Can corneal confocal microscopy be used as an imaging biomarker for neurodegeneration in children and adults with metabolic disease?

H GAD

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Can corneal Confocal Microscopy be used as an imaging biomarker for neurodegeneration in children and adults with metabolic disease?

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Directly related to the thesis

1. **Gad H**, Elgassim E, Mohammed I, Alhaddad AY, Aly HAHZ, Cabibihan JJ, Al-Ali A, Sadasivuni KK, Haji A, Lamine N, Khan A, Petropoulos IN, Ponirakis G, Kalteniece A, Ferdousi M, Azmi S, Alam U, Abuhelaiqa W, Jayyousi A, AlMohanadi D, Baagar K, Malik RA. Continuous glucose monitoring reveals a novel association between duration and severity of hypoglycemia and small nerve fiber injury in patients with diabetes. Endocr Connect. 2022 Nov 14;11(12):e220352.
2. **Gad H**, Al-Jarrah B, Saraswathi S, Mohamed S, Kalteniece A, Petropoulos IN, Khan A, Ponirakis G, Singh P, Khodor SA, Elawad M, Almasri W, Hendaus MA, Akobeng AK, Hussain K, Malik RA. Corneal confocal microscopy identifies a reduction in corneal keratocyte density and sub-basal nerves in children with type 1 diabetes mellitus. Br J Ophthalmol. 2022 Oct;106(10):1368-1372.
3. **Gad H**, Petropoulos IN, Khan A, Ponirakis G, MacDonald R, Alam U, Malik RA. Corneal confocal microscopy for the diagnosis of diabetic peripheral neuropathy: A systematic review and meta-analysis. J Diabetes Investig. 2022 Jan;13(1):134-147.
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Not in relation to the actual thesis

1. **Gad, H**., Mohammed, I., Saraswathi, S. *et al.* Corneal Langerhans cells in children with celiac disease. Sci Rep. 2022 Oct 31;12(1):18289.
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List of Poster Presentations related to this thesis

- **Hoda Gad**, Bara Al-Jarrah, Saras Saraswathi, Ioannis Petropoulos, Georgios Ponirakis, Adnan Khan, Parul Singh, Souhaila Al Khodor, Khalid Hussain, Mamoun Elawad, Wesam Almasri, Hatim Abdelrahman, Ahmed Elaww, Amel Khalifa, Fawziya Al-Khalaf, Goran Petrovski, Mahmoud Al Zyoud, Maryam Al Maadheed, Mohamed Hendaus-Rahal, Anthony Akobeng, Rayaz A Malik. Corneal Confocal Microscopy detects corneal nerve loss in children with Type

1 Diabetes Mellitus (T1DM) in Qatar. WCMQ 20th annual research retreat. 22nd Feb 2020.

- **Hoda Gad**, Bara Al-Jarrah, Saras Saraswathi, Ioannis Petropoulos, Georgios Ponirakis, Adnan Khan, Parul Singh, Souhaila Al Khodor, Khalid Hussain, Mamoun Elawad, Wesam Almasri, Hatim Abdelrahman, Ahmed Elaww, Amel Khalifa, Fawziya Al-Khalaf, Goran Petrovski, Mahmoud Al Zyoud, Maryam Al Maadheed, Mohamed Hendaus-Rahal, Anthony Akobeng, Rayaz A Malik. Acceptability and Tolerability of Corneal Confocal Microscopy (CCM) in Children in Qatar. WCMQ 20th annual research retreat. 22nd Feb 2020.
- **Hoda Gad**, Hajar Dauleh, Shiga Chirayath, Basma Haris, Houda Afyouni, Goran Petrovski, Saira Shehzad, Amel Khalifa, Fawziya Al-Khalaf, Ghassan Mohamadsalih, Parul Singh, Souhaila AlKodor, Mohamed A. Hendaus, Einas Elgassim, Farah Wahbeh, Fatima Sajjadi, Ioannis N. Petropoulos, Georgios Ponirakis, Khalid Hussain, Rayaz A. Malik. Corneal nerve changes in obese children and adolescents. Presented at the 32nd Neurodiab meeting, Bergen, Norway, 15-19 September 2022.
- **Hoda Gad**, Einas Elgassim, Adnan Khan, Ioannis N. Petropoulos, Georgios Ponirakis, Rayaz A. Malik. Evidence of early corneal nerve fibre regeneration with the weekly glucagon-like peptide 1 receptor agonist semaglutide. Presented at the 32nd Neurodiab meeting, Bergen, Norway, 15-19 September 2022.

DEDICATION

I dedicate this thesis to my beloved parents, husband, and children.

I also dedicate this work to Professor Rayaz Malik who had faith in me and believed that I could succeed in this.

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I wish to thank my examiners for their expertise and precious time to review this work.

DECLARATION

No portion of this work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

ALTERNATIVE THESIS FORMAT

The author has been granted permission to submit this Ph.D. thesis in an alternative format by her supervisor Professor Mark Slevin and Professor Rayaz A. Malik approved under the Manchester Metropolitan University, Faculty of Science and Engineering regulations, including sections which are in a format suitable for submission for publication or dissemination. The following chapters in this thesis have been published, submitted, or will be submitted for publication:

- Chapter 3: Published in J Diabetes Investig 2022
- Chapter 4: Published in J Diabetes Investig. 2020
- Chapter 5: Published in Br J Ophthalmol. 2021
- Chapter 6: Published in Endocr Connect. 2022
- Chapter 7: Submitted to Pediatric Neurology. 2023 (under review)
- Chapter 8: Will be submitted for publication

LIST OF ABBREVIATIONS

AN: Acanthosis nigricans

AGEs: Advanced Glycation End products

AB: Antibodies

AKD: Anterior keratocyte density

Anti-GAD: anti-glutamic acid decarboxylase

ALP: Alkaline phosphatase

ALT: Alanine transaminase

AST: Aspartate aminotransferase

BF%: Body fat percent

BMI: body mass index

CIDP: Chronic inflammatory demyelinating polyneuropathy

CCM : Corneal confocal microscopy

CASE IV: Computer assisted sensory evaluation, version-4

CGM: Continuous glucose monitoring

CNFD: Corneal nerve fibre density

CNBD: Corneal nerve branch density

CNFL: Corneal nerve fibre length

CNFT: corneal nerve fibre tortuosity

CI: Confidence interval

CV: Coefficient of variation

CAN: Cardiac autonomic neuropathy

CCL7: Chemokine (C-C motif) ligand 7

CONGA: Continuous overall net glycemic action

DM: Diabetes mellitus

DSPN: Distal symmetrical polyneuropathy

DPN: Diabetic peripheral neuropathy

DAG: Diacylglycerol

DN: Diabetic neuropathy

DN4: Douleur Neuropathique en 4

DNS: Diabetic neuropathy symptom

DPN^-: Without diabetic peripheral neuropathy

DPN^+: With diabetic peripheral neuropathy

DBP: Diastolic blood pressure

ECM: Extracellular matrix

ESC: Electrochemical skin conductance

FDA: Food and drug administration

FFM: Fat free mass

GlcNac: Uridine 5-diphosphate-N-acetylglucosamine

GLP-1 RA: Glucagon-like peprtide-1 receptor agonist

GV: Glucose variability

HOMA-IR: Homeostatic Model Assessment for Insulin Resistance

HRT III RCM: Heidelberg Retina Tomograph III Rostock Corneal Module

HRV: Heart rate variability

HAAF: Hypoglycaemia-associated autonomic failure

HC: Healthy control

HFD: High-fat-diet

HbA1c: Glycated haemoglobin

HDL-C: High-density lipoprotein-cholesterol

25-OHD : 25-hydroxycholecalciferol

IRs: Insulin receptors

IGF-I: Insulin-like growth factor-I

IRB: Institutional review board

IWL: Inferior whorl length

IGT: Impaired glucose tolerance

IENFD: Intraepidermal nerve fibre density

ICA: Anti-islet cell antibodies

IOFT: International Obesity Task Force

KD: Keratocyte density

LCFAs: long chain fatty acids

LDL-C: Low-density lipoprotein-cholesterol

LANSS: Leeds Assessment of Neuropathic Symptoms and Signs

MENA: the Middle East and North Africa

MNSI: Michigan neuropathy screening instrument

MD: Mean difference

MKD: Mid keratocyte density

MAGE: Mean amplitude of glycemic excursions

MFR: Ratio of Muscle-to-fat

µS: microSiemens

Na/K: Sodium/potassium

NO: Nitric oxide

NPQ: Neuropathic Pain Questionnaire

NPS: Neuropathic Pain Scale

NCS: Nerve conduction studies

NFD: Nerve fibre density

NBD: Nerve branch density

NCV: Nerve conduction velocity

NF-κB: Nuclear factor-kappa B

NDS: Neuropathy disability score

OxPhos: oxidative phosphorylation

pDPN: painful diabetic peripheral neuropathy

PROSPERO: International Prospective Register of Systematic Reviews

PKD: Posterior keratocyte density

PKC: Protein kinase C

PNS: Peripheral nervous system

QST: Quantitative sensory testing

QSART: Quantitative sudomotor axon reflex test

ROS: Reactive oxygen species

RCT: Randomized controlled trial

RAGE: Receptor for Advanced Glycation End product

SC: Schwann cell

SBNP: Sub-basal nerve plexus

SBP: Systolic blood pressure (SBP)

SD: Standard deviation of blood glucose

s.c.: Subcutaneous

T1DM: Type 1 diabetes mellitus

T2DM: Type 2 diabetes mellitus

TGFβ: Transforming growth factor beta

TCNS: Toronto clinical neuropathy score

TIR: Time in range

TBR: Time below range

TAR: time above range

TBW: Total body water

UENS: Utah early neuropathy scale

VEGF: Vascular endothelial growth factor

VPT: Vibration perception threshold

VLDL-C: very low-density lipoprotein cholesterol

WHO: World Health Organization

ABSTRACT

Corneal Confocal Microscopy (CCM) is a non-invasive ophthalmic imaging technique to quantify small nerve fibre morphology in patients with diabetic neuropathy and other metabolic peripheral neuropathies.

This thesis establishes that CCM is indeed a robust imaging technique which can identify early subclinical and clinical neuropathy in children with Type 1 diabetes mellitus (T1DM) and adults with T1DM and Type 2 diabetes mellitus (T2DM).

We demonstrate the utility of CCM in detecting neuropathy not only in patients with established neuropathy, but also in patients with diabetes without neuropathy. We also demonstrate a precise relationship between glycemic variability and hypoglycaemia with corneal nerve loss in patients with T1DM and T2DM.

This thesis provides further evidence for the utility of CCM as a surrogate endpoint of early small fibre neuropathy by demonstrating nerve damage in obese children with acanthosis nigricans (AN) (a sign of insulin resistance) and obese adults with and without T2DM. We also show nerve regeneration in individuals with obesity without T2DM after 3 months of treatment with the once weekly Glucagon-like peptide-1 receptor (GLP-1R) agonist semaglutide.

Chapter I- INTRODUCTION

The International Diabetes Federation (IDF) reports that 537 million individuals currently live with diabetes mellitus (1). Diabetes mellitus (DM) is defined as “a chronic metabolic disease, characterized by high blood glucose levels resulting from defects in insulin secretion and/or action” (2). There are two broad forms of diabetes based on etiology and pathophysiology: Type 1 (T1DM) and type 2 (T2DM) diabetes mellitus.

1.1. Type 1 diabetes Mellitus (T1DM)

Type 1 diabetes is an autoimmune disease of the pancreatic β-cells leading to destruction of the β-cells and insulin deficiency. It is associated with low quality of life, long-term complications and high costs for healthcare systems and patients (3). In a recent study a Markov model was applied to data on type 1 diabetes incidence and associated mortality to derive type 1 diabetes prevalence, incidence, associated mortality and life expectancy in 201 countries (3). In 2021, 8.4 million individuals had T1DM worldwide and of these 1.5 million were aged <20 years, 5.4 million were aged 20-59 years, and 1.6 million were aged ≥ 60 years. Furthermore, the model estimates placed global deaths due to T1DM at 175,000 in 2021 and of these, 35,000 were attributed to non-diagnosis, of which 14,500 were in sub-Saharan Africa and 8,700 were in South Asia. They also predicted that by 2040 the prevalence of T1DM will dramatically increase to 13.5-17.4 million cases (3).

The Arab region has a higher prevalence of diabetes than the global average, driven primarily by T2DM (4). Indeed, five of the top 10 countries with the highest prevalence of diabetes are in the gulf region: Kuwait (21.1%), Qatar

(20.2%), Kingdom Saudi Arabia (KSA) (20.0%), Bahrain (19.9%), and United Arab Emirates (19.2%). However, the incidence of T1DM in children and adolescents in KSA has also increased from 18.05/100000 in 1998 to 33.5/100000 in 2017 (4, 5). In 2015, Qatar had an incidence of T1DM of 33.49/100000, which was higher than Norway, United Kingdom, Canada, and the USA (5, 6). Indeed in 2020, the incidence was found to be even higher at 38.05/100000, based on a detailed biochemical, immunological and genetic study which undertook extensive antibody testing for anti-glutamic acid decarboxylase (GAD) antibodies (Ab), anti-islet cell Ab (ICA) and anti-insulin Ab (IAA) in Qatari children and adolescents (4, 5).

1.2. Type 2 diabetes Mellitus (T2DM)

Type 2 diabetes mellitus is characterized by insulin resistance and hyperinsulinemia and eventually glucose intolerance and hyperglycaemia. Globally, 462 million individuals are estimated to have type 2 diabetes (7). Between 1990 to 2017 the global incidence of T2DM increased from 228.5 to 279.1 and the prevalence increased from 4576.7 to 5722.1 (8). Diabetes affects the quality of life and is associated with significant morbidity and mortality. Rapid economic growth and urbanization has led to increased consumption of unhealthy diets and a sedentary lifestyle, resulting in obesity, and increased glucose and blood pressure levels (7). Approximately 60% of individuals with T2DM are overweight and 20% are obese (2). Early onset of T2DM in younger individuals is mostly associated with obesity and a sedentary lifestyle (7). In 2017, the estimated prevalence of T2DM in youth aged 10-19

years in the US was 670/100000 (9). A recent systematic review has shown that there are 41600 children and adolescents worldwide, newly diagnosed with T2DM (10). The incident cases of T2DM in children below 20 years of age in Iran in 2011 it was 22, in 2013 in Kuwait it was 32 and in 2016 in Qatar it was 45 (10). In 2020, there were 104 children with T2DM in Qatar with an incidence of 2.51 and prevalence of 23.7 (5). With the increasing prevalence of children who are overweight and obese, T2DM has become a global public concern (9).

Individuals with youth-onset diabetes are at increased risk of complications, with increased morbidity, and mortality, especially those who develop T2DM (9, 11). Diabetic nephropathy, retinopathy, peripheral neuropathy, cardiac autonomic neuropathy, arterial stiffness, and hypertension are prevalent in children and adolescents with T1DM and T2DM, with a higher prevalence in children and adolescents with T2DM (12).

1.3. Peripheral neuropathy (PN)

Peripheral neuropathy is characterised by damage to sensorimotor and/or autonomic nerves (13). The underlying pathology affects the axon and/or myelin sheath resulting in an axonal and demyelinating neuropathy (14). Diabetes is the most common cause of peripheral neuropathy worldwide, although other causes include infection, autoimmune diseases, genetic-disorders, cancer, bone marrow disorders, thyroid disease, alcoholism, toxins, medications, trauma and vitamin deficiencies. This thesis focuses on nerve

damage caused by T1DM, T2DM and obesity and the relationship with glucose variability.

Diabetic peripheral neuropathy is defined as "the presence of symptoms and/or signs of peripheral nerve dysfunction in people with diabetes after the exclusion of other causes" (13). Peripheral neuropathies are classified into diabetic and non-diabetic neuropathies (**Table 1)** (15).

Table 1. Classification of diabetic neuropathy

A. Diffuse neuropathy	**B. Mononeuropathy (mononeuritis multiplex)**	**C. Radiculopathy or polyradiculopathy**
<u>DSPN</u> • Small-fibre neuropathy • Large-fibre neuropathy • Mixed small/large-fibre neuropathy <u>Autonomic</u> Cardiovascular • Reduced HRV • Resting tachycardia • Orthostatic hypotension • Sudden death (malignant arrhythmia) Gastrointestinal • Diabetic gastroparesis • Diabetic enteropathy (diarrhea) • Colonic hypomotility (constipation) Urogenital • Diabetic cystopathy (neurogenic bladder) • Erectile dysfunction • Female sexual dysfunction Sudomotor dysfunction • Distal hypohydrosis/anhidrosis • Gustatory sweating Hypoglycemic unawareness Abnormal pupillary function	Isolated cranial or peripheral nerve (e.g. CN III, ulnar, median, femoral, peroneal) Mononeuritis multiplex (if confluent may resemble polyneuropathy)	Radiculoplexus neuropathy (a.k.a. lumbosacral polyradiculopathy, proximal motor amyotrophy) Thoracic radiculopathy Nondiabetic neuropathies common in diabetes Pressure palsies Chronic inflammatory demyelinating polyneuropathy Radiculoplexus neuropathy Acute painful small-fibre neuropathies (treatment-induced)

Adapted from the position statement by the ADA 2017 (15). DSPN: diabetic sensorimotor polyneuropathy, HRV: Heart rate variability.

The clinical presentation of peripheral neuropathy includes burning, shooting, stabbing, electric shock, compression sensation, painful cold or itching pain, numbness, paresthesia and autonomic symptoms (16). On clinical neurologic examination hypoalgesia, hyperalgesia, or allodynia may be evident. Patients with autonomic neuropathy may present with oversensitivity to light and blurred vision, dry eyes or mouth, palpitations, resting tachycardia, syncope, orthostatic dizziness, dysphagia, gastroparesis, constipation, diarrhea, urinary retention, erectile dysfunction, female sexual dysfunction, and sudomotor dysfunction (16).

1.4. Epidemiology of Diabetic peripheral Neuropathy

Diabetic peripheral neuropathy (DPN) is the most common type of neuropathy. In a population study of 4400 adults with diabetes from Belgium, 50% developed DPN after 25 years of follow-up (17). In more recent studies from the US and Europe, the prevalence of DPN was approximately 6-51% (18, 19). In the Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications (DDCT/EDIC) study, the prevalence of DPN in T1DM was 6% and increased to 30% after 13-14 years of follow-up (20). The prevalence of DPN in adults with T1DM in the Pittsburgh Epidemiology of Diabetes Complications was 34% and increased with age to 58% in those more than 30 years old (21).

In the SEARCH for Diabetes in youth study, the prevalence of DPN in youths with T2DM was 26% compared to youths with T1DM, with a prevalence of

8.2% (22). In the Action to Control Cardiovascular Risk in Diabetes trial (ACCORD) (23), the Veteran Affairs Diabetes trial (24) and the Bypass Angioplasty Revascularization Investigation 2 Diabetes trial (BARI 2D) (25), the prevalence of DPN in T2DM was 42%, 39%, and 51%; respectively. The differences in prevalence between studies may be attributed to different methods of assessing neuropathies.

1.5. Pathogenesis and Risk Factors for Diabetic Peripheral Neuropathy

Diabetic peripheral neuropathy is multifactorial with both metabolic and vascular insults playing a role (26). Hyperglycaemia, dyslipidaemia, and insulin resistance all contribute to mitochondrial dysfunction and excess formation of mitochondrial and cytosolic reactive oxygen species (27) leading to loss of axonal energy stores and axonal injury (26). Early nerve damage occurs in the unmyelinated C fibres **(Figure 1)** (28) followed by demyelination and axonal degeneration of the myelinated fibres (29).

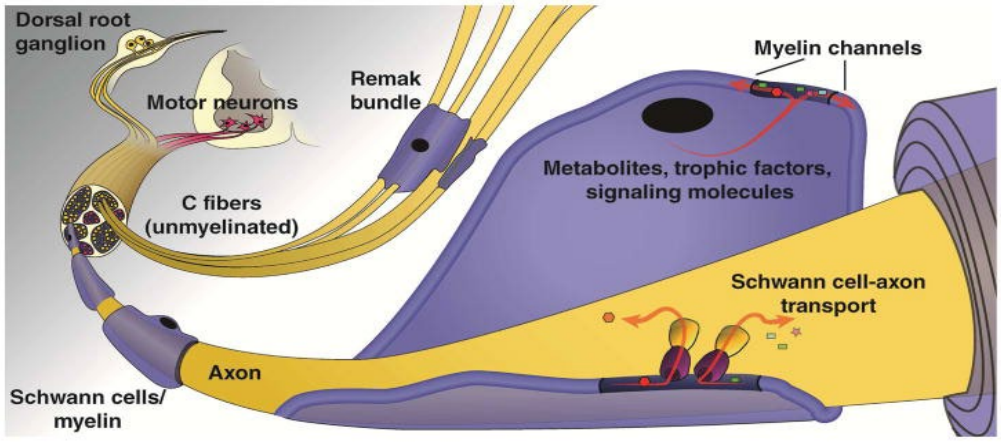

*Figure 1. The Peripheral Nervous System **(26)***

Tight glycemic control has been shown to prevent or delay the development of DPN in patients with T1DM, but with minimal to no impact in T2DM (30). In

the DCCT, intensive insulin therapy reduced the risk of neuropathy in T1DM by 60% at 6.5 years of follow-up (31), which persisted to 14 years, with a relative risk reduction of 30% (20). In T2DM the effect of intensive glycemic control is limited to an improvement in motor nerve conduction in the KUMAMOTO trial (32) and vibration perception threshold in the UK Prospective Diabetes Study (UKPDS) (33, 34).

In addition to age, duration of diabetes, and glucose control, diabetic neuropathy has been associated with modifiable cardiometabolic risk factors, including high triglycerides, body mass index (BMI), smoking and hypertension (35, 36).

1.5.1. Hyperglycaemia

A. Polyol pathway

Excess glucose is converted to sorbitol by aldose reductase, leading to an imbalance in cell osmosis and efflux of myoinositol and taurine. Myoinositol is required for sodium/potassium (Na/K) ATPase function, which leads to impaired nerve conduction. There is also depletion of the cellular stores of NADPH required for the generation of nitric oxide (NO) and antioxidant glutathione. As a result, cytoplasmic reactive oxygen species (ROS) mediate intracellular injury and cellular dysfunction (26).

B. Hexosamine and Protein Kinase C Pathways

Excess glucose induces glycolysis which disrupts several metabolic pathways and promote neuronal injury. Fructose-6-phophate, an intermediate of glycolysis, enters the hexosamine pathway forming uridine 5-diphosphate-N-

acetylglucosamine (GlcNac), which binds to serine/threonine residues promoting dysregulation of lipid homeostasis, inflammation, and tissue injury. Dihydroxy-acetone phosphate is converted to diacylglycerol (DAG) which causes neuronal protein kinase C (PKC) activation, with dysregulation of Na/K ATPase and altered gene expression of vascular endothelial growth factor (VEGF) and transforming growth factor beta (TGFβ), leading to vasoconstriction, hypoxia and nerve damage (26).

C. Advanced Glycation End products

Excess glucose reacts with proteins to form glycated residues to produce irreversible AGEs, causing cellular damage by cross-linking proteins and altering their function. Activation of the Receptor for AGE (RAGE) activates a downstream signaling cascade mediated by nuclear factor-kappa B (NF-κB) causing vasoconstriction, inflammation, and decreased neurotrophic factors (26).

D. Impaired insulin signaling

Whilst insulin does not control glucose transport into the PNS, it is a potent neurotrophic factor that promotes axonal growth. Insulin receptors (IRs) are highly expressed in enriched membranes of sensory and motor neurons and intraneural mitochondria. IRs are activated by insulin as well as insulin-like growth factor-I (IGF-I) which promotes a signaling cascade essential for cellular maintenance. Insulin deficiency and decreased C-peptide in T1DM downregulates insulin signaling, decreases gene expression of essential proteins and protein synthesis, promoting cellular injury. Exogenous

supplementation of insulin in T1DM restores glucose homeostasis and insulin-mediated signaling (26). In T2DM, metabolic syndrome (hypertension, hyperlipidemia, visceral adiposity, and impaired glucose regulation) promotes the onset and progression of DPN.

1.5.2. Dyslipidaemia

SC transports long chain fatty acids (LCFAs) from the extracellular space into the SC cytoplasm leading to LCFA overload and oxidative phosphorylation (OxPhos), with toxicity to neurons (26)

1.5.3. Obesity

Obesity is a chronic multifactorial disease (37) defined by the WHO as "excessive fat accumulation that might impair health" and is diagnosed at a BMI ≥30 kg/m^2 (38). The global prevalence of obesity increased from 11% in 2010 to 17% in 2020 and is expected to reach 20% by 2030 (39). In the Middle East, it is predicted that 1 in 5 men and 1 in 3 women will have a BMI ≥30 kg/m^2 by 2030 (39). The prevalence of obesity in children aged 5-9 years was 11% in 2020 and is estimated to reach 16% by 2030. In children aged 10-19 years, the prevalence of obesity was 7% in 2020 and is estimated to reach 11% by 2030 (39), with the Middle East likely to experience double these numbers by 2030. Qatar has the third highest rate of obesity in the MENA region (39).

Obesity increases the risk of metabolic disorders (T2DM and fatty liver) cardiovascular disease (hypertension, myocardial infarction, and stroke),

musculoskeletal disease (osteoarthritis), Alzheimer's disease, depression, cancer and reduces the quality of life (40).

Neuropathy is highly prevalent in individuals with diabetes but has also been reported in those with obesity without hyperglycaemia (41).

1.5.3.1. Obesity related neuropathy

Diabetes and obesity are the most common metabolic risk factors for neuropathy (41-45). Several studies have reported neuropathy in individuals with obesity regardless of glycemic status (41). A recent study has reported a 12% prevalence of neuropathy in obese individuals with normoglycaemia, 7.1% in prediabetes, and 40% in those with diabetes based on nerve conduction studies (46). Increased waist circumference and triglycerides are associated with neuropathy (42, 46). Abdominal obesity assessed by waist circumference was associated with neuropathy after adjusting for cardiometabolic risk factors such as HbA1c, blood pressure, HDL, LDL, and triglycerides (47). In a recent study, bariatric surgery reduced distal symmetrical polyneuropathy (DSPN) by 63% (48). Obesity is a proinflammatory state (49, 50) with multiple cytokines and chemokines playing a role in DSPN. Both C-C motif chemokine ligand 7 (CCL7) and CXCL10 are expressed in subcutaneous and visceral adipose tissue with CCL7 being upregulated in obesity (47). Several studies have shown an association between metabolic syndrome and polyneuropathy (41, 44, 45). In the SEARCH study, T1DM children with obesity, high triglycerides, LDL-C, and diastolic blood pressure were at increased risk of DN (51). In obese children and

adolescents there is a significant abnormality in nerve conduction compared to age-matched healthy controls (52).

1.6. Clinical assessment of peripheral neuropathy

There are several techniques for the diagnosis and evaluation of peripheral neuropathy and each has its advantages and disadvantages **(Table 2)**. The evaluation includes symptom questionnaires, the neuropathy disability score, quantitative sensory testing and electrophysiology. There are several validated tools to assess painful neuropathy e.g., the Neuropathic Pain Questionnaire (NPQ), the Neuropathic Pain Scale (NPS) and the McGill Pain Questionnaire (52). Patients are asked to self-report their pain symptoms and severity (52, 53). These tools are inexpensive and easy to use, but have limited sensitivity and high variability (53). Nerve conduction studies (NCS) are considered to be the gold standard in the diagnosis of neuropathy and are reproducible, but they assess only large fibres and require special equipment and trained staff to perform (48). Intraepidermal nerve fibre density (IENFD) evaluated in skin punch biopsy is an objective and sensitive technique to quantify sensory and autonomic small nerve fibre damage. However, the procedure is expensive, invasive and requires qualified personnel to perform (49). Quantitative sensory testing (QST) is a non-invasive technique that assesses both small and large nerve fibres by evaluating thermal and vibratory stimuli, respectively. However, it is subjective, requires special equipment and the reproducibility and sensitivity is highly variable (50). Vibration perception threshold (VPT) assesses large nerve fibre dysfunction and has limited utility

in detecting early neuropathy (12). The neuropathy disability score (NDS) can be simply and rapidly undertaken in the clinic and ranges from 0-10; where 0 is the minimum score and 10 is the maximum score (10, 51) with an NDS score more than 6 being associated with an increased risk of foot ulceration (51). Corneal confocal microscopy (CCM) is a rapid, non-invasive ophthalmic imaging technique that has high sensitivity and reproducibility for identifying DSPN (54) and other peripheral neuropathies (55).

Despite the wide availability and easy use of validated questionnaires and scales for the assessment of neuropathy and neuropathic pain (56, 57), they have variable sensitivity and specificity (58). They have several drawbacks including subjectivity, recall bias, lack of information on the history of pain and specificity to the affected site of pain rather than the whole body (56). Therefore, it is crucial to perform objective assessments to confirm the diagnosis of peripheral neuropathy e.g. NCS, QST, skin biopsy and CCM (59, 60).

Table 2. Clinical assessments of peripheral neuropathy

Examination type	Diagnostic test name	Advantages	Disadvantages
Clinical signs and symptoms	DN4, LANSS, NPQ, MNSI, DNS, TCNS, NDS, UENS	Easy to use, inexpensive	Limited sensitivity, high variability
Neurophysiology	NCS of motor and sensory nerves	Objective, widely available, reproducible	Only assess large fibres Requires special equipment
Skin punch biopsy	IENFD	Objective, gold standard to assess small fibres	Costly, time consuming, Risk of infection, Requires specialist equipment and personnel to quantify IENFD
Quantitative sensory testing (QST)	CASE IV, Biothesiometer, Thermoaesthesiometer, TSA neurosensory analyser	Easy to perform, rapid, non-invasive, examines small and large fibres	Reproducibility and sensitivity are variable Subjective, requires special equipment
Sudomotor function	Neuropad, Sudoscan, QSART, sympathetic skin response	Rapid, objective, easy to perform, simple, reproducible	Moderate sensitivity Uncertain interpretation
Vibration Perception	VPT	Simple, non-invasive technique that assesses large nerve fibre dysfunction	Limited value in detecting early neuropathy
Corneal Confocal Microscopy (CCM)	HRT III RCM	Objective, rapid, reproducible, assess small fibres	Costly, requires specialist equipment

Adapted from Petropolous IN et al (53), Azmi S et al (61), Basantsova NY et al (59), Tavakoli M et al (54).

1.7. Corneal Confocal Microscopy (CCM) and corneal layers

CCM allows the identification of very early neuropathy. This technique is a simple, rapid, non-invasive, reiterative, and cost-effective approach for quantifying small nerve fibre loss and repair. CCM provides cross-sectional images of all five layers of the cornea; epithelium, sub-basal layer, stroma, Descemet's membrane and endothelium **(Figure 2)** (62).

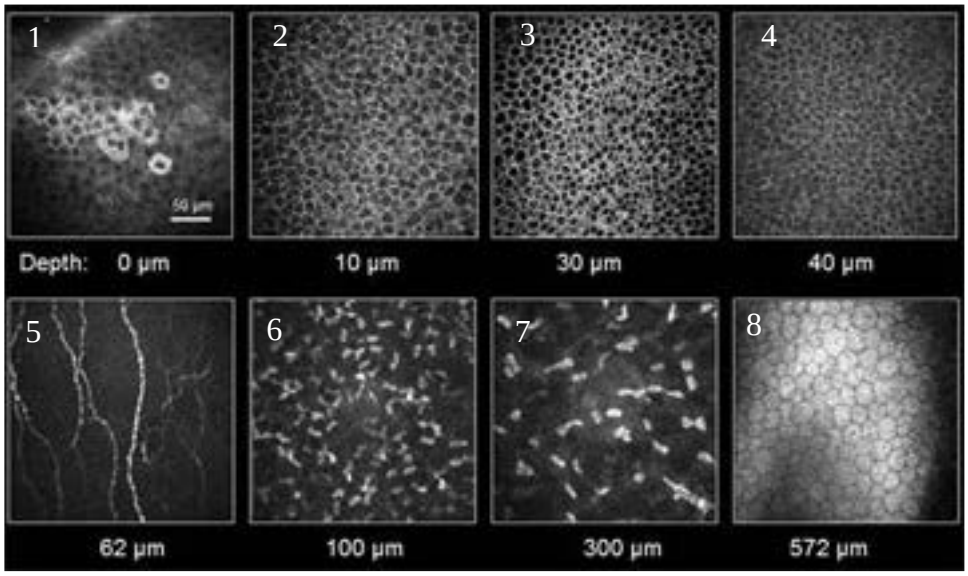

Figure 2. In vivo imaging with corneal confocal microscopy.

A cross-sectional view of human epithelium (1-4), sub-basal nerves (5), anterior and posterior stroma (6-7) and endothelium (8) (63).

A. Epithelium

The epithelium can be divided into three layers: superficial epithelial cells, wing cells, and basal cells, which vary in size and shape. The superficial cells are multi-cornered cells with a light border and bright nucleus of 10 µm thickness **(Figure 2.2)** (64, 65).

Wing cells are barely rounded cells with bright, thin borders and a bright nucleus and are 30-45 µm in diameter **(Figure 2.3)** (64, 66). Basal cells are

smaller, dark cells with a bright border that create a mosaic and they are 8-10 µm in size **(Figure 2.4)** (66).

B. Bowman's layer

Bowman's layer (anterior limiting membrane) does not consist of any cells but contains nerves perforating it **(Figure 2.5)**. It appears as a grey, inexpressive 8-10 µm thick layer (64, 65) that consists of chaotically orientated fibrils (67).

C. Stroma

The stroma comprises about 90% of the volume of the cornea and consists of collagen fibres (fibrils) (68) **(Figure 2.6, 2.7)**. It also contains stromal fibroblasts called keratocytes surrounded by an extracellular matrix (ECM) (67, 69). Keratocytes are hyper-reflective and spindle or osteoblast-shaped with a bright nucleus against a dark background.

Keratocytes play a major role in maintaining corneal transparency, homeostasis and corneal repair (70). Keratocytes are capable of synthesizing collagen, glycosaminoglycans and matrix metalloproteases (MMP) and play an important role in maintaining stromal homeostasis (71). Additionally, keratocytes provide the transparency of the cornea and reduces reflection of light through the crystallins in keratocytes (72).

The density of keratocytes decreases from the anterior stroma to the posterior stroma and they simultaneously increase in size (64). Keratocytes play a major role in corneal repair (73) via two proposed mechanisms: cell apoptosis or by developing into fibroblasts (74). Apoptosis is the initial response to injury induced by IL-1α from epithelial cells resulting in an acellular zone (74)

followed by keratocytes promoting MMP secretion, essential for collagen fibre formation (70, 71). Keratocytes induce the healing response by becoming activated and migrating to the injured area to differentiate into fibroblasts and myofibroblasts that secrete ECM (70). Differentiation into myofibroblasts is induced by TGFβ2 (transforming growth factor) released by epithelial cells (70, 75).

D. Endothelium

The endothelial layer consists of hexagonal-shaped cells with a black border and in some cells the nucleus can be observed as a dark spot (64) **(Figure 2.8)**. In patients with diabetes, corneal endothelial dysfunction is induced by metabolic stress (76, 77), manifested by thicker corneas, reduction in cell density and abnormal cell morphology (polymorphism and polymegathism) (76, 78), associated with a longer duration of diabetes (78).

1.8. Significance of alterations in corneal nerves:

Corneal nerve alterations have been reported in subjects with elevated glycated haemoglobin (HbA1c) levels (79). Furthermore, in 80 healthy control subjects reduced corneal nerve fibre density was related to elevated HbA1c, triglycerides and body mass index (80). There is evidence for significant corneal and intra-epidermal nerve fibre loss in subjects with impaired glucose tolerance (81) and recently diagnosed T2DM (82). In subjects with impaired glucose tolerance, lower baseline corneal nerve fibre length (CNFL) was associated with the development of T2DM and subjects who reverted to normal glucose tolerance showed a significant improvement in CCM

parameters (83). Furthermore, several studies have shown that a lower CNFL at baseline predicts the development of DSPN (84-86). Corneal nerve loss also precedes diabetic retinopathy and microalbuminuria, a finding which has significant implications for screening of diabetes complications and indeed the diagnostic cut-offs for diabetes (87). Several recent studies have also shown significant corneal nerve fibre loss (88, 89) and thinning of the retinal nerve fibre layer (90) in children and adolescents with T1DM (89, 91) without retinopathy, challenging the view that retinopathy is the earliest microvascular complication. Studies assessing the diagnostic value of CCM in T1DM and T2DM are summarized below in chapter 3. This thesis has assessed the diagnostic ability of CCM in the early detection of neuropathy in T1DM (chapter 4), T1DM and T2DM (Chapter 6) and obesity (Chapter 7, 8). Additionally, mechanisms related to nerve damage in diabetes such as alterations in keratocytes in T1DM (Chapter 5) and glucose variability and hypoglycaemia in both T1DM and T2DM are discussed in chapter 6.

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Chapter II: STUDY DESIGN AND METHODOLOGY

2.1. Hypotheses and aims

Corneal confocal microscopy (CCM) is a rapid, real-time, non-invasive ophthalmic imaging technique for the assessment of corneal nerve morphology.

2.2. Research Aims:

- To undertake a systematic review and meta-analysis assessing the diagnostic utility of CCM for sub-clinical (DSPN-) and established (DSPN+) diabetic neuropathy.
- To quantify corneal nerve morphology in children with type 1 diabetes mellitus (T1DM) compared to age-matched healthy controls using corneal confocal microscopy.
- To assess whether alterations in stromal keratocyte density are related to loss of corneal nerve fibres in children with T1DM.
- To investigate the relationship between continuous glucose monitoring metrics and corneal nerve changes in participants with T1DM and T2DM.
- To assess if there are alterations in corneal nerve morphology in obese children and adolescents with insulin resistance.
- To assess for evidence of nerve regeneration in obese subjects with and without diabetes after treatment with the weekly GLP-1 agonist, Semaglutide.

2.3. Study design

This thesis includes observational, cross-sectional, and prospective studies.

2.4. Study Approval

Research was conducted in accordance with the Declaration of Helsinki. Ethical approval was obtained from the institutional review board (IRB) at Sidra medicine, Hamad Medical Corporation (HMC) and Weill Cornell Medicine-Qatar (WCM-Q). Relevant approval letters in **Appendix 1.** Approval was granted by Manchester Metropolitan University (2023-53145-43100).

2.5. Participant enrollment

Pediatric participants of Arab ethnic origin with T1DM, obesity or healthy controls were recruited from Sidra medicine, Doha, Qatar. Adult participants with T1DM, T2DM, obesity and healthy controls were recruited from the national diabetes center in Hamad Medical Corporation, Doha, Qatar.

After carefully explaining the study, participants and their parents were given the opportunity to read the information sheet and consent forms, discuss and ask questions related to the study. Procedures, confidentiality issues, risk and benefits were explained to the participants and their parents. Participants who agreed to take part in the study were asked to sign an informed assent form to indicate their voluntary agreement to participate in the study, in addition to the informed consent form signed by their parents/guardians. Each adult participant provided informed consent prior to inclusion in the study. All participants were given a copy of the signed consent/assent forms for their records.

2.6. Inclusion criteria:

a. Children (age 8-18) with a documented diagnosis of T1DM and healthy volunteers.

b. Obese children (age 8-18) with BMI-for-age > +2SD (WHO- Growth Chart)

c. Adults (age 19-60) with T1DM or T2DM on insulin treatment

d. Obese adults (age 19-60) with BMI >25 kg/m^2, treated with Semaglutide 1.0 mg weekly injection with or without T2DM.

e. Ability to provide parental consent and child assent

2.7. Exclusion criteria:

a. Known disease of the cornea or history of trauma or surgery to the cornea within 6 months.

b. Other causes of neuropathy (malignancy, hypothyroidism, drugs, alcohol, chronic kidney failure, liver failure, connective tissue disease, amyloidosis, infectious disease such as Lyme disease, HIV and AIDS, vitamin B_{12} deficiency).

c. Serious illness that might affect the cornea and/or the nervous system.

d. Pregnant or planning to get pregnant in the subsequent 24 months for those treated with Semaglutide.

e. Hypersensitivity to GLP-1 agonist or any ingredient of the drug.

f. Contraindication to GLP-1 agonist therapy (pancreatitis, family history of thyroid carcinoma).

g. Failure to achieve parental consent or child assent.

2.8. Sample size determination

2.8.1. **Chapter 4 and 5:** The primary outcome measure was corneal nerve fibre pathology as assessed with CCM. A difference in corneal nerve fibre length of 2.7 mm/mm^2 has been demonstrated to be a clinically significant difference representing the difference between non-diabetic controls and individuals

with diabetes and mild neuropathy (1). The standard deviation for nerve fibre length (mm/mm^2) in non-diabetic adults has been reported to be 0.88 (2). Using these parameters, a power of 0.80 and a two-tailed alpha of 0.05, a minimum sample size of 16 was calculated per group. Given the lack of precision associated with this calculation we opted to increase the sample size to 20 per group, a number which is feasible within our clinical setting.

2.8.2. **Chapter 6:** This was a pilot study with no previously published data to use for the sample size calculation. A retrospective sample size calculation was undertaken based on the current findings. The primary outcome measure was corneal nerve fibre pathology as assessed with CCM. A difference in nerve fibre density of 6.13 fibre/mm^2 has been demonstrated to be a clinically significant difference representing the difference between mean nerve fibre density in children with obesity without acanthosis nigricans (AN) and children with obesity and acanthosis nigricans. The standard deviation for nerve fibre density (fibre/mm^2) in children with obesity without AN has been reported to be 6.14. Using these parameters, a power of 0.80 and a two-tailed alpha of 0.05, a minimum sample size of 16 was calculated per group. Given the lack of precision associated with this calculation we opted to increase the sample size to 20 per group.

2.8.3. **Chapter 7:** The primary outcome measure was corneal nerve fibre pathology as assessed with CCM and continuous glucose monitoring (CGM). A difference in nerve fibre length of 5.77 mm/mm^2 has been demonstrated to be a clinically significant difference representing the difference between mean nerve fibre

length in patients with diabetes with time below range (TBR) and healthy controls. The standard deviation for corneal nerve fibre length (mm/mm^2) in patients with diabetes and TBR has been reported to be 3.57. Using these parameters, a power of 0.80 and a two-tailed alpha of 0.05, a minimum sample size of 6 was calculated per group. Given the lack of precision associated with this calculation we opted to increase the sample size to 10 per group.

2.8.4. **Chapter 8:** This was an open label prospective observational study of patients attending clinic undergoing treatment with Semaglutide. The estimated sample size was assessed based on the change in CNFL from previous pilot data (unpublished). The study required a sample size of 31 (number of pairs) for each group (Obese with and without T2DM) to achieve a power of 80% and a level of significance of 5% (two sided), for detecting a CNFL mean difference of 1.35 mm/mm^2 between pairs and SD of 5.5 in obese participants without diabetes and a CNFL mean difference of 3.48 mm/mm^2 and a SD of 3.97 for obese participants with diabetes and effect size of 0.5 for both groups (with and without diabetes). To account for 10% loss to follow up, the study required 70 participants in total (35 participants in each group).

2.9. Study procedures

2.9.1. Medical history and demographic data

A full medical history was obtained from electronic medical records for all participants. Demographic data included date of birth, sex, and duration of disease.

Glycated hemoglobin (HbA1c %), lipid profile (total cholesterol (mmol/L), low-density and high-density lipoprotein cholesterol (LDL and HDL) (mmol/L) and triglycerides (mmol/L), liver function tests (bilirubin (μmol/L), total protein (g/L), Aspartate aminotransferase (AST) (IU/L), alanine transaminase (ALT) (IU/L)), and vitamin D level (25(OH)D (ng/ml)) were assessed as part of routine care and collected retrospectively from patients' records.

2.9.2. Anthropometry and body composition

All participants had their height (m), weight (kg) and BMI (kg/m^2) measured and recorded as part of their routine visit.

The weight and height of the obese children were measured using digital scales with light clothing, empty pockets and no shoes or socks. Weight (kg) was measured by the body composition analyzer (TANITA DC-430MAIII) and height (cm) by stadiometer (SECA model), and both were recorded to the nearest 0.1 g or cm, respectively (1). The cut-off points used to classify weight status was established by the International Obesity Task Force (IOFT)(2) and the WHO growth chart (3). Body composition was measured using the TANITA scale following the manual input of height, sex, and age of the participants. Children were asked to standstill with their feet touching all four metal plates of the scale. The following were measured by the Tanita-built-in equations: body fat percent (BF%), fat mass (kg), fat free mass (FFM) (kg), muscle mass (kg), total body water (TBW) (kg), TBW (%), and BMI (kg/mm^2). Ratio of Muscle-to-fat (MFR) was calculated manually using the muscle mass and fat mass. Height, weight, and BMI of the healthy control participants was measured as part of routine care and was collected from the electronic medical records **(Figure 3)**.

Figure 3. TANITA scale for body composition analysis.

2.9.3. Continuous Glucose Monitoring (CGM)

The FreeStyle Libre-1 CGM allows interstitial glucose measurements every minute for up to 14 days. After cleaning the back of the arm with an alcohol swab, the sensor applicator was applied to the sensor pack and the applicator was applied to the back of the arm where a fine plastic canula was inserted in the subcutaneous tissue to measure interstitial fluid glucose levels **(Figure 4)**.

Figure 4. Sensor applicator and pack.

The sensor was then linked to the mobile application using patient's or research phones. The mobile application (LibreLink) was linked to the study professional account to access the CGM data remotely. For those who did not have a smart phone, they were given a scanner to read their BG and data were extracted by linking the scanner device to the LibreView account to allow for data extraction. Patients were instructed to scan the sensor every 8 hours to avoid missing data. CGM is a minimally invasive procedure with the only potential complication being slight bruising where the canula is inserted. The sensor was worn for 4 days. Continuous glucose readings were accessed via LibreView account **(Figure 5)**. Patients were asked to stop sharing their data after 4 days.

Figure 5. Freestyle Libre sensor, scanner, and mobile application.

2.9.4. Neuropathy assessment

A. Corneal Confocal Microscopy (CCM)

Laser scanning in vivo corneal confocal microscopy was performed on all participants using the Heidelberg Retinal Tomograph (HRT III) with Rostock Corneal Module (Heidelberg Engineering, Heidelberg, Germany) **(Figure 6)**.

Both eyes of each participant were anaesthetized with two drops of Bausch & Lomb Minims ® (Oxybuprocaine hydrochloride 0.4% w/v). A drop of hypotears gel (Carbomer 0.2% eye gel) was used in both eyes. The technique of image capture and selection has been described in **Appendix 2**. Images from the Sub-basal nerve plexus (SBNP) layer (central and peripheral cornea), stromal layers and endothelial cells were captured. Images were analyzed using semi-automated nerve analysis software (CCMetrics; University of Manchester, Manchester, UK) (1). This software converts manual tracing of nerve tissues to measures of corneal nerve fibre density (CNFD) (no./mm^2), corneal nerve branch density (CNBD) (no./mm^2), corneal nerve fibre length (CNFL) (mm/mm^2), and corneal nerve fibre tortuosity (CNFT) (TC), with higher values indicating greater tortuosity. Keratocytes were analyzed using the CNBD metric to measure the average cell density (cells/mm^2) in each stromal layer (anterior, mid, and posterior).

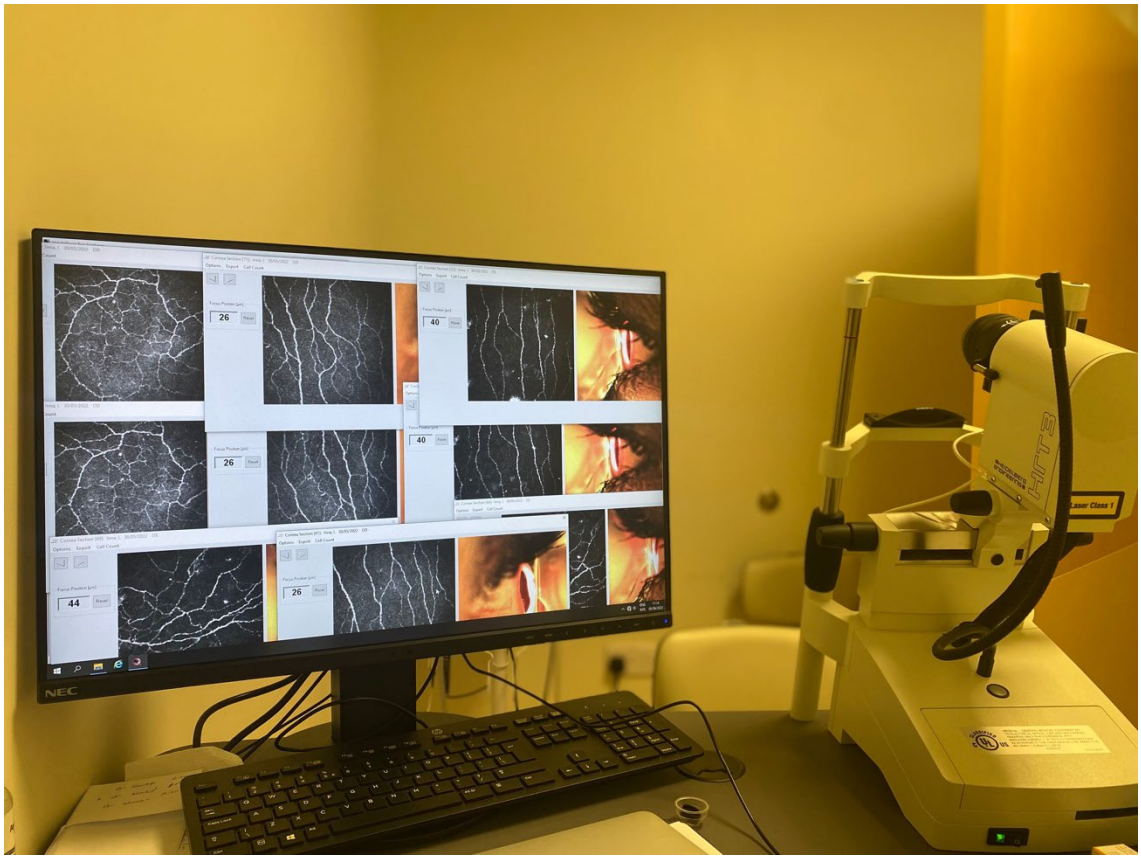


Figure 6. Corneal Confocal Microscopy (CCM) and CCM images.

B. Douleur Neuropathique en 4 (DN4) questionnaire

DN4 is a validated 10-items questionnaire for the diagnosis of neuropathic pain. Both validated English (3) and Arabic (4) versions were used as applicable. DN4 consists of 10 questions: 7 questions relating to the pain description (burning, painful cold, electric shocks) and associated abnormal sensations (tingling, pins and needles, numbness, itching) and 3 outcomes in the painful area for identifying hypoesthesia to touch and pin prick and allodynia to brushing. The scoring is based on a yes (1 point) or no (0 point) answer for each equally weighted question. The diagnosis of painful DPN (pDPN) is based on a DN4 questionnaire score of ≥4, which has a high sensitivity (80%) and specificity (92%) for pDPN (5).

C. Monofilament

Protective sensation was assessed using a 10 g monofilament (Semmes-Weinstein monofilament Examination) on both feet. Assessment was done by a single examiner in a quiet room and participants was instructed not to look where the examiner applies the filament. The filament was applied to the inner wrist of the participants to familiarize themselves with the pricking sensation. A sufficient force was then applied to cause the filament to bend on the site of the assessment for 2 seconds. The filament was applied twice to each site of the total 9 sites per foot **(Figure 7)**. Loss of protective sensation was recorded as "no feeling in ≥ 8 sites" (6).

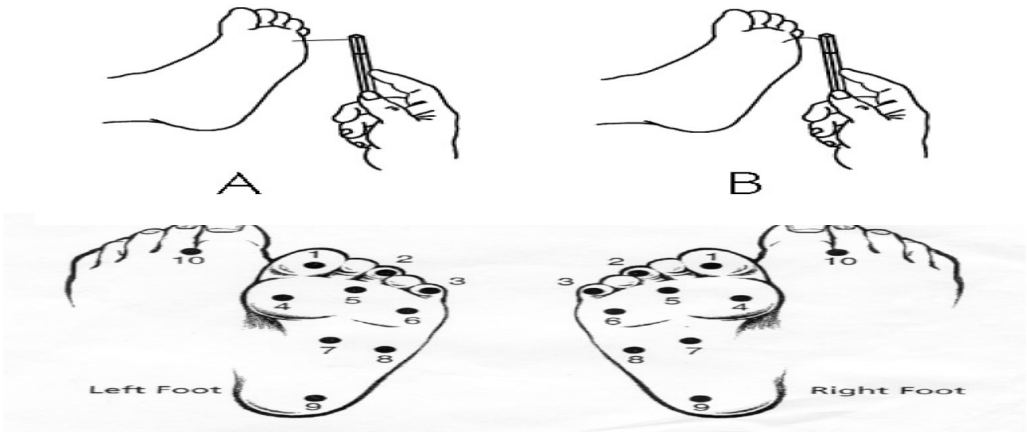


Figure 7. Monofilament examination sites.

D. Vibration perception threshold (VPT)

VPT was assessed in all obese participants by a single examiner. Participants were asked to remove their shoes, socks and relax for a few minutes before the examination. The examiner assured that participants had normal temperature in their lower limbs. The stimulator was applied on the pulp of the thumb to familiarize the participants with the vibration sensation before the actual measurements. The stimulator was then applied on the pulp of the big toe on both sides for each

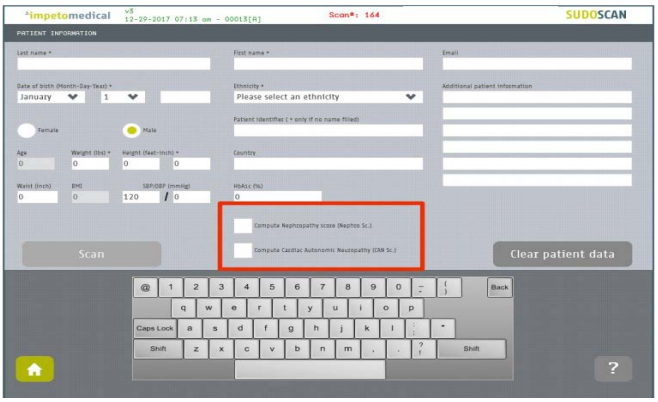
participant and the stimulus strength was increased slowly from zero until the vibration sensation was first perceived by indicating “yes”. Vibration sensation was recorded as an average for both feet in Volts (7). A VPT of ≥ 15V was considered as impaired vibration perception (8) **(Figure 8)**.

Figure 8. Vibration perception threshold.

E. Sudomotor function (Sudoscan)

Electrochemical skin conductance (ESC) using Sudoscan (Impeto Medical SAS) was measured in both hands and feet as described previously (9). Sudoscan evaluates sympathetic innervation based on sweat chloride concentration generated by the sweat gland in response to the voltage applied and is reported as ESC in microSiemens (µS).

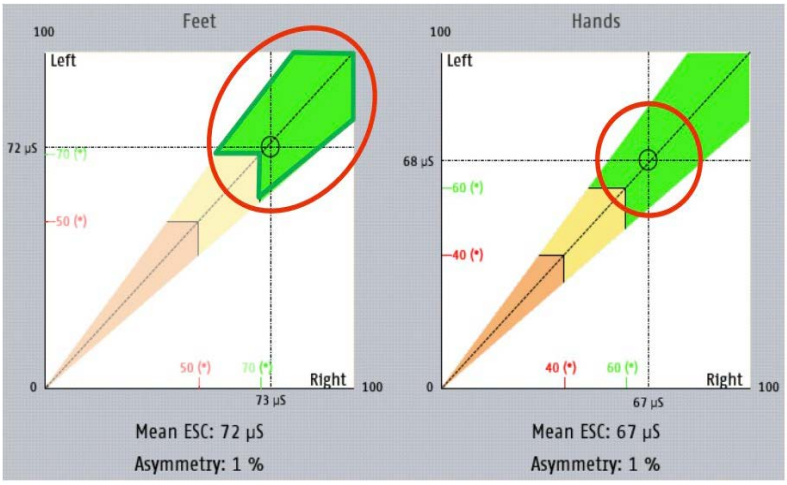
The following information was collected from each patient to input in SUDOSCAN before the examination: First name, last name, study ID, date of birth, sex, weight, and height.



The subject was then asked to take their shoes and socks off, stand with their bare feet on the foot sensor plates and place their hands on the hand sensor plates. Subjects were instructed to stand-still without movement for ~2 minutes **(Figure 9)**. For those who were unable to stand still for 2 minutes on the plates, they were asked to sit for the test.

Figure 9. Sudomotor function assessment using Sudoscan.

Results were recorded as the mean ESC for the hands and feet in micro Siemens (µS).



The key focus of this work was the use of corneal confocal microscopy as an ophthalmic imaging method to quantify corneal nerve damage in children and adults. Additionally, CCM was also used to assess potential mechanisms for nerve damage by assessing keratocytes.

The following chapters will present the data on the utility of CCM to detect early neuropathy in participants with diabetes and obesity.

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Chapter III – CORNEAL CONFOCAL MICROSCOPY FOR THE DIAGNOSIS OF DIABETIC PERIPHERAL NEUROPATHY: A SYSTEMATIC REVIEW AND META-ANALYSIS

Gad H, Petropoulos IN, Khan A, Ponirakis G, et al. Corneal confocal microscopy for the diagnosis of diabetic peripheral neuropathy: A systematic review and meta-analysis. J Diabetes Investig. 2022 Jan;13(1):134-147. doi: 10.1111/jdi.13643. Epub 2021 Aug 27. PMID: 34351711; PMCID: PMC8756328.

3.1. Abstract

INTRODUCTION: Corneal confocal microscopy (CCM) is a rapid non-invasive ophthalmic imaging technique that identifies corneal nerve fibre damage. Small studies suggest that CCM could be used to assess patients with distal symmetric polyneuropathy (DSPN).

AIM: To undertake a systematic review and meta-analysis assessing the diagnostic utility of CCM for sub-clinical DSPN ($DSPN^-$) and established DSPN ($DSPN^+$).

DATA SOURCES: Databases (PubMed, Embase, Central, ProQuest) were searched for studies using CCM in patients with diabetes up to April 2020.

STUDY SELECTION: Studies were included if they reported on at least one CCM parameter in patients with diabetes.

DATA EXTRACTION: Corneal nerve fibre density (CNFD), corneal nerve branch density (CNBD), corneal nerve fibre length (CNFL) and inferior whorl length (IWL) were compared between patients with diabetes with and without DSPN and controls. Meta-analysis was undertaken using RevMan V.5.3.

DATA SYNTHESIS: Thirty-eight studies including ~4000 participants were included in this meta-analysis. There were significant reductions in CNFD, CNBD, CNFL and IWL in $DSPN^-$ vs. controls ($P<0.00001$), $DSPN^+$ vs. controls ($P<0.00001$) and $DSPN^+$ vs. $DSPN^-$ ($P<0.00001$).

CONCLUSION: This systematic review and meta-analysis shows that CCM detects small nerve fibre loss in subclinical and clinical DPN and concludes that CCM has good diagnostic utility in DSPN.

KEYWORDS: CCM, Diabetic peripheral neuropathy, Diagnosis

3.2. Introduction

Distal symmetric polyneuropathy (DSPN) affects ~50% of patients with diabetes and leads to significant morbidity including neuropathic pain, erectile dysfunction, and foot ulceration (1). Currently, the diagnosis of DSPN in clinic relies on symptoms, loss of sensation to the 10g monofilament, neurological examination and occasionally electrophysiology (2). However, these methods do not reliably detect small nerve fibre damage which occurs in early DSPN (3).

In 2003, we showed that the ophthalmic technique of corneal confocal microscopy (CCM) can identify corneal small nerve fibre loss in patients with early and established DSPN (4). Subsequently we and others demonstrated good diagnostic utility for DPN (5-7), comparable to IENFD (8, 9). CCM also predicts incident DPN (8, 10) and identifies individuals at higher risk of developing DSPN (11). However, some studies have failed to demonstrate corneal nerve fibre loss in patients with and without DSPN (12, 13), which has been attributed to a small sample size (13) and variances in image acquisition and analysis protocols (14).

We have undertaken a systematic review and meta-analysis to generate a definitive single estimate for the diagnostic utility of CCM in sub-clinical and clinical DSPN.

3.3. Methods

3.3.1. Data Sources and Searches

This systematic review and meta-analysis is reported in accordance with MOOSE guidelines (15). The protocol was registered with the International Prospective Register of Systematic Reviews (PROSPERO) in November 2020 (CRD42018093498). Four databases were chosen to search for this systematic review: PubMed, EMBASE

(Ovid), CENTRAL and web of science (WoS)- (1900-present). In the PubMed and CENTRAL database both Mesh subject headings and keywords were searched; in Embase-(1988-present) Emtree subject headings and keywords were utilized. Numerous terms were tested for relevance and the final search strings for the three databases can be found in **Table 3**. Article language was limited to English and no date restrictions were set. A segment of the grey literature was searched through the use of dissertation and theses (ProQuest) and Clinicaltrials.gov. The databases were searched from inception to April 2020.

Table 3.Search details

PubMed
("microscopy, confocal"[MeSH] OR confocal microscopy[tiab] OR confocal microscopies[tiab] OR confocal microscope[tiab] OR confocal microscopic[tiab] OR (confocal[tiab] AND microscopical[tiab]) OR confocal image[tiab] OR confocal images[tiab] OR confocal imaging[tiab] OR confocal imagery[tiab] OR (confocal[tiab] AND picture[tiab]) OR (confocal[tiab] AND pictures[tiab]) OR CCM[tiab]) AND ("Cornea"[MeSH] OR cornea[tiab] OR corneas[tiab] OR corneal[tiab]) AND ("Peripheral Nervous System Diseases"[Mesh] OR "peripheral neuropathy"[tiab] OR "peripheral neuropathies"[tiab])
EMBASE (OVID: 1988-present)
1. exp confocal microscopy/ 2. (confocal microscop* or (confocal and microscopical) or confocal imag* or (confocal and picture*) or ccm).ab,ti. 3. 1 or 2 4. exp cornea/ 5. cornea*.ab,ti. 6. 4 or 5 7. exp peripheral neuropathy/ 8. (peripheral nervous system disease* or peripheral neuropath*).ab,ti. 9. 7 or 8 10. 3 and 6 and 9

CENTRAL	
#1	MeSH descriptor: [Microscopy, Confocal] explode all trees
#2	(confocal microscop* or (confocal and microscopical) or confocal imag* or (confocal and picture*) or ccm):ti,ab,kw
#3	#1 or #2
#4	MeSH descriptor: [Cornea] explode all trees
#5	cornea*:ti,ab,kw
#6	#4 or #5
#7	MeSH descriptor: [Peripheral Nervous System Diseases] explode all trees
#8	peripheral nervous system disease:ti,ab,kw
#9	peripheral nervous system diseases:ti,ab,kw
#10	peripheral neuropathy:ti,ab,kw
#11	peripheral neuropathies:ti,ab,kw
#12	(16-#11)
#13	(17, #6, #12)
WoS SCI-Expanded 1900-present	
	TS=(confocal microscopy OR "confocal microscopies" OR "confocal microscope" OR (confocal AND microscopic*) OR "confocal image" OR "confocal images" OR "confocal imaging" OR "confocal imagery" OR (confocal AND picture*) OR CCM) AND TS= (cornea*) AND TS=("peripheral neuropathy" OR "peripheral neuropathies") Indexes=SCI-EXPANDED Timespan=All years

ProQuest Dissertations & Theses
noft(confocal AND (microscop* OR image*)) AND noft(cornea*) AND noft(neuropath* OR diabet*)
Clinicaltrials.gov
(confocal) AND (cornea OR corneal) AND (diabetes OR diabetic OR neuropathy OR “nerve disorder”)

We included observational studies that reported on at least one of the following CCM parameters: corneal nerve fibre density (CNFD), corneal nerve branch density (CNBD), corneal nerve fibre length (CNFL), or inferior whorl length (IWL) in any of the following three groups: patients with type 1 and/or type 2 diabetes with distal symmetric polyneuropathy (DPN^+), without distal symmetric polyneuropathy ($DSPN^-$) and controls. Cross-sectional and longitudinal observational studies were included in this systematic review and meta-analysis. Narrative reviews, systematic reviews, correspondence, and case reports were excluded. Study country, age, diagnosis ($DSPN^+$, $DSPN^-$, control), duration of diabetes, HbA1c, software used for image analysis, CNFD, CNBD, CNFL and IWL were extracted when available. Studies using CCMetrics, ACCMetrics, ImageJ and other morphometric software to quantify CNFD, CNBD and CNFL were included. IWL was quantified using CCMetrics and ACCmetrics only. Data presented as median (IQR) was converted into mean ± SD using an online calculator and data presented as mean ± SEM was converted into mean ± SD using the RevMan calculator (18). HbA1c presented in (%) was also converted into (mmol/mol) using the NGSP calculator, where NGSP % must be between 3-20 (19).

Original studies that staged DSPN as per the diabetic neuropathy study group in Japan (DNSGJ) were classified as: $DSPN^-$ for stage I, $DSPN^+$ for stages II-V, for meta-analysis reporting purpose (20, 21). Stage I was reported as $DSPN^-$ and stages II-III were reported as $DSPN^+$ in this study (22). Patients classified according to the modified neuropathy disability score (NDS) were grouped as: scores between 0-2 ($DSPN^-$) and 3-10 ($DSPN^+$) (23, 24). No neuropathy was classified as $DSPN^-$ and mild-severe neuropathy was classified as $DSPN^+$ (25-28). No differentiation was made for either painful or painless DSPN and both were classified as $DSPN^+$ (29, 30). Where vibration perception threshold (VPT) was used, < 15V was classified as $DSPN^-$ and ≥15V as $DSPN^+$ (4).

3.3.2. Study Selection

After removal of duplicates, all citations were screened for relevance using the full citation, abstract and indexing terms, before excluding studies deemed as irrelevant. Where there was a lack of consensus a third (senior) author was consulted. The most recent and complete versions of the studies were reviewed for eligibility by two reviewers (HG and INP) according to the pre-specified inclusion and exclusion criteria. Full manuscripts of these potentially eligible citations were obtained. Two reviewers made the final inclusion and exclusion decisions independently and in case of disagreement, a third reviewer was consulted to resolve any conflicts. A flow chart of the search results was produced **(Figure 10)**. A data collection tool was developed to extract the data from each study. Data verification was undertaken by two reviewers (HG and INP). In the event of missing data, authors were emailed to obtain unpublished data.

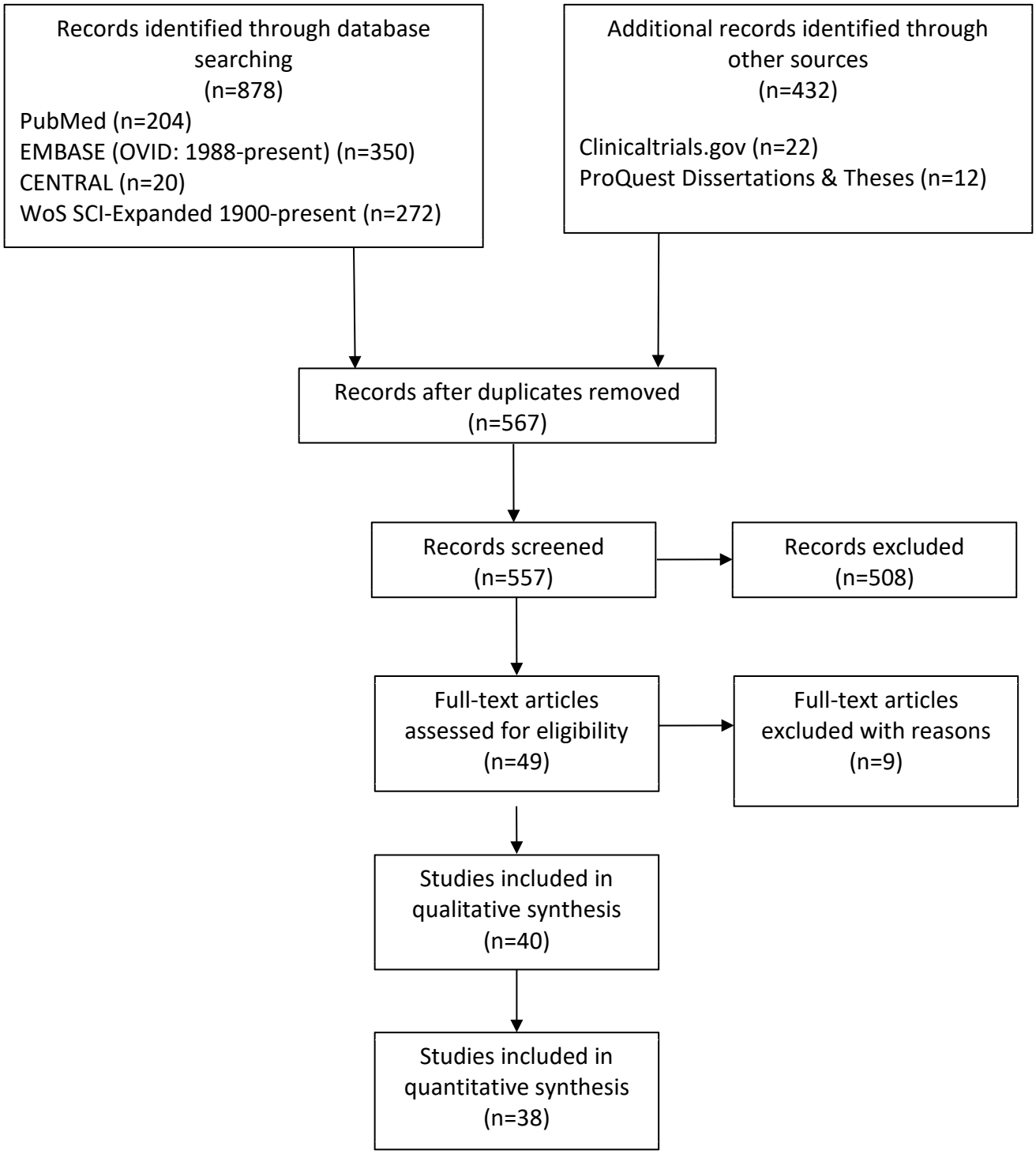


Figure 10. Flowchart of the included studies.

3.3.3. Data Extraction and Quality assessment

Included studies were assessed using the Cochrane Collaborations tool for assessing risk of bias (section 8.5) (31). The tool categorizes the risk of bias into high, moderate, low, or unclear risk. This tool assessed 6 domains: selection bias, performance bias, detection bias, attrition bias, reporting bias, and other bias, where applicable. Quality assessment was undertaken by two reviewers (AK and GP). If the risk of bias of a study was unclear, the effect of removing the study was checked and relevant outcomes were reported.

3.3.4. Data synthesis and Analysis

Meta-analysis was performed in RevMan (version 5.3) (32). Random effects meta-analysis was used in anticipation of heterogeneity due to differences in study population and type and duration of diabetes. The mean difference (MD) with 95% confidence interval (CI) was calculated for CNFD, CNBD, CNFL and IWL. Chi-squared (X^2) was used to test for difference between subgroups. The I^2 statistic was calculated, which is derived from Cochrane's chi-squared test Q and is used to describe the percentage of between-study variations attributed to variability in the true exposure effect (31). An I^2 value of 0-40% was classified as not important, 30-60% moderate, 50-90% substantial and 75-100% considerable (31).

3.3.5. Risk of Bias

Selection bias was assessed based on the study procedures, sequence generation and allocation concealment for the included studies **(Table 4)**. Inconsistency (such as reporting SE instead of SD) and lack of information (such as obtaining results from

figures) during data extraction from original articles were considered. The overall risk of bias for the assessed outcomes was unclear or low.

3.3.6. Sensitivity analysis

In the event of small study effects, sensitivity analysis was carried out to examine how the results of the meta-analysis change under different assumptions. For the sake of adjustment for heterogeneity and small study effects, we used comparison of fixed and random effects models **(10.4.4.1)** and the trim and fill strategy **(10.4.4.2)** as per the Cochrane recommendations (33).

A. Comparing fixed and random-effects estimates

Random effects meta-analysis was used for all study variables in anticipation of heterogeneity due to differences in study design and population. For variables that presented a significant publication bias (Egger's test $P<0.05$), we applied the fixed effects model to account for the presence of small study effects. Heterogeneity remained the same for CNFL, CNFD and IWL. Changing random effect to fixed effect led to a significant change in the effect size of the ImageJ subgroup from Z=1.91 (P=0.06) to Z=2.35 (P=0.02) and in the morphometry software subgroup from Z=1.81 (P=0.07) to Z=3.13 (P=0.002), however the overall effect size of all groups remained unchanged. Thus, the forest plot of CNBD in the $DSPN^{+}$ vs. $DSPN^{-}$ remained in the random effect model.

B. Trim and fill strategy

When removing the small studies to correct for the funnel plot asymmetry (Egger's test $P<0.05$), P-values for Egger's test remained the same for CNFD, CNBD, CNFL and IWL. All studies were included to calculate the overall effect size for the meta-analysis.

Table 4. Risk of bias assessment for non-randomized studies.

Study	Risk of Bias				
	Random sequence generation (selection bias)	Allocation concealment (selection bias)	Blinding of participants and personnel (performance bias)	Blinding of outcome assessment (detection bias)	Selective reporting (reporting bias)
Ahmed et al (34)	N/A	?	N/A	?	?
Alam et al (9)	N/A	?	N/A	-	?
Azmi et al (35)	N/A	N/A	?	-	-
Ishibashi et al (20)	N/A	N/A	N/A	N/A	-
Chen et al (36)	N/A	?	N/A	?	?
Ishibashi et al (21)	N/A	N/A	N/A	N/A	-
Brines et al (23)	N/A	N/A	N/A	N/A	-
Li et al (37)	N/A	?	N/A	?	?
Petropoulos et al (8)	N/A	?	N/A	-	?
Chen et al (8)	N/A	-	N/A	-	?
Ishibashi et al (24)	N/A	N/A	N/A	-	-
Xiong et al (25)	N/A	N/A	N/A	N/A	-
Pritchard et al (38)	N/A	?	N/A	?	?
Tummanapalli et al (39)	N/A	N/A	N/A	N/A	-
Ishibashi et al (40)	N/A	N/A	N/A	N/A	-
Petropoulos et al (41)	N/A	?	N/A	?	?
Petropoulos et al (42)	N/A	-	N/A	-	?
Pritchard et al (43)	N/A	-	N/A	-	?
Dehghani et al (44)	N/A	N/A	N/A	N/A	-
Ostrovski et al (45)	N/A	?	N/A	-	?
Tummanapalli et al (46)	N/A	N/A	N/A	N/A	-

Study	Risk of Bias				
	Random sequence generation (selection bias)	Allocation concealment (selection bias)	Blinding of participants and personnel (performance bias)	Blinding of outcome assessment (detection bias)	Selective reporting (reporting bias)
Tummanapalli et al (47)	N/A	N/A	N/A	N/A	-
Puttgen et al (29)	N/A	N/A	?	?	-
Lovblom et al (10)	N/A	?	N/A	?	?
Pritchard et al (48)	N/A	?	N/A	?	?
Ponirakis et al (49)	N/A	?	N/A	?	?
Edwards et al (50)	N/A	N/A	?	?	-
Quattrini et al (26)	N/A	N/A	N/A	-	-
Tavakoli et al (51)	N/A	N/A	N/A	-	-
Sivaskandarajah et al (52)	N/A	?	N/A	?	?
Tavakoli et al (27)	N/A	-	N/A	?	?
Hertz et al (28)	N/A	?	N/A	-	?
Kalteniece et al (53)	N/A	N/A	N/A	N/A	-
Kalteniece et al (30)	N/A	N/A	?	?	-
Dehghani et al (54)	N/A	N/A	N/A	N/A	-
Malik et al (4)	N/A	N/A	N/A	N/A	-
Ponirakis et al (55)	N/A	N/A	?	?	-
Andersen et al (12)	N/A	N/A	N/A	-	-

Table 5. Characteristics of the included studies.

Study	Country	Group	N	Age (years)	Duration of diabetes (years)	HbA1c % mmol/mol	CCM type	Software for image analysis	Assessment with CCM			
									CNFD	CNBD	CNFL	IWL
Ahmed et al (34)	Canada	DSPN+ DSPN- Control	33 56 64	50±14.3 34.9±14.8 38.9±17.6	31.4±13.5 17.6±14 N/A	8.7±2.1~ 72 7.4±1.3 ~ 57 NS	HRT-II	CCMetrics	√	√	√	
Ostrovski et al (45)	Canada	DSPN+ DSPN- Control	13 13 20	56.2±8.7 30.3±13.7 41.3±17.3	34.8±13 10.7±6.2 N/A	8.5±2.2 ~ 69 7.5±1.3 ~ 58 5.5±0.4 ~ 37	HRT-III	CCMetrics ACCMetrics	√	√	√	
Lovblom et al (10)	Canada	DSPN+ DSPN-	11 54	38±16 34±15	21±9 17±12	8.1±1.6 ~ 65 7.6±1.3 ~ 60	HRT-III	CCMetrics	√	√	√	
Sivaskandarajah et al (52)	Canada	DSPN+ DSPN- Control	33 63 64	48.5±13.7 32.7±13.6 38.3±16.4	32.3±13.1 17.3±12.2 N/A	8.4±1.6 ~ 68 7.5±1.2 ~ 58 5.6±0.4 ~ 38	HRT-III	CCMetrics	√	√	√	
Hertz et al (28)	Canada	DSPN+ DSPN- Control	14 12 20	NS NS 41.4±17.3	NS NS N/A	NS NS 5.5±0.4 ~ 37	HRT-III	CCMetrics	√	√	√	
Alam et al (9)	UK	DSPN+ DSPN- Control	31 30 27	53.3±11.9 38.8±12.5 41±14.9	37.2±13.1 17.2±12 N/A	8.5±1.5 ~69 8±1.3 ~ 64 5.5±0.3	HRT-III	CCMetrics	√	√	√	
Azmi et al (35)	UK	DSPN+ Control	29 32	61.9±12.3 47.7±1.6	46±13.9 N/A	8.3±1.3 5.7±0.6	HRT-III	ACCMetrics	√	√	√	
Chen et al (36)	UK	DSPN+ DSPN- Control	29 63 84	63±12 44±15 46±15	19.9±11.7 20±11.1 N/A	8.6±3.6 ~ 70.4±16 8±4.1 ~ 63.9±21.2 5.6 ~ 37.4±3.5	HRT-III	CCMetrics ACCMetrics	√	√	√	

Study	Country	Group	N	Age (years)	Duration of diabetes (years)	HbA1c % mmol/mol	CCM type	Software for image analysis	Assessment with CCM			
									CNFD	CNBD	CNFL	IWL
Brines et al (23)	UK	DSPN+ DSPN- Control	60 21 48	35.3±14.3 37.1±16.5 46.2±16.9	35.3±14.3 17.9±15.1 N/A	8.2±1.3 ~ 66 7.9±1.3 ~ 63 5.7±0.3 ~ 39	HRT-III	ACCMetrics	√	√	√	
Petropoulos et al (56)	UK	DSPN+ DSPN- Control	25 28 15	60.1±10.2 42.4±14.7 NS	24.8±19.5 16.2±9.3 N/A	7.6±1.5 ~ 60 NS 5.4±0.5 ~ 36	HRT-III	CCMetrics	√		√	√
Chen et al (8)	UK	DSPN+ DSPN- Control	17 46 26	59±11 44±13 44±15	39±14 23±15 N/A	8.5±1.3 ~ 69 8.2±1.4 ~ 66 5.5±0.3 ~ 37	HRT-III	CCMetrics ACCMetrics	√	√	√	
Petropoulos et al (41)	UK	DSPN+ DSPN- Control	61 50 47	56.5±13.2 44.2±15.6 52±13.2	35.33±14.3 23±14 N/A	8.4±1.8 ~ 68 7.9±1.7 ~ 63 5.6±0.3 ~ 38	HRT-III	CCMetrics	√	√	√	
Petropoulos et al (42)	UK	DSPN+ DSPN- Control	100 86 55	NS NS 51.7±11.4	34.4±17.3 24.2±21.2 N/A	7.9±1.6 ~ 63 7.7±1.6 ~ 61 5.5±0.3 ~ 37	HRT-III	CCMetrics ACCMetrics	√	√	√	
Ponirakis et al (49)	UK	DSPN+ DSPN-	46 64	60.75±8.9 45.5±14.4	36.5±14.4 22.25±13	8.6±0.4 ~ 70 7.62±0.48 ~ 60	HRT-III	CCMetrics	√	√	√	
Quattrini et al (26)	UK	DSPN+ DSPN- Control	44 10 15	59.3±17.25 43.5±10.2 55±18.5	NS NS N/A	8.01±2.32 ~ 64 7.16±1.26 ~ 55 NS	Confoscan-P4	Morphometric software	√	√	√	
Tavakoli et al (51)	UK	DSPN+ DSPN- Control	67 34 17	59±18.2 55±11.1 55±19.8	17.8±29.55 10.7±10.6 N/A	8.2±2.70 ~ 66 8.1±1.57 ~ 65 <6.5 <48	Confoscan-P4	Morphometric software	√	√	√	
Tavakoli et al (27)	UK	DSPN+ DSPN- Control	96 42 26	59±20 57±13 53±3	59±20 57±13 N/A	8.30±3.14 ~ 67 7.88±1.23 ~ 63 ~5.8 ~ 40	Confoscan-P4	CCMetrics	√	√	√	

Study	Country	Group	N	Age (years)	Duration of diabetes (years)	HbA1c % mmol/mol	CCM Type	Software for image analysis	Assessment with CCM			
									CNFD	CNBD	CNFL	IWL
Kalteniece et al (53)	UK	DSPN+ DSPN- Control	69 47 22	62.08±11.6 46.9±13.2 50.32±13.7	20.78±17.8 16.04±12.2 N/A	7.19±1.16 ~ 55 7.72±2.06 ~ 61 5.48±0.42 ~ 36	HRT-III	CCMetrics	√	√	√	√
Kalteniece et al (30)	UK	DSPN+ Control	140 30	65.09±1.13 61.2±1.33	21.8±2.05 N/A	7.5±0.17 ~ 58 5.63±0.06 ~ 38	HRT-III	CCMetrics	√	√	√	√
Malik et al (4)	UK	DSPN+ DSPN- Control	14 4 18	59.2±9.9 53±18.5 57.8±11.5	23.4±6.25 21.3±3.6 N/A	8.15±1.3 ~ 66 7.8±0.8 ~ 62 <6.5 ~ 48	Confoscan-P4	Morphometric software	√	√	√	
Ponirakis et al (55)	UK	DSPN+ DSPN- Control	33 41 70	64.1±1.79 44.3±2.19 41.8±1.63	37.6±3.2 23.3±2.03 N/A	7.9±0.26 ~ 63 7.5±0.18 ~ 58 5.29±0.12 ~ 34	HRT-III	ACCMetrics	√			
Puttgen et al (29)	Germany	DSPN+ Control	116 46	67.3±9 66±5.2	17.6±13 N/A	7.41±1.3 ~ 57 5.44±0.23 ~ 36	HRT-III	CCMetrics ACCMetrics	√	√	√	
Andersen et al (12)	Denmark	DSPN+ DSPN- Control	27 117 25	71.4±3.1 69.7±2.7 71.2±0.69	12.2±1.23 11.67±1.12 N/A	6.95±0.48 ~ 52 6.6±0.33 ~ 49 5.5±0.22 ~ 37	HRT-III	ACCMetrics	√	√	√	
Tummanapali et al (39)	Australia	DSPN+ DSPN- Control	28 35 34	NS	NS	8.45±0.5 ~ 69 7.59±0.6 ~ 59	HRT-III	ACCMetrics	√	√	√	√
Dehghani et al (44)	Australia	DSPN+ DSPN- Control	13 20 17	NS	NS	NS	HRT-III	CCMetrics ACCMetrics			√	
Tummanapalli et al (46)	Australia	DSPN+ DSPN- Control	23 27 29	47±15 32±10 37±11	22±13 15~±9 N/A	8.89±1.9 ~ 74 7.83±1.02 ~ 62 NS	HRT-III	ACCMetrics	√	√	√	√
Tummanapalli et al (47)	Australia	DSPN+ DSPN-	35 35	51±9.5 44.5±11	NS	8±1.4 ~ 64 8±2 ~ 64	HRT-III	ACCMetrics	√	√	√	√
Pritchard et al (48)	Australia	DSPN+ DSPN- Control	25 82 80	NS NS 37.0 ±17.8	NS	NS	HRT-III	CCMetrics			√	√
Edwards et al (50)	Australia	DSPN+ DSPN- Control	88 143 61	58±9 48±16 52±14	23±14 14±12 N/A	8.2±1.7 ~ 66 7.8±1.2 ~ 62 5.4±0.3 ~ 36	HRT-III	CCMetrics		√	√	√
Dehghani et al (54)	Australia	DSPN+ DSPN- Control	39 108 60	NS NS NS	NS NS N/A	NS	HRT-III	ACCMetrics	√	√	√	

Study	Country	Group	N	Age (years)	Duration of diabetes (years)	HbA1c % mmol/mol	CCM Type	Software for image analysis	Assessment with CCM			
									CNFD	CNBD	CNFL	IWL
Ishibashi et al (20)	Japan	DSPN+ DSPN- Control	55 23 28	56.4±14.1 48.1±10.6 50.2±7.41	9.6±16.3 5.8±5.8 N/A	8.03±3.0 ~ 64 7.7±2.11 ~ 61 5.6±0.26 ~ 38	HRT-III	ImageJ	√	√	√	
Ishibashi et al (21)	Japan	DSPN+ DSPN- Control	153 47 40	56.03±10.3 53.4±7.54 53.6±12.65	12.4±8.2 10.5±14.8 N/A	8.3±3.5 ~ 67 7.3±1.4 ~ 56 5.7±0.32 ~ 39	HRT-III	ImageJ	√	√	√	
Ishibashi et al (24)	Japan	DSPN+ DSPN- Control	115 47 45	54.4±19.1 52.4±9.6 52.8±4.7	7.9±11.4 5±4.5 N/A	9.06±4.4 ~ 76 8.5±1.4 ~ 69 5.5±0.03 ~ 37	HRT-III	ImageJ	√	√	√	
Ishibashi et al (40)	Japan	DSPN+ DSPN- Control	18 57 42	59.4±8.1 54.4±12.1 53.1±11.7	13.6±10.61 6.7±6.34 N/A	9±1.74 ~ 75 9.1±2.4 ~ 76 5.7±0.4 ~ 39	HRT-III	ImageJ	√	√	√	
Li et al (37)	China	DSPN+ DSPN- Control	79 49 24	70.15±7.34 67.12±6.01 68.3±5.19	12.58±7.28 9.79±7.09 N/A	7.94±1.86 ~ 63 7.07±0.96 ~ 54 5.88±0.82 ~ 41	HRT-II	CCMetrics ACCMetrics	√	√	√	
Xiong et al (25)	China	DSPN+ DSPN- Control	79 49 24	70.3±10 67.12±6.13 68.63±5.2	12.57±10.2 9.79±7.14 N/A	7.95±3.4 ~ 63 7.07±1.68 ~ 54 5.88±0.83 ~ 41	HRT-II	ImageJ	√	√	√	
Pritchard et al (38)	Australia, Canada, UK	DSPN+ DSPN-	16 74	51±14 42±16	29±16 15±12	8±1.1 ~ 64 7.9±1.2 ~ 63	HRT-III	CCMetrics			√	
Pritchard et al (43)	Australia, UK	DSPN+ DSPN- Control	48 100 60	57±11 43±16 46±15	34±16 20±15 N/A	8.6±1.8 ~ 70 8±1.2 ~ 64 5.5±0.3 ~ 37	HRT-III	CCMetrics		√	√	

Data presented as mean ± SD. CNFD: corneal nerve fibre density, CNBD: corneal nerve branch density, CNFL: corneal nerve fibre length, IWL: inferior whorl length, NS: not stated, N/A: not applicable

3.4.1. Corneal Nerve Fibre Density

DSPN^{+} vs. DSPN^{-}

Twenty-nine studies (4, 8-10, 12, 20, 21, 23-28, 34, 36, 37, 39-42, 45, 47, 49, 51-53, 55) with 3214 (1677 DSPN^{+} and 1537 DSPN^{-}) participants were included in the meta-analysis. CNFD (fibre/mm^2) was significantly lower in DSPN^{+} compared to DSPN^{-} (MD= -7.01, 95% CI -7.45 to 6.57, *P*<0.00001) (CCMetrics (MD=-6.83, 95% CI -7.82 to -5.84, *P*<0.00001), ACCMetrics (MD=-7.77, 95% CI -8.32 to -7.22, *P*<0.00001), ImageJ (MD= -3.48, 95% CI -4.64 to -2.33, *P*<0.00001) and morphometric software (MD=-11.40, 95% CI -15.42 to -7.38, *P*<0.00001)). There was a significant difference in the magnitude of CNFD reduction in the DPN^{+} group between studies (X^2=19.32, *P*=0.0002) **(Figure 11)**.

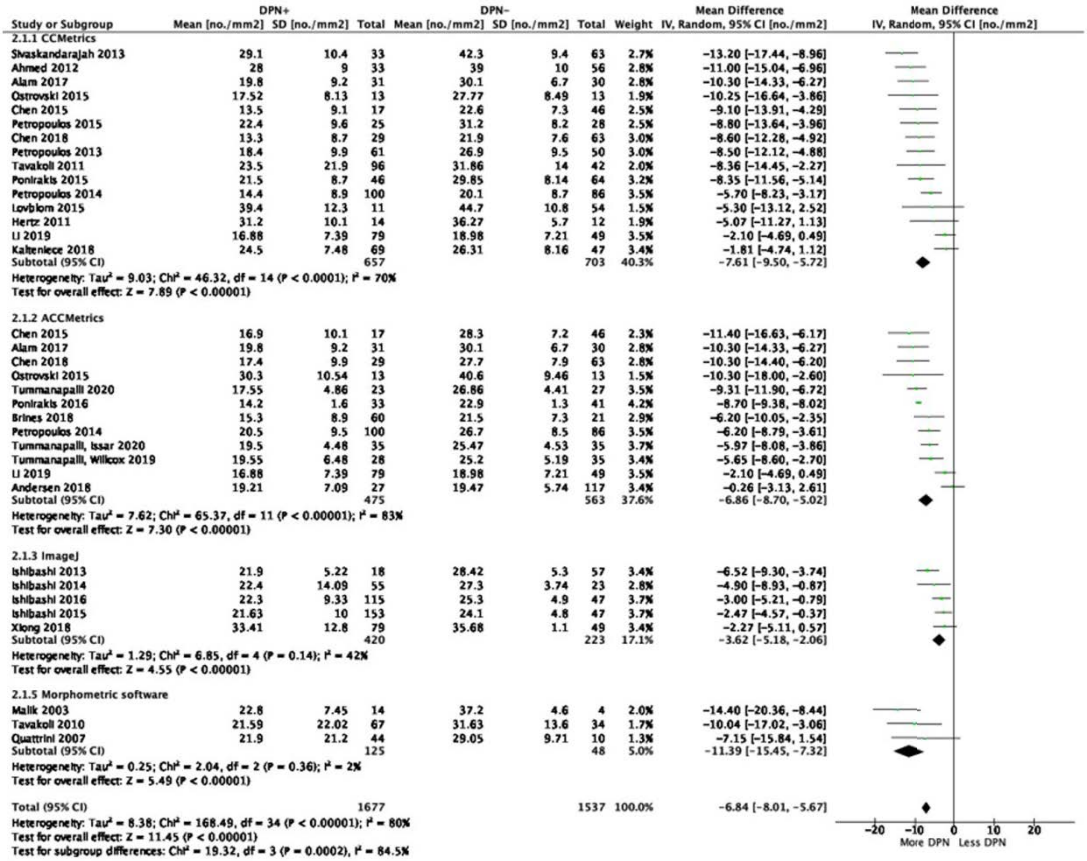


Figure 11. Forest plots of corneal nerve fibre density (CNFD) in patients with DSPN+ and without DSPN-.

DSPN$^+$ vs. Control

Twenty-nine studies (4, 8, 9, 12, 20, 21, 23-30, 34-37, 39-42, 45, 47, 51-54) with 3325 (1971 DSPN$^+$ and 1354 control) participants were included in the meta-analysis. CNFD (fibre/mm^2) was significantly lower in DSPN$^+$ compared to controls (MD=-11.94, 95% CI -12.25 to -11.62, *P*<0.00001) (CCMetrics (MD=-10.83, 95% CI -11.26 to -10.40, *P*<0.00001), ACCMetrics (MD=-13.75, 95% CI -14.26 to -13.25, P<0.00001), ImageJ (MD= -8.98, 95% CI -10.40 to -7.55, *P*<0.00001) and morphometric software (MD=-22.26, 95% CI -27.67 to -16.85, *P*< 0.00001)). There was a significant difference in the magnitude of CNFD reduction in the DPN$^+$ group between studies (X^2=15.50, *P*=0.001) **(Figure 12)**.

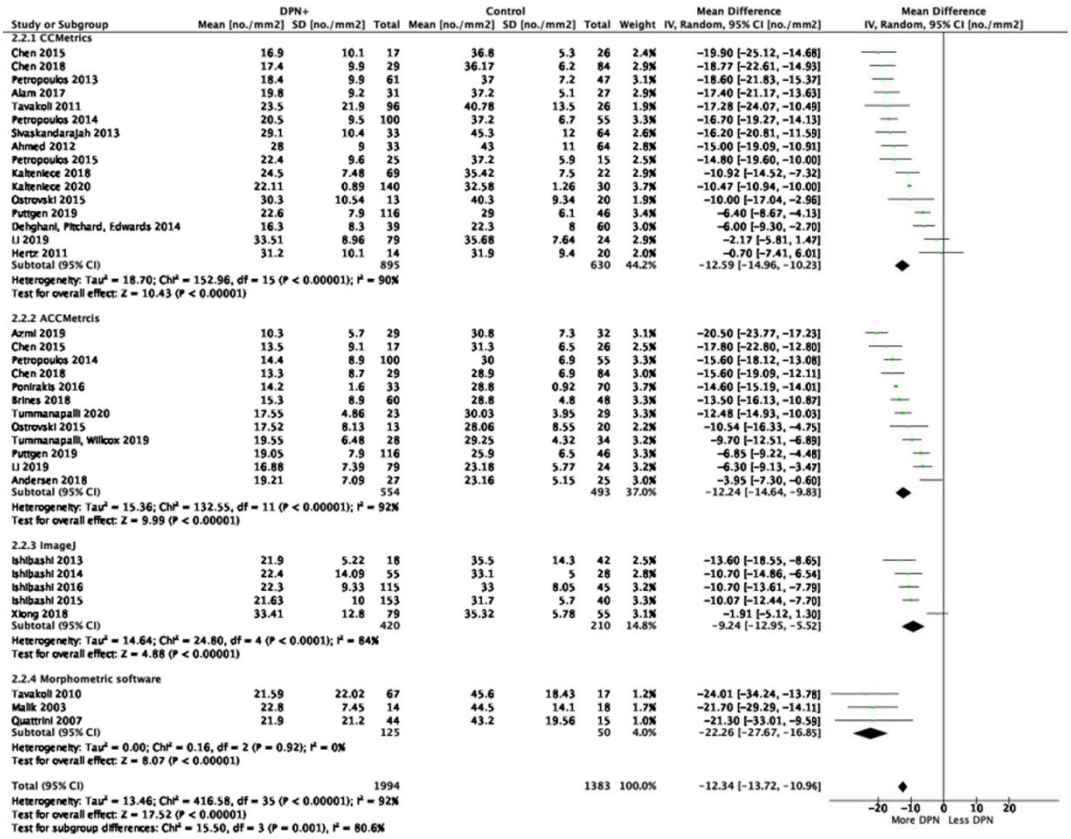


Figure 12. Forest plots of corneal nerve fibre density (CNFD) in patients with DSPN+ and healthy control.

DSPN⁻ vs. Control

Twenty-seven studies (4, 8, 9, 12, 20, 21, 23-28, 34, 36, 37, 39-42, 45, 47, 51-55) with 3035 (1620 DPN⁻ and 1415 control) participants were included in the meta-analysis. CNFD (fibre/mm^2) was significantly lower in DSPN⁻ compared to control (MD=-5.85, 95% CI -6.12 to -5.57, *P*<0.00001) (CCMetrics (MD=-5.76, 95% CI -6.15 to -5.37, *P*<0.00001), ACCMetrics (MD= -5.91, 95% CI -6.32 to -5.50], P<0.00001), ImageJ (MD= -5.89, 95% CI -7.13 to -4.65, *P*<0.00001) and morphometric software (MD=-11.07, 95% CI -16.34 to -5.80, *P*<0.0001)). There was no significant difference in the magnitude of CNFD reduction in the DSPN⁻ group between studies (X^2=4.01, *P*=0.26) **(Figure 13)**.

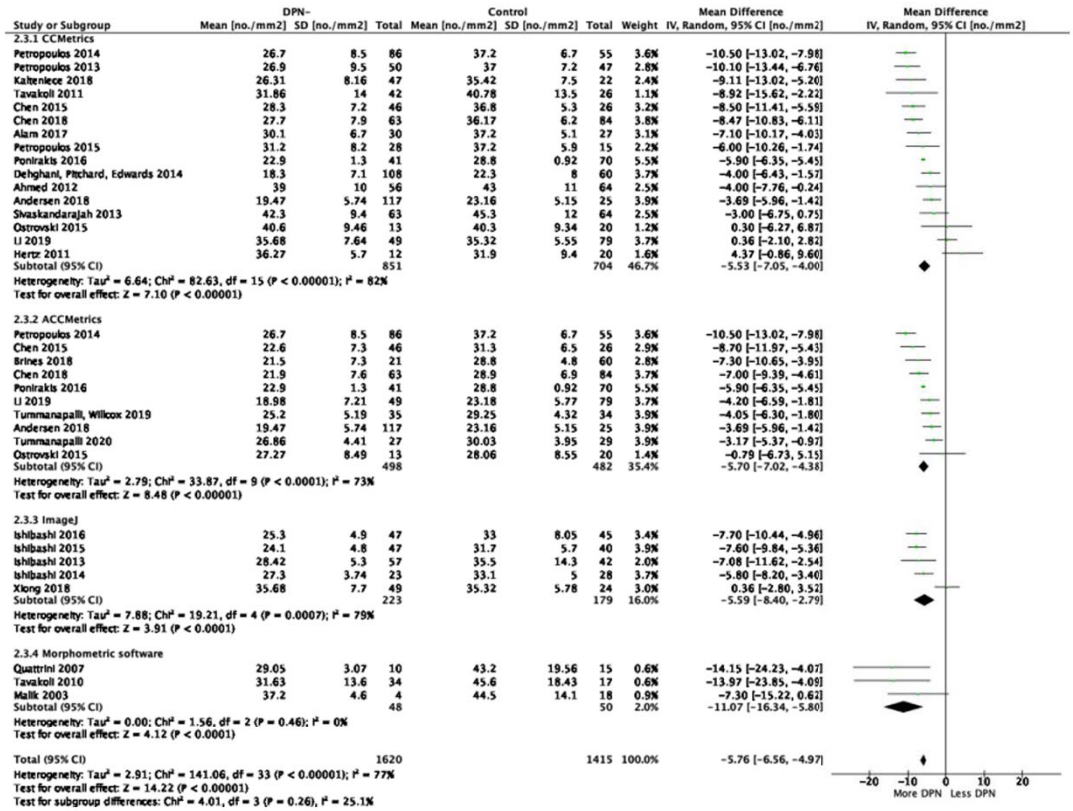


Figure 13. Forest plots of corneal nerve fibre density (CNFD) in patients without DSNP- and healthy control.

3.4.2. Corneal Nerve Branch Density

DSPN⁺ vs. DSPN⁻

Thirty studies (4, 8-10, 12, 20, 21, 23-28, 34, 36, 37, 39-43, 45, 47, 49-54) with 3552 (1763 DPN^+ and 1789 $DSPN^-$) participants were included in the meta-analysis. CNBD (branch/mm^2) was significantly lower in $DSPN^+$ compared to $DSPN^-$ (MD= -3.36, 95% CI -4.11 to -2.61, *P*<0.00001) (CCMetrics (MD=-10.37, 95% CI -12.56 to -8.18, *P*<0.00001) and ACCMetrics (MD=-8.20, 95% CI -10.20 to -6.20, *P*<0.00001). There was a significant difference in the extent of CNBD reduction in the $DSPN^+$ group between studies (X^2=30.97, *P*<0.00001), **(Figure 14)**.

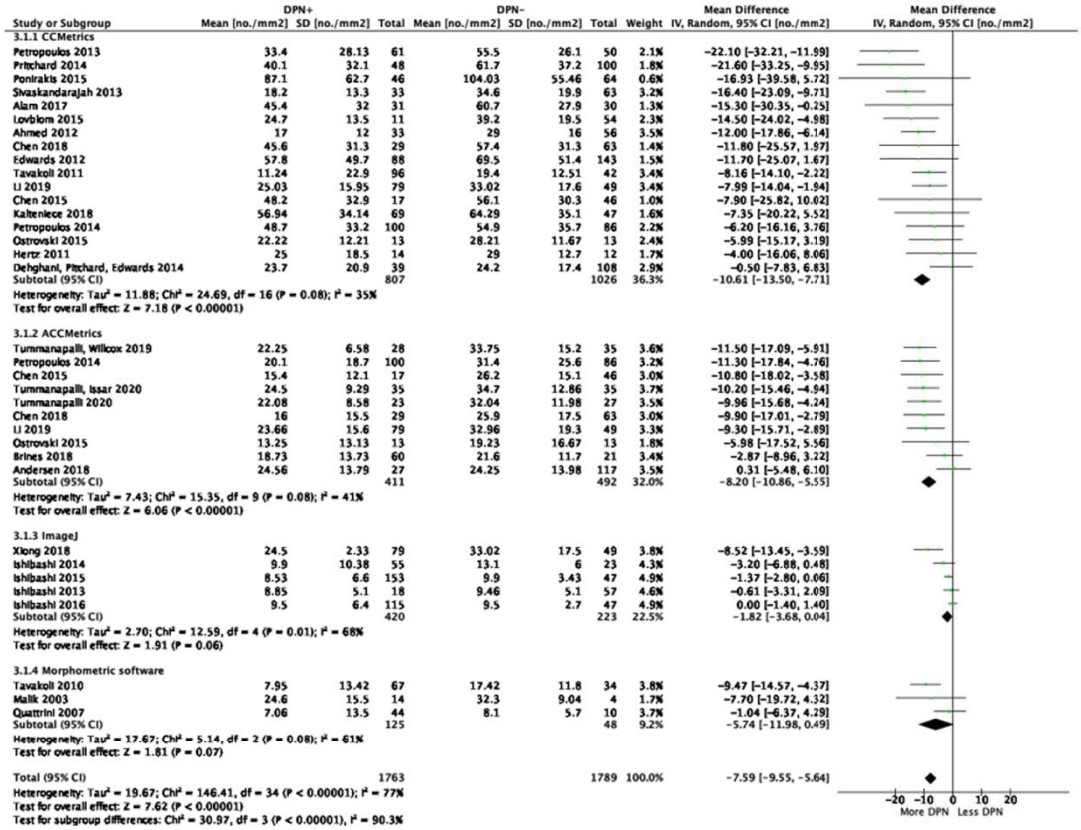
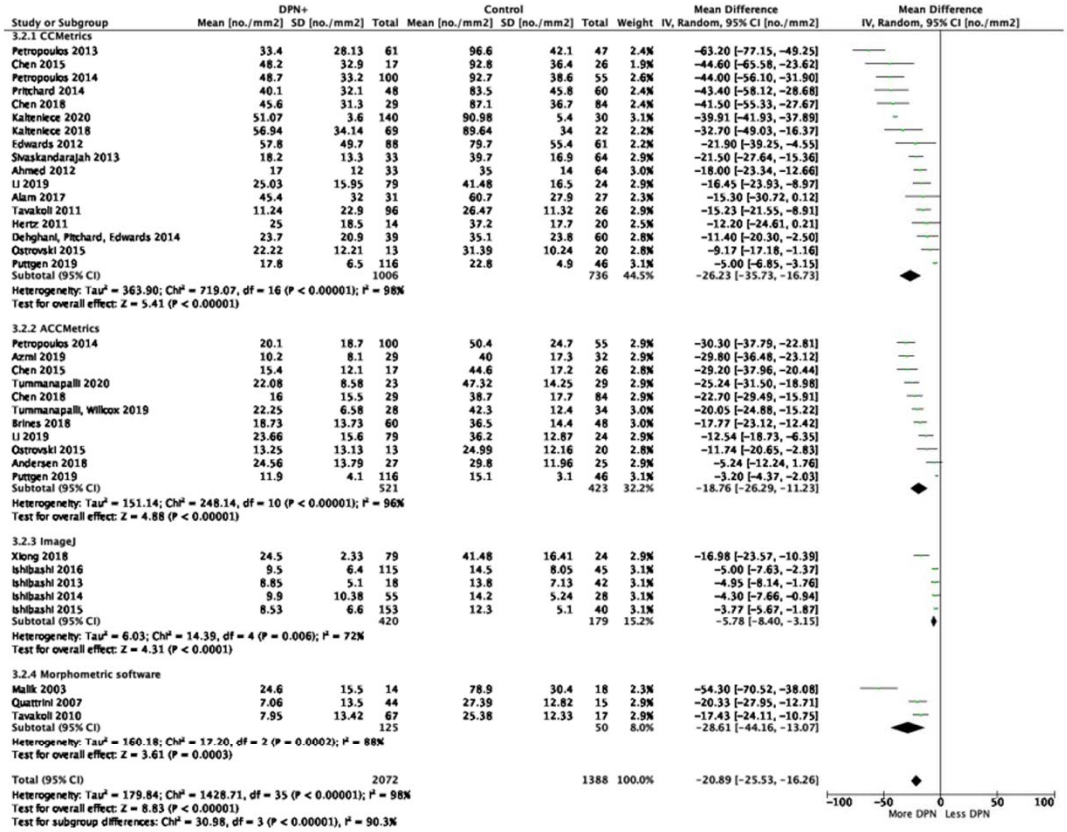


Figure 14. Forest plots of corneal nerve branch density (CNBD) in patients with DSPN+ and without DSNP-.

DSPN⁺ vs. Control

Thirty studies (4, 8, 9, 12, 20, 21, 23-30, 34-37, 39-42, 45, 47, 50-54) with 3460 (2072 DPN⁺ and 1388 control) participants were included in the meta-analysis. CNBD (branch/mm^2) was significantly lower in DSPN⁺ compared to controls (MD=-11.00, 95% CI -11.65 to -10.35, *P*<0.00001) (CCMetrics (MD=-20.87, 95% CI -22.05 to -19.68, *P*<0.00001), ACCMetrics (MD=-7.34, 95% CI -8.35 to -6.32, *P*<0.00001), ImageJ (MD= -4.79, 95% CI -6.05 to -3.53, *P*<0.0001) and morphometric software (MD=-21.81, 95% CI -26.61 to -17.01, *P*=0.0003)). There was a significant difference in the magnitude of CNBD reduction in the DSPN⁺ group between studies (X^2=30.98, *P*<0.00001) **(Figure 15)**.



15. Forest plots of corneal nerve branch density (CNBD) in patients with DSPN+ and healthy control.

DSPN- vs. Control

Twenty-six studies (4, 8, 12, 20, 21, 23-26, 28, 34, 36, 37, 39-43, 45, 47, 50-54) with 2813 (1606 DSPN- and 1207 control) participants were included in the meta-analysis. CNBD (branch/mm^2) was significantly lower in DSPN- compared to controls (MD=-6.37, 95% CI -7.31 to -5.44, *P*<0.00001) (CCMetrics (MD=-11.08, 95% CI -13.40 to -8.75, *P*<0.00001), ACCMetrics (MD= -11.17, 95% CI -13.46 to -8.88, *P*<0.00001), ImageJ (MD=-3.34, 95% CI -4.52 to -2.17, *P*<0.0001) and morphometric software (MD= -16.26, 95% CI -21.14 to -11.37, *P*=0.007)). There was a significant difference in the magnitude of CNBD reduction in the DSPN- group between studies (X^2=33.32, *P*<0.0001).

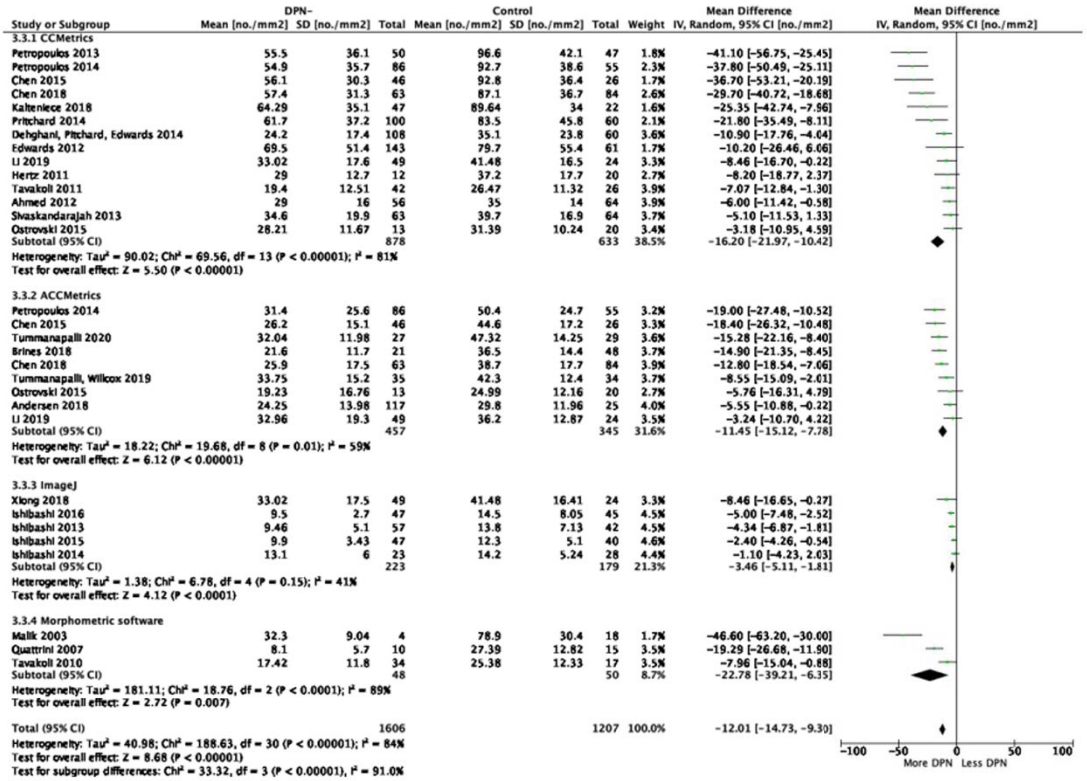


Figure 16. Forest plots of corneal nerve branch density (CNBD) in patients without DSNP- and healthy control.

3.4.3. Corneal Nerve Fibre Length

DSPN+ vs. DSPN-

Thirty-four studies (4, 8-10, 12, 20, 21, 23-28, 34, 36, 37, 39-45, 47-54, 57) with 3868 (1855 $DSPN^+$ and 2013 $DSPN^-$) participants were included in the meta-analysis. CNFL (mm/mm^2) was significantly lower in $DSPN^+$ compared to $DSPN^-$ (MD= -3.08, 95% CI -3.58 to -2.58, *P*<0.00001) (CCMetrics (MD= -3.74, 95% CI -4.49 to -2.99, *P*<0.00001), ACCMetrics (MD=-2.80, 95% CI -3.57 to -2.04, *P*<0.00001), ImageJ (MD= -1.57, 95% CI -2.06 to -1.09, *P*<0.00001) and morphometric software (MD=-3.49, 95% CI -5.63 to -1.35, *P*=0.001)). There was a significant difference in the magnitude of CNFL reduction in the $DSPN^+$ group between studies (X^2=25.42, *P*<0.00001) **(Figure 17)**.

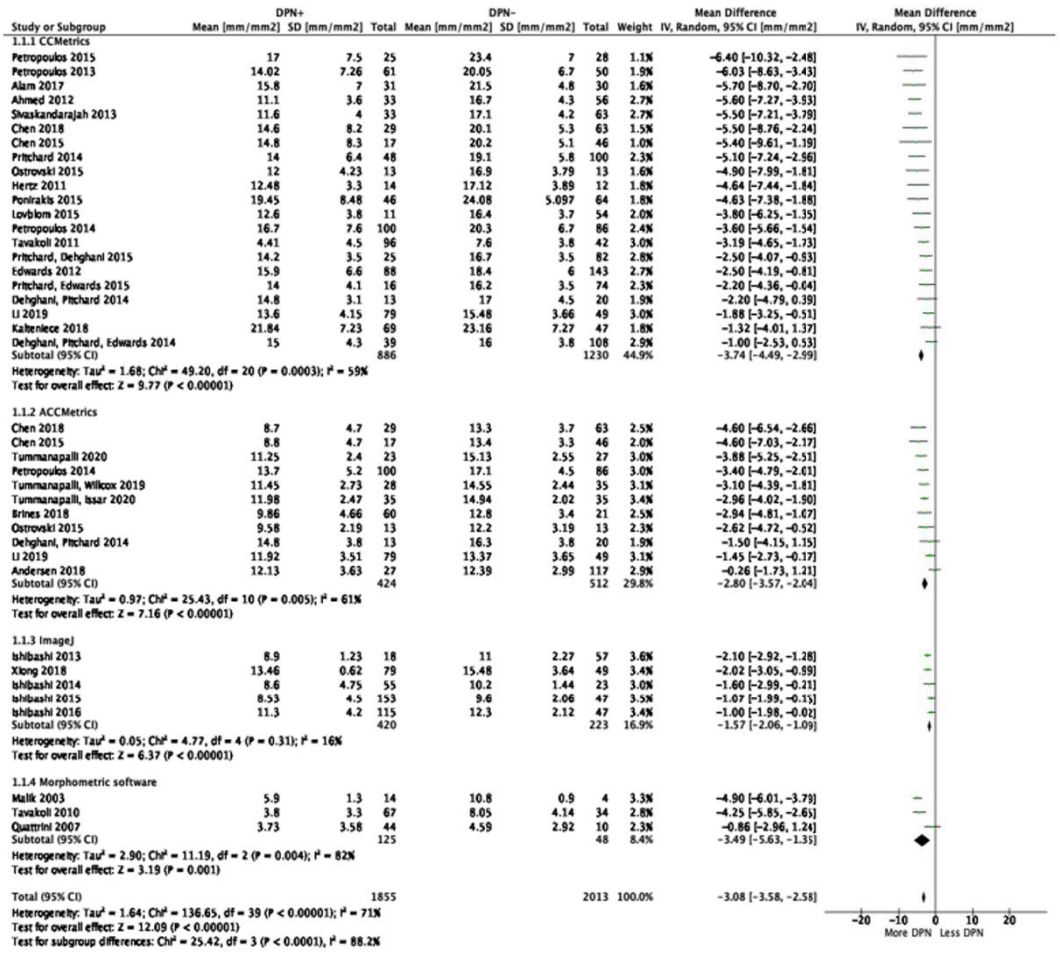


Figure 17.Forest plots of corneal nerve fibre length (CNFL) in patients with DSPN+ and without DSPN-.

DSPN⁺ vs. Control

Thirty-two studies (4, 8, 9, 12, 20, 21, 23-28, 30, 34-37, 39-45, 47, 50-52, 54) with 3459 (2036 $DSPN^+$ and 1423 control) participants were included in the meta-analysis. CNFL (mm/mm^2) was significantly lower in $DSPN^+$ compared to controls (MD=-6.05, 95% CI -6.77 to -5.34, *P*<0.00001) (CCMetrics (MD= -6.91, 95% CI -8.06 to -5.76, *P*<0.00001), ACCMetrics (MD= -5.49, 95% CI -7.03 to -3.95, *P*<0.00001), ImageJ (MD=-4.14, 95% CI -4.72 to -3.56, *P*<0.00001) and morphometric software (MD=-6.07, 95% CI -8.64 to -3.50, *P*<0.00001)). There was a significant difference in the magnitude of CNFL reduction between studies (X^2=19.59, *P*=0.0002) **(Figure 18).**

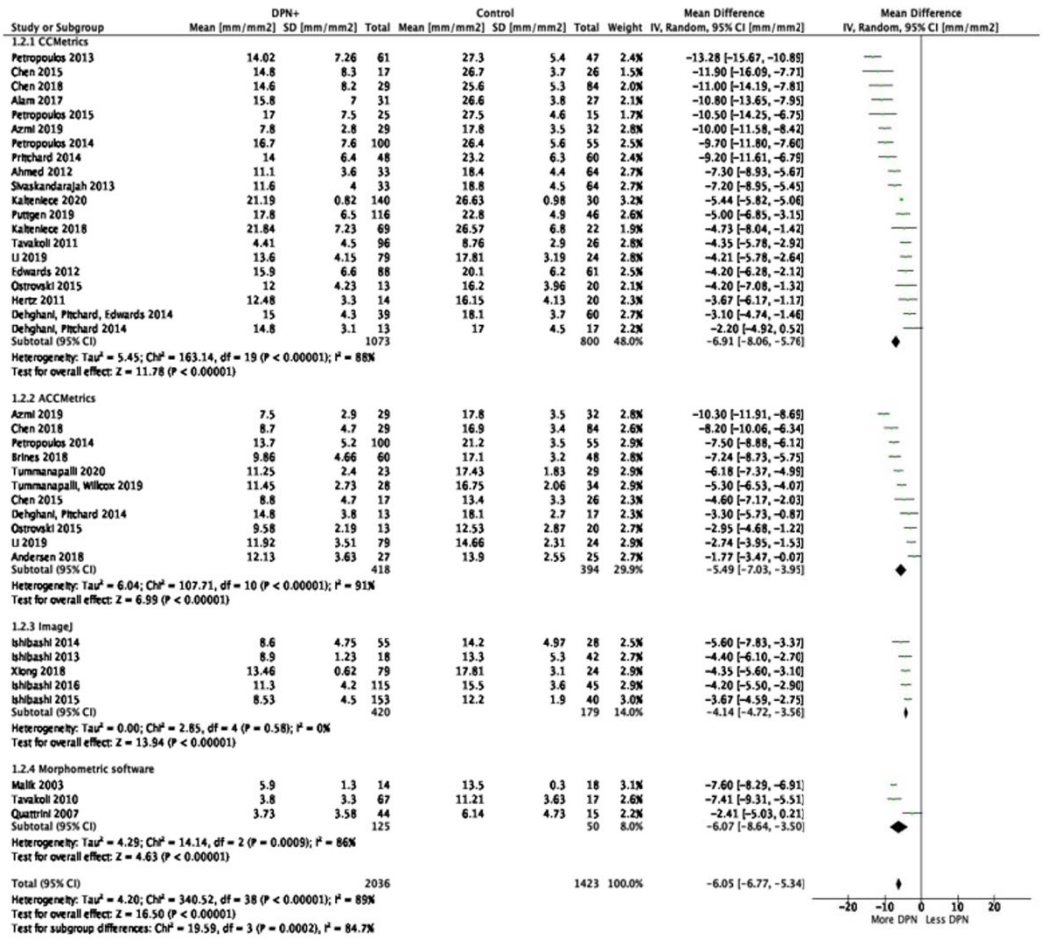


Figure 18. Forest plots of corneal nerve fibre length (CNFL) in patients with DSPN+ and healthy control.

DSPN⁻ vs. Control

Thirty studies (4, 8, 9, 12, 20, 21, 23-28, 34, 36, 37, 39-45, 47, 48, 50-54) with 3149 (1786 DSPN⁻ and 1363 control) participants were included in the meta-analysis. CNFL (mm/mm^2) was significantly lower in DSPN⁻ compared to controls (MD= -2.87, 95% CI -3.34, -2.40, *P*<0.00001) (CCMetrics (MD= -3.12, 95% CI -4.06 to -2.19, *P*<0.00001), ACCMetrics (MD=-2.63, 95% CI -3.43 to -1.83, *P*<0.00001), ImageJ (MD= -2.78, 95% CI -3.35 to -2.22, *P*<0.00001) and morphometric software (MD= -2.68, 95% CI -3.48 to -1.88, *P*<0.00001)). There was no difference in the magnitude of CNFL reduction in the DSPN⁻ group between studies (X^2=0.72, *P*=0.87), **(Figure 19).**

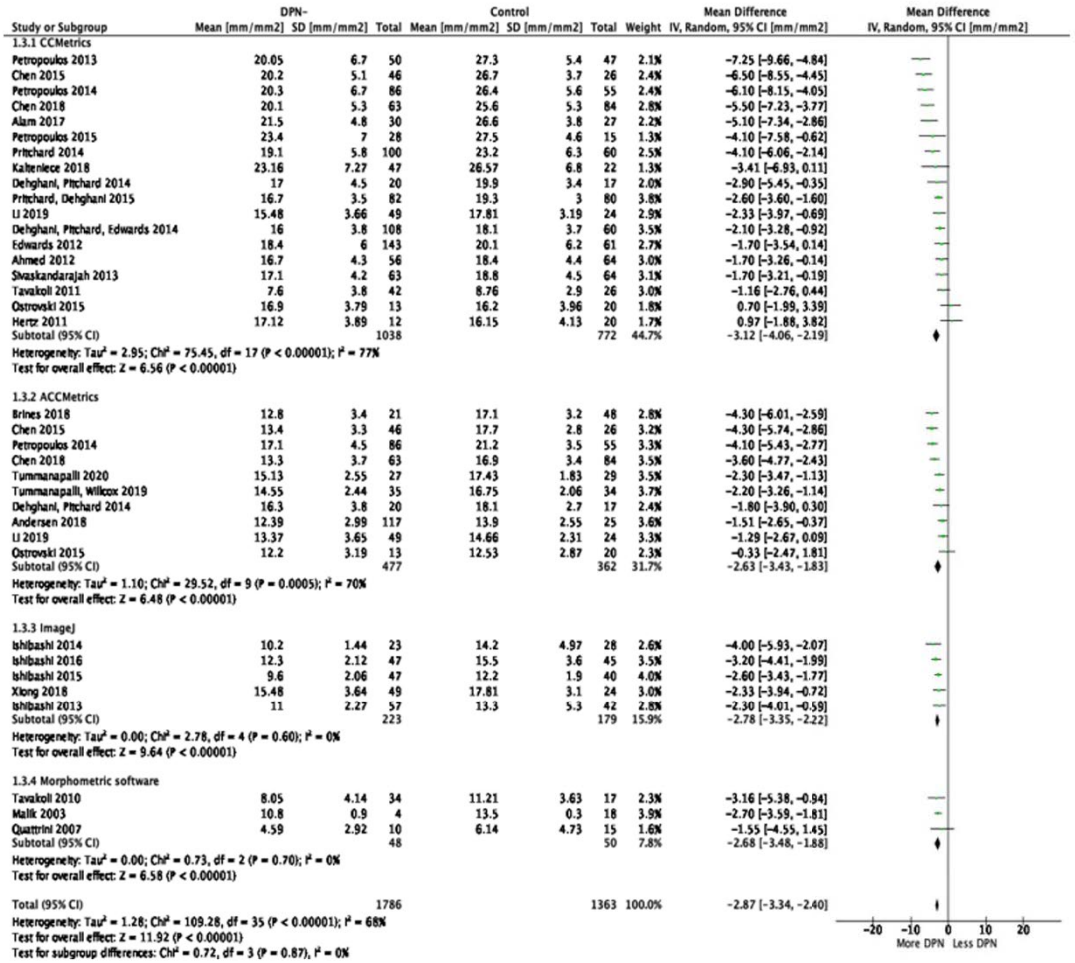


Figure 19.Forest plots of corneal nerve fibre length (CNFL) in patients without DSPN- and healthy control.

3.4.4. Inferior Whorl Length

DSPN⁺ vs. DSPN⁻

Six studies (8, 39, 47, 48, 53) with 459 (205 $DSPN^+$ and 254 $DSPN^-$) participants were included in the meta-analysis. IWL (mm/mm^2) was significantly lower in $DSPN^+$ compared to $DSPN^-$ (MD= -4.11, 95% CI -5.10 to -3.12, *P*<0.00001) (CCMetrics (MD=-3.42, 95% CI -5.47 to -1.36, *P*=0.001) and ACCMetrics (MD= -4.40, 95% CI -5.53 to -3.28, *P*<0.00001)). There was no significant difference in the magnitude of CNFL reduction in the $DSPN^+$ group between studies (X^2=0.68, *P*=0.41), **(Figure 20)**.

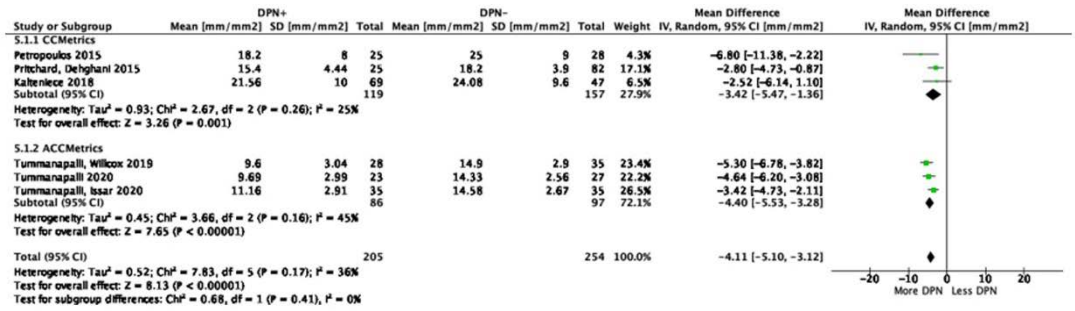


Figure 20. Forest plots of inferior whorl length (IWL) in patients with DSPN+ and without DSPN-.

DSPN⁺ vs. Control

Six studies (8, 30, 39, 47, 48, 53) with 520 (310 $DSPN^+$ and 210 control) participants were included in the meta-analysis. IWL (mm/mm^2) was significantly lower in $DSPN^+$ compared to control (MD=-10.36, 95% CI -13.30 to -7.42, *P*<0.00001) (CCMetrics (MD=-11.62, 95% CI -15.97 to -7.28, *P*<0.00001) and ACCMetrics (MD=-8.32, 95% CI -9.40 to -7.24, *P*<0.00001)). There was no significant difference in the extent of IWL reduction in the $DSPN^+$ group between studies (X^2=2.08, *P*=0.15), **(Figure 21).**

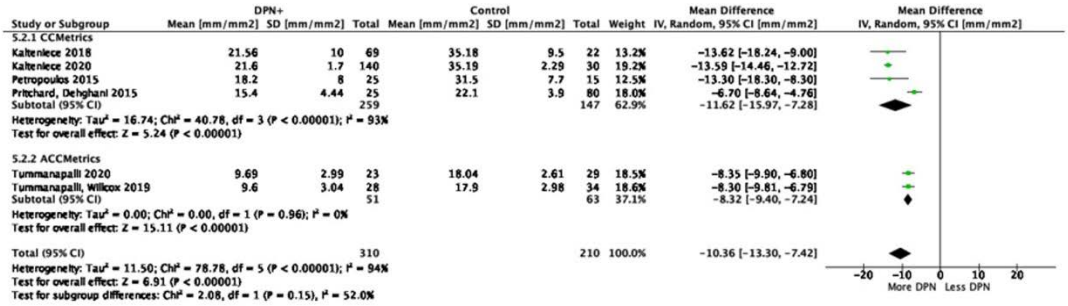


Figure 21.Forest plots of inferior whorl length (IWL) in patients with DSPN+ and healthy control.

DSPN- vs. Control

Five studies (8, 39, 47, 48, 53) with 399 (219 DSPN- and 180 control) participants were included in the meta-analysis. IWL (mm/mm^2) was significantly lower in the DSPN- group compared to controls (MD= -3.81, 95% CI -4.56 to -3.06, *P*<0.00001) (CCMetrics (MD=-4.43, 95% CI -5.56 t0 -3.29, *P*=0.003) and ACCMetrics (MD= -3.34, 95% CI -4.33 to -2.34, *P*<0.00001)). There was no significant difference in the extent of IWL reduction in the DSPN− group between studies (χ^2 = 2.11, *P* = 0.15), **(Figure 22)**.

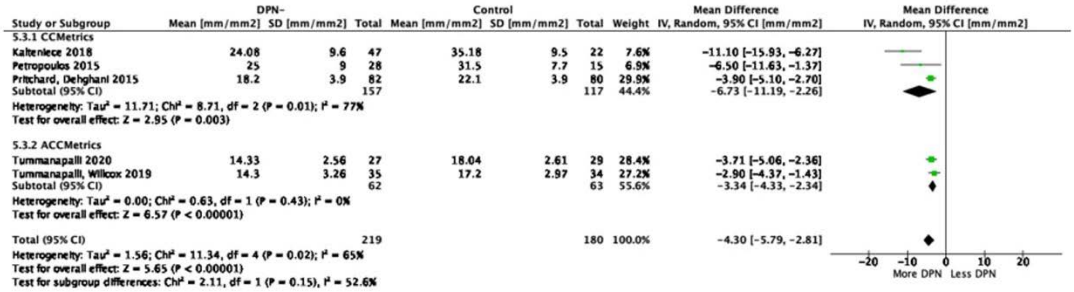


Figure 22. Forest plots of inferior whorl length (IWL) in patients without DSPN- and healthy control.

3.5. Discussion

In this large systematic review and meta-analysis of over 3000 participants, CCM demonstrates a consistent reduction in four major corneal nerve parameters in patients with DSPN compared to healthy controls and those without DSPN. Furthermore, we demonstrate a lesser but significant reduction in all corneal nerve parameters in patients without DSPN compared to controls, suggesting that CCM detects early sub-clinical DSPN. This is consistent with the demonstration of corneal nerve loss in subjects with impaired glucose tolerance (16), recently diagnosed type 2 diabetes (58) and children with type 1 diabetes (59). The greater corneal nerve loss in patients with DSPN compared to those without DSPN is consistent with studies showing that corneal nerve loss is associated with the severity of DSPN (4, 26, 37, 60, 61) and has good sensitivity and specificity for diagnosing DSPN (5-7). Both CNFD and IENFD have a comparable diagnostic performance for DSPN (8, 9, 62), although in a study of patients with recently diagnosed type 2 diabetes there were differences in the extent of small nerve fibre damage between CCM and skin biopsy (57). Additionally, a reduction in corneal nerve parameters is associated with incident DSPN (10, 57, 63) and greater corneal nerve loss (53) and augmented nerve branching (29) occurs in patients with painful diabetic neuropathy. CCM could act as a biomarker as defined by the NIH Biomarkers Definitions Working Group (64); it is non-invasive, easily measured, and produces rapid results with high sensitivity (5-7). It allows detection of subclinical DSPN, and there is minimal overlap in corneal nerve parameters between patients with and without DSPN and healthy people. In addition, CCM identifies those at risk of developing DSPN (10, 11, 57).

The outcomes of the current review extend considerably the findings of a previous systematic review and meta-analysis showing a reduction in CNFD, CNBD and CNFL in patients with and without DSPN compared to controls from 13 studies with 1680 participants (65) and a more recent trial sequential meta-analysis which showed a reduction in CNFD, CNBD and CNFL in patients with and without DSPN compared to controls in 13 studies with 1830 participants (14).

In the present review we have included IWL which has the potential to detect earlier nerve damage (56, 66, 67), especially in patients with painful diabetic neuropathy (30, 53).

The reliability of establishing a single estimate for the effect size of corneal nerve outcome measures from all the published studies may be affected by the inclusion of the same subjects from several studies, type of CCM used to acquire images, the mode of image acquisition and the image analysis tool used to quantify corneal nerve parameters. Additionally, 16 (42%) of the 38 studies included were from the same group, which may be considered an additional source of bias. This could not be overcome as by default the systematic review will select all published papers which fulfill the search criteria and as this group have pioneered and published the most on CCM, they were selected. Our analysis showed that the type of software used for image analysis had no significant influence on the heterogeneity of corneal nerve outcomes. Whilst the corneal nerve measure was lower when using automated (ACCMetrics) compared to manual (CCMetrics, ImageJ) software, the magnitude of difference in corneal nerve parameters between groups was comparable (42, 68).

Our sensitivity analysis shows no evidence of significant bias or heterogeneity. Although, this was expected given that there may be differences in corneal nerve parameters between patients with type 1 and type 2 diabetes (5, 7, 13) and in relation to HbA1c (69) and glycemic variability (70), presence of metabolic syndrome (71) and hypertension or hyperlipidemia (7, 72).

3.6. Conclusions

Corneal confocal microscopy is a rapid, non-invasive, and reproducible imaging technique to quantify small nerve fibre damage. Our systematic review and meta-analysis provides robust evidence that corneal confocal microscopy can be used to diagnose sub-clinical and established DSPN.

This chapter systematically summarized all studies on CCM and adults with DSPN. The meta-analysis demonstrated that in comparison to healthy controls, CCM detected changes in CNFD, CNBD, CNFL and IWL not only in patients with DSPN, but also in those without DSPN, suggesting that CCM could be an early marker of neurodegeneration. This chapter provides a base for the other chapters that focus on early corneal nerve changes in the pediatric population.

A recent study in which patients with T2DM underwent neurologic examination, quantitative sensory testing, electrophysiology, skin biopsy and CCM showed that CCM had the lowest sensitivity (CNFL 14.4% (9.8-20.2 95% CI) for diagnosing DSPN compared to other small fibre measures (73). However, this study assessed patients with a short duration of diabetes of ~ 5 years with excellent glycemic control (HbA1c 6.7%), which differs from the populations included in the meta-analysis with longer diabetes duration and worse glycemic control. Thus, whilst CCM has the lowest

sensitivity (CNFL 14.4%), the gold standard measures of IENFD (51.1%) and NCS (37.1%) also performed poorly. This therefore questions whether they used an optimal diagnostic-criteria for identifying DSPN and indeed they classified patients with DSPN based on the "probable DSPN definition" with either symptoms, signs and absent or reduced ankle reflex, which does not strictly follow the Toronto criteria. Badian et al, also recently found no relationship between CCM (wide-field CCM images) and IENFD loss (74) in patients with DSPN, similar to a study by Ziegler et al (58). However, both studies did show corneal nerve loss in diabetic patients, indicating that CCM has diagnostic potential confirming the findings of the current systematic review and meta-analysis. Ziegler et al. assessed well-controlled patients (HbA1c 6.8%, BP 134/74 mmHg) with minimal neuropathy (DSPN 11.4%, peroneal motor NCV (PMNCV) 42.6 m/s) and minimally reduced IENFD (10.6 vs. 8.3). Badian et al. (74) also studied patients with minimal or no DSPN with comparable NCV to control subjects (PMNCV 45.4 vs. 45.9 m/s, P=0.79); sural NCV (SNCV) was 44.5 vs. 45.7, P=0.32 and amplitude 6.1 vs. 7.1, P=0.33) and yet despite an almost normal HbA1c (6.1%) the IENFD was markedly reduced but was higher in the diabetic group compared to controls (0.9 vs. 0.8). Given that the IENFD was very low at 0.9 with a spread of ± 1 whilst CNFD ranged from 5 -20/mm^2, it is not surprising that there was no correlation between IENFD and CCM. The discrepancies can therefore be to a great extent explained by the unusual characteristics of the populations studied, in addition to the larger corneal area analysed, as well as different image-processing software algorithms used.

3.7. References

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Chapter IV – CORNEAL NERVE LOSS IN CHILDREN WITH TYPE 1 DIABETES MELLITUS WITHOUT RETINOPATHY OR MICROALBUMINURIA

Gad H, Al-Jarrah B, Saraswathi S, Petropoulos IN, et al. Corneal nerve loss in children with type 1 diabetes mellitus without retinopathy or microalbuminuria. J Diabetes Investig. 2020 Nov;11(6):1594-1601. doi: 10.1111/jdi.13313. Epub 2020 Jul 7. PMID: 32491258; PMCID: PMC7610109.

4.1. Abstract

Introduction/Aim

Corneal confocal microscopy is a rapid, non-invasive ophthalmic technique to identify sub-clinical neuropathy. The aim of this study was to quantify corneal nerve morphology in children with type 1 diabetes mellitus compared to age-matched healthy controls using corneal confocal microscopy.

Method

Twenty participants with type 1 diabetes mellitus (age 14±2 years, diabetes duration 4.08±2.91 years, glycated hemoglobin 9.3±2.1%) without retinopathy or microalbuminuria and 20 healthy controls were recruited from outpatient clinics. Corneal confocal microscopy was undertaken and corneal nerve fibre density (no./mm^2), corneal nerve branch density (no./mm^2), corneal nerve fibre length (mm/mm^2), corneal nerve fibre tortuosity and inferior whorl length (mm/mm^2) were quantified manually.

Results

Corneal nerve fibre density (22.73±8.84 vs. 32.92±8.59; *P*<0.001), corneal nerve branch density (26.19±14.64 vs. 47.34±20.01; *P*<0.001), corneal nerve fibre length (13.26±4.06 vs. 19.52±4.54; *P*<0.001) and inferior whorl length (15.50±5.48 vs. 23.42±3.94; *P*<0.0001) were significantly lower, whilst corneal nerve fibre tortuosity (14.88±5.28 vs. 13.52±3.01; *P*=0.323) did not differ between children with type 1 diabetes mellitus and controls. Glycated hemoglobin correlated with corneal nerve fibre tortuosity (*P*<0.006) and aspartate aminotransferase correlated with corneal

nerve fibre density (*P*=0.039), corneal nerve branch density (*P*=0.003), and corneal nerve fibre length (*P*=0.037).

Conclusion

Corneal confocal microscopy identifies significant sub-clinical corneal nerve loss, especially in the inferior whorl of children with type 1 diabetes mellitus without retinopathy or microalbuminuria.

Keywords: Type 1 diabetes mellitus, child, small fibre neuropathy

4.2. Introduction

Type 1 Diabetes Mellitus (T1DM) affects over half a million children worldwide (1, 2). Diabetes is associated with chronic microvascular complications in adults which increases morbidity and all-cause mortality (3). Diabetes is the main cause of distal symmetric polyneuropathy (DSPN) (4-6). Adults with DSPN present with a combination of symptoms such as numbness, pain, and tingling in the feet (7). The American Diabetes Association endorses screening for distal symmetric polyneuropathy (DSPN) at diagnosis of T2DM, 5 years after the diagnosis of T1DM and annually thereafter (8). Children and adolescents with T1DM rarely complain of neuropathic symptoms. However, a study of children with T1DM showed reduced motor and sensory nerve conduction velocities (24%) and at least one neuropathic symptom (60%) or sign (58%) (9) and in another study symptomatic neuropathy was present in 13.5%, whilst 22.5% had neurophysiological evidence of neuropathy (10) and 18% had impaired vibrotactile sense (11). Furthermore, in one study 36% had >2 abnormal autonomic function tests and 18.8% had severe autonomic neuropathy (12). In a prospective study abnormal nerve conduction velocity (NCV) was found in 31.6% at baseline which increased to 63.2% after 5 years (13) and in another study over 10 years the prevalence of clinical neuropathy increased from 6.5% to 16.1%, whilst NCV abnormalities increased from 17.7% to 46.8% (14). Whilst, neurophysiologic assessments are highly sensitive they are not easily performed in children (15). Vibration perception threshold (VPT) and tactile perception threshold tests are easy to perform but lack sensitivity for the early detection of DSPN (16).

There is a need for non-invasive sensitive screening tools for the early detection of neuropathy in children with diabetes.

Corneal confocal microscopy (CCM) is a rapid, non-invasive, and well-tolerated technique to detect and quantify neuropathy in adults with T1DM (17-24). An early study found no significant changes in CCM parameters in children with T1DM (15). However, a more recent study has shown a significant reduction in corneal nerve fibre measures in young children with T1DM with and without diabetic retinopathy (25). The aim of this study was to quantify corneal nerve morphology in the central cornea and inferior whorl of children with T1DM compared to age-matched healthy controls using CCM.

4.3. Methods

Twenty participants with T1DM and 20 age-matched healthy controls underwent CCM. Patients with a history of any other cause of neuropathy, malignancy, deficiency of B12 or folate, chronic renal failure, liver failure, connective tissue or systemic disease (rheumatoid arthritis, systemic lupus erythematosus, dermatomyositis, systemic scleroderma, Raynaud Phenomenon), previous corneal trauma or systemic disease that affects the cornea, surgery and a history of or current contact lens wear were excluded from the study. All participants provided assent and parental informed consent and the research adhered to the tenets of Declaration of Helsinki and was approved by Sidra Medicine and Weill Cornell Medicine Research Ethics Committee.

4.3.1. Image selection and quantification

Six central sub basal nerve plexus (SBNP) images were selected from the central cornea and corneal nerve fibre density (CNFD), (no./mm^2) corneal nerve branch

density (CNBD) (no./mm^2), corneal nerve fibre length (CNFL) (mm/mm^2), corneal nerve fibre tortuosity (CNFT) were quantified using manual CCMetrics. Six images centred on the inferior whorl and adjacent areas (upper right/left corner and lower right/left corners) were selected and the inferior whorl length (IWL) (mm/mm^2) was quantified utilizing the manual CNFL mode in CCMetrics **(Figure 23)** (26). The investigator was blind to the study group when performing CCM and analysing CCM images.

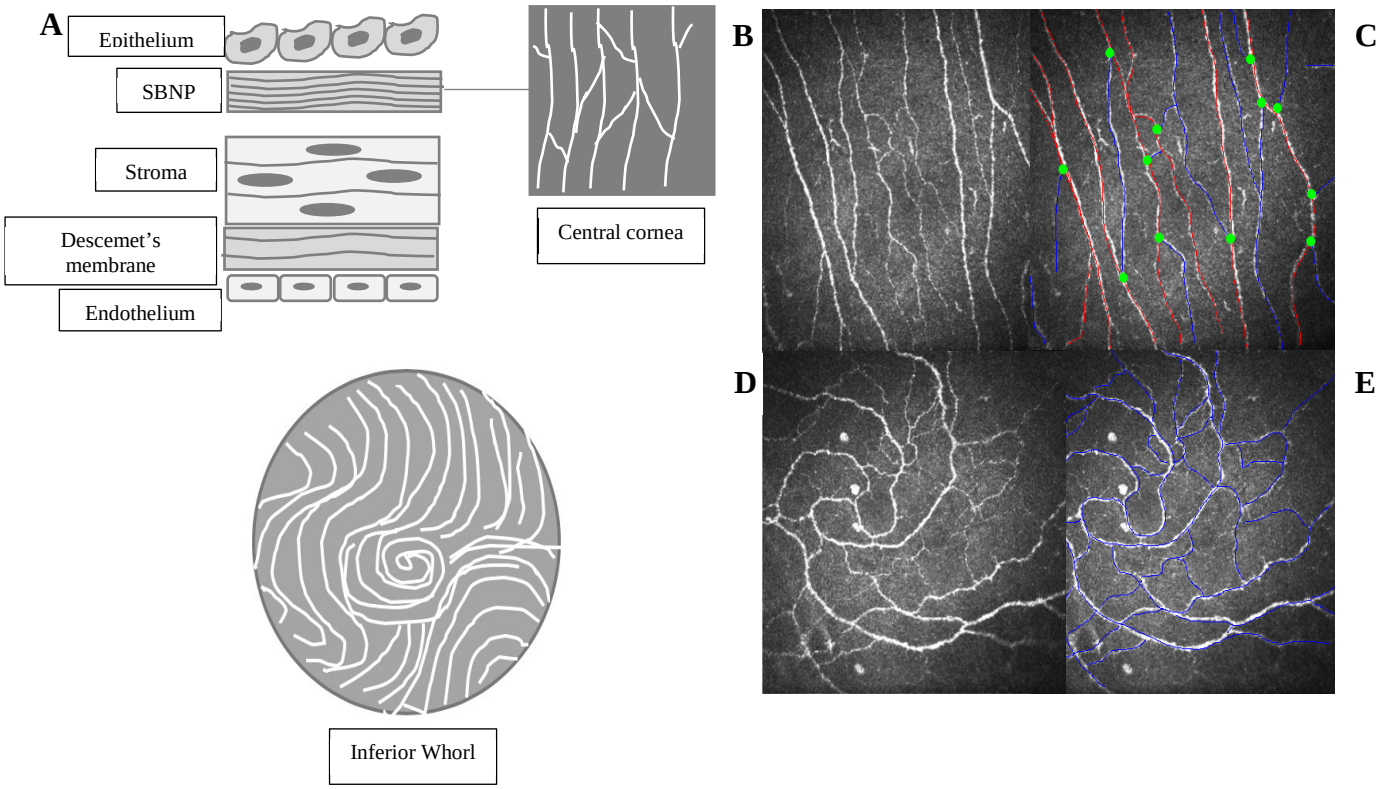

Figure 23. Central corneal sub-basal nerve plexus and inferior whorl.

(A) Schematic presentation of the sub-basal nerve plexus (SBNP) at the central and inferior whorl. (B) Nerve fibres at the central cornea, (C) tracing of the nerves using CCMetrics, (D) Nerve fibres at the inferior whorl (IW), (E) tracing of the IW using CCMetrics.

4.3.2. Statistical analysis

All statistical analyses were performed using IBM SPSS Statistics software Version 26 and $P<0.05$ was considered statistically significant. Normally distributed data were expressed as mean ± standard deviation and the means were compared using an independent sample t-test. Pearson correlation was undertaken to investigate the association between clinical parameters and corneal nerve fibre parameters. Graph prism version 8 was used to build dot plots.

4.4. Results

Twenty participants with T1DM and 20 healthy controls underwent CCM. Subjects with T1DM were slightly older ($P<0.02$) and taller ($P<0.02$) but had comparable weight and BMI. They also had a lower aspartate aminotransferase (AST) ($P<0.02$) but comparable bilirubin and alanine aminotransferase (ALT) **(Table 6)**.

Table 6. Clinical and laboratory measures in patients with T1DM and controls.

	Healthy (n=20)	T1DM (n=20)	*P*-value
Age	12.83±1.91	14.47±2.43	**0.02**
n (%)	11 (55)	9 (45)	N/A
Boys	9 (45)	11 (55)	
Girls			
Duration of T1DM	-	4.08±2.91	N/A
Height (m)	1.45±0.13	1.54±0.09	**0.02**
Weight (kg)	47.87±18.63	51.65±13.46	0.467
BMI (Kg/m^2)	22.26±5.47	21.68±5.09	0.733
HbA$_{1c}$ (%)	-	9.3±2.1	N/A
Bilirubin (µmol/L)	10.54±5.4	13.22±5.92	0.206
AST (IU/L)	24.83±5.45	20.44±4.23	**0.02**
ALT (IU/L)	15.08±4.03	16.44±3.74	0.339
25(OH)D (ng/ml)	23.88±8.96	18.16±8.56	0.085
Microalbuminuria n (%)			
Yes	-	0	N/A
No	-	11 (55.0%)	
Diabetic retinopathy n (%)			
Yes	-	0	N/A
No	-	8 (40.0%)	

Data are presented as mean ± SD. BMI: Body mass index, AST: aspartate aminotransferase, ALT: alanine aminotransferase.

Only 4 (20%) of the patients met the American Diabetes Association (ADA) criteria (>10 yrs. of age and >5 yrs. of diabetes) to undergo screening for microvascular complications. Eight (40.0%) underwent assessment for retinopathy and 11 (55.0%) underwent assessment for microalbuminuria of whom none had retinopathy or microalbuminuria.

CNFD (22.73±8.84 vs. 32.92±8.59; *P*=0.001), CNBD (26.19±14.64 vs. 47.34±20.01; *P*<0.001) and CNFL (13.26±4.06 vs. 19.52±4.54; *P*<0.001) were lower in patients with T1DM compared to healthy controls **(Figure 24A-C)**.

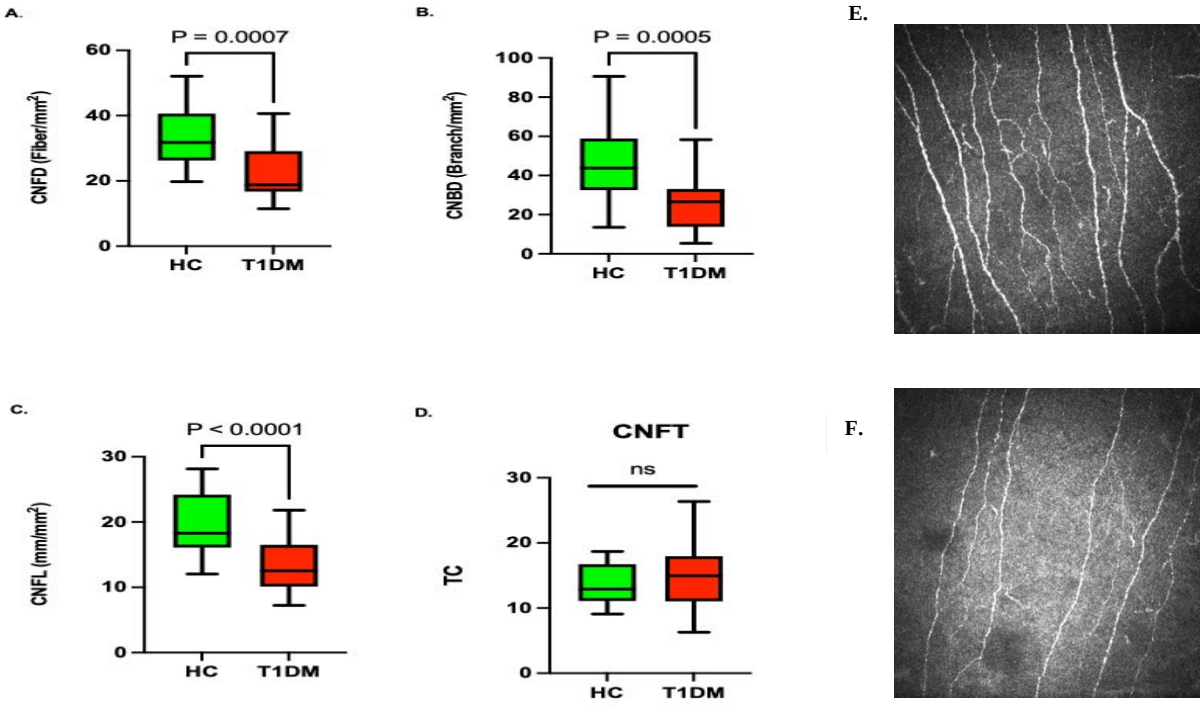

Figure 24. CCM parameters and images of the sub-basal plexus in children with T1DM and healthy controls.

(A) CNFD: Corneal nerve fibre density, (B) CNBD: corneal nerve branch density, (C) CNFL: corneal nerve fibre length, (D) CNFT: corneal nerve fibre tortuosity, (E) CCM image of corneal nerves in a healthy control, (F) CCM image of reduced corneal nerves in a child with T1DM.

CNFT did not differ between groups (14.88±5.28 vs. 13.52±3.01; *P*=0.323) **(Figure 24D)**. IWL was significantly lower in patients with T1DM (n=19) compared to controls (n=19) (15.50±5.48 vs. 23.42±3.94; *P*<0.0001) **(Figure 25 A-C)**. CNFD, CNBD, CNFL and IWL were >2SD lower than the mean of controls in 15%, 10%, 30% and 50% of patients with T1DM.

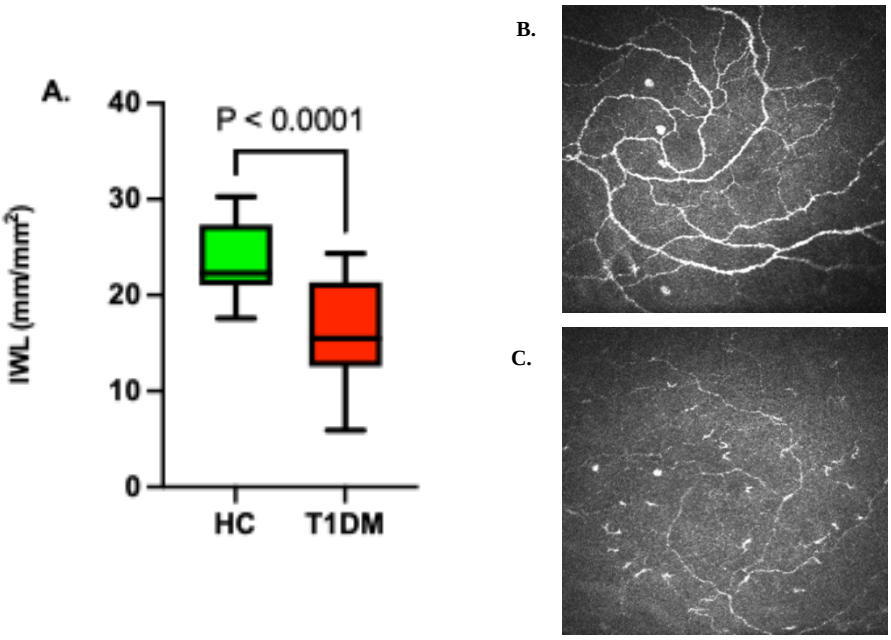

Figure 25. Inferior whorl length and CCM images of the inferior whorl in children with T1DM and healthy controls.

(A) IWL (Inferior whorl length) in healthy controls and children with TIDM, (B) CCM image of IWL in a healthy control and (C) CCM image of IWL in a child with T1DM.

Correlation between CCM parameters and clinical/laboratory measures

Age, duration of diabetes, height, BMI, 25(OH)D, bilirubin and ALT did not correlate with any CCM parameter (P>0.05). Glycated hemoglobin (HbA_{1c}) correlated significantly with CNFT (P<0.006) and AST correlated with BMI (P<0.01), CNFD (P=0.039), CNBD (P=0.003), and CNFL (P=0.037) **(Table 7)**.

Table 7. Correlations between CCM parameters and clinical and metabolic parameters.

	Age (years)	**Diabetes Duration (years)**	**HbA_{1c} (%)**	**Height (m)**	**BMI (Kg/m^2)**	**25(OH)D (ng/ml)**	**Bilirubin (µmol/L)**	**AST (IU/L)**	**ALT (IU/L)**
CNFD ($no./mm^2$)	-0.278 (0.235)	0.027 (0.915)	-0.300 (0.226)	-0.028 (0.907)	-0.396 (0.084)	0.072 (0.783)	-0.207 (0.41)	0.489 (**0.039**)	-0.121 (0.633)
CNBD ($no./mm^2$)	-0.144 (0.544)	0.108 (0.670)	0.221 (0.377)	0.040 (0.876)	-0.329 (0.157)	-0.002 (0.995)	0.033 (0.897)	0.666 (**0.003**)	0.129 (0.611)
CNFL (mm/mm^2)	-0.188 (0.428)	0.067 (0.791)	-0.036 (0.887)	0.053 (0.824)	-0.257 (0.275)	-0.099 (0.706)	-0.127 (0.615)	0.495 (**0.037**)	-0.013 (0.959)
CNFT (TC)	0.28 (0.231)	0.032 (0.899)	0.619 (**0.006**)	0.202 (0.392)	0.203 (0.39)	0.244 (0.344)	0.267 (0.284)	-0.165 (0.505)	-0.282 (0.256)
IWL (mm/mm^2)	0.195 (0.423)	0.029 (0.911)	-0.009 (0.974)	0.063 (0.798)	0.358 (0.133)	-0.380 (0.132)	-0.456 (0.066)	0.014 (0.957)	-0.001 (0.995)

Pearson correlation (r) was used. CNFD: Corneal nerve fibre density, CNBD: corneal nerve branch density, CNFL: corneal nerve fibre length, CNFT: Corneal nerve fibre tortuosity, IWL: inferior whorl length, BMI: body mass index, AST: aspartate aminotransferase, ALT: alanine aminotransferase.

4.5. Discussion

In the present study there is evidence of significant corneal nerve loss in children with T1DM without retinopathy or microalbuminuria. It is critical to detect and prevent nerve damage at the earliest stage of diabetic neuropathy as improvement in glycaemic control and other risk factors such as obesity, hypertension, dyslipidaemia may prevent nerve degeneration and promote nerve regeneration (27, 28).

Previous studies in adults with T1DM have found a significant reduction in central CNFD, CNBD and CNFL compared to healthy controls (18, 29-35) and in T1DM patients without retinopathy or microalbuminuria (34). Corneal nerve loss has good diagnostic utility for both diabetic somatic and autonomic neuropathy (22). Furthermore, a lower CNFL is associated with the development of clinical diabetic neuropathy (31, 36, 37), and a more rapid reduction in CNFL predicts the development and progression of diabetic neuropathy (38). Significant improvements in CNFD, CNBD, and CNFL have

been observed in T1DM patients after simultaneous pancreas and kidney transplantation (39, 40), omega-3 supplementation (41) and an improvement in multiple risk factors for diabetic neuropathy (42).

In the present study a significant reduction in central corneal nerve fibre parameters in young children with T1DM has been demonstrated, which is comparable with a previous study in children and young adolescents with T1DM (25, 43). Although our cohort of T1DM children had comparable age, clinical and metabolic characteristics with the previous study, they had lower CNFD, CNBD and CNFL (43). However, established risk factors for diabetic neuropathy in adults such as age, height, HbA_{1c}, duration of diabetes and BMI did not correlate with corneal nerve parameters, consistent with previous findings in adults with T1DM (35, 44). CNFT was not altered, in contrast with a study in adults with diabetes where nerve tortuosity was higher (45) and similar to findings by Ferdousi et al where CNFT was not lower in children with T1DM (43). A reduction in corneal nerves occurs, regardless of diabetes duration, in young patients with T1DM (35) and adults with T2DM (46).

The ADA has recommended initial screening for albuminuria and retinopathy in patients with T1DM aged over 10 years, after 3-5 years of diabetes (47). Whilst 20% of this cohort fulfilled the criteria for screening, none had microalbuminuria or retinopathy. Indeed, the significant corneal nerve loss in these children with T1DM without retinopathy or microalbuminuria agrees with previous findings in adults with T1DM (25, 34) and supports the thesis that neuropathy may precede retinopathy (48). It also argues for earlier screening of diabetic neuropathy in children with T1DM using CCM. AST was lower in our cohort with T1DM and

correlated with CNFD, CNBD, and CNFL. No relationship between AST and CCM has been observed in studies in adults with diabetes (21, 22, 30, 49). Whilst the association between body mass index and elevated AST is well established as a marker for liver injury in obese adults (50-53), in the present study AST was inversely correlated with BMI, which could be an incidental finding due to the small sample size.

The inferior whorl is distal to the central nerves and may allow the identification of earlier nerve damage (26, 54). Studies in adults with T1DM and T2DM have shown a greater reduction in IWL (49, 55), especially in those with painful diabetic neuropathy (56, 57). This is the first study in children with T1DM showing a marked reduction in IWL, with 50% having a reduction >2SD lower than the mean in controls.

A limitation of the current study is the cross-sectional design, relatively small number of subjects studied and the lack of additional measures of diabetic neuropathy. Prospective studies are needed to assess progression of corneal nerve abnormalities in relation to other complications and risk factors for diabetic neuropathy. There is a need to study children with T1DM with and without DSPN to compare the extent of nerve damage in relation to the severity of DSPN. We also lack a comparator group with retinopathy and microalbuminuria to investigate whether children with microvascular complications have more nerve damage.

Significant corneal nerve loss has been demonstrated in the central cornea and inferior whorl indicative of neuropathy in children with T1DM without microalbuminuria or retinopathy. This suggests that CCM could be used to screen for early sub-clinical neuropathy and to assess disease progression in children with T1DM.

To further explore potential mechanisms for early nerve damage in children with T1DM, changes in keratocyte density were assessed as keratocytes produce neurotrophic substances that maintain the integrity of corneal nerve fibres.

4.6. References

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Chapter V: CORNEAL CONFOCAL MICROSCOPY IDENTIFIES A REDUCTION IN KERATOCYTE DENSITY AND SUB-BASAL NERVES IN CHILDREN WITH TYPE 1 DIABETES MELLITUS

Gad H, Al-Jarrah B, Saraswathi S, Mohamed S, et al. Corneal confocal microscopy identifies a reduction in corneal keratocyte density and sub-basal nerves in children with type 1 diabetes mellitus. Br J Ophthalmol. 2021 Apr 30:bjophthalmol-2021-319057. doi: 10.1136/bjophthalmol-2021-319057. Epub ahead of print. PMID: 33931390.

5.1. ABSTRACT

Purpose: To assess whether alterations in stromal keratocyte density are related to loss of corneal nerve fibres in children with type 1 diabetes mellitus (T1DM).

Methods: Twenty participants with T1DM and 20 age-matched healthy controls underwent corneal confocal microscopy. Corneal sub-basal nerve morphology and corneal keratocyte density (KD) were quantified.

Results: Corneal nerve fibre density (CNFD) (P<0.001), corneal nerve branch density (CNBD) (P<0.001), corneal nerve fibre length (CNFL) (P<0.001) and inferior whorl length (IWL) (P<0.001) were lower in children with T1DM compared to healthy controls. Anterior (P<0.03) and mid (P=0.03) stromal keratocyte densities were lower with no difference in posterior KD in children with T1DM compared to controls. Age, duration of diabetes, height, weight, and BMI did not correlate with anterior, mid, or posterior KD. Inverse correlations were found between HbA_{1c} and posterior KD (r=-0.539, P=0.026), bilirubin with mid-KD (r=-0.540, P=0.025) and posterior KD (r=-0.531, P=0.028) and 25-hydroxycholecalciferol (25-OHD) with mid-KD (r=-0.583, P=0.018). CNFD, CNFL and IWL did not correlate with anterior (AKD), mid (MKD) or posterior (PKD) keratocyte densities.

Conclusion: This study demonstrates a reduction in corneal nerves and anterior and mid stromal keratocyte density in children with T1DM, but no correlation between corneal nerve and keratocyte cell loss.

5.2. INTRODUCTION

Type 1 diabetes mellitus (T1DM) affects over half a million children worldwide (1, 2). Diabetic neuropathy is a major complication in adults with T1DM resulting in neuropathic pain and foot ulceration (3, 4). Although, clinical neuropathy is rare, there are reports of neuropathy in children with T1DM (5-8). We have previously used corneal confocal microscopy (CCM) to identify significant corneal nerve loss in adults (9) and adolescents (10-12) with T1DM, even those without diabetic retinopathy (13) or microalbuminuria (14). In adults, corneal nerve loss is associated with painful diabetic neuropathy (15), has good diagnostic utility for diabetic neuropathy (9, 16) and predicts incident diabetic neuropathy (17, 18). The mechanisms underlying corneal nerve loss are complex, however in adults with diabetes, corneal nerve loss has been associated with age, HbA1c, body mass index (BMI), blood pressure, low-density lipoprotein (LDL) cholesterol and triglycerides (19-21). Our previous studies in children have shown no association between corneal nerve loss and the duration of diabetes, HbA1c or lipids (12, 13). This suggests that other factors may be important in the development of early corneal nerve damage.

The stroma comprises about 90% of the volume of the cornea and contains the stromal fibroblasts “keratocytes” (22) which maintain the integrity and mechanical stability of the cornea (23-25). Stromal keratocytes and activated fibroblasts have recently been shown to produce multiple pro-inflammatory and neurotrophic factors which have a dose-dependent effect on neurite outgrowth (26). Keratocytes play a role in nerve repair following nerve injury by two proposed mechanisms (27, 28): 1) secretion of matrix metalloproteases to promote collagen fibre formation; 2)

migration and activation to fibroblasts and myofibroblasts that secrete extracellular matrix important for tissue healing (29). We have previously used CCM to quantify alterations in the epithelium, stromal keratocytes and endothelium in adults (30) and adolescents (13) with diabetes. A reduction in anterior mid and posterior keratocyte density has been correlated with corneal nerve loss in adults with type 1 and type 2 diabetes (24). However, corneal nerve loss was found with preserved keratocyte density in adults without diabetic retinopathy with a reduction in keratocyte density only occurring in patients with diabetic retinopathy (30). Recently we have shown reduced corneal nerve and keratocyte densities in obese patients with and without diabetes with a correlation between corneal nerve fibre length and anterior keratocyte density and an improvement in both nerve and keratocyte densities and triglycerides and BMI, after bariatric surgery (31).

In children with T1DM we and others have shown loss of corneal nerves (11, 14), but with normal (13) or increased (11) keratocyte densities. To explore underlying associations between clinical and metabolic alterations and any change in anterior, mid, and posterior stromal keratocyte density and corneal nerve fibre morphology we have performed correlation analysis between these parameters in children with T1DM.

5.3. MATERIALS AND METHODS

5.3.1. Study subjects

Twenty participants with T1DM (age 14±2 years, diabetes duration 4.08±2.91 years, HbA1c 9.3±2.1%) and 20 age-matched healthy controls were recruited from outpatient clinics in Sidra Medicine and underwent CCM. The cohort in chapter IV is

also presented in this chapter. Patients with a history of any other cause of neuropathy, malignancy, deficiency of B_{12} or folate, chronic renal failure, liver failure, connective tissue, or systemic disease (rheumatoid arthritis, systemic lupus erythematosus, dermatomyositis, systemic scleroderma, Raynaud Phenomenon), previous corneal trauma or systemic disease that affects the cornea, surgery, and a history of or current contact lens wear were excluded from the study. All participants provided assent and parental informed consent and the research adhered to the tenets of Declaration of Helsinki and was approved by Sidra (150078-3) Medicine and Weill Cornell Medicine (17-00032) Research Ethics Committee.

5.3.2. Clinical Assessments

All participants underwent measurement of height (m), weight (kg), body mass index (BMI) (kg/m^2), liver function tests, 25-hydroxycholecalciferol (25-OHD) (ng/ml), and HbA1c (%).

5.3.3. Corneal Confocal Microscopy

Corneal confocal microscopy was undertaken using the Heidelberg Retina Tomograph Cornea Module (Heidelberg Engineering, Germany). Both eyes were anaesthetized with 2 drops of Bausch & Lomb Minims ® (Oxybuprocaine hydrochloride 0.4% w/v). A drop of hypotears gel (Carbomer 0.2% eye gel) was placed on the tip of the objective lens and a sterile disposable TomoCap was placed over the lens, allowing optical coupling of the objective lens to the cornea. Six images were selected from the sub basal nerve plexus (SBNP) in the central cornea and corneal nerve fibre density (CNFD) (fibres/mm^2) corneal nerve branch density (CNBD) (branches/mm^2), corneal nerve

fibre length (CNFL) (mm/mm^2) and corneal nerve fibr tortuosity (CNFT) were quantified manually using CCMetrics. Six images centered on the inferior whorl and immediately adjacent area were selected and inferior whorl length (IWL) (mm/mm^2) was quantified manually using the manual CNFL mode in CCMetrics. Six images of the anterior **(Figure 26A)**, mid **(Figure 26B)** and posterior **(Figure 26C)** stromal layer were selected and keratocyte density was quantified using the manual CNFD mode in CCMetrics (32). The investigator (HG) was blind to the study group when performing CCM and analyzing CCM images.

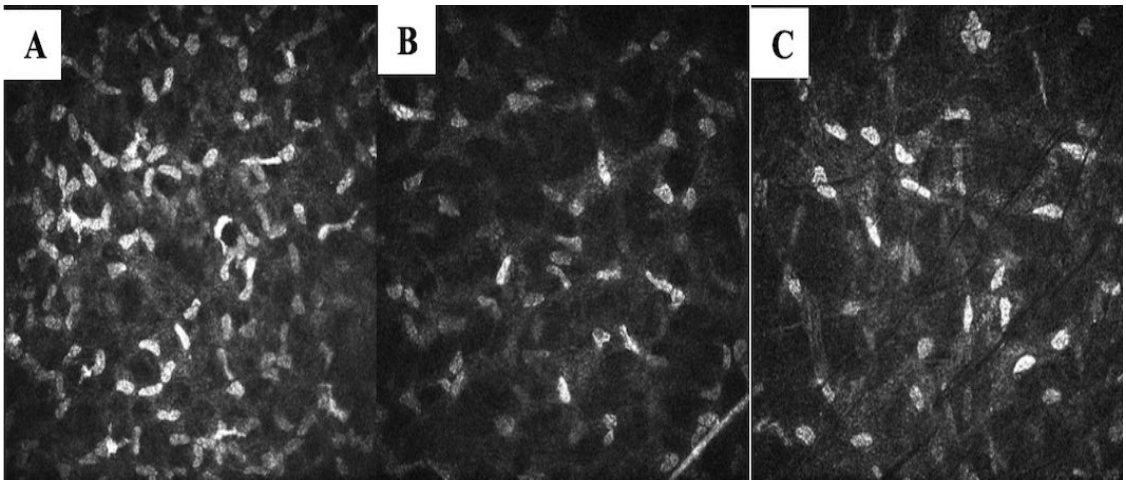

Figure 26. Images of the anterior (A), mid (B) and posterior (C) stroma with hyperreflective nuclei of the keratocytes.

5.3.4. Image Analysis

Keratocytes were counted manually using CCMetrics (Image Science, The University of Manchester, Manchester, UK) and the density (cells/mm^2) was derived as the number of cells per square millimeter of stroma using our previously established protocol (24).

5.3.5. Statistical Analysis

All statistical analyses were performed using IBM SPSS Statistics software Version 26 and P<0.05 was considered statistically significant. Normally distributed data were expressed as mean ± standard deviation and the means were compared using Independent t-test. Pearson correlation was undertaken to investigate the association between clinical parameters, corneal nerve fibre parameters and KD. Multiple logistic linear regression analysis was performed. The model included variables with *P-value* < 0.2 to account for statistically and clinically significant risk factors. Graph prism version 9 was used to build dot plots.

5.4. RESULTS

5.4.1. Clinical and Metabolic Characteristics

Clinical demographics are summarized in **Table 8**. Age (*P*=0.02) and height (*P*=0.02) were greater in patients with T1DM. There was no significant difference in weight, BMI, alanine aminotransferase, bilirubin or 25 OHD, but aspartate aminotransferase (AST) (*P*=0.019) was lower in participants with T1DM compared to controls.

Table 8. Clinical and corneal nerve parameters and keratocyte densities in patients with T1DM compared to healthy controls.

	Controls (n=20)	T1DM (n=20)	P-value
Age (Years)	12.83±1.91	14.47±2.43	**0.022**
Sex n (%)			
Boys	11 (55)	9 (45)	N/A
Girls	9 (45)	11 (55)	
Duration of diabetes (Years)	N/A	4.08±2.91	**N/A**
Height (m)	1.45±0.13	1.54±0.09	**0.020**
Weight (kg)	47.9±18.63	51.6±13.46	0.467
BMI (Kg/m^2)	22.26±5.47	21.68±5.09	0.733
HbA_{1c} (%)	N/A	9.29±2.06	N/A
AST (IU/L)	24.83±5.46	20.44±4.23	**0.019**
ALT (IU/L)	15.08±4.03	16.44±3.75	0.34
Bilirubin (µmol/L)	10.54±5.46	13.22±5.92	0.206
25 OHD (ng/mL)	59.77±22.45	45.41±21.39	0.085
CNFD (fibre/mm^2)	32.92±8.59	22.73±8.84	**0.001**
CNBD (no./mm^2)	47.34±20.01	26.19±14.63	**<0.001**
CNFL (mm/mm^2)	19.52±4.53	13.26±4.06	**<0.001**
CNFT (CT)	13.51±3.01	14.88±5.28	0.323
IWL (mm/mm^2)	23.43±3.94	15.51±5.48	**<0.001**
AKD (no/mm^2)	1231.24±193.38	1061.34±277.03	**0.035**
MKD (no/mm^2)	617.76±128.78	530.26±121.24	**0.038**
PKD (no/mm^2)	553.45±89.09	500.49±110.99	0.114

Data are presented as mean ± SD. BMI: body mass index, 25 OHD: 25hydroxycholecalciferol, AST: aspartate aminotransferase, ALT: alanine aminotransferase, CNFD: corneal nerve fibre density, CNBD: corneal nerve branch density, CNFL: corneal nerve fibre length, CNFT: corneal nerve fibre tortuosity, IWL: inferior whorl length, AKD: anterior keratocyte density, MKD: mid keratocyte density, PKD: posterior keratocyte density.

5.4.2. Corneal Confocal Microscopy

CNFD (*P*<0.001), CNBD (*P*<0.001), CNFL (*P*<0.001) and IWL (*P*<0.001) were lower and CNFT was comparable in children with T1DM compared to controls **(Table 8)** (14). Mid and posterior keratocyte densities were lower than anterior keratocyte density in both controls and children with T1DM. Anterior (*P*<0.03) and mid (*P*=0.03) keratocyte densities were lower with no difference in posterior keratocyte density in children with T1DM compared to controls **(Figure 27)**.

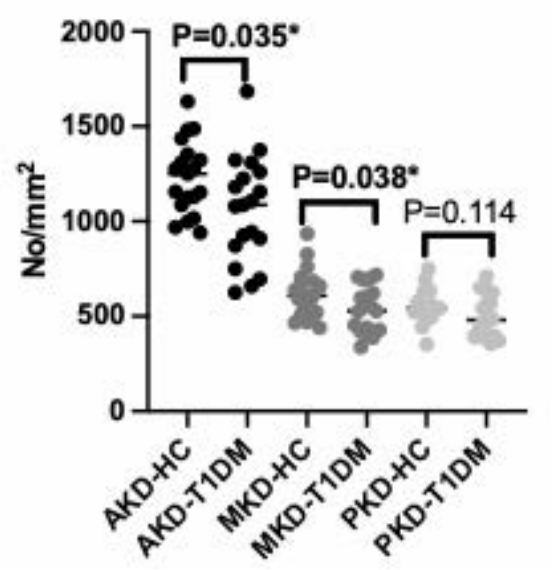

Figure 27. AKD, MKD, and PKD in children with T1DM compared to controls presented as mean and individual dot plots with significant difference.

AKD: anterior keratocyte density, MKD: mid keratocyte density, PKD: posterior keratocyte density, HC: Healthy control, T1DM: Type 1 diabetes mellitus.

5.4.3. Correlation

Age, duration of diabetes, height, weight, and BMI did not correlate with AKD, MKD or PKD **(Table 9)**. An inverse correlation was found between HbA_{1c} and PKD (r=-0.539, *P*=0.026), bilirubin with MKD (r=-0.540, *P*=0.025) and PKD (r=-0.531, *P*=0.028) and 25 OHD with MKD (r=-0.583, *P*=0.018). AST and ALT did not correlate with AKD, MKD or PKD. CNFD, CNFL and IWL did not correlate with AKD, MKD or PKD. There was a negative correlation between PKD with CNBD (=-0.49, *P*=0.030) and CNFT (r=-0.48, *P*=0.036).

Table 9. Correlation between anterior, mid, and posterior keratocyte densities with clinical demographics.

	Age (Years)	Duration of Diabetes (Years)	Height (m)	Weight (kg)	BMI (Kg/m^2)	HbA_{1c} (%)	Bilirubin (µmol/L)	25 OHD (ng/mL)
AKD (no/mm^2)	0.043	0.082	-0.161	-0.179	-0.120	-0.073	-0.178	-0.224
	(0.862)	(0.757)	(0.511)	(0.464)	(0.625)	(0.781)	(0.494)	(0.405)
MKD (no/mm^2)	0.071	-0.129	-0.152	0.036	0.114	-0.264	**-0.540**	**-0.583**
	(-.773)	(0.622)	(0.534)	(0.883)	(0.641)	(0.305)	**(0.025)**	**(0.018)**
PKD (no/mm^2)	0.095	0.004	0.064	0.223	0.247	**-0.539**	**-0.531**	-0.422
	(0.699)	(0.987)	(0.793)	(0.358)	(0.307)	**(0.026)**	**(0.028)**	(0.104)

Data is presented as Pearson Correlation (P-value). AKD: anterior keratocyte density, MKD: mid keratocyte density, PKD: posterior keratocyte density, BMI: body mass index, 25OHD: 25hydroxycholecalciferol.

5.4.4. Multiple Linear Regression

After multiple adjustments, sex, age, height, BMI, HbA_{1c}, ALT, AST, bilirubin, 25 OHD and corneal nerve parameters were not associated with AKD **(Table 10)**. Bilirubin (B=-11.190, 95% CI -19.48 to – 2.90, *P*=0.008) and 25 OHD (B=-3.400, 95% CI -5.72 to – 1.08, *P*=0.004) were independently associated with MKD (Table 3). HbA_{1c} (B=-30.641, 95% CI -53.39 to -7.89, *P*=0.008), AST (B=-11.35, 95% CI -22.38 to -0.311, *P*=0.044), bilirubin (B=-9.912, 95% CI -17.44 to -2.39, *P*=0.010), CNBD (B=-3.725, 95% CI -6.66 to -0.79, *P*= 0.013) and CNFT (B=-9.935, 95% CI -18.03 to -1.84, *P*=0.016) were associated with PKD **(Table 10).**

Table 10. Multivariate analysis of risk factors and corneal nerve parameters with anterior, mid, and posterior keratocyte density.

			Sex	Age (Years)	Height (m)	BMI (Kg/m²)	HbA1c (%)	Bilirubin (µmol/L)	AST (IU/L)	ALT (IU/L)	25 OHD (ng/mL)	CNFD (fibre/mm²)	CNBD (branch/mm²)	CNFL (mm/mm²)	CNFT (CT)	IWL (mm/mm²)
AKD	Adjusted	P-value														
		B (95%CI)	Not in Model	Not in Model	Not in Model	Not in Model	Not in Model	Not in Model	Not in Model	Not in Model	Not in Model	Not in Model	Not in Model	Not in Model	Not in Model	Not in Model
	Crude	P-value	0.236	0.862	0.511	0.464	0.625	0.494	0.193	0.815	0.405	0.948	0.350	0.600	0.310	0.576
		B (95%CI)	-155.82 (-423.67 to 112.02)	4.79 (-52.46 to 62.04)	-510.10 (-2113.83 to 1093.61)	-3.685 (-14.05 to 6.68)	-6.429 (-33.65 to 20.79)	-8.430 (-34.08 to 17.22)	-21.77 (-55.78 to 12.25)	4.61 (-36.59 to 45.81)	-2.640 (-9.236 to 3.956)	-0.489 (-16.10 to 15.131)	-4.255 (-13.60 to 5.09)	8.675 (-25.57 to 42.92)	-12.618 (-38.07 to 12.83)	-6.601 (-31.12 to 17.92)
MKD	Adjusted	P-value						**0.008**			**0.004**					
		B (95%CI)	Not in Model	Not in Model	Not in Model	Not in Model	Not in Model	-11.190 (-19.48 to – 2.90)	Not in model	Not in model	-3.400 (-5.72 to – 1.08)	Not in Model	Not in Model	Not in Model	Not in Model	Not in Model
	Crude	P-value	0.675	0.773	0.534	0.641	0.305	**0.025**	0.299	0.597	**0.018**	0.747	0.316	0.851	0.248	0.364
		B (95%CI)	24.609 (-97.04 to 146.26)	3.469 (-21.5 to 28.48)	-211.22 (-914.05 to [illegible])	2.681 (-9.24 to14.60)	-16.226 (-48.81 to 16.36)	-11.190 (-20.79 to -1.59)	-7.664 (-22.86 to 7.531)	4.531 (-13.350 to 22.412)	-3.400 (-6.12 to -0.68)	-1.061 (-7.88 to 5.75)	-1.993 (-6.07 to 2.08)	1.360 (-13.74 to16.55)	-6.258 (-17.29 to 4.78)	5.031 (-6.39 to16.45)
PKD	Adjusted	P-Value					**0.008**	**0.010**	**0.044**		0.063		**0.013**		**0.016**	0.163
		B (95%CI)	Not in Model	Not in Model	Not in Model	Not in Model	-30.64 1(-53.39 to -7.89)	-9. 912 (-17.44 to -2.39)	-11.35 (-22.38 to -	Not in Model	-2.095 (-4.302 to 0.112)	Not in Model	-3.725 (-6.66 to -0.79)	Not in Model	-9.935 (-18.03 to -1.84)	6.139 (-2.48 to 14.76)
	Crude	P-value	0.470	0.699	0.793	0.307	**0.026**	**0.028**	0.078*	0.796	0.104	0.584	**0.031**	0.936	**0.036**	0.207*
		B (95%CI)	38.636 (-71.57 to 148.85)	4.264 (-18.59 to 27.12)	82.028 (-567.61 to 731.68)	5.309 (-5.34 to 15.95)	-30.641 (-56.97 to – 4.30)	-9.912 (-18.63 to - 1.19)	-11.345 (-24.11 to 1.429)	-2.00 (-18.24 to 14.24)	-2.095 (-4.67 to 0.49)	1.639 (-4.56 to 7.84)	-3.725 (-7.06 to - 0.39)	0.537 (-13.29 to 14.37)	-9.935 (-19.15 to - 0.72)	6.139 (-3.75 to 16.03)

5.5. DISCUSSION

This study demonstrates a reduction in anterior and mid stromal keratocyte densities with no change in the posterior stromal keratocyte density and a loss of corneal nerves in children with T1DM. However, there is no independent association between keratocyte densities and corneal nerve parameters. Previous studies on corneal keratocytes in children are limited and whilst Szalai et al. did not quantify anterior or mid-stromal keratocyte densities, they found a significant higher posterior stromal keratocyte density, despite a loss of corneal nerves in adolescents with T1DM (11). Subsequently in a longitudinal study over 2 years, Deak et al. found no change in posterior keratocyte density despite a reduction in corneal nerves (13).

In adults with T2DM, Quadrado et al. found no difference in anterior, mid, or posterior keratocyte densities (33), whilst Bitirgen et al. found a significant reduction in anterior keratocyte density and corneal nerve parameters (30). Kalteniece et al. found a significant reduction in anterior, mid, and posterior keratocyte densities (24) and Ferdousi et al. found a significant reduction in the average stromal keratocyte density (25) which correlated with corneal nerve loss in adults with T1DM.

In the present study both anterior and mid keratocyte densities were reduced with no significant change in the posterior keratocyte density, although HbA1c was independently associated with PKD, in agreement with a study in adults by Kalteniece et al. (24). There was also an independent association between bilirubin with MKD and PKD and between AST and PKD. Bilirubin has been found to be protective in diabetic neuropathy (34-37) with an independent association between bilirubin levels and nerve conduction velocities (35), vibration perception (34) and diabetic

microvascular complications (34, 36). It has been suggested that this association with DSPN is mediated by anti-inflammatory and vascular protective effects of bilirubin (34, 36). However, we did not find any relationship between bilirubin levels and corneal nerve fibre parameters. A significant independent association was also found between mid-keratocyte density and 25 OHD levels. Vitamin D plays an important role in the integrity of gap junctions between epithelial cells and keratocytes (38). Thus, low levels of vitamin D may be associated with increased intercellular distance and reduced keratocyte density (38, 39). Posterior KD correlated inversely with CNBD, which is in contrast with findings in adults with T1DM where PKD correlated directly with CNBD (24). However, there was no correlation between corneal nerve and keratocyte cell loss.

A limitation of the current study is the cross-sectional design and relatively small cohort size of subjects studied. Nevertheless, we show an early loss of sub-basal corneal nerves and anterior and mid stromal keratocytes, and the latter was associated with HbA1c, AST, bilirubin and 25OHD. These associations warrant further studies to assess the mechanistic link between the risk factors for loss of corneal keratocytes and sub-basal nerves in T1DM.

The next chapter investigates other mechanisms beyond hyperglycaemia which may be related to nerve damage. CCM and continuous glucose monitoring (CGM) were assessed in diabetic adults to investigate whether different glycemic metrics have an impact on diabetic peripheral neuropathy.

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Chapter VI: CONTINOUS GLUCOSE MONITORING REVEALS A NOVEL ASSOCIATION BETWEEN DURATION AND SEVERITY OF HYPOGLYCAEMIA AND SMALL NERVE FIBRE INJURY IN PATIENTS WITH DIABETES

Gad H, Elgassim E, Mohammed I, Alhaddad AY, et al. Continuous glucose monitoring reveals a novel association between duration and severity of hypoglycemia and small nerve fiber injury in patients with diabetes. Endocr Connect. 2022 Oct 1:EC-22-0352. doi: 10.1530/EC-22-0352. Epub ahead of print. PMID: 36240043.

6.1. Abstract

Objective:

Continuous glucose monitoring has revealed that glycemic variability and low time in range are associated with albuminuria and retinopathy. We have investigated the relationship between glucose metrics derived from continuous glucose monitoring and a highly sensitive measure of neuropathy using corneal confocal microscopy in participants with type 1 and type 2 diabetes.

Methods:

A total of 40 participants with diabetes and 28 healthy controls underwent quantification of corneal nerve fibre density, corneal nerve branch density, corneal nerve fibre length and inferior whorl length and those with diabetes underwent continuous glucose monitoring for 4 consecutive days.

Results:

Corneal nerve branch density was significantly lower in patients with high glucose variability compared to low glucose variability (median (range) (25.0 (19.0 - 37.5) vs. 38.6 (29.2 - 46.9); P=0.007); in patients who spent >4% compared to <4% time below range (54-69 mg/dl) (25.0 (22.9 - 37.5) vs. 37.5 (29.2 - 46.9); P=0.045) and in patients who spent >1% compared to <1% time in severe hypoglycaemia (<54 mg/dl) (25.0 (19.8 - 41.7) vs. 35.4 (28.1 - 44.8); P=0.04). Duration in hyperglycaemia and severe hyperglycaemia showed no correlation with corneal nerve fibre density (P>0.05), corneal nerve branch density (P>0.05), corneal nerve fibre length (P>0.05) or inferior whorl length (P>0.05). However, duration in hypoglycaemia correlated with corneal nerve branch density (r=-0.34, P=0.03).

Conclusions:

Greater glucose variability and duration in hypoglycaemia, rather than hyperglycaemia are associated with nerve fibre loss in diabetes.

6.2. Introduction

Diabetic peripheral neuropathy (DPN) affects ~50% of people with T1DM and T2DM (1, 2). It has an insidious onset which can lead to painful diabetic neuropathy, erectile dysfunction, foot ulceration and lower limb amputation (1). Recognized risk factors for DSPN include poor glycemic control, obesity, hypertension, and dyslipidaemia (3, 4). However, HbA1c provides limited insight in the short-term variations in blood glucose which may affect nerve fibres (5). Continuous glucose monitoring (CGM) provides time in range (TIR) which is directly related to HbA1c, but also additional measures in relation to high and low blood glucose levels (6, 7).

Increased glycemic variability and low time in range (TIR) were associated with albuminuria and retinopathy, whilst neuropathy was associated with the standard deviation of blood glucose levels (SD) and mean amplitude of glycemic excursions (MAGE) (5). In a small proof of principle study, a higher mean glucose, M-value, and greater glycemic excursions were demonstrated in patients with painful compared to painless diabetic neuropathy (8). A recent systematic review demonstrated that a 10% increase in TIR was associated with a reduction in the prevalence of DSPN and cardiac autonomic neuropathy (9).

Severe iatrogenic hypoglycaemia can lead to neurological sequelae including cerebral dysfunction, seizures, and death and recurrent hypoglycaemia is associated with hypoglycaemia-associated autonomic failure (HAAF), reduced sympathetic neural responses and autonomic neuropathy (10-12). In a recent study higher MAGE and CV and especially nocturnal hypoglycaemia were associated with an increased risk of

DSPN (13). In a study of 80 adults with T1DM the standard deviation, coefficient of variation, mean amplitude of glycaemic excursion, percent time in level 1 (glucose 54-69 mg/dL) and level 2 (glucose < 54 mg/dL) hypoglycaemia, low blood glucose index and high blood glucose index were independently associated with cardiac autonomic neuropathy (CAN) (14). Sudomotor dysfunction, a measure of peripheral autonomic dysfunction (15) has also been independently associated with TIR in T1DM (16) and T2DM (17).

Corneal confocal microscopy (CCM) is a rapid non-invasive ophthalmic imaging technique that can identify early small nerve fibre loss in patients with DSPN (18) and has demonstrated comparable diagnostic utility to intra-epidermal nerve fibre density (IENFD) (4, 19, 20). A recent meta-analysis has confirmed the diagnostic utility of CCM in sub-clinical and clinical DSPN (21). In the current study we have investigated the relationship between different glucose metrics obtained using CGM and corneal nerve pathology using CCM in patients with type 1 and type 2 diabetes.

6.3. Methods

6.3.1. Patients

We recruited 68 participants (20 T1DM, 20 T2DM, and 28 healthy volunteers) between June 2021 to October 2021. Inclusion criteria were age ≥ 18 years and treatment with insulin. Exclusion criteria included vitamin B_{12} or folic acid deficiency, cancer, pregnancy, breast-feeding, or cardiac, liver, or renal dysfunction. Participants were also excluded if they had corneal pathology, allergy to eye-drops or previous ocular trauma or surgery in the past six months. The study was approved by the Ethics

Committee of Weill Cornell Medicine-Qatar, Hamad Medical Corporation, and Qatar University and was designed in accordance with the principles of the Helsinki Declaration. Written informed consent was obtained from all participants.

6.3.2. Basic and clinical demographics

Participants' height, weight, BMI, and blood pressure (BP) were measured. The lipid profile: total cholesterol (TC), triglyceride (TG), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C) and glycosylated hemoglobin A1c (HbA1c) were assessed only in participants with diabetes.

6.3.3. Continuous Glucose Monitoring (CGM)

The Freestyle Libre 1 system (Abbott) was used for subcutaneous interstitial continuous glucose monitoring. The sensor recorded glucose levels every 5 minutes for 4 consecutive days. The sensor was placed on the upper back part of the arm. The recommended target time in range (70-180mg/dL) (TIR) was >70% of the glucose readings (~16h 48min), time below range <70 mg/dL (TBR) <4% of the reading (~58min) (level 1 hypoglycaemia), TBR <54 mg/dL <1% of readings (~14min) (level 2 hypoglycaemia), time above range >180 mg/dL (TAR) <25% of the readings (~6h) (level 1 hyperglycaemia), TAR >250 mg/dL <5% of the readings (1h 12min) (level 2 hyperglycaemia). Glucose variability was defined as percent coefficient of variation (%CV) with a target ≤36%. Hypoglycaemia was defined according to continuous glucose reading of <70 mg/dl.

6.3.4. Corneal Confocal Microscopy (CCM)

Corneal confocal microscopy was undertaken using the Heidelberg Retina Tomograph Cornea Module (Heidelberg Engineering, Germany). Both eyes were anaesthetized with 2 drops of Bausch & Lomb Minims ® (Oxybuprocaine hydrochloride 0.4% w/v). A drop of hypotears gel (Carbomer 0.2% eye gel) was placed on the tip of the objective lens and a sterile disposable TomoCap was placed over the lens, allowing optical coupling of the objective lens to the cornea. Six images were selected from the sub basal nerve plexus (SBNP) in the central cornea and corneal nerve fibre density (CNFD) (fibres/mm^2) corneal nerve branch density (CNBD) (branches/mm^2) and corneal nerve fibre length (CNFL) (mm/mm^2) were quantified manually using CCMetrics. Six images centered on the inferior whorl and immediately adjacent area were selected and inferior whorl length (IWL) (mm/mm^2) was quantified manually using the manual CNFL mode in CCMetrics. The investigator was blind to the study group when performing CCM and analyzing CCM images.

6.3.5. Statistical analysis

All statistical analyses were performed using IBM SPSS Statistics software Version 27 and $P<0.05$ was considered statistically significant. Normality of the data was assessed using the Shapiro-Wilk test and by visual inspection of the histogram and a normal Q-Q plot. Data are expressed as mean and SD for the normally distributed variables and as median and range for the skewed variables. Inferential analyses were conducted for the corneal nerve parameters and clinical demographics between healthy control versus T1DM and T2DM using both parametric (ONE-WAY ANOVA) and non-parametric (Kruskal-Wallis 1-way ANOVA) tests. Differences between cardiometabolic

risk factors between participants with T1DM and T2DM were assessed using parametric tests (independent sample T-test) and non-parametric test (Mann-Whitney U) as appropriate. To investigate the differences between corneal nerve morphology in diabetic patients with different glycemic targets were investigated using ONE-WAY ANOVA for normally distributed data (CNFD and CNFL) and Kruskal-Wallis 1-way ANOVA for skewed data (CNBD). To investigate the association between corneal nerve parameters and clinical and CGM variables, Pearson and Spearman correlation were performed as appropriate. Graph prism version 9 was used to build dot plots.

6.4. Results

A total of 40 participants with diabetes aged 37-48 years and 28 healthy controls aged 24-49 years were enrolled in the study. Participants with diabetes and controls had comparable systolic blood pressure (SBP) mmHg (P=0.09), diastolic blood pressure (DBP) mmHg (P=0.24) and BMI kg/m^2 (P=0.06) **(Table 11)**. Participants with T2DM had higher triglycerides (P=0.002) and lower HDL (P=0.001), while those with T1DM had higher LDL levels (P=0.020) **(Table 11).** There was no difference in in the average CGM glucose or HbA1c between participants with T1DM and T2DM. Interstitial glucose was in range for 60% of participants with T1DM and T2DM (combined), 32% were above range and 8% were very high **(Figure 28)**. CNFD fibre/mm^2 (25.2 ± 5.87 vs. 26.4 ± 6.17 vs. 29.9 ± 6.02; P=0.02, CNBD branch/mm^2 27.0 (18.7 - 39.6) vs. 35.4 (27.1 - 50) vs. 56.2 (46.9 - 68.7); P<0.001, CNFL mm/mm^2 15.9 ± 3.91 vs. 17.6 ± 4.23 vs. 22.5 ± 3.57; P<0.001 and IWL mm/mm^2 14.9 ± 5.06 vs. 15.7 ± 7.29 vs. 20.8 ± 5.07; P=0.005 were significantly lower in participants with diabetes compared to controls **(Table 11)**.

Participants with T1DM and T2DM spent comparable time in range and above range (level 1 hyperglycaemia), while those with T1DM spent more time below range (level 1 hypoglycaemia) with approximately 4 hypoglycemic events over a period of 4 days **(Table 11)**.

Table 11. Demographics of diabetic patients and controls.

Demographics	**Healthy volunteers**	**T1DM**	**T2DM**	**P-Value**
Subjects, n	28	20	20	-
M:F ratio	22:6	13:7	15:5	-
Age (years)	35.4 ± 15.7	30.3 ± 8.91[II]	51.1 ± 8.93[^]	<0.001[*]
Diabetes duration (years) n(%) <10years 10-20years 21-40yeas	-	 6 (30) 9 (45) 5 (25)	 3 (15) 14 (70) 3 (15)	-
SBP (mmHg)	122 (120 - 136)	119 (115 - 123)	126.3 (120.7 - 138.3)	0.09
DBP (mmHg)	79.0 ± 10.6	74.4 ± 9.10	79.5 ± 11.1	0.24
BMI (kg/m^2)	27.0 ± 5.48	27.1 ± 6.67	30.6 ± 3.93	0.06
TC (mmol/L)	-	4.97 ± 1.07	4.25 ± 1.39	0.05
TG (mmol/L)	-	0.9 (0.8 - 1.57)	1.64 (1.46 - 2.35)	0.002[*]
HDL-C (mmol/L)	-	1.49 ± 0.36	1.11 ± 0.25	0.001[*]
LDL-C (mmol/L)	-	3.0 (2.2 - 3.6)	1.98 (1.8 - 2.4)	0.02[*]
Average CGM glucose (mg/dL)	-	179.6 ± 51	177.9 ± 44.8	0.91
HbA1c (%)	-	8.58 ± 2.1	9.12 ± 1.2	0.32
CNFD (fibre/mm^2)	29.9 ± 6.02	25.2 ± 5.87[$]	26.4 ± 6.17	0.020[*]
CNBD (branch/mm^2)	56.2 (46.9-68.7)	27.0 (18.7 - 39.6)[$]	35.4 (27.1 - 50)[^]	<0.001[*]
CNFL (mm/mm^2)	22.5 ± 3.57	15.9 ± 3.91[$]	17.6 ± 4.23[^]	<0.001[*]
IWL (mm/mm^2)	20.8 ± 5.07	14.9 ± 5.06[$]	15.7 ± 7.29[^]	0.005[*]
TIR % (min) (70-180 mg/dL)	-	45.9 ± 14.7 (662.5 ± 211.4)	53.9 ± 27.9 (776.2 ± 402.8)	0.27
TAR % (min) (181-250 mg/dL)	-	24.0±12.0 (344.9 ± 173.3)	30.0±18.2 (432.3 ± 261.8)	0.22
TBR % (min) (54-69 mg/dL)	-	4 (2 - 12) (120 (86 - 140))	0 (0 - 14) (0(0 - 240))	<0.001[*]
No. of hypoglycemic events	-	4.0 (3.0 - 7.0)	0 (0 - 7.0)	<0.001[*]

M: male, F: female, SBP: systolic blood pressure, DBP: diastolic blood pressure, BMI: body mass index, TC: total cholesterol, TG: triglycerides, HDL-C: high density lipoprotein-cholesterol, LDL-C: low-density lipoprotein cholesterol, HbA1c: glycated hemoglobin, CNFD: corneal nerve fibre density, CNBD: corneal nerve branch density, CNFL: corneal nerve fibre length, IWL: inferior whorl length, CGM: continuous glucose monitoring, TIR: time in range, TAR: time above range, TBR: time below range.
Data is expressed as mean ± SD or median (range).
[*]Significant difference between groups
[$]Significant difference between HC and T1DM
[^]significant difference between HC and T2DM
[II] Significant difference between T1DM and T2DM

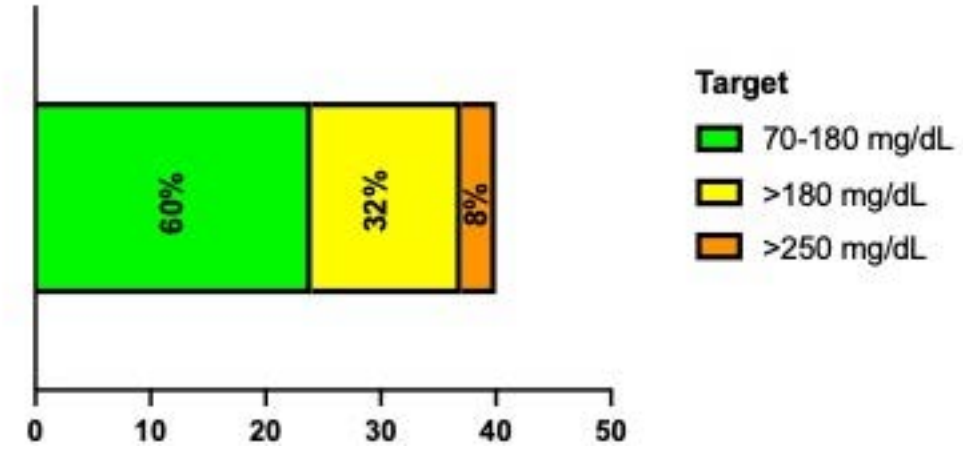

Figure 28. Distribution of T1DM and T2DM patients based on their interstitial glucose targets.

6.4.1. Corneal confocal microscopy (CCM) based on CGM

CNFD (P=0.50), CNBD (P=0.68), CNFL (P=0.71) and IWL (P=0.10) did not differ between patients who had diabetes duration for <10 years, >10 years or more than 20 years **(Table 12)**.

Table 12. Changes in corneal nerve morphology in relation to duration of diabetes and different glucose metrics on CGM.

Glycemic control indicators	CNFD	CNBD	CNFL	IWL
Duration of diabetes				
<10years (n=9)	27.2 ± 5.9	35.4 (25.0 - 50.0)	17.3 ± 2.17	15.5 ± 6.96
10-20years (n=23)	24.8 ± 5.64	29.2 (25.0 - 39.6)	16.3 ± 4.19	13.9 ± 5.57
21-40years (n=8)	26.9 ± 7.10	39.6 (18.7 - 55.2)	17.5 ± 5.62	19.3 ± 6.08
P-value	0.50	0.68	0.71	0.10
HbA1c %				
<7.5% (n=10)	26.5 ± 6.30	33.3 (25.0 - 43.7)	17.0 ± 2.44	16.6 ± 5.57
>7.5% (n=30)	25.5 ± 6.30	30.2 (25.0 - 40.6)	16.7 ± 4.56	14.9 ± 6.44
P-Value	0.67	0.89	0.85	0.47
GV (%CV)				
Low <36% (n=24)	26.2 ± 6.26	38.6 (29.2 - 46.9)	17.7 ± 4.64	5.63 ± 6.82
High>36% (n=16)	25.2 ± 5.65	25.0 (19.0 - 37.5)	15.4 ±2.75	14.9 ± 5.34
P-Value	0.62	0.007*	0.09	0.73
TIR (70-180 mg/dL)				
In range >70% (n=8)	26.7 ± 4.66	31.2 (26.0 - 44.8)	16.9 ± 3.0	14.3 ± 6.26
In range <70% (n=32)	25.6 ± 6.29	31.2 (25.0 - 40.6)	16.7 ± 4.37	15.6 ± 6.27
P-Value	0.64	0.75	0.91	0.59
TBR (54-69 mg/dL)				
Below range >4% (n=13)	27.0 ± 5.08	25.0 (22.9 - 37.5)	16.1 ± 2.31	14.0 ± 6.45
Below range <4% (n=27)	25.2 ± 6.35	37.5 (29.2 - 46.9)	17.1 ± 4.75	15.9 ± 6.11
P-Value	0.38	0.04*	0.51	0.35
TBR (<54 mg/dL)				
Severely below range >1% (n=9)	26.4 ± 6.6	25.0 (19.8 - 41.7)	15.7 ± 2.93	14.4 ± 6.63
Severely below range<1% (n=31)	25.6 ± 5.86	35.4 (28.1 - 44.8)	17.1 ± 4.38	15.6 ± 6.17
P-Value	0.79	0.04*	0.36	0.62
Hypoglycemic events				
>1 event (n=23)	26.1 ± 5.38	28.1 (25.0 - 39.6)	16.3 ± 3.51	14.6 ± 5.77
No events (n=17)	25.4 ± 6.83	37.5 (29.2 - 51.0)	17.4 ± 4.84	16.4 ± 6.78
P-Value	0.71	0.08	0.43	0.37
TAR (181-250 mg/dL)				
Above range >25% (n=22)	25.3 ± 6.92	36.5 (25.0 - 50.0)	16.9 ± 5.23	15.8 ± 6.18
Above range <25 (n=18)	26.3 ± 4.67	30.2 (25.0 - 37.5)	16.6 ± 2.19	14.8 ± 6.38
P-Value	0.61	0.44	0.83	0.62
TAR (>250 mg/dL)				
Severely above range >5% (n=31)	25.5 ± 6.33	31.2 (25.0 - 41.7)	16.7 ± 4.46	16.1 ± 6.48
Severely above range <5% (n=9)	26.7 ± 4.72	31.2 (27.1 - 43.7)	16.9 ± 2.75	12.5 ± 4.44
P-Value	0.59	0.97	0.89	0.14

CNFD: corneal nerve fibre density, CNBD: corneal nerve branch density, CNFL: corneal nerve fibre length, IWL: inferior whorl length, HbA1c: glycated hemoglobin, GV: glycemic variability, TIR: time in range, TBR: time below range, TAR: time above range. *Significant at P<0.05.

There was no difference in CNFD (P=0.67), CNBD (P=0.89), CNFL (P=0.85), and IWL (P=0.47) between participants with an HbA1c <8% or >8%. CNBD was significantly lower in patients with high GV compared to low GV (25.0 (19.0 - 37.5) vs. 38.6 (29.2 - 46.9); P=0.007). There was no difference in CNFD (P=0.62), CNFL (P=0.09) and IWL (P=0.73) between patients with high GV compared to low GV. There was no significant difference in CNFD (P=0.64), CNBD (P=0.75), CNFL (P=0.91) and IWL (P=0.59) between participants with diabetes who spent >70% time in range (TIR) (70-180 mg/dl) and <70% TIR. CNBD was significantly lower (25.0 (22.9 - 37.5) vs. 37.5 (29.2 - 46.9); P=0.04), with no difference in CNFD (P=0.38), CNFL (P=0.51) and IWL (P=0.35) in patients who spent >4% time below range (54-69 mg/dl) compared to <4% time below range. CNBD (25.0 (19.8 - 41.7) vs. 35.4 (28.1 - 44.8); P=0.04) was significantly lower, whilst CNFD (P=0.79), CNFL (P=0.36) and IWL (P=0.62) did not differ between patients who spent >1% compared to <1% time in severe hypoglycaemia (<54 mg/dl). CNFD (P=0.71), CNBD (P=0.09), CNFL (P=0.43) and IWL (P=0.37) did not differ between patients who had >1 hypoglycemic event compared to those who had no hypoglycemic events. CNFD (P=0.61), CNBD (P=0.44), CNFL (P=0.83) and IWL (P=0.62) did not differ between patients who spent >25% time in hyperglycaemia (181-250 mg/dl) compared to those who spent <25% time in hyperglycaemia. CNFD (P=0.59), CNBD (P=0.97), CNFL (P=0.89) and IWL (P=0.14) did not differ between patients who spent >5% time in severe hyperglycaemia (>250 mg/dl) compared to patients who spent <5% time in severe hyperglycaemia **(Table 12).** CNFD (P=0.11) did not differ significantly between patients in TIR, TAR or TBR compared to healthy controls. CNBD (P<0.0001) and CNFL (P<0.0001) were significantly lower in participants with diabetes

in TIR, TAR and TBR compared to healthy controls **(Figure 29A-G)**. There was no difference in CNFD (P=0.93), CNBD (P=0.24), and CNFL (P=0.61) between diabetic patients with TIR, TAR-Level 1, TAR-Level 2, TBR-Level 1 or TBR-Level 2 **(Figure 30A-C)**.

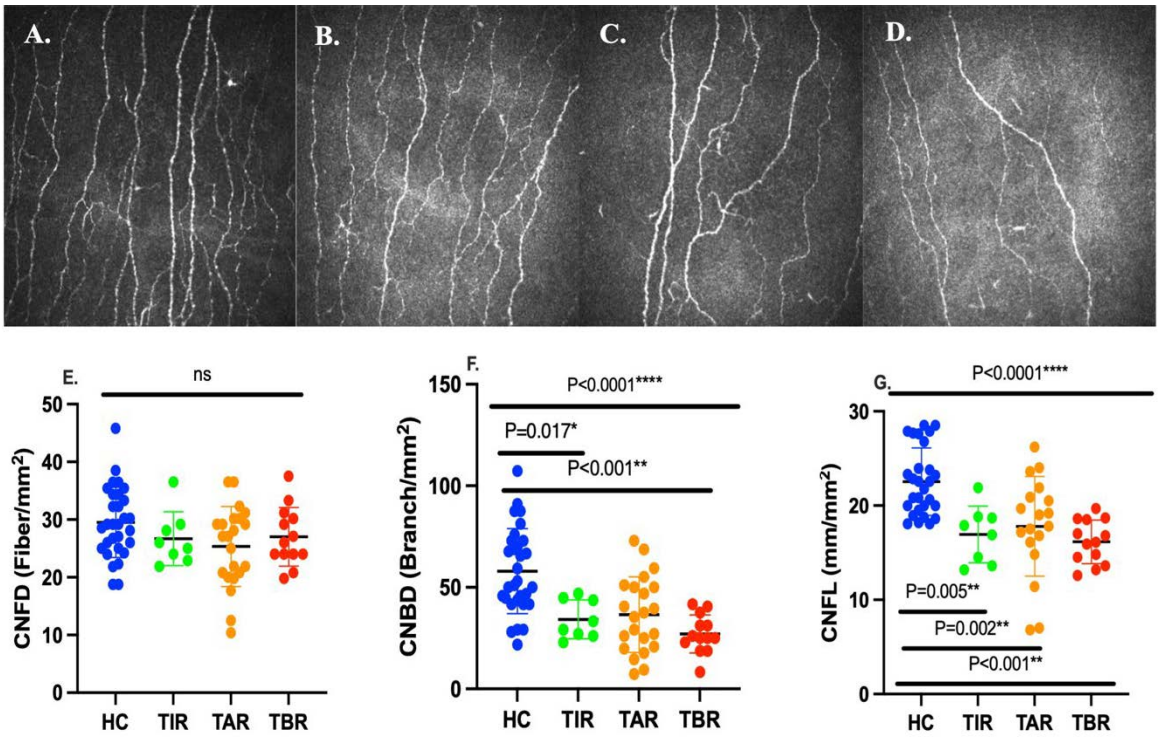

Figure 29. Corneal nerve fibre morphology and CCM parameters in diabetic patients compared to healthy controls based on glycemic targets.

CNFD: corneal nerve fibre density, CNBD: corneal nerve branch density, CNFL: corneal nerve fibre length, HC: healthy control, TIR: time in range, TAR: time above range, TBR: time below range

A. HC
B. Diabetic with TIR
C. Diabetic with TAR
D. Diabetic with TBR
E. CNFD in HC vs. diabetic in TIR, TAR, TBR
F. CNFD in HC vs. diabetic in TIR, TAR, TBR
G. CNFL in HC vs. diabetic in TIR, TAR, TBR

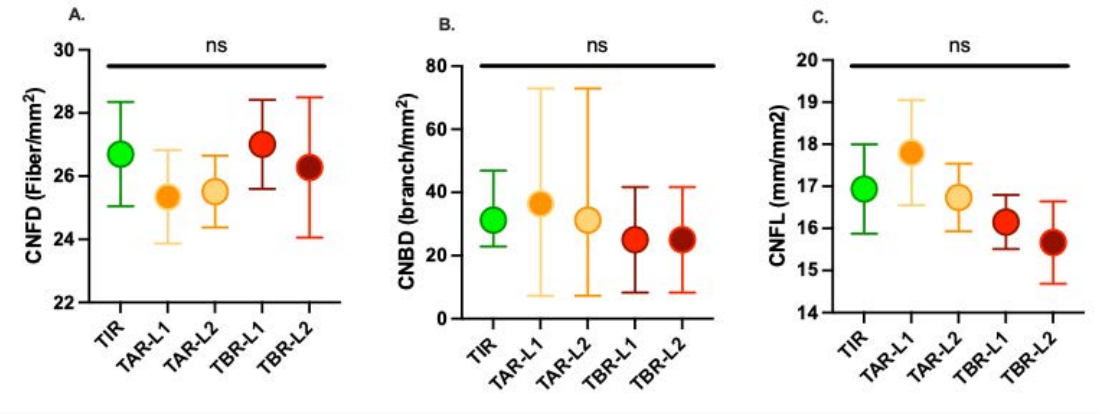

Figure 30. Corneal nerve fibre morphology and CCM parameters in diabetic patients based on different glycemic targets.

CNFD: corneal nerve fibre density, CNBD: corneal nerve branch density, CNFL: corneal nerve fibre length, HC: healthy control, TIR: time in range, TAR-L1: time above range-Level1, TAR-L2: time above range-Level2, TBR-L1: time below range-Level1, TBR-L2: time below range-Level2

6.4.2. Correlation between corneal nerve parameters and CGM indicators of glycaemia.

Duration of diabetes (years), plasma glucose, average interstitial glucose, and HbA1c did not correlate with CNFD (P>0.05), CNBD (P>0.05), CNFL (P>0.05) and IWL (P>0.05) **(Table 13)**.

Table 13. Correlation between CCM parameters and glycemic variables using CGM.

Glycemic indicators	CNFD (fibre/mm^2)	CNBD (branch/mm^2)	CNFL (mm/mm^2)	IWL (mm/mm^2)
Duration of diabetes (years)	0.11 (0.49)	0.10 (0.53)	0.01 (0.96)	0.004 (0.98)
Plasma Glucose (mmol/L)	0.19 (0.25)	-0.05 (0.77)	0.07 (0.66)	0.08 (0.64)
Average interstitial glucose (mg/dl)	-0.09 (0.55)	0.04 (0.79)	0.07 (0.66)	0.09 (0.57)
HbA1c (%)	-0.08 (0.63)	0.07 (0.67)	-0.04 (0.78)	-0.004 (0.98)
GV (%)	0.04 (0.79)	-0.39 (0.011)*	-0.28 (0.08)	-0.05 (0.76)
Duration in high glucose (min)	0.15 (0.35)	0.25 (0.12)	0.27 (0.09)	0.16 (0.337)
Duration in very high glucose (min)	-0.11 (0.48)	0.02 (0.88)	0.05 (0.74)	0.15 (0.35)
Duration in low glucose (min)	0.12 (0.46)	-0.34 (0.031)*	-0.23 (0.15)	-0.09 (0.59)
Number of hypoglycemic events (no)	0.07 (0.66)	-0.26 (0.11)	-0.19 (0.25)	-0.07 (0.65)

HbA1c: glycated hemoglobin, GV: glucose variability, CNFD: corneal nerve fibre density, CNBD: corneal nerve branch density, CNFL: corneal nerve fibre length

Glucose variability correlated significantly with CNBD (r=-0.398, P=0.01) **(Figure 31A)**, but did not correlate with CNFD (P>0.05), CNFL (P>0.05), or IWL (P>0.05). Duration in

hyperglycaemia and severe hyperglycaemia did not correlate with CNFD (P>0.05), CNBD (P>0.05), CNFL (P>0.05) and IWL (P>0.05). However, the duration in hypoglycaemia correlated significantly with CNBD (r=-0.34, P=0.03) **(Figure 31B)**, but not with CNFD (P>0.05), CNFL (P>0.05) and IWL (P>0.05). The number of hypoglycemic events did not correlate with CNFD (P>0.05), CNBD (P>0.05), CNFL (P>0.05) or IWL (P>0.05).

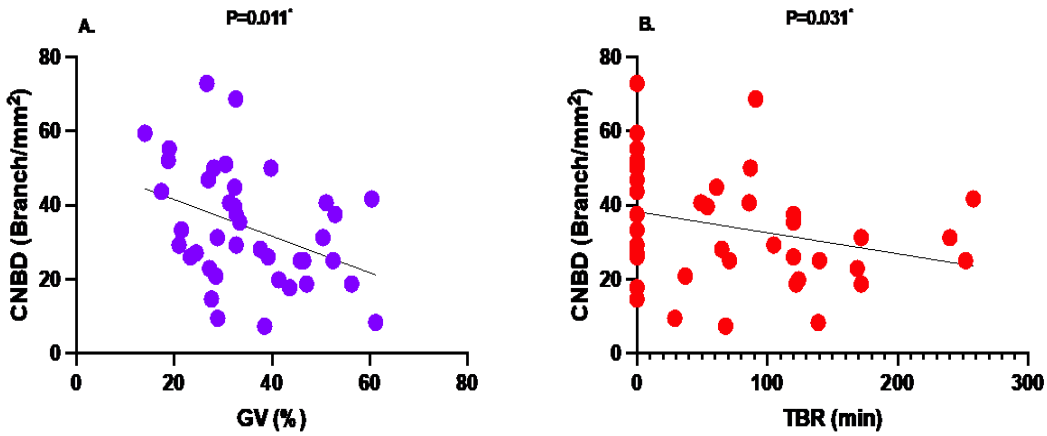

Figure 31. Correlation between CNBD vs. GV (%) and TBR (min) in patients with diabetes.

CNBD: corneal nerve branch density, GV: glucose variability, TBR: time below range

6.5. Discussion

We have demonstrated that increased glucose variability and hypoglycaemia detected using CGM are associated with lower corneal nerve branch density in patients with type 1 and type 2 diabetes. Several studies have reported an association between corneal nerve measures and the duration of diabetes (22, 23) and HbA1c (24, 25) in people with type 1 and type 2 diabetes. Several large clinical trials have

shown that improved glycemic control can prevent the development and progression of diabetic neuropathy in type 1 diabetes (26), but not type 2 diabetes (27-30). However, smaller interventional studies utilising CCM in type 1 and type 2 diabetes have demonstrated that lowering HbA1c is associated with an increase in corneal nerve parameters (31-34). Although, in patients with type 2 diabetes, we have recently shown that despite an improvement in HbA1c, in patients taking glucose lowering therapies associated with weight gain and hypoglycaemia there was a reduction in corneal nerve branch density (35).

Therefore, the relationship between glycemic control and complications is complex and whilst HbA_{1c} is an important measure of overall glucose control, it fails to capture the magnitude and frequency of glucose variations and indeed the contribution of hypoglycaemia. Indeed, intensive glycemic control is associated with an increased incidence of hypoglycaemia and adverse cardiovascular outcomes (36). Hence, there has been an increasing emphasis on defining the role of optimal glucose range and glucose variability in the development of diabetic complications (37). Diabetic neuropathy has been associated with an increase in the standard deviation of blood glucose (SD) and mean amplitude of glycemic excursions (MAGE) (5) and a recent study also demonstrated that TIR was associated with DSPN symptoms (38). A systematic review showed that a 10% increase in TIR was associated with a reduction in the prevalence of DSPN and cardiac autonomic neuropathy (9). Whilst higher MAGE and CV were associated with an increased risk of DSPN, there was also a significant association with the occurrence of nocturnal hypoglycaemia (13). Furthermore, in

adults with T1DM a range of indices of hypoglycaemia have been independently associated with cardiac autonomic neuropathy (CAN) (14).

Sudomotor dysfunction has also been independently related to nocturnal TIR in T1DM (16) and T2DM (17). In a recent study, glucose variability assessed by calculating the continuous overall net glycemic action (CONGA) and the percentage of time in normal and high range glucose was associated with nerve excitability and inferior whorl length but not corneal nerve fibre density or length in a cohort of patients with T1DM (39). We now show that increased glucose variability and time below range (TBR) were associated with small nerve fibre damage evidenced by lower corneal nerve branch density in patients with type 1 and type 2 diabetes. We believe the underlying mechanisms of nerve damage here are very different from the severe insulin induced experimental hypoglycemic neuropathy characterized by reduced motor and sensory nerve conduction velocities and a distal dying back axonal degeneration affecting motor more than sensory axons (40) and axonal degeneration (41) in the proximal sciatic rather than distal plantar nerves (42), with myelinated nerve fibre damage (43) in motor rather than sensory roots (44). Indeed, in a study of diabetic BB rats with insulin implants to induce moderate hypoglycaemia there was evidence of shorter and thinner intraepidermal nerve fibres (45). Thus, in the current study we show sensory small nerve fibre pathology characterized by a lower corneal nerve branch density in patients with level 1 (54-69 mg/dl) and level 2 (<54 mg/dl) hypoglycaemia. In a case report of a 26-year-old female with T1DM, frequent silent hypoglycaemia (average <60mg/dl) was associated with numbness and tingling in both hands and feet, which resolved with resolution of hypoglycaemia (46). Several mechanisms may

underlie hypoglycaemia-induced nerve injury, including reduced nerve blood flow and hypoxia (47-49) and a slowing of axonal transport (50).

We acknowledge limitations of the current study include the lack of prior power calculation, relatively small cohort size and short duration of CGM monitoring. However, a previous study in diabetic patients has shown that 3-days of CGM contributed to sustained improvement in HbA1c at 3 and 6 months (51). Nevertheless, CCM shows small nerve fibre damage in participants with diabetes with higher glucose variability and in those who spent a longer duration in hypoglycaemia. CGM, alongside CCM are highly sensitive technologies to explore the relationship between glycemic variability and nerve damage and provide novel insights into the development of diabetic neuropathy.

In the next chapter, we utilized CCM to assess children with obesity, as obesity is a major problem worldwide, especially in the Middle East. Previous studies have shown that obese adults have reduced corneal nerves, so the next chapter explored whether CCM can be used to detect early neuropathy in children with obesity.

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Chapter VII: EARLY CORNEAL NERVE LOSS IN CHILDREN WITH OBESITY AND ACANTHOSIS NIGRICANS

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7.1. Abstract

Background/aim: Obesity in adults is associated with peripheral neuropathy. Childhood obesity is highly prevalent in the MENA region and may also be associated with neuropathy. The aim of this study was to investigate if there was evidence of early small nerve fibre damage in obese children with acanthosis nigricans.

Material and methods: Children with obesity with and without acanthosis nigricans (AN) and healthy controls underwent body composition analysis, assessment of vibration perception threshold (VPT), monofilament sensitivity and corneal confocal microscopy (CCM). Corneal nerve fibre density (CNFD), branch density (CNBD), length (CNFL) and inferior whorl length (IWL) were quantified.

Results:

Forty-six participants with obesity (31 with AN and 15 without AN) aged 15 (14 - 17) years were compared to 20 healthy controls aged 13 (12 - 14) years. There was no difference in VPT, monofilament sensitivity and CCM measures between children with obesity and controls. However, children with AN had a significantly higher weight (P=0.02) and fat% (P=0.03) with lower CNFD (P=0.04) compared to children with obesity without AN.

Conclusion: Children with obesity and acanthosis nigricans have evidence of early corneal nerve loss, indicative of a sub-clinical neuropathy.

7.2. Introduction

The World Obesity Federation estimated that by 2025 there will be 206 million children and adolescents with obesity, and this will increase to 254 million by 2030(1). The prevalence of childhood obesity varies from 7.9% in the UAE, 14.7% in Qatar, 15.8% in Saudi Arabia to 19.9% in Kuwait (2). Childhood obesity is characterized by increased body fat mass with dyslipidaemia, hypertension, increased insulin resistance (IR), impaired glucose tolerance (IGT) and eventually type 2 diabetes mellitus (T2DM) (3, 4). Acanthosis nigricans (AN) in individuals with obesity is characterized by thickened skin and brown pigmentation on the neck, axillae, knees and elbows and is indicative of underlying IR, metabolic syndrome and an increased risk of developing T2DM (5). The severity of AN in children is associated with higher fasting insulin levels and Homeostatic Model Assessment for Insulin Resistance (HOMA-IR) score, indicative of insulin resistance(6).

Adults with impaired glucose tolerance (IGT) have evidence of neuropathy (7, 8) and children with obesity and IGT have abnormal nerve conduction studies (NCS) (4). Children and adolescents with T2DM have abnormal pin prick, light touch and vibration perception threshold (VPT) (9,10) and the incidence of neuropathy (VPT >20V) was two fold greater in children with T2DM (13.9/1000 patient years) compared to T1DM (7.8/1000 patient years) (11). In the SEARCH study adolescents with T2DM had a higher age-adjusted prevalence of peripheral neuropathy (17.7% vs 8.5%) compared to T1DM (12). Furthermore, the prevalence of DSPN based on the Michigan Neuropathy Screening Instrument (MNSI) was 3-fold higher in youth with

T2DM (22%) compared to T1DM (7%) and was associated with older age, longer duration of diabetes, smoking and lower HDL (13).

Corneal confocal microscopy enables the identification of early sub-clinical neuropathy. There is significant corneal and intra-epidermal nerve fibre loss in subjects with IGT (14) and recently diagnosed T2DM (15) and a lower corneal nerve fibre length predicts the development of DSPN (16). We have previously shown corneal nerve fibre loss in obese adults with and without diabetes and corneal nerve regeneration after bariatric surgery (17). The early identification of sub-clinical neuropathy is key to risk stratification and intervention to limit the development of overt and often irreversible neuropathy. In this study, we have undertaken an assessment of vibration perception threshold, monofilament sensitivity and CCM in children with obesity with and without AN.

7.3. Methods

7.3.1. Participants and Study Design

This study evaluated 66 participants aged 8-17. Forty-six participants with obesity were recruited from the pediatric endocrinology clinic and 20 healthy controls were recruited from the general pediatric clinics in a tertiary hospital in Qatar. Participants with a history of any other cause of neuropathy, malignancy, deficiency of B_{12} or folate, chronic renal failure, liver failure, connective tissue, or systemic disease (rheumatoid arthritis, systemic lupus erythematosus, dermatomyositis, systemic scleroderma, Raynaud phenomenon), previous corneal trauma or systemic disease affecting the cornea, and corneal surgery within 6 months of enrollment, were

excluded. All participants provided written assent and parental informed consent and the research adhered to the tenets of Declaration of Helsinki and was approved by the Weill Cornell Medicine-Qatar (WCM-Q) (20-0006) and Sidra Medicine (1542992) Research Ethics Committees.

7.3.2. Anthropometry

Weight (kg) was measured using the body composition analyzer (TANITA DC-430MAIII) and height (cm) using the stadiometer (SECA model) and both were recorded to the nearest 0.1 g or cm, respectively (18). The cut-off points to classify weight status were established using the International Obesity Task Force (IOFT)(19) and WHO growth chart (20). Body composition was measured using the TANITA scale following the manual input of height, sex, and age of the participants to derive the body fat percent (BF%), fat mass (kg), fat free mass (FFM) (kg), muscle mass (kg), total body water (TBW) (kg), TBW (%), and BMI (kg/m^2). The ratio of muscle-to-fat (MFR) was calculated manually using the muscle mass and fat mass. Height, weight, and BMI of the healthy control participants was measured as part of routine care and was collected from the electronic medical records. Participants were subdivided into those with and without acanthosis nigricans, based on established criteria (21). Classification was based on the darkness of skin in 5 locations and texture: neck, knee, axilla, elbow, and knuckles. A score of 0-4 was given where 0=absent; 1=present; 2=mild, 3=moderate; 4=severe. Score of texture was classified as follows: 0=smooth, 1-rough, 2= coarse, 3= extremely coarse.

7.3.3. Cardiometabolic panel assessments

Systolic (SBP) and diastolic (DBP) blood pressure, glycated hemoglobin (HbA1c), total cholesterol (TC), LDL cholesterol (LDL-C), HDL cholesterol (HDL-C), triglycerides (TG), and vitamin D (25-OH-D) were assessed as part of routine care for all participants who attended the obesity clinic and data were collected from their electronic medical records.

7.3.4. Neuropathy assessment

A. Vibration perception threshold (VPT)

Participants were asked to remove their shoes and socks and the stimulator was applied on the pulp of the big toe on both sides and the stimulus strength increased slowly from zero until the vibration was first perceived by indicating "yes". Vibration sensation was recorded as an average for both feet in volts (22). A VPT of **≥** 15V was considered to be impaired vibration perception (23).

B. Monofilament

A 10 g monofilament (Semmes-Weinstein monofilament) was applied with a sufficient force to cause the filament to bend at a total of 9 sites per foot, on both feet. Loss of protective sensation was recorded as "no feeling in **≥** 8 sites" (24).

C. Corneal Confocal Microscopy

Corneal confocal microscopy was undertaken using the Heidelberg Retina Tomograph III Rostock Cornea Module (Heidelberg Engineering, Heidelberg, Germany). Both eyes were anaesthetized with 2 drops of Bausch & Lomb Minims ® (Oxybuprocaine

hydrochloride 0.4% w/v). A drop of hypotears gel (Carbomer 0.2% eye gel) was placed on the tip of the objective lens and a sterile disposable TomoCap was placed over the lens, allowing optical coupling of the objective lens to the cornea. Six images were selected from the sub basal nerve plexus (SBNP) in the central cornea and corneal nerve fibre density (CNFD) (fibres/mm^2) corneal nerve branch density (CNBD) (branches/mm^2), and corneal nerve fibre length (CNFL) (mm/mm^2) were quantified manually using CCMetrics. Six images centered on the inferior whorl and immediately adjacent area were selected and inferior whorl length (IWL) (mm/mm^2) was quantified manually using the manual CNFL mode in CCMetrics. The investigator (HG) was blind to the study group when analyzing the CCM images.

7.3.5. Statistical Analysis

All statistical analyses were performed using IBM SPSS Statistics software Version 27 and $P<0.05$ was considered statistically significant. Normality of the data was assessed using the Shapiro-Wilk test and Q-Q plots. Data were expressed as mean ± standard deviation or median(range) based on their distribution. Comparison between healthy controls and children with obesity or between children with obesity with and without AN was performed using an independent t-test or Mann-Whitney U test, as appropriate. Pearson and spearman correlations were undertaken to investigate the association between clinical and anthropometric parameters and corneal nerve fibre metrics. Graph prism version 9 was used to build dot plots.

7.4. Results

Forty-six participants with obesity (26 boys and 20 girls) aged 15 (14 - 17) years were compared to 20 healthy controls. Obese children were grossly overweight compared

to healthy controls (weight 105.5 ± 29.1 *vs.* 47.9 ± 18.6, P<0.001) **(Table 14)**. Obese children were further sub-grouped into those with and without AN (31 with AN and 15 without AN). There was no difference in systolic BP (P=0.63), diastolic BP (P=0.86), HbA1c (P=0.82), total cholesterol (P=0.64), LDL-C (P=0.33), HDL-C (P=0.71), triglycerides (P=0.69) and vitamin D (P=0.42) between children with and without AN **(Table 15)**.

The weight of children with AN was significantly higher than that of children without AN (111.5 ± 31.2 *vs.* 93.2 ± 19.8, P=0.02) and children with AN gained weight at a younger age compared to those without AN (7.29 ± 4.42 vs. 9.83 ± 2.48 years, P=0.03) **(Figure 32A)**. BMI (41.4 ± 9.6 *vs.* 34.8 ± 5.06, P=0.02), fat% (47.7 (39.6 - 50.6) *vs.* 39.4 (36.1 - 43.9), P=0.03) and fat mass (54.0 ± 25.3 *vs.* 35.5 ± 12.9, P=0.002) were higher in children with AN compared to those without AN. Additionally FMR was significantly higher in obese children with AN (1.0 ± 0.62 *vs.* 0.67 ± 0.22, P=0.01) **(Figure 32A-F)**, but with no significant difference in muscle mass (P=0.81), FFM (P=0.95), TBW (P=0.75), TBW% (P=0.09) between children with and without AN **(Table 15)**.

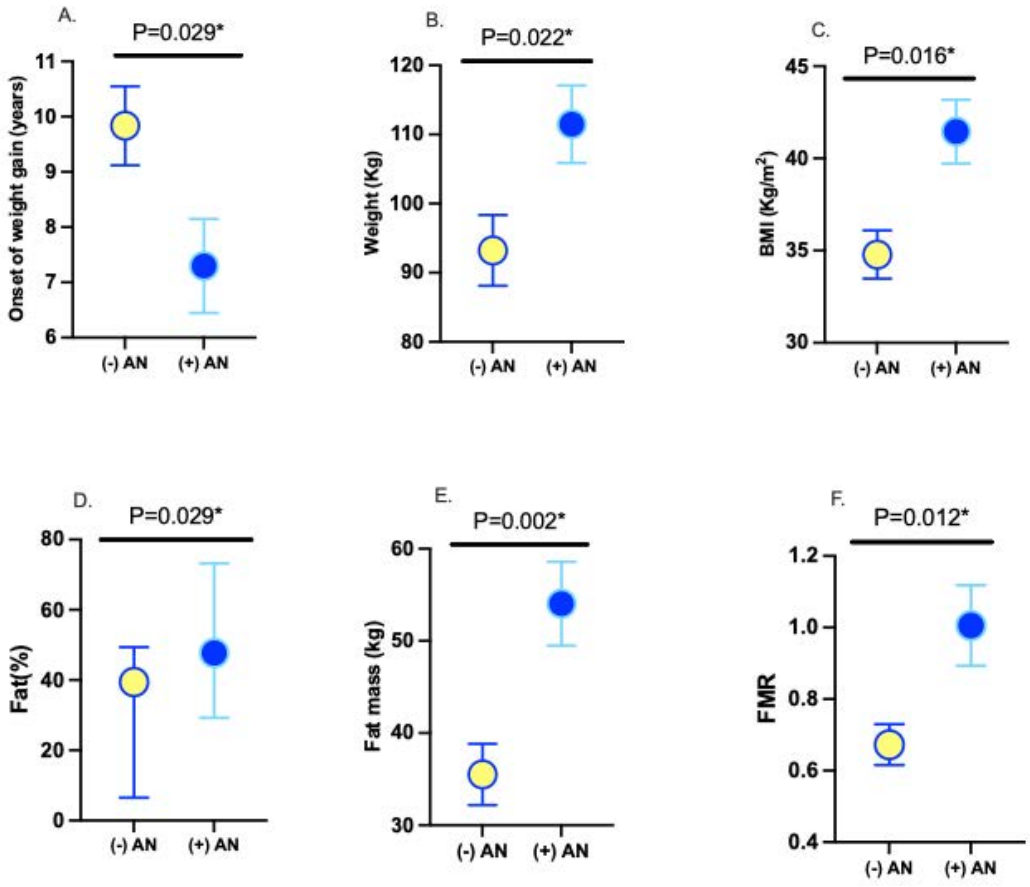

Figure 32. Body composition analysis in children with obesity with and without AN, (A) onset of weight gain, (B) weight, (C) BMI, (D) Fat%, (E) Fat mass, (F) FMR.

HC: healthy control; AN: acanthosis nigricans BMI: body mass index; FMR: fat-to-muscle ratio.

Table 14. Basic demographics and CCM parameters in children with obesity compared to healthy controls.

Characteristics	Healthy Controls (n=20)	Children with Obesity (n=46)	P-Value
Age (years)	13 (12 - 14)	15 (14 - 17)	0.014*
Weight (kg)	47.9 ± 18.6	105.5 ± 29.1	<0.001*
BMI (kg/m^2)	22.3 ± 5.47	39.3 ± 8.92	<0.001*
CNFD (fibre/mm^2)	27.8 ± 7.11	30.1 ± 7.36	0.25
CNBD (branch/mm^2)	53.8 ± 18.0	53.5 ± 26.6	0.97
CNFL (mm/mm^2)	20.5 ± 4.48	21.9 ± 5.82	0.35
IWL (mm/mm^2)	25.2 ± 4.56	23.7 ± 5.85	0.33

HC: healthy control; body mass index; CNFD: corneal nerve fibre density; CNBD: corneal nerve branch density; CNFL: corneal nerve fibre length; C=IWL: inferior whorl length.

Table 15. Clinical, metabolic, body composition and neuropathy analysis in children with obesity with and without AN.

Characteristics	(-) AN (n=15)	(+) AN (n=31)	P-Value
Age (years)	15 (12.7 - 17)	15 (14 - 16.5)	0.90
Onset of weight gain (years)	9.83 ± 2.48	7.29 ± 4.42	0.03*
Weight (kg)	93.2 ± 19.8	111.5 ± 31.2	0.02*
SBP (mmHg)	114.5 ± 9.26	112.8 ± 11.6	0.63
DBP (mmHg)	73.2 ± 6.05	72.7 ± 7.87	0.86
HbA1c (%)	5.5 (5.1 - 5.6)	5.4 (5.3 - 5.6)	0.82
TC (mmol/L)	3.98 ± 0.75	4.10 ± 0.69	0.64
LDL-C (mmol/L)	2.54 ± 0.77	2.76 ± 0.62	0.33
HDL-C (mmol/L)	1.2 (1 - 1.7)	1.1 (1 - 1.4)	0.71
TG (mmol/L)	1.4 (0.9 - 2)	1.0 (0.8 - 1.4)	0.69
25 OHD (ng/mL)	41.7 ± 23.4	35.5 ± 20.6	0.42
Fat (%)	39.4 (36.1- 43.9)	47.7 (39.6 - 50.6)	0.03*
Fat mass (kg)	35.5 ± 12.9	54.0 ± 25.3	0.002*
Muscle mass (kg)	55.8 ± 15.8	54.7 ± 14.4	0.81
FFM (kg)	56.9 ± 14.9	57.2 ± 15.2	0.95
TBW (kg)	42.2 ± 11.4	43.3 ± 10.5	0.75
TBW (%)	44.4 (41.9 - 47.2)	40.1 (37.2 - 45.1)	0.09
BMI (kg/m^2)	34.80 ± 5.06	41.4 ± 9.62	0.02*
FMR	0.67 ± 0.22	1.00 ± 0.62	0.01*
VPT (V)	2.85 ± 0.98	3.00 ± 1.04	0.63
CNFD ($fibre/mm^2$)	33.19±7.13	28.6 ± 7.09	0.04*
CNBD ($branch/mm^2$)	56.7 ± 30.3	51.9 ± 25.0	0.58
CNFL (mm/mm^2)	23.3 ± 5.76	21.3 ± 5.82	0.27
IWL (mm/mm^2)	24.2 ± 6.94	23.5 ± 5.51	0.75

*Significance at P<0.05

HC: healthy control; AN: acanthosis nigricans; SBP: systolic blood pressure; DBP: diastolic blood pressure; HbA1c: glycated hemoglobin; TC: total cholesterol; LDL-C: low-density-lipoprotein cholesterol; HDL-C: high-density-lipoprotein cholesterol; TG: triglycerides; 25 OHD: 25-hydroxy vitamin D; FFM: fat free mass; TBW: total body water; BMI: body mass index; FMR: fat-to-muscle ratio; VPT: vibration perception threshold; CNFD: corneal nerve fibre density; CNBD: corneal nerve branch density; CNFL: corneal nerve fibre length; IWL: inferior whorl length.

7.4.1. Peripheral neuropathy assessments

There was no significant difference in vibration perception threshold (VPT), sensitivity to the monofilament, CNFD, CNBD, CNFL and IWL in children with obesity compared to HC **(Figure 33A-E)**.

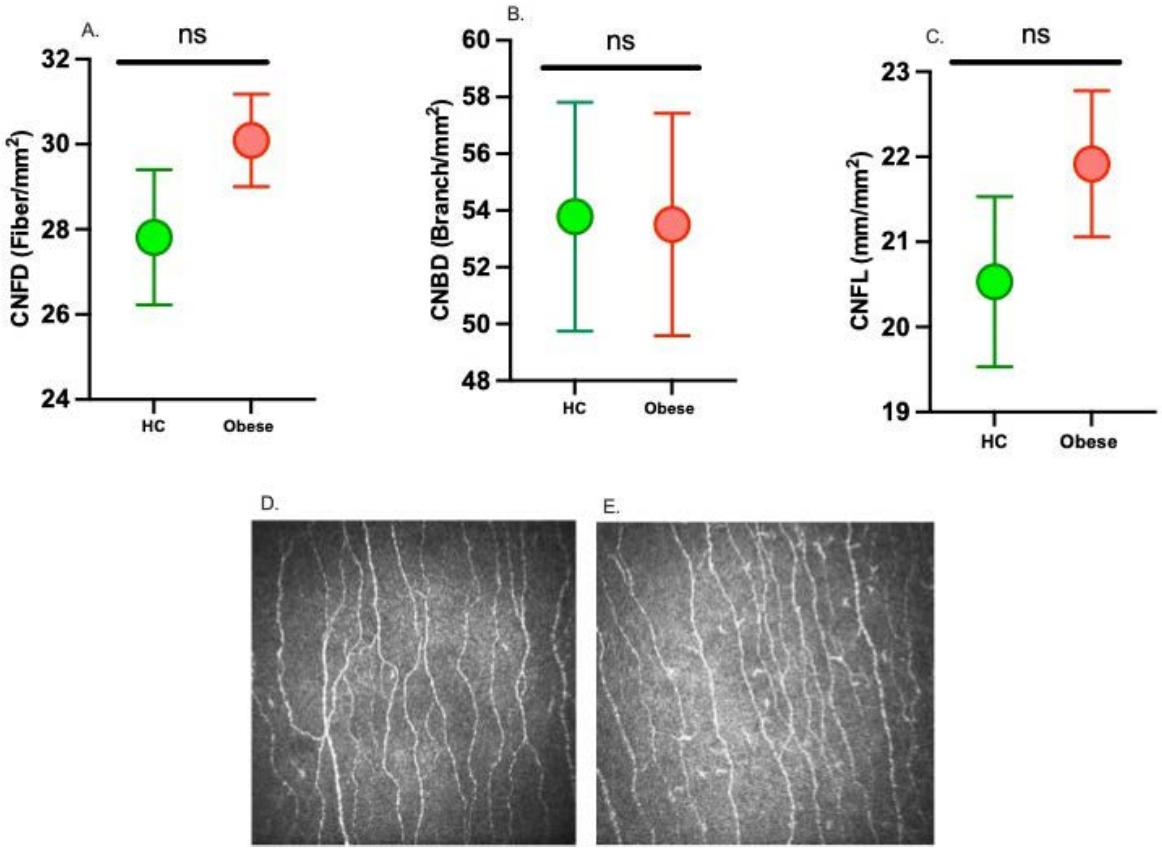

Figure 33. Corneal confocal microscopy measures (CNFD (A), CNBD (B) and CNFL (C)) and CCM image from a HC (D) and child with obesity (E).

HC: Healthy control; CNFD: corneal nerve fibre density; CNBD: corneal nerve branch density; CNFL: corneal nerve fibre length.

There was no significant difference in VPT or monofilament sensitivity between children with obesity with and without AN. CNFD was significantly lower, while CNBD, CNFL and IWL were comparable in children with obesity with and without AN **(Figure 34A-E)**.

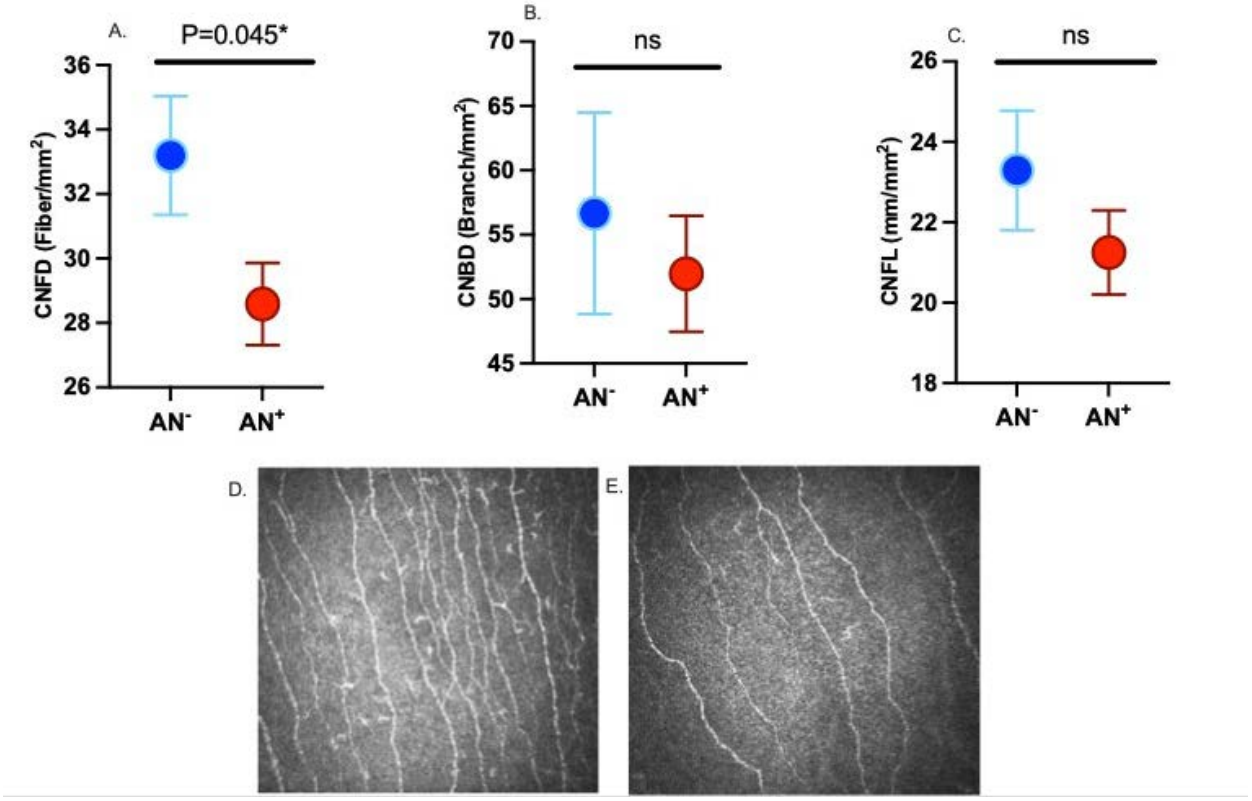

Figure 34. Corneal confocal microscopy measures (CNFD (A), CNBD (B) and CNFL (C)) and CCM image from a child with obesity without AN (D) and a child with obesity and AN (E).

AN: acanthosis nigricans; CNFD: corneal nerve fibre density; CNBD: corneal nerve branch density; CNFL: corneal nerve fibre length.

7.5. Discussion

The present study shows that children with obesity and acanthosis nigricans (AN) have corneal nerve loss, but with normal vibration perception and sensitivity to the monofilament, indicative of a sub-clinical neuropathy. These children also have greater weight, percentage body fat and fat-to-muscle ratio, which is consistent with previous studies in adults and children with AN (5, 25). A higher fat-to-muscle ratio is associated with fatty liver disease, metabolic syndrome and insulin resistance (26), and a high percentage body fat is associated with insulin resistance, higher triglycerides, visceral fat mass (27) and glucose dysregulation with a higher risk of developing T2DM (28).

Adults with insulin resistance have evidence of blunted corneal nerve regeneration following an improvement in glycemic control (29). Furthermore, an abnormal lipid profile is associated with neuropathy in subjects with IGT (30) and a higher BMI, total cholesterol and VLDL cholesterol are associated with neuropathy in patients with diabetes (31). High-fat-diet fed (HFD) rats have lower levels of synapsin-I protein, important for neurotransmission and neuronal plasticity (32) and suffer from inflammation mediated by long-chain fatty acids (LCFAs) (33) which induces Schwann cell endoplasmic reticulum (ER) dysfunction, mitochondrial depolarization and generation of reactive oxygen species (34). There is an increasing body of evidence linking lipid abnormalities to neuropathy (35) and we have previously shown that adults with obesity and abnormal lipoproteins and HDL functionality have evidence of corneal nerve loss (36, 37). Indeed, bariatric surgery was associated with an improvement in lipoprotein oxidation (38) and glycation (39), HDL functionality (40),

inflammation and insulin resistance (41) with corneal nerve regeneration (17, 42). We have also shown that subjects with impaired glucose tolerance (43), especially those with greater insulin resistance who develop type 2 diabetes (44) have greater corneal nerve loss. In the current study, whilst children with obesity and AN did not differ in relation to blood pressure, lipid profile and HbA1c, there is an increasing body of evidence showing that increased visceral obesity and alterations in adipokines perse are associated with diabetic neuropathy (45-49).

We acknowledge limitations of the study in relation to the cross-sectional design and small sample size. Furthermore, there was no objective assessment of insulin resistance e.g., via HOMA-IR or measurement of adipokines. Nevertheless, this is the first study to undertake corneal confocal microscopy to assess early neurodegeneration in children with obesity. Whilst children with obesity *per se* have normal corneal nerve morphology, those with acanthosis nigricans have evidence of early small nerve fibre degeneration. These observations warrant larger longitudinal studies to assess if sub-clinical corneal nerve loss predicts the later development of neuropathy in children with obesity, especially those with AN.

The next chapter explores the effect of the once weekly GLP=1 agonist, Semaglutide on peripheral neuropathy in adults with obesity with and without T2DM.

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Chapter VIII: An open-label obseRvational study of the Effect of semaglutide on peripheral neuropathy in obese participants with type 2 diabetes mellitus (T2DM) VERSus obesE participants without T2DM (REVERSE)

Hoda Gad, Rayaz A. Malik (unpublished data).

8.1. Abstract

Background/aim:

Corneal Confocal Microscopy (CCM) has been used to show early nerve fibre regeneration in patients with diabetes and obesity after bariatric surgery. Glucagon-like peptide-1 receptor (GLP-1R) agonists may have a beneficial effect on the central and peripheral nervous system. The primary objective of this study was to assess for evidence of nerve regeneration in obese patients with and without diabetes after treatment with the weekly GLP-1 agonist Semaglutide.

Methods:

This is an open label observational study. Thirty-two obese individuals (23 without T2DM and 9 with T2DM) who received Semaglutide 1.0mg weekly underwent body composition analysis using the TANITA scale, CCM to quantify corneal nerve fibre density (CNFD), branch density (CNBD) and length (CNFL), assessment of sudomotor function using Sudoscan and vibration perception threshold (VPT) before and at 3- and 6-months of treatment.

Results:

Weight and BMI were significantly reduced at 3 (P=0.04, P=0.04) and 6 (P=0.03, P=0.01) months after treatment with Semaglutide in obese individuals with diabetes. Weight was reduced at 3- and 6-months (P<0.001) with no change in BMI after Semaglutide treatment in obese individuals without diabetes. HbA1c was significantly reduced at 3-(P=0.02) and 6-months (P=0.04) in obese individuals with T2DM. HbA1c (P=0.025), total cholesterol (P=0.01) and LDL (P=0.01) were reduced with no change in triglycerides in obese participants without T2DM at 3-months. There was no

significant change in CNFD, CNBD, and CNFL after treatment in obese individuals with diabetes, but there was a significant increase in corneal nerve branch density 3 months after treatment with Semaglutide in the obese group without diabetes.

Conclusion:

Semaglutide once weekly results in weight loss and improvement in HbA1c in obese individuals with and without T2DM. There is no change in neuropathic symptoms, sudomotor function or vibration perception or corneal nerve parameters, except for early corneal nerve regeneration after 3 months of Semaglutide treatment in obese patients without diabetes.

8.2. Introduction

Obesity is a major contributor to the development of type 2 diabetes mellitus (T2DM) and much of the economic and health burden is due to the associated microvascular and macrovascular complications of T2DM (1). Diabetic peripheral neuropathy (DPN) affects 50% of patients with diabetes and is associated with foot ulcers and amputation (2). Not only hyperglycaemia (3), but also obesity (4-7), hyperlipidemia (8, 9), hypertension (10, 11), inflammation (12) and exercise (13-16) are associated with DSPN.

There are currently no FDA approved disease modifying therapies for DSPN (17). Corneal nerve morphology improved following treatment with Omega-3 fatty acids in patients with T1DM (18, 19). Intensive glycemic control in T2DM patients treated with Pioglitazone and exenatide or basal-bolus insulin were both associated with corneal nerve regeneration (20, 21). Early corneal nerve regeneration has also been detected following simultaneous pancrease and kidney transplantation in patients with T1DM (22, 23) and in obese individuals with T2DM following bariatric surgery (1, 24, 25).

Glucagon-like peptide 1 (GLP-1) receptor agonists reduce glucose, blood pressure and weight (26). GLP-1 agonists have also shown an improvement in nerve conduction (27-29) and intraepidermal nerve fibre density (IENFD) in T1DM mice. Liraglutide was associated with a lower incidence of lower limb amputation in patients with a diabetic foot ulcer (30).

Our aim was to assess the effect of the weekly subcutaneous GLP-1 agonist (Semaglutide-1.0mg) on peripheral neuropathy in obese participants with and without T2DM.

8.3. Methods

8.3.1. Study design and patient recruitment

This is an ongoing open label prospective observational study done as part of clinical care. We prospectively assessed 32 obese individuals with (n=9) and without (n=23) T2DM treated with once weekly s.c. Semaglutide 1.0 mg and compared them to 20 healthy controls. Assessments were undertaken pre, 3- 6-months post treatment. Patients with a history of corneal trauma or surgery in the past 6-months and those with history of retinopathy, nephropathy, or neuropathy due to diseases other than T2DM were excluded.

8.3.2. Body composition analysis

Height (cm), weight (kg), body mass index (BMI kg/m^2) were measured. Participants were asked to remove their shoes, empty their pockets, and step on the scale to complete bioimpedance analysis to calculate: weight (kg), fat (%), fat mass (kg), fat free mass (FFM)(kg), muscle mass (kg), total body water (TBW) (Kg) and (%), bone mass (kg), basal metabolic rate (Kcal), metabolic age (years), visceral fat rating, BMI (kg/m^2), and degree of obesity (%).

8.3.3. Blood tests

HbA1c, total cholesterol (TC), triglyceride (TG), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), bilirubin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), and 25-hydroxy vitamin D were assessed at each visit.

8.3.4. Neuropathy assessments

A. Corneal Confocal Microscopy

Corneal confocal microscopy was assessed using the Heidelberg Retina Tomograph (HRT III Rostock Cornea Module) (Heidelberg Engineering, Germany). Both eyes were anaesthetized with 2 drops of Bausch & Lomb Minims ® (Oxybuprocaine hydrochloride 0.4% w/v). A drop of hypotears gel (Carbomer 0.2% eye gel) was placed on the tip of the objective lens and a sterile disposable TomoCap was placed over the lens, allowing optical coupling of the objective lens to the cornea. Six images were selected from the sub basal nerve plexus (SBNP) in the central cornea and corneal nerve fibre density (CNFD) (fibres/mm^2) corneal nerve branch density (CNBD) (branches/mm^2) and corneal nerve fibre length (CNFL) (mm/mm^2) were quantified manually using CCMetrics. The investigator (HG) was blind to the study group when performing CCM and analyzing CCM images.

B. Douleur Neuropathique en 4 (DN4) questionnaire

Neuropathic pain was assessed using the Douleur Neuropathique en 4 (DN4) questionnaire. The diagnosis of painful DPN (pDPN) was based on a DN4 questionnaire score of ≥4, which has a high sensitivity (80%) and specificity (92%) for painful DPN (31).

C. Vibration Perception Threshold (VPT)

Vibration perception threshold (VPT) was measured using a Neurothesiometer (Horwell Scientific Laboratory Supplies) on the pulp of the large toe on both feet and the average value of both feet was recorded in Volts (V) with a cut-off of >15 V as abnormal (32).

D. Sudomotor function

Electrochemical skin conductance (ESC) using Sudoscan (Impeto Medical SAS) was measured in both hands and feet (33). Sudoscan evaluates sympathetic innervation based on sweat chloride concentration generated by the sweat gland in response to the voltage applied and is reported as ESC in microSiemens (μS) with a cut-off of >70 μS for feet and >60 μS for hands.

8.3.5. Statistical analysis

SPSS (Version 27.0, IBM SPSS statistics, Armonk, NY: IBM Corp) and Graph Prism (Version 9.0, GraphPad Software, La Jolla, CA, USA) were used for data analysis and visual presentation. Tests for normality of the data were done using the Shapiro-Wilk tests, visualization of histograms and Q-Q plots. To compare basic demographics between healthy controls and obese participants with and without T2DM, One-Way ANOVA or Kruskal-Wallis tests were used as appropriate. Independent t-test was used to compare between clinical demographics between obese participants with and without T2DM. The effect of Semaglutide at 3 and 6 months was assessed using the paired-t-test or Wilcoxon matched-pair signed-rank tests as appropriate. A *P* value of <0.05 was considered as statistically significant.

8.4. Results

8.4.1. Participant characteristics

We assessed 32 participants with obesity without T2DM (n=23), with T2DM (n=9) and 20 healthy controls. Body weight was higher in obese participants with and without

T2DM compared to HC (P<0.001) and obese individuals with T2DM weighed more than those without T2DM (P=0.03) **(Table 16)**, but had a comparable lipid profile. There was no significant difference in CNFD (P=0.18) (**Figure 35A**), but there was a significant difference in CNBD (0.05) (**Figure 35B**) and CNFL (**Figure 35C**) between groups. CNFL was significantly lower in obese with T2DM compared to healthy control (P=0.02) (**Figure 35C**) **(Table 16)**.

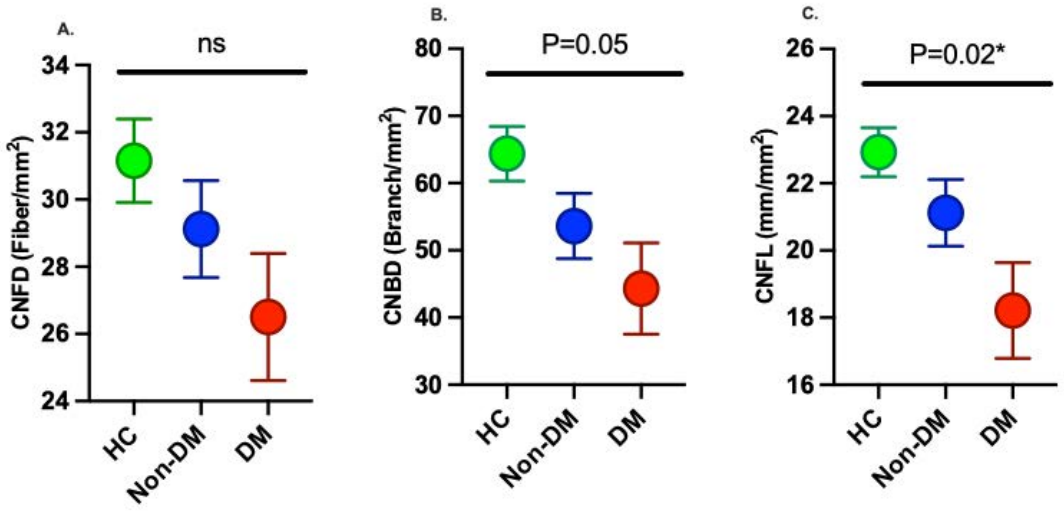

Figure 35. CCM pre Semaglutide treatment in obese individuals with and without T2DM compared to HC.

Table 16. Clinical, metabolic variables and CCM in obese individuals with and without T2DM versus healthy control.

Characteristics	HC (n=20)	Obese (n=23)	Obese with T2DM (n=9)	*P-value*
M:F ratio	15:5	14:9	6:3	N/A
Age (years)	35.7 ± 15.2	40.1 ± 7.62	36.2 ± 10.7	0.43
Weight (kg)	77.8 ± 14.1	99.5 ± 77.8[II&]	118.3 ± 26.9[^]	<0.001[*]
HbA1c (%)	-	5.40 (5.30 - 5.70)	8.50 (6.50 - 9.80)	<0.001[*]
TC (mmol/L)	-	5.30 ± 1.14	5.62 ± 1.15	0.50
LDL-C (mmol/L)	-	3.29 ± 0.06	3.82 ± 1.07	0.22
HDL-C (mmol/L)	-	1.39 ± 0.42	1.18 ± 0.36	0.18
TG (mmol/L)	-	1.36 ± 0.69	2.31 ± 2.67	0.32
CNFD (fibre/mm^2)	31.2 ± 5.56	29.1 ± 6.91	26.5 ± 5.68	0.18
CNBD (branch/mm^2)	64.4 ± 18.2	53.6 ± 23.3	44.3 ± 20.4	0.05
CNFL (mm/mm^2)	22.9 ± 3.3	21.1 ± 4.74	18.2 ± 4.28[^]	0.02[*]

HC: healthy control, T2DM: type 2 diabetes mellitus, HbA1c: glycated hemoglobin, TC: total cholesterol, LDL-C: low-density lipoprotein cholesterol, HDL-C: high-density lipoprotein cholesterol, TG: triglycerides, CNFD: corneal nerve fibre density, CNBD: corneal nerve branch density, CNFL: corneal nerve fibre length

[*]Significance between groups at P<0.005

[II]Significant difference between obese without T2DM and HC

[^]Significant difference between obese with T2DM and HC

[&]Significant difference between obese with and without T2DM

Metabolic changes in obese individuals without T2DM

Obese individuals without T2DM lost significant weight at 3 (P<0.001) and 6 (P<0.001) months after commencing semaglutide treatment with no further change in weight (P=0.17) or BMI (P=0.16) between 3 and 6 months. Percentage body fat was not reduced at 3 (P=0.52) or 6 (P=0.14) months of treatment. However, visceral fat was significantly reduced at 3 (P<0.001) and 6 (P<0.001) months of treatment with no further change in visceral fat between 3 and 6 months of treatment (P=0.28). Metabolic age was significantly reduced after 3 (P=0.01) and 6 months (P=0.03) of treatment with no further change in metabolic age between 3 and 6 months of treatment (P=0.59).

HbA1c was significantly improved after 3 (p=0.025), but not after 6 months of commencing treatment (P=0.19). Total cholesterol was significantly improved after 3 (P=0.01), but not after 6 months (p=0.27) of commencing treatment. Similarly, LDL was significantly improved after 3 (P=0.01) with no change after 6 months of commencing treatment (P=0.34) (P=0.49). HDL was significantly lower after 3 (P=0.01) with no change after 6 months (P=0.46) of commencing treatment. Triglycerides did not change after 3 (P=0.36) or 6 months (P=0.97) of commencing treatment **(Table 17)**.

Table 17. Clinical, metabolic and CCM variables pre and 3, 6 months post once weekly SC Semaglutide 1.0 mg in in obese individuals without T2DM.

Variables	Pre	3m-post MD (95% CI)	% Δ at 3m	6m-post MD (95% CI)	% Δ at 6m
Weight (kg)	99.5 ± 17.1	91.8 ± 16.8[a] 7.66 (5.79 - 9.55)	-7.85 ± 4.15	87.5 ± 17.6[b] 9.43 (6.49-12.4)	-10.1 ± 6.08
BMI (kg/m^2)	32.6 ± 3.84	30.2 ± 4.40 2.40 (2.13 - 4.23)	-7.56 ± 4.27	29.5 ± 5.14 3.18(2.13-4.23)	-10.0 ± 6.27
Fat (%)	34.3 ± 9.62	33.3 ± 8.66 0.94 (-2.09 - 3.97)	-1.16 ± 17.5	31.8 ± 8.61 2.61 (-1.0 - 6.23)	-6.03 ± 15.5
Visceral fat	11.7 ± 4.61	9.67 ± 4.10[a] 2.0 (1.22 - 2.78)	-17.1 ± 12.0	9.54 ± 4.70[b] 2.36 (1.74 - 2.98)	-22.1 ± 10.4
Metabolic age	51.5 ± 6.80	48.0 ± 8.95[a] 3.47 (0.90 - 6.03)	- 7.15 ± 9.9	46.7 ± 9.50[b] 4.82 (0.51 - 9.13)	-9.49 ± 11.9
HbA1c (%)	5.42 ± 0.44	5.29 ± 0.47[a] 0.13 (0.02 - 0.24)	-2.35 ± 4.30	5.22 ± 0.43 0.16 (-0.09 - 0.40)	-2.52 ± 8.6
TC (mmol/L)	5.30 ± 1.14	4.73 ± 0.87[a] 0.58 (0.19 - 0.97)	-9.08 ± 13.6	4.95 ± 0.95 0.35 (-0.30 - 1.0)	-3.12 ± 29.9
LDL-C (mmol/L)	3.39 ± 0.96	2.92 ± 0.88[a] 0.38 (0.09 - 0.67)	-9.96 ± 17.1	3.02 ± 0.96 0.27 (-0.32 - 0.86)	-3.08 ± 41.2
HDL-C (mmol/L)	1.39 ± 0.42	1.27 ± 0.31[a] 0.13 (0.04 - 0.22)	-7.18 ± 12.9	1.40 ± 0.41 0.06(-0.10-0.22)	-0.84 ± 19.3
TG (mmol/L)	1.36 ± 0.69	1.24 ± 0.56 0.12(-0.15-0.39)	1.96 ± 40.4	1.15 ± 0.51 0.01(-0.28-0.29)	16.8 ± 86.7
Sudoscan (µS)	67.6 ± 16.7	67.4 ± 19.3 0.15 (-10.4 - 10.7)	7.76 ± 43.4	65.8 ± 18.8 1.94 (-9.58 - 13.5)	3.11 ± 32.9
VPT (V)	4.31 ± 2.04	4.31 ± 3.15 0.0 (-1.13 - 1.13)	1.35 ± 35.9	4.18 ± 1.59 0.30 (-0.80 - 1.41)	5.55 ± 44.2
DN4	0.48 ± 0.98	0.05 ± 0.22 0.43 (-0.02 - 0.87)	Cannot be computed	0.0 ± 0.0[b] Not computed	Cannot be computed
CNFD (fibre/mm^2)	29.1 ± 6.91	30.2 ± 6.83 -1.07 (-2.49 – 0.34)	5.25 ± 12.6	30.9 ± 6.39 -0.65(-3.70 - 2.40)	3.41 ± 19.9
CNBD (branch/mm^2)	53.62 ± 23.0	61.6 ± 26.7[a] -8.00 (-15.4 to -0.57)	31.8 ± 69.7	62.4 ± 16.5 1.15 (-9.6 – 11.9)	5.45 ± 43.2
CNFL (mm/mm^2)	21.1 ± 4.73	22.2 ± 5.18 -1.13 (-2.44 - 0.18)	6.60 ± 15.3	22.9 ± 3.4 -0.09 (-1.60 – 1.42)	1.43 ± 14.2

BMI: body mass index, HbA1c: glycated hemoglobin, TC: total cholesterol, LDL-C: low-density lipoprotein, HDL-C: high-density lipoprotein, TG: triglycerides, VPT: vibration perception threshold, DN4: Douleur Neuropathique 4 Questions, CNFD: corneal nerve fibre density, CNBD: corneal nerve branch density, CNFL: corneal nerve fibre length.

a Significant difference between pre and 3-months post treatment.

b Significant difference between pre and 6-months post treatment.

% Δ at 3m: change after 3 months of treatment compared to baseline.

% Δ at 6m: change after 6 months of treatment compared to baseline.

Metabolic changes in obese individuals with T2DM

Weight was significantly reduced after 3 (P=0.04) and 6 months (P=0.03) of commencing semaglutide treatment. Similarly, BMI was significantly reduced after 3 (P=0.04) and 6 months (P=0.01) of treatment, with no further changes between 3 and 6 months of treatment. There was no significant improvement in Fat% after 3 months of treatment (P=0.17). Only 1 participant had Fat% measured after 6 months which was not suitable to compute inferential statistics. Visceral fat was not improved after 3 months of treatment (P=0.40). Only 1 participant had a visceral fat assessment at 6 months which was not suitable to compute inferential statistics. Metabolic age was not reduced after 3 months (P=0.40) and as after 6 months only 1 participant had metabolic age measured, it was not suitable to compute inferential statistics.

HbA1c was significantly improved after 3 (P=0.02) and 6 months (P=0.04) of commencing treatment, with no further improvements between 3 and 6 months of treatment (P=0.77).

Total cholesterol, nor LDL or HDL cholesterol did not change after 3 (P=0.13, P=0.36, P=0.80) or 6 months (P=0.58, P=0.48, P=0.55) of commencing treatment. Additionally, Triglycerides did not change after 3 (P=0.36) or 6 months (P=0.71) of treatment **(Table 18).**

Table 18. Clinical, metabolic and CCM variables pre and 3, 6 months post once weekly SC Semaglutide 1.0 mg in obese individuals with T2DM.

Variables	Pre	3m-post MD (95% CI)	% Δ at 3m	6m-post MD (95% CI)	% Δ at 6m
Weight (kg)	119 ± 24.4	114 ± 28.3[a] 4.90 (0.19 – 9.6)	-4.36 ± 4.32	109 ±3 6.3[b] 8.00 (1.46 – 14.5)	-7.69 ± 6.35
BMI (kg/m^2)	39.9 ± 8.97	36.9 ± 7.91[a] 2.09 (0.08 – 4.10)	-5.08 ± 4.75	36.95 ± 9.71[b] 3.40 (1.02 – 5.78)	-8.72 ± 6.04
Fat (%)	41.9 ± 7.24	35.4 ± 5.21 6.45 (-4.80 – 17. 7)	-14.3 ± 15.6	Cannot be computed	Cannot be computed
Visceral fat	16.0 ± 5.2	14.8 ± 5.8 1.25 (-2.73 – 5.23)	-8.26 ± 12.8	Cannot be computed	Cannot be computed
Metabolic age	57.5 ± 4.65	57.3 ± 4.19 0.25 (-0.55 – 1.05)	-0.39 ± .78	Cannot be computed	Cannot be computed
HbA1c (%)	8.10 ± 1.66	6.36 ± 0.88[a] 1.74 (0.41 – 3.07)	-19.7 ± 12.9	7.32 ± 2.40[b] 1.48 (0.04 – 2.92)	-17.4 ± 15.2
TC (mmol/L)	6.12 ± 0.95	5.46 ± 1.19 0.66 (-0.29 – 1.62)	-11.1 ±13.4	5.58 ± 1.17 0.27 (-0.90 – 1.44)	-2.97 ± 16.1
LDL-C (mmol/L)	4.4 ± 1.04	4.05 ± 0.79 0.38 (-0.73 – 1.48)	-7.18 ± 12.3	3.70 ± 1.22 0.29 (-0.68 – 1.26)	-5.98 ± 18.0
HDL-C (mmol/L)	1.20 ± 0.28	1.14 ± 0.30 0.06 (-0.11 – 0.23)	-4.87 ± 11.1	1.22 ± 0.47 -0.03 (-0.34 – 0.28)	4.21 ± 31.2
TG (mmol/L)	3.06 ± 3.5	1.56 ± 0.68 1.50 (-2.54 – 5.55)	-18.4 ± 38.7	1.38 ± 0.69 0.11 (-0.62 – 0.84)	-0.59 ± 48.4
Sudoscan (µS)	73.3 ± 7.70	69.4 ± 10.3 3.86 (-3.72 – 11.4)	-5.14 ± 11.9	75.2 ± 10.7 3.50 (-4.46 – 11.4)	-4.56 ± 9.92
VPT (V)	4.89 ± 1.53	4.07 ± 1.62[a] 0.82 (0.002 – 1.64)	-17.3 ± 14.7	4.00 ± 1.40 0.50 (-0.76 – 1.76)	-8.72 ± 18.6
DN4	0.86 ± 1.86	0.0 ± 0.0 0.86 (-0.87 – 2.6)	Cannot be computed	0.40 ± 0.89 -0.20 (-1.56 – 1.16)	Cannot be computed
CNFD (fibre/mm^2)	27.2 ± 5.22	27.8 ± 7.00 -0.60 (-5.00 – 3.81)	2.17 ± 14.9	28.3 ± 3.45 -2.60 (-9.43 – 4.23)	15.6 ± 34.2
CNBD (branch/mm^2)	47.9 ± 20.9	45.8 ± 27.0 2.08 (-7.05 – 11.2)	-8.81 ± 24.4	41.8 ± 19.4 0.69 (-18.7 – 20.1)	5.58 ± 40.6
CNFL (mm/mm^2)	18.9 ± 3.93	20.1 ± 4.89 -1.22 (-4.04 – 1.61)	7.25 ± 18.5	19.5 ± 4.26 -2.20 (-5.53 – 1.13)	15.7 ± 19.3

BMI: body mass index, HbA1c: glycated hemoglobin, TC: total cholesterol, LDL-C: low-density lipoprotein, HDL-C: high-density lipoprotein, TG: triglycerides, VPT: vibration perception threshold, DN4: Douleur Neuropathique 4 Questions, CNFD: corneal nerve fibre density, CNBD: corneal nerve branch density, CNFL: corneal nerve fibre length.

a Significant difference between pre and 3-months post treatment; b Significant difference between pre and 6-months post treatment.

% Δ at 3m: change after 3 months of treatment compared to baseline.

% Δ at 6m: change after 6 months of treatment compared to baseline.

8.4.2. Neuropathy assessments

Obese without T2DM

Sudoscan did not change after 3 (P=0.98) or 6 (P=0.73) months of treatment. DN4 improved after 6 months (P=0.04), but not significantly after 3 months (P=0.06). VPT did not change after 3 (P=1.0) or 6 (P=0.56) months of treatment **(Table 17)**.

Obese with T2DM

Sudoscan did not change after 3 (P=0.26) or 6 months (P=0.31) of treatment. VPT transiently improved after 3 months (P=0.049), but was again not significantly different from baseline after 6 months (P=0.30) of treatment. DN4 did not change after 3 (P=0.27) or 6 months (P=0.70) of treatment **(Table 18)**.

Corneal Confocal Microscopy

Obese without T2DM

There was no significant change in CNFD after 3 (P=0.13) or 6 months (P=0.66) of treatment. CNBD was significantly increased after 3 months (P=0.04) with no further change after 6-months (P=0.82)_of treatment. There was no significant change in CNFL after 3 (P=0.09) or 6 months of treatment (P=0.90) **(Table 17) (figure 36 A-C).**

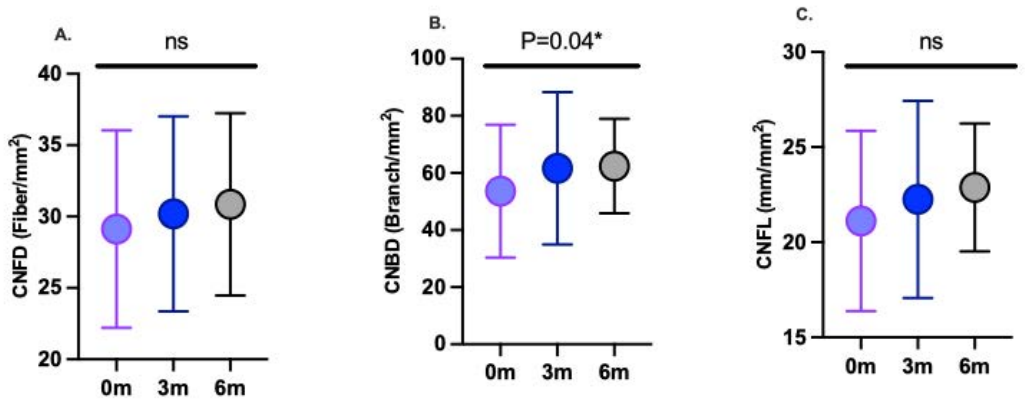

Figure 36. CCM parameters pre and post Semaglutide treatment in obese individuals without T2DM.

Obese with T2DM

There was no significant change in CNFD after 3 (P=0.75) or 6 months (P=0.37) of treatment. There was no significant change in CNBD after 3 (P=0.60) or 6 months (P=0.93) of treatment with no further change in CNBD between 3 and 6 months (P=0.45). There was no significant change in CNFL after 3 (P=0.33) and 6 months (P=0.15) of treatment with no further change between 3 and 6 months of treatment (P=0.44) (**Table 18) (Figure 37A-C).**

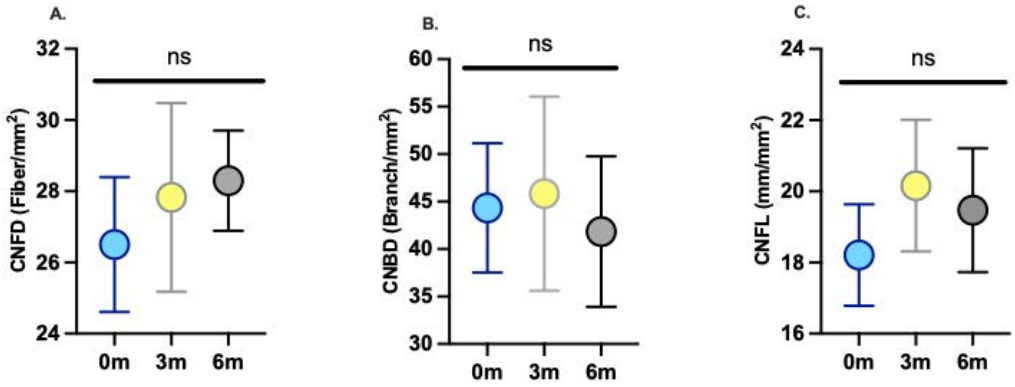

Figure 37. CCM parameters pre and post Semaglutide treatment in obese individuals with T2DM.

8.5. Discussion

This is the first study to assess the effect of the once weekly GLP-1 agonist Semaglutide 1.0 mg s.c. on peripheral neuropathy in obese individuals with and without T2DM. There was a reduction in weight in obese subjects with and without T2DM, which agrees with previous studies (1, 34). Additionally, BMI and HbA1c were significantly improved after 3 semaglutide treatment with no further improvements after 6 months in the obese group with T2DM. Improvement in HbA1c could also be due to existing hypoglycemic treatments. Only total cholesterol was improved in obese subjects without diabetes. High percentage body fat is associated with insulin resistance, high triglycerides, and visceral fat mass (39) and higher body fat percentage is linked to dysregulation of glucose metabolism and a higher risk of developing T2DM (40). Studies have shown that increased HbA1c and lipids are associated with small nerve fibre damage (8, 38) and there is evidence of a small fibre neuropathy in obese subjects without diabetes (35), obese subjects with impaired glucose tolerance (36) and subjects with IGT who develop T2DM (37). In the current study we show evidence of greater corneal nerve loss in obese subjects with T2DM compared to obese subjects without T2DM, although the former weighed significantly more and had overall poor glycemic control.

DN4 score, vibration perception threshold and sudomotor function did not improve after treatment with Semaglutide in obese individuals with and without diabetes, which agrees with previous studies of obese individuals following bariatric surgery (1, 41) and a study with once weekly exenatide combined with Pioglitazone (20). CCM has previously been used to detect small nerve fibr regeneration following

simultaneous pancreas and kidney transplantation (22, 42), treatment with ARA-290 (Cibinetide) (43-45), and Omega-3 (46). In the current study, there was a trend for improvement in all corneal nerve parameters, but only CNBD increased significantly 3- months after commencing Semaglutide in obese individuals without T2DM.

This is an ongoing study of patients attending the endocrinology clinic which has limitations including the open-label design, lack of selection criteria of patients and randomization and small sample size. The short-term follow-up period limits interpretation of the change in corneal nerve parameters, but warrants a larger randomized study with a longer duration.

In conclusion, we show that once weekly Semaglutide results in an improvement in weight, visceral fat, HbA1c, and lipid profile and may lead to corneal nerve regeneration.

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Chapter IX: DISCUSSION

9.1. Introduction

Diabetic peripheral neuropathy (DPN) is the most prevalent long-term complication of diabetes (1). DSPN has an impact on quality of life and an economic burden on the healthcare system (2). DSPN can lead to diabetic foot ulceration and amputation. It has a complex, multifactorial etiology and can develop not only in those with diabetes, but also in those with impaired glucose tolerance, insulin resistance and obesity (3). Currently there are no FDA approved therapies to prevent, slow or arrest progression of DSPN (4). Thus, early identification and risk factor modification is the key to managing DSPN.

Nerve conduction studies are the gold standard for the diagnosis of PN. However, they measure large fibre neuropathy (5), even though small fibre dysfunction may precede large fibre neuropathy. IENFD assesses small fibres, but skin biopsy is an invasive technique that requires expertise. CCM is a non-invasive, real-time, objective imaging technique that quantifies small nerve fibre damage and there is a good correlation between CCM and IENFD (6). CCM has been used to diagnose subclinical neuropathy in adults and children.

We now extend the use of CCM in detecting early neuropathy in children and adults with diabetes and obesity. We have also used CCM to assess its utility in detecting corneal nerve degeneration in relation to alterations in glucose variability assessed using continuous glucose monitoring and corneal nerve regeneration following treatment of adults with obesity with the GLP-1 agonist, Semaglutide. Previous studies have shown corneal nerve regeneration in patients with T1DM treated with continuous subcutaneous insulin infusion (7), simultaneous pancreas kidney

transplantation (8, 9) and patients with T2DM undergoing treatment with Exenatide and pioglitazone (10) or bariatric surgery (11).

9.2. Corneal confocal microscopy for the diagnosis of diabetic peripheral neuropathy: A systematic review and meta-analysis

In this systematic review and meta-analysis, we have shown that CCM has excellent diagnostic utility in detecting neuropathy not only in patients with confirmed DSPN, but also in those without apparent neuropathy compared to healthy controls. Our findings agree and build on previous systematic reviews and meta-analyses (12, 13). In addition, the present analysis included IWL, not assessed in previous meta-analyses. This meta-analysis included over 3000 patients, while previous analyses included less than 2000 patients (12, 13). A meta-analysis of RCTs is needed to provide a robust pooled estimate of the ability of CCM to detect nerve regeneration following interventions. Such a meta-analysis of RCTs may help to overcome limitations such as small cohort size and heterogeneity in patient selection and assessment and may aid in providing a robust rationale for the inclusion of CCM as an FDA end point for trials of new therapeutics for neurodegenerative disease, especially diabetic neuropathy. We are currently working on a meta-analysis of randomized controlled trials utilizing CCM to detect a change in corneal nerve measures in response to intervention (CRD42023319565).

9.3. Corneal nerve loss in children with type 1 diabetes mellitus without retinopathy or microalbuminuria

The present study shows early nerve damage manifested by reduced CNFD, CNBD, CNFL and IWL in children with T1DM without retinopathy or microalbuminuria compared to healthy controls. Our findings agree with previous data from adults (6, 14-16) and children (17) with T1DM. Corneal nerve changes were also related to glycated hemoglobin, which agrees with previous studies in adults where diabetes duration and glycated hemoglobin were independent predictors of nerve damage in T1DM (18, 19). The current study showed alterations in corneal nerve morphology in children with a short duration of diabetes, but relatively high HbA1c compared to other studies in children with T1DM (19, 20). A limitation of the current study is the cross-sectional study design and small sample size. A larger sample size and follow-up are needed to assess whether these young patients with sub-clinical corneal nerve loss will progress to clinical neuropathy. There is a need to include children with T1DM with DSPN to compare nerve damage in T1DM without DSPN and a group with retinopathy and microalbuminuria to assess if children with microvascular complications have more nerve damage.

9.4. Corneal confocal microscopy identifies a reduction in corneal keratocyte density and sub-basal nerves in children with type 1 diabetes mellitus

A previous study in children with T1DM showed no difference in keratocyte density in the posterior stroma compared to healthy control at baseline and after 2 years follow-up (19). In the present study we also show no difference in keratocyte density in the

posterior stroma, but we do demonstrate a significant reduction in keratocyte density in the anterior and mid stroma compared to healthy control, which agrees with studies in adults with diabetes (21) and severe obesity (22).

A limitation of the current study is the small sample size and the cross-sectional design of the study. However, this is the first study to simultaneously assess anterior, mid, and posterior keratocyte density in children and adolescents with T1DM and to relate it to corneal nerve fibre loss. A longitudinal study is warranted to assess a truly causal effect relationship between keratocyte density and corneal nerve changes.

9.5. Continuous glucose monitoring reveals a novel association between duration and severity of hypoglycaemia, and small nerve fibre injury in patients with diabetes

Hyperglycaemia and low time in range are established associations with neuropathy (23). Interestingly we now show for the first time, that lower corneal nerve branch density is associated with higher glycemic variability and especially in those who spend more time in level 1 and level 2 hypoglycaemia. This was a pilot study with CGM assessment undertaken for only 4 days in patients with T1DM and T2DM on insulin. This study was not powered to assess the relationship between hypoglycaemia and nerve damage. Rather it was designed to assess if AI algorithms could utilise physiological data like pulse pressure and continuous ECG to predict hypoglycaemia. The association of nerve damage with hypoglycaemia may have been driven by the increased risk of hypoglycaemia in patients treated with insulin. A longer duration of CGM wear (minimum of 14 days) may generate even more robust data. Further

longitudinal studies are warranted to assess the predictive ability of CGM for the development and progression of corneal nerve damage and diabetic neuropathy. We have been awarded product support from Abbott to supply CGM sensors to investigate the relationship between different glycemic metrics collected over a longer duration of CGM use to corneal nerve morphology in a larger population.

9.6. Early corneal nerve loss in children with obesity and acanthosis nigricans

Previous studies have assessed neuropathy in children with obesity using NCS only (24). Previously in adults we have shown that nerve regeneration after an improvement in glycemic control was attenuated in adults with insulin resistance (25). In the present study, we show for the first time that children with obesity and acanthosis nigricans, a clinical marker for insulin resistance, have evidence of greater corneal nerve loss compared to children without acanthosis nigricans. Children with obesity and acanthosis tended to gain weight at a younger age, weighed more and had a higher percentage body fat, and lower muscle-to-fat ratio. The current observation warrants further investigation of the effect of insulin resistance measured by HOMA-IR. Longitudinal studies may also help to establish if children with IR show more progressive loss of corneal nerves and hence the development of neuropathy. This is an ongoing study where we plan to recruit 160 obese children and follow them up for 1 year to assess the prognostic utility of CCM in detecting those at high risk of developing T2DM, especially those with AN.

9.7. An open-label obseRvational study of the Effect of semaglutide on peripheral neuropathy in obese participants with type 2 diabetes mellitus (T2DM) VERSus obesE participants without T2DM (REVERSE)

Previous studies of obese participants have shown that small nerve fibres can regenerate following bariatric surgery (11, 26). In the current study we show evidence of corneal nerve regeneration within 3 months of treatment with the GLP-1 agonist Semaglutide, associated with an improvement in weight, visceral fat and lipid profile in obese individuals without diabetes. This study assessed participants as part of routine care and we did not apply strict inclusion/exclusion criterion, nor did we compare against placebo. The current study continues to enroll more participants and we will repeat assessments at 12 and 24 months. There is a need for a randomized controlled study with more patients and a longer duration of follow-up to more robustly investigate the effect of Semaglutide on nerve regeneration. We are planning a clinical trial to assess the effect of once weekly semaglutide to semaglutide with diet and exercise in obese individuals with or without T2DM.

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Chapter X: APPENDIX – STUDY RELATED DOCUMENTS

Appendix 1. Research ethics approval letters

Document No.: HRP-522

Weill Cornell Medicine-Qatar

Approval

Rayaz Ahmed Malik, MD PhD
Professor of Medicine
Weill Cornell Medicine in Qatar
00974 4492-8998
ram2045@qatar-med.cornell.edu

July 22, 2018

Dear Dr Malik:

On July 12, 2018 the IRB approved the following through July 11, 2019 inclusive.

Type of review:	Initial
Title:	[1188894-3] Corneal Confocal Microscopy and the Gut Microbiome in Children with Type1 Diabetes and Celiac Disease.
Principal Investigator & affiliation:	Prof. Anthony Akobeng Sidra Medical Center
WCM-Q Investigator:	Rayaz Ahmed Malik, MD PhD
IRB Number:	17-00032
HHS grant title and ID, if any:	None
QNRF grant title and ID, if any:	None
Additional documents reviewed:	• Sidra IRB re-review determination letter 27.06.2018 (UPDATED: 07/4/2018)
Documents reviewed:	• Letter - Point-by-point response (UPDATED: 05/15/2018) • Protocol - Research Protocol version 1.4 dated 19 July 2018 (UPDATED: 07/19/2018) • Parental Permission Form – Parent Information sheet English & Arabic version 2.3 dated 19 July 2018 (UPDATED: 07/19/2018) • Parental Permission Form – Parent permission English & Arabic version 2.3 dated 19 July 2018 (UPDATED: 07/19/2018) • Child Assent – Child Information Sheet English version 2.3 dated 19 July 2018 (UPDATED: 07/19/2018) • Child Assent – Child Information Sheet Arabic version 2.3 dated 19 July 2018 (UPDATED: 07/22/2018) • Child Assent – Child Assent English & Arabic version 2.3 dated 19 July 2018 (UPDATED: 07/19/2018) • Data Collection - CRF version 1.4 dated 27 November 2017 (UPDATED: 12/10/2017) • Questionnaire/Survey – CCM Acceptability Questionnaire Arabic (UPDATED: 12/10/2017) • Questionnaire/Survey - CCM Acceptability Questionnaire (UPDATED:12/10/2017) • Application Form - HRP-201 FORM - Research Personnel (UPDATED: 12/10/2017) • Application Form - HRP-200 FORM - Initial Review Application (UPDATED: 12/10/2017)
Level of review:	Full board
Vulnerable population:	Children
Level of risk:	Minor increase over minimal risk

Before July 11, 2019, you are to submit a continuing review to request continuing approval or closure. If the IRB does not grant continuing review, approval of this protocol ends after July 11, 2019.

Copies of approved study documents are attached.

In conducting this study, you are required to follow the requirements in "INVESTIGATOR GUIDANCE: Investigator Obligations (HRP-800)."

Sincerely,

Manju Varghese, M.Pharm, CIP
IRB Specialist
IRB Office/Research
Weill Cornell Medicine-Qatar
T: +974 4492 8960
mav2040@qatar-med.cornell.edu

Cc:

Adeel A. Butt, MBBS, MS, FACP, FIDSA
WCM-Q IRB Chair
Professor of Medicine
Professor of Healthcare Policy and Research
Weill Cornell Medical College New York, NY and Doha, Qatar
Vice Chair, Department of Medicine
Director, Clinical Epidemiology Research Unit
Hamad Medical Corporation, Doha, Qatar
Email: aabutt@hamad.qa; aab2005@qatar-med.cornell.edu

سدرة للطب
Sidra Medicine

SIDRA MEDICINE

Tel: +974-4003-7747
Email: irb@sidra.org

Sidra IRB MOPH Registration: MOPH -Sidra-IRB-099
Sidra IRB DHHS Registration: IRB00009930
Sidra IRB MOPH Assurance: MOPH-A-Sidra-00100
Sidra IRB DHHS Assurance: FWA00022378

To: Anthony Kwaku Akobeng
Date: 27th June 2018
Protocol Title: Corneal Confocal Microscopy and the Gut Microbiome in Children with Type1 Diabetes and Celiac Disease.
IRB Protocol #: 1708012783
IRB Initial Approved Date: 17th September 2017
Expiration Date: 16th September 2018

The IRB has re-reviewed the submitted documents of the above-referenced protocol and has granted the study an **Expedited Review Approval** as a minimal risk study for the following categories:

- **Category 2:** Collection of blood samples by finger stick, heel stick, ear stick, or venipuncture.
- **Category 3:** Prospective collection of biological specimens for research purposes by noninvasive means.
- **Category 4:** Collection of data through noninvasive procedures.

It is the Principal Investigator's responsibility to obtain review and continued approval before the expiration date. You may not continue any research activity beyond the expiration date without approval by the Institutional Review Board. It is a condition of this approval that you report promptly to the IRB any serious, unanticipated adverse events experienced by subjects during this research, if they are directly related to the study protocol.

Amendments: If you wish to change any aspect of this study, such as the procedures, the consent forms, or the investigators, please communicate your requested changes to the IRB. Any new procedure is not to be initiated until the IRB approval has been given.

Sincerely yours,

Chiara Cugno

Chiara Cugno
Acting Chair, Sidra IRB

PO Box 26999
Doha - Qatar
Sidra.org

Weill Cornell Medicine-Qatar
Email: irb@qatar-med.cornell.edu
Tel: 00974-44928960
WCM-Q IRB Registration: MOPH-WCMC-Q-001
MoPH Assurance: IRB-A-WCM-2019-0004

Hamad Medical Corporation
Email: irb@hamad.qa
Tel: 00974-40256410
HMC-IRB Registration: MOPH-HMC-IRB-020
MoPH Assurance: IRB-A-HMC-2019-0014

Approval

Rayaz A Malik, MBChB, PhD
Professor of Medicine
Weill Cornell Medicine in Qatar
(+974) 4492 8998
Ram2045@qatar-med.cornell.edu

January 17, 2021

IRB of record:	WCM-Q
Type of Submission:	Continuing review
Title:	[1370493-13] A noninvasive monitor to predict hypoglycemia in diabetes patients
Principal Investigator (PI):	Rayaz Malik, PhD
WCM-Q IRB Number:	18-00024
HMC Site PI:	Dr. Khaled Baagar
Grant title and ID, if any:	A non-invasive monitor to predict hypoglycemia in diabetes patients NPRP11S-0110-180247
Documents reviewed and approved:	• HRP-202a FORM – Progress Report V3.1 dated 04Jan21(UPDATED: 01/5/2021) • HRP-204 FORM Prompt Reporting V1.0 dated 30Dec20 (UPDATED: 12/30/2020) • HRP-201 FORM Research Personnel list V.3.1 dated 30NOV2020 (UPDATED: 11/30/2020) • Protocol - 18-00024-Protocol-V1.4 - 19NOV2020 (UPDATED: 12/7/2020) • Consent Form - 18-00024-ICF- V1.3.- 19NOV2020 Arabic (UPDATED: 11/30/2020) • Consent Form - 18-00024-ICF- V1.3.- 19NOV2020 (UPDATED: 11/30/2020) • Data Collection - 4 day Diary Arabic version v1.1 19NOV2020(UPDATED: 11/25/2020) • Data Collection - 4 day Diary V1.1 19NOV2020 (UPDATED: 11/22/2020) • Eligibility Screening form-V1.1 dated 15Jun20 Arabic (UPDATED: 10/7/2020) • Eligibility Screening form-V1.1 dated 15JUN20 English (UPDATED: 06/28/2020) • CCM Acceptability Questionnaire V1 dated 15Jun20 Arabic (UPDATED: 10/7/2020)

Weill Cornell Medicine-Qatar
Email: irb@qatar-med.cornell.edu
Tel: 00974-44928960
WCM-Q IRB Registration: MOPH-WCMC-Q-001
MoPH Assurance: IRB-A-WCM-2019-0004

Hamad Medical Corporation
Email: irb@hamad.qa
Tel: 00974-40256410
HMC-IRB Registration: MOPH-HMC-IRB-020
MoPH Assurance: IRB-A-HMC-2019-0014

	• CCM Acceptability Questionnaire using V1 dated 15JUN20 English (UPDATED: 06/21/2020) • Advertisement - Email script (UPDATED: 06/28/2020) • Advertisement Document Arabic (UPDATED: 10/7/2020) • Advertisement Document English (UPDATED: 08/20/2020) • Data Collection Sheet (UPDATED: 06/28/2020)
Level of review & categories:	Full Board
Approved HMC enrollment:	40

Dear Dr. Malik,

On January 11, 2021, the WCM-Q approved the above referenced project from February 3rd, 2021 through February 2nd, 2022 inclusive.

The IRB also reviewed the reported lapse in QU IRB approval and determined that this information represents non-compliance which is neither serious not continuous. The IRB acknowledged that no research activities took place during the lapse of approval and requests that you submit the QU IRB approval once obtained.

On January 2nd, 2022, you are to submit a continuing review to request continuing approval or closure. If the IRB does not grant continuing review, approval of this protocol ends after February 2nd, 2022 inclusive. Copies of approved study documents are attached.

As part of PI's responsibilities, all research activities must be recorded in Cerner's medical records per visit for each subject involved in the study.

In conducting this study, you are required to follow the PI responsibilities stated below and the requirements in HRP-172 SOP - Reliance Agreement-IRB Submission Process for Investigators.

Weill Cornell Medicine-Qatar	مؤسسة حمد الطبية Hamad Medical Corporation
Email: irb@qatar-med.cornell.edu Tel: 00974-4928960 WCM-Q IRB Registration: MOPH-WCM-Q-001 MoPH Assurance: MOPH-A-WCM-Q-002	Email: irb@hamad.qa Tel: 00974-40256410 HMC-IRB Registration: MOPH-HMC-IRB-020 IRB-MoPH Assurance: IRB-A-HMC-2019-0014

Approval	
IRB of record:	WCM-Q
Title:	A non-invasive monitor to predict hypoglycemia in diabetes patients
Principal Investigator (PI):	Rayaz Malik
HMC IRB Number:	MRC-03-20-1252
HMC Site PI:	Khaled Ahmed Mohamed Baagar
WCM-Q Number:	18-00024
Grant title and ID:	A non-invasive monitor to predict hypoglycemia in diabetes patients NPRP11S-0110-180247
Level of review & categories:	Full Board
Vulnerable population & review category:	NA
Decision:	Acknowledgment for Approval
Approved HMC enrolment:	40

The HMC-IRB Acknowledges the approval from WCM-Q IRB dated 3rd February 2019 and subsequent amendment approvals. WCM-Q IRB has reviewed this study under the signed reliance agreement between HMC and WCM-Q.

2/10/2021

Weill Cornell Medicine-Qatar
Email: irb@hamad.qa Tel: 00974-40256410
HMC-IRB Registration: MOPH-HMC-IRB-020
IRB-MoPH Assurance: IRB-A-HMC-2019-0014

مؤسسة حمد الطبية Hamad Medical Corporation
Email: irb@hamad.qa Tel: 00974-40256410
HMC-IRB Registration: MOPH-HMC-IRB-020
IRB-MoPH Assurance: IRB-A-HMC-2019-0014

WCM-Q/HMC HRPP Oversight

1. **Ancillary reviews at (Relying Institution):**
   Relying institution PI must assure any ancillary reviews for human research protection reviews (hospital committees, pharmacy, nursing, radiation safety, etc.) are obtained and followed at the Relying Institution and obtain an acknowledgment from WCM-Q.
2. **External Sites :**
   This project cannot be carried out in other external sites until either one of the below conditions are met:
   a. IRB approval of the external site is granted. A copy should be forwarded to the WCM-Q office.
   b. IRB Authorization Agreement is signed with WCM-Q to rely on the IRB of this site.
3. **Ministry of Public Health (MoPH) approval for Clinical Trials:**
   As per the MoPH regulations, "A sponsor or an institution conducting clinical trials in Qatar shall not begin a clinical investigation subject to the Ministry of Public Health, Qatar review and approval until the Ministry of Public Health will provide the sponsor or institution in Qatar with a written determination, within 30 days after the Ministry of Public Health receives all the required documents". The office of HRPP will be in contact with MoPH to fulfill this requirement.
4. **Ministry of Public Health (MoPH), Qatar Assurance**
   Each institution engaged in human subjects' research must submit an Assurance to the Department of Research of the Ministry of Public Health (MoPH), Qatar. Samples and/or data cannot be shared with/received from an external site(s) before receiving MoPH approval of assurance.
   Please contact the IRB office in case of any questions.

Please contact the IRB office in case of any questions.

Sincerely,

Dr. Mohammed Bashir

Acting Chairman of Institutional Review Board:

Hamad Medical Corporation

Date:

M. Bashir
Dr. Mohammed Bashir
Sr. Consultant, Endocrine & Diabetes Medicine - HMC
038537

Signature:

Weill Cornell Medicine

Document No.: HRP-522

Approval

Rayaz A Malik, MBChB, PhD
Professor of Medicine
Weill Cornell Medicine in Qatar
(+974) 4492 8998
Ram2045@qatar-med.cornell.edu

August 24, 2020

Dear Dr Malik,

On August 23, 2020, the IRB approved the following through August 22, 2021 inclusive.

Type of submission:	Initial-Response/Follow-Up
Title:	[1574195-4] Early detection of small Fiber neuropathy in Obese Qatari Children and Adolescents with impaired Glucose Tolerance (IGT) and Type-2 Diabetes (FAT)
Lead Principal Investigator and Institution:	Khalid Hussain, MD Sidra Medicine
WCM-Q Principal Investigator:	Rayaz A. Malik, PhD
IRB Number:	20-00006
QNRF grant title and ID, if any:	None
Documents reviewed and approved:	• HRP-200 FORM Initial Application Form V1.1 dated 31May20 (UPDATED: 06/8/2020) • HRP-201 FORM – Research Personnel List V1.1 dated 31May20 (UPDATED: 06/8/2020) • Letter - Point by point response (UPDATED: 07/28/2020) • Study Protocol V1.2 dated 08Aug20 (UPDATED: 08/23/2020) • Data Collection Sheet (UPDATED: 08/9/2020) • Parental Permission Form V1.2 dated 08Aug20 Arabic (UPDATED: 08/9/2020) • Parental Permission Form V1.2 dated 08Aug20 English (UPDATED: 08/9/2020) • Child Assent – Arabic V1.1 dated 31May20 (UPDATED:0618/2020) • Child Assent – English V1.1 dated 31May20 (UPDATED:06/18/2020) • Child Information Sheet Arabic V1.1 dated 31May20 (UPDATED: 06/18/2020) • Child Information Sheet English V1.1 dated 31May20 (UPDATED: 06/18/2020) • Parental Information Sheet English V1.1 dated 31May2020 (UPDATED: 06/18/2020) • Parental Information Sheet Arabic V1.1 dated 31May2020 (UPDATED: 06/18/2020) • Advertisement – Invitation letter English (UPDATED:04/27/2020)

Weill Cornell Medicine

Document No.: HRP-522

	• Advertisement – Invitation letter Arabic (UPDATED:04/27/2020) • Questionnaire/Survey-DN4 Assessment tool -Arabic (UPDATED: 04/27/2020) • Questionnaire/Survey-DN4 Assessment tool – English (UPDATED: 04/27/2020) • CCM Acceptability Questionnaire V1.1 dated 31May20 Arabic (UPDATED: 07/16/2020) • CCM Acceptability Questionnaire V1.1 dated 31May20 English (UPDATED: 07/16/2020)
Additional documents reviewed:	• Letter - Approval Letter-2.pdf (UPDATED: 07/16/2020) • Sidra Approval Letter dated 23Apr20 (UPDATED: 04/27/2020)
Level of review:	Full Board

On July 22, 2021, you are to submit a continuing review to request continuing approval or closure. If the IRB does not grant continuing review, approval of this protocol ends after August 22, 2021.

Copies of approved study documents are attached. Please ensure to use the IRB stamped documents in the conduct of the research.

In conducting this study, you are required to follow the requirements in "INVESTIGATOR GUIDANCE: Investigator Obligations (HRP-800)."

Please also ensure to abide by the institutional requirements related to in-person human research activities detailed in the COVID-19 clinical research memo.

Sincerely,

Manju Varghese, M. Pharm, CIP
IRB Manager
IRB Office/Research
Weill Cornell Medicine-Qatar
Office: +974 4492 8960
Email: mav2040@qatar-med.cornell.edu

Cc:
Adeel A. Butt, MBBS, MS, FACP, FIDSA
WCM-Q IRB Chair
Professor of Medicine
Professor of Healthcare Policy and Research
Weill Cornell Medical College New York NY and Doha, Qatar
Vice Chair, Department of Medicine
Director, Clinical Epidemiology Research Unit
Hamad Medical Corporation, Doha, Qatar
Email: aabutt@hamad.qa ; aab2005@qatar-med.cornell.edu

Tel: +974-4003-7747
Email: irb@sidra.org

Sidra IRB MOPH Assurance: IRB-A-SIDR-2019-0020
Sidra IRB MOPH Registration: MOPH -Sidra-IRB-099
Sidra IRB DHHS Assurance: FWA00022378
Sidra IRB DHHS Registration: IRB00009930

23 April 2020

Approval

Dear Dr. Hussain,

On 23 April 2020, the IRB approved the following through 22 April 2021 inclusive.

Type of review:	Initial Review
Protocol Title:	Early detection of small Fiber neuropathy in Obese Qatari Children and Adolescents with impaired Glucose Tolerance (IGT) and Type-2 Diabetes (FAT)
Principal investigator:	Khalid Hussain
IRB number:	1542992
Sponsor/ Funding Agency:	Funded by Professor Rayaz A. Malik Start Up Fund
Grant title and ID, if any:	N/A
Documents reviewed:	• IRB Application Form(UPDATED: 04/22/2020) • IRB 404 Assent form (Child_Arabic (UPDATED: 04/7/2020) • IRB-404 Assent Form (Child)_English (UPDATED: 02/2/2020) • Data collection sheet (UPDATED: 12/22/2019) • CCM Acceptability Questionnaire_English(UPDATED: 02/18/2020) • CCM Acceptability Questionnare_Arabic (UPDATED: 04/7/2020) • Information sheet for parents/local guardians_English (UPDATED: 12/22/2019) • Information sheet for parents/local guardians_Arabic (UPDATED: 04/7/2020) • Information sheet for young people_English(UPDATED: 12/22/2019) • Information sheet for young children_Arabic (UPDATED: 04/7/2020)

Tel: +974-4003-7747
Email: irb@sidra.org

Sidra IRB MOPH Assurance: IRB-A-SIDR-2019-0020
Sidra IRB MOPH Registration: MOPH -Sidra-IRB-099
Sidra IRB DHHS Assurance: FWA00022378
Sidra IRB DHHS Registration: IRB00009930

	• Invitation letter_English (UPDATED: 12/22/2019) • Invitation letter_Arabic (UPDATED: 04/7/2020) • IRB-402 Parental Permission Form English_v1/15July2019 (UPDATED: 04/20/2020) • IRB 402 Parental permission form Arabic_v1/15Jul2019 (UPDATED: 04/7/2020) • SIDRA IRB Research Proposal_v1.0/15JUL2019 (UPDATED: 04/22/2020) • DN4 Questionnaire_English(UPDATED: 02/18/2020) • DN4 Questionnaire_Arabic (UPDATED: 04/7/2020) • Training and Credentials
Level of Review:	Expedited
Expedited categories:	2,4 and 7
Pediatric Category:	Research does not involve greater than minimal risk

Before 22 March 2021, you are to submit a :continuing review to request continuing approval or closure. If the IRB does not grant continuing review, approval of this protocol ends after 22 April 2021.

Copies of approved parental permission documents and assent documents are attached.

In conducting this study, you are required to follow Sidra's Policies and Procedures pertaining to Human Research Protection.

Other Institutions engaged in this human subject research must secure their IRB approvals and Assurance with MOPH for Protection of Human Subjects Involved in Research.

If you have questions or concerns, please call the IRB office at 4003-7747 or send an email to irb@sidra.org.

Sincerely yours,

Eileen McBride

Eileen McBride, MD
Vice Chair
Institutional review Board
Sidra Medicine
+974 40032957

29/06/2023
Project Title: CCM and metabolic neuropathies in children and adults

EthOS Reference Number: 53145

Ethical Opinion

Dear Hoda Gad,

The above application was reviewed by the Science and Engineering Research Ethics and Governance Committee and, on the 29/06/2023, was given a favourable ethical opinion. The approval is in place until 25/03/2025 .

Conditions of favourable ethical opinion

Application Documents

Document Type	File Name	Date	Version
Additional Documentation	Study related documents and approvals	23/02/2023	1
Recruitment Media	recruitment media	23/02/2023	1
Consent Form	Consent form	23/02/2023	1
Information Sheet	Information sheet	23/02/2023	1
Project Protocol	Study Proposal	10/04/2023	V1.0
Additional Documentation	Screen Shot 2023-06-06 at 9.02.58 AM	06/06/2023	V1.0
Additional Documentation	Assent form 20-00006_English-clean-V1.5.pdf (STAMPED)	06/06/2023	V1.5
Additional Documentation	English FAT CIS - clean-11JAN2021_v1.2.pdf (STAMPED)	06/06/2023	V1.2
Additional Documentation	English FAT PIS - clean - 11JAN_v1.2.pdf (STAMPED)	06/06/2023	V1.2.
Additional Documentation	IRB-402 Parental Permission Form English-clean-V1.6.pdf (STAMPED)	06/06/2023	V1.6
Additional Documentation	Approved advert-consent Arabic and English	06/06/2023	V1.0
Additional Documentation	CCM-MicrobiomeAssentChild_Hoda Gad_v1.2	06/06/2023	V1.2.
Additional Documentation	CCM-MicrobiomeChildInformationSheet_1.2	06/06/2023	V1.2
Additional Documentation	CCM-MicrobiomeConsentParent_v1.2	06/06/2023	V1.2.
Additional Documentation	CCMMicrobiomeParentInformationSheet_v1.2	06/06/2023	V1.2

The Science and Engineering Research Ethics and Governance Committee favourable ethical opinion is granted with the following conditions

Adherence to Manchester Metropolitan University's Policies and procedures

This ethical approval is conditional on adherence to Manchester Metropolitan University's Policies, Procedures, guidance and Standard Operating procedures. These can be found on the Manchester Metropolitan University Research Ethics and Governance webpages.

Amendments

If you wish to make a change to this approved application, you will be required to submit an amendment. Please visit the Manchester Metropolitan University Research Ethics and Governance webpages or contact your Faculty research officer for advice around how to do this.

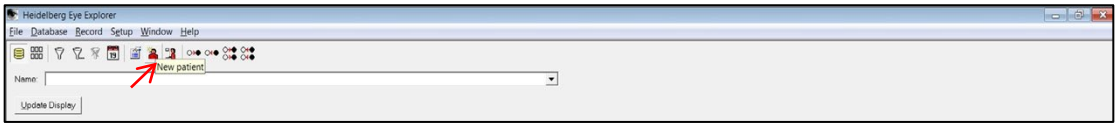
Appendix 2. Standard operating procedure for undertaking Corneal Confocal Microscopy (CCM)

Equipment Setup

The investigator is required to turn on the computer and follow the steps below:

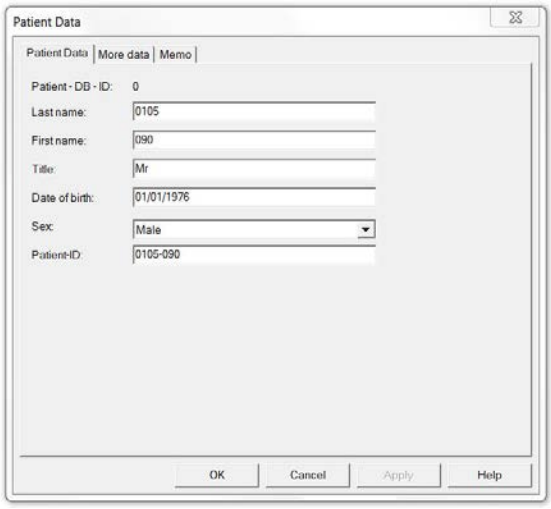
Click on the Heidelberg icon on the desktop to open Heidelberg Eye Explorer (HEYEX).

Click on the 'New Patient' icon to add the subject's details.

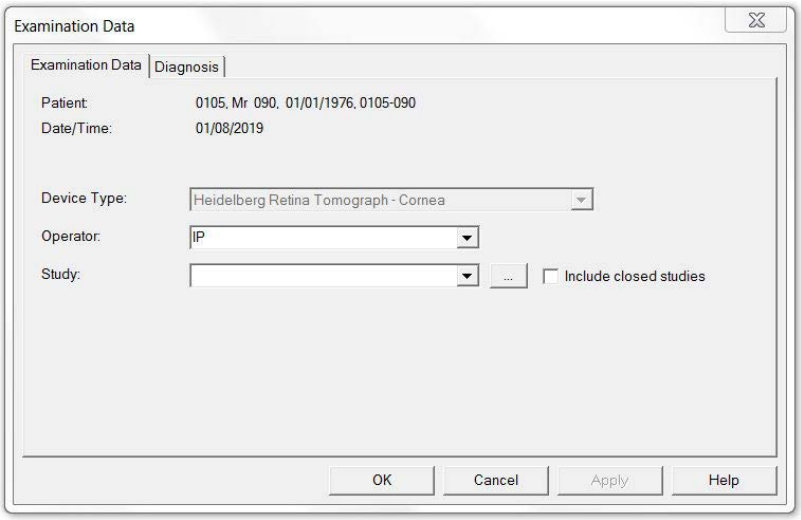


Enter subject details as required e.g. could be full name, initials and ID or ID as last name and study site ID as first name (the latter is particularly useful in multi-center trials).

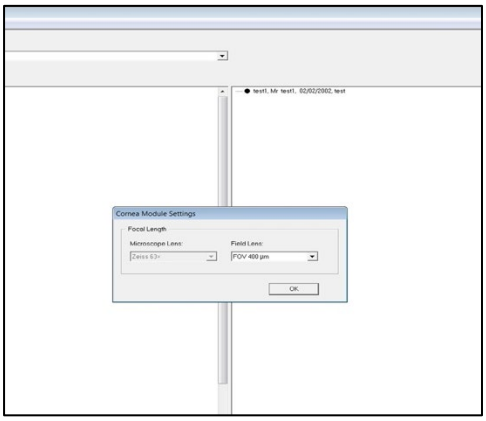
A date of birth is entered and verified using the identification card/passport (if available). Ensure that subject details entered on Heyex meet the requirements of your local ethics committee / institutional review board.



Select operator and study. At study initiation, type study name and select 'add'. Click 'OK'. Choose 'Study' and 'Operator' details to proceed to the live screen. Upon completion of the scan the subject can be re-identified in the database by searching for subject ID as last name or by the full ID.



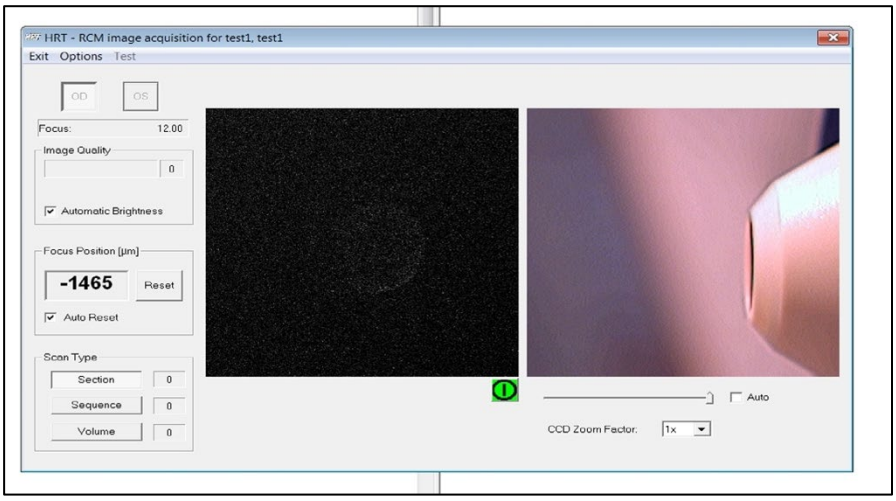
Click 'OK' and ensure the field of view (FOV) is set at 400µm.



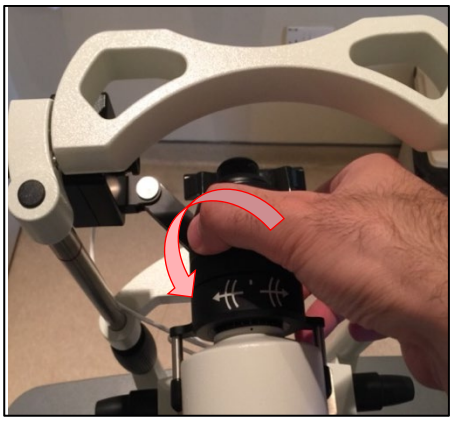
At this stage the live CCM screen will be activated. The image from the external CCD camera will be on the right and the CCM image will be on the left of the display screen.

The below preferred options/settings are required:

- Image quality = 0
- Focus position = -1465 µm (±100 µm).
- Scan type ‘section’
- Automatic brightness.

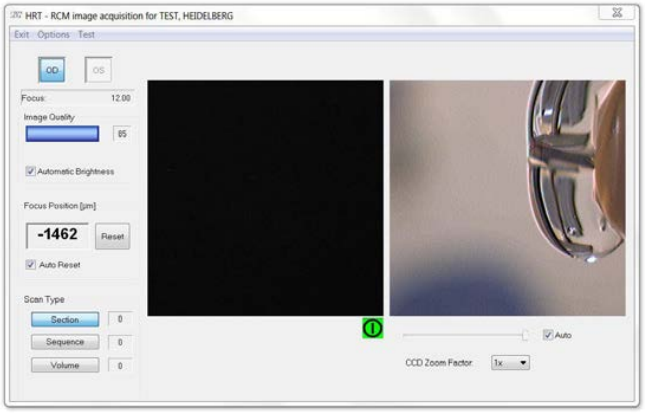


While standing behind the CCM, revolve the RCM lens anticlockwise (red arrow direction) until it stops (focus position normally > -1300µm).

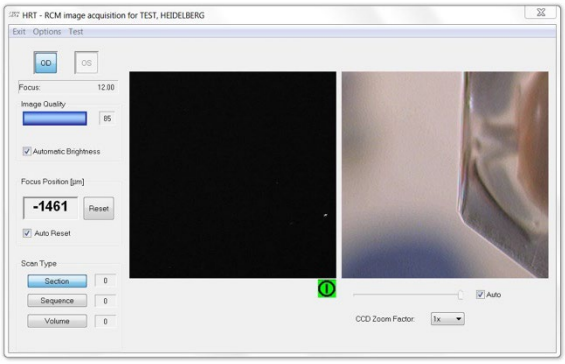


Prepare the subject for the CCM scan by instilling two drops/eye of Oxybuprocaine hydrochloride 0.4% and 1 pea-sized drop/eye of Viscotears.

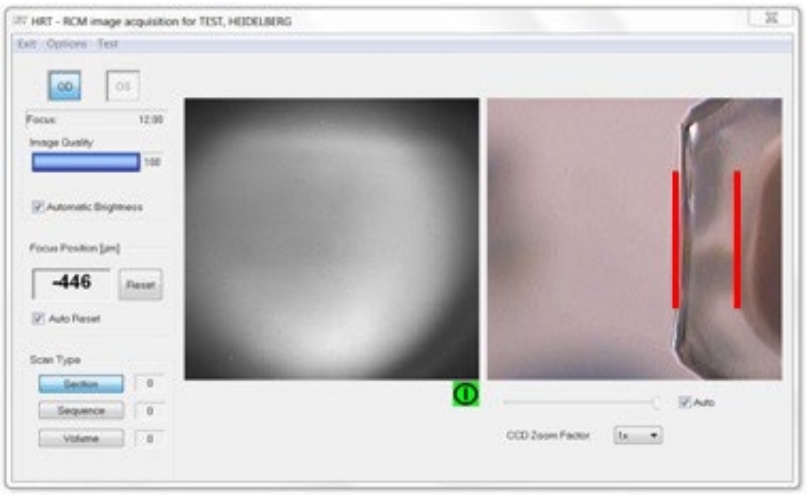
Apply Viscotears gel on the front of the lens. Clean and repeat if there are bubbles in the gel. An increase in image quality when the gel is applied is temporary due to the optical medium. <u>Ignore the change in image quality.</u>



Place the TomoCap over the lens on the microscope. Push until a clicking sound is heard.



The TomoCap surface should be perpendicular to the lens (red lines). Revolve the lens clockwise at normal speed for depth reset. Continue past the first focus plane (1st white screen).



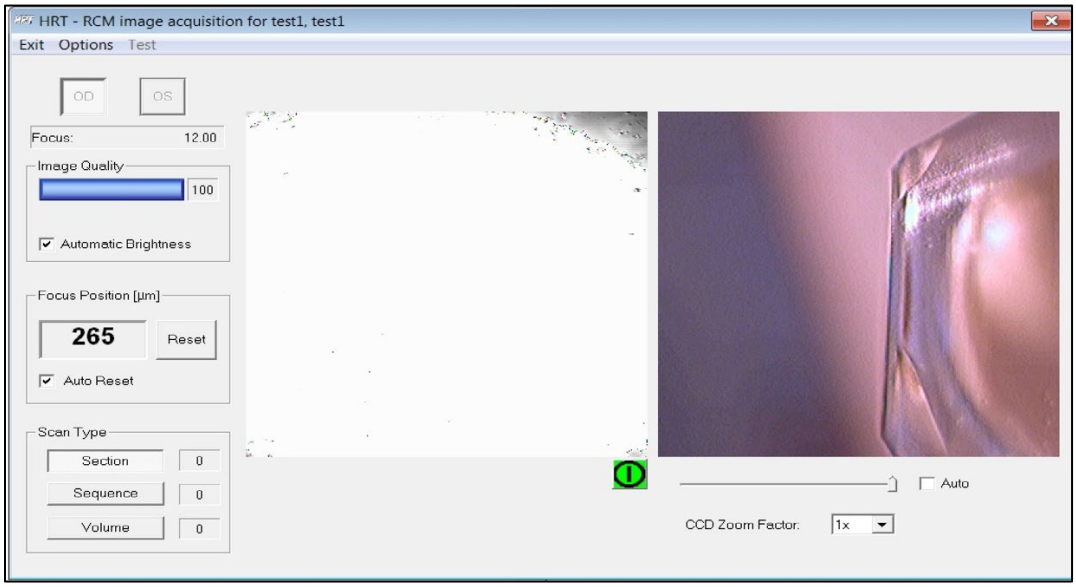
When the second focus plane (2nd white screen) is reached, the settings should match the ones below:

Image quality = 100%

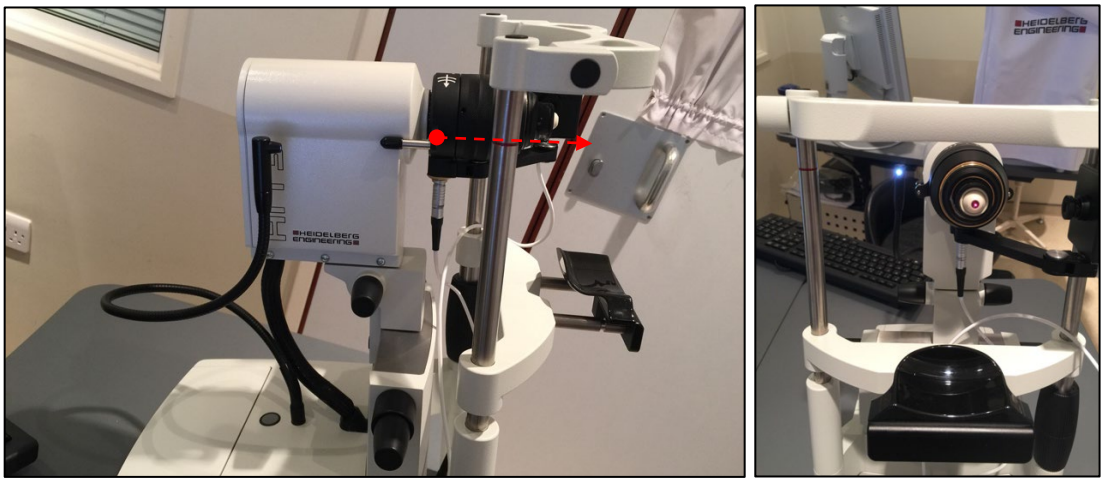
Focus position > 0 µm (usually 0-100 µm but occasionally can be higher </= 250 µm).

Click on “reset”, focus position will reset to 0 µm.

At this stage you are ready to perform the CCM scan.



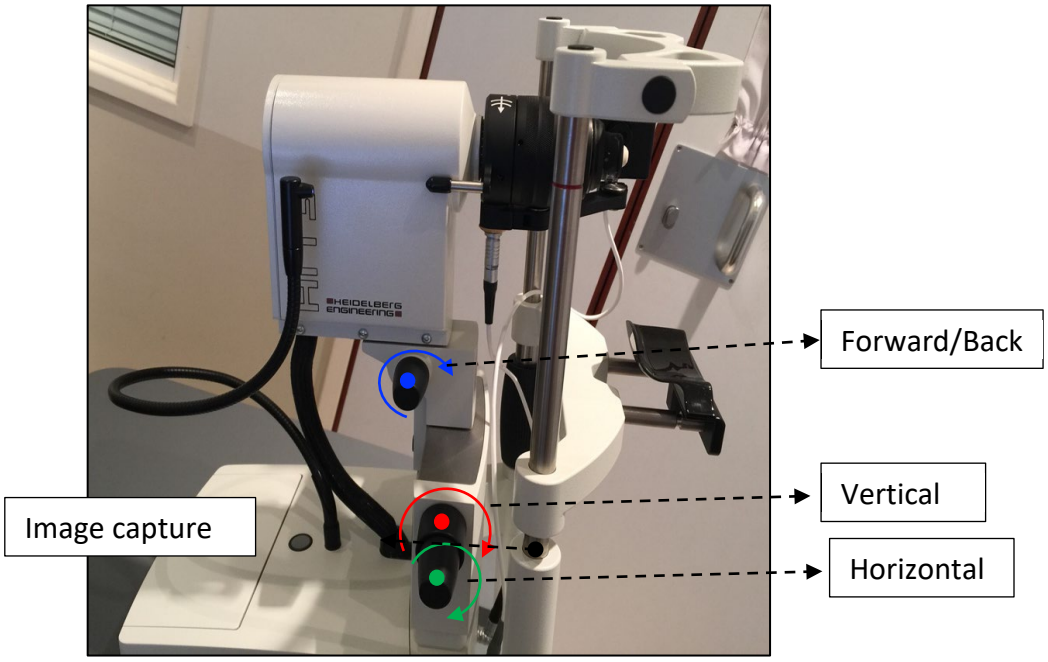
It is crucial that the subject is focused on the external fixation target (white light) for safety and image quality purposes. Use the dashed red line as a virtual guide to adjust the fixation target.



At this point, ask the subject to place their chin on the chinrest and gently push their forehead against the headrest. Ask them to fixate on the white light and keep their eye wide open. Explain to the subject that they should fixate on the white light with the eye not being examined and keep their eyes open during the scan which takes around 5 minutes. Their forehead should be constantly gently pushed against the headrest.

Inform the subject that the microscope is going to slightly touch their eye, but they will only feel a cool sensation. Reassure them that the scan will not cause any harm and there is no pain during or after the scan.

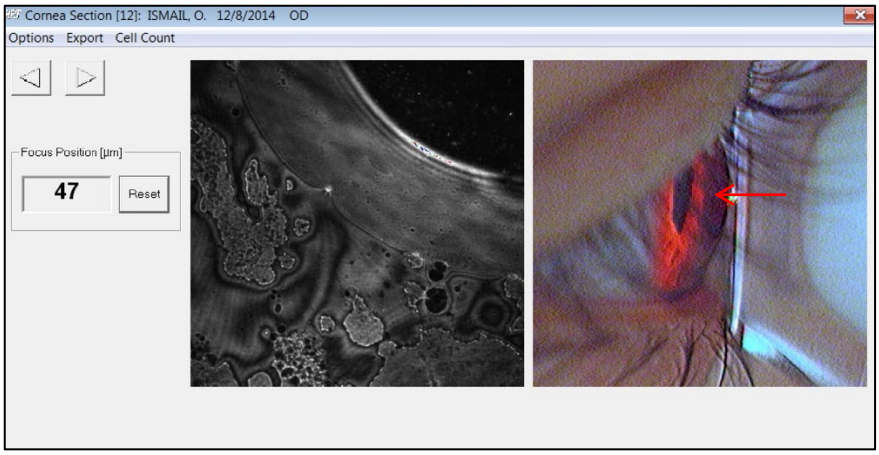
Use the knobs to position the CCM (horizontal-green, vertical-red and forward/ backwards-blue).



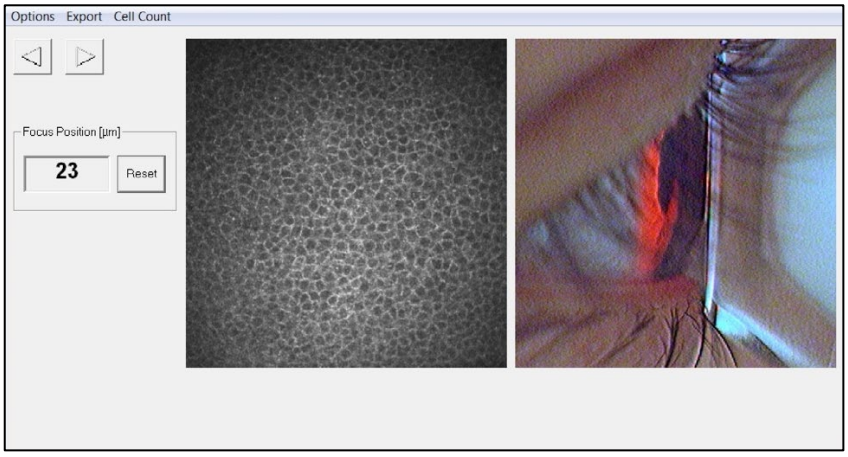
<u>How to capture the central subbasal nerve plexus.</u> The eye should be perpendicular to the TomoCap as shown in the image below. Assuming the head position is correct

(i.e., the entire eye is visible in the CCD image) use the external fixation target to micro-adjust the position of the eye. Move towards the subject's eye (blue knob) while observing the external camera window on your screen.

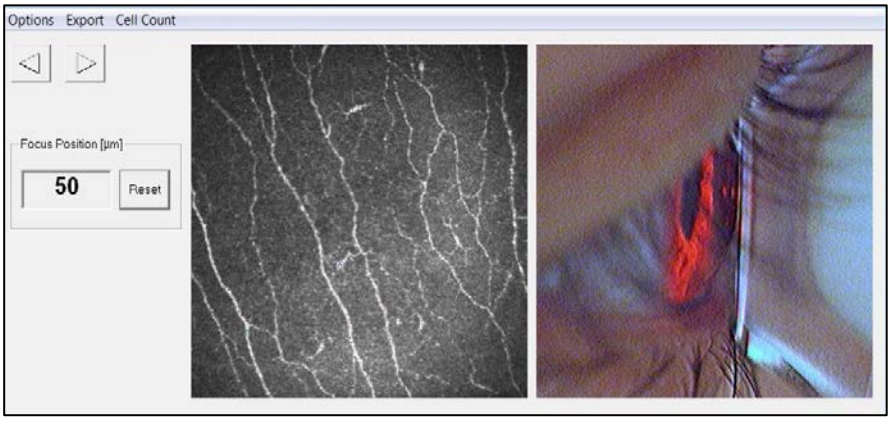
There will be two red dots, one on the TomoCap and one on the pupil. The position of the TomoCap should be adjusted so that they appear in the center of the cornea (same height with the center of the pupil).



The red dot will disappear when the TomoCap is coupled with the cornea. Once you achieve contact gently revolve the lens clockwise. The first visible layer is the epithelial cell layer. Capture 1-2 reference images.



Continue revolving your lens gently to the right to reach the subbasal nerve plexus (at approximately 50-80 μm).



In the vast majority of cases, the nerves at the apex are vertical and less tortuous compared to oblique / horizontal nerves in the periphery.

Ensure you capture enough images from the central / peri-central areas by slightly moving horizontally (green knob).

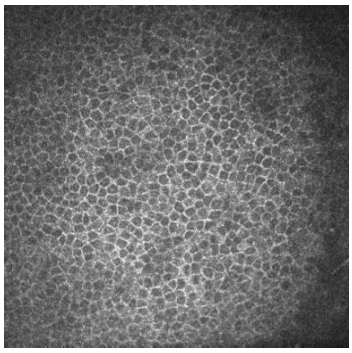
Gently move forward/back as required to optimize focus and avoid pressure lines.

Ensure that the subject maintains fixation on the white light.

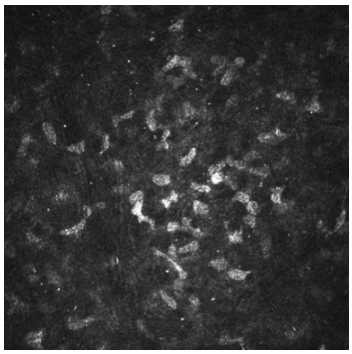
At the end of the scan, around 3 images per eye will be analyzed but the sample should be sufficient for accurate sampling (around 70-100 images / eye).

It is important to recognize the types of cells before and immediately after the subbasal nerve plexus. This will assist you in locating the area of interest accurately and minimize scanning time:

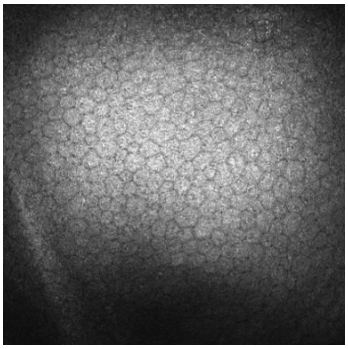
Epithelial cell layer = Outermost layer of the cornea. Hexagonal cells with bright border and dark nucleus. Revolve the lens gently clockwise until the nerves are in focus.



Stromal cell layer (or stroma) = Bright polymorphic cells against a dark background. Revolve the lens gently anticlockwise/counterclockwise to bring the subbasal nerves into focus.



Endothelial cell layer = Innermost corneal layer (after stroma). Hexagonal cells with bright nucleus and dark borders. Revolve the lens anticlockwise to bring the subbasal nerves into focus.



Pressure lines (red arrows in images A and C below) appear with increased pressure of the microscope on the cornea. Images with pressure lines are not ideal for analysis. The operator should monitor and adjust the position of the microscope as required to ensure minimal pressure. Common quality issues in CCM images are provided below:

Image A – Correctly focused on the subbasal nerve plexus. Increased pressure causes pressure lines (red arrow).

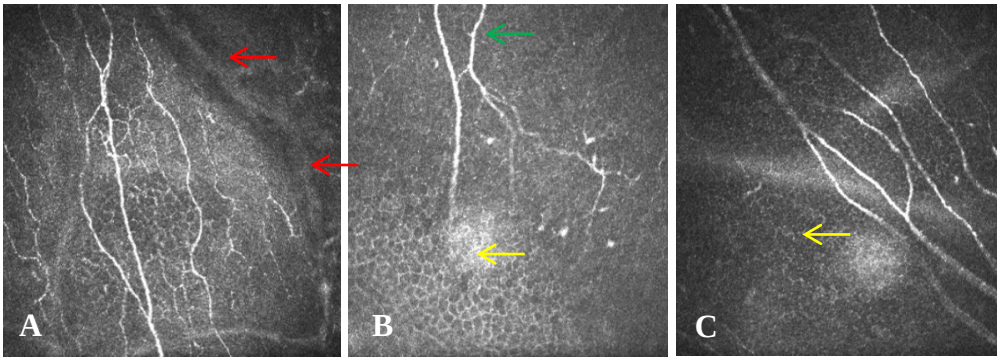
Solution: Gently move the microscope back and forward (blue knob) and re-focus.

Image B – The epithelial cell layer (yellow arrow) and subbasal nerves (green arrow) are visible in the same image. This indicates that the TomoCap is not perpendicular to the cornea. This results in a cross sectional view of the cornea.

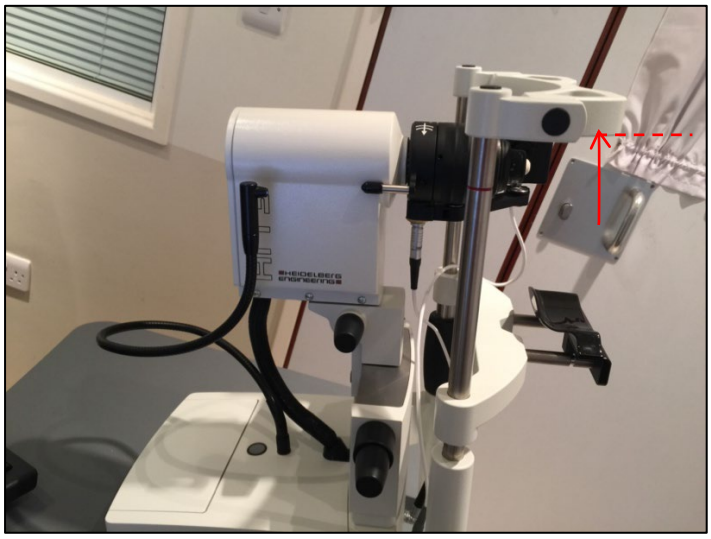
Solution: ensure your subject is fixating on the white light with their eyes open. Move the microscope as required (blue, red, green knob) and re-focus.

Image C – Poor focus and slight pressure lines.

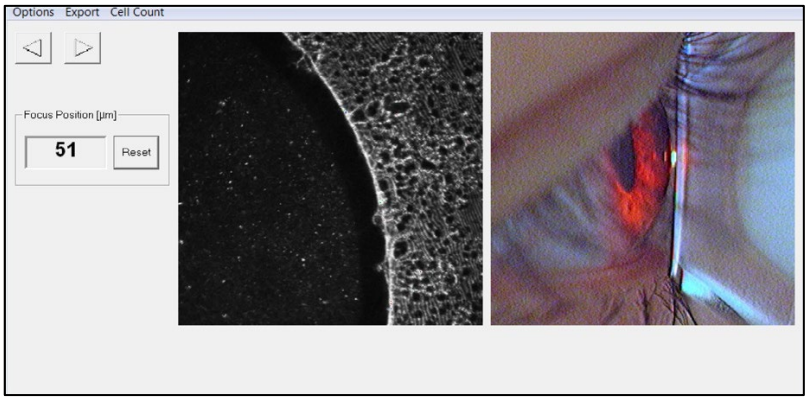
Solution: Gently move the microscope back and forward (blue knob) and re-focus.



How to capture the inferior whorl. Move the microscope back (ensure there is no contact with the eye). Adjust the white light and slightly move it up to the level of the dashed line and have the subject look up at the new position of the white light.

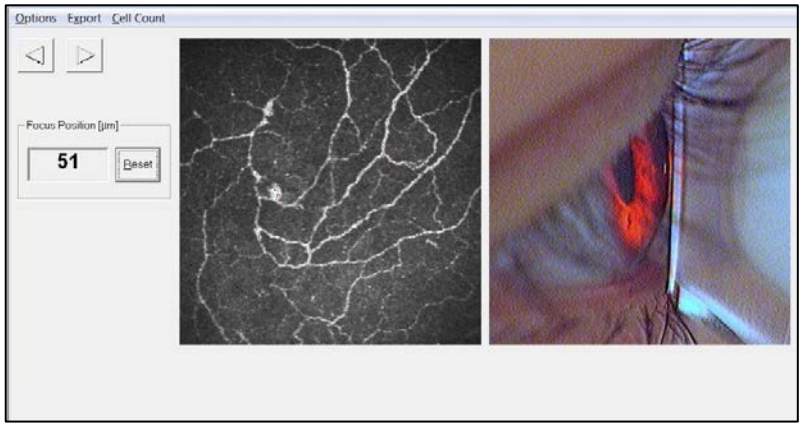


The inferior cornea will be perpendicular to the TomoCap. Aim to scan at the bottom end of the pupil (instead of the center for the central subbasal nerve plexus).

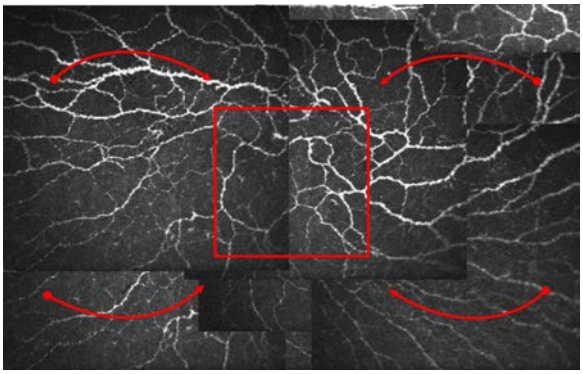


Follow the same procedure as for the central cornea (1.16 to 1.23).

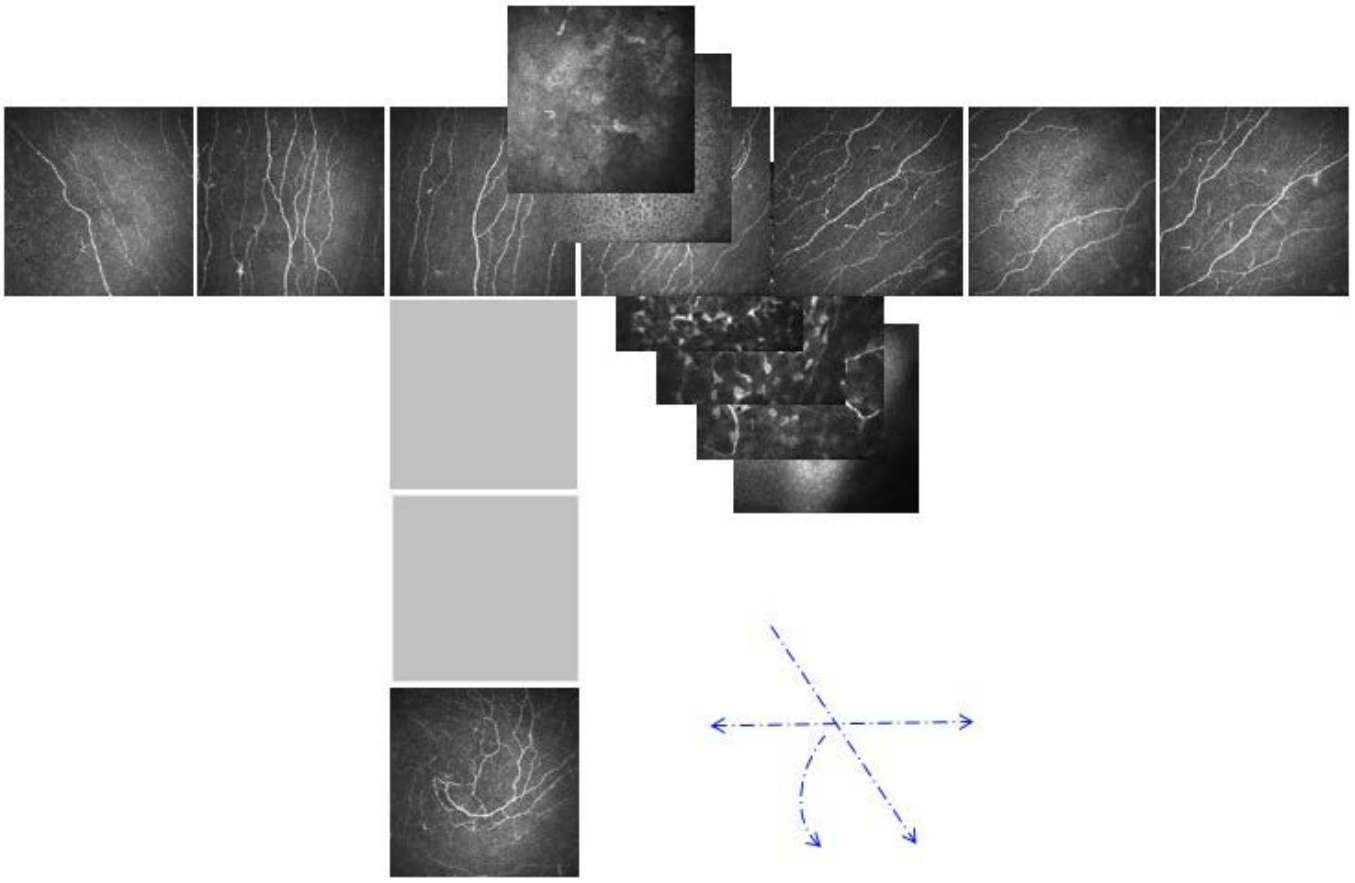
The inferior whorl will look as follows.



Sometimes the inferior whorl is not visible at first contact with the cornea. Use the diagram below (CCM image montage) to adjust positioning depending on the orientation of the nerves (it is the opposite for the fellow eye).



Ask the subject to sit back, “Reposition the microscope for the fellow eye”and repeat steps (1.16 to 1.23) to scan the fellow eye. A schematic of the scanning pattern is presented below.



To log follow-up examinations under the same subject, search for the subject identifier in the database. Select the subject of interest with single left click and choose the re-examine button. 

Using this function, the baseline and follow-up scans will be logged under the same subject.

Sometimes after a long scan (e.g., poor subject co-operation) the gel may ‘drop’ between the lens and the TomoCap resulting in a significant decrease in image quality

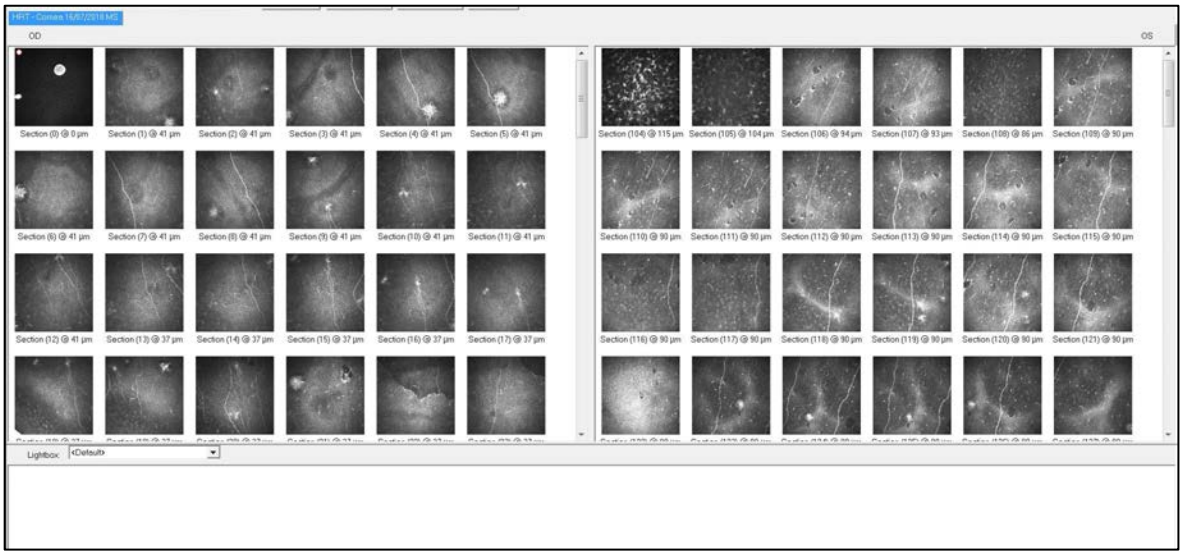
of the second eye scanned. Since the gel is the coupling agent between the TomoCap and the objective lens, the image quality decreases significantly. In this case set up the microscope again with a new TomoCap and gel to scan the other eye. An additional drop of anesthetic and added Viscotears is recommended.

Image acquisition demonstration

A demonstration of how to acquire CCM images is available via our YouTube channel https://youtu.be/QVZVOqnzUmI

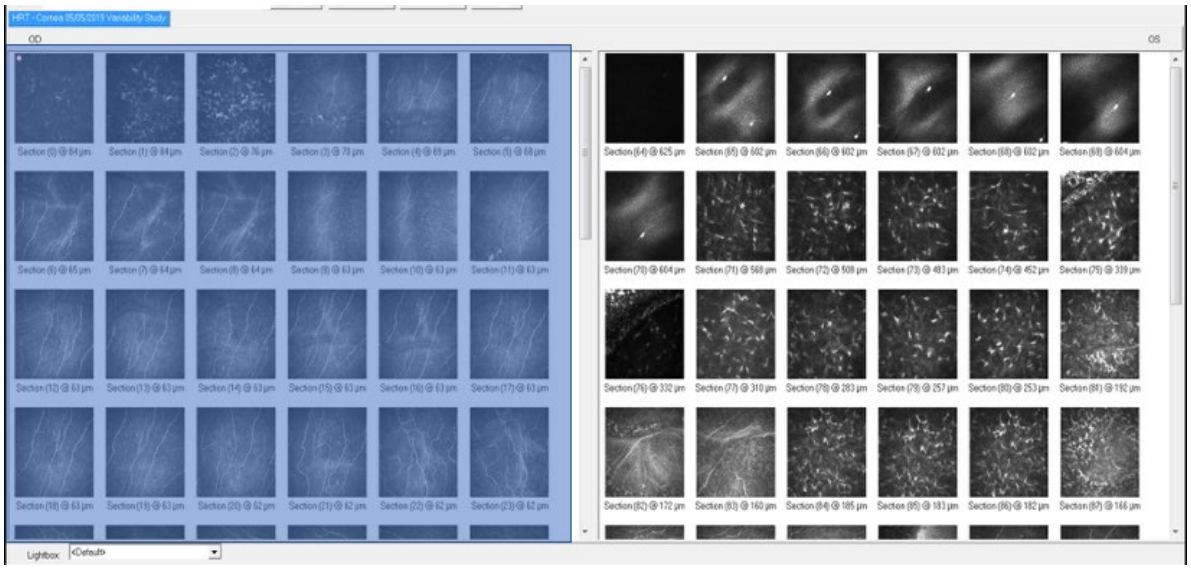
CCM Image Export

The images can be exported directly after the scan or at a later time. When the examination is complete the live image window can be closed. The images are automatically saved and the image preview panel appears. Once the subject has been located in the database, double click on the subject name and the image preview panel will appear. Please note, there may be more than 1 tab (blue) depending on the numbers of scans registered under the same subject.

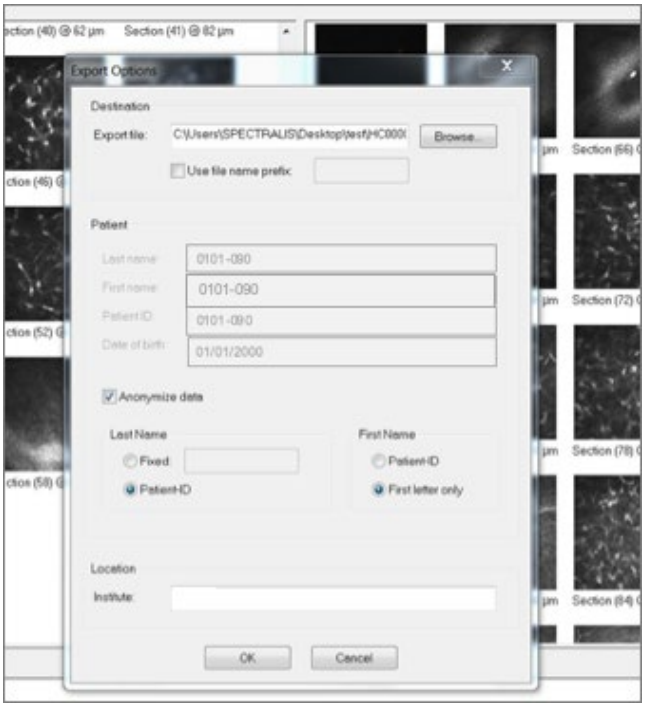


E2E files can be exported per eye per scan. Repeat the following steps for the other eye.

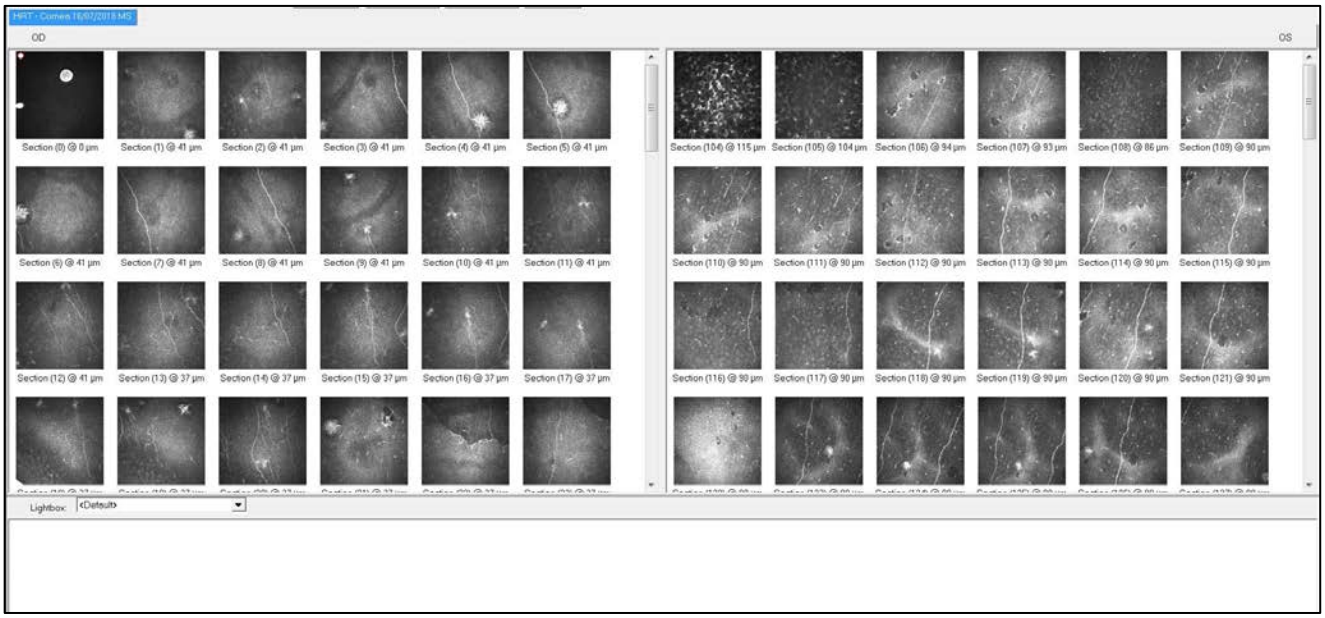
Click and drag with your mouse until all the images have been selected. The screen will appear as follows.



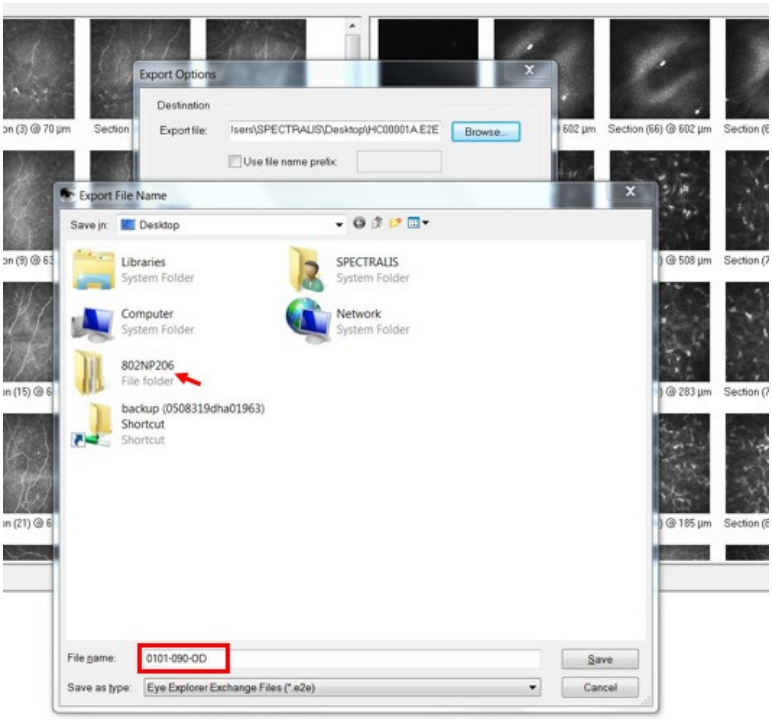
Right click and choose ‘export’. Select ‘Anonymize data’, select ‘Patient-ID’ under Last Name and under First Name. If there is an institute name manually replace by study site code.

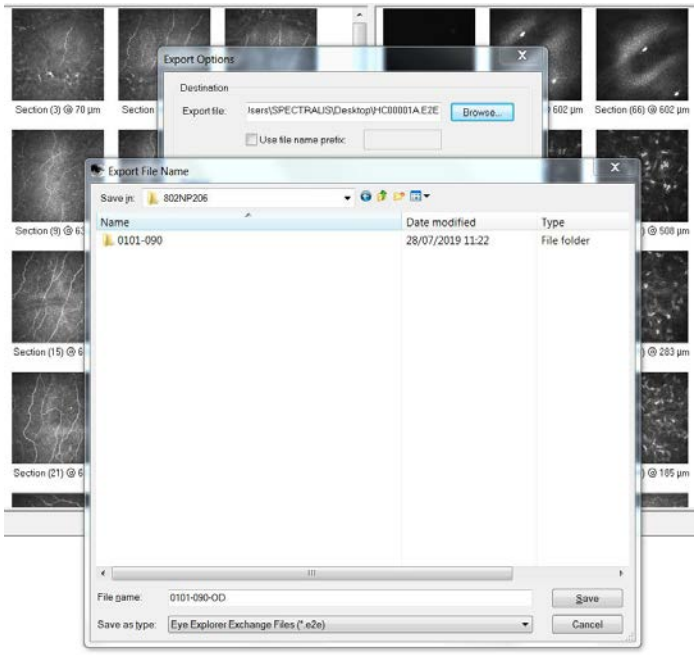


The database can be searched by subject ID, study etc. There are several filtering options.

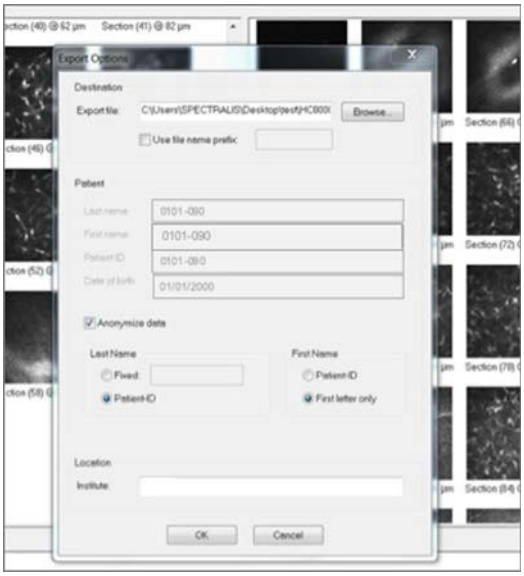


Choosing saving location - Click on ‘browse’ to change the default saving location e.g., save in a specific folder on the desktop as shown below (red arrow) or elsewhere. Confirm / enter file name (red outline).





Once the saving location has been chosen and the file name has been entered the following screen will re-appear. Click ‘OK’ to save the file.



Common problems for the investigator during scanning are:

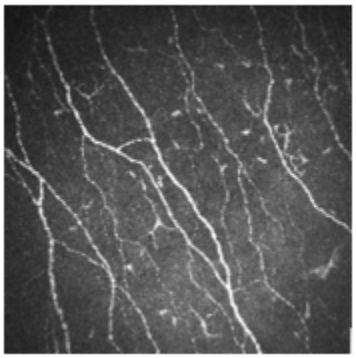
- Increased eye movement
- Extreme obesity
- Strabismus
- Poor vision
- Neurological disability (weakness/tremor etc.)
- Subject falling asleep during the scan
- Poor subject co-operation

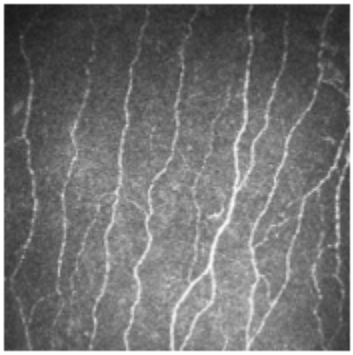
In the above cases the technician needs to adjust the microscope and the subject position to minimize image quality issues.

Image examples

Images with optimal quality (control and mild neuropathy)

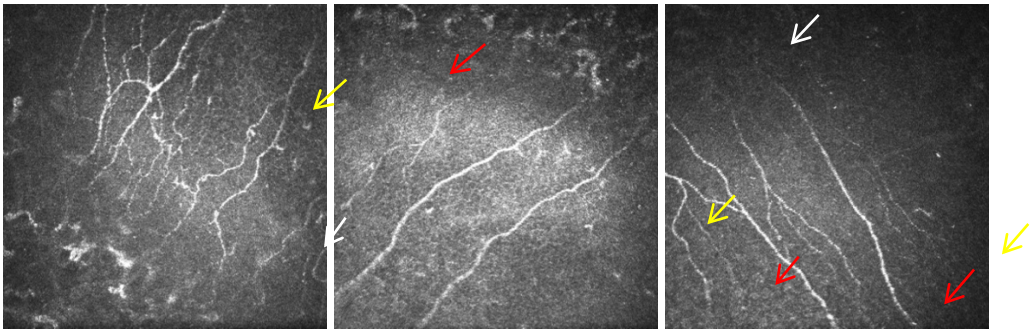
Well-focused, clearly visible nerve fibres, branches and underlying tissue.





<u>Images captured at an angle</u>

Poor subject co-operation results in increased eye movement, hence sub-optimal contact with the cornea. The images have the appearance of a cross-sectional image where the stroma (white arrow), nerves (yellow arrow) and epithelium (red arrow) are visible at the same time.

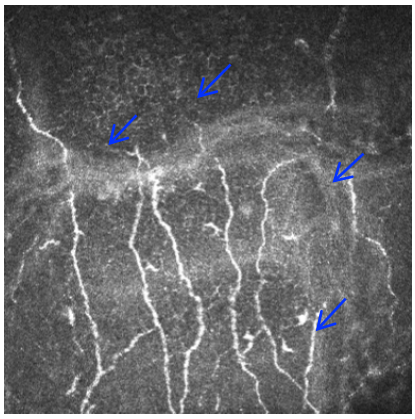


Images with pressure lines

When the cornea is thin, or the subject is not co-operating then excess pressure of the CCM TomoCap on the cornea will manifest as pressure lines in the image. From a clinical perspective, they are not associated with significant fluorescein staining on the slit lamp post-CCM examination, but they should best be avoided for comfort and image quality purposes. Note how the pressure lines (blue arrow) obstruct the view of the subbasal nerves. The non-uniform illumination and the semi-visible epithelial cells are also signs of excess pressure.

***** Pressure lines can be easily resolved by gentle adjustments of the microscope back and forward (blue knob) or an extra anesthetic drop in case of a sensitive subject.

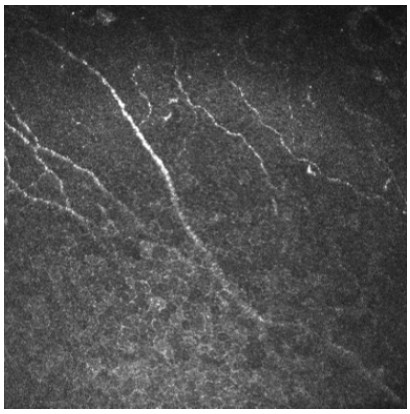
****** Other reasons include incomplete contact of the subject on the headrest/chinrest and the subject falling asleep.



Out of focus images.

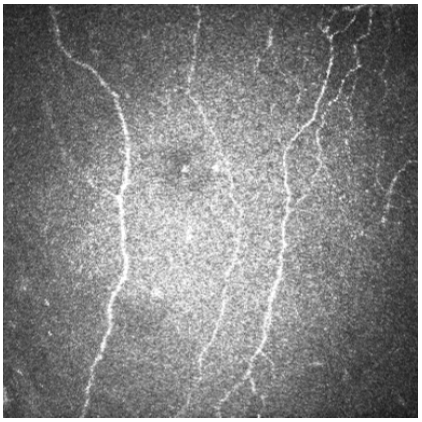
The image will look similar to a cross-sectional image due to the simultaneous viewing of nerves and epithelial cells. Note how the axons are clearly visible across an area and not the entire image. There are also no pressure lines. In this case gentle

movement of the lens will bring the subbasal nerves in full focus. Refocus the lens as needed.



Highly reflective/cloudy image

If the CCM is not calibrated properly before scanning or the scan takes too long and the gel drop falls to the bottom of the TomoCap the image may appear cloudy or bright at a particular location (usually centrally). If calibration and gel drop is checked and are not an issue, then underlying pathology or aging may be contributing to this artifact.



Equipment and Technician Set-Up Procedure

Purpose

To establish the requirements necessary to qualify multiple sites for acquiring study images using CCM for a study protocol. In order for the sites to be certified to perform CCM imaging on study subjects all sites will be required to complete equipment and technician certification.

In order to get certified the site will acquire CCM images on both eyes for one test subject.

Equipment

A CCM is a device for non-invasive corneal imaging solely distributed by Heidelberg Engineering GmbH (Heidelberg, Germany) under the commercial name 'Heidelberg Retinal Tomograph 3 with Rostock Corneal Module' (HRT III RCM). The equipment is composed of: an adjustable table, the CCM unit, RCM with revolving lens and knobs to adjust the focus position, external CCD camera to control the scanning position, external fixation target, chinrest and forehead-rest unit to minimize head movement and the TomoCap® .

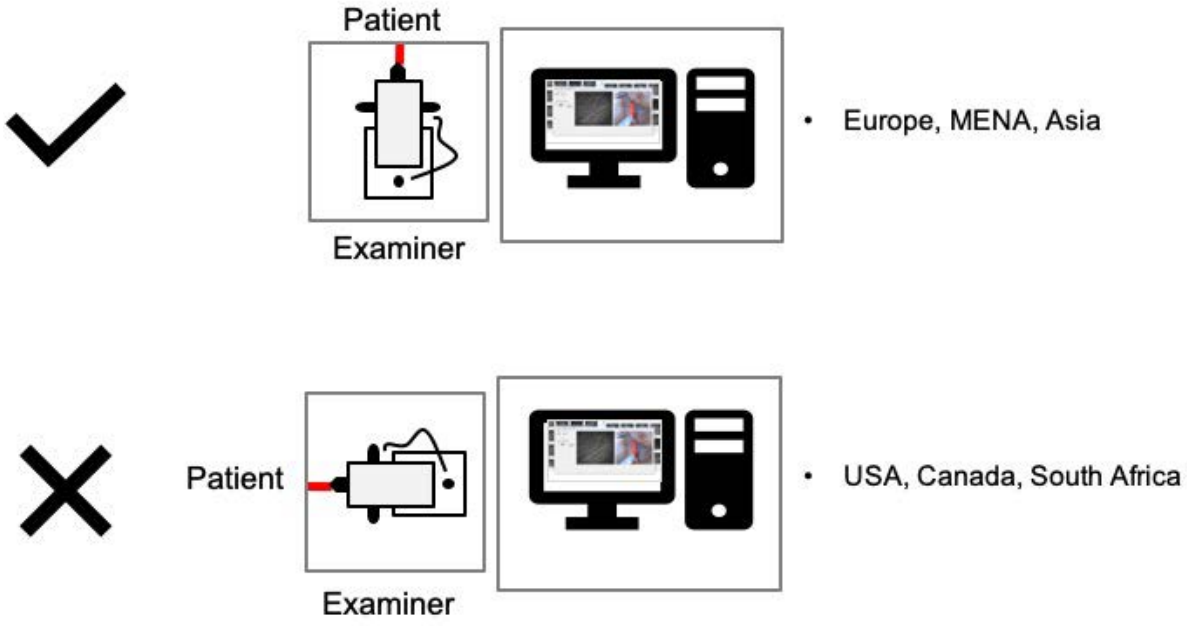
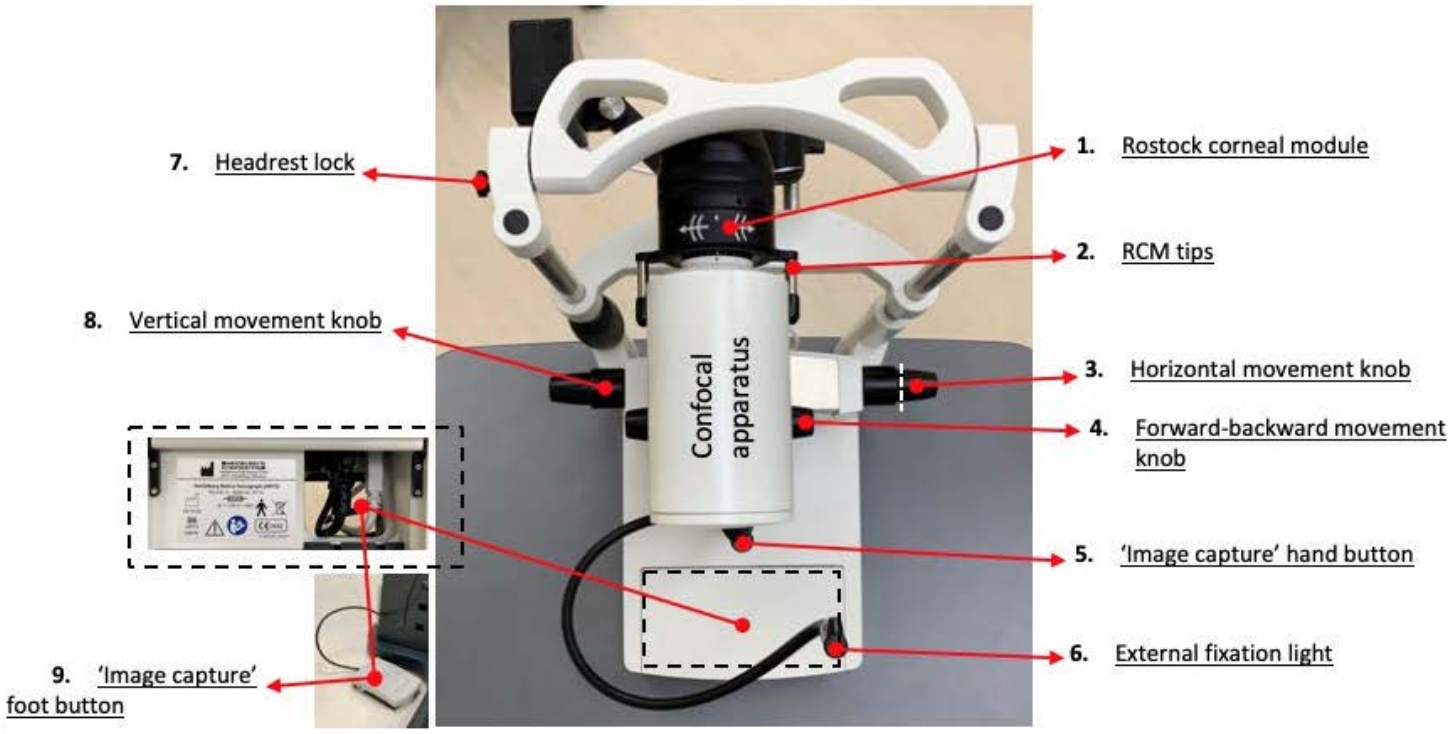


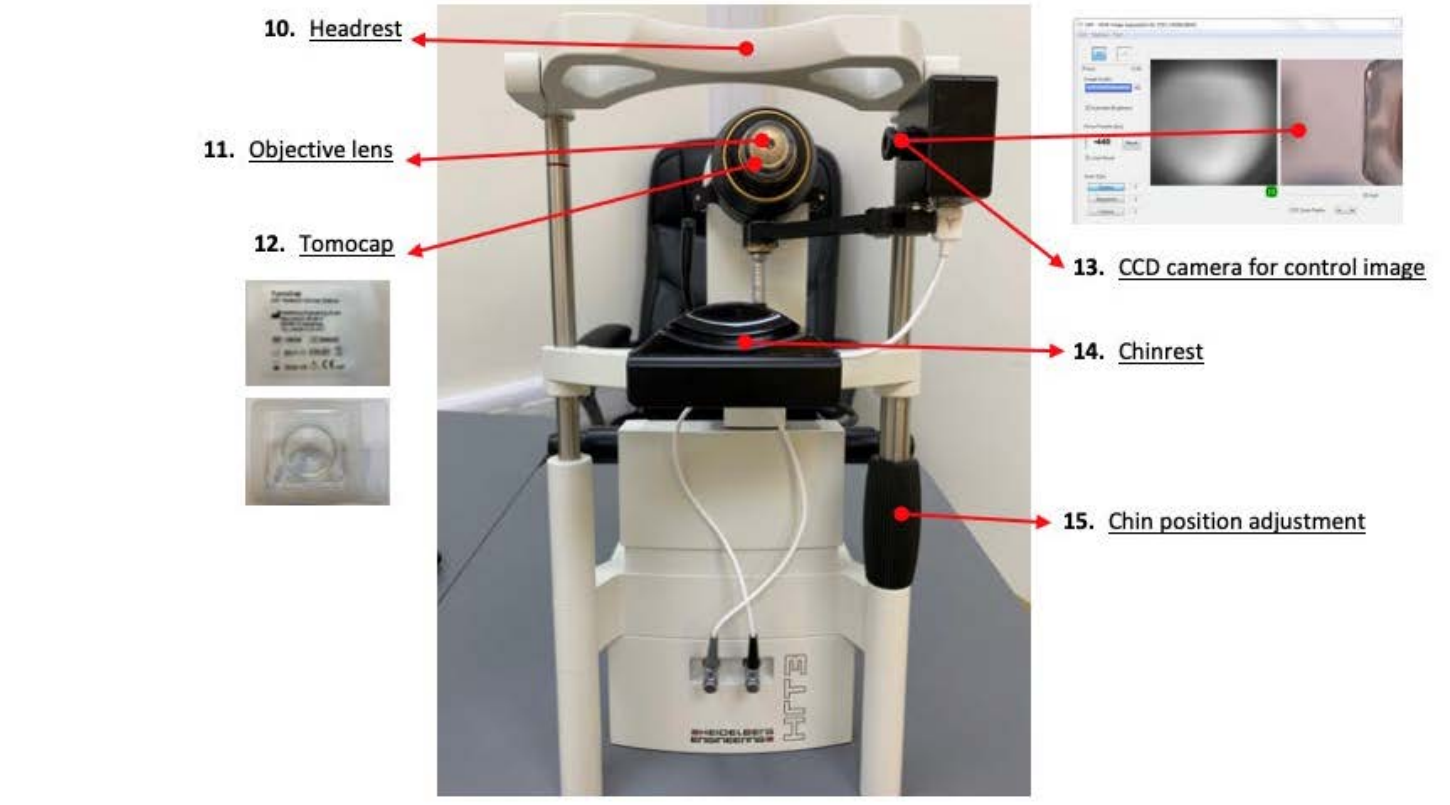
Figure 1. CCM set up in different regions. For an optimal scanning position, the patient and operator should be sitting across each other.

Technician

A CCM technician is a trained operator who performs a scan to obtain high resolution, microscopic images of the corneal layers. A CCM technician should have basic knowledge of corneal anatomy (types and appearance of layers, location), be able to set-up the CCM, anticipate and overcome technical difficulties (poor patient co-operation) to perform the scan and capture images suitable for quantitative analysis.

Corneal Confocal Microscope





Equipment Set-Up Checklist

☐ Operator and subject are able to sit across each other as in Figure 1.

All knobs (x6) are functional i.e. able to move the CCM unit:

- ☐ Horizontally [3]
- ☐ Vertically [8]
- ☐ Forward and backward [4]

☐ RCM [1] (black module) is in maximum contact with the HRT III unit.

- ☐ Press on the tips [2] of the RCM to confirm.

☐ The revolving part of the RCM [1] can move (revolve) manually to the maximum left / right position.

- ☐ This movement should be effortless i.e. the lens should not feel 'tight'.

The 'image capture' hand button [5] is functional:

- ☐ Produces a clicking sound when pressed.

☐ Captures images when pressed (can be verified during a scan without a subject).

☐ Ensure the foot button [9] is also connected to the unit.

☐ Repeat process with the foot button.

☐ The external CCD camera [13] faces the subject from the side

- ☐ The protective lens cover is removed.

☐ Verify with a test subject.

- ☐ Manually adjust the position, if needed.
- ☐ The CCM image appears sharp and focused [13] (see control image).
  - ☐ Adjust the lens manually if needed.

☐ Using the vertical black knob [15] the chinrest position [14] can be adjusted vertically.

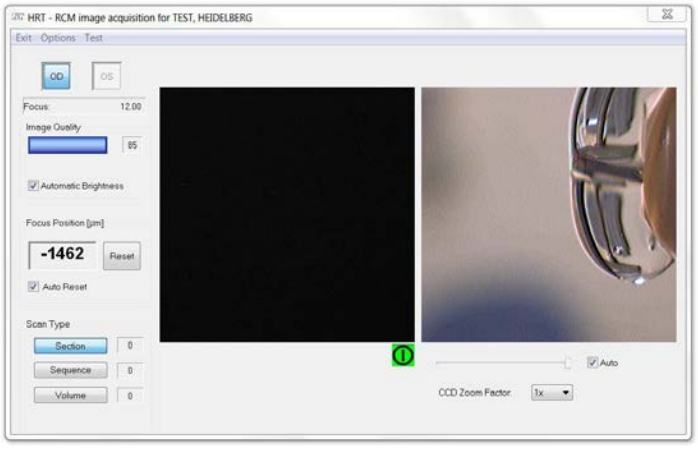
☐ The headrest [10] position can be locked [7].

Click the desktop icon to launch the Heidelberg Eye Explorer (for details see SOP CCM v4). Confirm the following steps can be completed:

- ☐ Click on ‘new examination’ 

☐ Enter subject details, click ‘OK’.

- ☐ Choose ‘operator’ and ‘study’, click ‘OK’.
- ☐ FOV at 400µm, click ‘OK’.
- ☐ The live window is activated.

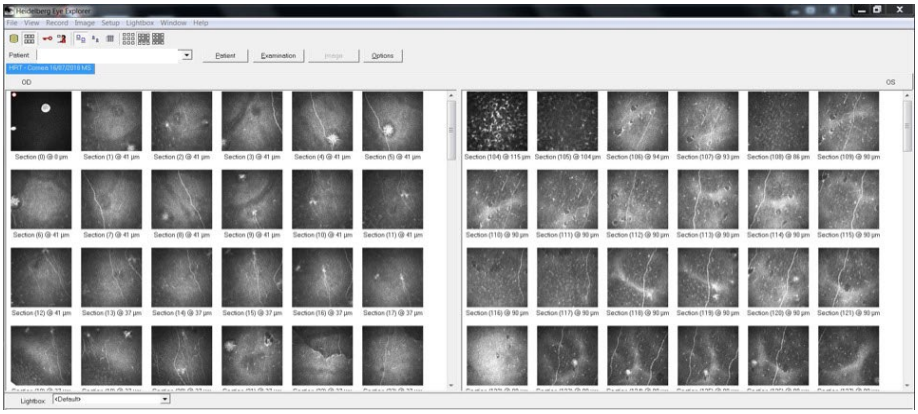


Technician Set-Up Checklist

*For a detailed visualized description refer to section 3 above.

- Technician has direct view of the subject and the monitor.
- Once the live window has been activated follow the steps below:
- Revolve the RCM lens anticlockwise until it stops.
- Image quality bar drops to ‘0’.
- Apply a drop (pea-size) of clear gel (bubble-free) on the tip of the microscope.
- Place a TomoCap 12 on top of the gel.
- Revolve the RCM lens clockwise until the first focal plane (1st white screen).
- Image quality increases to around ‘100’ and then drops again.

- Keep revolving until the second focal plane (2nd white screen).
- Image quality increases to '100'.
- Click on 'Reset'.
- Focus position is at 0 μm.
- Once the CCM is set up, prepare the subject for the scan:
- Instill 2 drops/eye of oxybuprocaine hydrochloride 0.4% (Bausch & Lomb, UK)
- Instill 1 drop/eye of Viscotears gel.
- Briefly explain the procedure to the subject and reassure them that they will not feel any pain during or after the scan.
- Ask the subject to rest their chin on the chinrest 14 and push their head against the headrest 10. They should remain in the same position during the scan.
- Adjust the chinrest position 15 if needed.
- Ask the subject to focus on the external fixation light and open their eyes 6.
- Using knobs 3, 4, 8 move the CCM towards the subject (micro-adjust the position if needed).
- Make minimal contact with the cornea (i.e. avoid pressure lines) and revolve the RCM lens clockwise at moderate speed.
- Capture images as per study protocol.
- Repeat the process for the second eye in the same subject (contralateral eye).
- When the scan is complete, close the examination window and image previews will appear on the panel.


Heidelberg Eye Explorer
File View Record Image Setup Lightbox Window Help
Patient
Patient
Examination
Image
Options
OD
OS
Lightbox
<Default>