM due to the interaction of genetic and multiple environmental factors (7-13). Overweight and obesity contribute to IR via several pathways including, an imbalance of hormones (e.g. increased leptin, reduced adiponectin and increased glucagon), increased cytokines (e.g. tumour necrosis factor-α, interleukin-6) and other inflammatory signals (e.g. nuclear factor-KB) (7;9;14-17). Crucially, increased release of non-esterified fatty acids (NEFAs) particularly from intra-abdominal adipose tissue in obesity, raises intracellular diacylglycerol and fatty acyl-co A, which reduce insulin post-receptor signalling (9). When insulin secretion is no longer sufficient to overcome IR, glucose intolerance progresses to T2DM. The decline in β-cell function appears to involve chronic hyperglycaemia per se (glucotoxicity), chronic exposure to NEFAs (lipotoxicity), oxidative stress (OS), inflammation, and amyloid formation (18-21). Patients with T2DM usually exhibit pancreatic α-cell dysfunction resulting in elevated (or non-suppressible) glucagon secretion in the presence of hyperglycaemia (22) and probably reduced prandial glucagon-like peptide-1 (GLP-1) secretion (23).

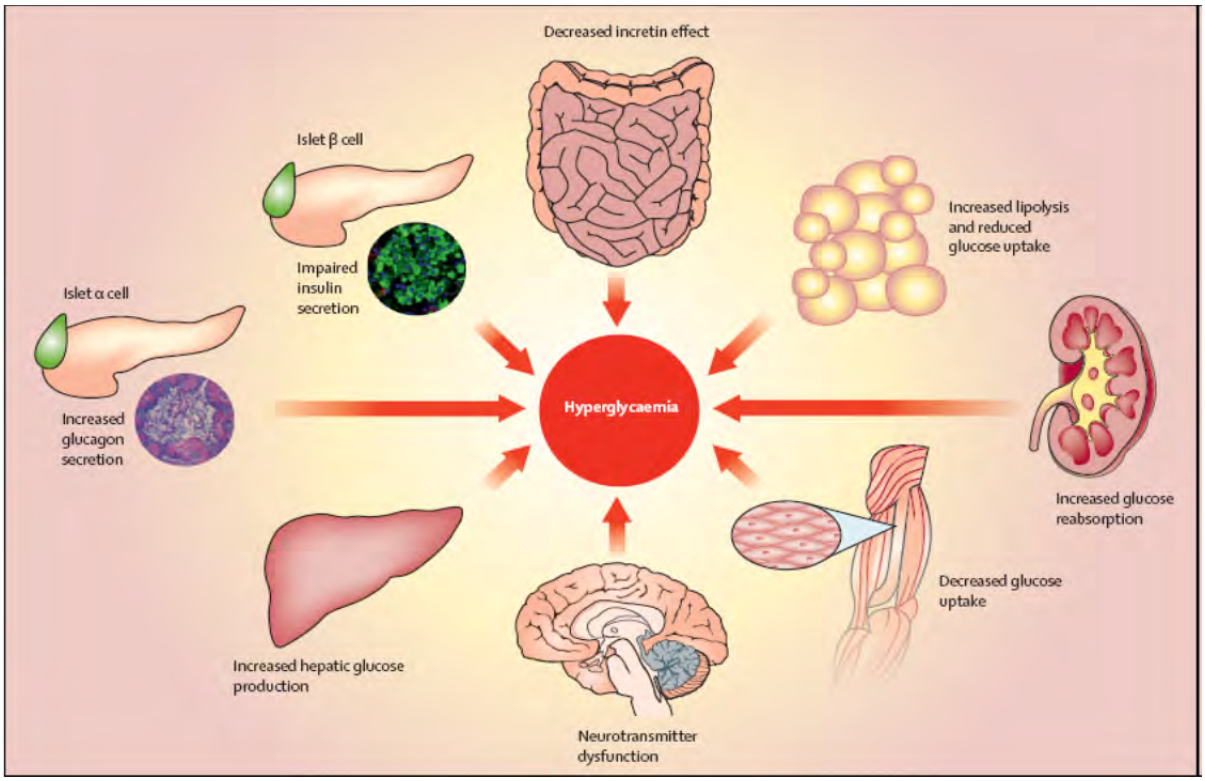


Figure 1-1: The complex pathophysiology of type 2 diabetes. Figure adapted from (24).

Most of the burden of T2DM is related to its micro and macro vascular complications. Microvascular complications have significant impact on morbidity, mortality and patients' quality of life. Diabetic retinopathy (DR) is one of the leading causes of blindness in the Western world. Diabetic nephropathy can lead to end-stage renal disease, which requires dialysis and/or renal transplantation and increases risk of vascular disease. Diabetic neuropathy (DN) results in the development of foot ulcers that can result in amputations, sexual dysfunction and many other unpleasant symptoms in addition to increased mortality. The presence of microvascular complications has been shown to have a negative impact on patient's quality of life (3;4;25). As a result, it is not surprising that there is great interest in improving understanding of the microvascular complications in patients with diabetes in order to develop effective strategies to treat, and ideally prevent, the development of such complications.

The main aim of treatment of T2DM is to prevent the development of vascular complications and reduce the morbidity and mortality of this condition. This is currently achieved by improving hyperglycaemia, strict blood pressure (BP) control, and lowering cholesterol levels. Due to the invariable progressive nature of T2DM, differently acting pharmacological agents are required at different stages of the disease to complement the benefits of life-style changes, which can be effective but are difficult to maintain (9;26). These agents include several classes of drugs that either improve insulin secretion from the β-cell (Sulphonylureas, meglitinides, GLP-1 analogues, dipeptidyl peptidase-4 (DPP-4) inhibitors), or improve insulin sensitivity (biguanides, thiazolidinediones), or reduce glucagon secretion and slow gastric emptying (GLP-1 analogues, amylin, DPP-4 inhibitors) or reduce glucose absorption from the gut (α-glucosidase Inhibitors) or overcome insulin resistance by providing exogenous insulin (27-36). Most of the initial improvements in glycaemia are not sustained because of continued β-cell dysfunction (37). Furthermore, many of these treatments have undesired side effects: hypoglycaemia, weight gain, gastrointestinal disturbances, peripheral oedema and potential cardiovascular effects (28).

But despite all these measures, DM-related vascular complications are still very common; hence there is a need for better understanding for the pathogenesis of these complications to develop new treatments.

1.2. Microvascular Complications

Diabetes-related microvascular complications (including DR, DN, and diabetic nephropathy) share a common pathogenesis that is driven by hyperglycaemia and have significant impact on patients' and health care systems.

The United Kingdom Prospective diabetes Study (UKPDS)-37 showed that the presence of microvascular complications resulted in worse general health, more mobility problems, more problems with usual activity, reduced vigour, more tension and mood disturbances (25). Similar results were found by other studies (3;4). Furthermore, the presence of microvascular complications has significant financial and social implications (38;39). The presence of diabetes related complications increases NHS costs for a particular patient by more than five-fold and social services costs 4 fold(2).

Hence, better understanding of the pathogenesis of these conditions is needed in order to develop strategies to reduce the burden and slow the progression or prevent the development of these complications.

1.2.1. Diabetic retinopathy

DR is the leading cause of blindness of people of working-age in the Western world and results in great morbidity and significant economic burden (40;41). The prevalence of DR is estimated to be between 40-50% in patients with T2DM with a higher prevalence in patients with type 1 diabetes, with similar results for US, Europe and the far east (40). The incidence of DR is much less well studied. In the Wisconsin Epidemiologic Study of Diabetic Retinopathy, 74% of patients developed DR over 10 years period with 64% of patients with DR at baseline progressing to more severe DR and

17% progressed to develop proliferative retinopathy (40). Over the same period 25% developed macular oedema (40). The 25-year follow up data from the same study showed that almost all (97%) patients develop DR and that 43% developed proliferative DR and 29% developed macular oedema (40;42).

Known risk factors for DR include increasing age, hyperglycaemia (1% decrease in HbA1c results in 40% reduction in the risk of DR, 25% reduction in the risk of progression to sight threatening diabetic retinopathy (STDR) and 15% risk reduction in blindness), hypertension (10 mm Hg decrease in systolic BP results in a decreased risk of retinopathy progression by 35%, need for laser therapy by 35%, and visual loss by 50%), diabetes duration, dyslipidemia, pregnancy, puberty, cataract surgery, obesity, alcohol consumption and genetic factors (e.g. Aldose reductase gene) (40;43).

Although the precise aetiology of DR remains debated, increased inflammation, OS, advanced glycation end-product (AGE) formation, activation of the polyol pathway and the renin-angiotensin system and perturbations of protein kinase C (PKC) (discussed below), result in direct cellular damage and functional and/or structural defects involving the microvasculature, which result in increased vascular permeability (resulting in macular oedema) or in ischaemic changes resulting in an increase in several factors such as vascular endothelial growth factor (VEGF), insulin-like-growth factor-1 (IGF-1), and erythropoietin which result in neovascularisation and the development of proliferative retinopathy (40;44-46).

DR can be classified into several categories according to severity **(Figure 1.2)**. In mild non-proliferative DR, microaneurysms and hard exudates might be present; 12% (within 1 year) and 30% (within 3 years) progress to proliferative DR (40). In preproliferative DR, there might be intra-retinal haemorrhages, soft exudates, venous beading and intra-retinal microvascular abnormalities (IRMA); 52% (within 1 year) and 71% (within 3 years) progress to proliferative diabetic retinopathy (40). In proliferative diabetic retinopathy neovascularisation of optic disc (NVD) or elsewhere (NVE), pre-retinal haemorrhage, or vitreous haemorrhage might occur; and requires urgent treatment (40). In

clinically significant macular oedema (CSMO), there retinal thickening within 500 μm from the centre of macula; hard exudates within 500 μm from the centre of macula with adjacent retinal thickening or retinal thickening of more than one optic disc area within one optic disc diameter from the centre of macula might occur (40).

DR is usually asymptomatic until the very late stages and systematic examination of the retina is an essential component of the care of patients with DM (40). Examination can be performed using several techniques such as indirect ophthalmoscopy and slit-lamp examination (40). Retinal imaging (particularly retinal photographs) is now more widely used for the diagnosis and screening of patients with DM (40). Retinal photographs have been shown to have a high sensitivity (90%) and specificity (97%) in detecting retinal lesions (40;47). Other imaging techniques include fluorescein angiography and optical coherence tomography (OCT). OCT is an optical biopsy of the retina, providing high-resolution, 3D images that closely approximate the histology of the retina and allows precise and reproducible measurements of retinal thickness, which are crucial for monitoring diabetic macular oedema **(Figure 1.3)** (40).

In addition to strict metabolic control (discussed below), several other treatments are also available. Ruboxistaurin, a PKC inhibitor, has been shown to reduce the risk of progression and need for laser treatment for diabetic macular oedema (48). Laser photocoagulation (panretinal or focal), remains the mainstay of treatment for STDR as it prevents visual deterioration, although restoring or improving visual loss is uncommon (49). The exact mechanism of how Laser works remains unclear, but it is thought that “burning” the retina reduces the production of VEGF (40). Vitrectomy has been the mainstay of surgical treatment for persistent vitreous haemorrhage and tractional retinal detachment; it reduces risk of retinal neovascularisation and macular oedema but increases the risk of iris neovascularisation and cataract formation (40;50). Ranibizumab (An anti-VEGF given as intraocular injection) have shown promising evidence to improve vision in patients with proliferative DR as well as CSMO (51;52).

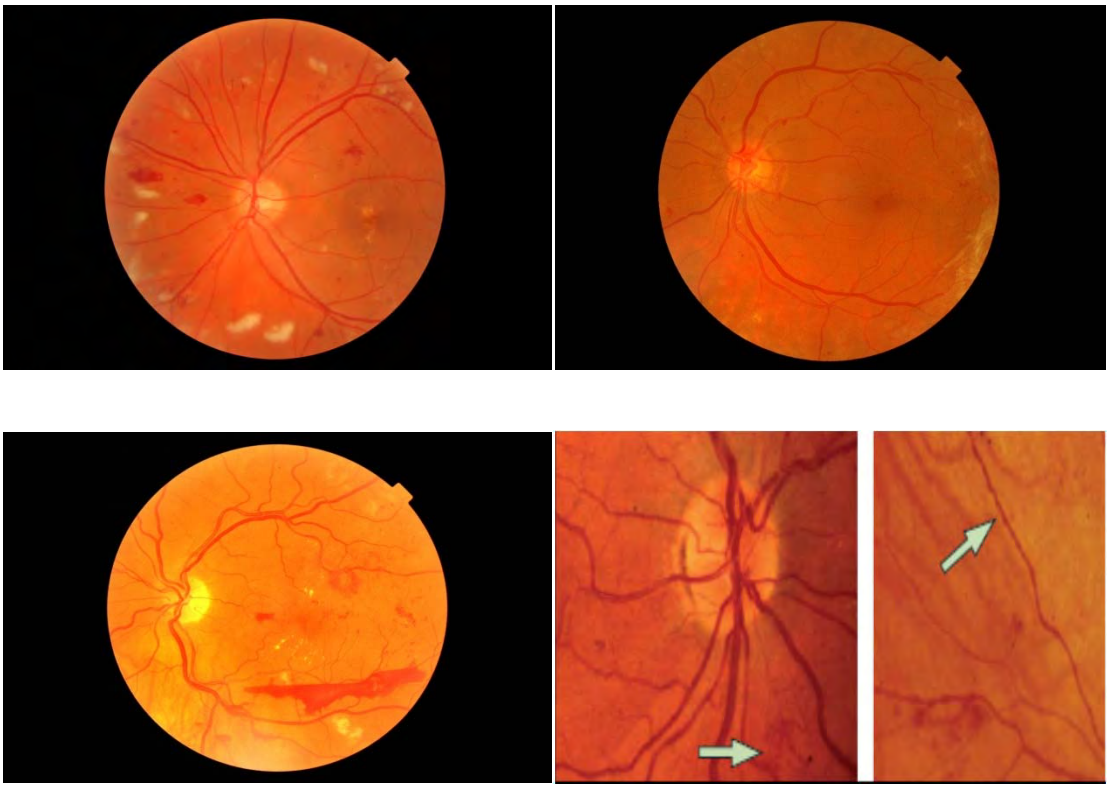


Figure 1-2: An example of different retinal lesions.

Right top showing soft exudates and intra retinal haemorrhages. Left top showing example of neovascularisation close to the disc. Left bottom showing a pre retinal haemorrhage and hard exudates and haemorrhages close to the macula. Right bottom showing IRMA and venous beeding (arrows).

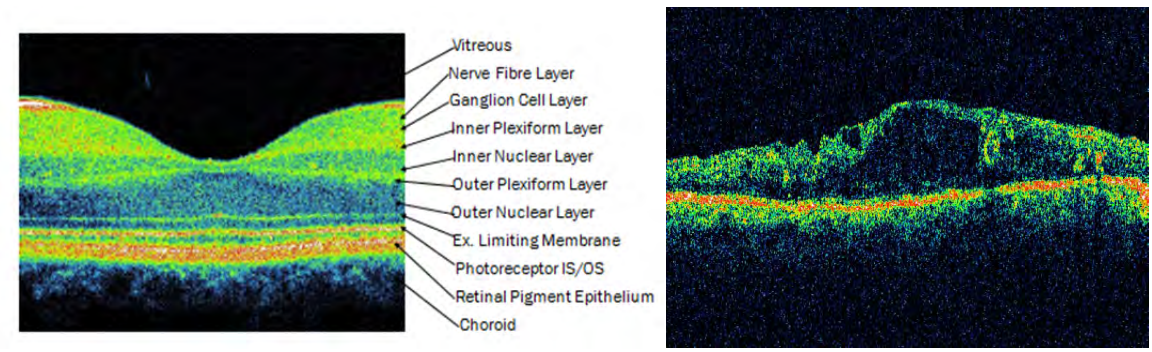


Figure 1-3: An example of OCT images.

Left side showing normal retina/macula with the anatomical layers. Right showing macular oedema.

1.2.2. Diabetic neuropathy

DN is the most common and difficult to treat complication of DM; resulting in great morbidity, mortality and significant economic burden (53;54). It is the most common form of neuropathy in the Western world and is the leading cause of non-traumatic amputations (53;54). The major morbidity of DN is the development of foot ulceration with subsequent increased risk of amputation (54).

DN can affect different aspects of the peripheral (diabetic peripheral neuropathy (DPN)) and the autonomic (diabetic autonomic neuropathy (DAN)) nervous systems. DPN and DAN often coexist(55). DPN is very common and the prevalence varies between 32% to 49% depending on the population examined and the methods used to diagnose it (56-60). DAN is also very common with a prevalence of 16-20% (55;61-64). Direct assessment of the cardiovascular sympathetic system, using radiolabeled analogues of norepinephrine ([123I] MIBG and [11C] HED), shows deficits of LV retention in subjects with T1DM or T2DM (65-69). Up to 40% of otherwise healthy patients with T1DM without deficits on cardiovascular reflex testing had abnormalities of [11C]HED retention affecting up to 8% of the left ventricle(67). The prevalence of DPN shows a significant correlation with age and diabetes duration (55;58;70;71).

DPN and DAN have a wide spectrum of manifestations and clinical features which reflect the heterogeneity of nerves affected and the widespread consequences of their dysfunction. The manifestations of DN can range from an imperceptible reduction in temperature perception in the feet to sudden cardiac death. Distal symmetrical sensorimotor polyneuropathy is the most common manifestation of diffuse DPN (72). Sensory deficits begin distally in the extremities and progress proximally resulting in the classical “stocking-glove” distribution (72). Initially, imperceptible loss of small nerve fibers can result in altered temperature perception, paresthesias, dysesthesias, and/or neuropathic pain (54). With progression of neuropathy large nerve fibers also become damaged which results in decreased light touch and proprioception sensations and ultimately muscle weakness (54). Painful DPN (PDPN) can affect up to 50% of patients with DPN and it is usually an

early manifestation of the disease (73). PDPN can have a significant impact on patients quality of life as it has a negative impact on sleep, functionality and mood (74). Although the pathophysiology of PDPN is not well understood, it is likely that abnormalities at multiple levels including small c-cell fibers, nerve roots, spinal cord and central nervous systems are likely to be involved (73). Other manifestations of DPN include polyradiculopathy, diabetic amyotrophy and mononeuritis multiplex (54). Similar to DPN, DAN has a wide variety of clinical manifestations. The earliest manifestations of peripheral autonomic neuropathy are likewise difficult to detect clinically, since they may be manifest solely as impaired peripheral vasomotor control, or decreased sudomotor function, which may progress to increased arterio-venous shunting (detectable by the presence of distended veins on the lower legs), severe oedema, neuroarthropathy (Charcot joints) and neuropathic ulceration. Cardiac involvement in DAN can results in reduced cardiovascular performance during exercise, impaired cardiac ejection fraction, abnormal systolic function, decreased diastolic filling, orthostatic hypotension and sudden cardiac death (54;75-78). DAN can also affect other systems including the gastrointestinal system (gastroperesis, diarrhea, constipation) and the genitourinary system (anaemia, erectile dysfunction, urinary incontinence) (54).

The contribution of hyperglycemia to the pathogenesis of microvascular complications, including DN, is beyond doubt (55;79;80). In addition, other metabolic factors, such as hypertension, dyslipidaemia and obesity are important in the development of DPN (55;81-83). The exact mechanisms by which these metabolic factors result in DN are uncertain but they follow common pathways described below.

DN is one of the most resistant complications to treatment. Good metabolic control remains the mainstay of DN treatment. Preventative measures against foot ulceration are also essential. There are several agents that are currently under development or in the early stages of clinical studies that are based on knowledge of the pathogenesis of DN, which hopefully will offer more effective therapy (84).

1.2.3. Diabetic nephropathy

Diabetes is the most common cause of end-stage renal disease (ESRD) (85) and about 40% of US adults with DM had some evidence of chronic kidney disease (CKD) (86). The presence of CKD in patients with DM also increases the annual cost of health care for those individuals by approximately 3 fold (86).

Diabetic nephropathy progresses slowly, starting with microalbuminuria, which develops into overt proteinuria in 20–40% of patients (85). About 20% of patients will have progressed to ESRD within 20 years after onset of overt proteinuria (85). The speed of progression from CKD is variable and largely dependent on BP and the degree of hyperglycaemia (87). Microalbuminuria occurs as a result of increased glomerular capillary pressure (85). As nephropathy advances, pore size of the glomerular basement membrane increases leading to proteinuria, followed by proliferation of mesangial cells precedes an increase in extracellular matrix and glomerular sclerosis resulting in worsening renal function (85).

Screening for diabetic nephropathy can be performed by measuring the albumin creatinine ratio (ACR) in random spot urine samples (86). Due to the variable nature of albumin excretion, two measurements (3 to 6 months apart) should be used before making the diagnosis of microalbuminuria to avoid false positives (such as following exercise, high salt diet, fever etc.) (86). Microalbuminuria is defined as the excretion of 30-299 mg/g creatinine (86). Assessment of microalbuminuria alone is not sufficient as patients with DM might develop severe kidney disease without the presence of microalbuminuria. Hence, assessment of estimated glomerular filtration rate (eGFR) alongside ACR is needed (86). CKD can be classified into 5 stages based on eGFR: ≥ 90 (stage 1), 60-89 (stage 2), 40-59 (stage 3A), 30-44 (stage 3B), 15-29 (stage 4), and <15 (stage 5) ml/min/1.73m^2 (www.renal org).

Diabetic nephropathy has several distinct phases starting with functional changes in the nephron at the level of the glomerulus, including glomerular hyper filtration and hyper perfusion, before the onset of any measurable clinical changes, followed thickening of the glomerular basement membrane, glomerular hypertrophy, and mesangial expansion take place (87). The pathogenesis of diabetic nephropathy is similar to other microvascular complications (discussed below). Haemodynamic changes occur as a result of the activation of various vasoactive systems, such as the renin–angiotensin–aldosterone (RAAS) and endothelin systems, and in response to the secretion of profibrotic cytokines, such as tissue growth factor β1 (TGF-β1), resulting in increased systemic and intra-glomerular pressure (87). In addition, hyperglycaemia induced OS and the activation of several metabolic pathways such as AGE, PKC and polyol results in the development of diabetic nephropathy (87).

The early haemodynamic changes result from decreased resistance in both the afferent and efferent arterioles of the glomerulus; with the afferent arteriole seems to have a greater decrease in resistance than the efferent, resulting in glomerular hyper perfusion and hyper filtration (87). Many factors have been involved in this defective auto regulation, including prostanoids, nitric oxide (NO), VEGF, TGF-1, and the RAAS (87). Blocking the RAAS has led to important clinical benefits in patients with diabetic nephropathy (87).

Another important factor in the early changes of diabetic nephropathy is changes in the protein nephrin. Nephrin, a protein found in podocytes, is crucial for maintaining the integrity of the intact dynamic filtration barrier (87). Patients with diabetic nephropathy have markedly reduced renal nephrin expression and increased nephrin excretion suggesting that nephrin excretion could be an early finding of podocyte injury (even before the onset of albuminuria) (87). Interestingly, RAAS blockers increase the low nephrin levels in patients with diabetes to those similar to control (87).

The role of genetics in pathogenesis of diabetic microvascular complications seems particularly important in the case of diabetic nephropathy (88). Abnormalities in eGFR and albuminuria

aggregate in families and several genes were identified using candidate genes approach and whole genome association studies (88). Genetic factors might contribute the ethnic difference described later in this chapter, but this issue has not been investigated in depth yet.

Strict metabolic control and the use of RAAS blockers form the fundamental part of managing patients with diabetic nephropathy (detailed below). Other lifestyle changes such as stopping smoking, weight loss and dietary modifications (low protein, low salt diet) have also been shown to be of benefit (86).

1.2.4. Pathogenesis

The development of microvascular complications is multi-factorial and there are common mechanisms to all the different complications, although three are also complications-specific mechanisms. Here, we concentrate on the common pathways that result in the development of microvascular complications. In summary, the main putative mechanism is that hyperglycaemia results in OS that results in the activation of multiple pathways which results in tissue, cellular and microvascular damage and further worsening of OS **(Figure 1.4)**.

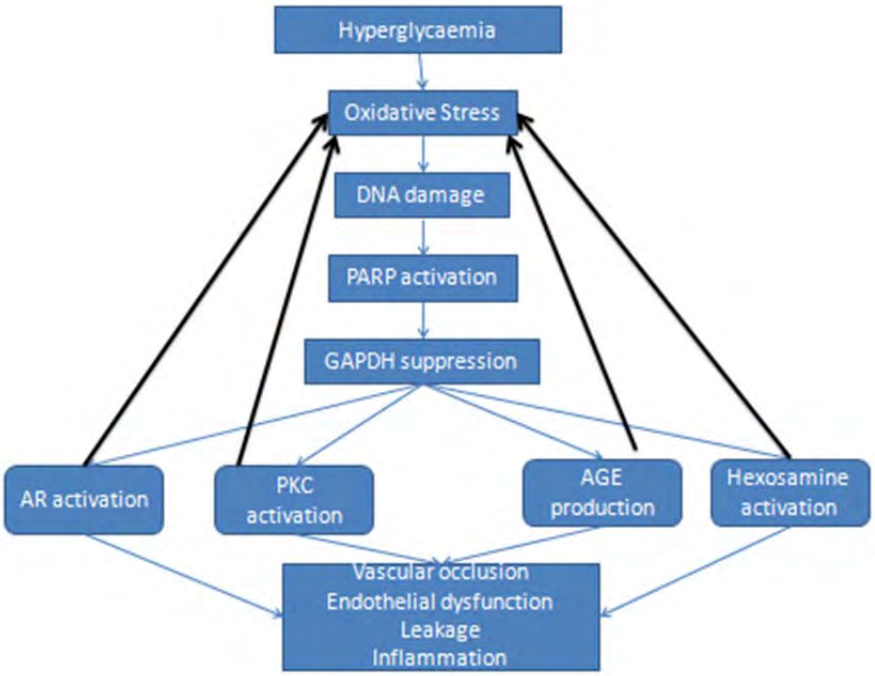


Figure 1-4: Summary of the mechanisms that relate hyperglycaemia to microvascular complications in patients with diabetes.

AR: Aldose Reductase, PK: Protein Kinase, AGE: Advanced Glycation End-products, PARP: poly(ADP-ribose) polymerase , GAPDH: glyceraldehyde-3 phosphate dehydrogenase.

1.2.4.1. Advanced glycation end-products

AGE can arise from intracellular oxidation of glucose to glyoxal, decomposition of 1-amino-1-deoxyfructose lysine adducts to 3-deoxyglucosone, and fragmentation of glyceraldehyde-3-phosphate and dihydroxyacetone phosphate to methylglyoxal (89). These three intracellular di-carbonyls react with amino groups of intracellular and extracellular proteins to form AGE.

AGE produce intracellular damage by different mechanisms **(Figure 1.5)** (90). AGE modify intracellular proteins, such as fibroblast growth factor, proteins involved in endocytosis and more importantly proteins involved in the regulation of gene transcription (89;90). AGE precursors can also diffuse out of the cell and modify extracellular matrix (such as types 1 and 4 collagen), which changes signalling between the matrix and the cell and causes cellular dysfunction (89-92). In

addition, AGE precursors diffuse out of the cell and modify circulating proteins in the blood such as albumin; which then bind to receptor of AGE (RAGE) causing the production of inflammatory cytokines, growth factors and adhesion molecules such as interleukin-1, insulin-like growth factor-1, platelet derived growth factor, tumour necrosis factor-α (TNF-α), TGF- β and vascular cell adhesion molecule-1 (VCAM-1) (89;90;93-95). Furthermore, AGE can stimulate the PKC pathway(89).

Intracellular hyperglycaemia is the primary event in AGE formation (89;96) and DM has a well-established association with raised AGE (97;98). Many of the therapeutic agents that are used in patients with T2DM lower AGE levels in vitro and in animal studies including ACE inhibitors, angiotensin-II blockers (99), thiazolidinediones (100), aspirin (101) and metformin (102). Animal trials showed that Inhibition of AGE production prevents the development and/or progression of the various microvascular complications (89;103-106).

In a recent study of experimental DN using streptozotocin (STZ)-diabetic mice epidermal axons, sural axons, Schwann cells, and sensory neurons within ganglia developed cumulative increases (in relation to diabetes duration) in RAGE mRNA along with progressive electrophysiological and structural changes which were milder in the $RAGE^{-/-}$ control (107); suggesting that AGE/RAGE are important in the development of DN but are not the sole mechanism. In addition, AGE were found to modify peripheral nerve myelin which made it susceptible to phagocytosis and resulted in segmental demyelination (108). AGE were also implicated in modifying the axonal cytoskeletal proteins (tubulin, neurofilament, and actin) resulting in axonal atrophy/degeneration (108). Furthermore, the glycation of extracellular matrix protein laminin also leads to impaired regenerative activity in DN (108). The interaction between AGE and RAGE might also affect the endo-neural vascular function as RAGE were found to be expressed in endothelial and Schwann cells (109). The use of AGE inhibitor (aminoguanidine) in diabetic rat models had beneficial effects on the development of retinopathy, nephropathy and neuropathy; particularly improvements in nerve conduction velocities and morphometric variables (110;111).

In humans, AGE levels were higher in patients with DM compare to control and correlated with HbA1c levels (112;113). AGE levels were also related to the development and severity of microvascular complications in humans with DM (114). In addition, serum AGE levels were related to the progression from micro- to macro-albuminuria and overt nephropathy (114). Furthermore, skin expression of AGE was shown to be an independent predictor or microvascular complications in the Diabetes Complications and Control Trial (DCCT) cohort (115). In fact, in the later study, AGE skin expression was better predictor of microvascular complications than HbA1c in the in the conventional treatment arm (115). Similar results were found in patients with T2DM, as serum AGE levels were significantly associated with the severity of retinopathy (116).

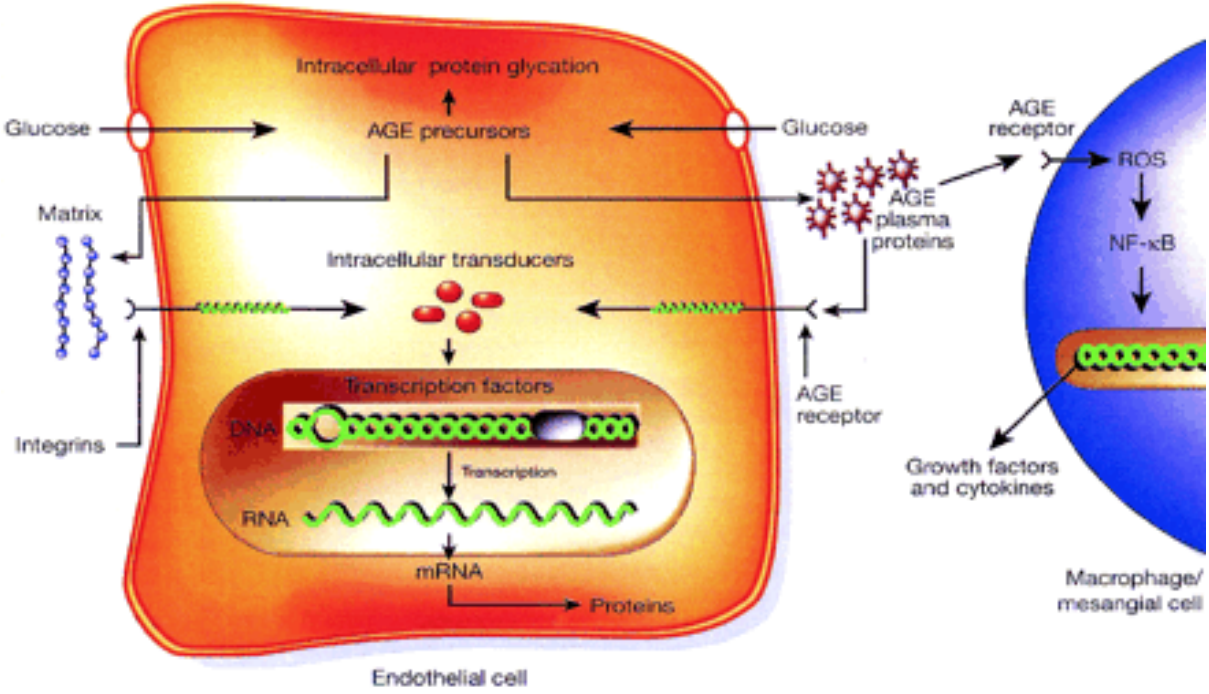


Figure 1-5: Mechanisms by which intracellular production of advanced glycation end-product (AGE) precursors damages vascular cells.

Covalent modification of intracellular proteins by dicarbonyl AGE precursors alters several cellular functions. Modification of extracellular matrix proteins causes abnormal interactions with other matrix proteins and with integrins. Modification of plasma proteins by AGE precursors creates ligands that bind to AGE receptors, inducing changes in gene expression in endothelial cells, mesangial cells and macrophages.

1.2.4.2. Protein Kinase C (PKC)

The PKC family consists of 10 isoforms (117). Intracellular hyperglycaemia results in an increased synthesis of diacylglycerol (DAG), which is a critical activating cofactor for PKC (90). PKC activation results in a variety of effects on gene expression resulting in decreased production of endothelial nitric oxide synthase (eNOS), increased endothelin-1, increased TGF- β, VEGF and increased plasminogen activator inhibitor-1 (**Figure 1.6**) (90). These changes are associated with vascular occlusion and increased endothelial permeability resulting in tissue damage (90). In addition, PKC activation results in increased NF-KB (which leads to pro-inflammatory gene expression) and increased NADPH oxidase resulting in OS (90).

Early animal studies have shown that PKC inhibition can prevent the development diabetic retinopathy and nephropathy (90;118;119). PKC inhibition (using LY333531) reverse the defects caused by an 8-week period of STZ-diabetic rats including deficits in sural nerve conduction velocity (SNCV), sciatic nerve and superior cervical ganglion blood flow and vascular responses in the mesenteric vascular bed (120). More recently, ruboxistaurin (a PKC inhibitor) has been shown to have a beneficial effect on vision, the progression of macular oedema and albuminuria levels in patients with T2DM (121-124).

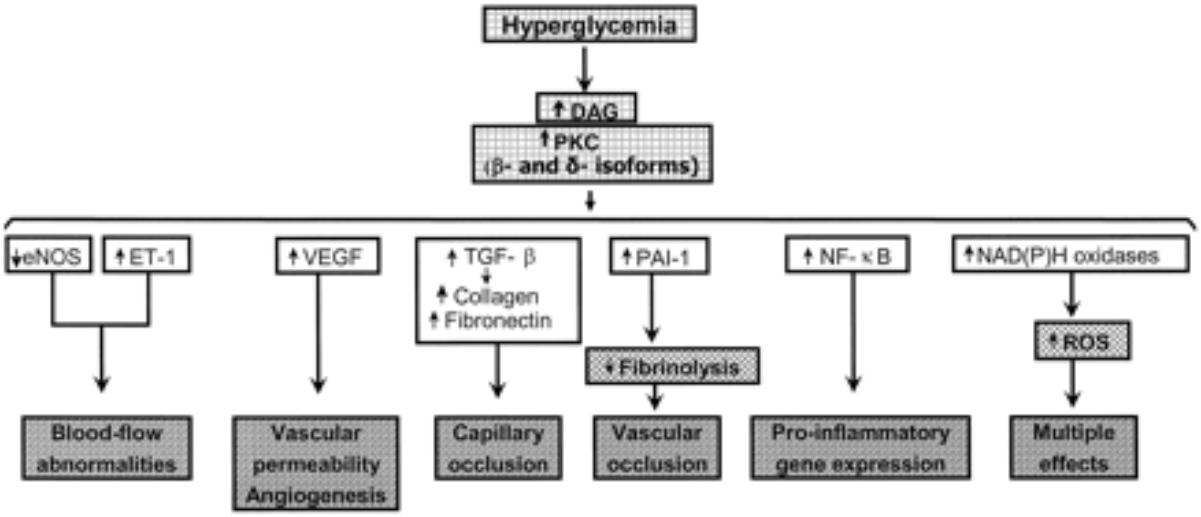


Figure 1-6: Consequences of hyperglycaemia-induced activation of protein kinase C (PKC).

Hyperglycaemia increases diacylglycerol (DAG) content, which activates PKC, primarily the b- and d-isoforms. Activation of PKC has a number of pathogenic consequences by affecting expression of endothelial nitric oxide synthase (eNOS), endothelin-1 (ET-1), vascular endothelial growth factor (VEGF), tissue growth factor-b (TGF-b) and plasminogen activator inhibitor-1 (PAI-1), and by activating NF-kB and NAD(P)H oxidases.

1.2.4.3. Polyol pathway

This pathway is the first of the mechanisms of microvascular complications to be described (89;125). Aldose reductase (AR), which is the first enzyme in the polyol pathway, catalyses NADPH-dependant reduction of a wide range of carbonyl compounds including glucose(**Figure 1.7**) (89). In patients without diabetes, only a very small proportion of glucose get metabolised via this pathway as AR has low affinity to glucose at normal concentrations (89). In the presence of hyperglycaemia, however, increasing amounts of glucose will be metabolised by this enzyme to sorbitol which is in turn metabolised to fructose by the enzyme sorbitol dehydrogenase (SDH) (89). SDH reduces NAD^+ to NADPH during this process (89). The oxidation of sorbitol by NAD^+ increases the cytosolic NADH:NAD^+ ratio, which results in inhibiting the enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) resulting in increasing concentrations of triose phosphate (89;126). Elevated triose phosphate levels could increase formation of AGE and DAG, thus activating PKC(89). The increase in NADH:NAD^+ ratio in hyperglycaemia is as a result of a marked decrease in the

absolute concentration of NAD^+ (as a result of consumption by activated poly(ADP-ribose) polymerase (PARP)), rather than reduction of NAD^+ to NADH (89;127). Activation of PARP is mediated by increased production of reactive oxygen species (ROS) and OS (89). Furthermore, activation of the polyol pathway exacerbates OS by causing reduction in the antioxidant defense system such as glutathione (GSH), taurine and myo-inositol (89;128).

The use of AR inhibitors has been shown to reduce or alleviate thermal hypoalgesia in STZ-rats and Ob/Ob mice (73) and to restore the low taurine transporter levels caused by hyperglycaemia in Schwann cells (129) confirming the important role for this pathway in the development of DN.

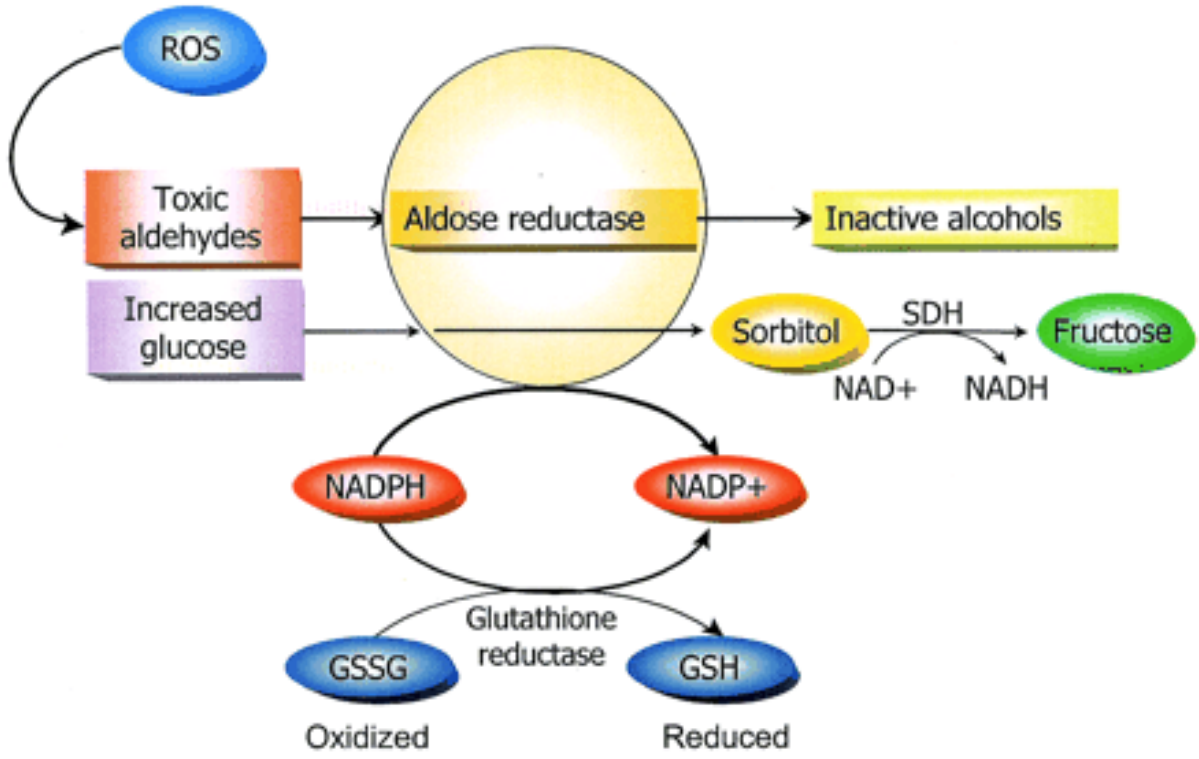


Figure 1-7: The polyol pathway.

Aldose reductase reduces toxic aldehydes produced by ROS into inactive alcohols and glucose into sorbitol using the cofactor NADPH, which is oxidised to NADP+. Sorbitol dehydrogenase (SDH) oxidises sorbitol into fructose using NAD+ as a cofactor. In hyperglycaemia, increased polyol pathway flux increases intracellular accumulation of sorbitol as well as fructose, whilst depleting NAD+ and NADPH, the latter of which is required for the regeneration of GSH.

1.2.4.4. Hexosamine Pathway

In cases of intracellular hyperglycaemia, most glucose is metabolized via glycolysis, to glucose-6 phosphate, then fructose-6 phosphate, and then through the rest of the glycolytic pathway (90). Some of that fructose-6-phosphate, however, gets converted to glucosamine-6 phosphate via an enzyme called glutamine:fructose-6 phosphate amidotransferase (GAFT) and finally to UDP (uridine diphosphate) *N*-acetyl glucosamine (90). *N*-acetyl glucosamine results in gene expression (90). For example, increased modification of the transcription factor Sp1 results in increased expression of PAI-1 which is involved in the development of microvascular complications.

1.2.4.5. Oxidative stress

The term OS refers to the situation of a serious imbalance between the production of free radicals and the antioxidant defense mechanisms, leading to potential tissue damage (130). Free radical species are a variety of highly reactive molecules that can be divided into different ROS, reactive nitrogen species (RNS) and reactive chlorine species (RCS). A common feature of cells that are damaged by hyperglycaemia is the presence of ROS/RNS causing OS (131;132).

There are four protein complexes (I, II, III, and IV) in the mitochondrial electron transport chain (90). Glucose metabolism through the tricarboxylic acid cycle (TCAC), generates electron donors (90). The main electron donors are NADH, which gives electrons to complex I, and $FADH_2$, which donates electrons to complex II (90). These electrons are passed to coenzyme Q, and then transferred to complex III, cytochrome-C, complex IV, and finally to molecular oxygen, which they reduce to water (90). Throughout the electron transport system ATP levels are precisely regulated (90). As electrons are transported some of the energy of those electrons is used to pump protons across the membrane at complexes I, III, and IV, which generates a voltage across the mitochondrial membrane (90). The energy from this voltage gradient drives the synthesis of ATP by ATP synthase;

alternatively, uncoupling proteins (UCPs) can move down the voltage gradient to generate heat to keep the rate of ATP generation constant (90).

In hyperglycaemia, there is more glucose being oxidized in the TCAC, which pushes more electron donors into the electron transport chain which results in the voltage gradient increase across the mitochondrial membrane (90;133) until a critical threshold is reached (90). At this point, electron transfer is blocked resulting in the back up of electrons generating superoxide which is degraded to hydrogen peroxide (which is then converted to H2O and O2) by the enzyme superoxide dismutase (SOD) (**Figure 1.8**) (90).

In experimental studies, abolishing the voltage gradient by using uncoupling protein-1 (UCP-1) results in lack of ROS production in hyperglycaemia (90;132). Similarly, hyperglycaemia does not increase ROS when superoxide is degraded by over-expressing the enzyme manganese SOD (MnSOD)(90). In endothelial cells that are deprived of mitochindrial DNA (ρ^0), the impact of hyperglycaemia on ROS production was completely lost (90). Similarly, in ρ^0 endothelial cells, hyperglycaemia completely fails to activate the polyol, PKC, and hexosamine pathways or AGE formation (90). Inhibiting of ROS production and normalising mitochondrial ROS levels prevents the activation of the AGE, PKC and polyol pathways by glucose (132). This suggests that diabetes-induced ROS and OS are important in stimulating the AGE, PKC and polyol pathways which results in the development of microvascular complications; although these same pathways also increase ROS production and OS.

All these factors suggest a crucial role for hyperglycaemia in the production of ROS and the role of ROS in activating the pathways that lead to microvascular complications. However, how does ROS activate those pathways? It was proposed that the key glycolytic enzyme GADPH plays an important role (90). This is based on the observation that in patients and animals with diabetes, the activity of GAPDH is reduced and that inhibition of GAPDH does not occur when ROS production is prevented by UCP-1 or MnSOD (90;131). When GAPDH activity is inhibited, the level of all the glycolytic

intermediates that is upstream of GAPDH increase, resulting in activation of the AGE and PKC pathways because the methylglyoxal (an AGE precursor) and DAG (a PKC activator) are formed from glyceraldehyde-3 phosphate. In addition, the levels of the glycolytic metabolite fructose-6 phosphate increase, which activates the hexosamine pathway (**Figure 1.9**)(90). Reduction in GADPH activity also increases intra-cellular glucose levels which activate the polyol pathway.

In addition to the excess in superoxide production, hyperglycaemia results in reduction in the antioxidant defense system such as GSH, vitamin E, vitamin C, alpha lipoic acid (ALA), and taurine amongst others (134). These antioxidants protect tissues from free radical damage, and are recycled or regenerated (134). GSH is by far the most important antioxidant in most mammalian cells. Hyperglycaemia induces GSH depletion and impairs GSH regeneration; GSH depletion has been linked to the development of diabetes complications including DN (135). Taurine is a β-amino acid (2-aminoethanesulfonic acid) with antioxidant properties (136;137). Taurine depletion is an important mediator of glucotoxicity and OS in peripheral nerves and other tissue (136;137). Nerve taurine replacement ameliorates deficits in nerve blood flow, NCV, and OS in experimental DN and counteracts OS (138;139). Furthermore, hyperglycaemia reduces the expression of taurine transporter in Schwann cells which is reversed by the use of antioxidants (129). The role of antioxidants depletion is summarized in **Figure 1.10.**

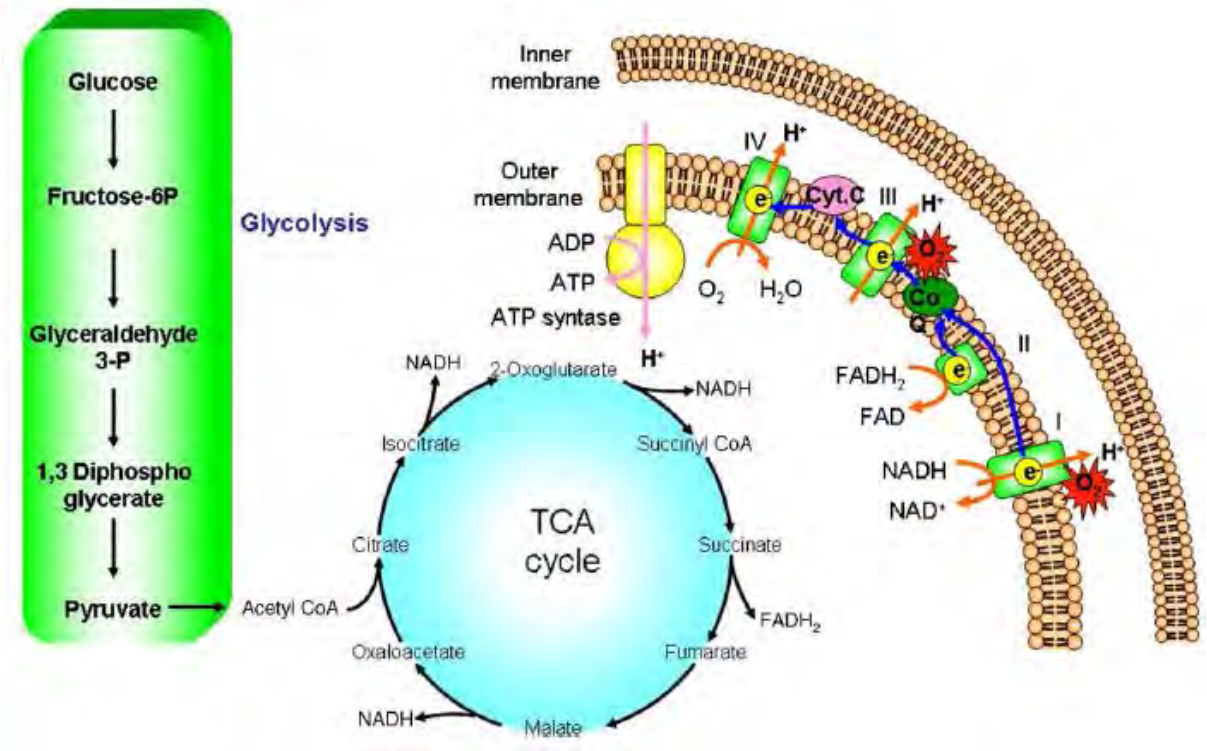


Figure 1-8: Production of superoxide by the mitochondrial electron-transport chain.

Increased hyperglycaemia-derived electron donors from the TCA cycle (NADH and FADH2) generate a high mitochondrial membrane potential (DmH+) by pumping protons across the mitochondrial inner membrane. This inhibits electron transport at complex III, increasing the half-life of free-radical intermediates of coenzyme Q (ubiquinone), which reduce O2 to superoxide

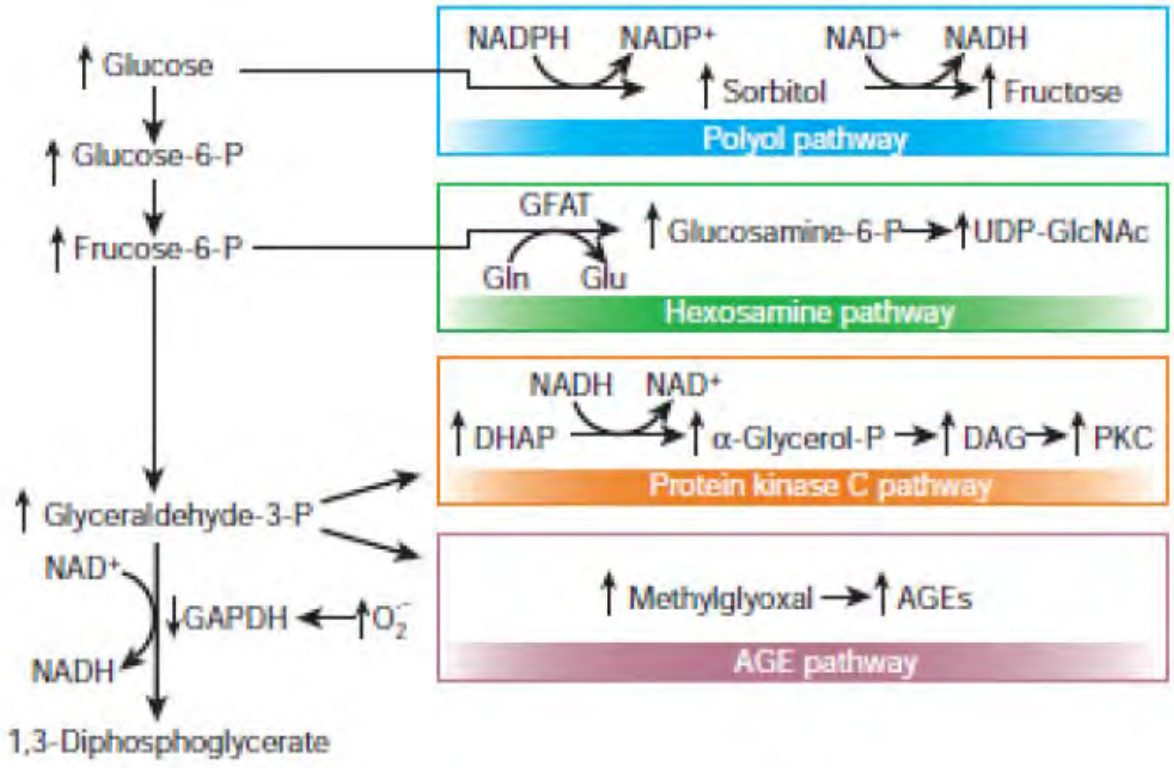


Figure 1-9: Potential mechanism by which hyperglycaemia-induced mitochondrial superoxide overproduction activates four pathways of hyperglycaemic damage.

Excess superoxide partially inhibits the glycolytic enzyme GAPDH, thereby diverting upstream metabolites from glycolysis into pathways of glucose overutilization. This results in increased flux of dihydroxyacetone phosphate (DHAP) to DAG, an activator of PKC, and of triose phosphates to methylglyoxal, the main intracellular AGE precursor. Increased flux of fructose-6-phosphate to UDP-N-acetylglucosamine increases modification of proteins by O-linked N-acetylglucosamine (GlcNAc) and increased glucose flux through the polyol pathway consumes NADPH and depletes GSH

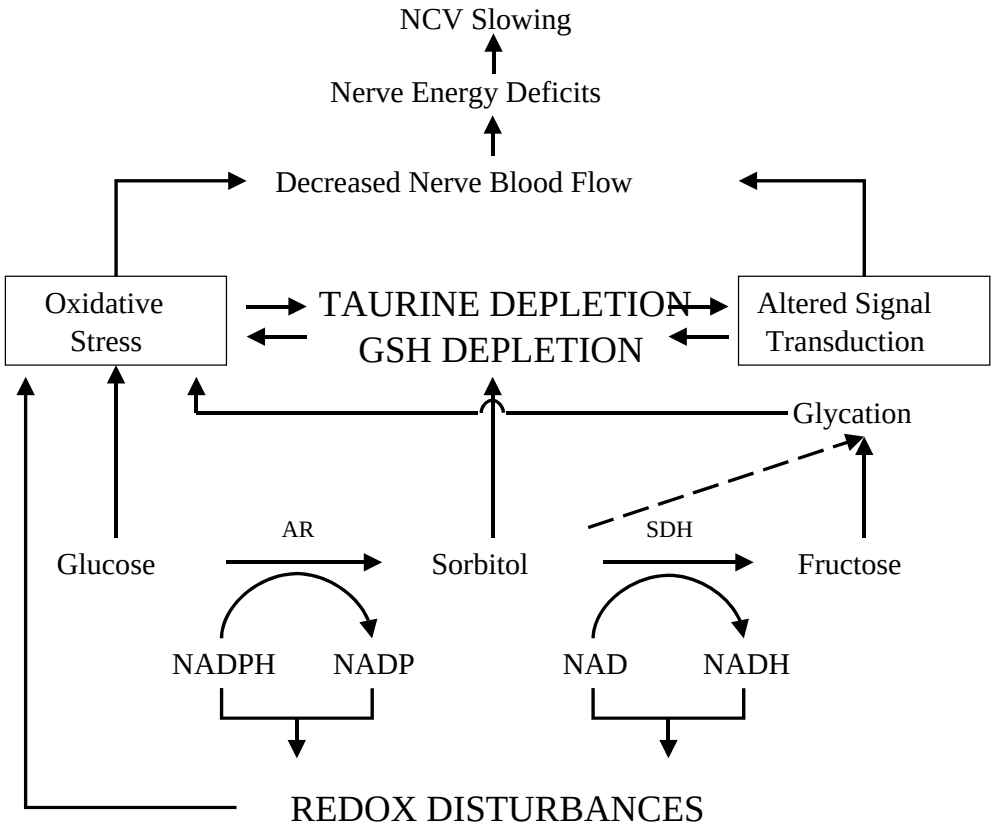


Figure 1-10: The role of antioxidants and potential mechanisms whereby activation of the aldose reductase pathway may exacerbate oxidative stress.

Activation of the aldose reductase (AR) pathway consumes NADPH and provokes intracellular sorbitol accumulation resulting in osmotic stress and the compensatory depletion of the organic osmolytes taurine and myo-inositol and perhaps depletion of reduced glutatione. In turn, this will promote oxidative stress and alterations in signal transduction pathways. Sorbitol oxidation by sorbitol dehydrogenase (SDH) promotes the formation of NADH ("pseudohypoxia") and leads to the formation of fructose which is a potent glycosylator. Increased oxidative stress results in disruption of vasoactive agents and reduced nerve blood flow, which compromises mitochondrial function, leading to nerve energy deficits and nerve conduction velocity (NCV) slowing.

1.2.4.6. Polymers of ADP-ribose polymerase (PARP)

As mentioned previously, GAPDH inhibition by ROS is an important step in activating the pathways leading to microvascular complications. However, experimentally, ROS can inhibit GADPH activity only at concentrations higher than those found in patients with DM, hence a different mechanism of GADPH inhibition was sought (90).

Poly(ADP-ribosyl)ation is the process by which polymers of ADP-ribose (PAR) are attached via an ester bond to glutamic acid, aspartic acid or lysine residues, mediated by the enzyme PARP (140). There are currently 18 known members of the PARP family, two of which, PARP1 and 2 are known to play a role in DNA repair (141). PARP1 binds as a homodimer to single-strand DNA breaks where it is activated and catalyses the cleavage of NAD+ forming nicotinamide and ADP-ribose, the polymers of which are added to nuclear proteins (142;143). Increased OS results in DNA damage and PARP1 activation (144-146). Although PARP1 plays a beneficial role in DNA repair, it is possible that hyper activation in diabetes leads to detrimental effects (143;146). Excess cleavage of NAD+ by PARP, would exacerbate the effect of increased flux through SDH which results in depleting NAD+ further, leading to OS (146). In addition NAD+ is required as a cofactor for the conversion of GAPDH. Hyperglycaemia-induced ROS inhibits GAPDH activity in vivo by modifying the enzyme with PARP (90;147-149). In summary, Increased ROS by hyperglycaemia results in breaks in DNA strands, hence activating PARP which results in increased PAR production which inhibits GADPH activity (90).

In models of diabetes increased PAR and PARP-1 activation is corrected by AR inhibitors(150). In addition PARP inhibition reduces OS and inducible NOS (iNOS) expression in high glucose-treated human Schwann cells (151) as well as improving thermal hypoalgesia, mechanical hyperalgesia, nerve conductivity and restoring IENF loss in animal models (150;152;153); which suggests an important role for PARP in the development of DN.

1.2.4.7. Cycloxygenase pathway

Prostaglandins are generated by cyclo-oxygenase (COX) from arachidonic acid. Two isoforms of the enzyme have been isolated in mammalian cells (154). COX-1 is constitutively expressed in most tissues and is involved in maintenance of cellular homeostasis, including regulation of vascular tone (154). In contrast, COX-2 is up-regulated by inflammatory, mitogenic, physical stimuli, and OS (155;156).Increased ROS production secondary to hyperglycaemia results in COX-2 mRNA induction and COX-2 protein expression (157;158). COX- 2 leads increased production of PGH_2, TXA2, and

$PGF_{2\alpha}$ and reduction in PGI_2, favouring vasoconstriction and ischemia (159). Selective COX-2 inhibition or COX-2 gene inactivation results in preventing deficits of NCV and nerve blood flow in experimental diabetes rats (160;161).

1.2.4.8. Na/K ATP channels

The Na/K ATPase is a ubiquitously expressed membrane pump that utilises ATP to export three Na^+ ions and import 2 K^+ ions (162). The Na^+ gradient across membrane is important for nerve impulses to travel and for the transport of molecules such as myoinositol and taurine. OS reduces the Na/K ATPase activity which could contribute to DN (162;163). Taurine, probably by an anti-oxidant mechanism, restores Na/K ATPase activity in the nerve of STZ-diabetic rats (164).

1.2.4.9. Mitogen activated protein kinases (MAPKs)

MAPKs are a group of serine/threonine kinases that are activated by phosphorylation in response to extracellular stimuli. There are three main groups of MAPKs: p38 MAPK, extracellular signal-regulated kinases (ERK, also known as p42/44 MAPK) and c-Jun N-terminal kinase/ stress activated protein kinase (JNK/SAPK, also known as p46/54 MAPK) (165). In Schwann cells, p42/44 MAPK activation is required for survival and proliferation as well as synthesis of growth factors such as nerve growth factor (NGF) (166). The p38 MAPK and JNK/SAPK mediate cellular stress as they are activated in response to oxidative and osmotic stress (167;168).

In DM, MAPKs have been seen as the transducers between hyperglycaemia and the biochemical stress in diabetic complications. Increases in p38 MAPK and JNK/SAPK phosphorylation have been observed in dorsal root ganglia (DRG) (169) and sciatic nerve (170) of STZ-diabetic rats as well as in hyperglycaemia-treated immortalized Schwann cells (171) and sural nerves from patients with end-stage DN undergoing lower-limb amputations (169). There is also an association between activation of p38 MAPK and JNK/SAPK and chronic pain in PDPN (172). In addition, P38 MAPK is a mediator of OS and is thought to be involved in AR regulation (173;174). AR inhibition reduces p38 activation and

specific inhibition of p38 MAPK also prevents the diabetes-induced reduction in motor and sensory NCV (170).

1.2.4.10. Lipids

Although the relation between lipids and macrovascular complications in patients with DM has been well recognised for a long time, such a relation between lipids and microvascular complications has only come to light recently. Oxidised LDL (oxLDL) is the product of the reaction between LDL and ROS. The proportion of LDL which is oxidised (oxLDL/apoB ratio) is associated with DN (175;176). OxLDL is cytotoxic and has been involved is endothelial dysfunction (176). OxLDLs exert effects on cells through lectin-like oxidized LDL receptor-1 (LOX-1) receptor on endothelial cells and CD36 on macrophages (176). Interestingly, LOX-1 expression is increased by hyperglycaemia and AGE and decreased by antioxidants, which suggest a synergistic role between glucose and lipids in producing cellular injury (176).

As mentioned above, the EDIC showed that the impact of intensive glycaemic control of the incidence of DPN persisted many years after the end of the DCCT despite that there was no difference in glycaemic control between the intensive and conventional arms after the end of DCCT. The exact mechanism of this is not clear but there were differences in the lipids between the conventional and intensive arms (177). Furthermore, in the EURODIAB study, serum lipids were an independent risk factor for the development of DN (81;82). Elevated triglyceride levels were also shown to be independently associated with Sural nerve myelinated fibre density after adjusting for drug history, diabetes duration, age and glycaemic control (178). These studies highlight the important role for lipids in the development of DN and that hyperglycaemia is not the only factor involved. This is supported further by the UKPDS and other studies in which there was no difference in the development of DN despite the presence of differences in glycaemic control between the intensive and conventional arms but there were no differences in lipids (176).

Total cholesterol, LDL, HDL and triglycerides have also been linked to the development of micro and frank albuminuria in cross-sectional studies and diabetic retinopathy in prospective studies (85).

1.2.5. Treatment of microvascular complications

The mainstay of the treatment of diabetes-related microvascular complications is intensive control of glucose, blood pressure and lipids.

Hyperglycaemia is probably the most important driving factor in the development of diabetes-related microvascular complications; in fact the cut-offs for glucose levels that define DM are chosen because microvascular complications are unlikely to occur under these levels (179). The importance of hyperglycaemia in the pathogenesis of microvascular complications has been emphasised further as interventional studies have shown that improvements in glycaemic control result in significant reduction in the risk of development and progression of microvascular complications. The DCCT examined whether intensive glucose control in patients with T1DM reduces the frequency and severity of microvascular complications (180). The intensive control group achieved lower HbA1c levels than the conventional control group (8.6 ±1.7 vs. 12.8 ±3.1 mmol/l respectively, $p<0.001$) and patients were followed up for a mean of 6.5 years(180). Intensive therapy reduced the adjusted mean risk for the development of retinopathy by 76% compared to conventional therapy (180). Intensive therapy has also slowed the progression of retinopathy by 54% and reduced the development of proliferative or severe non-proliferative retinopathy by 47% (180). In regard to nephropathy, intensive therapy reduced the presence of microalbuminuria by 39%, and that of albuminuria by 54% (180). The UKPDS has shown that a mean difference of 0.9% between the intensive and conventional treatment groups (over 10 years) resulted in 25% risk reduction in microvascular endpoints. Ten years following the end of the randomised intervention, this reduction in the microvascular complications endpoint persisted (24%, $p=0.001$)(181).

Similar to hyperglycaemia, there is a strong evidence to support the role of hypertension in the development of microvascular complications. The UKPDS 36, a prospective observational study aimed to determine the relation between systolic BP and the risk of macrovascular or microvascular complications in patients with T2DM, included 4801 patients who were randomised to either treatment or no treatment (182). For each 10 mmHg decrease in mean systolic blood pressure was associated with 13% reduction in the risk for microvascular complications (182). In the UKPDS 38, 1148 patients with T2DM were randomised to tight control of blood pressure (target blood pressure <150/85 mmHg) or less tight control aiming at a blood pressure of <180/105 mm Hg (183). In the tight control group there was 37% reduction in microvascular end points (183). In addition, after 9 years of follow up the group assigned to tight blood pressure control had a 34% reduction in risk of retinopathy deterioration by two steps and a 47% reduced risk of deterioration in visual acuity by three lines of the early treatment of diabetic retinopathy study (ETDRS) chart (183). The UKPDS-75 examined the impact of combined tight blood pressure and glucose control on the risk of diabetic complications over time in 4,320 newly diagnosed patients with T2DM (184). The relative risk of microvascular complications for the highest vs. the lowest HbA1c and blood pressure was 16.3 (95%CI: 7.3-36.1). Each 10mmHg reduction in systolic blood pressure was associated with 10% reduction in risk of microvascular disease(184).

The evidence and impact of hyperlipidaemia management on microvascular complications is rather limited. In a meta-analysis of 12 studies (7 of which were studies in patients with diabetes), the rate of decline in GFR was lower with statins compared with controls (185). The impact of statins on retinopathy and neuropathy is limited to very small studies that showing such benefit (85). There is, however, evidence that lowering triglycerides using fibrates has beneficial effect on neuropathy and retinopathy. In a subgroup of the Fremantle Diabetes Study (186), baseline fibrate use was lower in patients with DPN (4.7% vs. 1%, p<0.01)(186). Longitudinally, fibrate use (HR 0.52, 95%CI: 0.27–0.98] and statin use (HR 0.65, 95%CI: 0.46–0.93) were significant determinants of incident neuropathy (186). In retinopathy, two land mark studies showed a favourable impact of fibrates on

the progression of diabetic retinopathy, although this was not the primary outcome of these studies. In the ACCORD trial, in which 10215 participants were followed up for 4 years, fibrate use was protective against the progression of retinopathy (adjusted odds ratio, 0.60; 95% CI, 0.42 to 0.87; P=0.006)(187). In the FIELD study, in which 9800 were randomised into either fenofibrate or placebo, the requirement for first laser treatment for all retinopathy was significantly lower in the fenofibrate group than in the placebo group (absolute risk reduction 1.5% [0.7-2.3])(188). In the ophthalmology sub study, the primary endpoint of 2-step progression of retinopathy grade did not differ significantly between the two groups overall (46 [9.6%] patients on fenofibrate vs. 57 [12.3%] on placebo; p=0.19) or in the subset of patients without pre-existing retinopathy (43 [11.4%] vs. 43 [11.7%]; p=0.87)(188).

Angiotensin converting enzyme inhibitors (ACEi) have been shown to have a beneficial impact on microvascular complications. Treatment with ACEi improved neural and vascular dysfunction in STZ-diabetic rats and in patients with T2DM (189;190). ACEi have also shown to slow the decline in glomerular filtration rate(191). Similarly the MICRO-HOPE trial showed a 16% RRR in overt nephropathy and laser treatment (192).

Despite our current understanding of the pathogenesis of microvascular complications and despite good metabolic control, microvascular complications remain very common and important cause of morbidity and mortality in patients with diabetes. Hence, it is important to further our understanding of the pathogenesis of these complications to develop new effective treatments.

1.3. Ethnicity and Type 2 Diabetes

Exploring ethnic differences in relation to diabetes, obesity and vascular complications offer an opportunity to further our understanding of the pathogenesis of these conditions. In this chapter, I will focus mainly on the differences between White Europeans and South Asians, as these two ethnicities are relevant to my project.

1.3.1. Ethnicity and obesity

Obesity is a very common public health challenge. Ethnicity has a major impact on obesity prevalence and body fat distribution and it affects the relation between obesity and its complications.

Compared to White Europeans, South Asians seem to have a higher cardiovascular disease risk and higher prevalence of diabetes, hypertension and hyperlipidaemia at lower BMI values (193;194). In fact, South Asians have higher body fat percentages when compared to White Europeans despite having a similar BMI (195). Hence, international guidelines have recommended lower cut offs to diagnose overweight/obesity in South Asians compared to White Europeans based on BMI and waist circumference (196).

The prevalence of obesity in South Asians in the UK is 15%, 38% and 30% based on BMI ≥ 30 kg/m^2, waist hip ratio ≥ 0.95 and waist circumference ≥ 102 cm respectively (197). This is higher than the prevalence of obesity in England (approximately 24% based on BMI) (197). Furthermore, the cut-offs used to quote the above prevalence in South Asians are those for White Europeans and are not ethnicity specific. Using the lower ethnicity specific cut offs will results in even a higher prevalence of obesity amongst South Asians compared to White Europeans. Unpublished data from the United Kingdom Asian Diabetes Study (UKADS) showed that South Asians with type 2 diabetes had a much higher prevalence of obesity than White Europeans using ethnicity specific cut-offs (77% vs. 53% based on BMI and 99% vs. 87% based on waist circumference) (personal communication). This higher prevalence of obesity was despite that the South Asians had lower absolute BMI and waist circumference values.

1.3.2. Ethnicity and type 2 diabetes

Data from the Health Survey of England 2004 show that South Asians have a higher standardised risk of having diabetes compared to the general population (197). Pakistani women are over five times more likely than women in the general population to be diagnosed with diabetes (197). South Asian men are 3-4 times more likely to have diabetes compared to men in the general population (197). This tendency to have more diabetes in South Asians is even evident from the first decade of life(198). Even in patients without diabetes, South Asians exhibits more marked abnormalities in glucose metabolism (such as HOMA-IR, 2-hour post-load glucose levels, fasting glucose levels) than White Europeans, even thought the South Asians have lower adiposity measures (193). Based on glucose metabolism data, a BMI of 30 kg/m^2 in White Europeans was equivalent to that of 21 kg/m^2 in South Asians without diabetes (193); which further emphasise that South Asians are at much higher risk of obesity and its complications.

1.3.3. Ethnicity and Macrovascular Disease

Data regarding the impact of ethnicity on cardiovascular disease in patients with T2DM is very limited. However, there is a wealth of evidence in patients without diabetes that showed that South Asians are at higher risk of cardiovascular disease at younger age and lower adiposity compared to White Europeans (199). Furthermore, South Asians had higher mortality from coronary artery disease compared to White Europeans (199).

In South Asians men in the UK, the rates of ischaemic heart disease are 30–40% higher amongst than those in general population (194). The age-standardised mortality rate from coronary heart disease was also 50% higher in South Asians compared to the general population in England (194). Women are proportionally more affected than men. Similar to coronary artery disease, South Asians were found to develop cerebro-vascular disease at younger age and it was associated with 40% higher mortality compared to White Europeans (199;200). Unlike coronary and cerebro-vascular disease,

peripheral vascular disease seems to be less common in South Asians compared to White Europeans (with or without diabetes)(199;201;202).Interestingly, the prevalence of coronary artery disease was not different between South Asians with or without peripheral vascular disease(199).

1.3.4. Ethnicity and Microvascular Disease

This is an area of much interest and conflicting results. Epidemiological studies have compared the prevalence of all microvascular complications between South Asians and White Europeans with diabetes, but these studies had several limitations in regard to methodology and showed conflicting results in some instances. Furthermore, little is known for the mechanisms that underlies observed ethnic differences in diabetes microvascular complications.

Several studies examined the relation between diabetic nephropathy (both early and end stage diseases) and ethnicity. In regard to ESRD, all studies but one showed that South Asians were at higher risk of developing ESRD compared to White Europeans (**Table 1.1**) (203-208). However, the only study that showed no difference was the only prospective study while all others were retrospective/cross-sectional. Furthermore, all these studies were done in the UK (except one in Holland), with little or no data available from indigenous South Asians in their home countries or from the immigrant South Asian population around the globe. One cross-sectional study from India showed a prevalence of "overt" nephropathy as 8.1% (209). Another limiting factor in interpreting these studies, that adjustment for confounders has been very limited and variable between studies, and "big" risk factors such as obesity were not adjusted for.

Table 1-1: Summary of studies that compared end-stage renal disease between South Asians and White Europeans with type 2 diabetes.

RRT: renal replacement therapy

Study	Country	Design	Methodology	Results	Comments
Burden et al. 1992(203)	UK/Leicester	Retrospective	Case notes of patients receiving RRT 1979-1988	The incidence rate of end-stage renal failure in South Asians was 486.6 (95% CI: 185.1 to 788.1) cases per million person-years per year, compared to 35.6 (17 to 54.2) in White Europeans	Patients not referred for the local renal services or were receiving renal replacement in another centre would have been missed Limited adjustments
Lightstone et al 1995(206)	UK/ Leicester and London	Retrospective	Case notes of all patients receiving RRT in two centres in the UK 1982-1988	Age-adjusted incidence was 7 times higher amongst South Asians than White Europeans ($p<0.001$)	Same as above
Trehan et al	UK/North	Prospective	Consecutive	No difference in	Adjustment

2003(208)	West		patients who started RRT between 1 April 2000 and 31 December 2001 in the North West	diabetic nephropathy prevalence (21.9% vs. 24.9%) No differences after adjustment for possible confounders. The OR for diabetic nephropathy in South Asians 2.61 (0.70–9.71)	for much more confounders and better design than previous studies. Differences in social factors and age between the ethnicities were independent predictors
Chandie Shaw et al 2002(204)	Holland	Retrospective	Case-control study of patients who received RRT between 1990 and 1998 were identified through a national registry	The age adjusted relative risk of end-stage diabetic nephropathy in South Asians was 38 (95 % CI 16 to 91) compared with the native Dutch population	Diabetes duration was similar between the groups, but no further adjustment beyond age

In regard to earlier stages of diabetic nephropathy (i.e. microalbuminuria and proteinuria), the data is similar to ESRD and the quality of the studies is no better. Microalbuminuria and proteinuria has also been reported to be more common in South Asians than White Europeans in all but one study (for further details please refer to chapter 3) (210-214). The limitations of these studies are the same as for the studies of ESRD. All these studies are cross-sectional and all but one adjusted for possible confounders. Also, most of these studies are from the UK, apart from one from Holland and one from Australia. Studies from the South Asian homeland are scarce two studies reported a microalbuminuria prevalence of 25.5% (95% CI: 22.4 to 29%) and proteinuria in 16.2% (95% CI: 13.5 to 19.1%) (209;215).

Similar to nephropathy, DR prevalence was compared between South Asians and White Europeans in several studies, all of which were cross-sectional and in the UK (except one in Holland) compared (for more details please refer to chapter 3) (216-221). These studies showed conflicting results, while some have shown no difference between the ethnicities, others showed that South Asians were at higher risk of developing DR. The diagnosis of DR was based on very different criteria amongst the studies and the adjustment for confounders was very limited. In addition, further examination of ethnic differences based on the degree of DR (early vs. advanced) was not done except in one study. The predictors of diabetic retinopathy in South Asians are similar to those in White Europeans (such as diabetes duration, fasting plasma glucose, systolic BP, urinary albumin concentration and BMI) (222) and one study from India showed a DR prevalence of 17.5%, which is lower than that quoted in UK or Europe; but head-to-head comparison of these figures is not accurate (209).

Only three studies have examined the impact of ethnicity in DPN. All the three studies are from one group in the UK and they all showed that South Asians had lower risk of DPN, foot ulcerations, amputations and peripheral vascular disease (201;202;223;224). In a case-control study that was aimed to compare the risk of amputations between South Asians and White Europeans, the prevalence of DPN (based on the neuro disability score) was lower in South Asians (30% vs. 54%,

p=0.003) (201). However, no adjustment was made for differences between the ethnicities. Another study based on the same population and included 13409 White Europeans and 1866 South Asians with diabetes (type 1 and 2), aimed to assess the prevalence of foot ulceration (223). In this study, the some signs of DPN were more common and others were less common in South Asians on univariate analysis. South Asians were less likely than White Europeans to have abnormal vibration sensation (10.6% vs. 23.6%), abnormal temperature sensation (5.7% vs. 9.8%), absent ankle reflexes (31.6% vs. 37.6%), and abnormal neuropathy disability score (13.8% vs. 22.4%, all P < 0.0001)(223). However, South Asians had slightly higher neuropathy symptom score (31.0% vs. 24.9%) and monofilament insensitivity (20.9% vs. 16.5%) whereas pin-prick sensation was unchanged (16.7% vs. 17.2%)(223). Foot deformities were less common in South Asians (14.9% vs. 32.3%, P < 0.0001)(223). After adjustment for age, South Asians were less likely than White Europeans to have abnormal neuropathy disability score (17.6 vs. 21.8%), abnormal vibration sensation (14.2 vs. 22.8%) and abnormal temperature sensation (7.8 vs. 9.7%) (P < 0.0001 for all comparisons)(223). Abnormal pin-prick sensation, abnormal neuropathy symptom score, and 10-g monofilament insensitivity, however, were now worse in South Asians compared with White Europeans (223). No differences existed between Asians and Europeans for absent reflexes (223). The third study was also a cross-sectional study from a population drawn from primary care in Manchester which included age-matched 180 South Asians and 180 White Europeans with type 2 diabetes (224). There were significant differences in the metabolic profiles between the ethnicities (224). There was no difference in DPN prevalence based on the Neuro disability score (20% vs. 15%, p=0.2 for South Asians and White European respectively) or the Neuro symptoms score (2.2±2.2 vs. 2.2±2.1, p=0.9)(224). However, South Asians had better nerve conduction velocities compared to White Europeans (p=0.007) but there was no significant difference in the vibration perception threshold(224). Small fibre dysfunction was also more common in White Europeans (43% vs. 32%, p=0.03)(224). Adjustment for height and trans-coetaneous oxygen saturations removed the impact of ethnicity on DPN (large and small fibres) (224).

There is only one report that attempted to compare the prevalence of cardiac autonomic neuropathy (CAN) between South Asians and White Europeans (224). This report conducted a very limited assessment based on the heart rate response to deep breathing and postural blood pressure drop. There was greater change in heart rate in response to deep breathing in South Asians (10.8±7.4 vs. 8.5±5.3 bpm; p=0.002) while there was no difference in postural blood pressure change (224).

In summary, South Asians with type 2 diabetes possibly have higher risk of microvascular complications (except DN) compared to White Europeans. However, the studies are limited in number and mostly suffer from poor design or poor adjustment for possible confounders. Furthermore, many of these studies were conducted in primary care populations (particularly in regard to DN), and whether such relation between ethnicity and DPN in higher risk population exists is largely unknown. Studies comparing CAN between ethnicities with any degree of complexity are lacking. Hence, there is a need to conduct studies that examine the relation between ethnicity and microvascular complications, with particular emphasis on adjusting for potential confounders. In addition, there is a need to examine the relation between ethnicity and DPN in higher risk populations (such as secondary care). In addition, studies that examining the potential mechanisms underlying ethnic differences in microvascular complications are needed.

1.3.5. Ethnicity and other differences

In addition to the outlined above, South Asians and White Europeans differ in many other socio demographic and metabolic aspects that affect the relation between ethnicity, obesity and diabetes and its complications between the ethnic groups.

South Asians with type 2 diabetes have been reported to have lower smoking and lower alcohol intake compared to White Europeans (224), both of which are implicated in the development of macrovascular as well as microvascular complications.

In a cross-sectional survey of 500 patients with diabetes (232 White Europeans) from the UK, South Asians had lower income, less education, lower perceived knowledge of diabetes, less awareness of diet content, less awareness of diabetes complications and less awareness of the importance of adherence to treatment (225). All these factors might contribute directly or indirectly to the ethnic differences observed in patients with type 2 diabetes and its complications.

Differences in treatments between ethnicities have also been reported. South Asians are less likely to be prescribed anti-hypertensives and lipid lowering treatment compared to White Europeans, even in individuals who have the same cardiovascular risk (224;226). South Asians are also less likely to adhere to treatment regimens compared to White Europeans and more reluctant to start insulin treatment (226).

Lifestyle factors such as diet and exercise are also significantly different between the ethnic groups. Higher intakes of carbohydrate, saturated fatty acids, trans fatty acids and ω-6 polyunsaturated fatty acids, along with lower intakes of monounsaturated fatty acids and fibre have been reported in South Asians (226). Lower levels of physical activity have also been reported in South Asians (226).

Another factor that attracted a lot of attention is the high prevalence of vitamin D deficiency in South Asians compared to White Europeans (227). Our own data has shown that even within the same ethnicity, vitamin D deficiency is more common in South Asians with type 2 diabetes, compared to those without (228). Furthermore, type 2 diabetes was an independent predictor of hypovitaminosis D and hypovitaminosis D was an independent predictor of glycaemic control(228).

Other factors that might impact on the relation between ethnicity, obesity and type 2 diabetes are intrauterine factors. Low birth weight of Indian babies (mean <2.7 kg; which is lower by 1 kg than White European babies) was associated with more adiposity and poorer muscle mass compared with White Europeans babies (226).

Finally, there are multiple genetic factors that might be involved in explaining the ethnic differences in relation to diabetes and its complication (226).

1.4. Obstructive Sleep Apnoea

Obstructive sleep apnoea (OSA) is a common medical disorder that affects at least 4% of men and 2% of women (229). It is characterized by instability of the upper airway during sleep, which results in markedly reduced (hypopnea) or absent (apnea) airflow at the nose or mouth (229) (**Figure 1.11**). These apnea/hypopnea episodes are usually accompanied with oxygen desaturations and micro arousals that cause sleep fragmentation and reduction in slow wave and REM sleep (**Figure 1.11**) (229).

The American Academy of Sleep Medicine (AASM) guideline has defined sleep events as follows (230): Apnoea is defined as cessation or ≥ 90% reduction in airflow for a period of ≥ 10 seconds. Hypopnea is defined as ≥ 30% reduction in airflow for ≥ 10 seconds associated with ≥ 4% drop in oxygen saturations. Apnoeas are classified into obstructive or central based on the presence or absence of respiratory effort.

The apnea-hypopnea Index (AHI) is the average number apnea and hypopnea episodes per hour during sleep and is a marker of the severity of OSA (229). An apnoea hypopnea index (AHI) ≥ 5 events/hour is consistent with the diagnosis of OSA (231). OSA severity can 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 the nadir oxygen levels during sleep. OSA can be classified to mild, moderate and severe based on AHI 5- < 15, 15 - < 30 and ≥ 30 events/hours. The respiratory disturbance index (RDI) as another measure of OSA severity that includes the AHI in addition to respiratory effort–related arousal (RERA) (229). RERA is defined as a sequence of breaths characterized by increasing respiratory effort leading to an arousal from sleep, but that does not meet criteria for an apnea or hypopnea (229).

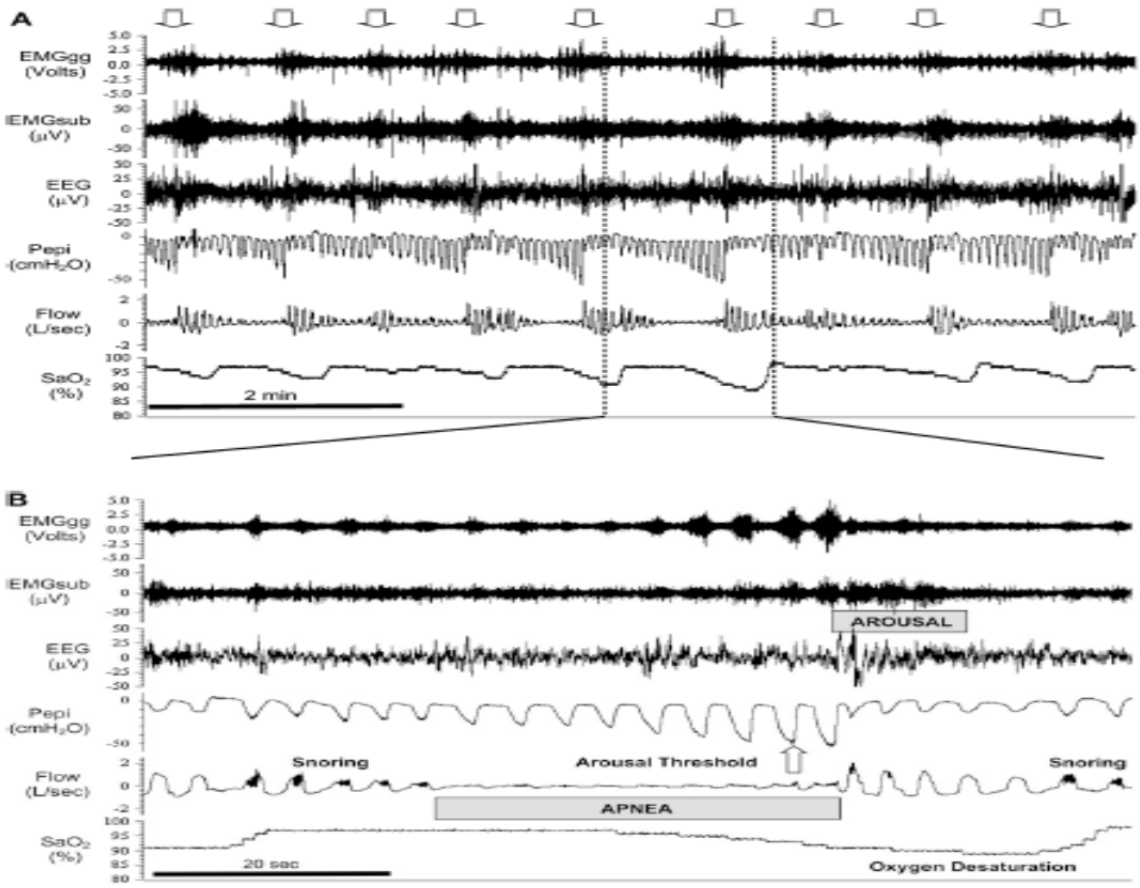


Figure 1-11: Polysomnographic tracings of OSA patient.

EMGgg: Electromyogram of the genioglossus muscle (intramuscular); EMGsub: EMG of the submental muscle (surface); EEG: electroencephalogram (C3–A2); Pepi: pressure at the level of the epiglottis; Flow: airflow measured via nasal mask and pneumotachograph; SaO2: arterial blood oxygen saturation measured via pulse oximetry at the finger. (A) An 8-minute segment during stage 2 sleep, during which the patient is experiencing sleep-disordered breathing. Note the repeated oxygen desaturations as a result of severely impaired (hypopnea) or absent (apnoea) airflow despite continual breathing efforts (Pepi) and the cyclical breathing pattern that ensues as the patient oscillates between sleep and arousal (downward pointing arrows). (B) An expanded segment during an obstructive event. Note: Evidence of snoring on the flow tracing, quantification of the arousal threshold, and progressive increases in EMGgg activity throughout the obstructive event, although occurring, were not sufficient to restore flow without arousal in this instance. Adapted from (232).

1.4.1. Epidemiology and Risk Factors

The prevalence of OSA varies considerably between studies, mainly due to differences in the population studied, study designs and the method and criteria used to diagnose OSA. A prevalence of 4% in men and 2% in women (229) has traditionally been quoted in many populations. Studies,

however, reported prevalence as low as 1% to as high as 28% (233). The prevalence from three well conducted studies with similar design from Wisconsin, Pennsylvania, and Spain showed an OSA prevalence of 17-26% in men and 9-28% in women and a prevalence of 9-14% and 2-7% for men and women with moderate to severe OSA (233). These studies used a two stage sampling design which allows some degree of estimate of the "self-selection" bias which is usually a significant problem in OSA studies.

The prevalence of OSA amongst population is affected by many other risk factors such as obesity, age, ethnicity and gender.

The large majority of epidemiological OSA studies took place in Western societies (particularly in the USA) and included mainly White European populations, hence data about the prevalence in other ethnicities is rather limited. In African-Americans, the available results are conflicting. While some studies showed higher (twice) adjusted OSA prevalence in Afro-Caribbean's (233;234), others did not show such a difference (235). Chinese have a high OSA prevalence (8.8% in men and 3.7% in women) despite that Chinese are less obese than Caucasians (236;237). This highlights the importance of other factors such as the anatomy of upper airways in the development of OSA. Chinese were shown to have more crowded upper airways with higher Mallampati score and shorter thyromental distance(238). Data regarding the prevalence of OSA in South Asians is also limited. I found only 3 papers in the literature that addressed this issue. In a semi-urban population in Delhi, the OSA prevalence was 3.7% (239), this rose to 9.3% in middle-age Urban Indians(240), and 19.5% in middle age Urban men (241).

The impact of gender on OSA status has been well recognised, men have 2 to 3 times increased risk of OSA compared to women (233). The exact mechanisms behind the gender differences in OSA prevalence are not clear but several possible factors have been proposed. Sex hormones have been blamed, particularly that men receiving testosterone replacement are at higher risk of OSA and that the prevalence of OSA in post-menopausal women is higher than those pre-menopausal and

hormone-replacement therapy reduces the risk of OSA in post-menopausal women (242-244). Differences in upper airway size and ventilator control between men and women have also been implicated but the results are conflicting (245). For detailed and excellent review for the underlying causes of gender differences in OSA please refer to(245).

Several studies have shown that the prevalence of OSA increases with age (243). In men, OSA (AHI ≥ 10 events/hour) was present in 3.2%, 11.3%, and 18.1% of the 20- to 44-year, 45- to 64-year, and 61- to 100-year age groups, respectively (246). In another study from Spain, the prevalence of any OSA was three times higher and the prevalence of moderate to severe OSA was 4 times higher in older patients (> 70 years old) compared to middle-aged participants (233). On the other hand, in the sleep health heart study, OSA prevalence increased with age but reached a plateau at the age of 65 years (**Figure 1.12**) (235). The relation with age seems to be due to changes in pharyngeal anatomy and upper airway collapsibility(243).

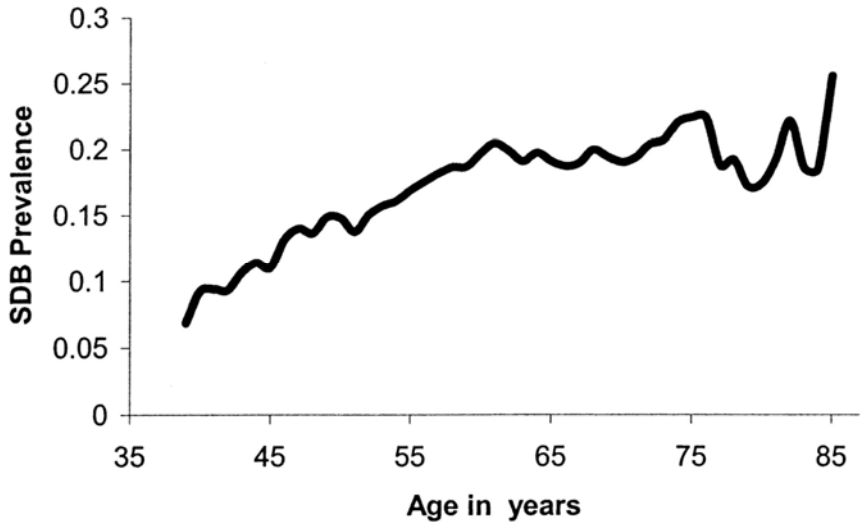


Figure 1-12: The relationship between age and SDB prevalence from the sleep heart health study **(235)**

Excess body weight has also been recognised to be an essential risk factor in patients with OSA, although not all OSA patients are obese or overweight. In the Wisconsin sleep study, each increase in BMI by one standard deviation, resulted in a 4-fold increase in OSA prevalence (247). Several

other studies have shown the strong link between OSA and excess body weight (233;236;237;241-243;246;248;249). Prospective studies showed that weight gain is associated with the development of or worsening pre-existing OSA (250-252). This was further supported by a randomised controlled trial which showed that weight loss (via life style modifications) improve/cure mild OSA (253). Surgical induced weight loss also resulted in significant improvements in OSA status (254). The mechanisms that link obesity to OSA are not entirely clear but several mechanisms have been proposed; weight gain can alter normal upper airway mechanics during sleep by increased parapharyngeal fat deposition resulting in a smaller upper airway, altering the neural compensatory mechanisms that maintain airway patency and reducing the functional residual capacity with a resultant decrease in the stabilizing caudal traction on the upper airway (243;249;255).

There are several other predisposing risk factors to OSA such as current smoking, excess alcohol intake and genetic factors (233;243).

1.4.2. OSA comorbidities

One of the major OSA associations is the relation with type 2 diabetes, this will discussed later in this chapter.

1.4.2.1. Hypertension

OSA has been associated with sustained hypertension and lack of the normal nocturnal dipping of blood pressure. Below are landmark studies in the field of OSA and hypertension but there are many others that I have not discussed here (233).

Non-dipping of blood pressure in OSA patients was examined prospectively in a subsample of 328 adults enrolled in the Wisconsin Sleep Cohort Study who completed 2 or more 24-hour ambulatory BP studies over an average of 7.2 years (256). After adjustment for a wide range of confounders, the adjusted OR (95%CI) of incident systolic non-dipping for baseline AHI 5-14.9 and ≥ 15, vs. AHI < 5, were 3.1 (1.3-7.7) and 4.4 (1.2-16.3), respectively (256). Nieto et al assessed the association between

OSA and hypertension in cross-sectional analysis of 6132 middle-aged and older persons (aged ≥ 40 years) from the Sleep Heart Health Study (257). After adjustment for BMI, neck circumference, WHR, alcohol and smoking, the OR (95%CI) for hypertension for the highest vs. the lowest category of AHI was 1.37 (1.03-1.83, p=0.005) (257). The associations of hypertension with OSA were seen in men and women, older and younger, all ethnic groups, and among normal-weight and overweight individuals (257). In another prospective study based on the Wisconsin Sleep Cohort Study, Peppard et al examined the relation between OSA and hypertension in 709 participants over 4 years (258). After adjustment for a wide range of confounders, and relative to an AHI of 0 events/h at base line, the OR for the presence of hypertension at follow-up were 1.42 (95%CI: 1.13-1.78), 2.03 (1.29-3.17) and 2.89 (1.46-5.64) for an AHI of 0.1 to 4.9, 5.0 to 14.9 and ≥15.0 events/h respectively (p=0002 for the trend) (258).

1.4.2.2. Road traffic accidents

Several cross-sectional studies using driving stimulators showed worse driving performance and increased risk of road traffic accidents in patients with OSA (259-262).There are 2 studies that objectively assessed the impact of undiagnosed OSA on having road traffic accidents.

In a sample of 913 employed adults in which motor vehicle accident history was obtained from a state-wide data base of between 1988 to 1993, men with OSA (AHI≥ 5 events/h) were significantly more likely to have at least one accident in 5 years compared to those without OSA (age and miles driven adjusted OR 4.2 for AHI 5-15, and 3.4 for AHI > 15) (233;263). Men and women combined with AHI > 15 (vs. no OSA) were significantly more likely to have multiple accidents in 5 years (OR 7.3) (233;263). Similar results were found in another case–control study from Spain (264). Interestingly, neither of these two studies showed a relation between reported sleepiness and the road traffic accidents nor there was a dose relation between OSA severity and the likelihood of involvement in an accident.

1.4.2.3. Cardiovascular mortality and morbidity

There are several cross-sectional and case control studies that showed a link/ an association between OSA and myocardial infarction; these studies will not be reviewed here in details due to their inherent limitations but a review of these can be found in (233). The two main landmark cross sectional studies that found an association between OSA and CVD are the Sleep Heart Health Study and the Wisconsin sleep study (265;266).

There are several prospective studies that linked OSA to cardiovascular disease, these studies have either used surrogate markers of OSA (such as snoring) or it compared patients with OSA who are using continuous positive airway pressure (CPAP) to those who declined or where non-compliant with CPAP treatment. Two large prospective studies that showed a 33-40% increase in CVD incidence in regular snorers vs. infrequent or non-snorers over 6-8 years of follow up, despite adjustment for possible confounders (267;268). One study did not show a relationship (269).

Three other studies used more accurate methods to diagnose OSA (i.e. polysomnography) (270-272). In a study of a 182 consecutive middle-aged men free of CVD at baseline who were followed for 7 years ; the incidence of CVD was 36.7% of patients with OSA vs. 6.6% subjects without OSA ($p < 0.001$) (270). OSA was an independent predictor of CVD (OR 4.9; 95% CI, 1.8–13.6) after adjustment for confounders (270). CPAP treatment was associated with lower incidence of CVD compared to those non-treated (56.8% vs. 6.7%, $p<0.001$)(270). In another prospective study in which men with OSA were followed for a mean of 10.1 years; patients with untreated severe OSA had a higher incidence of fatal cardiovascular events and non-fatal cardiovascular events than did untreated patients with mild-moderate OSA, simple snorers, patients treated with CPAP, and healthy participants (271). After adjustment for confounders, untreated severe OSA significantly increased the risk of fatal (OR 2·87, 95%CI 1·17–7·51) and non-fatal (3·17, 1·12–7·51) cardiovascular events compared with healthy participants (271). In another important prospective study, 1022 patients

were followed up for a median of 3.4 years (272). After adjustment for confounders, OSA was significant associated with stroke or death (hazard ratio, 1.97; 95%CI, 1.12-3.48; P=0.01) (272).

In addition to those observational epidemiological studies, two recent studies have also emphasised this relation between OSA and CVD. In 19 patients with stable coronary artery disease, patients with OSA had larger atherosclerotic plaque volume as assessed by intravascular ultrasound and AHI correlated positively with the plaque volume (r=0.6, p=0.01) (273). The role of the nocturnal events in OSA to the occurrence of myocardial infarction is further supported by a study that showed patients with OSA were more likely to develop acute myocardial infarction between 12 am and 6 am compared to patients matched for comorbidities but do not have OSA (32% vs. 7%, p=0.01)(274).

The impact of having OSA on mortality was examined in one landmark study, the Wisconsin Sleep Cohort(275). In this 18-year mortality follow-up, there was a step-wise reduction in survival with worsening OSA **(Figure 1.13)**. The adjusted hazard ratio (HR, 95% CI) for all-cause mortality with severe versus no OSA was 2.7 (1.3 to 5.7) after adjustment for possible confounders (275).

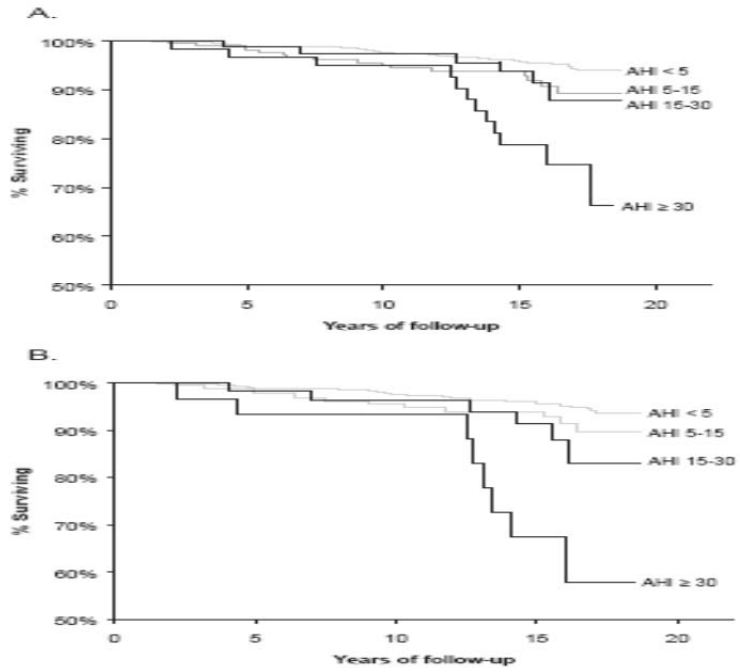


Figure 1-13: Kaplan-Meier estimates of survival probability according to OSA severity.

Long-rank test for differences in survival by SDB category: P < 0.00001. Adapted from (275)

1.4.2.4. Cognitive function

Very limited studies linked OSA to cognitive performance. In the Wisconsin Sleep Cohort Study, OSA severity (measured by AHI) was significantly but weakly related to diminished psychomotor efficiency, a factor reflecting the coordination of fine motor control with sustained attention and concentration, but OSA was not related to memory (233). They estimated that the impact of having AHI of 15 was approximately equivalent to the effect of 5 years of aging on psychomotor function. In another study from Denmark AHI ≥ 5 or was significantly associated with self-assessed concentration problems but not with memory (233).

1.4.2.5. Quality of life

Again data here is fairly limited. In the Wisconsin Sleep Cohort Study and the Sleep Heart Health Study, there was a linear association of OSA severity with decrements on the eight SF-36 scales (233).

1.4.3. Pathophysiology

OSA is a very complex disorder, and although that obesity and fat deposition around the neck plays an important role, there are many other important factors that contribute to the development of this condition (**Figure 1.14**).

1.4.3.1. Upper Airway anatomy

The human upper airway is a unique multipurpose structure involved in performing a variety of tasks such as speech, swallowing, and the passage of air for breathing (232). The airway, therefore, is composed of numerous muscles and soft tissue but lacks rigid or bony support (232). Most notably, it contains a collapsible portion that extends from the hard palate to the larynx which allows the upper airway to change shape and momentarily for speech and swallowing during wakefulness; but this feature also provides the opportunity for collapse at inopportune times such as during sleep (232).

Several imaging based studies showed that patients with OSA have smaller upper airway, resulting in an airway that is more prone to collapse (232). However, the interpretation of these studies is confounded by the fact they were performed during wakefulness and neural control during wakefulness is different from sleep. The closest study to assess upper airways during sleep was performed on anaesthetised patients and showed that increased collapsibility of the upper airway in OSA patients compared to those without OSA (276).

1.4.3.2. Upper Airway Dilators activity

Upper airway muscles (genioglossus) activity was found to be increased in OSA patients compared to age and obesity matched healthy controls (277), which suggests that these muscles are compensating for an underlying defect in the anatomy of the upper airway in patients with OSA (232). Interestingly, this muscle hyperactivity is resolved in CPAP treated patients (278). Sleep onset, is associated with greater reductions in upper airway muscles tone in OSA patients compared to controls, which explains the occurrence of apnoea/hypopnea episodes at sleep onset and during Rapid Eye Movement (REM) sleep (232;278). This reduction in upper airway muscle tone during sleep seems to be as a result of central lack of drive and local inhibitory reflexes that responds to changes in pressure in the upper airways (232).

1.4.3.3. Sleep Arousals

In patients with OSA, the majority of obstructive events are followed by an arousal, which restores airflow (232). Having an arousal, however, is not a must to restore airflow (232). Younes et found that arousals are incidental events that occur when thresholds for arousal are reached, and that arousals are not needed to initiate opening or to obtain adequate flow and that they likely increase the severity of the disorder by promoting greater ventilatory instability (279). Studies have found that the main reason for the occurrence of arousals in non-REM sleep is the negative intra-pleural pressure and respiratory effort, regardless of the cause that generated such a negative pressure (232;280). Patients with OSA seems to have higher arousal thresholds compared to people without

OSA; it is likely, however, that these differences in arousal pressure are not a primary defect in patients with OSA as CPAP treatment lowers the arousal threshold in OSA patients to levels similar to that in patients without OSA (232;281).

1.4.3.4. Ventilatory Control and Stability

Ventilatory control stability can be described using the engineering concept loop gain (232). In the context of ventilatory control, loop gain refers to the stability of the respiratory system and how responsive the system is to changes in breathing (e.g., arousal) (232). There are two principal components to loop gain: controller gain and plant gain. As it relates to respiratory control, controller gain refers to the chemo-responsiveness of the system (i.e., hypoxic and hypercapnic ventilatory responses). Plant gain reflects primarily the efficiency of CO2 excretion (i.e., the ability of a given level of ventilation to excrete CO2) (232). The physical separation of the sensors and effectors makes the ventilator feedback control system vulnerable to instability (232). An inherently high loop gain system is unstable (i.e., robust ventilator response to a respiratory stimulus) compared with a low loop gain system (i.e., dampened ventilatory response to an equivalent respiratory stimulus) (232). A commonly used analogy is the regulation of room temperature, whereby temperature will be prone to oscillation in a situation where there is a particularly sensitive thermostat and an overly powerful heater (i.e., high loop gain) (232). OSA patients were found to have elevated loop gain and suggest that ventilatory instability is an important mechanism contributing to OSA (232). Elevated loop gain would be expected to increase oscillations from the brainstem central pattern generator. One would predict that pharyngeal obstruction occurs when ventilatory motor output is at its nadir (i.e., when neural output to the upper airway muscles is low (232)). Also, elevated loop gain may also increase the ventilatory response to arousal, which may drive PaCO2 below the apnoea threshold during subsequent sleep. OSA could then occur depending on the prevailing upper airway mechanics (232).

1.4.3.5. Lung Volume

Several studies have shown that changes in lung volume affect upper airway muscles activity (232;282). Furthermore, the cross-sectional are of the upper airways was found to be related to the lung volumes during wakefulness in both healthy individuals and patients with OSA, with the relationship being stronger in OSA patients (232;283). During non-REM sleep, changes in lung volume were also related to the upper airways in patients with OSA and changes in lung volumes reduced airway collapsibility in OSA patients (232;284). The mechanisms that relate lung volume to upper airways are unclear but one mechanism supported by animal studies is the concept of a loss of caudal traction on upper airway structures during decreased lung volume (232). When lung volume is reduced there is a displacement of the diaphragm and thorax toward the head, which results in a loss of caudal traction on the upper airway, resulting in a more collapsible airway (232).

Due to space limitations, I won't be able to describe how all OSA risk factors exert their effect via the above described mechanisms, but I will just focus on obesity as an example. Fat distribution around the neck will increase the outside pressure on the upper airways. Fat infiltrating the structures and muscles around the upper airway will also increase upper airway collapsibility. Intra-abdominal fat causes restrictive defect on breathing which results in reduction in lung volumes (which in turn affects upper airway collapsibility as described above). Furthermore, obesity affects the chemosensitivity to O2 and CO2 and reduces ventilator drive. So we can see in this example how obesity affects several aspects of the OSA pathogenisis.

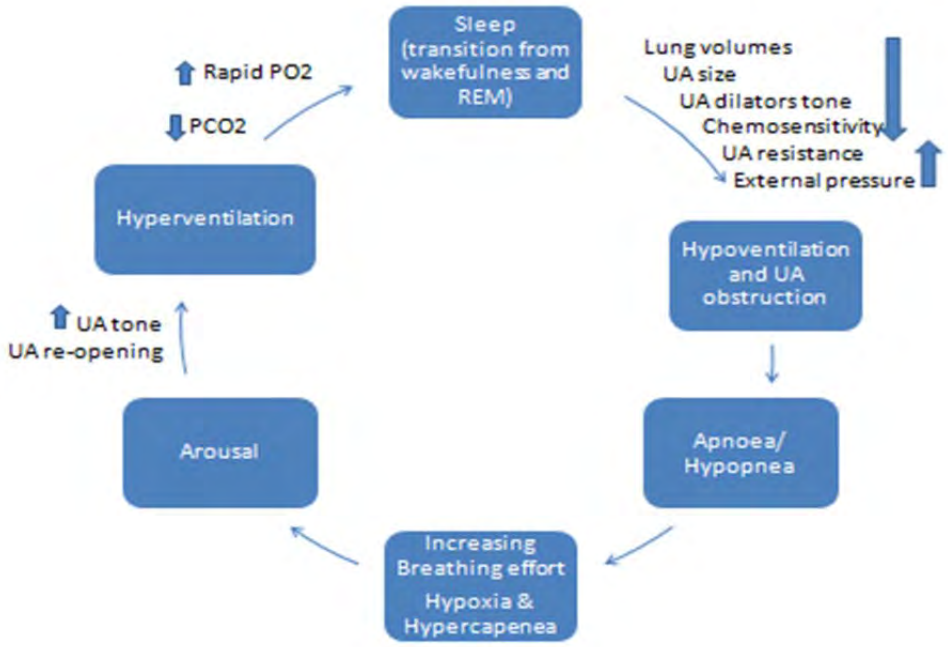


Figure 1-14: Summary of the pathogenesis of upper airway obstruction in patients with OSA.

UA: Upper Airways

1.4.4. Diagnosis

The diagnosis of OSA depends on a combination of clinical features and physiological studies.

1.4.4.1. Clinical Features

Good history and examination are still an essential part of the assessment of patients with OSA despite several reports showing the limited value of symptoms in predicting OSA (in one report, only one third of patients would have been identified clinically) (285;286). Having said that, the presence of abnormal breathing alone may not be enough to diagnose OSA. As a result the term OSA syndrome (OSAS rather than OSA) was coined in order to differentiate those with abnormal breathing pattern only from those with abnormal breathing patters that is associated with excessive daytime sleepiness and other features of OSA. The prevalence of OSA vary significantly whether excessive time sleepiness is included or not in the definition of OSA in the epidemiological studies

(287). For example, in the Wisconsin sleep cohort study, OSA (AHI > 5) prevalence was 29% vs. OSAS (AHI > 5 + excessive day time sleepiness) prevalence of 4% (247). However, it must be noted that patients appreciation of OSA symptoms (such as snoring, apnoeas, day time sleepiness, tiredness etc.) may not be accurate and the presence of a partner might help, though it may not eliminate, this under reporting of symptoms (287). The reasons for underreporting OSA symptoms is complex and might be related to several factors such as denial, neurocognitive impairment, and/or habituation to the symptoms (287).

Snoring is the most common symptom of OSA and it occurs in 95% of patients (287). Snoring, however, has a poor predictive value due to the high prevalence of snoring and the presence of many snorers who don't have OSA (287). Nonetheless, lack of snoring almost rules out OSA as only 6% of OSA patients have not reported snoring (287). Witnessed apnoeas are another important symptom that is usually reported by the partner. However, witnessed apnoeas do not correlate with disease severity and up to 6% of the "normal" population could have witnessed apnoeas without OSA (287). Other nocturnal symptoms such as choking (which is possibly a "proper" rather than a "micro" arousal to terminate apnoea), insomnia, nocturia and diaphoresis have been reported (287). Daytime symptoms include excessive daytime sleepiness (EDS), fatigue, morning headache and autonomic symptoms(287). OSA is a very important cause of EDS but up to 50% of the general population might suffer from EDS and its severity does not correlate with OSA severity (233;287).

1.4.4.2. Sleep studies

The gold standard to diagnose OSA is polysomnography that typically includes the recording of 12 channels such as EEG, electrooculogram (EOG), electromyogram (EMG), oronasal airflow, chest wall effort, Abdominal effort, body position, snore microphone, ECG, and oxyhaemoglobin saturation (287). The main problem with polysomnography is that it is time consuming and requires significant resources. Portable home based respiratory devices are another alternative (**Table 1.2**) (287;288). The main advantages are that they are less resources but they are associated with higher failure/loss

of lead rate compared to polysomnography (287). Pulse oximetry is another good way to diagnose OSA, it cannot however differentiate between obstructive and central apnoeas and it has a wide range of sensitivity (31-98%) and specificity (41-100%). The AASM recommend use a Type III device as a minimum (287).

Table 1-2: The AASM classification of portable polysomnography.

Adapted from (288)

	Type of Portable Polysomnography			
	Type I (attended)	Type II (unattended)	Type III (modified, for diagnosing OSA)	Type IV (1 or 2 channels)
Measurement channels (*n*)	≥ 7	≥ 7	≥ 4	≤ 2
Measurements	Electro-encephalogram Electro-oculogram Chin electro-myogram Electro-cardiogram Air flow Respiratory effort Pulse oximetry	Electro-encephalogram Electro-oculogram Chin electro-myogram Electro-cardiogram Air flow Respiratory effort Pulse oximetry	Electro-cardiogram Air flow 2 respiratory-effort channels Pulse oximetry Peripheral arterial tonometry*	Pulse oximetry Air flow or chest movement
Body position	Documented or objective	Possible	Possible	No
Leg movement	Electro-myogram or motion sensor	Optional	Optional	No
Personnel	Yes	No	No	No
Intervention	Possible	No	No	No

* Became available after 1994.

1.4.5. Management

OSA should be treated promptly and the aim of treatment is to reduce the morbidity and mortality associated with this condition. Weight loss and positional treatment (i.e. avoiding the position in which most episodes occur, which is usually the supine position) are important aspects of treatment. As with all obesity-related disorders, weight loss (regardless of the means) can result in significant improvements in OSA. In a randomised controlled trial of intensive life style intervention in 264 patients with OSA and T2DM the (Sleep AHEAD study) weight loss of 11 Kg on average in the treatment group resulted in a reduction in the AHI of about 10 events/hour (289). A recent study suggested that weight loss resulted in an increase in velopharyngeal airway volume and upper

airway length, which appear to influence the reduction in AHI (290). Similar results were found in a study of men with OSA, in which 10% weight loss resulted in improvements in the respiratory disturbance index by about 16 events/hour (291). Weight loss after bariatric surgery has also been associated with significant improvements in OSA severity (292).

Mandibular advancement devices (MAD) are effective in treating patients with mild to moderate OSA. They work by pulling the tongue forward or by moving the mandible and soft palate anteriorly, enlarging the posterior airspace, which results in opening in the airway and an increase in the airway size. MAD are considered as second line treatment for patients with mild to moderate OSA who could not tolerate CPAP (293).

Surgery has a limited role in patients with OSA and produces variable results (293). If the patients has upper airway obstruction (such as tonsils or tumours) then surgery is the most important aspect of treatment, otherwise its role is limited and usually associated with significant side effects (293).

CPAP is the mainstay of treatment for patients with OSA. CPAP works by providing a "pneumatic splint" by delivering an intraluminal pressure that is positive with reference to the atmospheric pressure and by increasing lung volumes (294). CPAP treatment has been shown to reduce AHI, reduce BP, improve sleepiness, improve quality of life, improve cognitive function, and reduce motor vehicle accidents (294). Furthermore, evidence from observational study suggests that CPAP treatment reduces the risk of CV events **(Figure 1.15)** (271). An in depth review about CPAP, its technical aspects and the evidence behind its use and its complications can be found in (294).

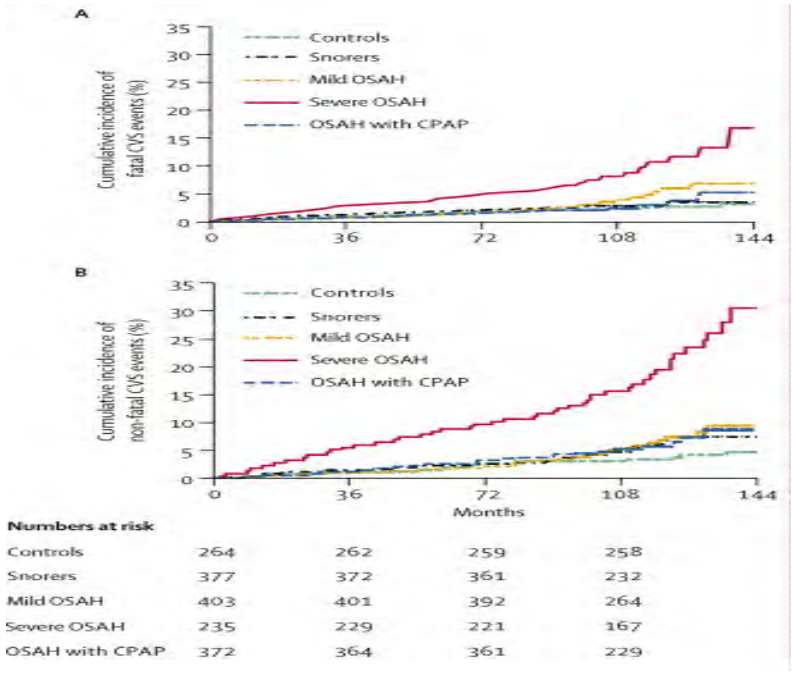


Figure 1-15: Cumulative percentage of individuals with new fatal (A) and non-fatal (B) cardiovascular events in each of the five groups studied

1.5. Obstructive Sleep Apnoea and Type 2 Diabetes

The risk factors for developing OSA and T2DM are similar (particularly obesity) and hence it is not surprising that there is a relationship between OSA and T2DM. However, not all obese patients have both conditions and many patients have one and not the other. Hence, understanding this relationship and the mechanisms that underpin this relation is important to understand the pathogenesis of these conditions. There are many studies that have examined the association between snoring, as a surrogate marker of OSA, and different aspects of glucose metabolism (295-304); here, however, I will mainly concentrate on the studies that validated the presence and severity of OSA using polysomnography (the gold standard). It must be noted, however, that OSA is a continuum from snoring (without OSA) to OSAS. Further details regarding the relationship between snoring and T2DM can be found in a recent review (305).

1.5.1. The impact of OSA on glucose metabolism

OSA has been associated with components of the metabolic syndrome and with IR independent of obesity (306). The prevalence of abnormal glycaemia in patients with OSA is much higher than those without OSA (up to 79%) (307;308).

In a cross sectional analysis in a subset of the Sleep Heart Health Study, Punjabi et al found that relative to those with a RDI < 5 events/hour, individuals with mild and moderate to severe OSA had adjusted OR of 1.27 (95% CI: 0.98 -1.64) and 1.46 (95% CI: 1.09 -1.97), respectively for fasting glucose intolerance (309). Sleep-related hypoxemia was also associated with glucose intolerance independently of age, gender, BMI, and waist circumference (309). In another study an AHI≥ 5/hour was associated with an increased risk of IGT or DM (OR: 2.15; 95% CI: 1.05-4.38) following adjustment for BMI and body fat (310). For a 4% decrease in oxygen saturation, the OR of worsening glucose tolerance was 1.99 (95% CI: 1.11 to 3.56) after adjusting for percent body fat, BMI, and AHI (310). Other studies also found similar results (311).

OSA (AHI ≥ 5 events/hour) has also been found to be associated with IR and that AHI and minimum nocturnal oxygen saturations were also independent determinants of IR; in both obese and non-obese individuals (312). Each additional AHI unit per sleep hour increased the fasting insulin level and HOMA-IR by about 0.5% (312). Vgontzas et al found that obese OSA patients were more insulin resistant compared to BMI-matched non-OSA patients (313). Similar results were found by other studies (314-320). In addition to its impact on IR, OSA was found to impair β-cell function (321). One study in mice also showed that intermittent hypoxia increases β-cell death (322).

It is of interest that a small recent study suggests that EDS might be, in part, responsible for the IR in patients with OSA (323). Barcelo et al studied 44 patients with OSA (22 with and 22 without EDS) matched for age, BMI and AHI, and 23 healthy controls (323). Patients with EDS had higher HOMA-IR compared with OSA patients without EDS or healthy controls ($p<0.001$ both comparisons) (323).

Interestingly, there was no difference in HOMA-IR between OSA patients without EDS and healthy controls (323). Glucose levels were significantly higher in patients with OSA and EDS compared to those with OSA without EDS and healthy controls (323). In support for the association between EDS and IR, CPAP treatment in the same study reduced the HOMA-IR and increased IGF-1 levels in patients with EDS, but did not modify any of these variables in patients without EDS (323).

The observed impact of OSA on glucose metabolism and IR results into an increased risk in developing T2DM in patients with OSA, which was shown in several prospective studies (295;324) (325-330).

All the above mentioned evidence is in OSA patients without diabetes, whether OSA has an impact on glucose metabolism in patients with known T2DM is less clear. Two observational studies have shown that OSA severity and HbA1c correlate positively in patients with T2DM after controlling for age, sex, race, body mass index, number of diabetes medications, level of exercise, years of diabetes and total sleep time (331;332). Furthermore, increasing severity of OSA was associated with poorer glucose control (332).

1.5.2. Prevalence of OSA in patients with T2DM

Several studies have examined the prevalence of un-diagnosed OSA in patients with T2DM; these results showed that OSA is very common in patients with T2DM but the there is significant variation in the actual prevalence of OSA between studies. This variation is likely to reflect the differences in population characteristics (primary vs. secondary care, long vs. short diabetes duration, ethnicity etc.) and the differences in the methods used and the criteria used to diagnose OSA.

Einhorn et al found a prevalence of OSA of 48% in a sample of 330 consecutive adults with T2DM recruited from a diabetes clinic in the USA (333). OSA (defined as AHI ≥ 10 events/hour) was diagnosed using a single-channel recording device that measures nasal airflow signal (333). West et

al found a lower prevalence of OSA in their patients with T2DM (23%) in a study of 1676 men recruited from a mixed primary and secondary care populations in Oxford in the UK (334).

In another study of 116 hypertensive men (21% with T2DM) from Sweden, Elmasry et al found a high prevalence of severe OSA (based on polysomnography) in patient with diabetes compared to those without (36% vs. 14.5%, P<0.05) (335).

In a randomly selected 165 patients from a teaching hospital diabetes clinic who had polysomnography in China, the prevalence of OSA (AHI ≥ 5 events/h) was 53.9% of participants and 32.7% had moderate/severe OSA (AHI ≥ 15/h) (336).

In another study of 306 patients from the USA that included a significant Afro-Caribbean population (19.1%) and had unattended polysomnography performed; over 86% of participants had OSA (AHI ≥ 5 events/hour) (337). A total of 30.5% of the participants had moderate OSA and 22.6% had severe OSA(337). In another study from secondary care in the UK, the prevalence of OSA in 52 consecutive patients with T2DM and obesity was 58% (331).

As a result of the high prevalence of OSA in patients with T2DM, the international diabetes federation (IDF) recommended screening for OSA in this high risk population (338). Data regarding the prevalence of OSA in South Asians with T2DM is lacking.

1.5.3. Impact of CPAP Treatment on T2DM and IR

As epidemiological studies suggest a relationship between OSA, obesity and T2DM, it is important to examine the impact of treating OSA on T2DM. The impact of CPAP on glycaemic control and insulin sensitivity in patients with T2DM has been examined in several studies. The results are, however, inconsistent.

Four months CPAP treatment resulted in improvements in insulin sensitivity in 10 obese patients with T2DM (339). Other studies found similar impact of CPAP on insulin sensitivity from as early as 2

days post-treatment (340;341). CPAP was also found to lower the 1-hour postprandial glucose levels in patients with T2DM, which was also associated with improvement in HbA1c (342). More recently, using continuous glucose monitoring (CGM), CPAP treatment was associated with less glucose variability and improved glucose control (343;344).

Not all studies have shown an improvement in IR in patients with OSA and T2DM. In a randomized placebo (sham CPAP) controlled trial of CPAP in 42 men with T2DM and OSA HbA1c and IR did not significantly change in either the therapeutic or placebo groups (345). The CPAP use per night, however, was 3.6 hours in the treatment group and 3.3 hours in the placebo group (345). Several other studies also showed negative effect of CPAP on IR and other glycaemic measures (346-348).

The above results show conflicting results. Most of these studies suggest that CPAP does not improve HbA1c in patients with T2DM, despite some of these studies showing positive improvements in IR and glucose levels following CPAP treatment. This lack of effect on HbA1c might be resulting from the relatively short duration of CPAP treatment or level of baseline HbA1C. Also, patients with T2DM included in these studies were of variable diabetes duration and the impact of CPAP treatment might be differential in relation to diabetes duration. Furthermore, the adherence to CPAP treatment has been variable. Also, all these studies included a relatively small number of patients.

1.5.4. Central Sleep Apnoea in DM

The relation between T2DM and OSA is not limited to the presence of obstructive apnoea but also related to central sleep apnoea (CSA) and periodic breathing. In a subgroup analysis of the Sleep Heart Health Study, there were significant differences in RDI, sleep stages, central apnoea index and periodic breathing between patients with and without DM. However, most of these differences lost their statistical significance after adjusting for age, sex, BMI, race, and neck circumference with the exception of percent time in REM sleep and prevalence of periodic breathing (349). In addition, CSA

was associated with DM though this association was not statistically significant (OR 1.42, 95% CI 0.80-2.55) (349). Similarly, Sanders and colleagues explored the relation between CSA and DM (350). They found a greater proportion of patients with DM had CSA compared to patients without DM. Also, a greater percentage of patients with DM exhibited periodic breathing (3.8% vs. 1.8%, DM vs. non-DM patients respectively, P=0.002) (350). The relation between CSA, periodic breathing and DM might be caused by the presence of autonomic neuropathy (350). Autonomic neuropathy has been associated with the development of OSA in patients with DM (351). Also, the sympathetic nervous system, in patients with DM, has been implicated in the central respiratory centre response to hypercapnic stimulus (352). The presence of autonomic neuropathy in other diseases such as multi-system atrophy (Shy-Drager syndrome) has also been implicated in the development of abnormalities in the central control of breathing (353).

1.5.5. Pathophysiology- OSA and T2DM

The mechanisms in which OSA can impact T2DM are not clear, but likely to be multi-factorial. There are several candidate mechanisms which I will discuss below (**Figures 1.16 and 1.17**).

1.5.5.1. Hormonal changes

As described above, OSA is associated with IR and β-cell dysfunction, both of which might lead to the development of T2DM. In addition to the above mentioned, OSA seems to affect glucose metabolism by causing changes to sleep structure and EDS (354;355).

OSA also appears to be associated with activation of the HPA axis and suppression of the GH axis (356-358), both of which contribute to increase IR. OSA is also associated with lower IGF-1, which can be reversed by one night of CPAP treatment (359;360).

Adiponectin has also been linked to the severity of OSA. Lam and colleagues showed that adiponectin levels correlate negatively with the severity of OSA independent of age, BMI and visceral fat volume (361), which contributes to worsening IR. In a subset of the Nurses' Health study,

adiponectin levels decreased with increasing frequency of snoring ($p<0.0001$, $p=0.002$ adjusted for age and BMI, $p=0.03$ adjusted for age, BMI and other confounders) (362). Several other studies suggested that adiponectin levels are lower in OSA patients, although short CPAP treatment did not seem to reverse this trend (363-367).

Inversely to adiponectin, leptin levels were shown to be higher in obese subjects with OSA compared to age and BMI matched obese subjects without OSA ($p<0.05$) (313). There are several studies that similarly showed increased leptin levels in patients with OSA (368-370).

Patients with OSA also have higher ghrelin levels compared to controls and 1-month CPAP treatment reduced the levels of acylated ghrelin but had no impact on unacylated ghrelin levels (371). Shorter duration of CPAP treatment (2-days) was also associated with reduction in ghrelin levels in another study (372).

Catecholamines are another possible mediator between OSA and glucose metabolism; several studies have shown a relation between OSA and elevated catecholamines levels since the 1980's (359;366;373;374). More recently, McArdle and colleagues showed that patients with OSA (AHI> 15 events/hour) had higher 24-h and nocturnal (12-h) urinary norepinephrine excretion compared to those without OSA (AHI< 5/hr) despite that the groups were matched for age, BMI and smoking status (359).

The hormonal changes that might relate OSA to T2DM are summarised in **Figure 1.16.**

1.5.5.2. Autonomic dysfunction

Sympathetic over activation plays an important role in the regulation of glucose and fat metabolism and the development of T2DM (375;376). OSA has been shown to be associated with increased sympathetic activity (366;377-380). It is likely that both, the recurrent hypoxia (377;381;382) and recurrent arousals (383) are contributing to the activation of the sympathetic system.

1.5.5.3. Inflammatory cytokines

OSA has been associated with elevated IL-6, TNF-α (313;366;384-386). These inflammatory markers have been related to adiposity and the development of IR (313).

1.5.5.4. Non-alcoholic steatohepatitis (NASH)

Non-alcoholic fatty liver disease (NAFLD) is a very common disorder that is associated with obesity, IR, T2DM and the metabolic syndrome (387;388). Two recent studies suggest the OSA might be a risk factor for the developing of histologically proven NAFLD and for progressing to NASH (319;389). Nocturnal desaturations were found to be associated with hepatic inflammation, hepatocyte ballooning, and liver fibrosis (319). Another study also found that subjects with histological NASH had significantly lower lowest desaturation, lower mean nocturnal oxygen saturation, and higher AHI compared with non-NASH controls (389).

1.5.5.5. Oxidative stress

Recurrent hypoxia and mitochondrial dysfunction in OSA results in the formation of ROS which results in cellular and DNA damage and oxidative stress (390). Oxidative stress has been implicated in IR and the impairment of insulin secretion (390-393). Many studies support OSA as a cause of oxidative stress (366;394-396). More detailed description of the impact of OSA on oxidative stress will be presented in the next section.

A summary of the mechanisms that relate OSA to T2DM are summarized in **Figure 1.17**.

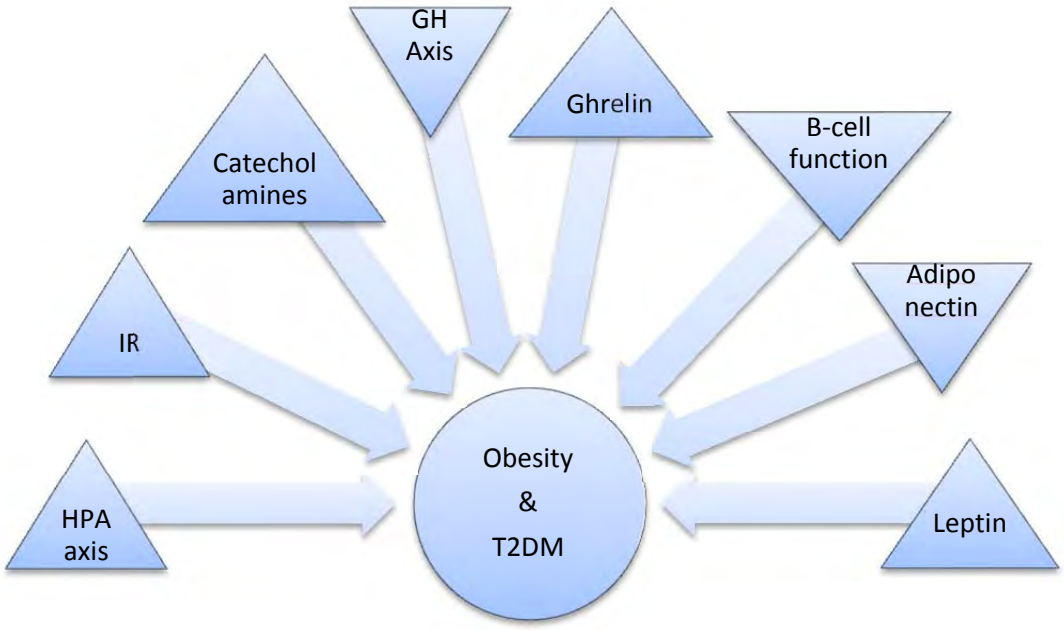


Figure 1-16: Hormonal Consequences of OSA.

The direction of the arrows represents the direction of change. IR: Insulin resistance.

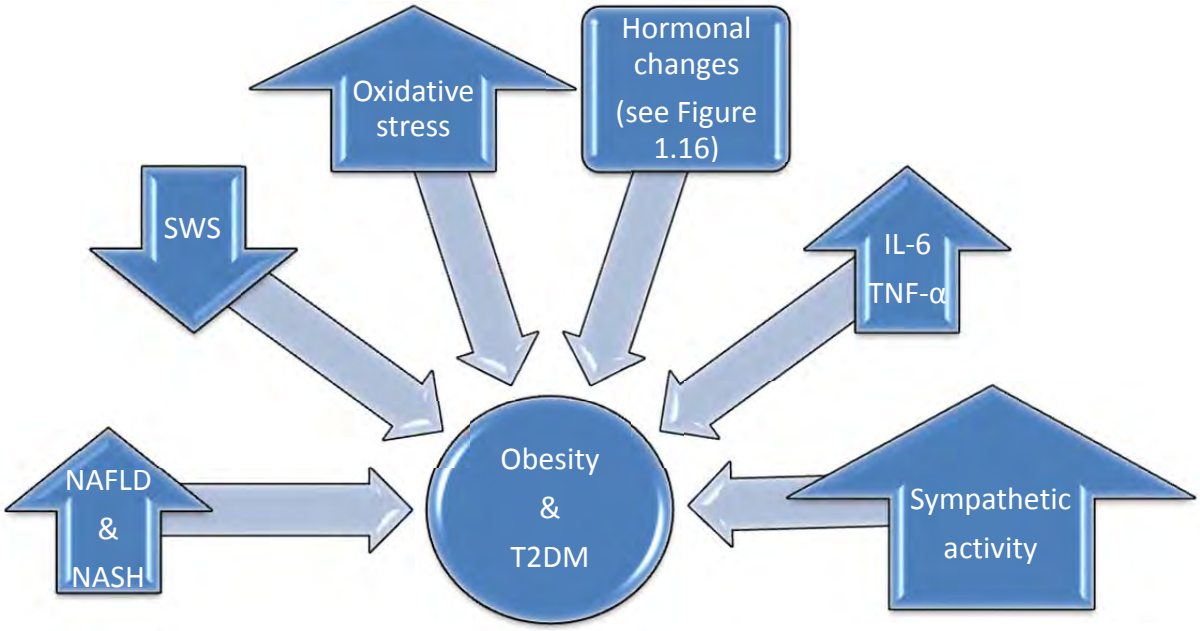


Figure 1-17: The mechanisms that relate OSA to the development of T2DM.

SWS: Slow wave sleep; NASH: Non-alcoholic steatohepatitis

1.6. OSA and Microvascular Complications in Patients with Type 2 Diabetes; A Hypothesis

I have discussed earlier in this thesis the pathogenesis of microvascular complications. Despite our improved understanding of the pathogenesis of these conditions and the improvement in metabolic control, these complications remain very common. Hence, better understanding of these conditions is vital in order to develop new treatments.

We have found that the OSA shares many of its molecular consequences with hyperglycaemia. We also found that OSA stimulates several of the pathways described in Figure 1. Hence, we hypothesised that OSA might be related to microvascular complications in patients with T2DM. In the section below I will detail the evidence that support our hypothesis.

1.6.1. OSA and hyperglycaemia

Hyperglycaemia is the main driver of the metabolic pathways that leads to microvascular complications. OSA is associated with hyperglycaemia and increased glucose variability as I discussed previously.

1.6.2. OSA and Hypertension

Hypertension is an important factor in the development and progression of microvascular complications. I have discussed earlier that OSA results in the development of sustained as well as nocturnal hypertension.

1.6.3. OSA and Advanced Glycation End-product

Hypoxia, which is one of the consequences of OSA, has been shown to result in increased AGE production and RAGE expression in animal and in-vivo studies (397;398). In addition, ischemia-

reperfusion injury (similar to the intermittent hypoxia that occurs in OSA) has been shown to increase the expression of RAGE (399). Only one study examined the association between AGE and OSA, and it found that AGE levels were higher in patients with OSA (AHI ≥ 5 events/hour) OSA compared to control but was less that those seen in patients with DM (400). Serum AGE levels correlated with OSA severity after adjustment for confounders (400).

1.6.4. OSA and Protein Kinase C Pathway:

Hypoxia and intermittent hypoxia have been shown to increase DAG levels (which drive the PKC pathway) In cell-based studies (401-406). Hypoxia seems to stimulate certain isoforms of the PKC family, particularly PKCα, PKCε and translocation of PKCδ (403).

As mentioned previously, PKC activation results in decreased eNOS production and increased ET-1, TGF- β, VEGF, PAI-1 and NF-KB. OSA has been shown to decrease eNOS and phosphorylated eNOS expression in freshly harvested venous endothelial cells in patients with newly diagnosed OSA (AHI ≥ 5 events/hour) by 59% and 94% respectively (407). CPAP treatment resulted is significantly increased expression of eNOS and phosphorylated eNOS (407).

The data regarding ET-1 in patients with OSA is not consistent and while some studies showed that OSA is associated with increased ET-1 levels (408-411), others did not (412;413).

Hypoxia is a major stimulant of VEGF production as angiogenesis is an important adaptive mechanism against ischemia (414;415). Hence, it is not surprising that OSA has been associated with increased VEGF levels in several studies (415-419). CPAP treatment lowers VEGF concentrations (415). VEGF levels correlate significantly with nocturnal desaturation (418).

OSA is also associated with hypercoagulability and increased levels of PAI-1 levels; however, whether OSA is an independent predictor of PAI-1 production remains controversial as many of the studies included small number of patients and they failed to control for a wide range of possible

confounders that are related to elevated PAI-1 levels (420-422). PAI levels were also found to correlate with OSA severity (422;423).

NF-KB is a pro-inflammatory transcription factor that is activated by PKC (see above) and that has been shown to be also activated in response to chronic intermittent hypoxia in cardiovascular tissue in vivo (366;424) and in vitro studies (425)and in human subjects (426). NF-KB has been proposed to play a central role in the relation between OSA and cardiovascular disease (427).

1.6.5. OSA and Hexosamine Pathway:

There is no data regarding the impact of OSA on the hexosamine pathway but OSA is related to some of the end results of this pathway such as PAI-1 as discussed above

1.6.6. OSA and Oxidative Stress

Repetitive episodes of re-oxygenation following hypoxia, as seen in OSA, simulate ischemia–reperfusion injury which results in the generation of ROS (428). Many markers have been used to demonstrate the relation between OSA and OS including: plasma, exhaled breath condensate and urinary 8-isoprostane levels; plasma levels of malondialdehyde (MDA); urinary o,o-dityrosine; plasma levels of TBARS206; urine levels of 8-hydroxy- 2'-deoxyguanosine (8-OhdG); and ROS production in monocytes, granulocytes, and neutrophils upon in vitro stimulation (366). IH has been associated with mitochondrial dysfunction, OS and increased ROS production (366;390;429;430). ROS levels have been shown to be 2-3 times higher in patients with OSA compared to healthy controls (390;431;432). In addition, multiple studies have shown increased oxidized lipids, DNA and carbohydrates in patients with OSA and animals exposed to intermittent hypoxia (390;394;433-438).

To summarize, hyperglycaemia increases ROS formation and oxidative stress. Oxidative stress plays a central role in the development of microvascular complications in patients with diabetes by increasing PARP activity and inhibition of GAPDH resulting in activating the AGE, PKC, hexosamine

and polyol pathways. OSA also results in ROS formation and cause OS. In addition, OSA stimulates the AGE and PKC pathways. OSA is very common in patients with T2DM and hence, OSA might play an important role in the development and/or progression of microvascular complications in patients with T2DM **(Figure 1.18)**.

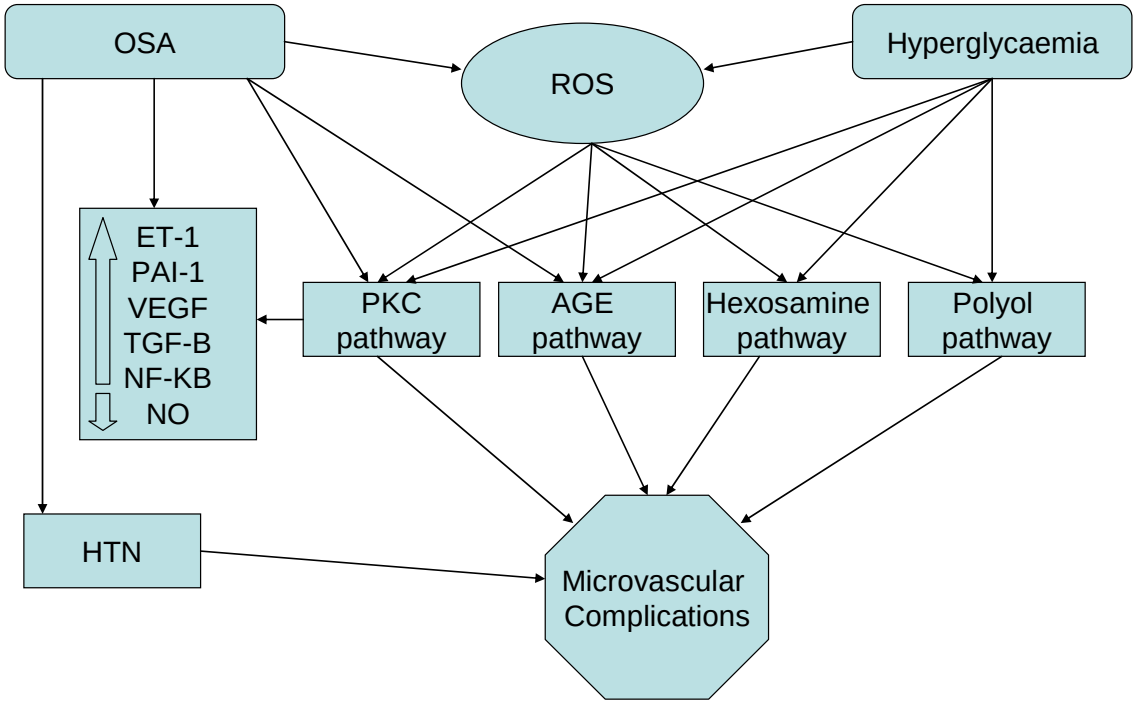


Figure 1-18: Possible mechanisms in which OSA can result in the development of microvascular complications

1.6.7. OSA and Microvascular Complications in Patients without Diabetes

If OSA stimulates the same pathways that are involved in the development microvascular complications as hyperglycaemia, as we described above, then patients with OSA (without diabetes) should develop such microvascular complications. In this section we review the literature regarding what is known about microvascular complications in patients with OSA without diabetes.

1.6.7.1. Nephropathy

OSA has been shown to be very prevalent in patients with end stage renal disease (30-80%) (439-441); however the relation between OSA and milder degree of renal impairment has only been studied in a very limited number of studies that showed conflicting results.

In a study of 505 men, OSA (defined as RDI ≥ 15 events/hour), serum cystatin-C correlated with RDI but this relation was lost after adjustment for possible confounders (442). Based on the Mayo clinic formula (and not MDRD), men with renal impairment had 2.1-fold greater odds of OSA even after adjustments for confounders (442). In a similar study by the same group lower eGFR (based on MDRD) was not associated with higher RDI (443).

In another in a cross-sectional study, of 91 obese adults, there was no significant difference in ACR between patients with vs. without OSA while significant difference existed in serum creatinine was significantly higher in the OSA group (444). Multivariate analysis showed that AHI was an independent predictor of serum creatinine in this study AHI (444).

In another study of 496 adults AHI was an independent predictor of ACR after adjustment for confounders (445). However, other studies which included smaller sample sizes showed no relation between proteinuria and OSA (446;447).

In order to address the issue of hypertension as a possible confounder for the effects of OSA on renal function a cross-sectional study of 62 untreated hypertensive patients with OSA and 70 hypertensive patients without OSA (AHI ≤ 5 events/hour), who were matched for a number of confounders, albuminuria was greater by 57% in patients with OSA vs. those without and ACR correlated with AHI and other markers of OSA severity (448). This relation remained significant following adjustment for confounders (448). This is supported by preliminary evidence suggesting that CPAP lowers the levels of ACR in OSA patients (449).

Despite the conflicting results, it seems that OSA is related to renal dysfunction and albuminuria independent of hypertension and BMI. Better designed cohort studies are needed.

1.6.7.2. Retinopathy

The relation OSA and retinopathy in patients without diabetes, has been studied scarcely in the literature. The main study that examined such a relation in a systematic way was the sleep heart health study (450). 2,927 subjects, aged 51 to 97 years, had retinal photographs taken and polysomnography performed (450). The overall prevalence of retinopathy was non significantly higher in people with higher RDI values (450). An increase of RDI from 0 to 10 was associated with a predicted decrease in arteriole-to-venule ratio of 0.01 (450). These results should be interpreted with caution as the participants had one 45° colour photograph of the retina of 1 randomly selected eye (DR is asymmetrical disease) was taken after 5 minutes of dark adaptation without the use of dilators (450).

OSA has been reported to be associated with other eye lesions such as OSA has been reported to be central serous chorioretinopathy, retinal vein thrombosis and ocular hypertension (451-454).

1.6.7.3. Peripheral Neuropathy

OSA and nocturnal hypoxia have been associated with the development of peripheral neuropathy in 3 small studies that included small number of participants ($n < 40$). In a case-control study, patients with severe OSA (AHI > 30 events/hour) had worse parameters (amplitude and velocity) during nerve conduction studies compared to age-matched controls (455). In another study, the prevalence of possible polyneuropathy was much higher in OSA patients (AHI≥ 10 events/hour) than that in age and BMI matched control (71% vs. 33%, $p<0.05$)(456). In addition, the amplitudes of the sensory nerve action potentials were significantly smaller in the OSA group (456). One small study of CPA showed that OSA treatment improves the amplitude of action potentials (457).

1.6.7.4. Autonomic Dysfunction

Amongst all microvascular complications, the evidence of association is strongest between OSA and CAN. CAN has been associated with OSA in subjects without diabetes (458).OSA has also been shown to be associated with increased sympathetic activity (359;377-380;383). It is likely that both, the recurrent hypoxia (377;381;382) and recurrent arousals (383) are contributing to the activation of the sympathetic system. Obese subjects with autonomic neuropathy develop more frequent and more prolonged hypopnoea/apnoea in comparison to obese subjects without autonomic dysfunction whether or not they had T2DM (459).

In summary, OSA activates the same pathways and has similar consequences to hyperglycaemia. Although the evidence is limited, OSA seems to be related to microvascular complications in patients without diabetes. OSA is very common in patients with T2DM and shares a major common risk factor i.e. obesity. It is plausible that OSA has an impact on the development and progression of microvascular complications in patients with T2DM. It is important to investigate such a relation as OSA treatment might have an impact on these costly complications to patients, society and health care delivery. Whether the addition of OSA to hyperglycaemia causes more oxidative stress and more activation of the AGE and PKC pathways compared to hyperglycaemia alone will need to be investigated.

1.7. Rationale of the project

As a result of the above mentioned, we have identified the following areas that needs further research:

1. Studies examining ethnic differences in microvascular complications are generally poorly designed and hardly ever offered any possible explanations for observed ethnic differences. Understanding the mechanisms behind ethnic differences is very important to improve the understanding the pathogenesis of microvascular complications.

2. The prevalence of OSA in South Asians with T2DM has never been reported before and whether there are ethnic and gender differences in OSA prevalence in patients with T2DM is unknown.
3. We have shown above that a relation between OSA and microvascular complications in patients with T2DM is plausible, but such a relation has never been explored before.
4. The impact of OSA on microvascular function and oxidative stress, which was examined in the general public, has not been examined in patients with T2DM. Hence, whether having OSA in patients with T2DM has any consequences, on top of that caused by hyperglycaemia, is unknown.

As a result I conducted a study to examine some of these areas. Due to the limited time and resources in regard to funding, staff and limitations of time, funding and staff, I could not conduct 4 different studies. So, we conducted a cross sectional study to answer the more important question (point 3 above) and we aimed to recruit similar numbers of South Asians and White Europeans so we can also answer 1 and 2 and we utilised the cohort recruited to answer point 4. Details of the design and methods are detailed in the second chapter.

2. Chapter two: Methods

2.1. Overview

As noted from the introductory chapter, we have identified several areas that need further research and exploration. In particular we identified the need for better understanding of the pathogenesis of microvascular complications. We have also shown the possible mechanisms that underpin our hypothesis that OSA could be related to diabetes-related complications and we identified the lack of data regarding the epidemiology of OSA in ethnic minorities with T2DM and the lack of data regarding the validity of the currently available OSA screening strategies in patients with T2DM. Furthermore we highlighted the lack of data to explain ethnic differences in microvascular complications that are reported in the literature.

Hence, we have conducted this project to increase our knowledge in regard to several of the above-mentioned points.

2.1.1. Hypothesis

Based on the above described information we hypothesised that:

1. OSA is associated with microvascular complications (nephropathy, retinopathy, peripheral neuropathy and autonomic neuropathy) in patients with T2DM.
2. OSA prevalence in South Asians with T2DM is different to that in White Europeans due to the differences in adiposity and fat distribution between the two ethnicities.
3. OSA is associated with increased oxidative stress and impaired microvascular regulation in patients with T2DM.

2.1.2. Primary aim:

Our primary aim is to assess the relationship between undiagnosed OSA and the presence and severity of the microvascular complications (nephropathy, retinopathy, peripheral neuropathy and autonomic neuropathy) in patients with T2DM.

2.1.3. Secondary aims:

1. To assess the relationship between ethnicity and microvascular complications in patients with T2DM and explore possible explanations for the observed ethnic differences (if any)

2. To assess the prevalence of OSA in patients with T2DM, particularly in South Asians as this has not been reported before.

3. To explore the possible underlying mechanisms for the relationship between OSA and microvascular complications (if such relationship is found).

2.1.4. Tertiary aims

1. To identify the clinical predictors of OSA in our patients and to assess the reliability of simple commonly used screening methods.

2. To assess the relationship between OSA and glycaemic control and cardiovascular risk factors in patients with T2DM.

2.1.5. Design and setting

We conducted an epidemiological, observational cross-sectional study of South Asians and White Europeans with T2DM. Ideally, to answer our primary aim, a cohort study of patients with T2DM, with and without OSA who are matched for a wide range of variables, would have been preferred. However, the limitation of resources (both financial and man power) and the need to perform this project within the 3 years time span of a PhD, which limits the time available to do prospective

studies, have persuaded us to perform a cross-sectional study. In order to improve on the design of our study, we have re examined our findings in a sub group of our study population (with and without OSA) which was matched for several variables (particularly in regard to obesity).

Patients were recruited from the out-patients diabetes clinic from two hospitals in the UK, Birmingham Heartlands Hospital (more than 80% of study participants) and the University Hospital of North Staffordshire. Birmingham Heartlands Hospital is one of the largest diabetes centres in the UK (24,000 patient visits/year from 8,500 patients with diabetes) and 40% of the clinic attendees are South Asians which allowed me to recruit from this difficult to reach ethnic group. Our diabetes centre serves an area that has a high proportion of South Asians, although the majority of the population are White Europeans. Both ethnicities live is the same geographical area, have similar deprivation scores and are referred to our clinic by primary care physicians using the same referral guidelines which are pre-agreed with our centre. Furthermore, there are no differences in clinic attendance rates between the ethnicities at our centre. Moreover our population has demographic characteristics similar to those described in previous reports in other localities in the UK (224). These factors suggest that our sample is representative. Ethnicity was determined using the UK decennial census by the study participants. Patients who originated from India, Pakistan, Sri Lanka or Bangladesh were considered to be of South Asian origin. Patients were approached in the waiting area of the outpatient department by I or Mrs. Safia Begum (a diabetes link worker), before they were seen by the clinicians and without looking at their notes or any prior knowledge of the patients circumstances. During the recruitment we avoided the use of any terms related to snoring. Patients were provided with the patient's information sheet and where contacted a week later to book the initial visit if the patient was agreeable.

For non-English speakers, and throughout the study, all communications were conducted by a researcher who is fluent in the participants' mother tongue language.

The project was approved by the Warwickshire Ethics committee and funded as part of a personal fellowship to myself from the National Institute for Health Research (NIHR). Further funding during the running of the project was obtained via competitive grant process from the UK Novo Nordisk Research Foundation and the Sanofi Aventis Excellence in Diabetes Award. The funding bodies had no influence on the design or the interpretation of the results of this study. All participants consented before joining the study.

2.1.6. Inclusion Criteria

1. Adult patients with T2DM
2. White Europeans and South Asian ethnic origins
3. Able to give consent

2.1.7. Exclusion Criteria

1. Past medical history of OSA or other sleep or respiratory disorder: as we do not know the impact of OSA treatment on our primary outcome, we have elected to exclude those participants. We have excluded patients with known respiratory disorders as they might be hypoxic and hence they might confound our results.
2. Sleeping tablets use: The use of sleeping tablets affects sleep architecture and hence might bias our results.
3. Pregnancy
4. Patients receiving dialysis: As end-stage renal disease impacts on patients sleep and is associated with fluid retention, cardiovascular disease and autonomic dysfunction, we excluded these patients in order not to confound our results (number excluded 7 patients).

We have kept our inclusion/exclusion criteria fairly liberal in order to facilitate recruitment and in order increase the external validity of our results.

In total, we have approached 691 patients, 425 were excluded (Type 1 diabetes (n=108), known OSA (n=8), known respiratory disorder, mostly asthma or COPD (n=93), ESRD (n=30), cancer patients (n=11), blind (n=9), declined to participate (n= 166)).

2.1.8. Data collected

1. **General and clinical:** Age, gender, ethnicity, diabetes duration, medications, past medical history, family history, smoking status, alcohol intake, education, employment.

2. **Anthropometry:** height, weight, Body mass index (BMI), waist circumference, hip circumference, waist–hip ratio (WHR), neck circumference, body composition, fat mass and bioimpedence. BMI, WC and hip circumference and neck circumference were measured twice and the average of the 2 measurements was entered to the database. Weight and body composition were measured using an automated body composition analyser (Tanita BC 420 S MA). Waist circumference was measured at the midpoint between the inferior border of the ribcage and the superior aspect of the iliac crest using an inelastic measuring tape (460). Hip circumference was measured horizontally at the widest circumference of the hips (461). Neck circumference was measured in the midway of the neck, between mid-cervical spine and mid-anterior neck, inelastic tape (462). In men with a laryngeal prominence (Adam's apple), it was measured just below the prominence (462). All circumferences were taken with the subjects standing upright, with the face directed straight, and shoulders relaxed (462).

3. **Metabolic:** BP, lipids, glycaemic control (HbA1c), thyroid function, liver function, bone profile (all these tests were performed in the respective hospital laboratories). Lipid profile, Electrolytes and urea were measured by colorimetric method (Roche diagnostics) and HbA1c was measured using high performance liquid chromatography (HPLC-Tosoh inc.) method. BP was measured by an automated device while the patient in sitting position and the left arm resting on a table. The 2

measurements were at least 10 minutes apart and the first measurement was after about 30 minutes after the start of the consultation. The average of the two readings was used in the database.

4. **Nephropathy:** Urinary albumin/creatinine ratio (ACR), estimated glomerular filtration rate (eGFR) and plasma urea and creatinine levels. These tests were performed in the respective hospital laboratories. ACR was estimated following measurement of urinary albumin by an immunoturbidimetric assay and urinary creatinine using a kinetic Jaffe assay.

5. **Retinopathy:** Assessed using the images from the retinal screening program.

6. **Peripheral Neuropathy Screening:** assessed using the Michigan Neuropathy Screening Instrument (MNSI) and the 10g monofilament test.

7. **Cardiac Autonomic dysfunction:** assessed using spectral analysis of heart rate variability.

8. **Sleep and Obstructive sleep apnoea:** Information regarding sleepiness and the risk of OSA were collected using the Berlin questionnaire and the Epworth sleepiness scale. Confirming the presence and the severity of OSA was done by performing home-based sleep study using a portable multi-channel respiratory device.

9. **Blood samples:** These were collected from consenting patients and were used to measure levels of 3-notrtyrosin (as a marker of nitrosative stress) and lipid peroxide (as a marker of oxidative stress). Plasma and serum samples were collected and were stored in -80 degrees freezer following centrifuge. These samples were collected following fasting when possible, but for patients who had recurrent hypoglycaemia, a non-fasting morning sample was collected.

10. **Microvascular assessment:** assessment of microvascular blood flow and endothelial function was performed by using Laser Speckle Contrast Imaging (LSCI) technique coupled with measurements of heating response and iontophoresis of acetylcholine (Ach) and Sodium Nitroprusside (SNP).

11. **Skin biopsies:** These were obtained to assess the intra epidermal nerve fibre density (IENFD), the presence of PAR in the nuclei and to semi-quantify pro-collagen 1 using immunostaining techniques. The biopsies were also examined for skin structure following staining with H&E.

All the data collected during one-to-one interview with the patient. The data were collected over 2-3 visits dependant o the time availability of the study participants. The blood samples, microvascular assessment and skin biopsies were performed in the 2nd or 3rd visit.

2.2. Nephropathy

Nephropathy was assessed using eGFR and ACR. Microalbuminuria is considered as the earliest clinical evidence of diabetic nephropathy and is defined as albumin excretion of ≥ 30 mg/day (463).

Albuminuria can be very variable and hence multiple measurements are required before confirming the diagnosis. In our study, as recommended by the guidelines, two abnormal early morning ACR measurements (out of three) within the last 6 months were required to diagnose microalbuminuria (463). An ACR > 2.5 mg/mmol in men and > 3.5 mg/mmol in women was considered abnormal in our study (464).

eGFR was calculated based on the MDRD equation (186 x (Creat / 88.4)$^{-1.154}$ x (Age)$^{-0.203}$ x (0.742 if female) x (1.210 if black), creatinine measured in mmol/L)(465). Diabetic nephropathy was diagnosed if eGFR was below 60 ml/min/1.73m^2 or ≥ 60 ml/min/1.73m^2 in the presence of albuminuria (465).

2.3. Retinopathy

DR was assessed using 2 x 45 degrees retinal images per eye as per the English National Screening programme guidelines (466). These images were obtained during the annual follow up at our diabetes clinic. Retinal images grading was performed in accordance with the English National Screening programme guidelines (R0: no retinopathy, R1: background retinopathy, R2: pre-proliferative retinopathy, R3: proliferative retinopathy, M0: no significant maculopathy, M1: maculopathy, P0: no photocoagulation, P1: photocoagulation) (466). A summary of the grades in the National screening programme can be found in **Table 2.1**. All images were first graded by a retinal grader. All abnormal images and 10% of normal images are then graded by a second grader. If discrepancy existed between the graders then a third senior grader/ophthalmologist would review the images and cast the final decision. In addition to the above process, all images were reviewed by myself and if I disagreed with any of the grades, Prof. Dodson (Consultant medical ophthalmologist and a Professor of medicine at Birmingham Retinal Screening Centre) would cast the final decision. All graders of the retinal images were blinded to the patient's OSA status. In patients who had doubtful lesions, the diagnosis of STDR made based on the assessment of the ophthalmologist in the outpatient department. In our study, we have definedSTDR as the presence of pre-proliferative or proliferative DR, maculopathy or photocoagulation (466). As the images were not done at the same time of the sleep study, the images date that is closer to the date of performing the sleep study (whether before or after the sleep study) was taken as the image to be entered in the database.

Table 2-1: Disease grading protocol in National Guidelines on Screening for Diabetic Retinopathy.

Adapted from(466).

Level 0	None	Lesions
Level 1	Background	Microaneurysm(s)
		Retinal haemorrhage(s) ± any exudate
Level 2	Pre-proliferative	Venous beading
		Venous loop or reduplication
		Intraretinal microvascular abnormality (IRMA)
		Multiple deep, round or blot haemorrhages
		(CWS—careful search for above features)
Level 3	Proliferative	New vessels on disc (NVD)
		New vessels elsewhere (NVE)
		Preretinal or vitreous haemorrhage
		Preretinal fibrosis ± tractional retinal detachment
Maculopathy (M)		Exudate within 1 disc diameter (DD) of the centre of the fovea
		Circinate or group of exudates within the macula
		Retinal thickening within 1 DD of the centre of the fovea (if stereo available)
		Any microaneurysm or haemorrhage within 1 DD of the centre of the fovea only if associated with a best VA of ≤ (if no stereo) 6/12
Photocoagulation (P)		Focal/grid to macula

2.4. Peripheral Neuropathy

In our study, we have assessed DPN using the Michigan Neuropathy Screening Instrument (MNSI) and the 10g monofilament sensation (as a marker of foot insensitivity).

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 (56;467-470). The questionnaire component (MNSIq) comprises 15 questions seeking to characterize sensory disturbance, but also peripheral vascular disease and general asthenia **(Figure 2.1a)**(467). 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 2.1b)**(467).

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.

In the MNSIe, 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), oedema and amputation. Having any abnormalities on inspection is scored as 1, while 0 is given for normal feet. Vibration Sensation was performed with the great toe unsupported. The tuning fork is placed over the dorsum of the great toe on the boney prominence of the Distal interphalangeal (DIP) joint. Patients, whose eyes are closed, will be asked to indicate when they can no longer sense the vibration from the vibrating tuning fork. In general, the examiner should be able to feel vibration from the hand-held tuning fork for 5 seconds longer on his distal forefinger than a normal subject can at the great toe (e.g. examiner's DIP joint of the first finger versus patient's toe). If the examiner feels vibration for 10 or more seconds on his or her finger, then vibration is

considered decreased. Vibration is scored as 1) present (score 0) if the examiner senses the vibration on his or her finger for < 10 seconds, 2) reduced if sensed for ≥ 10 (score=0.5) or 3) absent (score 1). The ankle reflexes are examined using an appropriate reflex hammer and was elicited in the sitting position with the foot dependent and the patient relaxed. For the reflex, the foot should be passively positioned and the foot dorsiflexed slightly to obtain optimal stretch of the muscle. The Achilles tendon should be percussed directly. If the reflex is obtained, it is graded as present (score 0). If the reflex is absent, the patient is asked to perform the Jendrassic maneuver (i.e., hooking the fingers together and pulling). Reflexes elicited with the Jendrassic maneuver alone are designated "present with reinforcement." (score 0.5). If reflex is absent, even in the face of the Jendrassic maneuver, the reflex is considered absent (score 1).

MNSI has been validated against the "gold standard" nerve conduction velocities. In the original publication, MNSI sensitivity and specificity were compared to the San Antonio Consensus Statement and the Mayo Clinic protocol (467). Of 29 patients with a clinical MNSIe > 2, 28 had neuropathy and 28 patients with an MNSIq of ≥ 7 had neuropathy (467). In another study, a MNSIe score > 2 had false positive and false negative values of 2.5%, specificity of 75% and sensitivity of 78.6% (471). In another study from Iran, a MNSIe score > 2 had a sensitivity of 65% and specificity of 83% compared to nerve conduction studies (472).

The MNSI also has been reported to have a high inter- and intra observer reproducibility (88.8% and 95% respectively) (471).

For the purpose of this study DPN was diagnosed if the MNSIe score was >2 and/or MNSIq score was ≥ 7 (469;473).

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 (473).

The sensitivity and specificity of the monofilament test varies considerably according to the populations studies and the methods used to diagnose DPN. In a recent study, the sensitivity of the monofilament test to diagnose clinically detectable DPN (diagnosed based on the DCCT protocol) was poor at 20%, while the specificity was 98% (473). The sensitivity of the monofilament test to predict amputations or foot ulceration, however, was much better at 62% with a specificity of 92% (473). A systematic review attempted to perform a meta-analysis to assess the sensitivity and specificity of the monofilament as a test for peripheral neuropathy, could not perform the meta-analysis due to the heterogenous nature of the studies. The sensitivity and specificity of these studies ranged from 41% to 93% and 68% to 100% respectively (474). Monofilament insensitivity, however, was found to be an independent predictor of foot ulceration in a prospective study of 1285 patients with diabetes free of ulcerations at baseline (OR 2.03, 95% CI 1.50 to 2.76)(475). Similar results were found by other studies (476;477).

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**	
	Absent	Present	Absent	Present
2. Ulceration	☐ 0	☐ 1	☐ 0	☐ 1

	Present	Present/ Reinforcement	Absent	Present	Present/ Reinforcement	Absent
3. Ankle Reflexes	☐ 0	☐ 0.5	☐ 1	☐ 0	☐ 0.5	☐ 1

	Present	Decreased	Absent	Present	Decreased	Absent
4. Vibration perception at great toe	☐ 0	☐ 0.5	☐ 1	☐ 0	☐ 0.5	☐ 1

Figure 2-1: The Michigan Neuropathy Screening Instrument

2.5. Cardiac Autonomic Neuropathy (CAN)

CAN was assessed using heart rate variability (HRV) and was analyzed using the continuous wavelet transform methods to generate numerical and graphical data using the ANX-3.0 software, ANSAR Inc., Philadelphia, PA. The R-R intervals were recorded and the HRV is plotted in the frequency domain to separate the respiratory frequency (Rfa, 0.15 to 0.4 Hz) from the low-frequency (Lfa, 0.04 to 0.15 Hz) components by spectral analysis. HRV and BP were recorded with the patient in sitting position during resting, deep breathing, Valsalva maneuver and standing position (478). Data

recorded included the E/I ratio, Valsalva ratio, 30:15 ratio, frequency domain analysis with respiratory adjustment (Lfa, Rfa and Lfa/Rfa), frequency domains without respiratory adjustment (very low frequency [VLF], low frequency [LF], high frequency [HF] and LF/HF ratio) and time domain analysis including standard deviation of normal sinus intervals (sdNN), square root of the mean of the squares of differences between adjacent normal sinus intervals (rmsSD), and percentage of differences between adjacent normal sinus intervals that are greater than 50 ms (pNN50).

For the purpose of this study, a diagnosis of CAN was made when 2 or more of the following tests were abnormal: E/I ratio, Valsalva ratio, 30:15 ratio and postural drop in BP (drop of 20mmHg in systolic or 10mmHg in diastolic BP) (479). Age-related normal values for E/I, Valsalva and 30:15 ratios were defined as previously reported (480).

The ANX incorporates the respiratory signal (ANSAR's patented MIT-based technology) into frequency analysis of HRV, which is very important to accurately locate parasympathetic activity. For example, during deep breathing the rate slows and parasympathetic activity moves into the low frequency area—0.04 - 0.10 Hz. The ANX also includes a proprietary algorithm using spline interpolation to correct for ectopic beats throughout the monitoring cycle.

The model of the computational methodology behind ANS monitoring is summarized in **Figure 2.2**. Conceptually, if the faster respiratory sinus arrhythmia signal and the slower mean heart rate changes could each be separated from the patient's cardiogram and analyzed independently, the result would yield a measure of Vagal outflow from the respiratory sinus arrhythmia and a measure of sympathetic activity from the changes in mean heart rate. Effectively this is what is accomplished in the frequency- or spectral-domain. Spectral analysis of respiratory sinus arrhythmia provides the indication of where in the frequency domain the Vagus is influencing the heart. This measure of Vagal outflow is labeled FRF: the Fundamental Respiratory Frequency. The FRF is equal to one over the breathing rate in breaths per minute in a normal, healthy individual. The FRF is then translated to the heart rate spectrum so that the respiratory frequency spectrum can be centered and the

parasympathetic activity computed. The parasympathetic activity is labeled the respiratory frequency area (RFa). From the low frequency portion of the heart rate spectrum the sympathetic activity (low frequency area, LFa) is computed by spectral analysis methods. Hence, the activity levels in both autonomic branches are computed non-invasively, independently, simultaneously, and quantitatively.

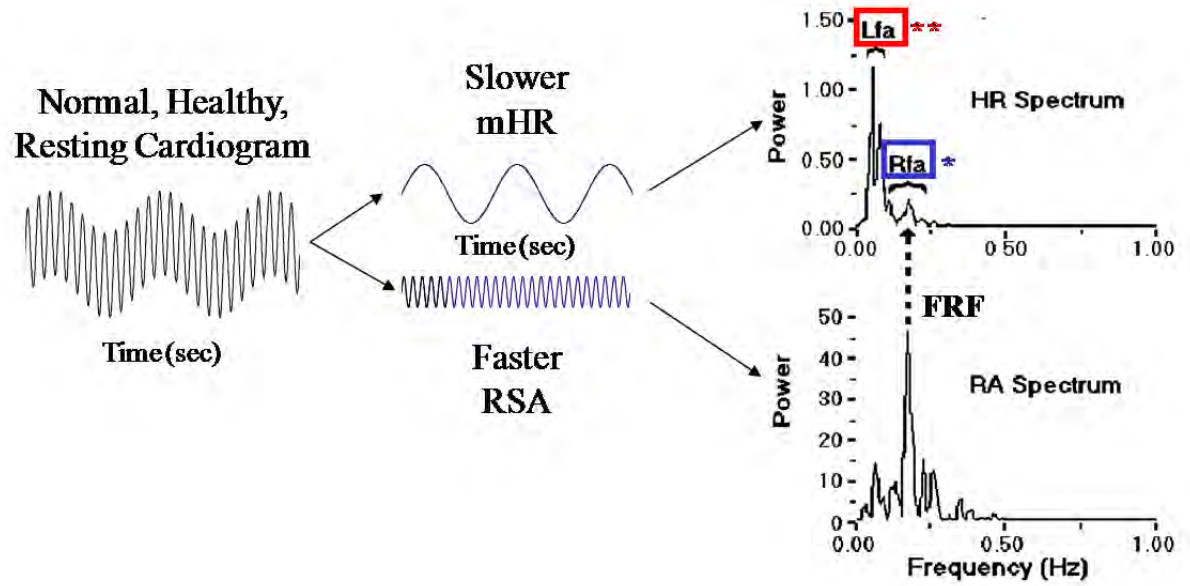


Figure 2-2: The principals of the ANX software.

mHR: mean heart rate, RSA: respiratory sinus arrhythmia, Rfa: parasympathetic, Lfa: sympathetic.

2.5.1. Parameters measured:

2.5.1.1. Fundamental Respiratory Frequency (FRF):

It is the frequency of the peak mode of the respiratory activity spectrum. In an otherwise healthy normal individual, FRF at rest is equal to one divided by the breathing rate in seconds per breaths. The FRF has been shown to be an indicator of the frequency range over which the parasympathetic nervous system is influencing heart rate control. This frequency is translated from the respiratory

activity spectrum to the heart rate spectrum to determine parasympathetic power from the RFa. The normal range for FRF during deep breathing is 0.09 to 0.15 Hz for 6 breaths per minute.

2.5.1.2. Low Frequency Area (LFa)

It is the area under the heart rate spectral curve over the frequency range from 0.04 Hz to 0.10 Hz or the lower limit of the RFa range. It is the power or tone of the sympathetic nervous system as mediated or driven by the parasympathetic system. At Baseline and during Standing the LFa is mostly sympathetic. During Deep Breathing, Valsalva, and the intervening baselines, the LFa is a changing mix of sympathetic and parasympathetic. During the initial baseline the LFa is should be between 0.5 and 10.0. An initial baseline LFa below 0.1 is a sign of cardiac sympathetic denervation. During the Valsalva challenge the LFa is expected to be > 28.0 for young healthy individuals, but it is age related.

2.5.1.3. Respiratory Frequency Area (RFa)

It is the area under the heart rate spectral curve over a frequency range centered on the FRF. The RFa is a measure of the parasympathetic power. This relationship holds regardless of the subject's respiratory activity, or whether the subject is free breathing or mechanically ventilated. During the baseline the RFa is expected to be > 0.5. An initial baseline RFa below 0.1 is generally accepted as a sign of cardiac parasympathetic denervation. During the Deep Breathing challenge the RFa is expected to be > 28.0 (approximately) for young healthy individuals, but it is age related

2.5.1.4. Lfa/Rfa Ratio

It is a measure of the Sympathovagal balance. At rest and while awake the Ratio in a young, healthy, normal patient should be near 2.0 in an otherwise normal and healthy individual; while asleep the Ratio should be about 0.5; the normal range is 0.4 to 3.0.

2.5.1.5. Other measures:

- **VLF**: The average European-standard Very Low Frequency area (milliseconds^2/Hz) for each phase of the study. It is a mixed measure of Barroreceptor reflex activity and Vascular oscillation activity.

- **LF**: The average European-standard Low Frequency area (milliseconds^2/Hz) for each phase of the study. It is a mixed measure of parasympathetic and sympathetic activity.

- **HF**: The average European-standard High Frequency area (milliseconds^2/Hz) for each phase of the study. It is a relative measure of parasympathetic activity.

- **LF/HF**: The average European-standard Ratio (unitless) for each phase of the study. It is an indication of sympathetic activity.**LF nu**: The average European-standard normalized Low Frequency area (LF/(LF+HF), unitless) for each phase of the study.

- **HF nu**: The average European-standard normalized High Frequency area (HF/(LF+HF), unitless) for each phase of the study.

- **TSP**: The average European-standard Total Spectral Power (LF+HF, milliseconds^2/Hz) for each phase of the study. It is an indication of total ANS activity.

- **sdNN**: The European-standard sample difference of the beat-to-beat (NN). A measure of HRV in milliseconds; more is better.

- **rmsSD**: The European-standard root mean square of sample difference in milliseconds. A measure of changing HRV; more is better.

- **pNN50**: The European-standard percent of consecutive beat-to-beat intervals that are greater than 50 milliseconds long (%). A measure of changing HRV; more is better

2.5.2. The report

A copy of the report generated by the ANX is in **Figure 2.3**.

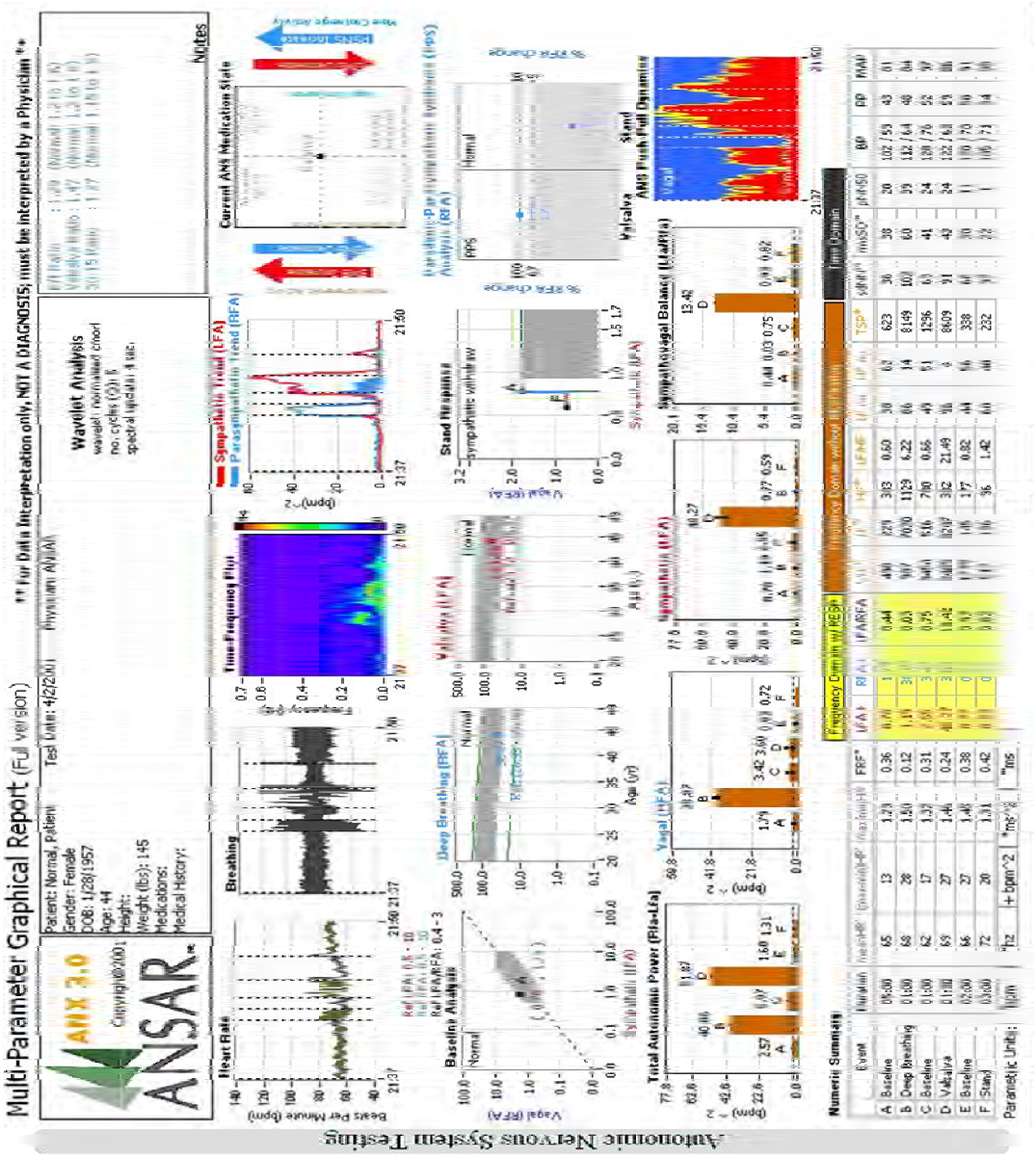


Figure 2-3: A copy of the report of the CAN test form a normal patient.

The results show "Sympathetic Withdrawal" during Stand. This is a false positive resulting from informing the patient that s/he is about to stand several seconds prior to standing. When this happens the nervous system begins to increase HR before standing in order to defeat orthostasis. The computer cannot read minds!! This is a graphical depiction of why the request to "Stand quickly" must not be given until the computer begins the stand portion of the clinical exam.

The top row from left to right:

- Patient demographics, medications, medical history, and signal processing and analysis technique.
- The standard ANS test ratios (E/I Ratio as a measure of ANS response to the Deep Breathing challenge, Valsalva Ratio, and the 30:15 Ratio as a measure of ANS response to the Stand challenge), notes from the technician indicating any anomalies during the study, and a count of the possible premature beats as detected from the ECG during the study.

The second row from left to right:

- A plot of the heart rate variability over the time course of the study, the more variability the better, to a point.
- A plot of the respiratory activity over the time course of the study, it provides the physician a means for validating the administration of the ANS study. The depth of respiration should increase during the deep breathing phase as compared to initial baseline. Again, the depth of respiration should increase during the Valsalva phase as compared to baseline and the individuals Valsalvas should be discernable.
- A 3-D (color) plot of the changing heart rate spectrum over the time course of the study. It contains all of the information in one plot.
- The "Trends Graph". A plot of the continuous, instantaneous LFa and RFA changes over the time course of the study. It displays the instantaneous changes in LFa and RFa throughout the study. It provides more detailed information than is available in the (average) numbers alone; see the yellow highlighted section of the table at the bottom of the report.

The third row from left to right:

- The “Baseline Analysis” plot. This plot aids in assessing balance between parasympathetic and sympathetic powers. The broken line indicates a Ratio of 1.0 for all values of LFa and RFa. Inside the gray area is normal, it indicates a ratio between 0.5 and 2.0. Outside the gray area but near the broken line is abnormal, but balanced.

- The “Deep Breathing Response (RFa)” plot. It aids in assessing parasympathetic responsiveness when challenged. The Deep Breathing RFa response is plotted against age. The solid black line indicates a normal RFa response adjusted for age. The gray area is within one standard deviation of the normal data and is still considered normal.

- The “Valsalva Response (LFa)” plot. It aids in assessing sympathetic responsiveness when challenged. The solid black line indicates a normal LFa response adjusted for age. The gray area is within one standard deviation of the normal data and is still considered normal.

- The “Stand Response” plot. It aids in assessing total autonomic responsiveness when challenged. RFa is plotted against Ratio (LFa/RFa). ‘A’ indicates the Initial Baseline LFa response and ‘F’ indicates the Stand LFa response. Inside the gray area is normal. Outside the gray area can indicate parasympathetic excess, Oothostatic Hypotension or some form of Syncope.

- The “ANS Push-Pull Dynamics” Plot. It displays the continuous, instantaneous changes in Ratio throughout the study.

The fourth row from left to right:

- A bar graph of the average “Total Autonomic Power (LFa + RFa)” as computed for each of the six challenges during the study. Total power should: 1) ↑ significantly for Deep Breathing (due to an RFa increase only), 2) ↑ significantly for Valsalva (due to an LFa increase only), 3) Stay the same or ↑ slightly for Standing (due to an LFa ↑ and a RFa ↓).

- A bar graph of the average parasympathetic power (RFa)" as computed for each of the six challenges during the study. RFa should: 1) ↑ significantly for Deep Breathing, 2) Stay the same or ↓ slightly for Valsalva, 3) Stay the same or ↓ for Standing.

- A bar graph of the average "Sympathetic power (LFa)" as computed for each of the six challenges during the study. LFa should: 1) Stay the same or ↓ slightly for Deep Breathing, 2) ↑ significantly for Valsalva, 3) ↑ for Standing.

- A bar graph of the average "Sympathovagal Power (LFa/RFa)" as computed for each of the six challenges during the study. Ratio should: 1) ↓ for Deep Breathing, 2) ↑ significantly for Valsalva, 3) ↑ for Standing

The fifth row from left to right:

A table showing the numerical results of the study for each of the six phases of the study including: the study phase, duration, mean heart rate, range heart rate (a measure of heart rate variability; the maximum minus the minimum heart rate (in beats per minute) for each phase of the study. Normal Ranges: Resting = 10 to 50 bpm; Deep Breathing, Valsalva, and Stand = 15 to 50 bpm), FRF, Lfa, Rfa, Lf/Rfa, VLF, LF, HF, LF/HF, LF nu, HF nu, TSP, sdNN, rmsSD, pNN50, blood pressure and mean arterial pressure (⅓Systolic+⅔Diastolic).

2.5.3. The protocol

Patients were instructed not to consume any caffeinated drinks prior to the test. The test is performed while the patients in sitting position. The operator has to connect the cardiac monitor to the patients as in **Figure 2.4**, place the BP cuff on and guide the patient through the different stages of the test (Baseline-5 minutes, deep breathing- 1minute, baseline-1 minute, Valsalva manoeuvre-2 minutes, baseline-2 minutes and Standing- 5 mintues). The Valsalva manoeuvre was performed by asking the patient to blow into a syringe connected to a gauge. We asked the patient achieve a pressure of 20mmHg and maintain it for about 10-15 seconds. If artefacts in hear or respiratory

rates appear, then the operator has to make sure there is no electrical interference from other equipment nearby (including mobile phones) and check that the leads are attached firmly to the skin. If all fail, then re-position the leads and re-run the test. A particular problem is the loss of lead contact with the skin following the Valsalva manoeuvre (too much blowing!), so the operator has to make sure all leads are firmly attached after the Valsalva.

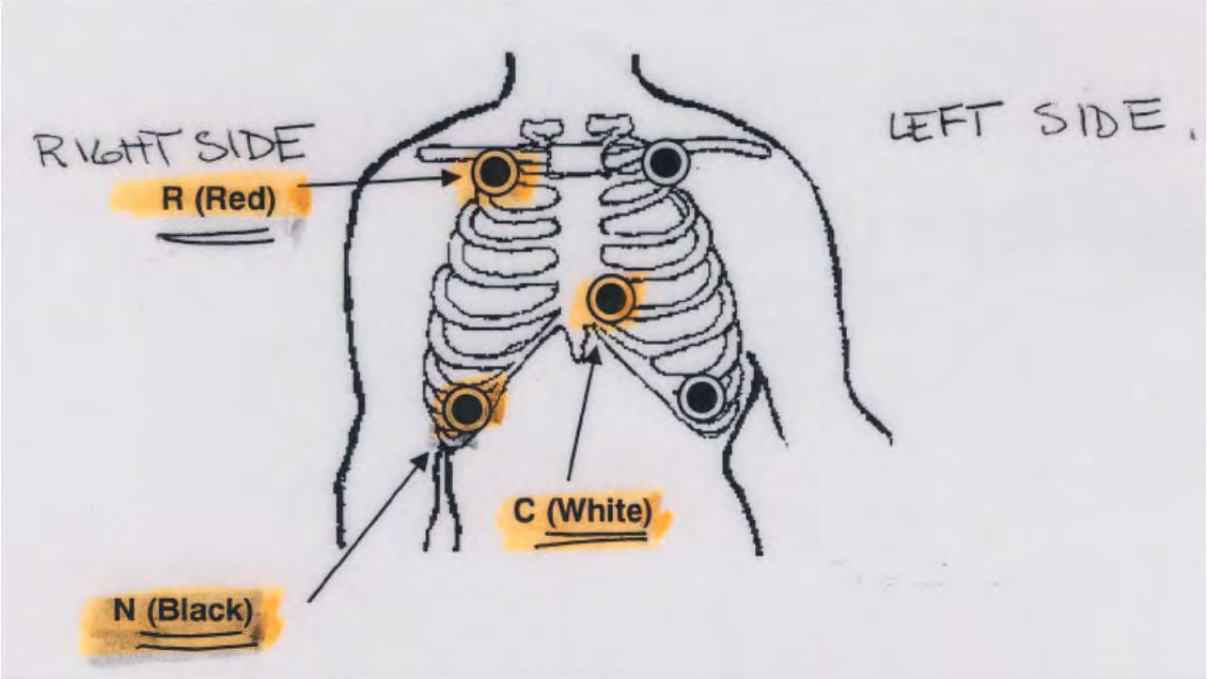


Figure 2-4: Lead positions for the CAN test.

2.6. Obstructive Sleep Apnoea Assessment

2.6.1. The Berlin Questionnaire

The Berlin Questionnaire was an outcome of the Conference on Sleep in Primary Care, which involved 120 U.S. and German pulmonary and primary care physicians and was held in April 1996 in Berlin, Germany (481). Questions were selected from the literature to reflect factors or behaviours that consistently predicted the presence of OSA (481). By consensus, the instrument focused on a limited set of known risk factors for OSA; one introductory question and four follow-up questions concern snoring; three questions address daytime sleepiness, with a sub-question about sleepiness

behind the wheel and one question concerns history of high blood pressure and obesity (based on BMI) (481).

A copy of the Berlin questionnaire can be found in **Figure 2.5**. The questionnaire is scored as follows:

Patients can be classified into High Risk or Low Risk based on their responses to the individual items and their overall scores in the symptom categories.

Categories and scoring protocol:

Category 1: items 1, 2, 3, 4, 5.

Item 1: if '**Yes**', assign **1 point.** Item 2: if '**c**' or '**d**' is the response, assign **1 point**

Item 3: if '**a**' or '**b**' is the response, assign **1 point.** Item 4: if '**a**' is the response, assign **1 point**

Item 5: if '**a**' or '**b**' is the response, assign **2 points**

Add points. Category 1 is positive if the total score is 2 or more points

Category 2: items 6, 7, 8 (item 9 should be noted separately).

Item 6: if '**a**' or '**b**' is the response, assign **1 point.** Item 7: if '**a**' or '**b**' is the response, assign **1 point**

Item 8: if '**a**' is the response, assign **1 point**

Add points. Category 2 is positive if the total score is 2 or more points

Category 3 is positive if the answer to item 10 is 'Yes' OR if the BMI of the patient is greater than 30kg/m^2.

High Risk: if there are 2 or more Categories where the score is positive

Low Risk: if there is only 1 or no Categories where the score is positive

The Berlin questionnaire performance in diagnosing OSA has varied a lot in the literature; this variation is due to several factors including the population studied (primary care vs. secondary care, healthy population vs. high risk population such as patients with coronary artery disease), the gold standard test (portable home-based studies vs. inpatient polysomnography), definition of OSA and the cut-offs used (AHI vs. RDI and 5 vs. 10 vs. 15 vs. 30 events/hour) and technical and methodological differences in the conduction of studies (number of nights over which the sleep study performed, the minimum acceptable sleep duration, definition of hypopneas etc.). In one primary care study of 744 participants that used portable home-based polysomnography as the gold standard, OSA based on RDI >5, >15 and >30 had a sensitivity of 86%, 54% and 17% respectively and a specificity of 77%, 97% and 97% respectively (481). The PPV was 89%, 97% and 92% respectively (481).

The study has been validated in multiple ethnicities. In a study in South Asians, using a modified Berlin questionnaire in which the cut of BMI was reduced to 25 instead of 30 kg/m^2, the sensitivity, specificity and PPV of the Berlin questionnaire were 86%, 95% and 96% respectively (482). In this study, the gold standard was in patient polysomnography (482).

In our project we have scored the Berlin questionnaire as outlined above with the modification of lower BMI in South Asians

The Berlin questionnaire has been validated as a screening tool for OSA in a variety of medical conditions such as myocardial infarction and stroke amongst others (483;484). Data regarding the validity of the Berlin questionnaire in patients with diabetes is lacking.

BERLIN QUESTIONNAIRE

Height (m) _____ Weight (kg)_____ Age_____ Male / Female

Please choose the correct response to each question.

CATEGORY 1

1. **Do you snore?**
   - ☐ a. Yes
   - ☐ b. No
   - ☐ c. Don't know

If you snore:

2. **Your snoring is:**
   - ☐ a. Slightly louder than breathing
   - ☐ b. As loud as talking
   - ☐ c. Louder than talking
   - ☐ d. Very loud – can be heard in adjacent rooms

3. **How often do you snore**
   - ☐ a. Nearly every day
   - ☐ b. 3-4 times a week
   - ☐ c. 1-2 times a week
   - ☐ d. 1-2 times a month
   - ☐ e. Never or nearly never

4. **Has your snoring ever bothered other people?**
   - ☐ a. Yes
   - ☐ b. No
   - ☐ c. Don't Know

5. **Has anyone noticed that you quit breathing during your sleep?**
   - ☐ a. Nearly every day
   - ☐ b. 3-4 times a week
   - ☐ c. 1-2 times a week
   - ☐ d. 1-2 times a month
   - ☐ e. Never or nearly never

CATEGORY 2

6. **How often do you feel tired or fatigued after your sleep?**
   - ☐ a. Nearly every day
   - ☐ b. 3-4 times a week
   - ☐ c. 1-2 times a week
   - ☐ d. 1-2 times a month
   - ☐ e. Never or nearly never

7. **During your waking time, do you feel tired, fatigued or not up to par?**
   - ☐ a. Nearly every day
   - ☐ b. 3-4 times a week
   - ☐ c. 1-2 times a week
   - ☐ d. 1-2 times a month
   - ☐ e. Never or nearly never

8. **Have you ever nodded off or fallen asleep while driving a vehicle?**
   - ☐ a. Yes
   - ☐ b. No

If yes:

9. **How often does this occur?**
   - ☐ a. Nearly every day
   - ☐ b. 3-4 times a week
   - ☐ c. 1-2 times a week
   - ☐ d. 1-2 times a month
   - ☐ e. Never or nearly never

CATEGORY 3

10. **Do you have high blood pressure?**
    - ☐ Yes
    - ☐ No
    - ☐ Don't know

Figure 2-5: The Berlin questionnaire

2.6.2. Epworth Sleepiness Score

The Epworth Sleepiness Score (ESS) was proposed as a measure of day time sleepiness (485). The multiple sleep latency test (MSLT) is also commonly used to assess daytime sleepiness, but as it is more time consuming and costly, the ESS offered a more practical alternative (486). It must be noted here that ESS measures excessive daytime sleepiness and not OSA; however, as OSA is a major cause of excessive daytime sleepiness, ESS was used as a screening method for OSA.

The ESS **(Figure 2.6)** is a brief, self-administered questionnaire that asks the patient to rate on a scale of 0 to 3 that chances that he/she would have dozed in 8 commonly encountered every day activity (486). Patents are asked to distinguish dozing from feeling tired. The ESS score can range between 0 and 24 (486).

ESS correlated significantly with sleep latencies measured during MSLT ($r=-0.5$, $p<0.01$) (485). ESS also correlated with different measures of OSA severity (486). ESS scores are higher in subjects with OSA compared to healthy people, and the higher ESS scores return to normal following CPAP treatment (485-487).

Total ESS is reliable in a test-retest sense over a period of months ($rho = 0.82$, $p < 0.001$) and has a high level of internal consistency (486). The reported sensitivity of ESS (> 10) to detect OSA (AHI ≥ 5) is about 66% (488). ESS was translated to several languages, data regarding the use of ESS in OSA patients with diabetes is lacking.

In this project an ESS > 10 was considered to be consistent with excessive daytime sleepiness (485).

Epworth Sleepiness Scale

Name: _____ Today's date: _____

Your age (Yrs): _____ Your sex (Male = M, Female = F): _____

How likely are you to doze off or fall asleep in the following situations, in contrast to feeling just tired?

This refers to your usual way of life in recent times.

Even if you haven't done some of these things recently try to work out how they would have affected you.

Use the following scale to choose the **most appropriate number** for each situation:

0 = would **never** doze
1 = **slight chance** of dozing
2 = **moderate chance** of dozing
3 = **high chance** of dozing

It is important that you answer each question as best you can.

Situation	**Chance of Dozing (0-3)**
Sitting and reading _____	___
Watching TV _____	___
Sitting, inactive in a public place (e.g. a theatre or a meeting) _____	___
As a passenger in a car for an hour without a break _____	___
Lying down to rest in the afternoon when circumstances permit _____	___
Sitting and talking to someone _____	___
Sitting quietly after a lunch without alcohol _____	___
In a car, while stopped for a few minutes in the traffic _____	

THANK YOU FOR YOUR COOPERATION

Figure 2-6: The Epworth Sleepiness Score

2.6.3. Portable Polysomnography

Home-based sleep studies were introduced as results of the large number of patients that need to be assessed for OSA and the rather limited facilities to have in-patients polysomnography (287). Home-based sleep studies do offer the advantage that the patents are sleeping in their own environment, and one report suggested better patient satisfaction (287). However, The lack of technician supervision means that dislodged leads are not replaced during the study, and consequently the likelihood of technically unsatisfactory studies is higher (287).

Home-based sleep studies were used in research as they allow the screening of large numbers at minimal cost. The sleep Heart Health study was the leading research project that has used portable home-based sleep studies (albeit the equipment at the time was much larger than now!) (489).

In our study we have used the Alice PDX (Philips Resporinics, USA) device to assess the presence of OSA **(Figure 2.7)**.

Figure 2-7: The Alice PDX

This equipment is a portable multi-channel diagnostic device that is used to perform home-based sleep studies to diagnose OSA and examine sleep structure and stages. The channels recorded are: flow pressure (via oral and nasal cannula), oral pressure (via thermistor), snoring signal (via the pressure flow oral/nasal cannulas), abdominal and thoracic movements (via zRIP effort belts), average and beat-to-beat oxygen saturations (via an oximeter), pulse rate and waveform (via an oximeter), body position (via 3D body position sensor), ECG, EEG and electro-oculogram (EOG). The device is operated by 3AA batteries. The machine is the size of the hand (12.7cm x 7.62cm x 5.08cm), lightweight (230 grams) and has colour coded input for easy hook-up. The recording can start and finish automatically (without the need of the patient to press any buttons) at a time that has been set-up via the machine software. Data are downloaded from the machine via a USB cable and scored manually using specific software (Sleepware).

The Alice PDX is compatible with AASM guidelines regarding portable home-based sleep studies. Alice PDX was compared to "Gold standard" in-patient polysomnography; the AHI correlated very well between the 2 methods ($r=0.93$, $p<0.001$) and the Bland-Altman plot suggested consistency between the 2 devices (http://www.healthcare.philips.com/asset.aspx?alt=&p=http://www.healthcare.philips.com/pwc_hc/main/homehealth/sleep/alicepdx/PDF/alicepdx_White_Paper_20091027.pdf)

In our study, the patient were taught how to fit the device on their own **(Figure 2.8)** and the device was pre-programmed to start recording at 10pm and to stop recording and 8am. The machine was returned by a taxi next morning (other times were used for specific patients). Patients were also asked to fill a sleep diary during that night to tell us about sleep time, wake up time, any interruptions to sleep and whether they had any problems with the machine.

Figure 2-8: The Alice PDX after being worn.

After receiving the machine, data was downloaded by the software and the resulting traces were analysed **(Figure 2.9)**. Analysis was performed in accordance with the AASM guidelines (230). Sleep studies with <4 hours of adequate recordings were repeated and excluded if the quality remained poor. Patients with predominantly CSA were excluded. An AHI ≥ 5 events/hour was consistent with the diagnosis of OSA (231). OSA severity was assessed based on the AHI, ODI (the number of oxygen desaturations of ≥ 4% per hour), the time spent with oxygen saturations < 90% and the nadir oxygen levels during sleep.

All sleep studies were double scored by Dr. Asad Ali (Consultant in respiratory medicine at the University Hospital of Coventry and Warwickshire) and I. If our scores differed by > 10% or we have classified the patient into different categories (even if the AHI difference was < 10%), then the sleep study was scored for a third time either by Dr. Ali or by Mr. Nicholls (senior Sleep technician at Birmingham Heartlands Hospital).

Studies in the literature excluded patients with inadequate recording duration from analysis, but the definition of inadequate varied between 2 and 6 hours. We have elected to choose 4 hours as the

cut off (which the case in the majority of the literature). The logic for excluding patients with sleep studies of less than 4 hour duration is that most apnoea/hypopnea events occur during transition from wakefulness to sleep and during REM sleep. In normal adults, REM occurs in more frequency and more prolonged duration during the second half of the night. Hence, in order not to interpret a sleep study as being "normal" before the patient enters REM sleep, we excluded patients with less than 4 hours of recording. However, patients with a recording < 4 hours who clearly had OSA were included in the analysis. In accordance with the AASM, apnoea was defined as cessation or ≥ 90% reduction in airflow for a period of at least 10 seconds. Hypopnea was defined as ≥ 30% reduction in airflow for ≥ 10 seconds associated with ≥ 4% drop in oxygen saturations. Apneas were classified into obstructive or central based on the continuation/presence of or absence of thoracic and abdominal efforts respectively.

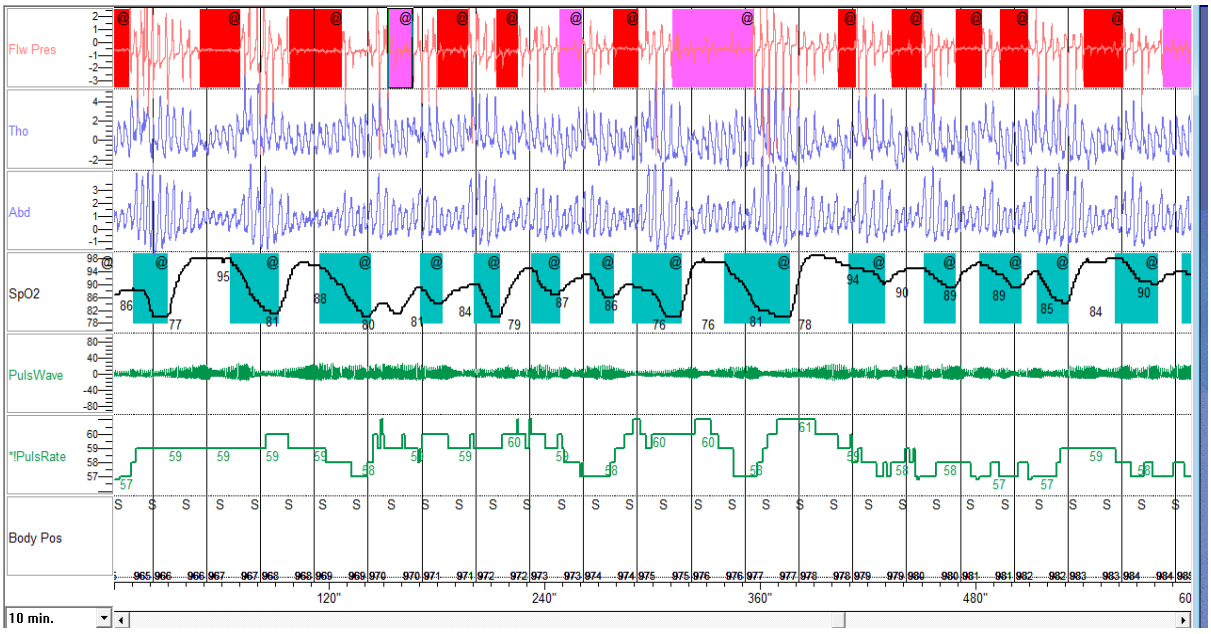


Figure 2-9: A screen shot of the data downloaded from Alice PDX showing evidence of apnoea, hypopnea and oxygen desaturations.

2.7. Serum Nitrotyrosine

The modification of tyrosine residues in proteins to 3-nitrotyrosine by peroxynitrite **(Figure 2.10)** or other potential nitrating agents has been detected in biological systems that are subject to nitrosative stress (490). 3-Nitrotyrosine is formed after a hydrogen ion is removed from tyrosine (to form tyrosyl) which interact with peroxynitrite to form 3-nitrotyrosine (490). While all tyrosine residues in proteins may theoretically be targets for nitration, the efficiency of tyrosine nitration is dependent on various biological conditions such as the local production and concentration of the reactive species, the existence and availability of antioxidants and scavengers, the accumulation of inflammatory cell and the presence of pro-inflammatory cytokines, as well as the proximity of these components (490). Peroxynitrite has been shown to be involved in the modification of a wide spread range of proteins and it disrupts several signalling pathways and has been implicated in a wide range of disorders, particularly cardiovascular disease, diabetes and their complications (490).

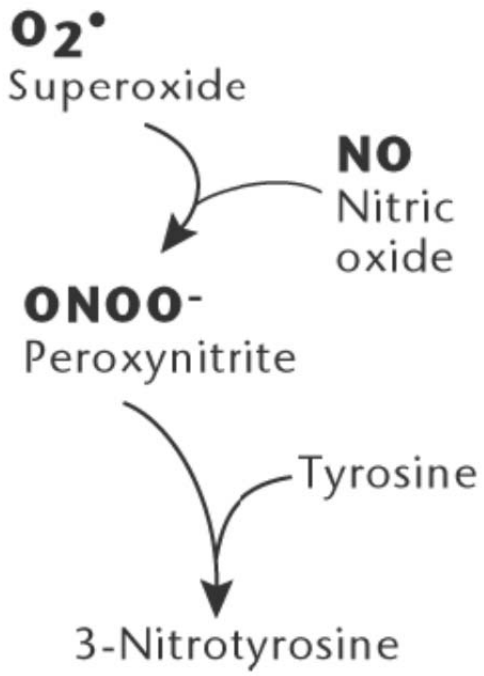


Figure 2-10: Nitrotyrosine formation

We have used a commercially available kit to emasure serum 3-nitrotyrosine levels (Oxiselect™, Cell Biolabs Incl). Cell Biolabs' Nitrotyrosine ELISA Kit is a competitive enzyme immunoassay developed for rapid detection and quantitation of 3-nitrotyrosine in protein sample. The quantity of 3-nitrotyrosine in protein sample is determined by comparing its absorbance with that of a known nitrated BSA standard curve. The kit has a nitrotyrosine detection sensitivity range of 20 nM to 8.0 µM.

The procedure was conducted in accordance with the manufacturer instructions. An example of the Nitrotyrosine ELISA curve provided by the manufacturer is in **Figure 2.11**. An example of the standard curve obtained by myself is in **Figure 2.12**.

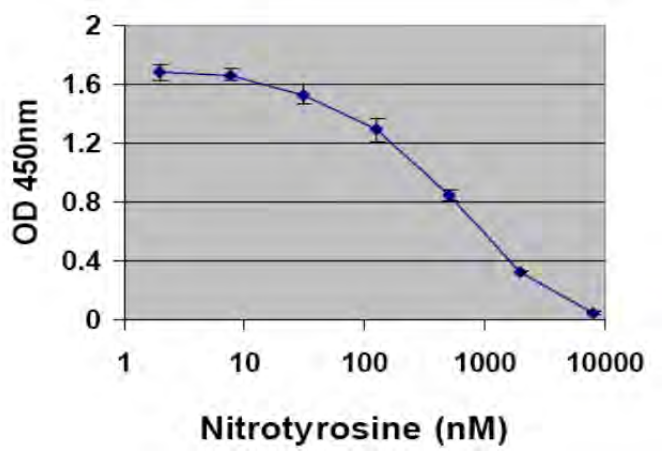


Figure 2-11: Nitrotyrosine ELISA curve provided by the manufacturer

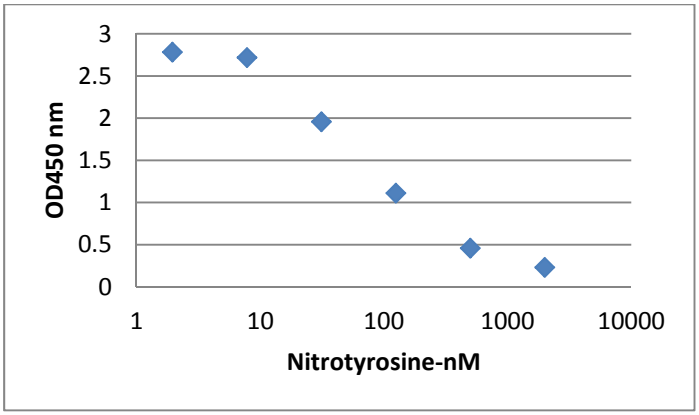


Figure 2-12: Nitrotyrosine ELISA standard curve from my plates.

2.8. Serum Lipid Peroxide

Samples were analysed for lipid peroxides using a modification of a method by el-Saadani et al (491).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.

I made up the reagent mix containing: 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).

I added 200ul of sample/blank to a cuvette and 2000ul of reagent mix, then incubated the mixture 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.

2.9. Skin Biopsy

Skin biopsies were performed in accordance to previously published protocols (492). Skin biopsies were done using 3-mm punch biopsy needles. The area was cleaned with alcohol and covered with a sterile sheet. Local anaesthetic (lignocaine 2%) was injected subcutaneously and 3X 3mm punch biopsies were taken. Then we applied pressure on the biopsy site to stop bleeding and the biopsy site was covered with an anti-septic and dressings. Patients were given extra dressings and told to contact myself if they have any worries or there were any signs of infections. Patients were instructed not wash the biopsy site for 24-hours. Pictures and safety visits were done on 1, 3, and 10 days post biopsy.

Biopsies were immersed in MCDB-153 (Sigma-Aldrich, Gillingham, Dorset, UK) culture medium containing 1.4mM $CaCl_2$ and 6mM glucose immediately after the biopsy was taken and was transferred to the university on ice. Two biopsies were cultured and one biopsy was frozen.

2.10. Frozen sections protocol

The biopsy was immersed in fixative (formal saline or glyofixx) in fridge for at least 18 hours. Next day, the biopsy was washed with 3X10 minutes changes of 0.1M Sorrenson's buffer in cryotubes and placed in 1ml of cryoprotectant solution at 4°C overnight. Next day, the biopsy was removed from cryoprotectant and placed in a drop of OCT on a small piece of foil (the biopsy must be orientated correctly, with epidermis perpendicular to silver foil, i.e. biopsy lying on its side). Next, the biopsy was frozen over dry ice (or alternatively using isopentane cooled in liquid nitrogen) and wrapped in a larger piece of foil, which had already been labelled and then the biopsy was stored in -80^{o}C freezer.

2.10.1. Solutions

2.10.1.1. Sorrenson's Buffer Stock

1. 7.176g sodium phosphate monobasic monohydrate was dissolved in 100mls distilled water (solution A).
2. 49.4g of sodium phosphate was dissolved in 750mls distilled water (solution B).
3. Solutions A & B were mixed and made up to 1L with distilled water. PH should be 7.6 (no adjustment with acid or base needed).
4. The mixture was diluted to 0.1M as required and stored for 6 months maximum at room temp.

2.10.1.2. Cryoprotectant solution.

1. 20mls Glyerol
2. 80mls 0.1M Sorrenson's buffer
3. Keeps for six months at 4°C

2.11. Immunohistochemistry

2.11.1. Intra Epidermal Nerve Fibre Density

IENFD is expressed as the number of intra-epidermal nerve fibre density per mm (493). Intra- and inter-observer variability for the assessment of IENFD demonstrates good agreement (493). IENFD was found to decline by age but not to be influenced by weight or height (493). A cut-off IENFD of ≤8.8/mm at the ankle was associated with a sensitivity of 77.2% and a specificity of 79.6% to diagnose DPN (493). The sensitivity and specificity to diagnose small fibre neuropathy improves to 88%-98% and 88.8%-95% respectively (493). IENFD was found to correlate inversely with thermal thresholds (493). There is no correlation between IENFD and neuropathy symptoms, although a recent study has demonstrated an inverse correlation with the severity of pain assessed using the VASmax (493). The American Academy of Neurology, American Association of Neuromuscular and Electrodiagnostic Medicine, and American Academy of Physical Medicine and Rehabilitation have concluded however that skin biopsy may be considered for the diagnosis of DPN, particularly SFN, with a level C recommendation (493-495). Additional morphological features of IENFD include the branch density, length and mean dendritic length; all show an early reduction which progresses with neuropathic severity (493).

Immuno-staining of IENFD was done on the free floating frozen sections in accordance with previously published protocols (496). **Figure 2.13** show an example of IENFD.

2.11.1.1. Cutting the sections protocol

Biopsies were transferred to the pathology department in liquid nitrogen. Beofre cutting the samples, the freezing microtome was turned on and allowed to cool down. I used OCT to build up a platform of frozen OCT on stage of microtome. A drop of OCT was put on top of the frozen OCT platform. The biopsy was remove from liquid nitrogen, and removed quickly from foil using forceps, taking care that it does not thaw out and placed on top of OCT platform. The biopsy was kept frozen

by using a freezer spray gently. Fifty µm thick sections were cut and collected with a fine paint brush and transferred to PBS. Sections were transferred to Glyofixx and fixed overnight at 4^{o}C.

2.11.1.2. The immunohistochemistry procedure

The immune-staining was performed using Vectastain Universal Elite ABC kit (Vector laboratories). Cut sections were placed in TBS (if staining is to follow) or stored in a glycol based antifreeze at -20^{o}C. Staining was carried out in 96 well plate (wash and dry plate first); sections transfer between wells was performed with the aid of a dissecting microscope, using a seeker or a thin paint brush. All incubations took place on a shaker plate and used 200-400 µl of solution per well. Sections were rinsed in TBS for 2X 10 minutes and place in 0.25% potassium permanganate for 15 minutes followed by a wash in TBS for 10 minutes. Sections were then placed in 5% oxalic acid for a maximum of 5 minutes (or till sections turn white if less than 5 minutes), followed by 2X10 minutes washes in TBS, then Incubated in 10% goat (blocking) serum (25µl of blocking serum + 2.5ml antibody diluents) for 4 hours followed by primary antibody (rabbit anti-human polycloncal PGP9.5 antibody (Biogenesis, 1:1200)) overnight. Next morning, sections were washed in TBS 2X10 minutes and incubated in biotinylated secondary antibody for 1 hour, then washed in TBS for 2X10 minutes. Endogenous peroxidise was then blocked using 1% hydrogen peroxide in 30% methanol/PBS solution for 30 minutes. Sections then were washed in PBS 2X5 minutes and incubated in streptavidin peroxidase conjugate for 1 hour, then wash in PBS 2X10 minutes. Sections were then incubated in DAB (made according to kit instructions from vector laboratories) until they appeared brown. The reaction was best monitored under a dissecting microscope. Sections were then washed in water and mounted in DPX after placing a drop of aqueous mounting medium on a microscope slide.

2.11.1.3. Solutions

2.11.1.3.1. TBS

8g Sodium Chloride + 3g Tris Base + 0.2g Potassium Chloride + 800ml distilled water. PH was adjusted to 7.6 using HCl and made up to 1L with distilled water

2.11.1.3.2. Secondary antibody

50µl blocking serum + 2.5ml antibody diluents + 50µl biotinylated secondary antibody

2.11.1.3.3. streptavidin peroxidise

This is the ABC reagent: 2 drops reagent A + 2 drops reagent B + 5m of antibody diluents. Prepare 30 minutes before use

2.11.1.3.4. Hydrogen peroxide

25µl hydrogen peroxide + 1.72ml PBS + 0.75ml ethanol

2.11.1.3.5. DAB

2.5 ml distilled water + 1 drop buffer (mix well) + 2 drops of DAB (mixed well) + 1 drop hydrogen peroxide (mixed well). This was made fresh for the procedure.

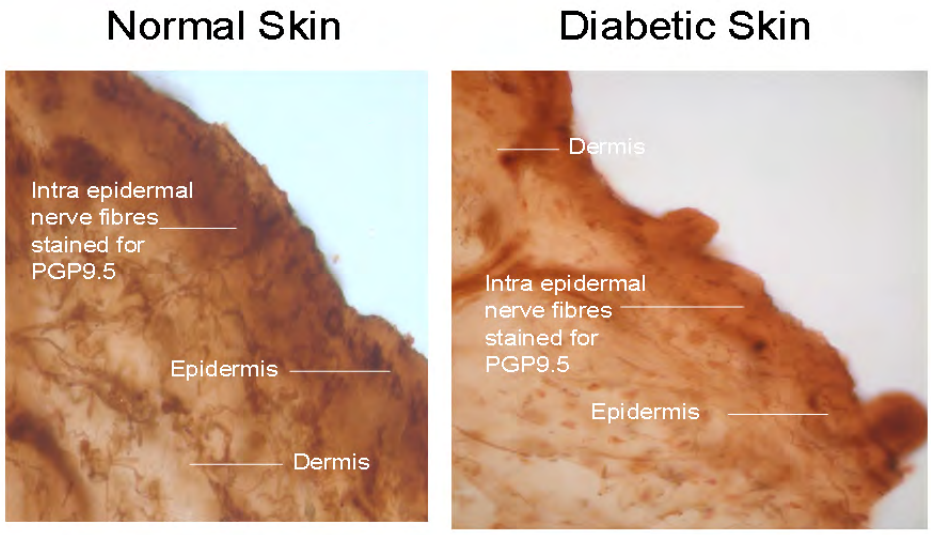


Figure 2-13: An example of immune-stained IENFD.

2.11.2. Poly (ADP-ribose) Polymerase

Polymers of ADP-ribose (PAR) are attached via an ester bond to glutamic acid, aspartic acid or lysine residues byPARP (140).PARP 1 and 2 are known to play a role in DNA repair (140). Increased OS results in DNA damage and PARP 1 activation (144-146). In diabetes, PARP 1 hyperactivation leads to detrimental effects (143;146). Excess cleavage of NAD+ by PARP, would exacerbate flux through SDH deplete NAD+ and increase OS (146). In addition NAD+ is required as a cofactor for the conversion of GAPDH. Hyperglycemia-induced ROS inhibits GAPDH activity in vivo by modifying the enzyme with PARP (131;147;497). In models of diabetes increased PAR and PARP-1 activation is corrected by aldose reductase inhibitors (150). In addition PARP inhibition reduces oxidative stress and prevents or reverses electrophysiological and behavioural deficits and IENFD loss in animal models (150;153;498); which suggests an important role for PARP in the development of DN.

PAR immune-staining was performed on the paraffin embedded sections according to previously published protocols (499). **Figure 2.14** show an example of PAR stained section from my study.

2.11.2.1. The immunohistochemistry procedure

The immune-staining was performed using Vectastain Universal Elite ABC kit (Vector laboratories). Slides were washed in 2 change of histoclear (5 minutes each) after turning the fume cupboard on, then dried thoroughly and transferred to 2 changes of ethanol 5 minutes each and dried again before transfer to hydrogen peroxide mixture (10 ml of hydrogen peroxide + 90 ml methanol), for 20 minutes in order to remove endogenous peroxidise. Slides were then washed in water for 10 minutes; excess water was dried and a circle was drawn around the sections with a PAP pen. Blocking serum (35µl per section) was added for 20 minutes followed by adding the primary antibody (1:400, 35µl per section) (Enzo Life Sciences, Exeter, UK) for 40 minutes. The slides were then washed 3 X 5 minutes in TBS and excess fluid from the back of the slide and around the section were dried. The sections were then incubated with the biotinylated secondary antibody for 30 minutes (35µl per section, prepared similar to the IENFD section above) followed by 3 washes (5

minutes each) in TBS. The slides were then dried of excess fluid and incubated with the ABC for 30 minutes (35µl per section, prepared similar to the IENFD section above). The sections were then washed 3 X 5 minutes in TBS and excess fluid dried and the sections were incubated in DAB (Vector Laboratories) (35µl per section, prepared similar to the IENFD section above) till they turned brown (no more than 10 minutes, the brown colour may not be visible to the naked eye as it depends on the amount of PAR-stained nuclei, examine under the microscope if in doubt). The slides then were washed in 2 changes of water and counter-stained with haematoxylin for 2 minutes, then washed in water for 2-3 minutes till the haematoxylin stain appeared blue (might need to be examined under the microscope). Then the slides were dehydrated in 2 changes of ethanol followed by 2 changes of histoclear (5 minutes each) and dried thoroughly between the changes before mounting the cover slips using a drop of DPX (avoiding air bubbles) and left overnight to dry and DPX to harden.

The solutions for this procedure are the same of those for the staining of IENFD (detailed above).

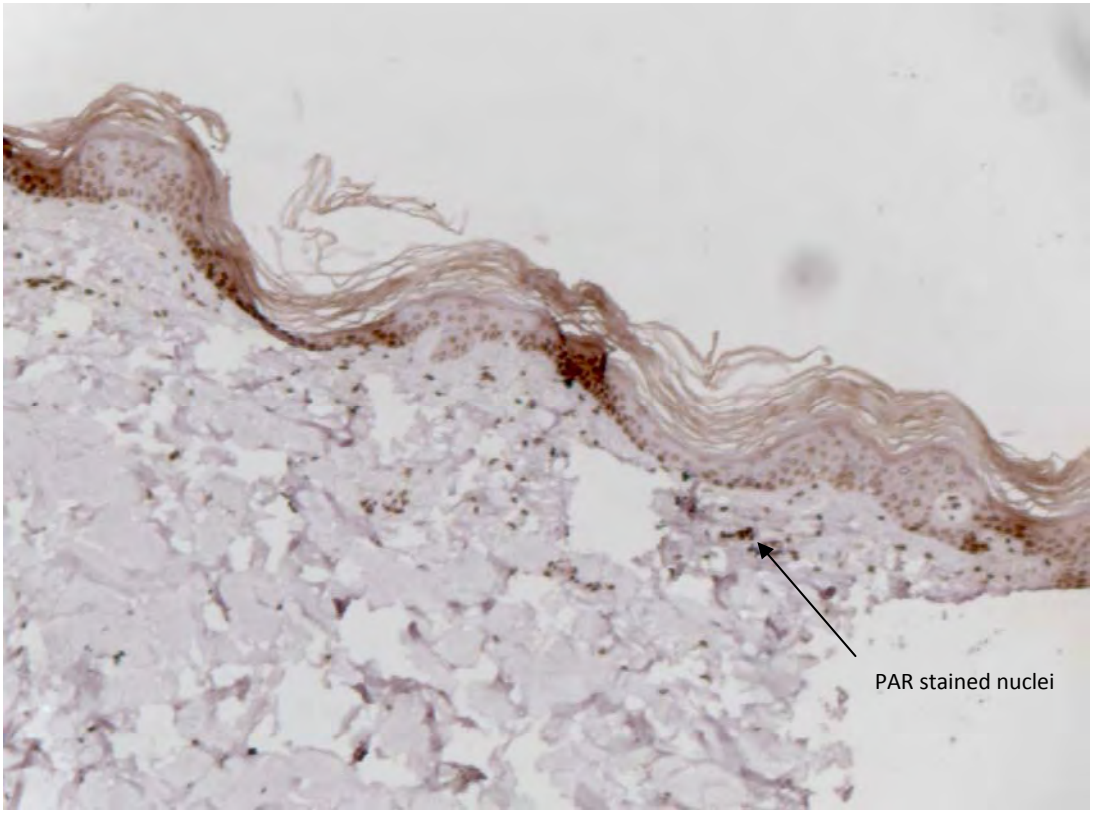


Figure 2-14: An example of PAR stained section from my samples.

2.12. Image analysis

Images for IENFD were captured using a Ziess Axioshop plus microscope and an Axiovision 4.4 program and analysed using Scion image software. All sections were scored blindly and the final score was the average of at least 3 sections.

IENFD presented as the number of nerve fibres per mm. Nerve fibres were calculated in the epidermis above the basement membrane. A nerve fibre that branches inside the epidermis will be counted as one; while a nerve fibre that branches below the basement membrane will count as two. IENFD analysis was performed in accordance with previous studies (492;496).

PARP was presented as the percentage of the stained nuclei. The number of stained and unstained endothelial nuclei were counted in ten fields from each section, using 400X magnification resulting in 300-400 nuclei being assessed as described previously (499).

2.13. Microvascular regulation

Microvascular and endothelial assessment was performed using LSCI (Moor Instruments Ltd, Devon, UK) (500). LSCI is a full-field technique that is used to record real time images of microvascular blood flow. In LSCI, speckles are formed by the backscatter of laser light, and moving particles cause fluctuations in the speckle pattern which is related to the speed of the illuminated particles (such as blood cells) (501). When there is a high level of movement (fast flow) the changing pattern becomes more blurred and the contrast in that region reduces accordingly. The contrast image is processed to produce a colour-coded image that correlates with blood flow in the tissue. The main strength of this technique is video frame rate blood flow images (25 per second) enabling tracking of fast transient blood flow changes that could not be seen by conventional laser imaging techniques.

Microvascular blood flow (measured in arbitrary perfusion units (APU) or flux) was assessed at the left mid thigh level under standardized conditions (502). 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 (502).

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.

Local heating leads to a temperature-dependent increase in skin cutaneous flow and achieves a maximal vasodilatation between 42 and 44 degrees (502). This vasodilatation corresponds to the maximal vasodilator capacity of the vessels (502). Heating-evoked vasodilatation is mediated by at least two independent mechanisms: an initial peak relies predominantly on local sensory nerves and is mediated by an axon reflex thought to be dependent on calcitonin-gene-related peptide and substance P followed by a plateau that is mediated largely by NO (502). Ach-induced vasodilatation is mainly dependent on prostanoids as well as NO and axonal reflexes (502).

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 (503). 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, Ach and SNP measurements was 0.9, 0.7, 0.9, and 0.8. The CV for the baseline, heating, Ach and SNP measurements were of 7%, 6%, 14%, and 10% respectively. The ICC for the ratios of baseline, Ach and SNP 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.

2.14. 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 independent t-test or the Mann-Whitney test. Categorical variables were compared using the Chi-square test. The Bonferroni correction was applied in the case of multiple comparisons.

Differences between independent groups were assessed by analysis of variance (ANOVA) with post-hoc analysis or the Kruskal–Wallis test. Analysis of covariance (ANCOVA), was used to assess the impact of covariates on the differences between several independent groups.

Correlations between continuous variables were performed using the Pearson or Spearman tests. Partial correlation was used to assess the impact of possible continuous confounders on the correlation between two continuous variables. To assess whether a categorical or scale variable is independently associated with a particular outcome, logistic regression (the backward method) was used if the outcome was a dichotomous variable and linear regression (the backward method) was used if the outcome was a continuous variable. 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. A $p < 0.1$ was used to retain variables in the model. To assess the relation between OSA severity and different study outcomes, AHI quartiles, ODI quartiles, nadir nocturnal oxygen saturation and time spent with oxygen saturation < 80% (as a dichotomous variable) were used in the regression models. Non-normally distributed data was normalized by log or square root transformation before being used in the analysis when normality was an assumption of the statistical test used. A p value < 0.05 was considered significant unless stated otherwise.

All assumption of statistical tests used in this thesis were adhered to. In ANCOVA the assumptions of homogeneity of regression slopes (by running the ANCOVA and examining the interaction between

the covariate and the fixed factor) and independence of the covariate and fixed factors were examined and adhered to. In regression models, we assessed multicollinearity 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 some models, the impact on estimates for the main exposure variable (OSA or other OSA severity related measures or ethnicity depending on the model) were minimal which suggests that the collinearity had had limited impact on models estimates for the main exposure. 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 the predictor off interest in that particular model , 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.

3. Chapter Three: OSA in Patients with T2DM: Epidemiology, Ethnicity, Clinical predictors and Screening

3.1. Introduction

As discussed in chapter one, OSA and T2DM share common risk factors such as obesity and age, hence it is no surprise that OSA is very common in patients with T2DM. The reported prevalence of OSA in T2DM varies significantly in the literature from 23% to 86% (331;333-337). This variation is mainly due to differences in the populations studied, the methods of diagnosing OSA (polysomnography, portable devices, oximetry, symptoms) and the definition and cut offs used to diagnose OSA (AHI vs. RDI vs. ODI, cut offs 5, 10, 15, 30 events/h). Data from the UK regarding the prevalence of OSA in T2DM is mainly from one study that used primary care population and used a combination of symptoms and oximetry to diagnose OSA, and this study showed an OSA prevalence of 23% in patients with T2DM (334). Ethnicity also has a significant impact on the prevalence of OSA in patients with T2DM (336;337) but the prevalence of OSA in South Asians with T2DM has not been explored before.

Due to the high prevalence of OSA in patients with T2DM, the IDF recommended the use of the OSA symptoms, the Berlin questionnaire and the ESS to screen for OSA (338). Although these methods are well validated in the general public and in several ethnicities, there validity in patients with T2DM has not been explored.

3.2. Hypothesis

1. OSA prevalence in patients with T2DM would differ between South Asians and White Europeans

Rationale: Obesity is a major risk factor for OSA. South Asians and White Europeans are known to have significant differences in adiposity and its distribution. Hence, it is likely that OSA prevalence will be different between the ethnic groups.

2. The Berlin questionnaire and ESS are not good screening tools for OSA in patients with T2DM

Rationale: Hyperglycaemia and T2DM are strongly associated with obesity and hypertension, which are essential components of the Berlin questionnaire, and are quite commonly associated with non-specific symptoms such as tiredness (essential components of the Berlin and ESS). Hence we hypothesised that the performance of these screening tools in patients withT2DM is unsatisfactory.

3.3. Aims

The primary aim of this study is to examine the prevalence of OSA in patients with T2DM and to compare that prevalence between South Asians and White Europeans. Secondary aims included studying the possible reasons behind any ethnic differences observed in OSA prevalence and to assess the validity of OSA symptoms, the Berlin questionnaire and ESS as screening methods for OSA in patients with T2DM.

3.4. Methods

Methods will be briefly reviewed here; please refer to chapter 2 for more details.

We conducted an epidemiological, observational cross-sectional study of South Asians and White Europeans with T2DM. Patients were recruited from the out-patients diabetes clinic from two hospitals in the UK, Birmingham Heartlands Hospital (more than 80% of study participants) and the University Hospital of North Staffordshire. Patients with past medical history of OSA or any other respiratory disorders, receiving renal replacement therapy, using sleeping tablets or pregnant were excluded. Data were collected during one-to-one interviews. For non-English speakers all communications were conducted by a researcher who is fluent in the participants' mother language.

The presence of OSA was examined using a portable multichannel respiratory device (Alice PDX, Philips Resporinics, USA); an AHI ≥ 5 events/hour was consistent with the diagnosis of OSA (231). OSA severity was assessed based on the AHI, ODI, time spent with oxygen saturations < 90%, time spent with oxygen saturations < 80% and the nadir nocturnal oxygen saturation during sleep. OSA

was classified into mild (AHI 5- <15), moderate (15- <30) and severe (≥30). The Berlin questionnaire (with a BMI cut off of 25 kg/m^2 in South Asians) was used to assess the risk of having OSA and the ESS was used to detect EDS (please refere to chapter 2 for more details). The project was approved by the Warwickshire Ethics committee as described in chapter 2 and all participants were consented before joining the study.

This was a secondary analysis of the study that will be presented later examining the relationship between OSA and microvascular complications in patients with T2DM. Detailed statistical methods are presented in chapter 2, but to assess the clinical utility of the Berlin questionnaire and the ESS in diagnosing OSA, sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were used, and the analysis was performed in South Asians and White Europeans separately.

3.5. Results

Two hundred and sixty six patients were recruited in total. 30 sleep studies were excluded because of poor quality (either too short recording or loss of signal) and 2 patients were excluded because they predominantly had central, rather than obstructive, sleep apnoea. That left 234 for analysis.

The baseline characteristics of the study population are summarised in **Table 3.1**. This data suggest that our study population is generally of obese, middle to older age and has relatively a long duration of diabetes with good metabolic control and high use of anti-hypertensives and lipid lowering treatment. The use of insulin is also high, probably not surprising as the population was recruited from secondary care and has long diabetes duration. The study population was balanced from gender and ethnicity point of view.

Table 3-1: Summary of study population characteristics.

Data presented as n (%), mean ± SD or median (IQR).

Variable	
Demographics	
Male	135 (57.7%)
White European	129 (55.1%)
Age (years)	57.18 ± 11.65
Consume alcohol	65 (27.8%)
Current or ex smoker	94 (40.2%)
Metabolic/Biochemistry	
Diabetes duration (years)	11.0 (6.0-16.2)
Systolic BP (mmHg)	129.0 (120.5-138.1)
Diastolic BP (mmHg)	78.5 (71.4-84.5)
HBA1c (%)	7.98 (7.19-9.20)
Total cholesterol (mmol/l)	3.7 (3.3-4.4)
HDL (mmol/l)	1.10 (0.90-1.30)
LDL (mmol/l)	1.7 (1.3-2.3)
Triglycerides (mmol/l)	1.7 (1.2-2.4)
TSH (mU/l)	1.7 (1.2-2.3)
eGFR (ml/min/1.73m^2)	86.14 ± 26.41
Adiposity	
Height (cm)	165.76 ± 11.22
BMI (kg/m^2)	33.1 (29.0-37.5)
Waist circumference(cm)	112.25 (101.50-123.50)
Hip circumference (cm)	111.0 (102.0-123.0)
Waist hip ratio	0.99 (0.94-1.03)
Neck circumference (cm)	41.0 (38.0-44.5)
Cardiovascular disease	
History of IHD	47 (20.1%)
History of stroke/TIA	24 (10.3%)
History of PVD	11 (4.7%)
Medications	
Aspirin	156 (66.7%)
Lipid lowering treatment	196 (83.8%)
Anti hypertensives	190 (81.2%)
Number of anti hypertensives	2.0 (1.0-2.0)
RAS inhibitors	166 (70.9%)
Oral Anti Diabetes Treatment	218 (93.2%)
Metformin	205 (87.6%)
Sulphonylurea	88 (37.6%)
Glitazones	36 (15.4%)
DPP-4 inhibitors	17 (7.3%)
GLP-1 analogue	26 (11.1%)
Insulin	125 (53.4%)
Insulin dose	77.0 (52.0-112.5)

3.5.1. OSA epidemiology

One hundred and fifty one patients had OSA (64.5%), of which 90 (38.5%), 35 (15%) and 26 (11.1%) were mild, moderate and severe OSA respectively. OSA severity parameters are summarised in **Table 3.2**.

Table 3-2: Summary of OSA severity parameters.

Data presented as median (IQR)

OSA parameters	
Apnoea Hypopnea Index (events/hour)	7.2 (2.6-15.3)
Epworth Sleepiness Scale	8.0 (4.0-13.0)
Proportion of time spent with oxygen saturations < 90% (%)	1.3 (0.2-7.2)
Oxygen de-saturation Index (events/hour)	6.4 (2.6-13.9)
Nocturnal nadir oxygen saturation (%)	83.0 (77.0-88.0)

3.5.1.1. The gender effect

Similar to the general public, OSA prevalence was higher in men (n=135) than women (n=99) in patients with T2DM (74.8% vs. 50.5%, p <0.001). The relationship between gender and OSA severity parameters is summarised in **Table 3.3**. Men have more moderate to severe OSA, higher AHI and higher ODI than women.

Table 3-3: The relationship between gender and OSA severity

		Men	Women	P
OSA severity	Normal	25.2%	49.5%	< 0.001
	Mild	40.7%	35.4%	
	Moderate	20.7%	7.1%	
	Severe	13.3%	8.1%	
Apnoea Hypopnea Index (events/hour)		8.1 (4.9-19.4)	5.1 (1.4-11.0)	< 0.001
Epworth Sleepiness Scale		7.0 (3.0-13.0)	8.0 (4.0-12.0)	0.983
Proportion of time spent with oxygen saturations < 90% (%)		1.1 (0.1-6.5)	1.5 (0.2-7.8)	0.728
Oxygen de-saturation Index (events/hour)		8.3 (3.4-16.0)	5.4 (1.6-10.7)	0.007
Nocturnal nadir oxygen saturation (%)		82.0 (78.0-87.2)	83.0 (76.0-88.5)	0.651

3.5.1.2. The ethnicity effect

The prevalence of OSA was higher in White Europeans compare to South Asians with T2DM (75.2% vs. 51.4%, $p < 0.001$). The relation between ethnicity and OSA severity parameters is summarised in **Table 3.4**. White Europeans seem to have more moderate to severe OSA, higher AHI and higher ODI than South Asians.

Table 3-4: The relationship between ethnicity and OSA severity

		South Asians	White Europeans	P
OSA severity	Normal	48.6%	24.8%	< 0.001
	Mild	36.2%	40.3%	
	Moderate	9.5%	19.4%	
	Severe	5.7%	15.5%	
Apnoea Hypopnea Index (events/hour)		5.1 (1.4-11.5)	8.5 (4.9-20.6)	< 0.001
Epworth Sleepiness Scale		6.0 (1.5-12.5)	8.0 (5.0-13.0)	0.983
Proportion of time spent with oxygen saturations < 90% (%)		0.5 (0.0-2.9)	3.5 (0.5-13.7)	0.728
Oxygen de-saturation Index (events/hour)		4.9 (1.5-10.9)	8.5 (4.1-18.4)	0.007
Nocturnal Oxygen nadir saturations (%)		84.0 (80.0-89.0)	82.0 (76.0-86.0)	0.651

3.5.1.3. The gender ethnicity interaction

The higher prevalence of OSA in White Europeans compared to South Asians with T2DM seems to be present only in men and not in women **(Table 3.5).** This difference in OSA prevalence was mainly due to higher prevalence of moderate to severe OSA in White Europeans **(Table 3.5).** Similarly, the higher prevalence of OSA in men compared to women was mainly present in White European rather than South Asians (**Table 3.6**)

Table 3-5: A comparison of OSA prevalence between South Asians and White Europeans in men and women.

		South Asians	White Europeans	P
Men				
OSA prevalence		54.1%	91.9%	< 0.001
OSA severity	Normal	54.9%	8.1%	< 0.001
	Mild	36.1%	44.6%	
	Moderate	11.5%	28.4%	
	Severe	6.6%	18.9%	
Women				
OSA prevalence		47.7%	52.7%	0.621
OSA severity	Normal	52.3	47.3%	0.711
	Mild	36.4	34.5%	
	Moderate	6.8	7.3%	
	Severe	4.5	10.9%	

Table 3-6: A comparison of OSA prevalence between men and women in South Asians and White Europeans.

		Women	Men	P
South Asians				
OSA prevalence		47.7%	54.1%	0.519
OSA severity	Normal	52.3%	45.9%	0.807
	Mild	36.4%	36.1%	
	Moderate	6.8%	11.5%	
	Severe	4.5%	6.6%	
White Europeans				
OSA prevalence		52.7%	91.9%	< 0.001
OSA severity	Normal	47.3%	8.1%	< 0.001
	Mild	34.5%	44.6%	
	Moderate	7.3%	28.4%	
	Severe	10.9%	18.9%	

3.5.1.4. Possible explanations for the ethnic differences in OSA prevalence

In order to explore possible explanations for the ethnic differences observed, we examined whether there are ethnic differences in OSA risk factors that were measured in our study such as age, obesity, renal function, alcohol intake and smoking **(Table 3.7)**. These results show a worse OSA risk profile in White Europeans compared to South Asians, which might contribute to the higher prevalence of OSA in this ethnic group.

Table 3-7: A comparison of some OSA risk factors between South Asians and White Europeans with T2DM.

	South Asians	White Europeans	P value
Consume alcohol	2.9%	48.1%	< 0.001
Current or ex smoker	28.6%	49.6%	0.001
Age (years)	54.5 ± 12.5	59.3 ± 10.4	0.002
eGFR (ml/min/1.73m^2)	89.9 ± 25.8	83.1 ± 26.6	0.047
Height (cm)	164.1 ± 8.9	167.1 ± 12.7	0.037
BMI (kg/m^2)	30.2 (26.8-33.8)	35.2 (31.5-41.3)	< 0.001
Waist circumference (cm)	103.5 (96.5-115.4)	117.0 (109.1-130.5)	< 0.001
Waist hip ratio	1.00 (0.95-1.03)	0.99 (0.93-1.04)	0.245
Neck circumference (cm)	39.0 (36.7-41.7)	43.0 (39.4-47.0)	< 0.001
Diabetes duration (years)	11.0 (6.0-18.0)	11.0 (5.0-16.0)	0.411

In order to explore possible explanations for the gender ethnic interaction observed, we examined whether there are ethnic differences in OSA risk factors are affected by gender (**Table 3.8**). These results show that White European men have worse OSA risk profile compared to White European women and South Asian men which might contribute to the higher prevalence of OSA in White European men. However, despite that South Asian men had a worse OSA risk profile compared to South Asian women; there was no significant difference in OSA prevalence between South Asian men and women. This suggests that there are other factors that might contribute to the gender/ethnic differences observed.

Table 3-8: A comparison of some OSA risk factors between South Asians and White Europeans with T2DM classified by gender. SA: South Asians; WE: White Europeans

	Men			Women			P value	P value
	SA (n=61)	WE (n=74)	P value	SA (n=44)	WE (n=55)	P value	SA men vs. women	WE men vs. women
Alcohol	4.9%	62.2%	<0.001	0%	29.1%	<0.001	0.263	<0.001
Current or ex smoker	44.3%	55.4%	0.197	6.8%	41.8%	<0.001	<0.001	0.127
Age (years)	56.8 ± 12.6	60.0 ± 9.8	0.103	51.4 ± 11.8	58.5 ± 11.3	0.003	0.028	0.419
eGFR (ml/min/1.73m^2)	88.3 ± 24.9	82.0 ± 23.2	0.127	92.1 ± 27.1	84.5 ± 30.7	0.200	0.459	0.588
Height (cm)	169.2 ± 6.8	174.1 ± 7.6	<0.001	156.9 ± 6.0	157.8 ± 12.2	0.662	<0.001	< 0.001
BMI (kg/m^2)	28.5 (25.6-32.3)	33.9 (30.9-39.5)	<0.001	32.4 (29.5-35.9)	37.0 (34.0-47.0)	<0.001	0.001	0.006
Waist circumference (cm)	102.0 (97.0-113.5)	117.6 (109.2-132.0)	<0.001	108.5 (95.6-117.1)	116.5 (109.0-129.0)	0.001	0.305	0.790
Waist hip ratio	1.02 (0.98-1.05)	1.02 (0.99-1.08)	0.174	0.97 (0.93-1.01)	0.92 (0.88-0.96)	<0.001	<0.001	<0.001
Neck circumference (cm)	40.6 (38.9-43.0)	45.2 (42.4-48.5)	<0.001	37.0 (35.0-38.6)	39.2 (38.0-43.0)	<0.001	<0.001	<0.001
Diabetes duration (years)	11.0 (6.5-20.0)	11.0 (6.0-16.2)	0.450	9.5 (6.0-14.5)	10.0 (5.0-15.0)	0.649	0.146	0.298

To assess whether these differences in OSA risk factors between the ethnicities are responsible for the higher prevalence of OSA in White Europeans, we have used logistic regression models of increasing complexity. The outcome measure was having OSA and the predictors were the variables reported in Table 6 **(Table 3.9)**. Only measures of obesity (particularly neck circumference) removed the significance of the ethnicity impact on OSA **(Table 3.9)**.

Table 3-9: The impact of possible confounders on the relation between ethnicity and OSA prevalence.

The OR are the odds for having OSA in White Europeans vs. South Asians with T2DM.

	OR	95% CI	P value
Unadjusted: Ethnicity	2.863	1.646-4.978	< 0.001
Ethnicity + Gender	3.136	1.755-5.602	< 0.001
Ethnicity + Gender + Smoking	3.592	1.950-6.614	< 0.001
Ethnicity + Gender + Smoking + Alcohol	3.106	1.538-6.273	0.002
Ethnicity + Gender + Smoking + Alcohol + Age	2.888	1.417-5.889	0.004
Ethnicity + Gender + Smoking + Alcohol + Age + Height	2.755	1.340-5.664	0.006
Ethnicity + Gender + Smoking + Alcohol + Age + Height + eGFR	2.759	1.325-5.747	0.007
Ethnicity + Gender + Smoking + Alcohol + Age + Height + eGFR + Waist circumference	1.433	0.634-3.240	0.387
Ethnicity + Gender + Smoking + Alcohol + Age + Height + eGFR + Neck circumference	1.237	0.537-2.849	0.617
Ethnicity + Gender + Smoking + Alcohol + Age + eGFR + BMI	1.340	0.592-3.036	0.483
Ethnicity + BMI	2.083	1.140-3.808	0.017
Ethnicity + waist circumference	1.828	0.993-3.364	0.053
Ethnicity + Neck circumference	1.421	0.759-2.659	0.272
Ethnicity + Gender + BMI	1.900	1.002-3.601	0.049
Ethnicity + Gender + waist circumference	1.950	1.031-3.689	0.040
Ethnicity + Gender + Neck circumference	1.603	0.827-3.107	0.163
Ethnicity + Gender + Age + BMI	1.525	0.779-2.986	0.219
Ethnicity + Gender + Age + waist circumference	1.582	0.808-3.095	0.181
Ethnicity + Gender + Age +Neck circumference	1.302	0.645-2.629	0.461

3.5.2. The utility of available OSA screening methods

The sensitivity, specificity, PPV and NPV of the Berlin questionnaire and the ESS and the presence of snoring in diagnosing OSA at cut offs of AHI ≥ 5, 10, 15 events/hours in patients with T2DM are summarised in **Tables 3.10**. White Europeans and South Asians were analysed separately.

Table 3-10: The utility of snoring, the Berlin questionnaire and the ESS in diagnosing OSA in White Europeans and South Asians with T2DM.

WE: White Europeans (n=129), SA: South Asians (n=105)

	Snoring		Berlin		ESS	
	SA	WE	SA	WE	SA	WE
OSA (AHI ≥ 5 events/hour)						
Sensitivity	88.5%	91.0%	83.0%	85.0%	43.0%	37.0%
Specificity	30.4%	11.0%	37.0%	28.0%	78.0%	56.0%
PPV	59.0%	77.3%	58.4%	78.1%	67.6%	72.0%
NPV	70.0%	27.3%	67.9%	37.5%	56.3%	22.8%
OSA (AHI ≥ 10 events/hour)						
Sensitivity	94.0%	94.0%	90.0%	89.0%	58.0%	48.0%
Specificity	27.0%	12.0%	34.0%	25.0%	78.0%	68.0%
PPV	37.2%	45.4%	36.4%	47.6%	52.9%	54.0%
NPV	90.0%	72.7%	89.3%	75.0%	81.7%	63.3%
OSA (AHI ≥ 15 events/hour)						
Sensitivity	100.0%	100.0%	94.0%	91.0%	63.0%	44.0%
Specificity	24.0%	14.0%	30.0%	24.0%	73.0%	64.0%
PPV	20.5%	39.1%	19.5%	39.0%	29.4%	40.0%
NPV	100%	100.0%	96.4%	83.3%	91.5%	68.3%

3.6. Discussion

Our study showed a high prevalence of OSA in patients with T2DM (approx 65%), consistent with previous studies (23%-86%) (331;333-337). The difference in OSA prevalence in our study compared to other studies is mainly due to differences in population characteristics, study design and the criteria used to diagnose OSA. For example, our OSA prevalence of 64.5% is lower than that of Foster et al (86%) (337). But the study by Foster et al included only obese patients (we had no BMI entry criteria in our study) and had a significant proportion of the study population of Afro-Caribbean origin (Afro-Caribbeans have a higher prevalence of OSA) (337). On the other hand, we have a higher

prevalence of OSA than that reported by West et al (23%), because West et al sample was mainly from primary care in Oxford and used oximetry to diagnose OSA (334). In Einhorn et al OSA prevalence was lower than ours (48%) but he used single channel respiratory device rather than a multichannel and OSA was defined as ≥ 10 events/hour (rather than 5 as in our study) (333).

Our study showed that the prevalence of OSA is higher in men with T2DM compared to women. Although this has been reported in the general public (233), data regarding the impact of gender on OSA prevalence in patients withT2DM has not been reported before. We also reported the novel finding of a high OSA prevalence of just over 50% in South Asians, which is still lower than that of White Europeans with T2DM. Interestingly, the gender differences in OSA prevalence only existed in White Europeans while South Asian men had only a slightly non significant higher prevalence of OSA compared to South Asian women. This is occurred despite that South Asian men having worse OSA risk profile compared to South Asian women, suggesting that other factors apart from major OSA risk factors (such as age and obesity) play an important role in the pathogenesis of OSA in patients with T2DM.

The reasons behind the above-mentioned ethnic differences are not clear but we attempted to clarify some of the reasons behind such differences using the data collected in our study. Our data showed that White Europeans had a more adverse OSA risk profile than South Asians (such as age, obesity, smoking, alcohol intake etc.). Similarly men had a more adverse OSA risk profile than women. Any of these risk factors could explain the lower prevalence of OSA in South Asians and in women. Logistic regression analysis, however, suggested that only obesity measures (particularly neck circumferences) are the main reasons behind the higher OSA prevalence in White Europeans with T2DM. Neck circumference seems to play a more important role than BMI and waist circumference in explaining ethnic differences as it was the only variable that removed the statistical significance from the relationship between ethnicity and OSA when ethnicity and adiposity were the only variables included in the model. This is further supported by similar OSA prevalence between

South Asian men and White European women (54% vs. 53%) who also happened to have similar neck circumferences (40.6 vs. 39.2 cm) despite that the White European women had higher BMI, waist circumference and alcohol intake compared to the South Asian men.

This does not exclude that other factors might contribute to the ethnic difference in OSA prevalence that we have not measured in our study (such as upper airway anatomy, upper airway muscle tone, ventilator drive, lung volumes etc). The underlying mechanisms of the lower prevalence of OSA in South Asians with T2DM and the gender differences need to be examined in further studies in more depth taking into account the factors not measured by this study. Based on our findings, White European men are by far the highest risk group of having OSA in our study and hence may be need to be the groups to be targeted for screening.

Due to the high prevalence of OSA in patients with T2DM, IDF recommended screening for OSA in this high risk population using the Berlin questionnaire and ESS (338). Although the Berlin questionnaire and ESS are well validated in the general public (please see chapter 2), their use and accuracy in patients with T2DM have not been examined. Furthermore, a cheap screening method is needed due to the large number of patients with T2DM that need to be screened for OSA, as polysomnography is expensive and time consuming. Our data suggest that neither snoring, nor the Berlin questionnaire nor the ESS is a good screening method to diagnose or rule out OSA (defined as AHI ≥ 5 events/hour). However, the Berlin questionnaire and snoring are very useful to rule out the diagnosis of moderate to severe OSA (AHI ≥ 15 events/hour). In fact our data suggest that lack of history of snoring rules out the diagnosis of moderate to severe OSA in South Asians or White Europeans with T2DM (NPV 100% in both ethnicities). These results are similar to a previous report that showed that only 6% of OSA patients have not reported snoring; but this study was in non-diabetics (287). It is interesting that our results showed that the use of the Berlin questionnaire showed no advantage in terms of sensitivity, specificity, PPV and NPV in diagnosing OSA over that offered by the presence/absence of snoring. Although snoring is included in the Berlin questionnaire,

other questions in the questionnaire are related to tiredness, hypertension and obesity. As patients with T2DM are generally obese, the vast majority are prescribed anti-hypertensives, and many have tiredness for reasons other than OSA. Hence, it is "easier" for patients with T2DM to score "high risk" on the Berlin questionnaire which diminishes its performance. This could explain the much worse performance of the Berlin questionnaire in our study compared to studies in the general public (sensitivity 86%, specificity 77%, PPV 89%) (481). Snoring was also better than the ESS, even in patients with moderate to severe OSA. Our data, in part, do not support the IDF recommendation as the Berlin questionnaire and ESS performed poorly as screening test for OSA. This however depends on the intention of the clinician treating the patient. So, if the clinician is interested in finding only patients with moderate to severe OSA, then asking about snoring or the use of the Berlin questionnaire is a useful screening tool to reduce the number of patients requiring polysomnography. On the other hand, if the clinician is interested only in patients with OSA and EDS, then the ESS is a useful measure of EDS. Finally, none of these screening methods is good in diagnosing OSA if AHI cut off of 5 was used.

It is important in our study to ascertain that our clinic population is representative and that the South Asians and White Europeans in our study are comparable. We believe that our study population is representative to that of the general South Asians and White Europeans with type 2 diabetes for several reasons. Both ethnicities (in our study) live in the same compact geographical area; they have similar standards of living and have similar deprivation scores. The referral guidelines, which are agreed between our diabetes specialists and the primary care trust, are the same for both ethnicities and the same referral criteria apply to both ethnicities, hence we do not believe that there are any referral differences between South Asians and White Europeans. We have also explored the issue of non- attendance in our diabetes clinic and found no ethnic differences in the "did not attend" rate. This is probably reflected in that the proportion of South Asians in our study (45%) is close to their prevalence in the clinic (40%). In addition, we approached similar numbers of South Asians and White Europeans to be recruited to the study and the response

rate was similar in the two ethnic groups (approximately 65%, excluding those who were not eligible to enter the study). Further evidence that our sample is representative comes from the similar characteristics (such as age, BMI, height, HbA1c, history of cardiovascular disease etc) in the South Asians and White Europeans in our study to those in another report from a different region in the UK (224).

The main limitation of our study is the lack of matching between South Asians and White Europeans. However, matching the ethnicities for such variables (particularly obesity) might reduce the external validity of our findings as such "matched" population may not be representative of "real life" in which these two ethnicities are very different. Additionally there is the possibility of self-selection bias (ie patients with OSA preferentially agreeing to participate based on their ethnicity) which cannot be entirely excluded. However, patients attending a general diabetes clinic were approached about willingness to enrol in the study and we are unaware of an ethnicity-mediated self-selection bias which would affect our conclusions. Furthermore, there were no ethnic differences in declining to participate in the study. Our study has several advantages over other reported studies. Our study is the largest in the UK that used multichannel respiratory device to diagnose OSA; previous work from the UK either used questionnaires or single channel devices. Another advantage to this work is that we included a wider population compared to other studies, such as including both genders while some studies included men only; and including South Asians which have not been included in any previous study in this area of research.

In summary, our study showed a high prevalence of OSA patients with T2DM, with White European men being the highest risk group. We also showed that South Asians with T2DM have a high prevalence of OSA, albeit this prevalence is lower than that of White Europeans. The interaction between ethnicity and gender was important in regard to OSA prevalence as the higher prevalence of OSA in White Europeans compared to South Asians was only true for men but not women. We also found that differences in obesity accounted for the ethnic differences in OSA prevalence.

Further studies examining other mechanisms that could explain these ethnic differences in OSA prevalence are needed, particularly focusing on the OSA underlying mechanisms such as upper airway size, upper airway muscle tone and ventilator drive. Snoring and the Berlin questionnaire were very good in ruling out moderate to severe OSA, but not useful in diagnosing OSA of any severity. Further research is needed to identify screening methods/algorithms that help clinicians reduce the number of sleep studies needed in the view of the high prevalence of OSA in patients with T2DM.

4. Chapter four: Obstructive Sleep Apnoea and Diabetic Peripheral Neuropathy

4.1. Introduction

DPN is common and results in great morbidity, mortality and significant economic burden (54). Known DPN risk factors include increasing age and the duration and degree of the antecedent hyperglycemia (58;70) as well as hypertension, dyslipidemia, and obesity (79;82). Putative mechanisms for DPN include increased oxidative/nitrosative stress, AGE formation, activation of the hexosamine and polyol pathways and perturbations of PKC activation, resulting in direct cellular damage and functional and/or structural defects involving the extra-cellular matrix and/or microvasculature (497;504). Despite our improved understanding of the pathogenesis of DPN, disease-modifying treatments are still lacking (with the exception of improved glycemic control) (79;504). Hence, improved understanding of DPN pathogenesis is important in order to identify new treatments (504).

4.2. Hypothesis

OSA and nocturnal hypoxemia are associated with DPN and impaired perception of 10g monofilament sensation in patients with T2DM

Rationale: OSA has similar molecular consequences to hyperglycaemia (366;400;505); many of these molecular consequences are involved in the pathogenesis of DPN.

4.3. Aims

The primary aim of this study is to explore the interrelationships of OSA, nocturnal hypoxemia and DPN in subjects with T2DM. Secondary aims of this study include:

1. Exploring the relationship between OSA and the "at risk foot" in patients with T2DM.
2. Exploring the relationship between OSA severity, nocturnal hypoxemia severity and DPN severity in patients with T2DM.

3. To assess the interrelationship between OSA, nocturnal hypoxemia and small fibre neuropathy in patients with T2DM.

4.4. Methods

We conducted an observational cross-sectional study in adults with T2DM. Patients with respiratory disease (including pre-diagnosed OSA, chronic obstructive pulmonary disease (COPD), asthma etc), 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 the details 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).

Data collected included demographics, anthropometrics, metabolic indices and renal function (eGFR using the MDRD equation). Sleep assessment included the use of sleep diaries and the ESS.

DPN was assessed using the Michigan Neuropathy Screening Instrument (MNSI) (56;468-470;506). DPN was diagnosed if the MNSI examination (MNSIe) score was >2 and/or MNSI questionnaire (MNSIq) score was ≥ 7 (469;473). Foot insensitivity was assessed by using a 10-g monofilament and defined as < eight correct responses (473). For more details about the MNSI and the monofilament test, please refer to Chapter 2.

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 (230). 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 apnoea

hypopnea index (AHI) ≥ 5 events/hour was consistent with the diagnosis of OSA (231). 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.

Small fibres were assessed using intra-epidermal nerve fibre density (IENFD) by obtaining skin biopsies as outlined in Chapter 2. OSA scorers were blinded to the patient's DPN status.

Details of statistical analysis can be found in Chapter 2. 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.

For detailed methodology, please refer to Chapter 2.

4.5. Results:

We recruited 266 patients; 32 were excluded (30 for poor sleep recording quality and 2 because of having central sleep apnoea), leaving 234 patients for analysis.

Of these 234 patients, 57.7% were men and 55.1% White Europeans and 44.9% South Asians. The overall prevalence of DPN was 47.9%. The overall prevalence of OSA was 64.5%. Of the 151 patients with OSA, 60% had mild (AHI 5 to < 15 events per hour), 23% had moderate (AHI 15 to < 30) and 17% had severe (AHI ≥ 30) OSA.

As expected, patients with OSA (OSA+) were older, had longer diabetes duration and higher systolic BP, BMI, waist and neck circumference and were sleepier compared to those without OSA (OSA-) (**Table 4.1**). In addition, OSA+ patients exhibited more lipid abnormalities and consumed more alcohol (**Table 4. 1**).

Table 4-1: Participant characteristics in relation to OSA status.

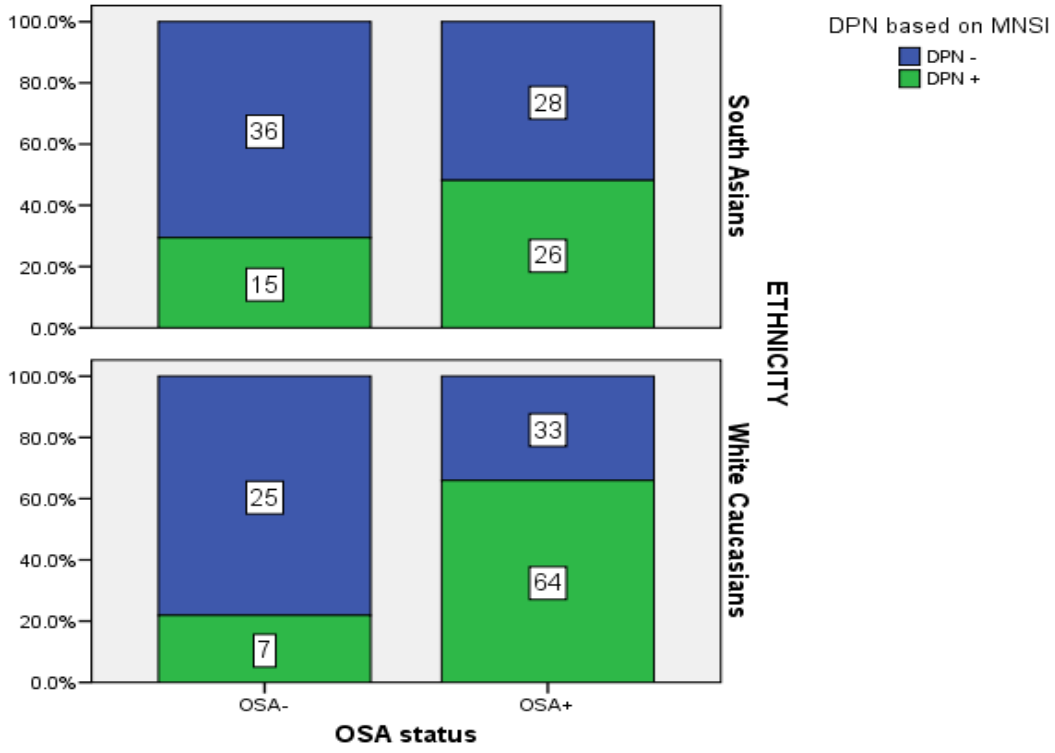
Data presented as median (IQR) or mean (SD). GFR: Glomerular Filtration Rate, TIA: Transient Ischaemic Attack, PVD: Peripheral Vascular Disease. Analysis performed using the Chi-square test for categorical variables, the independent t test for normally distributed variables and the Mann-Whitney U test for non-normally distributed variables.

	OSA- (n=83)	OSA+ (n=151)	P value
Male	41.0%	66.9%	< 0.001
White Europeans	38.6%	64.2%	<0.001
Age (years)	54.71 (11.94)	58.53 (11.29)	0.016
Diabetes Duration (years)	9.00 (5.00-15.00)	11.0 (7.00-17.00)	0.018
Body Mass Index (kg/m^2)	30.20 (27.30-35.00)	34.40 (30.90-39.50)	< 0.001
Waist circumference (cm)	105.5 (96.00-115.00)	116.0 (107.50-125.50)	< 0.001
Hip (cm)	106.0 (98.0-117.00)	114.0 (105.00-125.0)	< 0.001
Waist hip ratio	0.97 (0.93-1.02)	1.01 (0.96-1.05)	0.002
Neck circumference (cm)	38.00 (36.50-41.25)	43.00 (39.00-46.00)	< 0.001
Height (cm)	163.5 (8.3)	167.8 (10.0)	0.001
Systolic blood pressure (mmHg)	125.5 (115.0-135.5.0)	130.0 (123.5-140.0)	0.002
Diastolic blood pressure (mmHg)	78.50 (71.0-85.00)	78.00 (71.00-84.50)	0.884
HbA1c (%)	7.70 (7.00-8.70)	8.27 (7.27-9.33)	0.049
Total cholesterol (mmol/L)	3.70 (3.40-4.50)	3.70 (3.30-4.30)	0.573
Triglycerides (mmol/L)	1.50 (1.00-2.10)	1.80 (1.30-2.50)	0.034
HDL (mmol/L)	1.19 (0.93-1.41)	1.08 (0.90-1.23)	0.016
Estimated GFR (ml/min/1.73 m^2)	92.92 (25.16)	82.41 (26.41)	0.003
TSH	1.63 (1.00-2.200)	1.72 (1.20-2.35)	0.319
Epworth sleepiness score	5.0 (2.0-12.0)	8.0 (4.0-13.0)	0.003
Smoking (current or ex-smoker)	38.6%	41.1%	0.708
Alcohol (drinks alcohol)	14.5%	35.1%	0.001
Metformin	94.0%	84.1%	0.028
Sulphonylureas	42.2%	35.1%	0.285
Glitazones	14.5%	15.9%	0.771
Oral anti-diabetes treatment	97.6%	90.7%	0.047
Incretin-based therapy	13.3%	21.9%	0.107
Insulin	41.0%	60.3%	0.005
Insulin dose (total daily units)	61.00 (35.00-88.00)	80.00 (56.00-118.00)	0.007
ACE inhibitors /Angiotensin II blockers	48.2%	45.7%	0.714
Beta blockers	19.3%	25.8%	0.258
Calcium channel blockers	19.3%	33.8%	0.019
Alpha blockers	3.6%	9.9%	0.083
Diuretics	21.7%	43.7%	0.001
Anti-hypertensive agents	73.5%	85.4%	0.025
Lipid lowering treatment	85.5%	82.8%	0.584

Anti platelets drugs	60.2%	70.2%	0.122
Stroke or TIA	7.2%	11.9%	0.276
Ischemic heart disease	16.9%	21.9%	0.398
PVD	1.2%	6.6%	0.061
Albuminuria	24.3%	43.2%	0.007
Sight threatening retinopathy	20.5%	47.6%	< 0.001

The overall prevalence of DPN was significantly higher in OSA+ compared to OSA- patients (59.6% vs. 26.5%, p<0.001, respectively). This relationship between OSA and DPN was present irrespective of ethnicity (**Figure 4.1**). The prevalence of DPN was higher in patients with OSA whether they were White Europeans (66% vs. 21.9%, p<0.001) or South Asians (48.1% vs. 29.4%, p=0.049) (**Figure 4.1**).

Figure 4-1: The relationship between OSA and DP in ethnicity subgroups.



Similarly, the prevalence of DPN was higher in patients with OSA regardless of gender, although the relationship was stronger in men. In women the prevalence of DPN in patients with OSA was 56% compared to a prevalence of DPN in patients without OSA of 32.7% (p=0.019). In men the respective

figures were 61.4% vs. 17.6%, p<0.001. The relationship between OSA and DPN existed in South Asian men, White European men and White European women, but not South Asian women (**Table 4.2**).

Table 4-2: A summary of the impact of the ethnicity gender interaction on the relationship between OSA and DPN.

Data presented as the proportion of patients with DPN in the respective OSA groups.

	OSA-	OSA+	P value
South Asian men (n=61)	21.4%	51.5%	0.016
White European men (n=74)	0%	66.2%	0.003
South Asian women (n=44)	39.1%	42.9%	0.802
White European women (n=55)	26.9%	65.5%	0.004

The overall prevalence of foot insensitivity was 37.3%. Foot insensitivity was significantly higher in OSA+ compared to OSA- patients (50.0% vs. 14.5%, p<0.001, respectively). The prevalence of an abnormal monofilament test was also more common in patients with OSA whether they were White Europeans (57.3% vs. 15.6%, p<0.001) or South Asians (37% vs. 13.7%, p=0.006). The higher prevalence of foot insensitivity in OSA patients was true in men (54.0% 14.7%, p<0.001) as well as women (42.0% vs. 14.3%, p=0.002). For the impact of ethnicity gender interaction on the relationship between OSA and foot insensitivity, please refer to **Table 4.3**.

Table 4-3: A summary of the impact of the ethnicity gender interaction on the relationship between OSA and foot insensitivity**.**

Data presented as the proportion of patients with impaired perception to 10g monofilament in the respective OSA group.

	OSA-	OSA+	P value
South Asian men (n=61)	17.9%	45.5%	0.030
White European men (n=74)	0%	58.2%	0.008
South Asian women (n=44)	8.7%	23.8%	0.171
White European women (n=55)	19.2%	55.2%	0.006

Patients with OSA had more abnormalities on all aspects of the neurological examination (**Table 4.4**). Interestingly, all patients who had foot ulceration in our sample also had OSA (**Table 4.4**). Based on

the MNSIq, patients with OSA had a higher prevalence of skin hypersensitivity (32.5% vs. 13.3, p=0.001). A previous history of "open sore on the foot" was also more common in OSA+ patients (27.2 vs. 7.2%, p<0.001); consistent with findings using the monofilament (**Table 4.4**). The rest of the MNSIq components were not significantly different between OSA+ and OSA- patients (**Table 4.4**).

Table 4-4: The relationship between OSA status and components of the MNSI and monofilament perception.

Data presented as % of abnormal test/response in the particular OSA group. MNSIe: the examination component of MNSI. MNSIq: the questionnaire component of MNSI. *These questions are not scored as part of the MNSIq. P < 0.01 and < 0.033 were considered significant when comparing the components of MNSIe and MNSIq respectively.

	OSA- (n=83)	OSA+ (n=151)	P values
MNSIe			
Inspection	41.0	66.7	<0.001
Ulcers	0	5.3	0.032
Ankle reflexes	30.1	58.0	<0.001
Vibration	22.9	60.0	<0.001
10g monofilament	14.5	50.0	<0.001
MNSIq			
Are you legs and/or feet numb?	38.6	48.3	0.150
Do you ever have any burning pain in your legs and/or feet?	45.8	51.0	0.446
Are your feet too sensitive to touch?	13.3	32.5	0.001
Do you get muscle cramps in your legs and/or feet?*	61.4	72.8	0.072
Do you ever have any prickling feelings in your legs or feet?	43.4	52.3	0.190
Does it hurt when the bed covers touch your skin?	8.4	12.6	0.173
When you get into the tub or shower, are you able to tell	4.9	12.5	0.056
Have you ever had an open sore on your foot?	7.2	27.2	< 0.001
Has your doctor ever told you that you have diabetic	18.1	29.8	0.049
Do you feel weak all over most of the time?*	45.8	41.1	0.485
Are your symptoms worse at night?	41.0	39.7	0.854
Do your legs hurt when you walk?	56.6	62.9	0.346
Are you able to sense your feet when you walk?	8.4	16.6	0.084
Is the skin on your feet so dry that it cracks open?	36.1	47.0	0.108
Have you ever had an amputation?	2.4	7.9	0.088

In order to assess whether the relationship between OSA and DPN is secondary to, independent of, the differences observed in baseline characteristics, logistic regression (the backward method) was used (**Table 4.5**). Despite some attenuation by adiposity measures, OSA remained independently associated with DPN (OR 2.744, 95% CI 1.458-5.164, p=0.002) after adjustment (**Table 4.5**). Replacing BMI with waist circumference or waist/hip ratio in the model did not change the significant relationship between OSA and DPN. Other independent associations in addition to OSA included adiposity measures [waist circumference (OR 1.029, 95% CI 1.009-1.050, p=0.005), BMI (OR 1.041, 95% CI 1.002-1.081, p=0.037)], insulin use (OR 2.866, 95% CI 1.596-5.146, p<0.001) and age (OR 1.041, 95% CI 1.014-1.069, p=0.003). Inserting OSA in the model as a 3-category (no OSA, mild OSA and moderate to severe OSA) rather than a dichotomous (no OSA and OSA) variable, demonstrated that both mild (OR 2.936, 95% CI 1.475-5.843, p=0.002) and moderate to severe OSA (OR 2.455, 95% CI 1.131-5.328, p=0.023) were independently associated with DPN.

In order to assess whether any of the OSA or nocturnal hypoxemia parameters can predict DPN, we have repeated the logistic regression after removing OSA and inserting AHI (as quartiles), ODI (as quartiles), time spent with sats < 80% (as binary variable) and nadir nocturnal oxygen saturations separately into separate models. Using AHI quartile 1 (AHI<2.90) as the reference point showed that quartile 2 (AHI 2.90 to 7.59) (OR 2.432, 95% CI 1.052-5.620, p=0.038), quartile 3 (AHI 7.60-16.09) (OR 3.969, 95%CI 1.656-9.512, p=0.002) and quartile 4 (AHI ≥16.01) (OR 3.016, 95%CI 1.252-7.264, p=0.014) were all independent predictors of DPN after full adjustment. Nadir nocturnal oxygen saturation (OR 0.960 (95% CI 0.927-0.994), p=0.022) was also an independent predictor of DPN. ODI quartiles were independent predictors of DPN (p=0.038), with only quartile 3 (ODI 6.65-14.39) reaching statistical significance when considering quartile 1 (ODI < 2.70) as the reference point (OR 3.346, 95% CI 1.408-7.949, p=0.006). Time spent with sats <80% was not independently associated with DPN (OR 1.873 (95%CI 0.935-3.749), p=0.077).

Table 4-5: Assessing the impact of possible confounders on the association between OSA and DPN (based on MNSI) using different logistic regression models (Backward method).

Model	Nagelkerke R Square	Odds ratio	95% confidence interval	P value
Unadjusted: OSA	0.131	4.091	2.277-7.350	<0.001
Model 1	0.160	3.799	2.098-6.878	<0.001
Model 2	0.188	3.391	1.839-6.252	<0.001
Model 3	0.206	3.162	1.705-5.865	<0.001
Model 4	0.218	2.915	1.560-5.446	0.001
Model 5	0.239	2.815	1.502-5.274	0.001
Model 6	0.245	2.649	1.404-4.998	0.003
Model 7	0.256	2.744	1.458-5.164	0.002
Model 8	0.286	2.603	1.364-4.968	0.004

Model 1: OSA + age

Model 2: OSA + age + ethnicity + gender + diabetes duration

Model 3: OSA + age + ethnicity + gender + diabetes duration + BMI

Model 4: OSA + age + ethnicity + gender + diabetes duration + BMI + waist circumference

Model 5: includes the variables that are different between patients with and without OSA as indicated in table 4.1. Model 5 includes: OSA + age + ethnicity + gender + diabetes duration + BMI + height + systolic BP + HbA1c + triglycerides + HDL + eGFR + alcohol + oral anti-diabetes treatments + insulin + anti-hypertensive agent use (ACE inhibitors, angiotenisn II blockers, beta blockers, alpha blockers, calcium antagonists and diuretics were included in the model individually).

Model 6: As for model 5 but BMI and waist circumference inserted together into the model.

Model 7: OSA + ethnicity + age + gender + alcohol intake + smoking + BP (systolic and diastolic) + diabetes duration + HbA1c + Total cholesterol + HDL + triglycerides + TSH + eGFR + oral glucose lowering treatments (including metformin, sulphonylurea, glitazones, and DPP-4 inhibitors combined) + insulin+GLP-1 analogues + anti-hypertensive agents (ACE inhibitors, angiotenisn 2 blockers, beta blockers, alpha blockers, calcium antagonists and diuretics were included in the model individually) + anti-platelets (aspirin and clopidogrel combined) + lipid lowering therapy (including statins, ezetimibe and fibrates combined) + peripheral vascular disease + height + obesity (BMI and waist circumference inserted separately)

Model 8: As for model 7 but BMI and waist circumference inserted together into the model

Using the monofilament test to detect the “at risk foot” as an outcome, OSA remained independently associated with foot insensitivity (OR 4.147, 95% CI 1.904-9.034, p<0.001, Nagelkerke R Square 0.360) after adjustment as in **Table 4.5**. Similar to DPN, both mild (OR 5.035, 95% CI 2.160-11.735, p<0.001) and moderate to severe (OR 3.174, 95% CI 1.295-7.779, p=0.012) OSA were independently associated with the “at risk foot” after adjustment. AHI quartiles where also independently associated with abnormal 10g monofilament sensation; with quartile 1 as the reference, quartile 2 (OR 4.080, 95%CI 1.431-11.633, p=0.009), quartile 3 (OR 4.788, 95%CI 1.633-14.042, p=0.004), and quartile 4(OR 3.757, 95%CI 1.296-10.890, p=0.015) were all independent predictors of the “at risk foot”. ODI, nadir nocturnal oxygen saturation and time spent with oxygen saturations < 80% were not independent predictors after adjustment.

In addition, OSA and AHI quartiles were independently associated with MNSIe components. To assess the relationship between OSA and the MNSIe components, we repeated the logistic regression models as performed in **Table 4.5**, but changed the outcome measure to the aspect of clinical examination of interest. OSA was independently associated with reduced/absent ankle jerk reflex (OR 2.932, 95% CI 1.528-5.626, p=0.001). OSA was also independently associated with reduced/absent vibration sensation (OR 3.429, 95% CI 1.745-6.737, p<0.001). OSA was also independently associated with having an abnormality on foot inspection (OR 2.309, 95% CI 1.285-4.147, p=0.005). 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. Furthermore, with quartile 1 being the reference, AHI quartiles were independent predictors of reduced/absent ankle jerk reflex; quartile 2 (OR 2.270, 95%CI 1.001-5.146, p=0.05), quartile 3 (OR 2.071, 95%CI 0.893-4.798, p=0.090), quartile 4 (OR 4.093, 95%CI 1.634-10.250, p=0.003). AHI quartiles were also independent predictors of reduced vibration sensation; quartile 2 (OR 3.135, 95%CI 1.256-7.824, p=0.014), quartile 3 (OR 5.493, 95%CI 2.052-14.705, p=0.001) and quartile 4 (OR 2.848, 95%CI 1.065-7.615, p=0.037). AHI quartiles were not independently associated with abnormal foot inspection.

In order to assess the relationship between OSA and hypoxemia severity, we divided MNSIe into 3 categories (MNSIe score categories: <2, 2- <4 and ≥ 4) and compared OSA and hypoxemia measures across these groups. Patients characteristics across MNSIe categories are summarised in **Table 4.6**. MNSIe categories correlated significantly with OSA metrics and nocturnal hypoxemia severity, independently of age, obesity, diabetes duration, gender and eGFR in the case of AHI (**Table 4.7**). There was also a significant trend of higher DPN prevalence in patients with lower nadir oxygen saturations during sleep (**Figure 4.2**, p=0.022 for the trend). There was no significant increase in DPN prevalence across AHI categories (60%, 57.1% and 61.5% for mild, moderate and severe OSA respectively).

Table 4-6: Participants characteristics in relation to MNSIe categories.

Data presented as median (IQR) or mean±SD. GFR: Glomerular Filtration Rate. P value for the trend

	Group 1: < 2 (n=90)	Group 2: 2 - < 4 (n=100)	Group 3: ≥ 4 (n=44)	**P value**
Male	51.1%	54.0%	79.5%	
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)	23.3%	30.0%	31.8%	0.475
eGFR	90.1±27.1	87.4±24.4	75.1±26.8	0.009

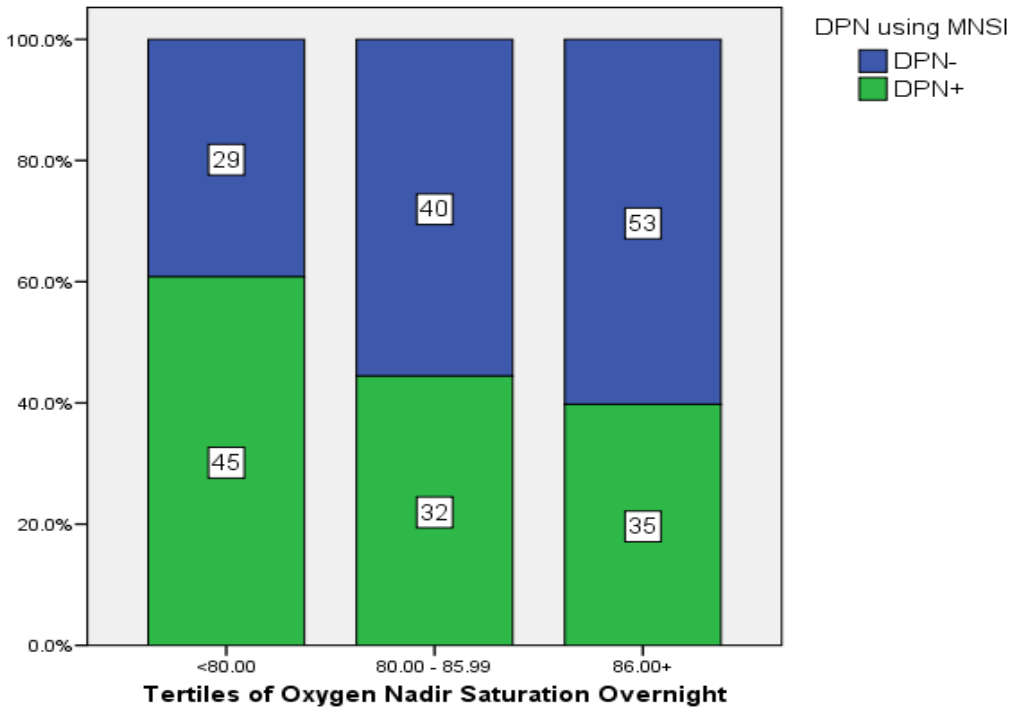
Table 4-7: The relationship between DPN severity based on the MNSIe score and OSA and nocturnal hypoxemia severity using the Kruskal-Wallis H test.

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 of the analysis performed. Data in the adjusted analysis presented as mean (95% confidence interval).

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)
Adjusted p value	0.020	0.075	0.890	0.262

Figure 4-2: The relationship between DPN prevalence (based on MNSI) and OSA severity as represented by the nadir oxygen saturation during sleep.

Numbers in the bars represents number of patients. The p value for the trend is 0.022. Analysis was performed using the Chi-square test.



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 (**Table 4.8**) DPN prevalence remained higher in the OSA+ group (52.9% vs. 24.3%, 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 (42.9% vs. 12.9%, p<0.001). After adjustment as in **Table 4.5**), OSA remained independently associated with DPN (OR 3.359, 95% CI 1.532-7.366, p=0.002, Nagelkerke R Square 0.255) or at risk foot (based on monofilament perception) (OR 5.297, 95%CI 1.909-14.696, p=0.001, Nagelkerke R Square 0.380).

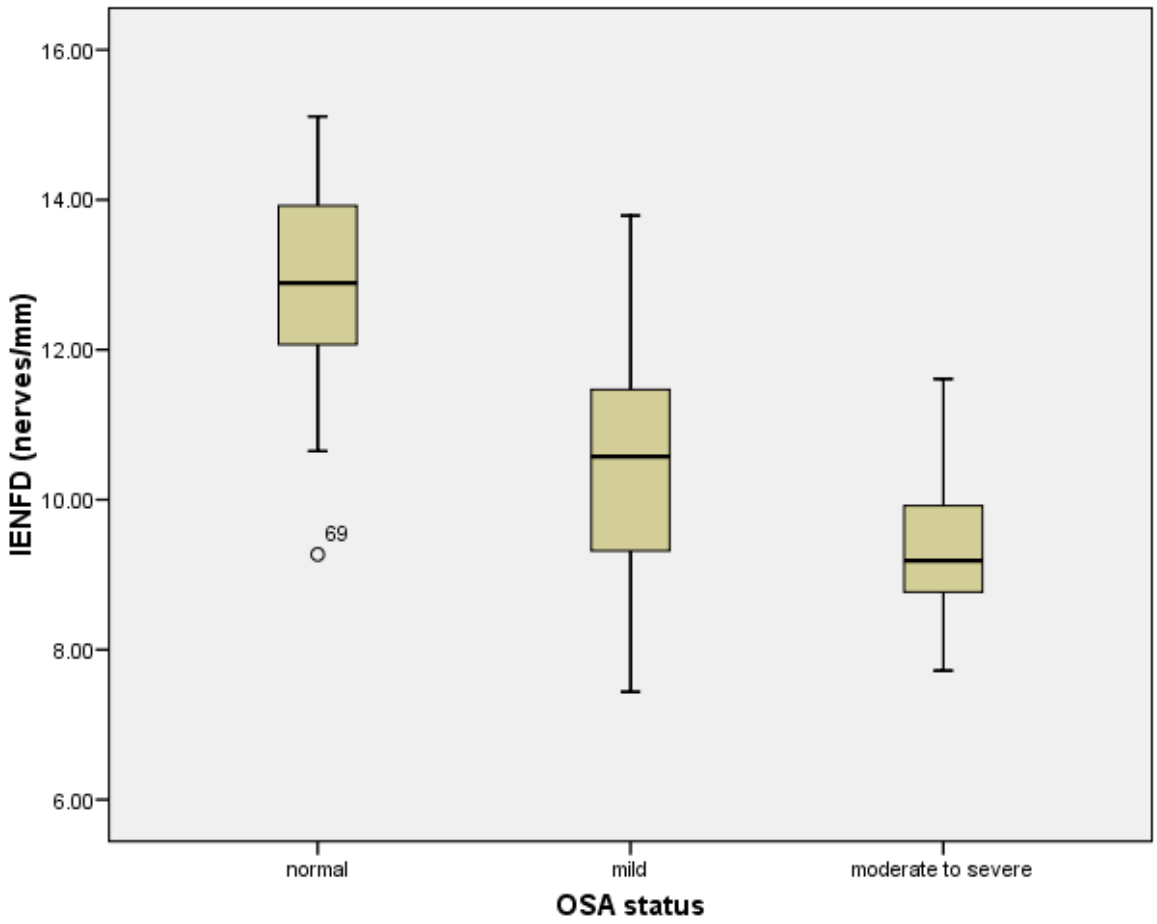
Table 4-8: The characteristics of patients in the matched subgroup in relation to OSA status.

Data presented as median (IQR) or mean (SD). GFR: Glomerular Filtration Rate, PVD: Peripheral Vascular Disease. The main aim for this subgroup is to match for BMI and diabetes duration.

	OSA- (n=70)	OSA+ (n=70)	P value
Male	47.1%	71.4%	0.003
White Europeans	31.4%	60%	0.001
Age (years)	55.1±12.2	59.8±10.2	0.015
Diabetes Duration (years)	10.0 (6.0-15.0)	10.0 (6.0-15.0)	0.876
Body Mass Index (kg/m^2)	30.0 (26.9-33.9)	31.3 (27.8-33.8)	0.394
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)	0.083
Diastolic blood pressure (mmHg)	78.5 (70.1-85.6)	78.0 (73.1-82.1)	0.877
HbA1c (%)	7.7 (7.1-8.7)	8.0 (7.0-9.1)	0.683
Total cholesterol (mmol/L)	3.7 (3.3-4.4)	3.6 (3.1-4.2)	0.319
Triglycerides (mmol/L)	1.6 (1.1-2.3)	1.7 (1.2-2.4)	0.759
HDL (mmol/L)	1.19 (1.0-1.39)	1.05 (0.90-1.17)	0.011
Estimated GFR (ml/min/1.73 m^2)	90.3±23.2	84.7±24.1	0.159
TSH	1.7 (1.1-2.2)	1.6 (1.0 vs. 2.1)	0.684
Epworth sleepiness score	5 (1-11)	8 (3-13)	0.027
Smoking (current or ex-smoker)	41%	41%	1.0
Alcohol (drinks alcohol)	11.4%	32.9%	0.002
Oral anti-diabetes treatment	97.1%	92.9%	0.245
Insulin	44%	54%	0.237
Lipid lowering therapy	84.3%	78.6%	0.385
Anti-hypertensives	72.9%	80%	0.319
PVD	1.4%	4.3%	0.310

IENFD results were available in 48 patients (9 with no OSA, 22 mild OSA and 17 moderate to severe OSA). Patients with OSA (AHI ≥ 5) had lower IEFD than those without OSA (12.8 ± 1.9 vs. 10.1 ± 1.5, p<0.001), similar results were found for OSA defined as AHI ≥ 10 (n=22) vs. those with AHI < 10 (n=26) (11.1 ± 2.0 vs. 9.9 ± 1.6, p=0.02). There was also a significant trend of lower IENFD with worsening OSA status (p<0.001) (**Figure 4.3**).

Figure 4-3: The relationship between IENFD and OSA status severity.



IENFD correlated with AHI (r=-0.51, p<0.001), and ODI (r=-0.33, p=0.02) but not with time spent with oxygen saturation < 80% or nocturnal nadir oxygen saturation.

Following adjustment for age, gender, ethnicity, diabetes duration, HbA1c, eGFR, BP, BMI and insulin use, OSA (AHI ≥ 5) (B=-2.39, p=0.002), OSA (AHI ≥ 15) (B=-1.62, p=0.01) AHI (B= -2.3, p=0.001), and ODI (B=-1.99, p=0.007) were all independently associated with IENFD .

4.6. 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 (333;337). The DPN prevalence in our cohort is also similar to previous studies (56-58;60).

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) (please see chapter 2 for more details). It is worth noting that all patients with a history of 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.

We have shown that gender and ethnicity has a major impact on OSA prevalence in T2DM. OSA was associated with DPN and foot insensitivity in both ethnicities and men and women, although this relationship was stronger in White Europeans and in men. Interestingly, examining the impact of gender ethnicity interaction showed that OSA was not associated with either DPN or foot insensitivity in South Asian women, while the relationship existed in South Asian men, White

European women and White European men. The lack of relationship between OSA and foot insensitivity in South Asian women is likely to be related to the small proportion of patients in this category who had impaired perception to 10g monofilament (15%). Hence, we needed a larger cohort of South Asian women to detect such a relationship. The lack of relationship between OSA and DPN in South Asian women could also be in part due to the small sample size of patients in this category and the lower DPN prevalence in South Asians. Larger studies are needed to assess the impact of the gender ethnicity interaction on the relationship between OSA and DPN and confirm our findings.

The independent association between OSA, AHI, ODI and IENFD confirms the association between OSA and DPN which was found based on clinical parameters of DPN (the MNSI). Furthermore, the association between OSA and IENFD suggests that OSA is associated with small fibres neuropathy in addition to larger fibre dysfunction (such as detected by vibration and 10g monofilament perception).

There are several possible explanations for a relationship between OSA and DPN (please see chapter 2 for more details). OSA has been shown to increase AGE production (400) and has been associated with altered PKC signaling (403;406), which plays an important role in cellular response to hypoxia (507). OSA is associated with decreased endothelial nitric oxide synthase and increased endothelin-1 levels (410;508). OSA is also associated with hypercoagulability (increased plasminogen activator inhibitor-1) (421) and inflammation (NF-KB)(366). The repetitive episodes of re-oxygenation following hypoxemia in OSA patients simulate ischemia–reperfusion injury which results in the generation of reactive oxygen species (366;390). Furthermore, OSA has been recently identified as a "missed" cause in patients with idiopathic peripheral neuropathy (509;510). 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.

However, the relative importance of intermittent and/or sustained/chronic hypoxemia in the association observed between OSA and DPN is unclear. The correlation between DPN severity and AHI, ODI, and nocturnal hypoxemia measures on the one hand and that between serum nitrotyrosine abundance, lipid peroxide levels and OSA severity and nocturnal hypoxemia on the other suggest that both types of hypoxemia might play a role. Indeed patients with COPD (who have sustained hypoxemia) are known to be at increased risk of peripheral neuropathy (511).

We also report that mild and moderate to severe OSA and AHI and nadir nocturnal oxygen saturations are all independently associated with DPN. It is also apparent that worsening hypoxemia is associated with higher DPN prevalence and that worsening DPN is associated with worsening OSA and hypoxemia measures. Nonetheless, there was no increase in DPN 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.

An intriguing finding is that our population was not excessively sleepy as assessed by the Epworth Sleepiness Score (ESS); even in patients with OSA the median ESS 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 (please see chapter 3 for more details).

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 glycaemic indices in some studies (343;344); others did not show a benefit (345;348). 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.

The main limitation of our study is its cross-sectional nature, hence causation cannot be proven. We have used home-based portable multi-channel respiratory devices rather than in-patient overnight polysomnography. However, this approach is well established and validated (512). The MNSI is not the "gold standard" for diagnosing or staging DPN but it has been validated against nerve conduction studies (506;513) and has been used widely in landmark studies (56;468;469;473). 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.

In conclusion, we have identified a novel association between DPN, the at risk foot, IENFD and OSA in patients with T2DM. 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 OSA treatment on DPN.

5. Chapter five: Obstructive Sleep Apnoea and Sight threatening diabetic retinopathy in Patients with Type 2 Diabetes

5.1. Introduction

DR is the leading cause of blindness of patients at working-age in the Western world and results in great morbidity and significant economic burden (40;41). The overall prevalence of DR is estimated to be between 40-50% in patients with diabetes (40). Known risk factors for DR include increasing age, hyperglycaemia, hypertension, diabetes duration, dyslipidemia, pregnancy, puberty, cataract surgery, obesity, alcohol consumption and genetic factors (eg. Aldose reducatase gene) (40;43). Although the precise aetiology of DR remains debated, but increased inflammation, OS, AGE formation, activation of the polyol pathway and the rennin-angiotensin system and activation of PKC, result in direct cellular damage and functional and/or structural defects involving the microvasculature, which result in increased vascular permeability (resulting in macular oedema) or in ischemic changes resulting in an increase in several factors such as VEGF, insulin-like-growth factor-1 (IGF-1), and erythropoietin which result in neovascularisation and the development of proliferative retinopathy (40;44-46). Despite the improvement in controlling metabolic/systemic and vascular risk factors, DR remains very common and a significant proportion of those with DR will progress to sight threatening diabetic retinopathy (STDR), requiring Laser as the standard treatment(40). Hence, improved understanding of DR pathogenesis and risk factors is important in order to identify new treatment targets/strategies.

5.2. Hypothesis

OSA and nocturnal hypoxemia are associated with STDR and impaired perception of 10g monofilament sensation in patients with T2DM

Rationale: OSA is associated with several pathways that are involved in the pathogenesis of STDR such as oxidative stress, PKC activation and AGE formation (366;400;505).

5.3. Aims

The primary aim of this study is to explore the interrelationships of OSA and STDR in subjects with T2DM. Secondary aims of this study include:

4. Exploring the relationship between nocturnal hypoxemia and STDR
5. Assessing the relationship between OSA and macular thickness in patients with T2DM.

5.4. Methods:

The methods of this study are similar to those in Chapter 4. OSA was assessed by performing a single overnight home-based cardio-respiratory sleep study using a portable multi-channel device (Alice PDX, Philips Respironics), please refer to Chapters 2 and 4 for more details. The presence of DR/STDR was assessed using 2 x 45 degrees digital retinal images per eye as per the English National Screening programme guidelines (466), please refer to Chapter 2 for more information. A subgroup of patients (n=51) had OCT based on clinical need, either to monitor treatment of macular oedema or in cases when the diagnosis is unclear on retinal photographs (514). Data from this sub-group was used to correlate OSA severity with foveal and macular thickness as measured by OCT. OCT was performed using Zeiss Stratus.

Details of statistical methods can be found in chapter 2. In order to further explore the impact of baseline differences on the associations observed, a sub sample of 69 patients with and 69 without OSA were group matched for a variety of risk factors.

5.5. Results:

Two hundred and sixty six patients were recruited. 8 did not attend the appointment for the digital retinal photography repeatedly, 30 patients had poor quality recordings and 2 patients had predominantly CSA were excluded; leaving 226 patients for analysis. Of these 226 patients, 57.1% were men and 54.0% White Europeans and 46.0% South Asians.

The overall prevalence of OSA was 63.3% which was also more common in White Europeans than South Asians (73.8% vs. 51.1%, p<0.001, respectively). Of the 143 patients with OSA, 60.8% were mild (AHI 5 to < 15 events per hour), 23.1% were moderate (AHI 15 to < 30) and 16.1% were severe (AHI ≥ 30). The clinical characteristics of this study population are similar to that in **Table 4.1**. As expected, patients with OSA (OSA+) were older and had higher systolic BP, BMI, waist and neck circumferences and were sleepier compared to those without OSA (OSA-) (**Table 4.1**). In addition, OSA+ patients exhibited higher triglycerides, lower HDL levels and consumed more alcohol (**Table 4.1**). The use of oral hypoglycemic agents was similar between OSA+ and OSA- patients apart from metformin use which was slightly higher in the OSA- group. The use of antihypertensive agents and insulin was higher in OSA+ patients (**Table 4.1**). There was no difference in the use of anti-platelet or lipid-lowering therapy between OSA+ and OSA- patients. =

The overall prevalence of STDR was 37.6%, DR prevalence was 62.8% (R0 37.2%, R1 45.1%, R2 9.7%, R3 8.0%) and maculopathy prevalence was 33.2%. There were no significant differences in the prevalence of STDR, DR or maculopathy prevalence between South Asians and White Europeans. The prevalence of STDR, pre-proliferative and proliferative DR and maculopathy was higher in OSA+ patients (**Table 5.1**). The prevalence of background retinopathy was lower in the OSA group (**Table 5.2**). A subgroup analysis by ethnicity showed that these differences remained true in both South Asians and White Europeans (**Figure 5.1**)

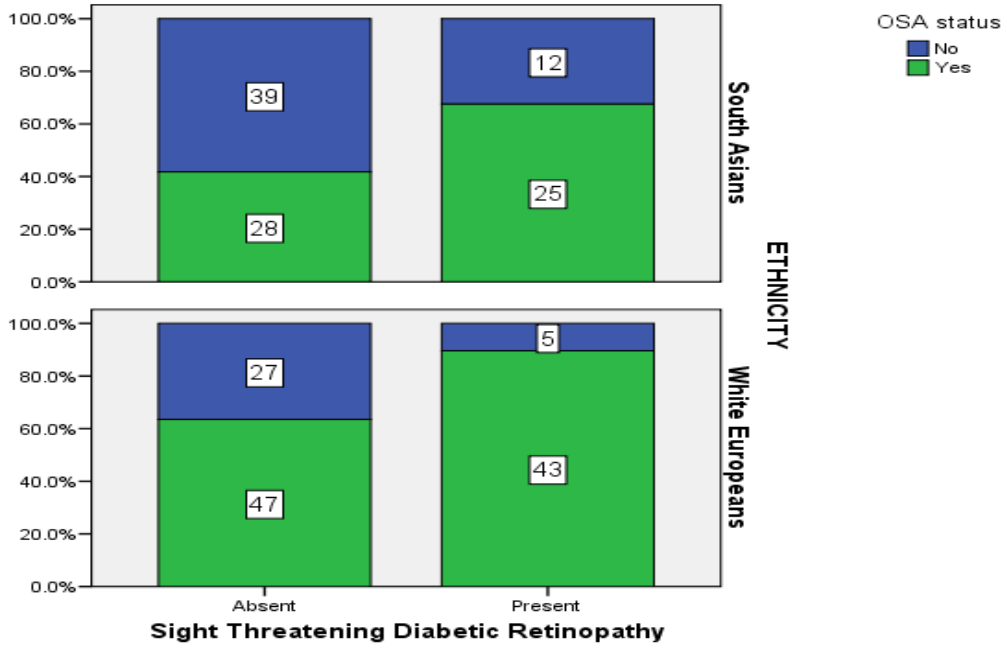
Table 5-1: The relation between OSA status and sight threatening diabetic retinopathy, retinopathy and maculopathy (unadjusted analysis).

Data presented as prevalence of DR in the respective OSA group.

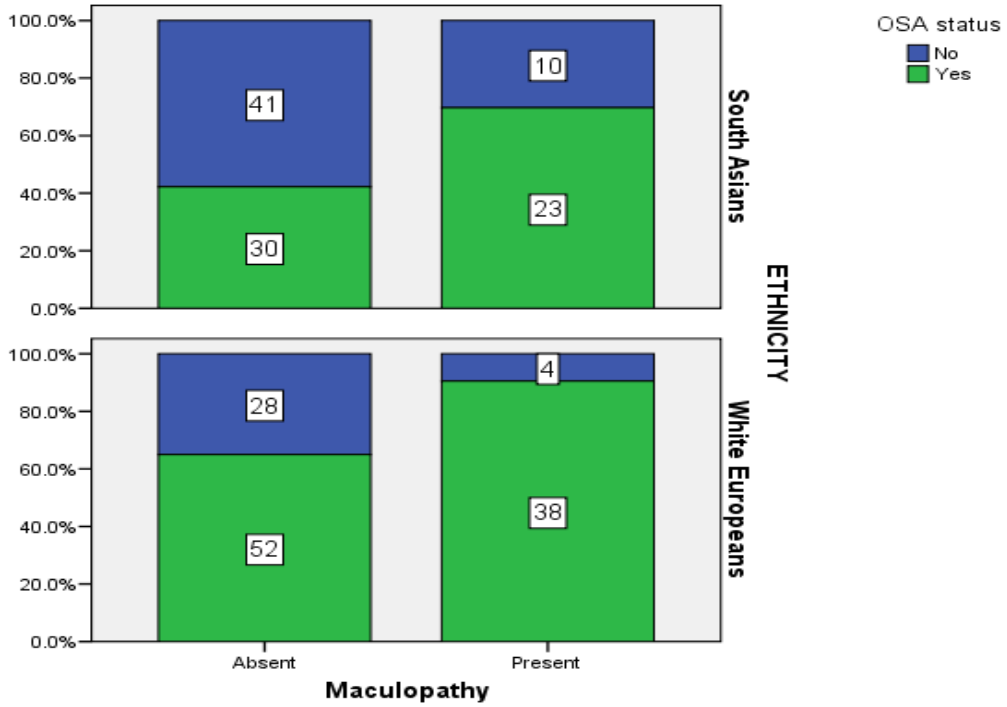
		OSA-	OSA+	P values
Sight threatening diabetic retinopathy		20.5%	47.6%	<0.001
Retinopathy status	R0	45.8%	32,2%	0.001
	R1	49.4%	42.7%	
	R2	1.2%	14.7%	
	R3	3.6%	10.6%	
Maculopathy		16.9%	42.7%	<0.001

Figure 5-1: The relation between sight threatening diabetic retinopathy, retinopathy and maculopathy and OSA in South Asians and Europeans with type 2 diabetes.

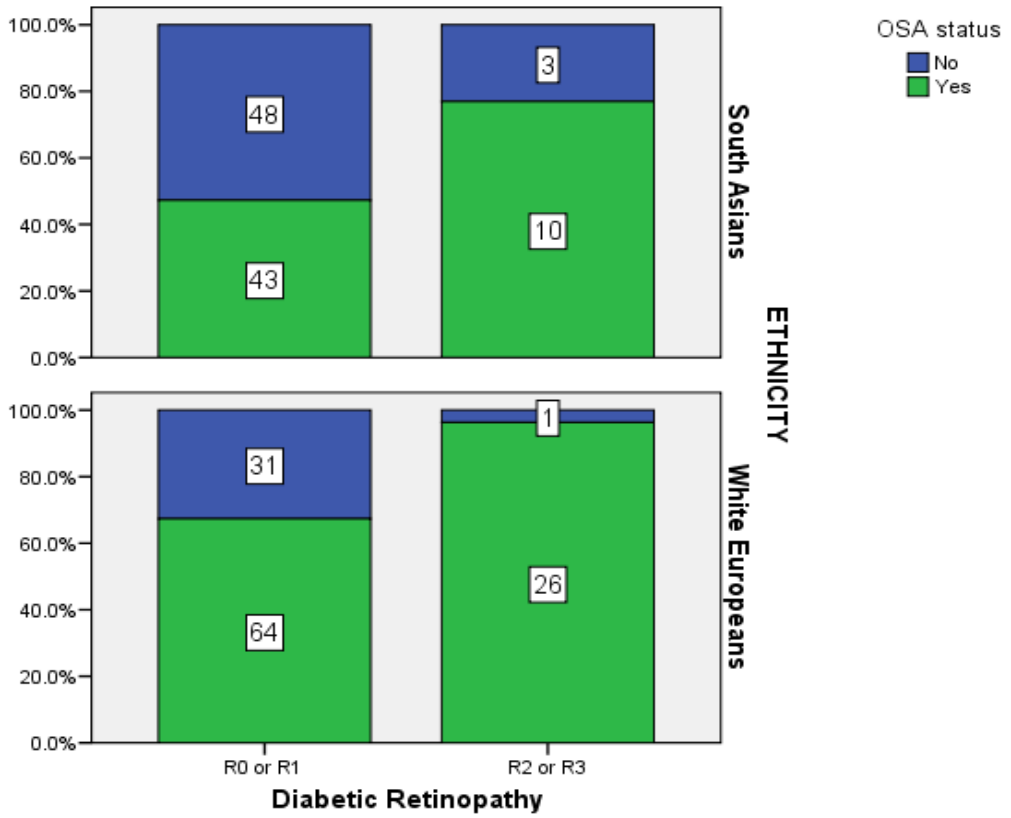
Numbers in bars represent absolute counts. The p values are for the difference between OSA+ and OSA- patients.



P= 0.012 in South Asians and 0.001 in White Europeans



P= 0.009 in South Asians and 0.002 in White Europeans.



P=0.045 in South Asians and 0.003 in White Europeans.

The impact of gender on the relationship between OSA and DR is summarised in **Table 5.2**. The relationship between OSA and STDR and maculopathy does not seem to be affected by gender. The relationship between OSA and retinopathy status followed the same pattern in men and women, with patients with OSA having more R2 and R3 and less R1 and R0; but the relationship in women was stronger than that in men (**Table 5.2**)

The impact of gender ethnicity interaction on the relationship between OSA and DR is summarised in **Table 5.3**. On the whole, OSA patients had more STDR, maculopathy, R2 and R3 and less R0 and R1 regardless of ethnicity and gender, but the relationship between OSA and advanced retinopathy (R2 or R3) was weaker in South Asian men compared to other ethnic/gender groups.

Table 5-2: The impact of gender on the relationship between OSA and DR.

		OSA-	OSA+	P values
Men (n=129)				
Sight threatening diabetic retinopathy		17.6%	44.2%	0.006
Retinopathy status	R0	38.2%	31.6%	0.218
	R1	52.9%	43.2%	
	R2	2.9%	14.7%	
	R3	5.9%	10.5%	
Maculopathy		11.8%	40.0%	0.003
Women (n=97)				
Sight threatening diabetic retinopathy		22.4%	54.2%	0.001
Retinopathy status	R0	51.0%	33.3%	0.008
	R1	46.9%	41.7%	
	R2	0%	14.6%	
	R3	2.0%	10.4.%	
Maculopathy		20.4%	47.9%	0.004

Table 5-3: The impact of the gender ethnicity interaction on the relationship between OSA and DR

		OSA-	OSA+	P values
South Asian men (n=60)				
Sight threatening diabetic retinopathy		17.9%	37.5%	0.092
Retinopathy status	R0	35.7	25	0.570
	R1	53.6	62.5	
	R2	3.6	9.4	
	R3	7.1	3.1	
Maculopathy		10.7%	37.5%	0.017
White European men (n=69)				
Sight threatening diabetic retinopathy		16.7%	47.6%	0.213
Retinopathy status	R0	50.0%	34.9%	0.443
	R1	50.0%	33.3%	
	R2	0%	17.5%	
	R3	0%	14.3%	
Maculopathy		16.7%	41.3%	0.238
South Asian women (n=44)				
Sight threatening diabetic retinopathy		30.4%	61.9%	0.036
Retinopathy status	R0	47.8%	28.6%	0.05
	R1	52.2%	42.9%	

	R2	0%	19%	
	R3	0%	9.5%	
Maculopathy		30.4%	52.4%	0.139
White European women (n=53)				
Sight threatening diabetic retinopathy		15.4%	48.1%	0.011
Retinopathy status	R0	53.8%	37%	0.199
	R1	42.3%	40.7%	
	R2	0%	11.1%	
	R3	1.9%	5.7%	
Maculopathy		11.5%	44.4%	0.008

In order to assess whether the relationship between OSA and STDR is secondary to or independent of the differences observed in baseline characteristics (as outlined in **Table 4.1**), logistic regression models (backward method) were used (**Table 5.4**). Despite adjustment for a wide range of possible confounders, OSA remained an independent predictor of STDR (**Table 5.4**). In addition to OSA (OR 3.628, 95% CI 1.753-7.510, p=0.001), other independent predictors of STDR included: diabetes duration (OR 1.115, 95% CI 1.064-1.169, p<0.001), HbA1c (OR 1.355, 95% CI 1.085-1.694, p=0.007) and the use of anti-hypertensives (OR 3.100, 95% CI 1.140-8.424, p=0.027).

Similar results were found in regards to maculopathy (M1) or advance retinopathy (R2 or R3). After adjustment for possible confounders as in **table 5.4**, OSA remained an independent predictor of maculopathy (OR 3.320, 95% CI 1.591-6.926, p=0.001) and advanced retinopathy (OR 6.065, 95% CI 1.914-19.226, p=0.002).

In order to assess the relationship between STDR and OSA metrics and hypoxemia measures, we used the same logistic regression model as in **Table 5.4** but replace OSA with the OSA/hypoxemia measure of interest. Replacing OSA with AHI quartiles showed that AHI quartiles were independent predictors of STDR (p=0.031). Using AHI quartile 1 (AHI < 2.90) as the reference point, quartile 3 (7.06-16.09) (OR 3.689, 95% CI 1.472-9.246, p=0.005) and quartile 4 (≥ 16.10) (OR 2.668, 95% CI 1.052-6.763, p=0.039) were independently associated with STDR; while quartile 2 (2.90-7.59) (OR 1.638, 95% CI 0.660-4.063, p=0.287) was not a predictor of STDR.

Similar to STDR, AHI quartiles were independent predictors of maculopathy (p=0.04) when using the same model as in **Table 5.4**. Using quartile 1 as the reference point, AHI quartiles 3 (OR 3.761, 95% CI 1.472-9.608, p=0.006) and 4 (OR 2.583, 95% CI 0.999-6.677, p=0.05) were associated with maculopathy, while quartile 2 (OR 1.785, 95% CI 0.703-4.531, p=0.223) was not an independently associated with maculopathy.

Unlike STDR and maculopathy, only AHI quartile 4 was an independent predictor of advanced retinopathy (R2 or R3) (OR 7.824, 95% CI 1.874-32.657, p=0.005), when quartile 1 was taken as the reference point. AHI quartile 2 (OR 3.882, 95% CI 0.876-16.677, p=0.074), and quartile 3 (OR 4.024, 95% CI 0.932-17.382, p=0.062) were not independently associated with advanced retinopathy.

Similar to AHI, ODI tertiles 2 (4.10-11.39) and 3 (≥ 11.4) were independently associated with STDR (OR 3.46, 95%CI 1.47-8.16, p=0.005 and OR 3.25, 95%CI 1.28-8.23, p=0.013 for ODI tertiles 2 and 3 respectively) and maculopathy (OR 3.26, 95%CI 1.36-7.85, p=0.008 and OR 2.88, 95%CI 1.11-7.49, p=0.030 for ODI tertiles 2 and 3 respectively) and Advanced DR (OR 5.24, 95%CI 1.38-19.86, p=0.015 and OR 6.67, 95%CI 1.67-26.55, p=0.007 for ODI tertiles 2 and 3 respectively) when tertile 1 (<4.1) was taken as the reference point.

Time spent with oxygen saturation < 80% and nadir nocturnal oxygen saturation were not independent predictors of STDR, maculopathy or advanced DR.

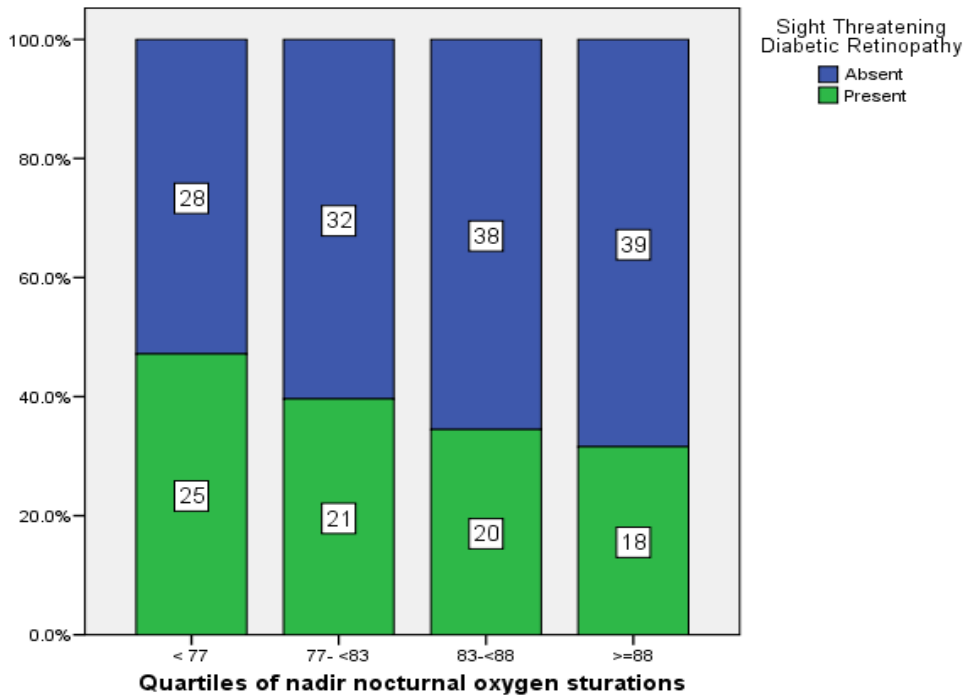
Similarly to STDR and maculopathy, ODI quartile, time spent with oxygen saturation < 80% and nadir nocturnal oxygen saturation were not independent predictors of maculopathy using the same logistic regression model.

Table 5-4: Assessing the impact of possible confounders on the association between OSA and STDR, maculopathy and advanced retinopathy using different logistic regression models Backward method).

The odds ratios (OR) reported are the odds for having STDR in OSA+ compared to OSA- patients. The model is adjusted for OSA + ethnicity + age + gender + alcohol intake + smoking + BP + diabetes duration + HbA1c + Total cholesterol + HDL + Triglycerides + eGFR + oral glucose lowering treatments (including metformin, sulphonylurea, glitazones, and DPP-4 inhibitors combined) + insulin + GLP-1 analogues + anti-hypertensives (ACE inhibitors, angiotenisn 2 blockers, beta blockers, alpha blockers, calcium antagonists and diuretics combined) + anti-platelets (aspirin and clopidogrel combined) + lipid lowering therapy (including statins, ezetimibe and fibrates combined) + obesity + recruitment site. Models 1, 2, and 3 included BMI, waist circumference and waist/hip ratio respectively

Model	Nagelkerke R Square	Odds ratio	95% confidence interval	P value
Sight Threatening Diabetic Retinopathy				
Unadjusted: OSA	0.10	3.520	1.882-6.583	<0.001
Model 1	0.345	3.628	1.753-7.510	0.001
Model 2	0.345	3.628	1.753-7.510	0.001
Model 3	0.360	3.684	1.766-7.685	0.001
Maculopathy				
Unadjusted: OSA	0.099	3.666	1.889-7.117	< 0.001
Model 1	0.343	3.320	1.591-6.926	0.001
Model 2	0.343	3.320	1.591-6.926	0.001
Model 3	0.343	3.320	1.591-6.926	0.001
Advanced DR (R2 or R3)				
Unadjusted: OSA	0.123	6.645	2.272-19.433	0.001
Model 1	0.337	6.065	1.914-19.226	0.002
Model 2	0.337	6.065	1.914-19.226	0.002
Model 3	0.344	5.218	1.620-16.806	0.006

Figure 5-2: The relation between STDR and OSA severity as represented by the nadir nocturnal oxygen saturation during sleep. Numbers in the bars represents number of patients.



There was no difference in STDR prevalence between patients with mild or moderate to severe sleep apnea (48.8% vs. 47.3%). However, there was a non-significant trend of increasing STDR prevalence amongst patients with the worse hypoxia as measured by nocturnal nadir oxygen saturations (**Figure 5.2**). Based on the subgroup of 51 patients who had OCT (32 with 19 without OSA, 59% male, 45% White Europeans, aged 58.8 (11.6) years, diabetes duration 15.1 (8.6) years, HbA1c 8.7 (1.6)%, systolic BP 131 (17) mmHg, BMI 32,3 (5.7) kg/m^2), foveal thickness correlated with AHI (r=0.31, p=0.03) and time spent with oxygen saturations below 80% (r=0.29, p=0.045). Using linear regression and after adjusting for gender, ethnicity, age at diabetes diagnosis, BMI, mean arterial pressure, and diabetes duration; foveal thickness remained associated with AHI (R^2=0.27, B=0.001, p=0.049) and time spent with oxygen saturation < 80% (R^2=0.31, B=0.002, p=0.012).

Although the logistic regression models showed that OSA is independently associated with STDR, we wanted to explore that further by matching patients for major STDR risk factors as much as possible. We have managed to group match 69 patients with and 69 patients without OSA for (OSA- vs. OSA+):

systolic BP (128.01±12.22 vs. 127.06±12.69 mmHg, p=0.665), diabetes duration (10.0 (5.5-15.5) vs. 11.0 (6.5-15.0) years, p=0.540) age (56.24±10.93 vs. 58.46±9.43 years, p=0.203), BMI (31.22±6.15 vs. 31.01±4.57 kg/m^2, p=0.819), waist circumference (105.58 ± 12.83 vs. 106.35±9.17 cm, p=0.683) and HbA1c (8.13±1.54 vs. 8.29±1.39 %, p=0.509). Despite the matching, STDR (43.5 vs. 18.8%, p=0.002), maculopathy (39.1% vs. 14.5%, p=0.001) and advanced retinopathy (21.7% vs. 5.8%, p=0.007) remained more common in OSA+ patients compared to patients with OSA, confirming the findings of the logistic regression models.

5.6. Discussion

T2DM and OSA frequently co-exist and can result in a range of metabolic and physiological perturbations implicated in the pathogenesis of STDR. We therefore studied possible interrelationships between OSA and STDR in a cohort of patients attending hospital clinics in the UK. Our report identifies OSA as a novel independent predictor of STDR, maculopathy and pre-proliferative and proliferative DR in patients with T2DM.

The population in our report comprises subjects attending a large inner city, hospital-based diabetes clinic in which the known duration of diabetes was approximately 10 years and many of the subjects already exhibited established diabetes complications such as proteinuria. As we have explained in the previous chapter, the OSA prevalence in our sample although high (63.4%) is consistent with other studies (333-335;337). For more in depth comparison with published literature, please refer to Chapter 4.

The overall prevalence of STDR and DR in our cohort (38% and 64% respectively) is higher than that reported in the literature (STDR 5-15% and DR 40-50%) (40;515). This is mainly due to the differences in studies population as our population is selected from the outpatients department of a secondary centre and had long diabetes duration while the other studies were population based studies.

As expected, patients with OSA differed from those without OSA in regards to multiple demographic and metabolic factors including age, diabetes duration, adiposity measures, lipid profiles and renal function. OSA was also more common in men. Patients with OSA had higher HbA1c and blood pressure and were prescribed more insulin (including higher insulin doses) and more anti-hypertensive agents. Nevertheless, although these differences contributed to the observed relationship between OSA and STDR, maculopathy and pre-proliferative and proliferative DR, OSA remained an independent predictor of these outcomes after adjustment for possible confounders. Furthermore foveal and central macular thicknesses were independently associated with OSA severity as measured by AHI and ODI.

The relationship between OSA and STDR, maculopathy and advanced DR was consistent across both ethnic groups and men and women in our cohort. This relationship however was stronger in White European compared to South Asians. The lack of association between OSA and advanced DR in South Asian men is likely to reflect the small sample size and the small number of events (i.e. advanced DR). The association between OSA and STDR, maculopathy and advanced DR was stronger in South Asian and White European women compared to men of the same ethnicity. This occurred despite that women had a better metabolic profile than men with the same ethnicity (see Chapter 3 for more details). The exact mechanisms behind these findings are not clear and the results of subgroup comparisons need to be treated cautiously due to the small sample size. But one possible mechanism is that OSA impact on the development or progression of DR might be more important in patients with better metabolic control, while in patients with worse metabolic control OSA might play a lesser role as hyperglycaemia and hypertension might have a more prominent role in driving the development of such complications. Larger studies are needed to explore these differences and studies examining the impact of different degrees of hyperglycaemia, hypertension and obesity on the relationship between OSA and DR would be of interest.

Interestingly in our results, although OSA was an independent predictor of pre-proliferative and proliferative DR, the prevalence of background retinopathy was actually lower in the OSA group (despite that the OSA patients had increased risk factors prevalence to retinopathy). This might suggest that OSA does not contribute to the development of DR in patients with diabetes, but might results in a more rapid progression from early to advanced DR compared to patients without OSA. Two previous studies examined the relation between OSA and DR in patients with type 2 diabetes, they have both shown similar results to ours but our study differs in several aspects. In the first study, Shiba et al, have used a highly selected population of 219 Japanese patients with type 2 diabetes, which were undergoing vitreous surgery for a variety of indications, and compared the ODI between patients with proliferative and non-proliferative DR (516). They have used pulse oximetry to calculate the ODI. The results showed that patients with proliferative DR had higher ODI compared to those with non-proliferative DR. After adjustment for age, HbA1c and the presence of hypertension, higher oxygen saturations were found to be protective against proliferative retinopathy (516). The second study, which has similar methodology to ours, examined the link between OSA and DR included 118 men from primary and secondary care in the UK (517). The study population was stratified using questionnaires and pulse oximetry, which was followed by a portable multichannel respiratory device to diagnose OSA. DR was assessed using 2-field images to assess DR. The results were similar to our study in that OSA was independently associated with DR and maculopathy after adjusting for age, BMI, diabetes duration and history of hypertension and OSA patients had less background retinopathy (517). Our study extends and adds to the results of those previous studies as we have not restricted our entry criteria to one gender and we included both White Europeans and South Asians. In addition, unlike the previous studies, our study population was recruited from the waiting area of our diabetes clinic and all the patients were examined for OSA using the multi-channel respiratory devices. Furthermore, our study population was well characterised compared to the previous studies which allowed us to adjust for a much wider range of possible confounders.

There are several possible explanations for a relationship between OSA and STDR (please refer to Chapter 2 for more details). Although the precise aetiology of DR remains debated, putative mechanisms include increased inflammation, OS and several other pathways leading to cellular and microvascular damage resulting in increased vascular permeability (macular oedema) or in ischemic changes (proliferative DR) (40).Data from human studies and animal models of OSA/intermittent hypoxia demonstrate that OSA activates several of these pathogenetic pathways. For example, OSA has been shown to increase AGE production (400) and has been associated with altered PKC signaling (including PKCα and PKCε and PKCδ) (403;406), which plays an important role in cellular response to hypoxia (507;518). OSA has been shown to be associated with decreased endothelial nitric oxide synthase and increased endothelin-1 levels (410;519). OSA is also associated with hypercoagulability (increased plasminogen activator inhibitor-1) (421) and inflammation (NF-KB) (366). Furthermore, OSA has been associated with increased VEGF and erythropoietin, which are major aetiological factors in the development of proliferative DR. Although hypoxemia markers were not independent predictors of STDR in our study, this might reflect the relatively uncomplicated grading system used the English National Screening programme; this is further supported by the relationship found between hypoxemia and foveal thickness when OCT (more quantitative measure than retinal images) was used.

Despite these molecular consequences of OSA, changes similar to proliferative retinopathy or maculopathy have not been described in OSA patients without diabetes, which suggests that the molecular consequences of OSA might be amplified when impacting on a tissue that is already damaged by hyperglycaemia. OSA, however, has been associated with several ocular pathologies in patients without diabetes, including generalised arterioral narrowing in the retina (as measured by lower arteriole-to-venule ratio) (450). Associations between OSA and retinal venous occlusion, central serous choriretinopathy, optic neuropathy and glaucoma have all been hinted in the literature (520-523).

The main limitation of our study is the use of home-based portable multi-channel respiratory devices rather than in-patient overnight polysomnography. However, this approach for the diagnosis of OSA is well established (512;524). Additionally, home-based sleep studies help to avoid the "first night effect" which is commonly encountered with hospital-based polysomnography as a result of sleeping in a new environment. Our study is cross-sectional, hence causation cannot be proven. As described above, our sample population is also drawn from secondary/tertiary diabetes centres; hence we cannot necessarily extend our conclusions to other patient populations. We have used 2-field images to assess DR, rather than 7-field images, which might result in missing peripheral retinal lesions. This is unlikely to affect the results unless patients with OSA preferably develop more peripheral lesions compared to patients without OSA (or vice versa), but there is no evidence to support this argument.

In conclusion, we have identified a novel association between OSA and STDR, advanced DR and maculopathy in patients with T2DM. Prospective studies are required to determine the role of OSA and intermittent hypoxia in the development and progression of STDR/DR/maculopathy and the impact of OSA treatment on these diabetes-related complications.

6. Chapter six: Obstructive Sleep Apnoea and Diabetic Nephropathy in Patients with Type 2 Diabetes

6.1. Introduction

Diabetic nephropathy is the most common cause of end-stage renal disease (ESRD) requiring dialysis (85) and is very common as it affects about 40% of patients with diabetes (86). Furthermore, diabetic nephropathy is an independent risk marker for all-cause mortality and adverse cardiovascular (CVD) events (86;525;526). As 20% of patients will have progressed to ESRD within 20 years after onset of overt proteinurea (85) diabetic nephropathy is also very costly as the annual cost of health care for patients with diabetic nephropathy is approximately 3 folds compared to those without nephropathy (86).

The pathogenesis of diabetic nephropathy is similar to other microvascular complications (as described in the introduction). Haemodynamic changes occur as a result of the activation of various vasoactive systems, such as RAAS and endothelin systems resulting in increased systemic and intraglomerular pressure, and microalbuminuria (87). The early haemodynamic changes result from decreased resistance in both the afferent and efferent arterioles of the glomerulus; with the afferent arteriole seems to have a greater decrease in resistance than the efferent, resulting in glomerular hyperperfusion and hyperfiltration (87). Many factors have been involved in this defective autoregulation, including prostanoids, NO, VEGF, TGF-1, and the RAAS (87). Another important factor in the early changes of diabetic nephropathy, changes in the protein nephrin. Nephrin, a protein found in podocytes, is crucial for maintaining the integrity of the intact dynamic filtration barrier (87). Patients with diabetic nephropathy have markedly reduced renal nephrin expression and increased nephrin excretion suggesting that nephrin excretion could be an early finding of podocyte injury (even before the onset of albuminuria) (87). Furthermore, the role of genetic susceptibility has been increasingly recognised in patients with diabetic nephropathy (88). The speed of progression from CKD is variable and largely dependent on BP and the degree of hyperglycaemia (87).

Diabetic nephropathy shares the same risk factors as CVD and hence multi-factorial management targeting multiple CVD risk factors is an essential part of the management of these patients (527;528). Improvements in glycaemic control, BP control and lipid levels have all been shown to be beneficial in patients with diabetic nephropathy (180-182;184;185). ACEi have been shown to play an essential role in the management of patients with diabetic nephropathy and slow the decline in GFR (87;191). Other life style changes such as stopping smoking, weight loss and dietary modifications (low protein, low salt diet) have also been shown to be of benefit (86).

However, despite intensive metabolic control, diabetic nephropathy remains very common and many patients with diabetes still progress to ESRD. Hence, better understanding of the pathogenesis and risk factors of this condition is needed in order to prevent the development or slow the progression of this complication.

6.2. Hypothesis

OSA is associated with diabetic nephropathy in patients with T2DM.

Rationale: OSA is associated with hypertension, impaired microvascular regulation, increased AGE levels, activation of PKC and increased oxidative stress in the general public, all of which are risk factors for the development and/or progression of diabetic nephropathy.

6.3. Aims

The primary aim of this study is to explore the interrelationships between OSA and nocturnal hypoxemia and diabetic nephropathy in subjects with T2DM. A secondary aim is to explore the relationship between OSA and albuminuria and renal function in patients with T2DM.

6.4. Methods

This is a secondary analysis that examined the relationship between OAS and diabetic peripheral neuropathy. For more details please refer to Chapters 2 and 4 of this thesis. Diabetic nephropathy was assessed using eGFR and albumin/ creatinine ratio (ACR). Albuminuria is considered as the earliest clinical evidence of diabetic nephropathy and is defined as albumin excretion of ≥ 30 mg/day (463). In our study, two abnormal early morning ACR measurements (out of three) within the last 6 months were required to diagnose albuminuria, due to the variable nature of albumin secretion (86;463). An ACR > 2.5 mg/mmol in men and > 3.5 mg/mmol in women was considered abnormal in our study (464). eGFR was calculated based on the MDRD equation ($186 \times (Creat / 88.4)^{-1.154} \times (Age)^{-0.203} \times (0.742$ if female), creatinine measured in mmol/L)(465). Diabetic nephropathy was diagnosed if eGFR was below 60 ml/min/1.73m^2 or ≥ 60 ml/min/1.73m^2 in the presence of albuminuria (465;529). eGFR was included in the definition of diabetic nephropathy as some patients with DM might develop severe kidney disease without the presence of albuminuria (86).

More detailed methods and statistical methods can be found in Chapter 2.

6.5. Results

Two hundred and sixty six patients were recruited. Data regarding eGFR was available in the whole cohort, but only 221 patients provided enough urinary samples to make the diagnosis of albuminuria. OSA data was available on 234 patients out of 266 (30 had inadequate sleep studies and 2 had central sleep apnoea), of which 234 patients had eGFR available and 199 patients provided urinary samples.

Of these 234 patients, 57.7% were men and 55.1% White Europeans and 44.9% South Asians. The overall prevalence of OSA was 64.5%. Of the 151 patients with OSA, 60% had mild (AHI 5 to < 15 events per hour), 23% had moderate (AHI 15 to < 30) and 17% had severe (AHI ≥ 30) OSA. The overall prevalence of diabetic nephropathy, eGFR<60 and albuminuria were 39.7%, 15% and 36.2%

respectively. Of the patients with albuminuria, 72% had microalbuminuria (26.1% of the total sample) and 28% had microalbuminuria (10.1% of the total sample).

As described in previous chapters, patients with OSA (OSA+) were older, had longer diabetes duration and higher systolic BP, BMI, waist and neck circumference and were sleepier compared to those without OSA (OSA-) (**Table 4.1**). In addition, OSA+ patients exhibited more lipid abnormalities and consumed more alcohol (**Table 4.1**).

The relationship between OSA and diabetic nephropathy is summarised in **Table 6.2**. Patients with OSA had lower eGFR and higher prevalence of diabetic nephropathy and albuminuria and higher ACR levels (**Table 6.2**). Diabetic nephropathy (58% vs. 28.6%, p= 0.003 and 54.5% vs. 32%, p=0.05 for South Asians and White Europeans respectively) and albuminuria (50% vs. 26.5%, p=0.016 and 38.7% vs. 20.0%, p=0.088 for South Asians and White Europeans respectively) remained more common in patients with OSA regardless of ethnicity, although the relationship was stronger in South Asians.

Table 6-1: The relationship between OSA and diabetic nephropathy.

	OSA-	OSA+	P value
eGFR (n=234)	92.9 ± 25.2	82.4 ± 26.4	0.006
Diabetic nephropathy (n=201)	29.7%	55.9%	< 0.001
eGFR < 60 (n=234)	8.4%	18.5%	0.038
Albuminuria (n=199)	24.3%	43.2%	0.007
ACR (mg/mmol) (n=213)	0.73 (0.27-2.90)	1.4 (0.40-6.63)	0.004

The impact of gender, ethnicity and gender ethnicity interaction on the relationship between OSA and diabetic nephropathy is summarised in **Tables 6.2, 6.3 and 6.4.** The relationship between diabetic nephropathy, albuminuria, and OSA is similar both ethnicities although the relationships is stronger in South Asians (**Table 6.2**). The relationship between OSA and diabetic nephropathy was stronger in women (**Table 6.3**). The analysis of ethnicity gender interaction shows that most of the relationship between OSA and diabetic nephropathy and albuminuria is with South Asian women, the relationship was significantly weaker in the other groups (**Table 6.4**).

Table 6-2: The impact of ethnicity on the relationship between OSA and diabetic nephropathy

	OSA-	OSA+	P value
South Asians			
eGFR (n=105)	94.0 ± 22.53	86.11 ± 28.17	0.118
Diabetic nephropathy (n=99)	28.6%	58.0%	0.003
eGFR < 60 (n=105)	3.9%	14.8%	0.057
Albuminuria (n=99)	26.5%	50.0%	0.016
ACR (mg/mmol) (n=101)	0.92 (0.49-3.15)	3.20 (0.90-11.63)	0.009
White Europeans			
eGFR (n=129)	91.23 ± 29.17	80.36 ± 25.29	0.045
Diabetic nephropathy (n=102)	32.0%	54.5%	0.050
eGFR < 60 (n=129)	15.6%	20.6%	0.615
Albuminuria (n=100)	20.0%	38.7%	0.088
ACR (mg/mmol) (n=112)	0.50 (0.00-2.45)	1.00 (0.27-4.07)	0.047

Table 6-3: The impact of gender on the relationship between OSA and diabetic nephropathy

	OSA-	OSA+	P value
Men			
eGFR (n=135)	90.61 ± 24.82	82.90 ± 23.70	0.107
Diabetic nephropathy (n=119)	40.0%	53.9%	0.187
eGFR < 60 (n=135)	5.9%	18.8%	0.072
Albuminuria (n=117)	40.0%	44.8%	0.646
ACR (mg/mmol) (n=91)	1.73 (0.58-9.88)	1.35 (0.40-7.30)	0.851
Women			
eGFR (n=99)	94.52 ± 25.52	81.44 ± 31.42	0.025
Diabetic nephropathy (n=82)	22.7%	60.5%	0.001
eGFR < 60 (n=99)	10.2%	18.0%	0.266
Albuminuria (n=82)	13.6%	39.5%	0.008
ACR (mg/mmol) (n=122)	0.55 (0.00-1.15)	1.8 (0.43-6.69)	0.002

Table 6-4: The impact of gender ethnicity interaction on the relationship between OSA and diabetic nephropathy

	OSA-	OSA+	P value
South Asian women			
eGFR (n=44)	94.23 ± 20.21	89.85 ± 33.39	0.598
Diabetic nephropathy (n=41)	13.0%	66.7%	<0.001
eGFR < 60 (n=44)	4.3%	9.5%	0.599
Albuminuria (n=41)	8.7%	55.6%	0.001
ACR (mg/mmol) (n=43)	0.60 (0.00 0.95)	3.75 (0.91-11.59)	<0.001
White European women			
eGFR (n=45)	94.78 ± 29.85	75.35 ± 28.97	0.018
Diabetic nephropathy (n=41)	33.0%	55.0%	0.162
eGFR < 60 (n=55)	15.4%	24.1%	0.418
Albuminuria (n=41)	19%	25%	0.645
ACR (mg/mmol) (n=48)	0.45 (0.00-3.10)	1.0 (0.12-2.42)	0.343
South Asian men			
eGFR (n=61)	93.77 ± 24.64	83.73 ± 24.55	0.117
Diabetic nephropathy (n=58)	42.3%	53.1%	0.412
eGFR < 60 (n=61)	3.6%	18.2%	0.112
Albuminuria (n=58)	42.3%	46.9%	0.728
ACR (mg/mmol) (n=58)	2.00 (0.63-14.29)	2.30 (0.900-21.13)	0.839
White European men			
eGFR (n=74)	75.85 ± 21.71	82.50 ± 23.45	0.506
Diabetic nephropathy (n=61)	25.0%	54.4%	0.338
eGFR < 60 (n=74)	16.7%	19.1%	1.0
Albuminuria (n=59)	25.0%	43.6%	0.630
ACR (mg/mmol) (n=64)	0.65 (0.23-1.80)	1.00 (0.30-6.15)	0.299

There was no worsening in eGFR or ACR levels with increasing OSA severity (**Table 6.5**), while the presence of albuminuria and diabetic nephropathy increased with worsening OSA (**Table 6.5).**

Table 6-5: The relationship between OSA severity and diabetic nephropathy.

	OSA-	Mild OSA	Moderate to severe OSA	P value for trend
eGFR (n=234)	92.9 ± 25.2	82.1 ± 26.3	82.9 ± 26.8	0.014
eGFR < 60 (n=234)	8.4%	18.9%	18%	0.087
Albuminuria (n=199)	24.3%	41%	46.8%	0.008
Diabetic nephropathy (n=201)	29.7%	54.4%	58.3%	0.001
ACR (mg/mmol) (n=213)	0.75 (0.28-2.98)	1.73 (0.50-10.65)	1.50 (0.33-5.45)	0.028

There was a weak but significant relationship between AHI and nocturnal hypoxemia metrics on one hand and eGFR and ACR levels on the other (**Table 6.6**), but this relationship was lost after adjusting for age.

Table 6-6: correlations between OSA metrics and eGFR and ACR.

Data presented as correlation coefficient (p value)

	AHI	ODI	Time spent with oxygen saturations< 80%	Nocturnal nadir oxygen saturation
eGFR	-0.176 (0.007)	-0.173 (0.009)	-0.113 (0.089)	0.090 (0.178)
ACR	0.132 (0.055)	0.121 (0.080)	0.186 (0.007)	-0.085 (0.221)

In order to explore whether the higher prevalence of diabetic nephropathy and/or albuminuria in patients with OSA is cofounded by between groups differences observed in **Table 4.1**, logistic regression (the backward method) was used (**Table 6.7**). OSA remained an independent predictor of diabetic nephropathy (OR 2.169, 95%CI 1.025-4.591) after adjustment. Other independent predictors of diabetic nephropathy included age (OR 1.074, 95% CI 1.034-1.116, p<0.001), diabetes duration (OR 1.109, 95% CI 1.052-1.169, p<0.001), HbA1c (OR 1.385, 95% CI 1.061-1.809, p=0.017), triglycerides (OR 1.459, 95% CI 1.072-1.986, p=0.016) and BMI (OR 1.059, 95% CI 1.006-1.115, p=0.028). OSA remained an independent predictor of nephropathy after adjusting for waist/hip ratio instead of BMI (OR 2.496, 95%CI 1.200-5.194, p=0.014). However, adjusting for waist circumference instead of BMI makes the association between OSA and nephropathy borderline (OR 1.992, 95% CI 0.935-4.247, p=0.074) and waist circumference was an independent predictor of diabetic nephropathy (OR 1.033, 95% CI 1.008-1.059, p=0.010). OSA was not an independent predictor of albuminuria following adjustment. Independent predictors of albuminuria were: diabetes duration (OR 1.116, 95% CI 1.056-1.180, p<0.001), male gender (OR 2.369, 95% CI 1.082-5.185. p=0.031), HbA1c (OR 1.395, 95% CI 1.070-1.820, p=0.014), triglycerides (OR 1.588, 95% CI 1.159-2.176, p=0.004), eGFR (OR 0.971, 95% CI 0.956-0.987, p<0.001), systolic BP (OR 1.026, 95% CI 1.003-1.051,

p=0.028), use of oral glucose lowering agents (OR 9.223, 95% CI 1.457-58.384, p=0.018), the use of anti-hypertensives (OR 3.405, 95% CI 1.088-10.659, p=0.035).

In order to assess the relationship between OSA severity and nocturnal hypoxemia severity and diabetic nephropathy and/or albuminuria, the same logistic regression model was used as in **Table 6.5** after replacing OSA with variable of interest. OSA severity (**Table 6.8**), AHI quartiles (**Table 6.9**), ODI, and nadir nocturnal oxygen saturation were not independent predictors of diabetic nephropathy or albuminuria after adjustment. There was a trend towards an independent relationship between the worst quartile of nocturnal nadir oxygen saturation (with oxygen saturation < 77%) (OR 2.524, 95% CI 0.935-6.810, p=0.068) and diabetic nephropathy when the quartile with the least hypoxemia (nadir saturations ≥ 87%) was taken as the reference point. Time spent with oxygen saturation < 80% (as a binary variable those who spent ≥ 0.1% of time with oxygen saturation < 80% vs. those who spent 0%) was independently associated with diabetic nephropathy (OR 2.854, 95% CI 1.202-6.777, p=0.017) and albuminuria (OR 2.738, 95% CI 1.157-6.476, p=0.022)

The prevalence of nephropathy decreased with increasing nadir nocturnal oxygen saturations (p=0.001) **(Figure 6.1).**

Table 6-7: Assessing the impact of possible confounders on the association between OSA and diabetic nephropathy and albuminuria using different logistic regression models (Backward method).

The odds ratios (OR) reported are the odds for having the outcome in OSA+ to OSA- patients. The adjusted model included (in no particular order) OSA + ethnicity + age + gender + alcohol intake + smoking + BP + diabetes duration + HbA1c + Total cholesterol + Triglycerides + HDL + oral glucose lowering treatments (including metformin, sulphonylurea, glitazones, and DPP-4 inhibitors combined) + insulin+GLP-1 analogues + anti-hypertensives (ACE inhibitors, angiotenisn 2 blockers, beta blockers, alpha blockers, calcium antagonists and diuretics combined) + anti-platelets (aspirin and clopidogrel combined) + lipid lowering therapy (including statins, ezetimibe and fibrates combined) + BMI.

	Nagelkerke R Square	OR	95% CI	P
Diabetic nephropathy (n=201)				
Unadjusted	0.085	2.997	1.630-5.511	< 0.001
Adjusted	0.411	2.169	1.025-4.591	0.043
Albuminuria (n=199)				
Unadjusted	0.050	2.366	1.250-4.479	0.008
Adjusted	0.420	1.643	0.687-3.931	0.265

Table 6-8: The relationship between OSA severity and diabetic nephropathy and albuminuria. Adjustment as in Table 6.8

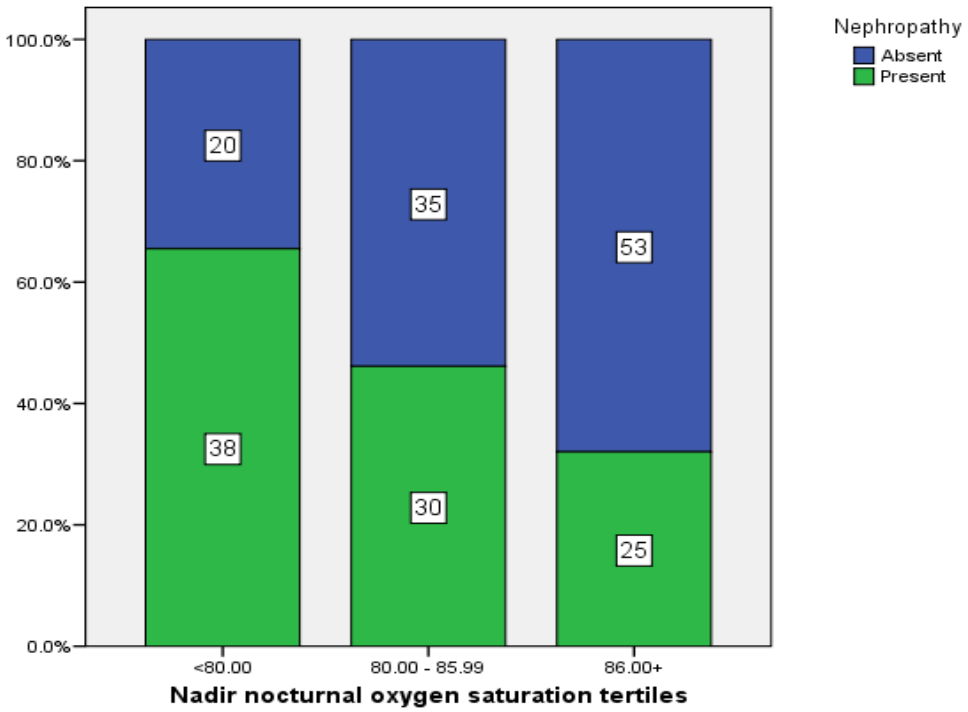
	Nagelkerke R Square	OR	95% CI	P
Diabetic nephropathy (n=201)				
Unadjusted	0.086			
Mild OSA		2.823	1.449-5.499	0.002
Moderate to severe OSA		3.309	1.547-7.076	0.002
Adjusted	0.391			
Mild OSA		2.154	0.955-4.860	0.065
Moderate to severe OSA		2.194	0.868-5.547	0.097
Albuminuria (n=199)				
Unadjusted	0.053			
Mild OSA		2.164	1.078-4.344	0.030
Moderate to severe OSA		2.738	1.254-5.980	0.012
Adjusted	0.383			
Mild OSA		1.637	0.685-3.911	0.267
Moderate to severe OSA		2.467	0.927-6.562	0.070

Table 6-9: The relationship between AHI and diabetic nephropathy/albuminuria. Adjustment as in Table 6.8

	Nagelkerke R Square	OR	95% CI	P
Diabetic nephropathy (n=201)				
Unadjusted	0.061			
Quartile 2		1.788	0.816-3.918	0.146
Quartile 3		2.743	1.230-6.120	0.014
Quartile 4		3.105	1.349-7.142	0.008
Adjusted	0.391			
Quartile 2		1.312	0.471-3.658	0.604
Quartile 3		1.622	0.565-4.657	0.369
Quartile 4		2.215	0.760-6.451	0.145
Albuminuria (n=199)				
Unadjusted	0.043			
Quartile 2		1.383	0.599-3.194	0.448
Quartile 3		2.386	1.043-5.459	0.039
Quartile 4		2.419	1.025-5.709	0.044
Adjusted	0.383			
Quartile 2		1.093	0.385-3.104	0.868
Quartile 3		2.307	0.796-6.689	0.124
Quartile 4		2.557	0.864-7.565	0.090

Figure 6-1: The relationship between nadir nocturnal oxygen saturations and diabetic nephropathy.

p=0.001 for the trend



Using multilinear regression, OSA and hypoxia measures were not independent predictors of eGFR, while age and obesity were. Similar results were obtained for ACR levels.

6.6. Discussion

Our data show that OSA was independently associated with diabetic nephropathy in patients with T2DM, although this relationship was mainly driven by an association between OSA and diabetic nephropathy in women rather than men. Our data also showed that patients with T2DM and OSA have lower eGFR, more albuminuria and higher ACR. OSA severity (as judged by AHI and ODI) correlated with eGFR and ACR levels, but these correlations were lost after adjustment. Nocturnal hypoxemia was also independently associated with diabetic nephropathy and albuminuria.

We found that ethnicity gender interaction impacted on the relationship between OSA and diabetic nephropathy, with the relationship being strongest in South Asian women. The reasons/mechanisms behind such ethnic gender interactions are unclear and require further study. Nonetheless, it is important to note that the number of patients in each category of the ethnicity gender interaction is small and hence we need to be cautious not to over interpret such findings.

There are several mechanisms that could explain the relationship between OSA and diabetic nephropathy, particularly the impact of OSA on OS, AGE production, PKC activation, VEGF and NO production, all of which are important in the pathogenesis diabetic nephropathy and have been shown to be affected in patients with OSA but without T2DM (please see Chapter 1 of the thesis for more details). This is further supported in our data by the relationship between diabetic nephropathy/albuminuria and nocturnal hypxemia and the increased levels of serum nitrotyrosine and plasma lipid peroxide (indicating increased nitrosative and oxidative stress) and the impaired microvascular regulations in OSA patients (please see later chapters for more details).

OSA has been shown to be associated with nephropathy in patients without diabetes. OSA has been shown to be very prevalent in patients with ESRD (30-80%) (439-441); OSA has also been shown to

be associated with lower eGFR and that eGFR correlated negatively with AHI (442;443). In addition, OSA has been associated with increased microalbuminuria in obese subjects without diabetes (444;445). Even when studies conducted in hypertensive subjects, OSA remained an independent predictor of albuminurea, suggesting that the role of OSA is over and above that of BP (448). This is further supported by preliminary evidence suggesting that CPAP lowers the levels of ACR in OSA patients (449). Please refer to the introductory chapter for more details about these studies.

Our data show that OSA was not an independent predictor of albuminuria after adjustment despite that OSA was an independent predictor of diabetic nephropathy. This might reflect our relatively small sample size as we might be under powered to adjust for such a wide range for variables. This is further supported that the p value of the OR for patients with moderate to severe OSA and patients in the highest AHI quartiles in regard to predicting albuminuria was < 0.1 suggesting a possible sample size impact. Another possible explanation for the lack of independent association between OSA and eGFR and albuminuria is that we excluded patients with ESRD from the study and patients with ESRD are known to have high prevalence of OSA and hence their exclusion might have masked an independent relationship between OSA and eGFR. Differences in ACE inhibitors or angiotensin II blockers might have also affected the relationship between OSA and diabetic nephropathy in our study. The proportion patients prescribed these medications, however, was similar between patients with and without OSA. Nonetheless, the efficacy of blocking the rennin angiotensin aldosterone system may not be the same in patients with and without OSA; this will require further study.

There was a significant univariate relationship between OSA severity and diabetic nephropathy severity (as judged by eGFR and ACR levels), which became non-significant following adjustment. Nonetheless, the prevalence of diabetic nephropathy and albuminuria increased in a stepwise manner across AHI quartiles and across OSA severity even though that this trend was not significant after adjustment. Furthermore, time spent with oxygen saturation < 80% was independently associated with diabetic nephropathy and albuminuria' adding to that the trend towards an

independent relationship between nadir nocturnal oxygen saturation quartiles and diabetic nephropathy; we can conclude that the severity of OSA and nocturnal hypoxemia might be associated with diabetic nephropathy in patients with T2DM, although our sample size did not allow us to show and independent relationship following adjustment (except in the case of time spent with oxygen saturation < 80%).

In our study, the relationship between OSA and diabetic nephropathy seems to be weaker than that between OSA and DPN and STDR. A possible explanation is that the kidneys might be more resistant to hypoxic damage compared to the nerves or the retina. Another important possibility is that genetic factors play an important in the pathogenesis of diabetic nephropathy (88) while the role of genes in the development of DR and DPN is much less established.

Similar to what discussed in the previous chapter, the main limitations of this study that it is cross-sectional and hence causation cannot be examined. We also have used portable multi-channel respiratory devices to diagnose OSA which are not the “Gold standard” but their use has been well established in the literature as described previously.

In summary, we found and independent association between OSA and diabetic nephropathy and between nocturnal hypoxemia and diabetic nephropathy and albuminuria. We found no such a relationship with OSA severity or AHI, mainly because of our sample size. Further work examining the relationship between OSA and diabetic nephropathy is needed, particularly prospective study examining the impact of OSA on the natural history of this albuminuria and diabetic nephropathy in patients with T2DM.

7. Chapter seven: Obstructive Sleep Apnoea and Cardiac Autonomic Neuropathy

7.1. Introduction

DN is the most common complication of DM resulting in great morbidity, mortality and significant economic burden (53;54). DN can affect different aspects of the peripheral (DPN) and the autonomic (DAN)) nervous systems. DPN and DAN often coexist (55). DAN is very common in patients with DM; this high prevalence, however, varies between different studies depending of the population studied and the methods of diagnosing DAN. In the Diabetes Control and Complication Trial (DCCT), which comprised a cohort of 278 healthy patients with T1DM, the prevalence of DN (defined as an abnormal neurological examination plus either abnormal nerve conduction studies in two nerves or abnormal autonomic function testing) was 39% (56). Abnormalities of cardiovascular reflex testing can be identified in 16-20% of subjects with DM (55;61-64). In the Eurodiab IDDM Complications Study, abnormalities of heart rate variability (HRV) were detected in approximately 19% of study participants (61). Direct assessment of cardiovascular sympathetic system, using radio-labeled analogues of norepinephrine ([123I] MIBG and [11C] HED), showed deficits of LV retention in subjects with T1DM or T2DM, with up to 40% of otherwise healthy patients with T1DM without deficits on cardiovascular reflex testing having abnormalities of [11C]HED retention affecting up to 8% of the left ventricle (65-69). Similar to DPN, age, glycaemic control and diabetes duration are major risk factors for DAN (71;530).

DAN has a wide spectrum of manifestations and clinical features which reflects the heterogeneity of nerves affected and the widespread consequences of their dysfunction. The manifestations of DAN can range from dry skin in the feet (due to sudomotor dysfunction) to sudden cardiac death (530). DAN typically occurs as a system-wide disorder affecting all parts of the ANS; this is in part because the vagus nerve (the longest of the autonomic nerves) accounts for 75% of all parasympathetic activity, and DAN manifests first in longer nerves, even early effects of DAN are widespread (530).The earliest manifestations of peripheral DAN are likewise difficult to detect clinically, since they may be manifest solely as impaired peripheral vasomotor control, or decreased sudomotor

function, which may progress to increased arterio-venous shunting (detectable by the presence of distended veins on the lower legs), severe edema, neuroarthropathy (Charcot joints) and neuropathic ulceration. Cardiac involvement in DAN can result in reduced cardiovascular performance during exercise, impaired cardiac ejection fraction, abnormal systolic function, decreased diastolic filling, arrhythmias, orthostatic hypotension and sudden death (54;75-78;530). DAN could also affect other systems including the gastrointestinal system (gastroperesis, diarrhoea, constipation) and the genitourinary system (anaemia, erectile dysfunction, urinary incontinence) (54).

The pathogenesis of DAN is complex and similar to that of other microvascular complications as discussed in previous chapters, including metabolic damage to nerve fibers, neurovascular insufficiency, autoimmune damage, and neurohormonal growth factor deficiency (530) caused by several different factors such as the activation of polyol pathway, PKC activation, increased oxidative and nitrosative stress, immune mechanisms, reduction in neurotrophic growth factors, increased AGE production and PARP activation (497;530-535).

Current treatment of DAN and CAN is largely limited to improved glycaemic control and symptomatic treatment of DAN consequences (such gastrointerstinal or urinary symptoms). Hence there is need for better understanding of DAN pathogenesis to improve treatments.

As we have described in previous chapters and in Chapter 1, OSA has been shown to have a significant impact on many of the metabolic and biological pathways which are related to the development of DAN; in fact OSA has been shown to be associated with sympathetic over-activation in patients without T2DM (please see Chapter 1) (359;377-380;383;458). The relationship between DAN and OSA can be bi-directional, although OSA might cause DAN by different mechanisms including OS and increase inflammation as discussed in Chapter 1, DAN might worsen or cause OSA as ANS is involved in ventilator control and upper airways muscular tone (536), both of which play an important role in the pathogenesis of OSA as described in Chapter 1.

7.2. Hypothesis

OSA is associated with CAN and CAN is associated with worsening OSA in patients with T2DM.

Rationale: OSA is associated with the activation of several pathways that are involved in the pathogenesis of CAN such as oxidative stress, the polyol pathway and PKC. The ANS is involved in the control of ventilatory drive and upper airway tone, hence abnormalities in the ANS could result in increase upper airway collapsibility.

7.3. Aims

The primary aim is to examine the interrelationship between OSA and CAN in patients with T2DM. A secondary aim is to assess the impact of CAN on hypopnea/apnoea events duration in patients with T2DM.

7.4. Methods

This is a secondary analysis to the project that examined the relation between OSA and DPN in patients with T2DM. For more details about the methods, please refer to chapters 2 and 4.

OSA was assessed as previously described, using a single night multi-channel respiratory device. CAN was diagnosed using HRV during baseline, deep breathing, valsalva and standing; and was analyzed using the continuous wavelet transform methods to generate numerical and graphical data using the ANX-3.0 software, ANSAR Inc., Philadelphia, PA. For more in-depth details please see chapter 2. For the purpose of this study, a diagnosis of CAN was made when 2 or more of the following tests were abnormal: E/I ratio, Valsalva ratio, 30:15 ratio and postural drop in BP (drop of 20mmHg in systolic or 10mmHg in diastolic BP) (479;537). Age-related normal values for E/I, Valsalva and 30:15 ratios were defined as previously reported (480). All patients were advised not to consume caffeine for at least 2 hours before the test, and adjustment for baseline respiratory rate was performed using the above mentioned software as per published guidelines (537).

Statistical and more detailed methods can be found in Chapter 2.

7.5. Results

CAN results were available on 225 patients, 199 of which had overnight sleep studies and were included in this analysis. One hundred and twenty four patients had OSA (62.3% of the total cohort), of which 79 (39.7%), 24 (12.1%) and 21 (10.6%) patients had mild, moderate and severe OSA respectively. The prevalence of CAN was 42.7% (n=85). The 114 patients without definite CAN include 61 patients (30.7%) who have one abnormal test, but those were classified in the non-CAN group in this study.

7.5.1. The relationship between OSA and CAN parameters

Patients' characteristics are summarized in **Table 7.1**. As in previous chapters, OSA patients were older, had longer diabetes duration, were more obese and had an adverse metabolic profile compared to patients without OSA.

Table 7-1: Participants characteristics in relation to OSA status.

Data presented as median (IQR) or mean (SD). GFR: Glomerular Filtration Rate.

	OSA- (n=75)	OSA+ (n=124)	P value
Male	44%	66.1%	0.002
White Europeans	34.7%	60.5%	<0.001
Age (years)	54 (12)	58 (11)	0.038
Diabetes Duration (years)	9.0 (5.0-15.0)	11.0 (7.0-16.7)	0.031
Body Mass Index (kg/m^2)	30.2 (27.0-34.6)	34.4 (30.1-39.6)	<0.001
Waist circumference (cm)	103.5 (95.5-115.0)	114.0 (106.1-125.0)	<0.001
Hip (cm)	105.0 (97.0-117.0)	113.0 (103.5-124.4)	0.001
Waist hip ratio	0.97 (0.93-1.02)	1.01 (0.96-1.05)	0.007
Neck circumference (cm)	38.0 (36.5-41.2)	42.0 (39.0-45.6)	<0.001
Systolic blood pressure (mmHg)	124.5 (113.5-135.5)	131.8 (123.5-140.7)	0.001
Diastolic blood pressure (mmHg)	78.5 (71.0-84.5)	78.5 (71.6-84.5)	0.502
HbA1c (%)	7.6 (6.9-8.7)	8.2 (7.3-9.4)	0.028
Total cholesterol (mmol/L)	3.7 (3.4-4.5)	3.7 (3.3-4.3)	0.704
Triglycerides (mmol/L)	1.4 (1.0-2.1)	1.8 (1.3-2.5)	0.023
HDL (mmol/L)	1.2 (0.9-1.5)	1.1 (0.9-1.2)	0.037
Estimated GFR (ml/min/1.73 m^2)	94.6 (25.1)	84.0 (25.8)	0.005

Epworth sleepiness score	5.0 (2.0-12.0)	8.0 (4.0-13.0)	0.009
Smoking (current or ex-smoker)	38.7%	36.3%	0.737
Alcohol (drinks alcohol)	10.7%	32.3%	0.001
Oral anti-diabetes treatment	97.3%	91.1%	0.121
Incretin-based therapy	10.7%	19.4%	0.106
Insulin	41.3%	59.7%	0.012
Insulin dose (total daily units)	62.0 (36.0-88.0)	80.0 (56.0-119.0)	0.009
ACE inhibitors /Angiotensin II blockers	65.3%	71.8%	0.340
Beta blockers	17.3%	23.4%	0.310
Calcium channel blockers	17.3%	32.3%	0.021
Alpha blockers	2.7%	9.7%	0.061
Diuretics	17.3%	41.9%	<0.001
Anti-hypertensives	72%	82.3%	0.088
Anti-platelet drugs	61.3%	71.8%	0.126
Lipid lowering therapy	84%	84.7%	0.898

CAN prevalence was non-significantly higher in patients with OSA compared to those without (46.0% vs. 37.3%, p=0.233). This difference was mainly due to a higher prevalence of CAN in patients with OSA of South Asian ethnicity (49% vs. 32.7%, p=0.1) rather than White Europeans (46.2% vs. 44%, p=0.849). The relationship between OSA and CAN was also affected by gender. In men, patients with OSA had a trend toward higher prevalence of CAN compared to patients without OSA (48.8% vs. 30.3%, p=0.071), while there was no such relationship in women (42.9% vs. 40.5%, p=0.825). The interaction between ethnicity and gender impacted on the OSA relationship to CAN; with South Asian men the only group that showed difference in CAN prevalence between patients with and without OSA (**Table 7.2**)

Table 7-2: The impact of ethnicity gender interaction on the relationship between OSA and CAN

	OSA-	OSA+	P value
South Asian men (n=59)	25.0%	51.6%	0.036
White European men (n=56)	60.0%	47.1%	0.664
South Asian women (n=39)	42.9%	44.4%	0.921
White European women (n=45)	42.9%	37.5%	0.714

There was no stepwise increase in CAN prevalence across OSA categories (37.3% vs. 46.8% vs. 44.4% for normal vs. mild vs. moderate to severe OSA respectively, p=0.475). There was also no increase in CAN prevalence across AHI quartiles (<2.90 vs.2.90 - 7.59 vs.7.60 - 16.09 vs. ≥16.10) (37.0% vs. 50.0% vs. 42.0% vs. 41.5% quartiles 1 to 4 respectively, p=0.589).

Examining the relationship between individual HRV parameters and OSA showed that patients with OSA had lower Valsalva and 30:15 ratios (only significantly in the case of Valsalva ratio) (**Table 7.3**). The Valsalva ratio also worsened with increasing OSA severity (**Table 7.2)**. None of the ratios worsened significantly across AHI quartiles.

Table 7-3: The relationship between single CAN parameters and OSA, OSA severity and AHI quartiles.

AHI quartiles represent the following groups AHI < 2.90 (n=54), 2.90 - 7.59 (n=54), 7.60 - 16.09 (n=51) and ≥ 16.10 (n=41). Postural hypotension presented as the % who had postural hypotension in the respective category.

	E/I ratio	Valsalva ratio	30:15 ratio	Postural hypotension
OSA-	1.09 (1.06-1.16)	1.30 (1.13-1.66)	1.25 (1.10-1.74)	5.3%
OSA+	1.08 (1.04-1.14)	1.20 (1.08-1.35)	1.18 (1.08-1.37)	12.1%
P value	0.178	0.006	0.056	0.12
OSA-	1.09 (1.06-1.16)	1.30 (1.13-1.66)	1.25 (1.10-1.74)	5.3%
Mild OSA	1.08 (1.05-1.15)	1.21 (1.07-1.37)	1.16 (1.05-1.39)	15.2%
Moderate to severe OSA	1.08 (1.04-1.12)	1.18 (1.10-1.34)	1.20 (1.11-1.34)	6.7%
P value	0.213	0.022	0.096	0.09
AHI quartile 1	1.10 (1.06-1.17)	1.29 (1.12-1.64)	1.31 (1.09-1.85)	7.4%
AHI quartile 2	1.09 (1.05-1.15)	1.20 (1.09-1.52)	1.15 (1.09-1.39)	7.4%
AHI quartile 3	1.08 (1.04-1.14)	1.21 (1.06-1.36)	1.18 (1.07-1.37)	16.3%
AHI quartile 4	1.08 (1.04-1.13)	1.20 (1.11-1.34)	1.20 (1.10-1.40)	7.3%
P value	0.642	0.221	0.221	0.359

Comparing the spectral analysis and frequency and time domain analysis between patients with and without OSA showed that both sympathetic and parasympathetic tones to be withdrawn in patients with OSA (**Table 7.4**). But the sympathetic/parasympathetic ratio remained the same, suggesting that sympathetic and parasympathetic are weakened to the same degree in patients with OSA. After adjustment for age, gender, ethnicity and BMI, diabetes duration and alcohol intake only Valsalva Rfa (B=-0.257, p=0.024), standing Lfa (B=-0.223, p=0.017), standing Rfa (B=-0.242, p=0.008) and Valsava SDNN (B=-0.099, p=0.023) remained significant, while the rest of the parameters in **table 7.4** were not associated with OSA after adjustment.

Table 7-4: The relationship between OSA and frequency and time domain analysis.

The table shows respiratory adjusted parameters and the SDNN. Other frequency and time domains showed similar results/patterns.

	OSA-	OSA+	P value
Baseline Lfa	0.97 (0.44-2.13)	0.54 (0.22-1.50)	0.015
Baseline Rfa	0.54 (0.21-1.47)	0.35 (0.15-0.72)	0.015
Baseline Lfa/Rfa	2.3 (1.29-5.43)	2.71 (1.52-5.36)	0.552
Deep breathing Lfa	0.60 (0.27-1.84)	0.50 (0.18-1.40)	0.204
Deep breathin Rfa	3.94 (0.83-10.10)	2.75 (0.61-6.80)	0.124
Deep breathin Lfa/Rfa	0.19 (0.07-0.95)	0.22 (0.09-0.63)	0.473
Valsalva Lfa	24.23 (3.72-64.48)	11.96 (2.16-25.61)	0.007
Valsalva Rfa	2.75 (0.60-8.59)	1.29 (0.28-3.4400)	0.003
Valsalva Lfa/Rfa	19.13 (7.00-49.24)	19.41 (8.36-47.01)	0.554
Standing Lfa	1.16 (0.43-2.99)	0.71 (0.17-1.47)	0.002
Standing Rfa	0.44 (0.17-1.39)	0.27 (0.13-0.61)	0.010
Standing Lfa/Rfa	4.64 (2.13-9.43)	4.24 (2.31-9.35)	0.992
Baseline SDNN	24.50 (18.00-42.00)	21.00 (14.00-31.00)	0.003
Deep breathing SDNN	38.00 (24.75-58.50)	32.00 (18.00-50.00)	0.039
Valsalva SDNN	69.50 (42.25-105.75)	57.00 (31.00-78.00)	0.011
Standing SDNN	30.50 (20.75-41.00)	25.00 (17.00-35.00)	0.018

The correlations between AHI, ODI, time spent with oxygen saturation <80% and nadir nocturnal oxygen saturation and the HRV and spectral analysis data showed that several parameters of autonomic function worsened with increasing severity of OSA and hypoxemia measures (**Table 7.5**). The majority of these correlations were modest. In order to simplify the results I focused on the

results of the HRV standardized ratios and the respiratory adjusted values of the frequency and time domain analysis during Valsalva and standing.

Table 7-5: The correlations between HRV and spectral analysis data and OSA and hypoxia severities.

Data presented as correlation coefficient (r) and p value.

		AHI	Time spent with oxygen saturation < 80%	ODI	Nadir nocturnal oxygen saturation
E/I ratio	r	-.093	-.143	-.066	.083
	P	.189	.047	.361	.248
Valsalva ratio	r	-.140	-.240	-.148	.229
	P	.048	.001	.039	.001
30:15 ratio	r	-.094	-.160	-.105	.219
	P	.188	.026	.141	.002
Valsalva Lfa	r	-.156	-.191	-.167	.158
	P	.028	.008	.020	.028
Valsalva Rfa	r	-.149	-.141	-.163	.146
	P	.035	.051	.023	.042
Standing Lfa	r	-.211	-.125	-.191	.130
	P	.003	.082	.007	.070
Valsalva SDNN	r	-.132	-.195	-.134	.139
	P	.064	.007	.062	.053
Valsalva RMSSD	r	-.120	-.202	-.102	.166
	P	.093	.005	.157	.021
Valsalva PNN50	r	-.146	-.183	-.147	.186
	P	.041	.011	.040	.009
Standing SDNN	r	-.191	-.177	-.202	.189
	P	.007	.014	.005	.008
Standing RMSSD	r	-.113	-.150	-.137	.205
	P	.114	.038	.055	.004
Standing PNN50	r	-.170	-.119	-.148	.137
	P	.017	.099	.039	.056

In order to assess whether the association observed in **Table 7.5** are independent of possible confounders, I conducted several linear regression models (Backward method). The predictors included in the model were age, diabetes duration, BMI, gender, ethnicity and alcohol intake, as these are just considered as significant risk factors of CAN. Following adjustment, AHI remained negatively associated with standing Lfa, baseline LF, Valsalva LF, standing LF, standing SDNN and

standing PNN50 (**Table 7.6**). Nadir nocturnal oxygen saturation) was independently and positively associated with Valsalva HF, Valsalva RMSSD, Valsalva PNN50, standing SDNN and standing RMSSD following adjustment (**Table 7.7**). Time spent with oxygen saturation < 80% (as a binary variable; those who spent 0% of the time with oxygen saturation < 80% vs. those who spent ≥0.1%) was independently inversely associated with Valsalva ratio (B= -0.046, p= 0.017), deep breathing RMSSD (B= -0.122, p=0.021), deep breathing PNN50 (B= -0.251, p=0.013), Valsalva SDNN (B= -0.094, p=0.046) and Valsalva RMSSD (B= -0.094, p=0.046).

Table 7-6: The relationship between AHI and parameters of CAN after adjustment for age, diabetes duration, BMI, gender, ethnicity and alcohol intake.

Only significant associations after adjustment are shown.

		R^2	B	P
Standing Lfa	Unadjusted	0.057	-0.340	0.001
	Adjusted	0.247	-0.281	0.003
Valsalva HF	Unadjusted	0.024	-0.264	0.031
	Adjusted	0.138	-0.229	0.055
Standing LF	Unadjusted	0.039	-0.331	0.005
	Adjusted	0.188	-0.288	0.052
Standing SDNN	Unadjusted	0.032	-0.092	0.012
	Adjusted	0.091	-0.102	0.023
Standing PNN50	Unadjusted	0.025	-0.153	0.027
	Adjusted	0.063	-0.192	0.006

Table 7-7: The relationship between nadir nocturnal oxygen saturation and CAN parameters after adjustment for age, diabetes duration, BMI, gender, ethnicity and alcohol intake.

Only significant associations after adjustment are shown.

		R^2	B	P
Valsalva ratio	Unadjusted	0.055	0.124	0.001
	Adjusted	0.188	0.088	0.014
30:15 ratio	Unadjusted	0.062	0.173	<0.001
	Adjusted	0.136	0.163	0.002
Valsalva SDNN	Unadjusted	0.041	0.266	0.005
	Adjusted	0.193	0.173	0.050
Valsalva RMSSD	Unadjusted	0.033	0.271	0.011
	Adjusted	0.117	0.220	0.033
Valsalva PNN50	Unadjusted	0.039	0.484	0.006
	Adjusted	0.132	0.348	0.041
Standing SDNN	Unadjusted	0.036	0.201	0.008
	Adjusted	0.082	0.176	0.019

7.5.2. The relationship between CAN and OSA parameters

There were little differences between patients with and without CAN, apart from that patients with CAN were 4 years older and had longer diabetes duration; and a higher proportion of patients with CAN received insulin treatment (in (**Table 7.8**).

Table 7-8: Participants characteristics in relation to CAN status.

Data presented as median (IQR) or mean (SD). GFR: Glomerular Filtration Rate.

	CAN- (n=114)	CAN+ (n=85)	P value
Male	57%	58.8%	0.799
White Europeans	49.1%	52.9%	0.594
Age (years)	54.5 (11.9)	58.8 (11.2)	0.011
Diabetes Duration (years)	10.0 (5.0-13.3)	13.0 (8.5-20.0)	< 0.001
Height (cm)	165.8 (9.2)	166.7 (10.0)	0.543
Body Mass Index (kg/m^2)	32.9 (28.6-37.6)	33.1 (28.2-37.3)	0.955
Waist circumference (cm)	110.0 (99.9-121.0)	112.5 (100.4-123.8)	0.597
Hip (cm)	109.0 (100.7-121.6)	111.0 (102.0-123.0)	0.550
Waist hip ratio	0.99 (0.95-1.03)	0.99 (0.94-1.05)	0.932
Neck circumference (cm)	40.1 (37.5-43.8)	41.0 (38.0-44.5)	0.347
Systolic blood pressure (mmHg)	129.3 (120.5-138.4)	129.0 (119.0-138.8)	0.852
Diastolic blood pressure (mmHg)	80.5 (75.9-85.5)	75.5 (69.3-82.3)	0.002
HbA1c (%)	7.9 (7.2-8.8)	7.9 (7.1-9.7)	0.432
Total cholesterol (mmol/L)	3.7 (3.3-4.5)	3.7 (3.3-4.2)	0.352
Triglycerides (mmol/L)	1.6 (1.2-2.2)	1.8 (1.2-2.5)	0.276
HDL (mmol/L)	1.1 (0.9-1.4)	1.1 (0.9-1.3)	0.338
Estimated GFR (ml/min/1.73 m^2)	92.4 (24.1)	82.0 (27.4)	0.005
Smoking (current or ex-smoker)	36.8%	37.6%	0.907
Alcohol (drinks alcohol)	22.8%	25.9%	0.616
Anti-plaelets	66.7%	69.4%	0.682
Oral anti-diabetes treatment	94.7%	92.9%	0.599
GLP-1 analogues	9.6%	8.2%	0.731
Insulin	44.7%	63.5%	0.009
Insulin dose (units)	74.0 (51.5-120.0)	80.0 (55.0-101.3)	0.80
Lipid lowering therapy	84.2%	84.7%	0.924
Anti hypertensives	75.4%	82.5%	0.241

There were no significant differences in OSA parameters between patients with and without CAN, although there was a non-significant trend towards more hypoxemia and longer apnoeas in patients with CAN (**Table 7.9**).

Table 7-9: Comparison of OSA parameters across CAN groups

	CAN-	CAN+	P value
AHI	6.3 (2.3-13.7)	6.8 (3.2-13.1)	0.747
ODI	5.6 (2.5-14.1)	6.7 (3.1-12.6)	0.515
Time spent with oxygen saturation < 80% (minutes)	0.0 (0.0-0.0)	0.0 (0.0-0.1)	0.077
Nadir nocturnal oxygen saturation (%)	83.0 (79.0-88.0)	82.0 (75.5-87.0)	0.173
Mean apnoea duration (seconds)	14.5 (11.7-18.2)	15.9 (11.8-21.1)	0.117
Mean hypopnoea duration (seconds)	20.3 (16.6-26.4)	20.8 (16.8-27.2)	0.443
Mean apnea hypopnoea duration (seconds)	18.8 (15.9-23.5)	19.9 (15.5-23.9)	0.203

Interestingly, we found an inverse modest correlation between some HRV and spectral analysis parameters and the duration of the respiratory events (particularly apnoeas), suggesting that better autonomic function is associated with shorter apneas (**Table 7.10**). Of particular interest are the associations between duration of apneas and the Lfa/Rfa ratio suggesting that higher ratios (sympathetic predominance) were associated with shorter apneas (**Table 7.10**). These associations occurred despite that there was little difference between patients with and without CAN in regard to obesity, alcohol intake or smoking which are major risk factors for OSA. Furthermore, patients with and without CAN were matched for gender and ethnicity which are also known to affect OSA. The main possibly confounding effect for these associations comes from the differences in age between groups and possibly insulin treatment; hence we have used linear regression to adjust for those factors. Most of the significant correlations were confounded by age and insulin treatment. However, the mean duration of respiratory events remained significantly associated with several

measurements of CAN, including baseline Lfa/Rfa (B= -4.074, p=0.007), standing Lfa (B=-2.433, p=0.004), standing Lfa/Rfa (B= -2.253, p=0.017).

Table 7-10: A summary of the correlations between HRV and spectral analysis and duration of apnoeas and hypopneas. Data presented as r and p values.

Only significant associations are shown.

		Mean duration of apnoeas	Mean duration of hypopneas	Mean duration of apnoeas and hypopneas
Valsalva ratio	r	-.199	-.096	-.124
	P	.006	.188	.088
30:15 ratio	r	-.168	-.056	-.090
	P	.021	.439	.216
Valsalva Lfa	r	-.222	-.150	-.184
	P	.002	.039	.011
Valsalva Rfa	r	-.198	-.103	-.151
	P	.006	.156	.037
Standing Lfa	r	-.267	-.201	-.209
	P	< .001	.005	.004
Standing Rfa	r	-.141	-.057	-.080
	P	.052	.434	.272
Standing Lfa/Rfa	r	-.147	-.190	-.184
	P	.042	.008	.011
Valsalva RMSSD	r	-.182	-.118	-.159
	P	.012	.106	.028
Valsalva PNN50	r	-.183	-.102	-.147
	P	.011	.160	.043
Standing SDNN	r	-.178	-.095	-.149
	P	.014	.190	.040
Standing RMSSD	r	-.217	-.140	-.176
	P	.003	.053	.015

7.6. Discussion

Unlike our results in the previous chapters, the presence of OSA was not associated with a higher prevalence of CAN in patients with T2DM. OSA, however, was associated with abnormalities in certain autonomic function tests, particularly sympathetic and parasympathetic withdrawal without disturbing the sympathetic parasympathetic balance. OSA severity and hypoxemia severity also correlated with several parameters of sympathetic and parasympathetic function, suggesting that

worsening OSA and/or hypoxemia is associated with worsening autonomic function. Most of these associations became non-significant following adjustment for possible confounders (particularly age and obesity), but several associations remained significant following adjustment, which suggest that OSA is associated with cardiac autonomic function abnormalities independent of possible confounders, although this relationship is only modest. Interestingly, despite that OSA parameters were associated with several sympathetic and parasympathetic parameters; there was no single significant association between the sympathetic/parasympathetic balance and any of the OSA parameters. Furthermore, our study showed that the mean duration of the apnoea/hypopnea events correlated significantly with HRV and spectral analysis parameters suggesting that worse autonomic function was associated with more prolonged respiratory events, despite adjustment for possible confounders.

This association between OSA and respiratory events on one hand and CAN parameters on the other, suggests that the relationship between OSA and CAN in patients with T2DM can be bi-directional in which there is a vicious circle of more prolonged respiratory events resulting in worsening OSA and worsening hypoxia resulting in worsening CAN parameters which in turn results in worse OSA and more prolonged apneas and so on (**Figure 7.1**).

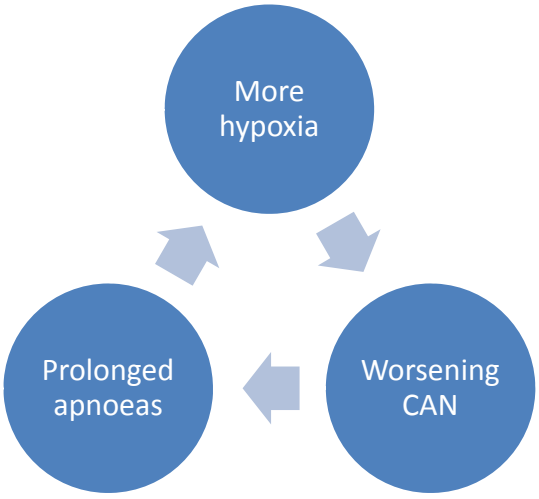


Figure 7-1: The proposed relationship between CAN and OSA in patients with T2DM

In patients without T2DM, OSA has been shown consistently to be associated with autonomic neuropathy and increased sympathetic tone (359;377-380;383;458). Both recurrent hypoxia (377;381;382) and recurrent arousals (383) have been shown to contribute to the activation of the sympathetic system in OSA patients. Obese subjects with autonomic neuropathy develop more frequent and more prolonged hypopnoea/apnoea in comparison to obese subjects without autonomic dysfunction whether or not they had T2DM (459). Unlike the studies in patients without T2DM, the studies examining the relationship between OSA and CAN in patients with T2DM have not been consistent in showing such a relationship, and some of these studies showed that patients with DAN had worse/longer respiratory events (538-541). It must be noted however that these studies were very small in numbers (< 20 patients per group) and there was no adjustment for any possible confounders. Furthermore, the CAN assessment in these studies was very limited and the definition of DAN differed significantly between the studies. Hence, our study adds to the literature as it is the largest to date and included patients of South Asian ethnicity and patients had extensive assessment of cardiac autonomic function.

The lack of similar associations in patients with T2DM could be explained by the longer diabetes duration in our cohort. Diabetes duration is a major risk factor of CAN, and although sympathetic predominance is an early sign of CAN in patients with T2DM, as diabetes progresses, both sympathetic and parasympathetic damage occur. Hence, any early sympathetic predominance in patients with OSA early in the course of T2DM might have been lost as the disease progressed. It is also possible that OSA does not result in the development of CAN in the context of T2DM, but only aggravate it further and speed up the progression of CAN if CAN was initiated by the hyperglycaemia. This is supported by our finding of lack of higher prevalence of CAN in patients with OSA but with the associations observed between CAN frequency and time domains parameters and OSA parameters despite adjustment for diabetes duration. In other words, it is plausible that the development of CAN in patients with T2DM is a function of the hyperglycaemia, but OSA speed up the progression of CAN. This is important as further studies will need to examine the interaction of

OSA and CAN on traditional well established CAN consequences such as cardiovascular disease and mortality.

We found an interesting impact of ethnicity and gender on the relationship between OSA and CAN. We found that CAN prevalence is no higher in patients with OSA compared to patients without OSA in the total cohort. However, South Asian men with OSA had significantly higher prevalence of CAN than those without OSA. The reason for the ethnicity/gender difference is not clear. The small sample size might play a role, and this could be a "chance" finding. Another possibility is that OSA might play a more prominent role in the development of CAN in South Asians as they have less CAN risk factors (such as age and obesity) compared to White Europeans. This, however, does not explain why South Asian women have no difference in CAN prevalence between patients with and without OSA. Finally, we have used normative values for HRV ratios that are published in the literature to identify CAN. These normative values may not apply to South Asians as the original study from which normative values were obtained did not include any South Asians.

The mechanisms relating OSA to CAN are similar to those linking OSA to other microvascular complications. As indicated in the introduction, oxidative and nitrosative stress are essential components in the pathogenesis of CAN in patients with T2DM. The association between CAN and nadir nocturnal oxygen saturation supports the role of hypoxia in the pathogenesis of CAN and hypoxia can increase oxidative and nitrosative stress.

The limitations to this study are similar to previous studies, the main limitation being its cross-sectional nature, hence causation cannot be proven. A strength of this study is the extensive autonomic function assessment and the well characterization of this cohort.

In summary, OSA in patients with T2DM is not associated with increased CAN prevalence but there is an association between OSA and hypoxia parameters on one hand and parameters of autonomic function on the other. Further studies examining the relationship between OSA and CAN in patients

with ”early” diabetes and the impact of OSA on the development and progression of CAN prospectively are needed. Studies using the impact of OSA on cardiac sympathetic innervation using imaging techniques would be of interest as our results suggested a relationship between sympathetic predominance and the duration of apnoea/hypopnea events. Finally, studies assessing the impact of CPAP on CAN in patients with T2DM are needed.

8. Chapter eight: Obstructive Sleep Apnoea and Microvascular and Endothelial Function in Patients with Type 2 Diabetes

8.1. Introduction

The endothelium has several important roles including regulating vascular tone, platelet activity, leukocyte adhesion, and angiogenesis by producing NO and other regulatory factors (542). Endothelial dysfunction results in reduction in NO and induces pro-inflammatory changes that promotes atherosclerosis (542). Endothelial dysfunction is characterized by impaired endothelium-dependent vasodilation which is usually associated with a pro-inflammatory, proliferative, and pro-coagulatory milieu that promotes atherosclerosis (543).

Endothelial and microvascular dysfunction have been associated with cardiovascular disease (542;544). Endothelial dysfunction has also been associated with cardiovascular disease risk factors (such as hypertension, insulin resistance, smoking etc.) and cardiovascular disease progression and predicts future cardiovascular events (542;545-549). Furthermore, treating cardiovascular disease risk factors (anti-hypertensives, statins, anti-inflammatory, exercise, anti-oxidants etc.) have been shown to improve endothelial function and such improvement predicted better prognosis (542;550). Hence, there is a strong clinical evidence that endothelial dysfunction contributes to the pathogenesis of cardiovascular disease and improvements in endothelial function is associated with better outcomes (542).

Loss of NO bio-availability (reduced production, increased destruction or reduced activity) is the key manifestation of endothelial dysfunction (542). Multiple factors have been highlighted to reduce NO bioavailability. Endogenous inhibitors of endothelial nitric oxide synthase (eNOS) (such as asymmetrical dimethyl arginine) are elevated, and contribute to endothelial dysfunction in a wide range of pathological states (including hyperglycaemia) (542;551). Endoplasmic reticulum stress (which is associated with inflammatory changes) has also been implicated in endothelial dysfunction, particularly in patients with obesity and T2DM (542;552). Adropin, an important regulator of energy homeostasis and insulin sensitivity that is produced in liver and brain, is another factor that has recently been recognised to regulate eNOS activity (553;554). In addition to eNOS bioavailability

other factors, such as abnormal function of junction gap proteins, can result in abnormal vascular function and increased permeability (542).

DM is a well recognised cause of endothelial dysfunction (543). In diabetes there is an imbalance between endothelial factors that result in vasodilatation (such as NO, prostacyclin, Bradykinin) and those that result in vasoconstriction (thromboxane A2, endothelin, angiotensin II) resulting in endothelial permeabilization, platelet aggregation, leukocyte adhesion, and cytokine production (543) and eventually atherosclerosis. Furthermore, and as discussed in chapter 1, endothelial dysfunction is the end path in which hyperglycaemia and OS resulting in microvascular complications (90;543).

OSA is also a well recognised cause of endothelial dysfunction as well (555). OSA has been shown to be associated with impaired flow-mediated vasodilatation, reduced NO levels, increased endothelin levels and increased inflammation and OS independent of obesity (555-558). However evidence for any impact of OSA on endothelial function in patients with T2DM is lacking.

8.2. Hypothesis

OSA is associated with abnormalities in microvascular regulation and endothelial function in patients with T2DM

Rationale: OSA is associated with microvascular complications in patients with T2DM. OSA is associated with PKC activation, AGE production and oxidative stress; all of which are known to impact endothelial and microvascular function.

8.3. Aims

The primary aim of this study is to assess the relationship between OSA and nocturnal hypoxemia and microvascular and endothelial function in patients with T2DM. A secondary aim is to assess the

relationship between microvascular complications and microvascular blood flow regulation in patients with T2DM.

8.4. Methods

For details about study participants, recruitments and measurements please refer to previous chapters. We have utilised the same cohort of patients that was studied in the OSA and microvascular complications study. All study participants were contacted and all those who agreed were consented to have the test.

OSA was assessed using home-based multichannel respiratory device as detailed in chapter 2.

Microvascular /endothelial function was assessed using Laser Speckle Contrast imaging (LSCI) combined with inotophoresis of sodium nitroprusside (SNP) and acetylcholine (Ach) and heating as detailed in chapter 2 (Methods). Patients who were already started on CPAP by the time of conducting the endothelial assessment were excluded.

Statistical methods can be found in Chapter 2.

8.5. Results

Seventy nine patients agreed to have the LSCI, 8 were excluded because they were already receiving CPAP, leaving 71 (24 OSA-) for analysis. Out of the 47 patients with OSA, 28 were mild and 19 moderate to severe.

The characteristics of the study participants in regard to OSA status are summarized in **Table 9.1**. The differences between OSA+ and OSA- patients in this subsample are similar to those in the larger cohort. There were more males and more White Europeans in the OSA+ group. There was also a trend that OSA+ patients are older, heavier and with higher BP and longer diabetes duration. There were no differences between the groups in regard to glycaemic control, lipid profile, renal function and the prescription of glucose lowering, BP lowering and lipid lowering treatments.

Table 8-1: 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	37.5%	68.1%	0.014
White Europeans	33.3%	63.8%	0.015
Age (years)	56.0±10.1	60.6±11.3	0.094
Diabetes Duration (years)	10.0 (7.2-15.7)	14.0 (6.0-20.0)	0.271
Body Mass Index (kg/m^2)	30.7 (28.0-35.8)	34.4 (31.1-36.6)	0.063
Systolic blood pressure (mmHg)	125.5 (112.5-132.6)	132.0 (121.5-139.0)	0.076
Diastolic blood pressure (mmHg)	77.6±9.106	76.9±9.5	0.244
HbA1c (%)	7.4 (6.6-8.4)	7.6 (7.1-8.6)	0.381
Total cholesterol (mmol/L)	3.7 (3.3-4.4)	3.7 (3.1-4.1)	0.284
Triglycerides (mmol/L)	1.6 (1.0-2.7)	1.7 (1.2-2.2)	0.775
HDL (mmol/L)	1.10 (0.94-1.54)	1.08 (0.90-1.29)	0.447
Estimated GFR (ml/min/1.73 m^2)	86.1±25.1	78.8±24.7	0.780
Epworth sleepiness score	7.0 (3.2-13.5)	8.5 (3.0-12.2)	0.655
Smoking (current or ex-smoker)	45.8%	34.0%	0.333
Alcohol (drinks alcohol)	12.5%	36.2%	0.036
Oral anti-diabetes treatment	95.8%	89.4%	0.354
Insulin	45.8%	53.2%	0.557
Lipid lowering therapy	87.5%	87.2%	0.975
Anti-hypertensives	75%	89.4%	0.114
Anti-platelets	62.5%	74.5%	0.296

In order to assess whether this subsample is representative to that in the total cohort, we compared the patients who agreed to have LSCI performed with those who declined in patients with and without OSA (**Table 9.2**). On the whole there were no differences between patients who had and those who did not have LSCI across a wide range of demographic, clinical and biochemical characteristics, except a lower HbA1c in patients with OSA who agreed to have LSCI compared to those who declined stratified by their OSA status. This suggests that our subsample is representative of the total cohort.

Table 8-2: Comparison of the characteristics of patients who had Laser Speckle Contrast Imaging performed (LSCI+) and those who did not (LSCI-) in relation to OSA status.

Data presented as median (IQR) or mean (SD). GFR: Glomerular Filtration Rate.

	Patients without OSA			Patients with OSA		
	LSCI+ (n=24)	LSCI- (n=59)	P value	LSCI+ (n=47)	LSCI- (n=97)	P value
Male	37.5%	42.4%	0.682	68.1%	68.0	0.996
White Europeans	33.3%	40.7%	0.533	63.8%	62.9%	0.912
Age (years)	56.0±10.1	54.2±12.7	0.539	60.6±11.3	57.3±11.3	0.100
Diabetes Duration (years)	10.0 (7.2-15.7)	9.0 (5.0-13.0)	0.194	14.0 (6.0-20.0)	11.0 (7.0-17.0)	0.214
Body Mass Index (kg/m^2)	30.7 (28.0-35.8)	30.0 (26.8-34.6)	0.567	34.4 (31.1-36.6)	33.9 (30.1-39.5)	0.733
Systolic blood pressure (mmHg)	125.5 (112.5-132.6)	122.0 (115.0-137.5)	0.849	132.0 (121.5-139.0)	130.0 (123.5-141.0)	0.897
Diastolic blood pressure (mmHg)	77.6±9.106	77.0±10.7	0.820	76.9±9.5	78.8±10.5	0.309
HbA1c (%)	7.4 (6.6-8.4)	7.7 (7.2-8.8)	0.141	7.6 (7.1-8.6)	8.5 (7.7-9.7)	0.006
Total cholesterol (mmol/L)	3.7 (3.3-4.4)	3.7 (3.4-4.6)	0.896	3.7 (3.1-4.1)	3.7 (3.3-4.4)	0.186
Triglycerides (mmol/L)	1.6 (1.0-2.7)	1.4 (1.1-2.1)	0.868	1.7 (1.2-2.2)	1.9 (1.3-2.6)	0.079
HDL (mmol/L)	1.10 (0.94-1.54)	1.20 (0.90-1.40)	0.932	1.08 (0.90-1.29)	1.07 (0.90-1.21)	0.545
Estimated GFR ($ml/min/1.73\ m^2$)	86.1±25.1	95.7±24.9	0.115	78.8±24.7	83.6±27.5	0.308
Epworth sleepiness score	7.0 (3.2-13.5)	5.0 (1.0-11.0)	0.492	8.5 (3.0-12.2)	8.0 (5.0-14.0)	0.328
Smoking (current or ex-smoker)	45.8%	35.6%	0.385	34.0%	42.3%	0.344
Alcohol (drinks alcohol)	12.5%	15.3%	0.746	36.2%	35.1%	0.895
Oral anti-diabetes treatment	95.8%	98.3%	0.506	89.4%	90.7%	0.796
Insulin	45.8%	39.0%	0.565	53.2%	64.9%	0.175
Lipid lowering therapy	87.5%	84.7%	0.746	87.2%	79.4	0.251
Anti-hypertensives	75%	72.9%	0.843	89.4%	82.5%	0.281

The relationship between microvascular complications in patients with T2DM and microvascular regulation is summarised in **Table 9.3**. Patients with DPN, STDR and diabetic nephropathy showed evidence of impaired baseline and Ach-induced and SNP-induced vasodilatation. Patients with STDR and diabetic nephropathy also showed also evidence of impaired heating response.

Table 8-3: The relationship between microvascular regulation and microvascular complications in patients with T2DM.

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. DPN: diabetic peripheral neuropathy; STDR: sight threatening diabetic retinopathy; DN: diabetic nephropathy; Ach: acetylcholine; SNP: sodium nitroprusside.

	DPN- (n=37)	DPN+ (n=34)	P value
Flux			
Baseline	29.50 (20.60-38.05)	20.25 (13.95-30.98)	0.004
Heating	165.70 (139.90-203.55)	148.95 (128.35-194.60)	0.272
Ach	116.80 (98.75-159.50)	94.05 (66.80-122.08)	0.014
SNP	133.60 (77.00-188.65)	102.80 (57.68-133.28)	0.008
Conductance			
Baseline	0.29 (0.22-0.44)	0.20 (0.15-0.32)	0.004
Heating	1.75 (1.43-2.07)	1.61 (1.27-1.98)	0.290
Ach	1.29 (1.06-1.68)	0.97 (0.73-1.28)	0.005
SNP	1.44 (0.90-2.01)	1.09 (0.60-1.42)	0.007
Flux in relation to maximum vasodilatation			
Baseline	0.19 (0.12-0.26)	0.15 (0.09-0.20)	0.014
Ach	0.72 (0.62-0.90)	0.62 (0.40-0.82)	0.043
SNP	0.89 (0.72-1.06)	0.56 (0.40-0.82)	0.002
	STDR- (n=41)	STDR+ (n=28)	P value
Flux			
Baseline	31.50 (19.15-38.05)	22.00 (15.78-29.38)	0.020
Heating	165.70 (142.45-203.75)	139.95 (120.35-181.45)	0.040
Ach	116.80 (92.10-153.85)	94.05 (70.48-116.35)	0.036
SNP	135.10 (76.55-173.70)	102.80 (65.50-128.13)	0.015
Conductance			
Baseline	0.31 (0.19-0.42)	0.20 (0.17-0.29)	0.043
Heating	1.81 (1.51-2.07)	1.50 (1.23-2.01)	0.081
Ach	1.29 (1.04-1.60)	0.97 (0.75-1.28)	0.031
SNP	1.44 (0.90-1.96)	1.09 (0.72-1.41)	0.025
Flux in relation to maximum vasodilatation			

Baseline	0.16 (0.12-0.23)	0.16 (0.12-0.20)	0.566
Ach	0.72 (0.59-0.87)	0.63 (0.46-0.84)	0.456
SNP	0.84 (0.53-0.99)	0.64 (0.49-0.92)	0.149
	DN- (n=35)	DN+ (n=31)	P value
Flux			
Baseline	31.70 (16.70-40.70)	23.60 (17.10-30.00)	0.069
Heating	172.70 (147.40-212.20)	143.80 (122.60-171.70)	009
Ach	119.20 (94.60-151.90)	98.70 (68.90-116.40)	0.017
SNP	133.60 (79.10-181.00)	111.40 (73.10-129.20)	0.111
Conductance			
Baseline	0.31 (0.18-0.42)	0.25 (0.18-0.32)	0.141
Heating	1.79 (1.58-2.09)	1.57 (1.22-1.90)	0.040
Ach	1.29 (1.07-1.59)	1.01 (0.75-1.29)	0.021
SNP	1.40 (0.89-1.96)	1.22 (0.78-1.47)	0.174
Flux in relation to maximum vasodilatation			
Baseline	0.16 (0.12-0.23)	0.16 (0.12-0.22)	0.695
Ach	0.72 (0.61-0.90)	0.63 (0.40-0.85)	0.289
SNP	0.78 (0.47-1.05)	0.76 (0.53-0.94)	0.944

Patients with OSA had lower microvascular blood flux at baseline and following Ach and SNP iontophoresis (**Table 9.4**). Maximal vasodilatation (following 44C heating) was not different between groups. After adjustment for BP and for maximal vasodilatation, baseline and Ach and SNP induced flux remained lower in OSA+ patients (**Table 9.4**).

Table 8-4: Assessment of microvascular blood flow and endothelial function in with T2DM 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. Ach: acetylcholine; SNP: sodium nitroprusside

	OSA- (n=24)	OSA+ (n=47)	P value- unadjusted
Flux			
Baseline	35.60 (26.75-42.37)	30.80 (15.70-20.70)	< 0.001
Heating	168.90 (133.20-209.05)	154.60 (129.30-192.80)	0.405
Ach	130.95 (99.07-177.52)	99.30 (68.90-120.20)	0.01
SNP	146.50 (117.05-204.62)	103.00 (62.30-135.10)	0.001
Conductance			
Baseline	0.40 (0.28-0.48)	0.20 (0.16-0.31)	< 0.001
Heating	1.82 (1.43-2.03)	1.66 (1.28-2.07)	0.368
Ach	1.43 (1.09-1.83)	1.07 (0.75-1.29)	0.002
SNP	1.61 (1.15-2.14)	1.16 (0.62-1.41)	0.001
Flux in relation to maximum vasodilatation			
Baseline	0.22 (0.16-0.29)	0.14 (0.10-0.17)	< 0.001
Ach	0.81 (0.67-0.90)	0.63 (0.43-0.77)	0.005
SNP	0.93 (0.77-1.13)	0.57 (0.41-0.89)	< 0.001

OSA severity (based on AHI and ODI) correlated negatively with baseline, Ach-induced and SNP-induced flux and nocturnal hypoxemia correlated positively with SNP-induced flux even when adjusted to BP and maximal vasodilatation (**Table 9.5**).

Table 8-5: The relationship between OSA severity, hypoxia severity and microvascular and endothelial function parameters.

		AHI	ODI	Nadir Nocturnal Oxygen Saturation
Flux				
Baseline	r	-0.408	-0.337	0.215
	p	<0.001	0.004	0.072
Heating	r	-0.125	-0.135	0.106
	p	.300	0.262	0.377
Ach	r	-0.242	-0.200	0.101
	p	.042	0.095	0.401
SNP	r	-.324	-0.366	0.256
	p	.006	0.002	0.031
Conductance				
Baseline	r	-0.400	-0.310	0.182
	p	0.001	0.008	0.128
Heating	r	-0.115	-0.101	0.068
	p	0.342	0.402	0.575
Ach	r	-0.297	-0.221	0.088
	p	0.012	0.064	0.465
SNP	r	-0.356	-0.362	0.247
	p	0.002	0.002	0.038
Flux in relation to maximum vasodilatation				
Baseline	r	-0.360	-0.295	0.155
	p	0.002	0.012	0.197
Ach	r	-0.229	-0.192	0.052
	p	0.054	0.108	0.667
SNP	r	-0.351	-0.395	0.279
	p	0.003	0.001	0.018

In order to adjust for baseline differences between patients with and without OSA, we applied linear regression models (backward method), with the flux being the outcome and ethnicity, gender, age, diabetes duration, BMI and OSA as the predictors (**Table 9.6**). The predictors were chosen as these are major factors that can affect endothelial dysfunction. Following adjustment OSA remained an independent predictor of lower baseline and SNP-induced flux. This remained the case even after adjustment for BP and maximal vasodilatation. Repeating the same model but replacing OSA with parameters of OSA severity and nocturnal hypoxemia severity showed similar results in that AHI and ODI were independent predictors of baseline and SNP-induced flux even after adjustment for BP and maximal vasodilatation (higher AHI or ODI associated with lower flux). Nadir nocturnal oxygen saturation was an independent predictor of SNP-induced flux only, which remained significant after adjustment for BP and maximal vasodilatation (lower nadir oxygen saturation associated with lower flu).

Table 8-6: The adjusted analysis of the impact of OSA and nocturnal hypxemia on microvascular blood flow and endothelial function in patients with T2DM.

Data presented as B and p value. The analysis was performed using blood flow as the outcome and ethnicity, gender, age, 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.

	OSA	AHI	ODI	Nadir nocturnal oxygen saturation
Flux				
Baseline	B= -0.203,p<0.001	B=-0.207, p<0.001	B=-0.160, p=0.004	B=0.123, p=0.259
Heating	B=-0.038, p=0.343	B=-0.049, p=0.222	B=-0.038, p=0.348	B=0.061, p=0.429
Ach	B=-0.093, p=0.098	B=-0.045, p=0.441	B=-0.018, p=0.753	B=-0.024, p=0.832
SNP	B=-0.245, p<0.001	B=-0.189, p=0.003	B=-0.201, p=0.002	B=0.331, p=0.010
Conductance				
Baseline	B=-0.223, p<0.001	B-0.212, p<0.001	B=-0.162, p=005	B=0.107, p=0.346
Heating	B=-0.044, p=0.290	B=-0.050, p=0230	B=-0.029, p=0.511	B=0.030, p=0.709
Ach	B=-0.099, p=0.075	B=-0.046, p=0.425	B=-0.016, p=0.796	B=-0.039, p=0.724
SNP	B=-0.227, p<0.001	B=-0.193, p=0.003	B=-0.202, p=0.002	B=0.311, p=0.017
Flux in relation to maximum vasodilatation				
Baseline	B=-0.202, p<0.001	B=-0.193, p<0.001	B=-0.107, p=0.063	B=0.065, p=0.562
Ach	B=0.061, p=0.265	B=-0.03, p=0.811	B=016, p=0.768	B=-0.085, p=0.414
SNP	B=-0.211, p<0.001	B=-0.167, p=0.003	B=-0.177, p=0.003	B=0.282, p=0.014

8.6. Discussion

Our results show a novel association between OSA and nocturnal hypoxemia and microvascular function and endothelial function including endothelial dependent and independent vasodilatation in patients with T2DM. Furthermore, OSA severity (as measured by AHI and ODI) and nocturnal hypoxemia severity correlated with microvascular/endothelial function suggesting worsening microvascular/endothelial function with worsening OSA severity and worsening nocturnal hypoxemia. These results support our hypothesis that OSA is related to microvascular complications in patients with T2DM and suggest a possible mechanism to this relationship.

Microvascular/endothelial dysfunction has been implicated in the pathogenesis of diabetes-related microvascular complications (559;560). This is supported by our data showing impaired microvascular regulation in patients with microvascular complications. The impairment of Ach-induced and SNP-induced flux is suggestive of reduced action or increased destruction of NO in addition to deficits in prostanoids action/secretion or low capillary density. The impaired heating response is suggestive of impaired neural function, NO secretion/function or capillary damage. Increased oxidative/nitrosative stress has also been linked to the development and progression of microvascular complications as discussed previously (561;562).

Our data are consistent with a role for impaired microvascular blood flow regulation as a link between OSA with DPN and other microvascular complications. Patients with OSA had lower blood flow at baseline and following stimulation with Ach and SNP. These differences (with exception of Ach) persisted even when adjusted for maximal vasodilatation, MAP, age, BMI, diabetes duration, gender and ethnicity. Our data also show that AHI, ODI and nadir nocturnal oxygen saturations are also independent predictors of microvascular blood flow regulation in patients with T2DM and correlate with microvascular /endothelial function measures. The impaired response to SNP might be in part due to impaired response to nitric oxide secondary to OS (563).

Our results suggest that OSA affects different aspects of microvascular endothelial function. On one hand our data show that OSA is associated with reduce baseline flux. Baseline flux, however, is usually the most variable and least reproducible measurement obtained by this technique and hence many investigators suggested presenting these values adjusted to maximal vasodilatation (502;563). On the other hand the heating response, which is dependent on neurogenic axon reflex and NO action (502;563), was not different between patients with and without OSA suggesting that there is no additional capillary structural damage (such as capillary density) caused by OSA in patients with T2DM . Ach-induced flux represents the action of several players including NO, prostanoids and axon reflexes (502;563). The reduction in Ach-induced flux results from either an impaired production or impaired action of these factors. If the Ach-induced flux was only due to impaired secretion of NO, then providing NO via another route (SNP in our study) would have corrected the flux. The fact the flux in OSA+ patients remained lower than that in OSA- patients after SNP iontophoresis suggests that impaired NO action (with or without impaired production) is a major contributor to the impaired microvascular/endothelial function in patients with OSA. This further supported by that the SNP-induced flux was reduced in the face of comparable heating response between groups, suggesting that reduced SNP response is secondary to reduced NO action rather than reduction in production or capillary structural deficit (in which case heating response would have been different between groups as well). In the next chapter we show that there is increased Oxidative and nitrosative stress in patients with OSA and T2DM, factors that are known to impair NO action.

Although several lines of evidence showed a relation between OSA and impaired microvascular/endothelial function (555) , The impact of OSA on microvascular blood flow regulation in patients with T2DM has not previously been reported. Furthermore, assessment of endothelial function using Laser imaging techniques in patients with OSA (without diabetes) is very limited. In one previous report, impaired brachial artery flow mediated dilatation was identified in obese non-diabetic OSA patients (564). 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; consistent with our results. However their findings differed from those reported herein in that the response to Ach and SNP was not impaired by OSA (564). These findings suggest that when OSA is complicated by diabetes/hyperglycaemia there are additional deficits of vasoactive agent metabolism or action, which is thought to mediate the pathogenic effects of oxidative/nitrosative stress in the development of microvascular complications (565).

The main limitation of our study is the differences observed between patients with and without OSA, particularly in regard to age, ethnicity and adiposity. Having said that, the relationship between OSA (and its metrics) and microvascular/endothelial function remained significant after adjusting for several possible confounders. Nonetheless, matching the groups for several variables and/or having larger sample size that will allow more adjustments and would have been desirable. Differences in ethnicities (and hence in skin colour) might have affected our results, but the equation that is used by the software to calculate flux has a factor that would adjust for several variables that might affect the results such as the distance between the Laser and the skin, the angle of the Laser beam etc, but including differences in skin colour.

Our study has several strengths. This is first study to assess the impact of OSA on microvascular/endothelial function in patients with T2DM. Our study population was well characterised which allow us to adjust for baseline differences and improve our understanding of the data. Our LSCI protocol results were highly reproducible which suggest that our protocol was very consistent and that differences observed in measurements are unlikely to represent artefact (Please see Chapter 2 for more details).

In summary, OSA, OSA severity and nocturnal hpoxemia are associated with microvascular/endothelial function deficits, particularly impaired action of NO in patients with T2DM. The relationship between OSA and microvascular/endothelial abnormalities is a possible mechanism contributing to the relationship observed between OSA and microvascular complications in patients with T2DM. Further prospective studies are needed to assess the impact of OSA on

microvascular/endothelial function in patients with T2DM. Furthermore, interventional studies to assess the impact of OSA treatment on endothelial function are warranted.

9. Chapter nine: Obstructive Sleep Apnoea and Oxidative Stress in Patients with Type 2 Diabetes

9.1. Introduction

In the previous chapters we have shown that OSA was associated with microvascular complications in patients with T2DM and that OSA is associated with impaired microvascular regulation.

In the first chapter we explained how the generation of ROS and RNS coupled with GAPDH inhibition and PARP activation, play an important role in the development of microvascular and endothelial dysfunction resulting in microvascular complications in patients with T2DM.

We have shown in the previous chapter that OSA is associated with impaired microvascular regulation in patients with T2DM. OSA is also a well recognised to cause OS and is associated with ROS production in the general population; but whether OSA results in OS in patients with T2DM is unknown. Hence, it is plausible that increased OS and PARP activation might play a role in explaining the relationship observed between OSA, impaired microvascular regulation and microvascular complications in our study.

9.2. Hypothesis

OSA is associated with increased nitrosative stress, oxidative stress and PARP activation in patients with T2DM

Rationale: OSA is associated with microvascular complications and impaired microvascular regulation. One possible mechanism for the relationship between OSA and microvascular complications is increased oxidative stress, nitrosative stress and PAR activation (please see chapter 1 for details).

9.3. Aims

The primary aim of this study is to assess whether OSA is associated with increased oxidative and nitrosative stress and PARP activation in patients with T2DM. A secondary aim was to assess PARP

activation and markers of oxidative and nitorsative stress between patients with and without microvascualr complications.

9.4. Methods

This is a secondary analysis of the project examining the relationship between OSA and microvascular complications in patients with T2DM. All patients were approached and fasting blood sample and a skin biopsy were obtained from consented patients. Patients who are at high risk of hypoglycaemia (either know to have hypoglycaemia unawareness or taking insulin treatment) provided the blood sample without fasting.

OSA was assessed using a home-based portable multi-channel respiratory device as explained previously. Serum 3-nitrotyrosine levels were assayed as a marker of nitrosative stress as explained in the methods chapter (chapter 2). Plasma lipid peroxide levels were measured as a marker of OS as explained in the methods chapter (chapter 2).

Skin biopsies were obtained, stored and immuno-stained for PAR as explained in details in the methods chapter (chapter 2).

Statistical methods can be found in Chapter 2.

9.5. Results

One hundred and two blood samples were obtained. Fifty patients consented and provided skin biopsies.

9.5.1. Oxidative/nitrosative stress

The participants characteristics who provided a blood sample in accordance to their OSA status is summarised in **Table 9.1**. The differences between patients with and without OSA in this subsample were broadly similar to those existed in the total cohort. Patients with OSA were older and heavier

but had similar HbA1c and lipid profile. OSA patients had a trend of longer diabetes duration but similar treatments profile.

Table 9-1: The characteristics of patients who had undergone serum nitrotyrosine and lipid peroxide 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	34.5%	65.8%	0.004
White Europeans	55.2%	67.1%	0.257
Age (years)	54.8±12.1	59.0±11.0	0.095
Diabetes Duration (years)	10.0 (4.5-12.0)	12.0 (6.0-20.0)	0.076
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)	0.118
Diastolic blood pressure (mmHg)	78.3±8.9	77.2±10.1	0.627
HbA1c (%)	7.4 (6.8-8.5)	8.0 (7.1-9.1)	0.203
Total cholesterol (mmol/L)	3.8 (3.2-4.3)	3.7 (3.2-4.3)	0.772
Triglycerides (mmol/L)	1.6 (1.2-2.3)	1.8 (1.3-2.5)	0.661
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	0.203
Epworth sleepiness score	7.0 (4.0-14.0)	8.0 (3.2-11.7)	1.0
Smoking (current or ex-smoker)	44.8%	41.1%	0.731
Alcohol (drinks alcohol)	17.2%	38.4%	0.040
Oral anti-diabetes treatment	96.6%	94.5%	0.668
Insulin	44.8%	58.9%	0.197
Lipid lowering therapy	82.8%	82.2%	0.946
Anti-hypertensives	69%	89%	0.014

A comparison between the study participants who provided a blood sample and those who did not can be found in **Table 9.2**. Apart from slight over representation of South Asians in the group of patients without OSA who provided a blood sample, the remaining characteristics were similar suggesting that this sub sample is representative of the total cohort.

Table 9-2: Comparison of the characteristics of patients who had serum nitrotyrosine lipid peroxide measured (A) and those who did not (B) in relation to OSA status.

Data presented as median (IQR) or mean±SD. GFR: Glomerular Filtration Rate.

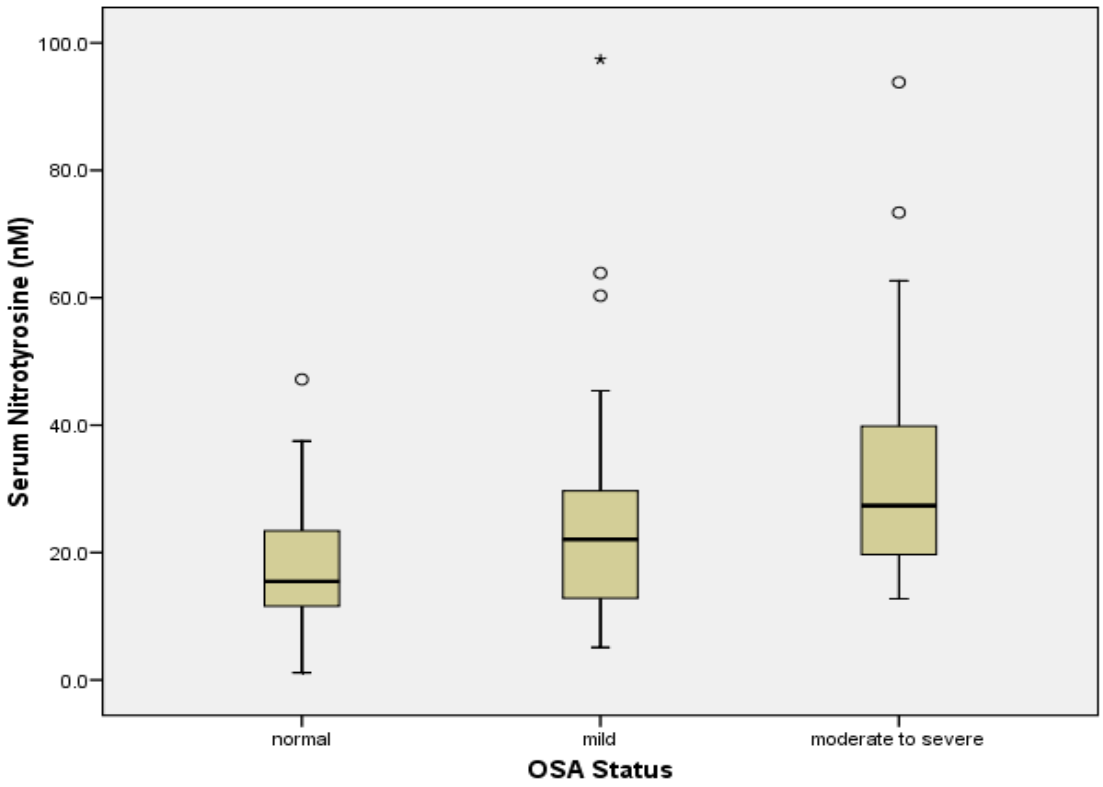
	Patients without OSA			Patients with OSA		
	A (n=29)	B (n=54)	P value	A (n=73)	B (n=78)	P value
Male	34.5%	44.4%	0.379	65.8%	67.9%	0.775
White Europeans	55.2%	29.6%	0.023	67.1%	61.5%	0.474
Age (years)	54.8±12.1	54.7±12.0	0.970	59.0±11.0	58.1±11.6	0.656
Diabetes Duration (years)	10.0 (4.5-12.0)	9.0 (5.7-15.2)	0.738	12.0 (6.0-20.0)	11.0 (8.0-17.0)	0.810
Body Mass Index (kg/m^2)	31.0 (28.1-35.2)	30.0 (26.7-34.7)	0.522	34.6 (31.1-39.8)	33.8 (30.8-37.9)	0.332
Systolic blood pressure (mmHg)	127.0 (120.5-137.5)	121.2 (113.0-136.0)	0.278	131.5 (123.7-140.5)	130.0 (123.5-138.9)	0.509
Diastolic blood pressure (mmHg)	78.3±8.9	76.6±10.8	0.477	77.2±10.1	79.2±9.9	0.219
HbA1c (%)	7.4 (6.8-8.5)	7.7 (7.1-9.0)	0.364	8.0 (7.1-9.1)	8.3 (7.4-9.9)	0.113
Total cholesterol (mmol/L)	3.8 (3.2-4.3)	3.7 (3.4-4.6)	0.852	3.7 (3.2-4.3)	3.7 (3.3-4.3)	0.982
Triglycerides (mmol/L)	1.6 (1.2-2.3)	1.3 (1.0-2.1)	0.341	1.8 (1.3-2.5)	1.9 (1.3-2.5)	0.393
HDL (mmol/L)	1.1 (0.9-1.5)	1.2 (1.0-1.4)	0.477	1.1 (0.9-1.2)	1.1 (0.9-1.2)	0.854
Estimated GFR ($ml/min/1.73\ m^2$)	89.7±24.7	94.6±25.5	0.401	82.2±27.6	82.6±25.4	0.447
Epworth sleepiness score	7.0 (4.0-14.0)	5.0 (1.0-11)	0.087	8.0 (3.0-12.0)	8.0 (5.0-14.0)	0.103
Smoking (current or ex-smoker)	44.8%	35.2%	0.390	41.1%	41%	0.993
Alcohol (drinks alcohol)	17.2%	13%	0.597	38.4%	32.1%	0.417
Oral anti-diabetes treatment	96.6%	98.1%	0.651	94.5%	87.2%	0.120
Insulin	44.8%	38.9%	0.600	58.9%	61.5%	0.741
Lipid lowering therapy	82.8%	87.0%	0.597	82.2%	83.3%	0.853
Anti-hypertensives	69%	75.9%	0.493	89%	82.1%	0.224

Nitrotyrosine levels were higher in patients with (n=47) DPN compared to those without (n=55) [25.56 nM (17.68-35.78) vs. 19.45 nM (11.45 -29.61), p=0.011] and in patients with (n=36) STDR compared to those without (n=64) (19.68 (12.73-29.58) vs. 28.50 (19.10-37.10), p=0.038). Serum nitrotyrosine levels were also non-significantly higher in patients with diabetic nephropathy (n=43) compared to those without (n=42) (19.50 (12.17-29.70) vs. 24.22 (17.68-37.17), p=0.174).

Patients with OSA had higher serum nitrotyrosine levels compared to those without OSA [23.53 nM (16.67 -36.07) vs. 15.49 nM (11.53 -24.28), 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 9.1**). Post-hoc analysis showed significant differences between moderate to severe OSA and mild OSA (p=0.035) 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).

Figure 9-1: 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).

P value for the trend p < 0.001, p= 0.035 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.



Serum nitrotyrosine levels correlated with OSA severity and nocturnal hypoxemia measures [AHI (r=0.380, p<0.001), time spent with oxygen saturations <80% (r=0.227, p=0.022), ODI (r=0.353, p<0.001) and nadir nocturnal oxygen saturation (r=-0.214, p=0.031)]. All correlations remained significant following adjustment for age, BMI and diabetes duration (r= 0.378, 0.374 and 0.262 and p < 0.001, < 0.001 and < 0.001 for AHI, ODI and nadir nocturnal oxygen saturation respectively).

Using linear regression, and after adjustment for OSA, ethnicity, age, gender, alcohol intake, smoking, BP, diabetes duration, HbA1c, Total cholesterol, HDL, Triglycerides, eGFR, oral glucose lowering treatments, insulin, GLP-1 analogues, anti-hypertensives, anti-platelet agents, lipid lowering therapy and obesity, OSA (AHI ≥ 5) (p=0.003), OSA (AHI ≥ 15) (p=0.005), AHI (B=0.280,

p<0.001), ODI (B=0.267, p<0.001), and nadir nocturnal oxygen saturation (B= -0.330, p=0.006) were all independent predictors of nitrotyrosine levels. Other independent predictors of nitrotyrosine included age (B=0.011, p=0.001) and insulin use (p=0.027).

Lipid peroxide levels were higher in patients with DPN (n=43 with and 56 without DPN) (21.14 (3.86-42.48) vs. 12.20 (2.90-24.55), p=0.014) with a non-significant trend of higher lipid peroxide in patients with diabetic nephropathy and sight threatening retinopathy. 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 multivariate adjustment for age, gender, ethnicity, diabetes duration, BMI, HbA1c, insulin use, anti-hypertensives and alcohol intake using the backward method, OSA (AHI ≥ 5) (B=1.07, p=0.04) and time spent with oxygen saturation < 80% (B=1.08, p=0.049) were independently associated with plasma lipid peroxide levels. AHI, OD and nadir oxygen saturation were not associated with lipid peroxide levels after adjustment. Other independent associations of lipid peroxide included HbA1c (B=0.61, p=0.001) and triglycerides (B=1.33, p<0.001).

9.5.2. Poly (ADP-ribose) (PAR)

Fifty patients consented and provided skin biopsies. Out of the 50 who provided skin biopsies, 8, 23 and 19 patients had no, mild and moderate to severe OSA (OSA defined as AHI ≥ 5). In order to balance the group sizes and due to the small sample size in the groups without OSA, we have used an AHI cut of 10 (26 and 24 patients had AHI < 10 and ≥ 10 respectively) to compare groups. An AHI cut off of 10 is commonly used in the literature and is used by some as the cut off to start CPAP

treatment; hence using this cut off is meaningful (566). Thirty four patients (68%) had evidence of DPN (based on MNSI).

Participants characteristics in relation to OSA status (AHI < and ≥ 10) are summarised in **Table 9.3**. The groups were largely similar apart from a trend of higher BMI in the AHI ≥ 10.

Table 9-3: The characteristics of patients who had undergone skin biopsies in relation to OSA status.

Data presented as median (IQR) or mean (SD). GFR: Glomerular Filtration Rate.

	AHI < 10 (n=26)	AHI ≥ 10 (n=24)	P value
Male	73.1%	75.0%	0.877
White Europeans	76.9%	83.3%	0.571
Age (years)	59.9 ±10.7	61.0 ± 10.6	0.715
Diabetes Duration (years)	12.0 (10.0-16.3)	15.0 (7.5-22.3)	0.633
Body Mass Index (kg/m^2)	33.9 ± 9.0	37.8 ± 8.0	0.112
Waist hip ratio	1.00 ± 0.07	1.01 ± 0.07	0.421
Systolic blood pressure (mmHg)	133.0 ± 16.2	132.5 ± 20.8	0.930
Diastolic blood pressure (mmHg)	80.6 ± 10.9	77.2 ± 7.1	0.203
HbA1c (%)	8.33 ± 1.51	8.15 ± 1.23	0.640
Total cholesterol (mmol/L)	3.7 ± 0.6	4.0 ± 1.2	0.273
Triglycerides (mmol/L)	1.7 ± 0.9	2.0 ± 0.8	0.224
HDL (mmol/L)	1.2 ± 0.4	1.1 ± 0.3	0.550
Estimated GFR (ml/min/1.73 m^2)	89.2 ± 27.02	74.4 ± 28.2	0.065
Epworth sleepiness score	8.5 (4.0-12.3)	10.0 (8.0-14.0)	0.151
Smoking (current or ex-smoker)	53.8%	41.7%	0.389
Alcohol (drinks alcohol)	57.7%	50.0%	0.586
Oral anti-diabetes treatment	96.2%	87.5%	0.260
Insulin	61.5%	54.2%	0.598
Lipid lowering therapy	88.5%	100%	0.086
Anti-hypertensives	76.9%	100%	0.012
Anti platelets	69.2%	66.7%	0.846
Diabetic peripheral neuropathy	59.3%	76%	0.199
Diabetic nephropathy	36.0%	60.9%	0.085
Sight threatening diabetic	33.3%	56.5%	0.10

The percentage of PAR stained nuclei was non-significantly higher in patients with DPN (55.4 ± 18.9 vs. 62.9 ± 16.8, p=0.164 for patients without (n=16) and with (n=34) DPN respectively) and significantly higher in patients with diabetic nephropathy (55.2 ± 17.6 vs. 67.1 ± 16.7, p=0.024 for patients without (n=25) and with (n=21) diabetic nephropathy respectively) and STDR (54.0 ±17.2 vs. 69.8 ±15.0, p=0.002 for patients without (n=27) and with (n=21) STDR respectively).

Patients with AHI ≥ 10 had a higher percentage of PAR stained nuclei than those without OSA (68.1 ± 15.5 vs. 53.5 ± 16.8, p=0.002). There was a non-significant trend of increased percentage of PAR stained nuclei between patients with AHI < 5, AHI 5 to < 15 and ≥ 15 (p=0.096) (**Figure 9.2**). Examples of PAR stained images can be found in **Figure 9.3.**

Figure 9-2: The relation between PAR and OSA severity.

There is a trend of increased percentage of PAR stained nuclei between patients with no OSA (n=8), mild OSA (n=23) and moderate to severe OSA (n=19), p=0.096 for the trend.

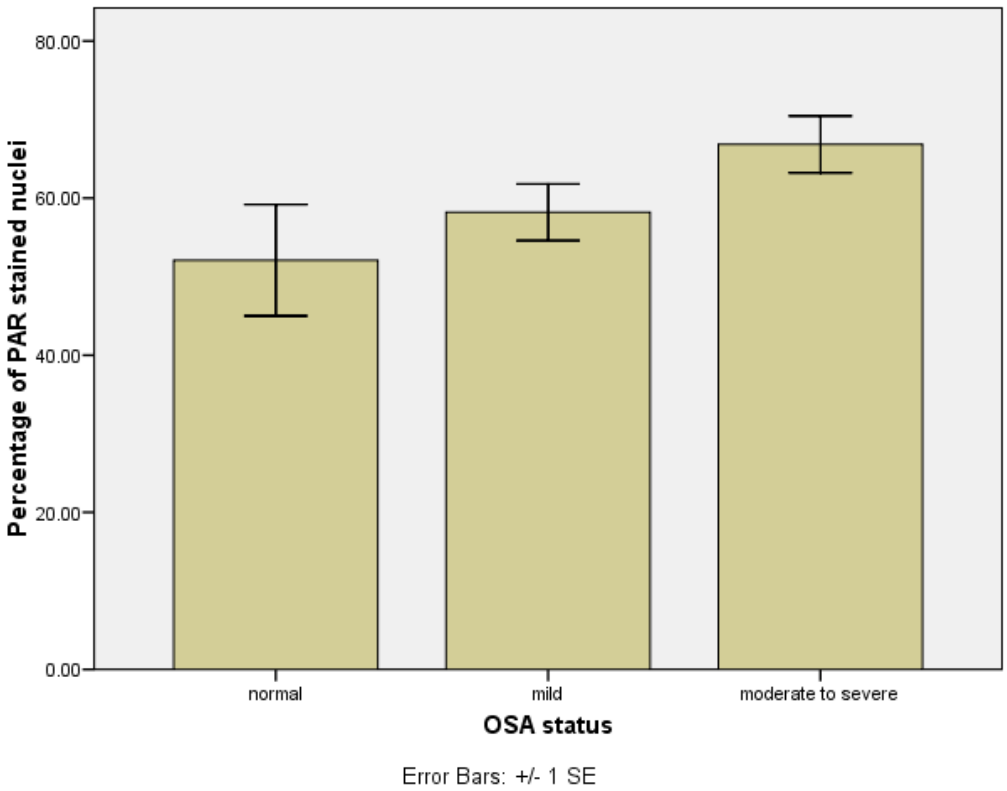
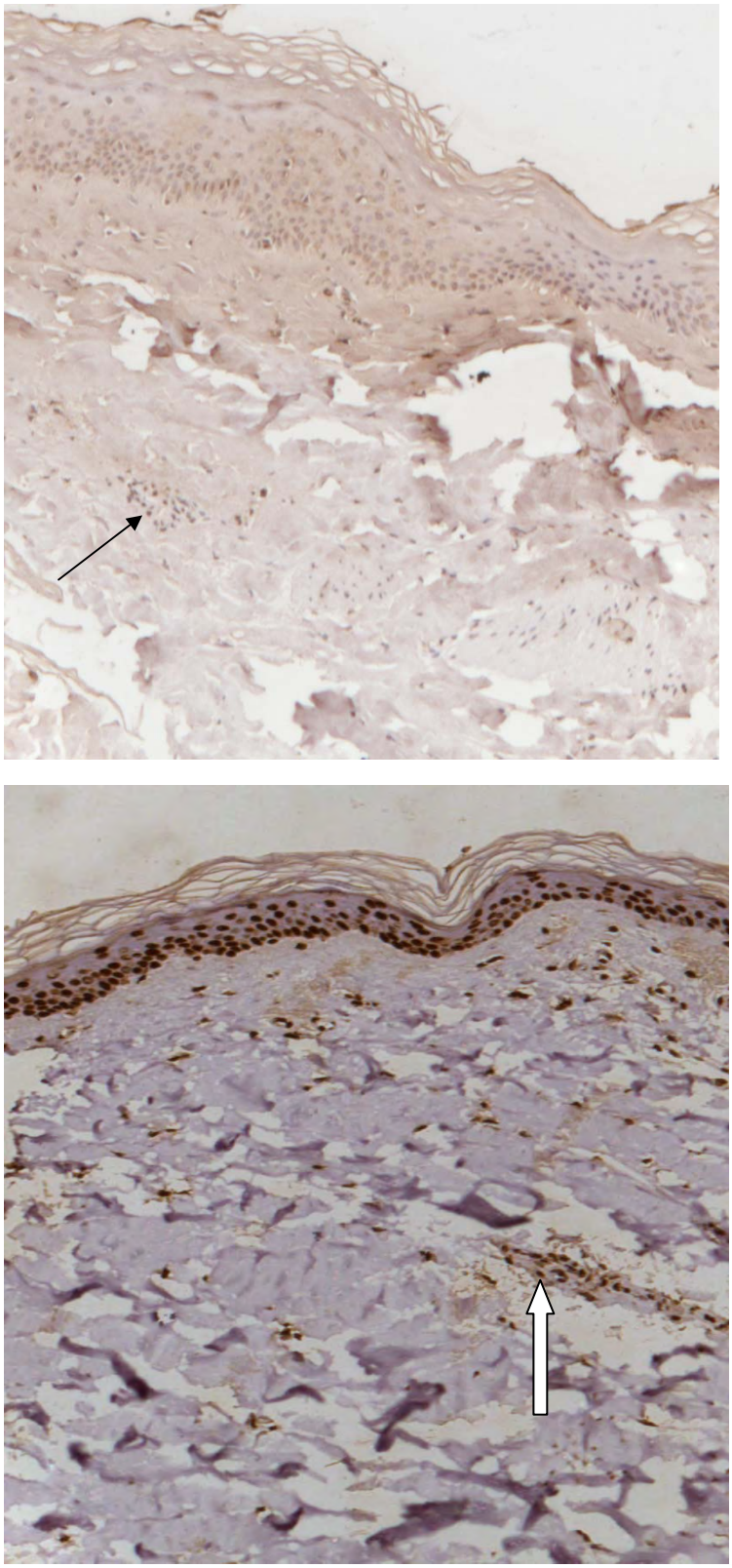


Figure 9-3: Examples of images of PAR stained nuclei from patients without (upper) and with (lower) OSA.

Thin arrow: PAR negative nuclei, Thick arrow: PAR positive nuclei. The actual counting took place under the microscope using greater magnification (X400).



The percentage of PAR stained nuclei correlated significantly with OSA severity and nocturnal hypoxemia severity (**Table 9.4).**

Table 9-4: The relationship between percentage of PAR stained nuclei and OSA and hypoxemia severities.

Data presented as correlation coefficients and p values. This analysis is unadjusted.

		AHI	ODI	Time spent with oxygen saturation < 80%	Nadir nocturnal oxygen saturation
% PAR stained nuclei	r	0.336	0.280	0.303	-0.176
	p	0.017	0.049	0.032	0.22

Using linear regression (backward method), and after adjusting for age, BMI, diabetes duration, HbA1c and the use of anti-hypertensives, lipid lowering therapy and insulin treatment, an AHI ≥ 10 remained independently associated with percentage of PAR stained nuclei (R=0.489, B=13.86, p=0.003). Using the same model, AHI was also independently associated with percentage of PAR stained nuclei (R=0.417, B=12.6, p=0.028). ODI (p=0.075) and nadir nocturnal oxygen saturation (p=0.350) were not independent predictors of percentage of PAR stained nuclei.

9.6. Discussions

Our results showed increased oxidative and nitrosative stress in patients with OSA and T2DM and that this relationship between OSA and OS and nitrosative stress was independent of possible confounders. Furthermore, OSA severity and hypoxemia severity were independently associated with oxidative/nitrosative stress severity. We have also shown an increase in DNA damage/repair as shown by increased PAR stained nuclei that patients with OSA and T2DM that was independent of possible confounders. To our knowledge, we are the first to report the above mentioned associations.

As oxidative/nitrosative stress play an essential role in the pathogenesis of diabetic microvascular complications (please see chapter 1), then our results support that the relationship between OSA

and microvascular complications and microvascular regulation that we have observed in our study could be in part secondary to the increased oxidative/nitrosative stress observed in patients with OSA and T2DM.

The higher levels of serum nitrotyrosine, plasma lipid peroxide and PAR activation in patients with microvascular complications compared to those without supports the role played by these factors in the development of diabetic microvascular complications (please refer to chapter 1 for more details).

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 the pathogenesis of DPN (562;567) by reducing nerve perfusion and impairing vascular reactivity of epineurial arterioles (561;568). 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 (569). It is associated with the development of thermal hyper- and hypoalgesia, mechanical hypoalgesia, tactile allodynia, and small sensory nerve fiber degeneration (568). More recently the inhibition of nitrosative stress has been shown to result in improvement of experimental neuropathy in diabetic rodent models (570). To our knowledge, this is the first report of an association of OSA with nitrosative stress in patients with T2DM. The significant correlation between serum nitrotyrosine and nocturnal hypoxemia measures, suggests that nitrosative stress is a potential mechanistic link between OSA and microvascular complications including DPN. Another report in patients **without** diabetes showed that endothelial expression of nitrotyrosine correlated with AHI despite adjustment for age and adiposity (508).

Poly(ADP-ribosyl)ation is the process by which polymers of ADP-ribose (PAR) are attached via an ester bond to glutamic acid, aspartic acid or lysine residues, mediated by the enzyme PARP (140). There are currently 18 known members of the PARP family, two of which, PARP1 and 2 are known to play a role in DNA repair (141). Increased OS results in DNA damage and PARP1 activation (144-146).

Although PARP1 plays a beneficial role in DNA repair, it is possible that hyperactivation in diabetes leads to detrimental effects (143;146). Excess cleavage of NAD+ by PARP, would exacerbate the effect of increased flux through SDH which results in depleting NAD+ further, leading to OS (146). In addition NAD+ is required as a cofactor for the conversion of GAPDH. Hyperglycemia-induced OS inhibits GAPDH activity in vivo by modifying the enzyme with PARP (90;147-149). PARP inhibition reduces OS and inducible NOS (iNOS) expression in high glucose-treated human Schwann cells (151) as well as improving thermal hypoalgesia, mechanical hyperalgesia, nerve conductivity and restoring IENF loss in animal models (150;152;153); which suggests an important role for PARP in the development of microvascular complications. Our data showing increased PAR in patients with OSA and T2DM compared to those without OSA suggest that PARP activations is a possible mechanism linking OSA to the development/progression of microvascular complications in patients with T2DM.

The main limitation of our study is its cross-sectional nature, so causation cannot be proven. It would be of much interest to assess whether this increase in oxidative and nitorsative stress and PARP activation results in the development or faster progression of diabetic microvascular complications prospectively. Another limitation to our study is the relatively small sample size which limits the amount of adjustments that can be performed; nonetheless the associations observed remained significant after adjustment for a number of main confounders.

In summary, OSA and nocturnal hypoxemia are associated with increased oxidative and nitrosative stress and PAR activation in patients with T2DM independently of possible confounders. This association might explain the cross-sectional association observed between OSA and/or nocturnal hypoxia and diabetic microvascular complications in patients with T2DM. Further studies assessing this association prospectively and assessing the impact of OSA treatment on oxidative/nitrosative stress and PARP activation are needed.

10. Chapter ten: Ethnic differences in microvascular complications: possible explanations for observed differences

10.1. Introduction

I have reviewed in the introductory chapter the evidence for ethnic differences in the prevalence and progression of microvascular complications. I have also highlighted that the results of published literature were conflicting in regard to diabetic nephropathy and DR; with most, but not all, studies showing South Asians to be at higher risk of having these complications compared to White Europeans. On the other hand, DPN has been consistently reported to be less common in South Asians compare to White Europeans in 3 studies from the same team/centre. There are no data that compared CAN prevalence between South Asians and White Europeans.

The lower prevalence of DPN in South Asians is surprising as diabetes-related complications have predominantly a vascular etiology and South Asian patients are at higher risk of cardiovascular disease compared to White Europeans with T2DM (571;572), hence it would be expected that all microvascular complications to be more common in South Asians.

Possible explanations for the epidemiological differences in diabetes-related microvascular complications have not been explored in the literature. With the exception of one study that attempted to explore the reasons for the lower DPN prevalence in South Asians and implicated differences in height, peripheral vascular disease (PVD) and transcutaneous partial pressure of oxygen (TCpO2) (224). This report, however, did not adjust for a wide range of well established risk factors of DPN including obesity.

I have also shown in the previous chapters, that OSA prevalence differed between South Asians and White Europeans with T2DM and that OSA and nocturnal hypoxemia are associated with diabetes-related microvascular complications. Hence, it is plausible that

ethnic differences in OSA and hypoxemia might explain the relationship between ethnicity and microvascular complications.

Furthermore, I have shown in the last 2 chapters that microvascular regulation, nitrosative stress, oxidative stress and PAR activation are associated with microvascular complications in patients with T2DM. Differences in any of these parameters might contribute to the observed ethnic differences in microvascular complications.

10.2. Hypothesis

1. Ethnic difference in OSA and nocturnal hypoxemia might explain ethnic differences in microvascular complications

Rationale: OSA prevalence is lower in South Asians compared to White Europeans with T2DM. OSA and nocturnal hypoxemia are associated with microvascular complications in patients with T2DM

2. CAN prevalence is different between ethnicities.

Rationale: Ethnic differences in CAN prevalence has not been explored before. As all other microvascular complications are different between ethnicities, we expect CAN to be no exception.

3. DPN prevalence is lower in South Asians compared to White Europeans with T2DM, while the prevalence of DR and diabetic nephropathy are higher in South Asians.

Rationale: Data from published literature suggest this relationship between ethnicity and microvascular complications

10.3. Aims

The aims of this study are:

1. Compare the prevalence of DPN, DR, diabetic nephropathy and CAN between South Asians and White Europeans with T2DM.

2. If ethnic differences are found, to explore the contribution of OSA and nocturnal hypoxemia to the observed ethnic differences

3. Compare microvascular regulation between South Asians and White Europeans with T2DM.

4. Compare nitrosative stress, oxidative stress and PAR activation between South Asians and White Europeans with T2DM.

10.4. Methods

This is a cross-sectional study that utilised the cohort examined in previous chapters for the relationship between OSA and microvascular complications in patients with T2DM.

The methodology of this study is detailed in the previous chapters.

Assessments of microvascular complications, microvascular regulation, oxidative stress, nitrosative stress and PAR activation as detailed previously.

Statistical methods can be found in Chapter 2.

10.5. Results

Two hundred and sixty six patients were recruited. For the primary aim of comparing the prevalence of diabetic microvascular complications, all patients (n=266) were included. For

exploring the impact of OSA on ethnic differences, 234 patients who had available OSA data were included (please see chapter 4 for more details). For details about the patients who had blood samples for oxidative/nitrosative stress markers measurements and microvascualr regulation, please see chapter 11.

South Asians were shorter and younger but had similar duration of known diabetes and similar glycaemic control (HbA1c). South Asians also had lower adiposity measurements, systolic BP, smoking and alcohol intake (Table 1). The prescription of anti-hypertensive and incretin-based treatment was also lower in South Asians (**Table 10.1**). The prevalence of other microvascular complications and past medical history of coronary artery disease was similar between ethnicities, while South Asians had lower prevalence of PVD (**Table 10.1**) which is consistent with previous reports (223).

Table 10-1: Summary of Baseline Characteristics in Relation to Ethnicity.

Data are presented as median (IQR) or mean (SD) depending on data distribution. The percentages represent % of participants in the ethnic group. BP: Blood Pressure, STDR: Sight threatening diabetic retinopathy defined as pre-proliferative or proliferative retinopathy or maculopathy or previous laser treatment. TIA: Transient Ischaemic Attack. PVD: Peripheral Vascular Disease

	South Asians (n=126)	White Europeans (n=140)	P value
Age (years)	54.9 ± 12.5	59.2 ± 10.8	0.003
Male (%)	60.3	56.4	0.521
Smoking (current or ex-smoker)	30.2	50.7	0.001
Alcohol (%)	2.4	47.9	<0.001
Diabetes Duration (years)	11.0 (7.0-18.0)	10.5 (5.0-16.0)	0.153
BMI (kg/m^2)	30.1 (26.5-33.6)	35.3 (31.8-41.4)	< 0.001
Waist circumference (cm)	103.2 (96.9-113.1)	117.2 (109.2-129.4)	< 0.001
Hip circumference (cm)	(102.5 (97-112.25)	120.0 (108.6-131.75)	< 0.001
Waist hip ratio	1.00 ± 0.06	0.99 ± 0.11	0.276

Neck circumference (cm)	39.0 (36.9-41.8)	43.0 (39.6-47.0)	< 0.001
Height (cm)	164.5 ± 8.9	167.1 ± 12.3	0.051
Neck height ratio	0.24 ± 0.02	0.26 ± 0.03	<0.001
Systolic BP (mmHg)	126.0 (115.5-138.5)	130.0 (124.5-140.0)	0.008
Diastolic BP (mmHg)	77.5 (70.0-84.5)	79.0 (73.6-85.9)	0.032
Mean arterial pressure (mmHg)	93.7 (86.6-99.9)	96.7 (91.1-103.3)	0.004
HbA1c (%)	8.0 (7.16-9.20)	8.0 (7.23-9.19)	0.868
Total cholesterol (mmol/L)	3.7 (3.3-4.3)	3.7 (3.3-4.4)	0.637
Triglycerides (mmol/L)	1.8 (1.1-2.4)	1.7 (1.3-2.5)	0.550
HDL (mmol/L)	1.1 (0.9-1.2)	1.1 (0.9-1.3)	0.217
TSH (mU/L)	1.6 (1.0-2.3)	1.9 (1.4-2.3)	0.024
Estimated GFR ($mL/min/1.73m^2$)	90.0 ± 25.5	83.7 ± 27.1	0.053
Oral anti diabetes agent (%)	91.3	92.9	0.632
Insulin (%)	50.0	57.9	0.199
Insulin dose (units)	80.0 (44.0-118)	67.0 (52.0-97.5)	0.649
GLP-1 analogues (%)	4.0	17.1	0.001
Lipid lowering treatment (%)	81	85	0.379
Anti-hypertensive therapy	74.6	84.3	0.05
Anti-platelet agents (%)	65.6	64.5	0.847
Ischemic heart disease (%)	23.0	17.1	0.231
CABG (%)	13.5	10.0	0.375
Stroke/ TIA (%)	9.5	10.7	0.748
PVD (%)	2.4	8.6	0.029

As indicated in previous chapters, OSA prevalence, OSA severity and nocturnal hypoxemia severity were more pronounced in White Europeans compared to South Asians with T2DM (please see chapter 3 for details).

10.5.1. Ethnic differences in microvascular complications prevalence

Ethnic differences in diabetic nephropathy and DR are summarised in **Table 10.2**. South Asians had higher eGFR and higher ACR compared to White Europeans, but there was no difference in the prevalence of diabetic nephropathy between ethnicities. The prevalence of STDR and maculopathy were similar between ethnicities but South Asians had more background (R1) and less pre-proliferative and proliferative DR (R2 or R3) retinopathy than White Europeans.

However, this lack of difference in the prevalence of diabetic nephropathy and DR between ethnicities seems to be due to a gender ethnicity interaction (**Table 10.2**). While in women the prevalence of DR and diabetic nephropathy was similar or higher in South Asians compared to White Europeans; White Europeans men had higher prevalence of DR and diabetic nephropathy compares to South Asian men (**Table 10.2**).

Table 10-2: Summary of ethnic differences in diabetic nephropathy and retinopathy status in patients with T2DM.

eGFR: estimated glomerular filtration; ACR: Albumin creatinine ratio; STDR: sight threatening diabetic retinopathy; SA: South Asian; WE: White European.

	Numbers included in the analysis (SA/WE)	South Asians	White Europeans	P value
Total cohort				
eGFR (mL/min/1.73m^2)	126/140	90.0 ± 25.5	83.7 ± 27.1	0.053
ACR mg/mmol/l	121/122	1.73 (0.57-6.15)	0.93 (0.26-4.15)	0.024
Albuminuria	115/113	39.1%	34.5%	0.470
Diabetic nephropathy	116/116	44.8%	48.3%	0.599
STDR	126/140	36.5%	35%	0.798

Maculopathy		126/140	32.5%	30.7%	0.749
Retinopathy status	R0	126/140	33.3%	42.1%	0.087
	R1		53.2%	37.9%	
	R2		7.9%	10.7%	
	R3		5.6%	9.3%	
Women					
Albuminuria		41/41	29.3%	22.0%	0.448
Diabetic nephropathy		41/41	36.6%	43.9%	0.499
STDR		44/53	45.5%	32.1%	0.177
Maculopathy		44/53	40.9%	28.3%	0.192
Retinopathy status	R0	44/53	38.6%	45.3%	0.752
	R1		47.7%	41.5%	
	R2		9.1%	5.7%	
	R3		4.5%	7.5%	
Men					
Albuminuria		58/59	44.8%	42.4%	0.789
Diabetic nephropathy		58/61	48.3%	52.5%	0.648
STDR		60/69	28.3%	44.9%	0.052
Maculopathy		60/69	25.0%	39.1%	0.088
Retinopathy status	R0	60/69	30.0%	36.2%	0.031
	R1		58.3%	34.8%	
	R2		6.7%	15.9%	
	R3		5.0%	13.0%	

DPN was more common in White Europeans (n=129) compared to South Asians (n=105) (55.0% vs. 39.0%, P=0.015). Foot insensitivity as assessed by abnormal monofilament perception was also more common in White Europeans (46.9% vs. 25.7%, P=0.001) (**Table**

10.3). White Europeans had more abnormalities on all aspects of neuropathy examination (**Table 10.3**) and consistent with our findings with the monofilament, reported more open sores on the foot (12.4% vs. 26.4%, P=0.008). Analysis of patient symptom scores demonstrated that symptoms consistent with sensory deficit were not different between ethnic groups whereas pain/discomfort symptoms related to were non-significantly more common in South Asians. There was no gender effect on the relationship between ethnicity and abnormal 10g monofilament perception. In DPN, however, the gender effect was similar to that observed in DR and diabetic nephropathy in that there was no difference in DPN prevalence between South Asian and White European women (40.9% vs. 47.3%, p=0.52) while the prevalence of DPN was significantly higher in White European men compared to South Asians (60.8% vs. 37.7%, p=0.008).

Table 10-3: Ethnic Differences in Components of the MNSIe and Monofilament Perception.

Data are presented as % of abnormal test/response in the particular ethnic groups. MNSIe: the examination component of MNSI. P < 0.01 was considered significant following the Bonferroni correction. Statistical analysis in this table represents univariate analysis with no adjustments.

	South Asian (n=105)	White Europeans (n=129)	*P* values
Inspection	50.5	63.3	0.049
Ulcers	1.9	4.7	0.25
Ankle reflexes	37.1	57.0	0.003
Vibration	34.3	57.0	0.001
10g monofilament	25.7	46.9	0.001

The prevalence of CAN was not different between the ethnic groups (40.8% vs. 43.3%, p=0.724 for South Asians vs. White Europeans respectively). Spectral analysis and frequency

domain parameters including 30:15 ratio, Baseline Lfa, Baseline Rfa, Deep breathing Lfa, Standing Lfa and Standing LF were more preserved (higher) in South Asians, but all these differences were abolished after adjustment for age (data not shown).

10.5.2. Possible explanations for ethnic differences

We only found an impact of ethnicity on DPN and foot insensitivity prevalence, so we have focused here on exploring the underlying causes for the ethnic differences observed in DPN and foot insensitivity prevalence.

In order to determine whether ethnicity was an independent predictor of DPN, logistic regression models were used (**Table 10.4**). The association between ethnicity and DPN remained significant despite adjusting for a wide range of known DPN risk factors and possible confounders including: age, gender, alcohol intake, smoking, mean arterial pressure (MAP), diabetes duration, HbA1c, lipids, eGFR, glucose lowering therapies, anti-hypertensive and, anti-platelet agents, lipid lowering therapy, height and history of PVD (**Table 10.4**). This association, however, was abolished after adding adiposity, OSA or hypoxemia measures to the models (**Table 10.4**), suggesting that ethnic-differences in DPN prevalence can be mainly explained by the differences in adiposity and OSA between the ethnic groups. Even in models that adjusted for one possible confounder (adiposity or OSA related), the relationship between ethnicity and DPN was abolished.

Using foot insensitivity (10g monofilament) as the outcome measure in the regression models (as in **Table 10.4**) showed similar results in that ethnicity remained and independent predictor of foot insensitivity after adjustment and the addition of adiposity measure (BMI, waist circumference, neck circumference) abolished this relationship between ethnicity and foot insensitivity. Unlike DPN, OSA and hypoxia measures did not abolish the relationship

between ethnicity and foot insensitivity, despite that OSA and hypoxemia measures remained independent predictors of foot insensitivity.

Table 10-4: Assessing the Impact of Possible Confounders on the Association Between Ethnicity and DPN (based on MNSI) using Logistic Regression Models with Increasing Complexity.

The odds ratios reported are the odds for having DPN in White Europeans to South Asians. MAP: mean arterial pressure, eGFR: Estimated Glomerular Filtration Rate, PVD: Peripheral Vascular Disease, BMI: Body Mass Index.

Model	Nagelkerke R^2	Odds ratio	95% confidence interval	*P* value
Unadjusted: Ethnicity	0.034	1.911	1.132-3.225	0.015
Model 1*	0.149	1.926	1.104-3.357	0.021
Model 2*	0.206	1.080	0.492-2.371	0.85
Model 3*	0.216	1.071	0.492-2.331	0.86
Model 4*	0.200	1.523	0.802-2.892	0.20
Model 5*	0.216	1.093	0.481-2.481	0.83
Model 6*	0.215	1.313	0.632-2.728	0.47
Model 7*	0.219	1.290	0.615-2.704	0.50
Model 8*	0.209	1.286	0.597-2.769	0.52
Model 9*	0.204	1.228	0.571-2.642	0.600
Model 10*	0.239	0.944	0.409-2.187	0.89
Ethnicity + BMI**	0.047	1.593	0.900-2.819	0.11
Ethnicity + waist circumference**	0.066	1.408	0.788-2.515	0.25
Ethnicity + waist/hip ratio**	0.062	2.006	1.178-3.417	0.01
Ethnicity + Neck circumference**	0.070	1.382	0.773-2.472	0.28
Ethnicity + OSA**	0.141	1.466	0.839-2.560	0.18
Ethnicity + AHI quartiles**	0.139	1.410	0.800-2.484	0.24
Ethnicity + nadir nocturnal oxygen saturations**	0.096	1.581	0.918-2.722	0.10
Ethnicity + time spent with saturations < 80%**	0.089	1.625	0.950-2.779	0.08

Model 1: Ethnicity + age + gender + alcohol intake + smoking + MAP + diabetes duration + HbA1c + total cholesterol + triglycerides + eGFR + insulin + glucose lowering treatments[$]+ anti-hypertensives[£]+ anti-platelets[@]+ lipid lowering therapy^ + PVD + Height

Model 2: Model 1 + BMI

Model 3: Model 1 + waist circumference

Model 4: Model 1 + waist hip ratio

Model 5: Model 1 + neck circumference

Model 6: Model 1 + OSA

Model 7: Model 1 + AHI quartiles (Q1 <2.90, Q2 2.90-7.59, Q3 7.60-16.09, Q4 ≥ 16.10 events/hour)

Model 8: Model 1 + nadir nocturnal Oxygen saturations

Model 9: Model 1 + Time spent with Oxygen saturations < 80%

Model 10: Model 1 + BMI + OSA

[$]Adjustment for glucose lowering treatments included adjustment for metformin, sulphonylurea, glitazones and incretin-based therapy individually.

[£]Adjustment for anti-hypertinsives included ACE inhibitors, angiotenisn 2 blockers, beta blockers, alpha blockers, calcium antagonists and diuretics individually.

[@]Anti-platelets included aspirin and clopidogrel combined

^Adjustment for lipid lowering therapy included statins, ezetimibe and fibrates combined.

*Logistic regression the backward method was used

**Logistic regression the enter method was used

10.5.3. The impact of OSA on ethnic differences in microvascular complications prevalence

In previous chapters we have shown that OSA is associated with microvascular complications in patients with T2DM. In last paragraph we have also shown that OSA explained ethnic differences in DPN prevalence. Hence, we wanted to assess whether ethnic differences in microvascular complications differ by OSA status (**Table 10.5**). Interestingly, the prevalence of STDR and DPN were non-significantly higher in South Asians compared to White Europeans in patients without OSA. In patients with OSA, however, STDR prevalence was equal between ethnicities and DPN prevalence was higher in White Europeans.

Table 10-5: Summary of ethnic differences in diabetic nephropathy and retinopathy status in patients with T2DM.

STDR: sight threatening diabetic retinopathy; SA: South Asian; WE: White European.

	Numbers included in the analysis (SA/WE)	South Asians	White Europeans	P value
In patients without OSA				
Diabetic nephropathy	49/25	28.6%	32.0%	0.760
STDR	51/32	23.5%	15.6%	0.385
DPN	51/32	29.4%	21.9%	0.449
In patients with OSA				
Diabetic nephropathy	50/77	58%	54.5%	0.702
STDR	53/90	47.2%	47.8%	0.944
DPN	54/97	48.1%	66.0%	0.032

10.5.4. Ethnicity and microvascular regulation

For characteristics of patients who had the microvascular function examined in comparison to the rest of the cohort, please refer to the previous chapter. The characteristics of South Asians and White Europeans who had microvascular regulation are summarised in **Table 10.6**. South Asians had similar diabetes duration, HbA1c and lipid profile to White Europeans but South Asians were younger and had lower adiposity measures (**Table 10.6**).

Table 10-6: Summary of patients characteristics who had microvascular function assessment in relation to Ethnicity.

Data are presented as median (IQR) or mean (SD) depending on data distribution. The percentages represent % of participants in the ethnic group. BP: Blood Pressure. TIA: Transient Ischaemic Attack. PVD: Peripheral Vascular Disease

	South Asians (n=34)	White Europeans (n=44)	P value
Age (years)	55.20 ± 10.71	62.50 ± 9.96	0.003
Male (%)	55.9%	56.8%	0.934
Smoking (current or ex-smoker)	23.5%	54.5%	0.006
Alcohol (%)	0%	50%	< 0.001
Diabetes Duration (years)	12.94 ± 7.91	13.61 ± 7.55	0.703
BMI (kg/m^2)	31.60 ± 5.09	38.47 ± 11.73	0.001
Waist circumference (cm)	106.05 ± 11.99	119.47 ± 16.19	< 0.001
Hip circumference (cm)	108.36 ± 10.67	122.11 ± 21.11	0.001
Waist hip ratio	0.98 ± 0.07	0.99 ± 0.08	0.693
Neck circumference (cm)	39.09 ± 4.12	42.63 ± 3.85	< 0.001
Systolic BP (mmHg)	127.40 ± 18.39	132.57 ± 17.77	0.213
Diastolic BP (mmHg)	75.85 ± 9.39	78.66 ± 8.95	0.183
HbA1c (%)	8.08 ± 1.62	7.80 ± 1.22	0.414
Total cholesterol (mmol/L)	4.0 ± 1.27	3.79 ± 0.88	0.393
Triglycerides (mmol/L)	1.91 ± 1.41	1.87 ± 0.92	0.894
HDL (mmol/L)	1.10 ± 0.31	1.21 ± 0.35	0.136
Estimated GFR ($mL/min/1.73m^2$)	86.27 ± 23.93	78.80 ± 24.83	0.183
Oral anti diabetes agent (%)	91.2%	93.2%	1.0
Insulin (%)	44.1%	54.5%	0.361
Insulin dose (units)	69.0 ± 37.45	78.08 ± 43.41	0.508
Lipid lowering treatment (%)	82.4%	93.2%	0.138
Anti-hypertensive therapy	79.4%	90.9%	0.148

Anti-platelet agents (%)	70.6%	70.5%	0.990
Ischemic heart disease (%)	17.6%	25%	0.435
CABG (%)	14.7%	9.1%	0.441
Stroke/ TIA (%)	2.9%	15.9%	0.128
PVD (%)	2.9%	9.1%	0.380

There were significant differences in microvascular function between South Asians and White Europeans (**Table 10.7**). White Europeans had higher flux following heating (maximum vasodilatation) but lower Acetylcholine response when taken as a proportion of maximum vasodilatation (**Table 10.7**). However, as there were significant ethnic differences in OSA prevalence and severity we needed to explore whether the observed ethnic differences in microvascular function are related OSA status. Analysing ethnic differences by microvascular function by OSA status showed some interesting results (**Tables 10.8 and 10.9**). In patients without OSA, South Asians had lower baseline, heating-induced, and endothelial dependent and independent flux compared to White Europeans; while in patients with OSA there were no differences in microvascular function between ethnicities except higher heating-induced flux in White Europeans.

Table 10-7: Assessment of microvascular blood flow and endothelial function in South Asians and White Europeans with type 2 diabetes.

Data presented as median (IQR) or ratios. Blood flux was measured in arbitrary perfusion units (APU). Conductance is the measure of dividing flux by the mean arterial pressure.

	South Asians (n=34)	White Europeans (n=44)	P value-unadjusted
Flux			
Baseline	24.55 (17.40-36.63)	26.85 (16.53-36.93)	0.832
Heating	140.05 (106.70-167.58)	174.00 (141.78-205.88)	0.007
Ach	111.00 (82.63-132.95)	107.20 (76.63-142.93)	0.832
SNP	96.60 (66.85-138.55)	125.70 (77.48-169.38)	0.174
Conductance			
Baseline	0.27 (0.19-0.42)	0.28 (0.18-0.38)	0.739
Heating	1.54 (1.20-1.88)	1.77 (1.50-2.17)	0.029
Ach	1.25 (0.80-1.36)	1.07 (0.79-1.57)	0.778
SNP	1.07 (0.76-1.50)	1.25 (0.77-1.80)	0.314
Flux in relation to maximum vasodilatation			
Baseline	0.19 (0.13-0.26)	0.16 (0.10-0.22)	0.103
Ach	0.78 (0.63-0.91)	0.63 (0.51-0.81)	0.036
SNP	0.81 (0.47-1.00)	0.77 (0.52-0.94)	0.614

Table 10-8: Assessment of microvascular blood flow and endothelial function in South Asians and White Europeans with type 2 diabetes but without OSA.

Data presented as median (IQR) or ratios. Blood flux was measured in arbitrary perfusion units (APU). Conductance is the measure of dividing flux by the mean arterial pressure.

	South Asians (n=16)	White Europeans (n=8)	P value-unadjusted
Flux			
Baseline	30.35 (22.10-40.23)	39.80 (32.48-72.50)	0.045
Heating	144.75 (101.75-181.53)	193.75 (174.73-246.08)	0.011
Ach	114.30 (84.83-166.78)	171.0 (116.30-205.05)	0.093
SNP	134.35 (81.88-176.50)	200.60 (138.58-278.23)	0.023
Conductance			
Baseline	0.33 (0.24-0.45)	0.42 (0.33-0.85)	0.120
Heating	1.63 (1.27-1.95)	1.99 (1.84-2.84)	0.013
Ach	1.29 (0.86-1.81)	1.68 (1.28-2.18)	0.136
SNP	1.47 (0.94-2.03)	2.02 (1.59-2.94)	0.032
Flux in relation to maximum vasodilatation			
Baseline	0.22 (0.16-0.28)	0.22 (0.18-0.29)	0.881
Ach	0.82 (0.68-0.91)	0.76 (0.65-0.89)	0.569
SNP	0.91 (0.77-1.25)	0.93 (0.80-1.12)	1.0

Table 10-9: Assessment of microvascular blood flow and endothelial function in South Asians and White Europeans with type 2 diabetes but with OSA.

Data presented as median (IQR) or ratios. Blood flux was measured in arbitrary perfusion units (APU). Conductance is the measure of dividing flux by the mean arterial pressure.

	South Asians (n=18)	White Europeans (n=36)	P value-unadjusted
Flux			
Baseline	20.55 (16.30-29.70)	24.00 (15.70-33.28)	0.588
Heating	139.65 (106.70-162.13)	162.80 (132.25-200.75)	0.042
Ach	104.65 (74.60-117.65)	95.90 (70.48-127.68)	0.956
SNP	76.40 (61.65-117.95)	114.80 (70.53-151.43)	0.075
Conductance			
Baseline	0.22 (0.17-0.32)	0.25 (0.17-0.36)	0.869
Heating	1.49 (1.12-1.82)	1.69 (1.32-2.09)	0.132
Ach	1.14 (0.74-1.30)	0.97 (0.76-1.31)	0.620
SNP	0.84 (0.64-1.30)	1.21 (0.68-1.52)	0.147
Flux in relation to maximum vasodilatation			
Baseline	0.15 (0.12-0.25)	0.15 (0.09-0.19)	0.279
Ach	0.67 (0.62-0.89)	0.61 (0.42-0.76)	0.095
SNP	0.58 (0.40-0.90)	0.67 (0.48-0.90)	0.582

10.5.5. Ethnicity and nitrosative stress and oxidative stress

Patients who consented to give blood samples were similar to the rest of the study cohort (please see previous chapter). The characteristics of South Asians and White Europeans who consented for blood sampling were similar to those reported in **Table 12.1.** There were no differences in serum nitrotyrosine levels (21.54 (12.74-29.47) vs. 21.08 (14.82-37.80), p=0.616) or plasma lipid peroxide levels (13.42 (1.83-30.80) vs. 16.06 (3.45-30.29), p=0.646) between South Asians (n=39) and White Europeans (n=67) with T2DM. Analysing the results by OSA status still showed no significant differences between ethnicities.

10.6. Discussion

Our study demonstrates for the first time that lower adiposity, less OSA and nocturnal hypoxemia are potential explanatory factors for the lower DPN prevalence in South Asians (compared to White Europeans) patients with T2DM. We also described that differences in microvascular regulation might contribute to the ethnic differences in DPN.

Contrary to some of the published literature, the prevalence of STDR or diabetic nephropathy was not higher in South Asians (please see chapter 1 for more details). This is despite that White Europeans were older and more obese than South Asians, which suggests that South Asians are at increased risk of these complications at younger age and lower adiposity. However, there was an impact of gender on the relationship between ethnicity and STDR, with White European men having higher prevalence of STDR, DR and Maculopathy compared to South Asian men; while the opposite occurred in women. The explanation for such gender impact is not clear. One explanation, however, is that OSA is more common and more severe in White European men compared to South Asian men, while there is no difference in OSA between White European women and South Asian women (as shown in chapter 3). As we have shown that OSA is associated with microvascular complications in the previous chapters, it is plausible that OSA gender/ethnicity differences might contribute to the gender ethnicity interaction that we have observed.

Our data demonstrating a lower prevalence of DPN and foot ulceration in South Asian subjects are consistent with other reports (201;223;224). Interestingly, however, the overall prevalence of neuropathic symptoms ascribable to DPN was comparable across ethnicities with similar percentages reporting symptoms consistent with a sensory loss but (non-

significantly) more South Asian patients reporting neuropathic pain (pin prick sensation). It is known, however, that South Asians have a higher prevalence of vitamin D deficiency (573), which might contribute to differences in some symptoms, particularly generalized weakness.

Our results show that obesity, OSA and hypoxemia removed the relationship between ethnicity and DPN. These findings support out finding in a previous chapter showing an independent association between OSA and hypoxemia and DPN. The mechanisms that underpin such a relationship have been detailed in chapter 1.

Maximal skin blood flow of the lower limb on heating was reduced but endothelial function preserved in South Asian compared to White Europeans with T2DM. In contrast there was no significant differences in the response to SNP. Heating to 44C leads to maximal vasodilatation which corresponds the maximal vasodilatory capacity (502). This response to heating is largely mediated by nitric oxide (NO) (502). This suggests that the difference in the heating response between the two ethnicities is mainly related to differences in NO secretion/action. Deficits in NO and heating responses have been associated with increased prevalence of cardiovascular disease (574) and so our findings might have relevance for the higher cardiovascular disease risk observed in South Asians with T2DM (571).

In contrast to the heating response, the response to Ach (when measured as a ratio of maximal vasodilatation) was greater in South Asians, suggesting relative preservation of endothelial dependent vasodilatation. The response to Ach is thought to reflect both an axon reflex as well as prostaglandin-mediated component (502) which might therefore be of relevance to the lower prevalence of DPN and foot complications in these subjects. In patients ***without*** diabetes the heating response has been reported to be not different (574) while the SNP response is lower (575) in South

Asians compared to White Europeans when measured on the forearm. Our data are also consistent with the finding of relative preservation of TCpO2 in South Asian subjects with diabetes (224).

However, when stratified by OSA status, South Asian had worse baseline and heating-induced flux and endothelial dependent and independent vasodilatation; which is consistent with the higher CVD risk observed in South Asians at younger age and lower adiposity. On the other hand, in patients with OSA there were hardly any differences in microvascular regulation/function between ethnicities, suggesting the OSA might confound and remove ethnic differences in microvascular function. This is could be because the severity of OSA was worse in White Europeans compared to South Asians (as discussed in Chapter 3). This OSA impact on the ethnic differences in microvascular regulation is further supported by the fact that DPN and DR prevalence was non-significantly higher in South Asians compared to White Europeans in patients without OSA; while the DPN prevalence was significantly higher in White Europeans and DR prevalence was not different between ethnicities in patients with OSA.

It is important in our study to ascertain that our clinic population is representative and that the South Asians and White Europeans in our study are comparable. We believe that our study population is representative to that of the general South Asians and White Europeans with T2DM for several reasons. Both ethnicities (in our study) live in the same compact geographical area; they have similar standards of living and have similar deprivation scores. The referral guidelines, which are agreed between our diabetes specialists and the primary care trust, are the same for both ethnicities and the same referral criteria apply to both ethnicities, hence we do not believe that there are any referral differences between South Asians and White Europeans. We have also explored the issue of non- attendance in our diabetes clinic and found no ethnic differences in the “did not attend” rate. This is probably reflected in that the proportion of South Asians in our study (45%) is close to their

prevalence in the clinic (40%). In addition, we approached similar numbers of South Asians and White Europeans to be recruited to the study and the response rate was similar in the two ethnic groups (approximately 65%, excluding those who were not eligible to enter the study). Further evidence that our sample is representative comes from the similar characteristics (such as age, BMI, height, HbA1c, history of cardiovascular disease etc) in the South Asians and White Europeans in our study to those in another report from a different region in the UK (224).

The main limitation of our study is the cross-sectional nature of the study, and hence causation cannot be proven. Another limitation is the differences in baseline parameters between the ethnicity groups, which might have affected the associations observed in our study. However the principal findings of our study remained highly significant after adjusting for a wide range of possible confounders. Furthermore, matching the ethnicities for such variables (particularly obesity) might reduce the external validity of our findings as such "matched" population may not be representative of "real life" in which these two ethnicities are very different. Additionally there is the possibility of self-selection bias (ie patients with microvascular complications preferentially agreeing to participate) which cannot be entirely excluded. However, patients attending a general diabetes clinic were approached about willingness to enrol in the study and we are unaware of an ethnicity-mediated self-selection bias which would affect our conclusions. This is further supported by the similar prevalence of diabetic retinopathy between the two ethnicities. The MNSI is not the "gold standard" for diagnosing DPN but it has been validated against nerve conduction studies (506;513) and has been used widely in land mark studies(56;468;469;473;576). 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.

In summary, clinical signs consistent with DPN are reduced in South Asian compared to White Europeans patients with T2DM receiving hospital-based diabetes care. South Asians had similar prevalence of DR and diabetic nephropathy to White Europeans despite that White Europeans were older and more obese. Differences in adiposity, OSA and nocturnal hypoxemia appear to explain the ethnic differences in DPN prevalence, which suggest a role for these factors in the pathogenesis of DPN. Preserved endothelial dependent vasodilatation in South Asians might contribute to the lower DPN prevalence observed in this ethnic group. In patients without OSA, microvascular function was impaired in South Asians compared to White Europeans, while in OSA patients there were little differences between ethnicities. OSA seems to play a major role in determining the relationship between ethnicity and microvascular complications and microvascular function in patients with T2DM. Prospective and interventional studies are needed.

11. Summary and Future Directions

11.1. Summary of findings

Type 2 diabetes is very common and results in significant morbidity and mortality. Microvascular complications contribute significantly to the morbidity and mortality of the disease. Despite intensive glycaemic and metabolic control, diabetes microvascular complications remain very common. Hence, better understanding of the pathogenesis of these complications is needed in order to develop effective treatments that slow the progression or prevent the development of these complications.

In this thesis, I examined the relationship between microvascular complications and obstructive sleep apnoea and ethnicity. In addition, I also examined the potential pathogenetic pathways which could be responsible for these relationships.

I have shown that OSA is very common in patients with type 2 diabetes and I have reported for the first time a lower prevalence of OSA in South Asians with type 2 diabetes compared to White Europeans, with White European men being the highest risk group. This ethnic difference in OSA was accountable for by “traditional” OSA risk factors such as age and adiposity. The Berlin questionnaire and Epworth Sleepiness Score, which are commonly used to screen for OSA in the general public, showed poor sensitivity and specificity in my study cohort, suggesting that appropriate methods to screen for OSA in patients with type 2 diabetes need to be developed.

I have also shown a novel association between OSA and the microvascular complications of diabetes including diabetic peripheral neuropathy, cardiac autonomic neuropathy, diabetic retinopathy, and diabetic nephropathy. OSA was also associated with lower intra epidermal nerve fibre density in skin biopsies and macular oedema based on ocular computer tomography.

In addition I have shown that OSA is associated with increase oxidative stress, increased nitrosative stress, poly (ADP-ribose) polymerase activation and impaired microvascular regulation in patients with type 2 diabetes. These associations might explain the epidemiological relationship between OSA and microvascular complications in patients with Type 2 diabetes. Theses associations have not been described before in these patients.

I have also shown that there are ethnic differences in the prevalence of microvascular complications between South Asians and White Europeans and that there are ethnic gender interactions in the ethnic differences observed.

There are several limitations to this work. Most importantly, due to the cross-sectional nature of the study causation cannot be proven. In addition, patients with and without OSA differed in many complications-related risk factors, particularly obesity, which might account for the differences observed between patients with and without OSA. Nonetheless, the association between OSA and microvascular complications remained significant despite adjustment for a wide range of possible confounders, particularly age and obesity.

The work described in thesis raises several important questions and highlights the need for future projects.

11.2. Future observational epidemiological studies

In the first instance, the relationship between OSA and microvascular complications needs to be confirmed by conducting prospective observational studies to show whether obstructive sleep apnoea results in the development or progression of microvascular complications. Such studies should also be conducted in patients with type 1 diabetes who are becoming increasingly obese and hence at increased risk of obstructive sleep apnoea. These epidemiological studies need to take into account important factors such the impact of gender and ethnicity. Studies also need to take into

account that the impact of obstructive sleep apnoea on microvascular complications might be different according to diabetes duration.

The methods used to assess diabetic peripheral neuropathy in this work were mainly clinically based (which mostly reflects large fibre function) although in some patients intra epidermal nerve fibre density (which reflects small fibre function) was also assessed. The relationship between obstructive sleep apnoea and diabetic peripheral neuropathy needs to be explored using a wider variety of methods such as nerve conduction velocities (large fibre), quantitative sensory testing (both large and small fibre) and sudomotor function (small fibre). Examining the relationship between obstructive sleep apnoea and painful diabetic neuropathy, and the development and healing of diabetic foot ulceration is also of great interest.

The diagnosis of cardiac autonomic neuropathy needs to be explored using different modalities, particularly overnight heart rate variability as this may yield different results to the current study which assessed heart rate variability during the day.

Future studies examining the relationship between obstructive sleep apnoea and diabetic retinopathy also need to use more in-depth methods. Ocular coherence tomography should be used to assess maculopathy and assess the distribution of oedema (central vs. peripheral). More sophisticated retinal images using more fields than used in my study (such as 7 fields) and using more detailed scoring protocols will be needed.

Studies examining the relationship between obstructive sleep apnoea and diabetic nephropathy need to include patients of all disease severity, unlike our study that excluded patients with advanced disease.

11.3. Future mechanistic studies

The pathogenic mechanisms that might contribute to the relationship between obstructive sleep apnoea and microvascular complications require further exploration. Potential mechanisms include

assessing advanced glycation end-products, PKC, the anti oxidant defence systems, vascular endothelial growth factor and inflammatory markers amongst others. As we already have serum and plasma samples stored, we will be able to explore some of these possible mechanisms.

Further mechanistic work should also include examining the changes occurring at tissue level. This might be easier to be performed using animal models of obstructive sleep apnoea/intermittent hypoxia.

11.4. Future Interventional studies

The impact of obstructive sleep apnoea treatments, such as mandibular advancement devices and continuous positive airway pressure ventilation on the development, progression and regression of microvascular complications need to be examined in prospective randomised controlled trials. These trials need to assess the impact of treatment in primary and secondary prevention cohorts (i.e. in patients without and with the complication of interest) and need to take into account the impact of ethnicity.

In the trial to assess the impact of obstructive sleep apnoea on diabetic neuropathy, the best primary outcome measure would be intra epidermal nerve fibre density and the trial should include patients with less than severe neuropathy (i.e. detectable sural nerve potentials). In a retinopathy trial, macular thickness, as measured by ocular coherence tomography, visual acuity, and the need for Laser treatment are appropriate outcomes. In a nephropathy study, changes in eGFR would be an appropriate outcome. Heart rate variability and respiratory adjusted spectral analysis are appropriate in the cardiac autonomic neuropathy trial

11.5. Other future studies

Obstructive sleep apnoea is known to be associated with hypertension, cardiovascular disease and mortality in the general public, but such data are lacking in patients with type 2 diabetes. Hence,

longitudinal studies are also needed to assess the relationship between obstructive sleep apnoea and mortality and cardiovascular disease in these patients. Furthermore, studies assessing the relationship between obstructive sleep apnoea and the impact of its treatment on hypertension (particularly resistant hypertension) in patients with type 2 diabetes would be important.

Obstructive sleep apnoea is very common in patients with type 2 diabetes. However my data also showed that current screening methods are inadequate; further studies are therefore needed to identify and test appropriate screening tools.

The issue of obstructive sleep apnoea and glycaemic control is of interest, although I have not focused much on this aspect in my work. The literature is limited and provides conflicting results but most of the studies lack good design. Well designed randomised controlled trials of appropriate duration and including the right target population are needed to solve this issue. In addition, not only is the impact of obstructive sleep apnoea on glycaemic control is important, but its impact on other glycaemic measures such as post prandial glycaemia, glucose variability and even hypoglycaemia are also important to study.

Another important aspect that needs to be examined is the natural history of obstructive sleep apnoea in patients with type 2 diabetes. We know that obstructive sleep apnoea is very common in patients with diabetes but we do not know whether it gets better, worsens or stays the same over time.

I have already started to plan address some of these issues. I am currently in the process of submitting a grant to assess the impact of a mandibular advancement device in patients with mild obstructive sleep apnoea on glycaemic control in patients with type 2 diabetes. As part of this study I will also assess the impact of these devices on hypertension, microvascular regulation and oxidative stress. I am also currently in the process of re inviting the patients who contributed to the current

study to be re evaluated as this might give insights into the impact of obstructive sleep apnoea on the progression of microvascular complications.

In addition, I intend to apply for an NIHR Clinician Scientist Fellowship in order to conduct a prospective study assessing the natural history of obstructive sleep apnoea in type 2 diabetes and its impact on the natural history of diabetic peripheral neuropathy. Imbedded within this study will be two randomised controlled studies to assess the impact of a mandibular advancement device (for mild obstructive sleep apnoea) and continuous positive airway pressure ventilation (for moderate to severe apnoea) on patients with mild diabetic peripheral neuropathy. The impact on other microvascular complications will be assessed as secondary outcome in this study.

I have also set up collaboration with Professor Naresh Punjabi, the principal investigator of the Sleep Heart Health Study, to utilise his longitudinal study to assess whether having type 2 diabetes and obstructive sleep apnoea increases mortality and cardiovascular disease.

In addition, I have set up collaboration with Professor Martin Stevens and leading basic scientists at the University of Birmingham to assess the impact of intermittent hypoxia on neuropathy in rodent models which should help us further our understanding of the underlying mechanisms linking obstructive sleep apnoea to diabetes-related complications and might also provide us with early evidence regarding whether reversing hypoxia has any impact on peripheral neuropathy.

I believe that the field of obstructive sleep apnoea in patients with type 2 diabetes is still in its infancy and a lot of work still needs to be done in order to understand the interactions between those two very common conditions. The results of the current work in concert with my proposed future research could have significant implications for the care of patients and help to reduce the impact of the devastating chronic complications of diabetes.

Appendix: Publications

Here I include a list of selected publications that are relevant to this thesis

1. Tahrani AA, Zeng W, Shakher J, Piya MK, Hughes S, Dubb K, Stevens MJ. Cutaneous Structural and Biochemical Correlates of Foot Complications in High-Risk Diabetes. Diabetes Care. In press (This is relevant to the methods chapter)
2. Tahrani AA, Ali A, Raymond NT, Begum S, Dubb K, Mughal S, Jose B, Piya M, Barnett AH, Stevens MJ. Obstructive Sleep Apnea and Diabetic Neuropathy: a Novel Association in Patients with Type 2 Diabetes. Am J Respir Crit Care Med. 2012 Jun [Epub ahead of print] PubMed PMID: 22723291. (This paper include the results from chapters 3,4,8 and 9)
3. Kahal H, Tahrani AA, George JT, Barlow IM, Malik MA. Obstructive sleep apnoea; a rare cause of pseudophaeochromocytoma. QJM. 2011 Nov 10. [Epub ahead of print] PubMed PMID: 22075007. (This is relevant to chapter 1)
4. Zeng W, Tahrani A, Shakher J, Varani J, Hughes S, Dubb K, Stevens MJ. Effects of a synthetic retinoid on skin structure, matrix metalloproteinases, and procollagen in healthy and high-risk subjects with diabetes. J Diabetes Complications. 2011 Nov-Dec;25(6):398-404. Epub 2011 Nov 4. PubMed PMID: 22055260; PubMed Central PMCID: PMC3240843. (This is relevant to the method chapters)
5. Kempler P, Amarenco G, Freeman R, Frontoni S, Horowitz M, Stevens M, Low P, Pop-Busui R, Tahrani A, Tesfaye S, Várkonyi T, Ziegler D, Valensi P; on behalf of the Toronto Consensus Panel on Diabetic Neuropathy*. Gastrointestinal autonomic neuropathy, erectile-, bladder- and sudomotor dysfunction in patients with diabetes mellitus: clinical impact, assessment, diagnosis, and management. Diabetes Metab Res Rev. 2011 Jul 11. doi: 10.1002/dmrr.1223. [Epub ahead of print] PubMed PMID: 21748841. (This is relevant to chapters 1 and 2)

6. Tahrani AA, Bailey CJ, Del Prato S, Barnett AH. Management of type 2 diabetes: new and future developments in treatment. Lancet. 2011 Jul 9;378(9786):182-97. Epub 2011 Jun 24. Review. PubMed PMID: 21705062. (This is relevant to chapter 1)
7. Piya MK, Shivu GN, Tahrani A, Dubb K, Abozguia K, Phan TT, Narendran P, Pop-Busui R, Frenneaux M, Stevens MJ. Abnormal left ventricular torsion and cardiac autonomic dysfunction in subjects with type 1 diabetes mellitus. Metabolism. 2011 Aug;60(8):1115-21. Epub 2011 Feb 18. PubMed PMID: 21306747; PubMed Central PMCID: PMC3142285. (This is relevant to the methods chapter)
8. Tahrani AA, Askwith T, Stevens MJ. Emerging drugs for diabetic neuropathy. Expert Opin Emerg Drugs. 2010 Dec;15(4):661-83. Epub 2010 Aug 27. Review. PubMed PMID: 20795891. (This is relevant to chapter 1)
9. DeVille-Almond J, Tahrani AA, Grant J, Gray M, Thomas GN, Taheri S. Awareness of obesity and diabetes: a survey of a subset of British male drivers. Am J Mens Health. 2011 Jan;5(1):30-7. Epub 2010 Apr 21. PubMed PMID: 20413385. (This is relevant to chapter 1)
10. Tahrani AA, Ball A, Shepherd L, Rahim A, Jones AF, Bates A. The prevalence of vitamin D abnormalities in South Asians with type 2 diabetes mellitus in the UK. Int J Clin Pract. 2010 Feb;64(3):351-5. Epub 2009 Oct 27. PubMed PMID: 19863680. (This is relevant to chapters 1 and 10).

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