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Nielsen, Thomas Arendt

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**THE FUNCTION OF THE AUTONOMIC
NERVOUS SYSTEM IN TYPE 1 DIABETES
AND IN RESPONSE TO PHYSIOLOGICAL
AND PHARMACOLOGICAL MODULATIONS**

**BY
THOMAS ARENDT NIELSEN**

DISSERTATION SUBMITTED 2023



AALBORG UNIVERSITY
DENMARK

THE FUNCTION OF THE AUTONOMIC NERVOUS SYSTEM IN TYPE 1 DIABETES AND IN RESPONSE TO PHYSIOLOGICAL AND PHARMACOLOGICAL MODULATIONS

Ph.D. thesis
by
Thomas Arendt Nielsen



AALBORG UNIVERSITY
DENMARK



AALBORG UNIVERSITY HOSPITAL

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PhD supervisor:: Professor Christina Brock, DVM, PhD,
Mech-Sense, Department of Gastroenterology
& Hepatology, Aalborg University Hospital, Aalborg,
Denmark

PhD co-supervisors: Professor Asbjørn Mohr Drewes, MD, PhD, DMSc,
EDPM, R.
Director of Mech-Sense & Centre for Pancreatic
Diseases, Department of Gastroenterology & Hepatology,
Aalborg University Hospital, Aalborg, Denmark

Professor Henrik Vorum, MD, PhD,
Department of Ophthalmology Aalborg University
Hospital, Aalborg, Denmark

PhD committee: Clinical Professor Jacob Udby Blicher (chair)
Aalborg University, Denmark

Senior Research Fellow and Consultant Autonomic
Neurophysiologist Peter O. Julu
Queen Mary University of London, UK

Clinical Research Associate Professor Steffen Hamann
University of Copenhagen, Denmark

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CV

THOMAS ARENDT NIELSEN

Born 1990, Aalborg, Denmark



CURRENT POSITION AND WORK ADDRESS

2021-2022 Department of Gastroenterology &
Hepatology, Aalborg University
Hospital, Aalborg, Denmark
2022- Department of Ophthalmology, Aalborg University Hospital,
Aalborg, Denmark

EDUCATION

2011-2018 Cand. Med. (MD), The Faculty of Medicine, Aalborg University

PUBLISHED PAPERS

1. Brock C, Wegeberg AM, Nielsen TA, Karout B, Hellström PM, Drewes AM, Vorum H. The Retinal Nerve Fiber Layer Thickness Is Associated with Systemic Neurodegeneration in Long-Term Type 1 Diabetes. *Transl Vis Sci Technol.* 2023 Jun 1;12(6):23. doi: 10.1167/tvst.12.6.23. PMID: 37367720.
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ENGLISH SUMMARY

Type 1 diabetes is a chronic autoimmune disease caused by the immune-mediated destruction of insulin-producing pancreatic beta cells, whereas type 2 diabetes is characterized by insulin resistance alone or combined with insufficient insulin production. Both types result in hyperglycemia. Prolonged hyperglycemic exposure can lead to several life-impairing microvascular complications, such as diabetic nephropathy, neuropathy, and retinopathy.

Diabetic neuropathy is the most common complication of diabetes and can affect both peripheral and autonomic nerves and cause, e.g., distal symmetrical polyneuropathy (DSPN) and diabetic autonomic neuropathy (DAN). Whereas DSPN affects sensorimotor neurons in a “glove and stocking” pattern, DAN results in dysfunction and impaired regulation of several organ systems and autonomically innervated muscles. Diabetic neuropathies can be painful as well as non-painful.

Glucagon-like peptide 1 receptor agonists (GLP-1 RA's) are used in the treatment of type 2 diabetes by stimulating insulin secretion and inhibiting glucagon secretion, resulting in a reduction of glucose levels. GLP-1 RA's have several extra-pancreatic effects, including anti-inflammatory, cardioprotective, and neuroprotective effects. In mice, topical administration of a GLP-1 RA has shown to prevent thinning of the neuroretina and promote neurogenesis in retinal cells. In humans, GLP-1 RA's are known to increase heart rate through autonomic stimulation mechanisms not yet fully understood. In this thesis, we investigate the effects of the GLP-1 RA liraglutide in individuals with long-term type 1 diabetes to assess the anti-inflammatory and neuroprotective effects, independently of the anti-hyperglycemic effects.

The overall aims of this thesis were to study:

- 1) The effects of long-term type 1 diabetes on autonomic nerves involved in neurocardiac regulation (Study I, III, and IV)
- 2) The effects of long-term type 1 diabetes on sympathetic nerves innervating the superior and inferior tarsal muscles (Study I)
- 3) The effects of the GLP-1 RA liraglutide on heart rate variability (HRV) and the retinal nerve fiber layer thickness in individuals with long-term type 1 diabetes and confirmed DSPN (Study II and IV)

Paper I investigated the relationship between the sympathetically innervated tarsal muscles' response to phenylephrine (assessed as changes in palpebral fissure height) and HRV measures. The study aimed to examine if the phenylephrine-induced response in palpebral fissure height was associated with HRV measures and thus could be a potential marker of autonomic dysfunction in individuals with type 1 diabetes and DSPN. The phenylephrine-induced changes in palpebral fissure height were associated with autonomic function, diabetic retinopathy stage, and disease duration.

Paper II assessed, in a randomized, double-blinded, placebo-controlled study, whether liraglutide influenced the retinal nerve fiber thickness due to anti-inflammatory and possible neurotropic actions using optical coherence tomography. The thickness of the inferior, superior, nasal, temporal, and peripapillary retinal nerve fiber layer thickness were compared in individuals with type 1 diabetes and DSPN, who were randomized to receive either liraglutide or placebo. Liraglutide did not induce measurable changes in the retinal nerve fiber layer thickness both on group level and in the subgroups constituted by proliferative and non-proliferative retinopathies.

Paper III assessed HRV in resting conditions (24-h.) and ultra-short-term HRV to assess the physiological modulation in response to tonic cold pain exposure (cold pressor test) in participants with long-term type 1 diabetes and verified DSPN. The main findings revealed adynamic sympathetic and parasympathetic HRV responses in the diabetes group when compared with healthy controls.

Paper IV investigated the effect of liraglutide/placebo on heart rate and HRV modulation before, during, and after stimulation by tonic cold pain exposure (cold pressor test). Liraglutide increased heart rate when compared to placebo. Furthermore, modulation by tonic cold pain unmasked alterations in the sympathetic response and showed an unaltered parasympathetic response, indicative of sympathetic activation.

The fundamental and clinically relevant knowledge generated in this thesis provided new insight into the effects of type 1 diabetes on autonomic nerve function, as well as the effect of liraglutide on the neurocardiac regulation and human retinal nerve fibers.

DANSK RESUME

Type 1 diabetes er en kronisk autoimmun sygdom, forårsaget af immunmedieret destruktion af de insulinproducerende beta-celler i bugspytkirtlen, hvorimod type 2 diabetes er karakteriseret af insulinresistens og insuffICIENT insulinproduktion. Begge typer medfører i forhøjet blodsukker. Langvarig eksponering for forhøjet blodsukker niveauer kan medføre flere mikrovaskulære komplikationer, såsom diabetisk nefropati, neuropati og retinopati.

Diabetisk neuropati rammer ca. 50% af personer med diabetes og anses for at være den, der forvolder flest belastende symptomer fra både perifere og autonome nerver. De diabetes inducerede nervebeskadigelser kan føre til udvikling af distal symmetrisk polyneuropati (DSPN), samt diabetisk autonom neuropati (DAN). DSPN påvirker perifere sensomotoriske nerver, og kan medføre nedsat sensibilitet, nedsat muskelkraft og kan medføre smerter, hvorimod DAN fører til dysregulering af de fleste organsystemer samt de autonomt innerverede muskler. Diabetisk polyneuropati kan hos nogen medføre neurogene smerter men ikke hos andre.

Glukagon lignende peptid-1 receptoragonister (GLP-1RA'er) anvendes i behandlingen af type 2-diabetes, ved at stimulere frigivelsen af insulin og hæmme frigivelsen af glukagon, hvilket medfører en reduktion af blodsukkerniveauet.

Udover at virke på cellerne i bugspytkirtlen, har GLP-1 RA'er vist sig, at have anti-inflammatoriske, kardioprotektive samt neuroprotektive virkningsmekanismer. Topikal administration af øjendråber indeholdende GLP-1-RA'er har i mus vist sig at forebygge udtynding af nethinden, samt fremme nydannelsen af nerveceller i nethinden. GLP-1 RA'er har konsistent vist at øge hjerterefrekvensen, omend den/de underliggende mekanisme(r) endnu ikke er fuldt belyst. I denne afhandling, undersøgte vi effekten af GLP-1 RA liraglutid i individer med langvarig type 1 diabetes, for at undersøge den anti-inflammatoriske og neuroprotektive effekt uafhængigt af den anti-hyperglykæmiske virkning.

Det overordnede formål med studierne der indgår i PhD afhandlingen er at studere:

- 1) Effekten af langvarig type 1 diabetes på de autonome nerver som er involveret i den neurokardielle regulering (Studie I, III og IV)
- 2) Effekten af langvarig type 1 diabetes på de sympatiske nerver der innervierer de superiore og inferiore tarsale muskler (Studie I)
- 3) Effekten af liraglutid (en GLP-1 RA) på hjerterefrekvensvariation (HRV) samt på det retinale nervefiber lags tykkelse i patienter med langvarig type 1 diabetes og verificeret distal symmetrisk polyneuropati (Studie II og IV).

Artikel 1 undersøgte forholdet mellem de sympatisk innerverede tarsale musklers respons på phenylephrin (ændringer i øjenspalte højde) samt hjerterefrekvensparametre. Det blev undersøgt om den phenylephrin-inducerede respons på øjenspalte højden var associeret med hjerterefrekvensparametre og om den dermed potentielt kunne være en markør for dysfunktioner af sympatiske nerver hos personer med type

1-diabetes og verificeret polyneuropati. Studiet fandt, at de phenylephrin-inducerede ændringer i øjenspalte højden var associeret med den sympatiske funktion, graden af diabetisk retinopati, samt sygdomsvarigheden.

Artikel 2 undersøgte, i et randomiseret, double-blindet, placebo-kontrolleret studie, effekten af liraglutid på det retinale nervefiber lag. Studiet sammenlignede liraglutid og placebo's respektive effekt på tykkelsen af den inferiore, superiore, nasale, temporale, samt peripapillære retinale nervefiber lags tykkelse ved brug af optisk koherens tomografi. Liraglutid medførte ikke målbare ændringer i den retinale nervefiber lags tykkelse, hverken på gruppeniveau eller i den proliferative, eller non-proliferative undergruppe.

Formålet med artikel 3, var at vurdere effekten af langvarig type 1-diabetes på hjertevariabilitet i hvile, samt som respons på smerte-stimulering med is-vand. Studiet sammenlignede hjertevariabilitets responserne blandt personer med langvarig type 1-diabetes og raske forsøgsparticipanter, og fandt adynamiske sympatiske og parasympatiske-responser i diabetesgruppen.

I artikel 4 undersøgte, i et randomiseret, double-blindet, placebo-kontrolleret studie, effekten af liraglutid på hjertefrekvens og hjertevariabilitets parametre, som følge af smerte-stimulering med is-vand. Studiet sammenlignede effekten af liraglutid og placebo på personer med langvarig type 1-diabetes og DSPN og bekræftede at behandling med liraglutid øgede hjertefrekvensen. Stimulation is-vand tydeliggjorde ligeledes ændringer i det sympatiske respons, men ikke i parasympatiske respons.

Denne PhD afhandling bidrager til en øget klinisk og fundamental indsigt i effekten af langvarig type 1-diabetes på de autonome nervefunktioner, samt virkningen af liraglutid på den neurokardielle regulering samt på det retinale nervefiber lag.

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LIST OF PAPERS

This thesis is based on the following papers:

- I. Nielsen TA, Andersen CU, Vorum H, Riahi S, Segal R, Drewes AM, Karmisholt J, Jakobsen PE, Brock B, Brock C. Palpebral Fissure Response to Phenylephrine Indicates Autonomic Dysfunction in Patients With Type 1 Diabetes and Polyneuropathy. *Invest Ophthalmol Vis Sci*. 2022 Aug 2;63(9):21. doi: 10.1167/iovs.63.9.21. PMID: 35980646; PMCID: PMC9404365.
- II. Nielsen TA, Segal R, Uggerhøj Andersen C, Vorum H, Drewes AM, Jakobsen PE, Brock B, Brock C. Liraglutide Treatment Does Not Induce Changes in the Peripapillary Retinal Nerve Fiber Layer Thickness in Patients with Diabetic Retinopathy. *J Ocul Pharmacol Ther*. 2022 Jan-Feb;38(1):114-121. doi: 10.1089/jop.2021.0055. Epub 2021 Dec 16. PMID: 34918951
- III. Nielsen TA, Lundbye-Christensen S, Dimitrova YK, Riahi S, Brock B, Drewes AM, Brock C. Adynamic response to cold pain reflects dysautonomia in type 1 diabetes and polyneuropathy. Accepted for publication in *Scientific Reports*.
- IV. Nielsen TA, Lundbye-Christensen S, Dimitrova YK, Riahi S, Brock B, Drewes AM, Brock C. Cold pain exposure reveals heart rate variability alterations in liraglutide-treated individuals with type 1 diabetes. Submitted for publication in *Communications Medicine*

The above papers will be referred to as study I-IV throughout the thesis. Study I and II are published, and peer-reviewed original papers. Study III has been accepted for publication. Study IV have been submitted for publication.

ABBREVIATIONS

ANS: Autonomic nervous system

CPT: Cold pressor test

CAN: Cardiovascular autonomic neuropathy

DAN: Diabetic autonomic neuropathy

DPN: Diabetic peripheral neuropathy

DSPN: Distal symmetrical polyneuropathy

T1DM: Type 1 diabetes mellitus

T2DM: Type 2 diabetes mellitus

PFH: Palpebral fissure height

Δ PFH: Difference in PFH between phenylephrine-treated and untreated eye

RNFL: Retinal nerve fiber layer thickness

DPN: Diabetic peripheral neuropathy

DR: Diabetic retinopathy

PDR: Proliferative diabetic retinopathy

NPDR: Non-proliferative diabetic retinopathy

HR: Heart rate

HRV: Heart rate variability

VLF: Very low-frequency band (0.0033-0.04 Hz)

Δ VLF: Difference in VLF parameters between baseline and follow-up

LF: Low-frequency band (0.04-0.15 Hz)

Δ LF: Difference in LF parameters between baseline and follow-up

HF: High-frequency band 0.15-0.4 Hz)

Δ HF: Difference in HF parameters between baseline and follow-up

LF/HF: Low-frequency/high-frequency ratio

SDNN: Standard deviation of NN intervals

SDANN: Standard deviation of all NN intervals for each 5-minute for 24 hours

SDNNI: Mean of the standard deviation of all NN intervals for each 5-minute interval for 24 hours

RMSSD: Root mean square of successive RR interval differences

CBS: Cardiac sensitivity to the baroreflex

CVT: Cardiac vagal tone

GLP-1: Glucagon-like peptide 1

GLP-1 R: Glucagon-like peptide 1 receptor

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

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1 INTRODUCTION

Type 1 diabetes is a chronic, metabolic disease known to cause microvascular complications such as retinopathy, neuropathy, and nephropathy. In type 1 diabetes (T1DM), the most frequent type of neuropathy is diabetic peripheral neuropathy (DPN), with a prevalence of 17,5% and an incidence of 8,8 per 1000 person-years^{1,2}. DPN takes on two different forms: diabetic distal symmetrical polyneuropathy (DSPN) and diabetic autonomic neuropathy (DAN)^{3,4}. While DSPN affects sensorimotor neurons in the extremities, in a “glove and stocking” pattern, DAN leads to impairment and dysregulation of the autonomic nervous system (ANS)^{5,6}. When diabetic neuropathy affects the ANS, deterioration in the normal functions and regulation of, e.g., cardiovascular, gastrointestinal, urogenital, sudomotor, and neurovascular functions may appear, resulting in a reduction in quality of life⁶⁻⁸. Impairment of cardiovascular autonomic control in individuals with diabetes mellitus is known as cardiovascular autonomic neuropathy (CAN). In individuals with CAN, ANS regulation is altered, resulting in an impaired ability of the heart rate to adapt to different conditions and maintain homeostasis. Manifestations of CAN include sinus tachycardia, abnormal blood pressure regulation, orthostatic hypotension, dizziness, syncope, exercise intolerance, asymptomatic myocardial ischemia, and infarction⁹. CAN is a serious yet common complication of diabetes mellitus, associated with increased overall mortality and morbidity¹⁰⁻¹⁴. Routine clinical examinations based on existing clinical guidelines do not include a thorough investigation of the autonomic nervous system, even though CAN is associated with life-threatening complications¹⁵.

Several methods exist for assessing the autonomic nervous system, including evaluating cardiovascular autonomic reflex, heart rate variability (HRV), pupillomotor-, sudomotor- and pilomotor functions¹⁶⁻¹⁸. To diagnose CAN, cardiovascular autonomic reflex tests (CARTs) are needed, however, HRV is widely used to evaluate the autonomic nervous function in 24-hour Holter recordings (gold standard), but recently short-term recordings have also been used, and even ultra-short epochs of 2 minutes or less, is now being introduced.

Regulation of the heart rate results from complex autonomic interaction with primarily the brainstem, and as such, the ANS and the sympathetic-vagal balance has a significant role as the heart's modulator, allowing for rapid dynamic adaptation to changes in the internal and external environment, to maintain homeostasis. Different parameters of the heart rate variability (HRV) reflect the sympathetic and parasympathetic effects on the sinoatrial node for the regulation of heart rate¹⁹. Based on the electrical conductivity of the heart, HRV is adopted as a specific, sensitive, and reliable method for assessing autonomic dysfunction in people with T1DM^{4,20,21}. It uses the beat-to-beat variations (R-R interval) of consecutive heartbeats to quantify the functional status of the physiological control of the heart, allowing an evaluation of the ANS as a function of time and frequency. Reduction in HRV is a sign of imbalanced sympathetic-vagal regulation (autonomic dysfunction) and can be used as a marker of

CAN in T1DM, even before the onset of the symptoms^{22,23}. There is, however, an unmet clinical need to develop provocative tests that could reveal early signs of CAN because the current diagnostic procedure is based on abnormal reflex testing, where CAN is in its early development or manifest.

Modulation of the ANS is possible in several ways, such as by controlled deep and slow breathing activating the physiological baroreceptor response²⁴ or by electrical stimulation of the vagal nerve²⁵. Furthermore, registered pharmacological drugs are known to block sympathetic (e.g., beta-blockers)/parasympathetic (e.g., atropine) activity or enhance sympathetic (e.g., phenylephrine, adrenalin, or dopamine)/parasympathetic (e.g., choline esterase inhibitors or neostigmine) activity. However, glucagon-like peptide-1 receptor agonists (GLP-1 RA's) have also been shown to modulate the autonomic nervous system²⁶. GLP-1 RA's act as neuropeptides by regulating the ANS and the cardiovascular system²⁷, and when secreted by the hypothalamic nuclei, GLP-1 has been shown to increase parasympathetic activity²⁸. Furthermore, GLP-1 RA's have been shown to have antioxidative, anti-inflammatory, and neuroprotective effects on central and peripheral nerves^{29,30}, which may affect HRV. A recently published preclinical study found that GLP-1 receptor agonists even exert neurotrophic influence on retinal cells³¹, underpinning its multifaceted actions.

The fundamental knowledge generated during this Ph.D. project will contribute to a better understanding of autonomic nervous system function in individuals with type 1 diabetes and the effect of the GLP-1 receptor agonist, liraglutide, on the neurocardiac regulation and possible neurotrophic effects on retinal nerve fibers.

The hypotheses of this PhD project are as follows:

- 1) Long-term type 1 diabetes causes neuropathy to the peripheral sympathetic nerve fibers innervating the superior and inferior tarsal muscles³².
- 2) Treatment with liraglutide increases peripapillary retinal nerve fiber layer thickness by inducing nerve regeneration and restoring vascularization³³.
- 3) Exposure to tonic cold pain causes enhanced sympathetic drive and attenuates the dynamic sympatico-vagal response in participants with long-term type 1 diabetes when compared to healthy controls.
- 4) Treatment with liraglutide leads to increased heart rate and sympathetic drive in situational dynamic heart rate variability measures, evident as increased low-frequency and very low-frequency content.

In summary, the objectives of this PhD project are:

- To investigate the relationship between the sympathetically innervated tarsal muscle response to phenylephrine and HRV measures in individuals with long-term T1DM and polyneuropathy (Paper I)³²
- To assess associations between the tarsal muscle response to phenylephrine and the degree of diabetic retinopathy, disease duration, as well as the degree of diabetic retinopathy and HRV measures in individuals with long-term T1DM and DSPN (Paper I)³²

- To compare the effect of liraglutide on retinal nerve fiber layer thickness following 26 weeks treatment in comparison to placebo in individual with long-term T1DM and DSPN (Paper II)³³
- To assess the dynamic HRV alterations in response to cold tonic pain exposure in healthy and in individuals with long-term T1DM and DSPN (Paper III)
- To compare 24-hour HRV measures, baroreflex sensitivity and cardiac vagal tone in individual with long-term T1DM and DSPN and healthy (Paper III)
- To assess and compare the dynamic HRV responses to tonic cold pain exposure in individuals with long-term T1DM and DSPN treated following 26 weeks of treatment with liraglutide and placebo (Paper IV)

2 BACKGROUND

2.1 DIABETES MELLITUS AND ITS COMPLICATIONS

Diabetes mellitus is a chronic metabolic disease characterized by elevated blood glucose levels. It can lead to serious damage to the heart, blood vessels, eyes, kidneys, and nerves. According to WHO, diabetes affects up to 422 million individuals worldwide, and it has been estimated that up to 1.5 million deaths are directly attributed to diabetes³⁴.

Diabetes is caused by impairment in insulin secretion or action or a combination of the two. However, there are primarily two types of diabetes: type 1 and type 2 diabetes. Whereas type 1 diabetes is characterized by the autoimmune destruction of insulin-producing pancreatic beta cells, resulting in relative or absolute insulin deficiency, type 2 diabetes is characterized by insulin resistance alone or combined with insufficient insulin production.

Type 1 diabetes typically presents in childhood, and the incidence increases with age and peaks around 10-14 years³⁵. Within the last decades, the global incidence of type 1 diabetes has risen by 3.4% annually³⁶. Both genetic, epigenetic, and environmental factors are involved in developing type 1 diabetes³⁷. Symptoms of type 1 diabetes include polyuria, polydipsia, weight loss, fatigue, and vision changes, and diabetes is diagnosed by the presence of elevated blood glucose levels. The progression of diabetes mellitus is associated with the development of microvascular complications, such as retinopathy, neuropathy, and nephropathy³⁸. Hyperglycemia and HbA1c levels above 7% have been shown to increase the risk of developing such microvascular complications³⁹.

2.1.1 DIABETIC RETINOPATHY

Diabetic retinopathy (DR) is a degenerative neurovascular disease affecting retinal microvasculature. In most developed countries, DR is the leading cause of blindness in working-aged adults^{40,41}. DR affects approximately 93 million people worldwide, of which 17 million are believed to have proliferative DR⁴⁰. In a large Danish population-based cohort study of individuals with type 1 diabetes, the 25-year prevalence of diabetic retinopathy was 97%, and the incidence of proliferative retinopathy was 42.9%⁴². The prevalence of DR increases with disease duration and poor glycemic- and blood pressure control⁴³. Screening for DR and improved glycemic control significantly reduces the risk of diabetes-induced blindness⁴⁴.

Diabetic retinopathy ranges from non-proliferative diabetic retinopathy (NPDR) to proliferative diabetic retinopathy (PDR). The severity of diabetic retinopathy can be staged using the International Clinical Diabetic Retinopathy Disease Severity scale⁴⁵.

NPDR is staged as mild, moderate, or severe based on the number of microaneurysms/intraretinal hemorrhages and the presence of venous beading and/or intraretinal microvascular abnormalities (see Fig. 1). PDR is characterized by the presence of neovascularization arising from the retina or optic disc and is associated with preretinal or vitreous hemorrhages. Furthermore, subsequent fibrosis may lead to tractional retinal detachment.

The development of PDR is caused by progressive retinal ischemia, which releases local growth factors, such as vascular endothelial growth factors, resulting in retinal neovascularization⁴⁶.

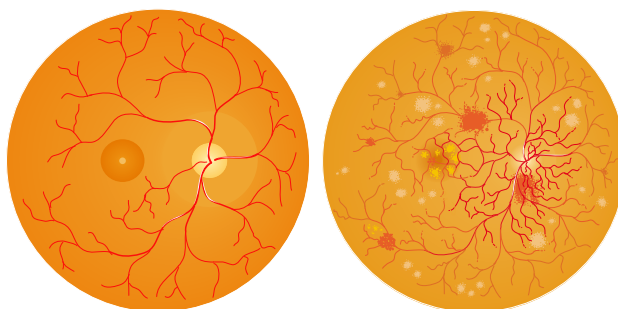


Figure 1: Graphic visualization of the retina without retinopathy (left) and with diabetic retinopathy (right), showing intraretinal hemorrhages, cotton wool spots, hard exudates, and the formation of new blood vessels (intraretinal microvascular abnormalities/neovascularization). Graphics supplied by Servier Medical Art, licensed under a Creative Commons Attribution 3.0 unported license.

Although DR has classically been considered a microvascular disease, diabetes has also been shown to cause retinal neurodegeneration in type 1-^{47,48} and type 2 diabetes^{49,50}. These changes have been referred to as retinal diabetic neuropathy or diabetic retinal neurodegeneration^{51,52}. Growing evidence suggests that thinning of retinal neuronal and axonal layers precedes the presence of clinically visible microvascular changes of DR^{53,54}. Retinal thinning within the macula has even been shown in pre-diabetes⁵⁵.

Diabetic retinal neurodegeneration is primarily caused by dysfunction of the retinal neovascular unit⁵¹. Damage occurs early in disease progression, and dysfunction of the neovascular unit results in blood-retinal-barrier breakdown and vascular leakage caused by secretion of various factors, including vascular endothelial growth factors and pro-inflammatory cytokines^{56–58}. As a result, apoptosis of retinal neurons results in thinning of retinal nerve fiber layers (RNFL), eventually causing loss of vision^{51,58,59}. Diabetic retinal neurodegeneration may not only be an early indicator of DR but is more likely a factor in the development of DR and thus contributes to the microvascular changes^{52,60}. Consequently, early therapeutic neuroprotective strategies, e.g., GLP-1 RA's³⁰, may not only affect diabetic retinal neurodegeneration but could, as such, also be involved in preventing the progression of DR^{30,61,62}.

Interestingly, diabetic retinal neurodegeneration is associated with diabetic peripheral- and autonomic neuropathy⁶³⁻⁶⁷ and has been shown to predict the development of diabetic peripheral neuropathy (DPN)⁶⁸.

2.1.2 DIABETIC NEUROPATHY

Diabetic neuropathy is the most common chronic complication of diabetes¹³ and affects up to 50% of individuals with diabetes⁶⁹. It is a heterogeneous disorder that affects the peripheral, central, and autonomic nervous system in a length-dependent manner and is associated with a reduction in quality of life¹³ and high healthcare costs⁷⁰. Diabetic neuropathies can be painful as well as non-painful.

The pathogenesis of diabetic neuropathy is complex, multifactorial, and only partially understood. It involves several vascular, metabolic, inflammatory, and immune-mediated mechanisms, which result in oxidative stress, inflammatory stress, and ischemia⁷¹. Prolonged exposure to hyperglycemia is a key factor in developing diabetic neuropathy⁷¹. Other factors associated with the development of diabetic neuropathy is associated with disease duration, obesity, dyslipidemia, mitochondria dysfunction, and hypertension⁷¹.

There are several manifestations of diabetic neuropathy⁷², however, and for the sake of the scope of this thesis, the primary focus will be on distal symmetric polyneuropathy (DSPN), diabetic autonomic neuropathy (DAN), and cardiovascular autonomic neuropathy (CAN).

2.1.2.1 DISTAL SYMMETRICAL POLYNEUROPATHY

DSPN is a symmetrical, length-dependent, sensorimotor polyneuropathy affecting the peripheral sensorimotor neurons⁷². It is defined by the presence of symptoms and/or signs of peripheral nerve dysfunction in individuals with diabetes after other causes have been excluded¹³. The diagnosis of DSPN is in principal clinical and can be made after clinical examination, including pinprick, vibration, and temperature perception¹³. According to the Toronto criteria, confirmed DSPN requires the presence of nerve conduction abnormalities and symptoms and signs of neuropathy³.

In type 1 diabetes, the 20-year prevalence of DSPN is around 20%¹³. It is the most common variety of neuropathy and accounts for up to 75% of diabetic neuropathies^{72,73}. About half of the individuals with DSPN experience symptoms ranging from numbness, tingling, and pain, most commonly in the toes, feet, and legs¹³. The other half, however, may be asymptomatic (loss of protective sensation), and patients may present with painless foot ulcers. In type 1 diabetes, tight glycemic control has been shown to reduce the incidence of DSPN by up to 78%^{39,74}.

According to the type of sensory fiber (small or large fibers) involved, symptoms may vary. Early symptoms caused by the involvement of small sensory fibers may include

neuropathic pain, dysesthesia, paresthesia, hyperalgesia, and allodynia¹³. The involvement of large sensory fibers may cause numbness, tingling, and loss of proprioception⁷⁵.

2.1.2.2 DIABETIC AUTONOMIC NEUROPATHY

Damage to the autonomic nerves as a complication of diabetes is known as diabetic autonomic neuropathy (DAN). Such autonomic dysfunction impairs the regulatory ability of the ANS, which consequently impairs the continuous regulation of one or more organ systems, including cardiovascular, gastrointestinal, urogenital, ocular, and sudomotor function⁷⁶.

As such, the symptoms are pleomorphic and may include hypo/hyperglycemia unawareness, resting tachycardia, exercise intolerance, orthostatic hypotension, gastroparesis, constipation, diarrhea, neurogenic bladder, erectile dysfunction in men/lacking lubrication in women, dysfunction of sweating and thermoregulation well as impaired pupillary function¹⁵. Such complications have a significant impact on the quality of life¹⁵.

2.1.2.3 CARDIOVASCULAR AUTONOMIC NEUROPATHY

The prevalence of CAN in type 1 diabetes varies from 17-66%⁷⁷, and in individuals with DPN, more than 50% will have CAN⁷⁸. The key factor for the development of CAN is hyperglycemia⁷⁴; however, there are several risk factors for developing CAN, including increased age, disease duration, poor glycemic control, obesity, hypertension, dyslipidemia, smoking, as well as the presence of microvascular complications such as diabetic retinopathy, neuropathy, and nephropathy/microalbuminuria^{11,15,79}.

One manifestation of DAN is cardiovascular autonomic neuropathy (CAN) which is a serious complication of diabetes mellitus. CAN results from impairment of the autonomic regulation of the cardiovascular system, affecting heart rate control and vascular dynamics, thus challenging the maintenance of cardiovascular homeostasis^{3,11}. CAN has significant clinical implications, including increased morbidity and mortality¹⁰⁻¹⁴, and is associated with a range of cardiovascular symptoms and complications. One of the most common symptoms of CAN is orthostatic hypotension, which presents as dizziness and potentially syncope in response to postural changes⁹. In healthy, a compensatory vasomotor reflex mediated by baroreceptors primarily located in the aortic arch and carotid sinus increases sympathetic activity and decreases parasympathetic activity in response to a sudden drop in blood pressure, and thus maintains continuous cerebral blood flow. In diabetes however denervation of efferent sympathetic nerves results in reduced vasoconstriction in peripheral vascular beds, and thus this response is compromised¹². Other symptoms include resting tachycardia and exercise intolerance, as well as asymptomatic myocardial ischemia and infarction, which has caused the nickname

“the silent killer”⁹. Damage to autonomic nerve fiber results in an impaired ability of the heart rate to adapt to different conditions and maintain homeostasis.

Diabetes affects autonomic nerves in a length-dependent manner^{77,80}. The vagal nerve, which accounts for approximately 75% of parasympathetic activity, is the longest parasympathetic nerve, and thus, CAN often affects this nerve first¹². Manifestations of parasympathetic denervation are resting tachycardia, as well as decreased parasympathetic activity. As a result, sympathetic predominance is present in early CAN with parasympathetic neuropathy⁸¹, and later sympathetic nerves are also affected¹². Thus, CAN is associated with impairment of autonomic modulation and reduced baroreflex sensitivity⁸², which can even be observed in subclinical stages^{83,84}.

2.1.2.4 DIABETIC CENTRAL NEUROPATHY

As for the peripheral and autonomic nervous systems, the central nervous system is also affected by diabetes. Diabetes has been associated with accelerated cortical atrophy, reduced gray matter and cerebellar volume, as well as atrophy of somatosensory and motor cortices^{85–87}. Moreover, cervical spine atrophy has been shown in individuals with diabetes and is more severe in individuals with DSPN than those without^{88,89}. Whether or not changes in the central nervous system is a primary phenomenon or occur due to adaptive shrinking as a consequence of peripheral neuropathy remains controversial⁹⁰. However, not only structural volumetrics are affected by diabetes; reductions in central transmission and EEG abnormalities have been shown in type 1 diabetes^{91,92}. Decreased connectivity between the thalamus and superior frontal, middle frontal, and precentral cortex has also been shown in individuals with T1DM and DSPN⁹³. Furthermore, type 1 diabetes has been associated with cognitive impairment^{94,95}.

2.1.3 DIABETIC NEPHROPATHY

Diabetic nephropathy is another common microvascular complication of diabetes and is the most common cause of chronic kidney disease, affecting up to 30% of individuals with type 1 diabetes⁹⁶. Diabetic nephropathy is characterized by persistent (micro)albuminuria and progressive decline in renal function. Diabetic nephropathy progresses through several stages, including hyperfiltration, microalbuminuria, macroalbuminuria, and eventually leads to end-stage kidney disease⁹⁷.

2.2 THE AUTONOMIC NERVOUS SYSTEM

The ANS regulates involuntary physiologic processes. Thus, the ANS is deeply involved in every aspect of our daily life. Its ability to maintain homeostasis through changes in the sympathetic and parasympathetic branches is necessary for the appropriate functioning of most organ systems, including the cardiovascular system⁹⁸.

The ANS comprises three anatomically distinct divisions, the sympathetic, parasympathetic, and enteric nervous systems.

Activation of the sympathetic nervous system involves a “fight-and-flight” response resulting in, e.g., increased heart rate, dilated pupils, and inhibition of peristaltic movement, whereas activation of the parasympathetic nervous system involves a “rest and digest” response, which slows down the heart rate, contracts the pupils and stimulates peristaltic movements.

The ANS has traditionally been thought to be reciprocally balanced by two opposing branches, the sympathetic and parasympathetic divisions. However, as proposed by *Berntson et al.* as the doctrine of autonomic space, the relationship between parasympathetic and sympathetic outflow can also be described in a dimensional space, where both branches can be co-activated and co-inhibited simultaneously⁹⁹, in both a linear and non-linear fashion¹⁰⁰. Even though this theory is more complex, it emphasizes that the ANS is not a zero-sum system.

The primary focus of the thesis will be on neurocardiac autonomic regulation.

2.2.1 CARDIAC AUTONOMIC NERVOUS SYSTEM

In both sympathetic preganglionic and parasympathetic preganglionic and postganglionic neurons, acetylcholine is the primary neurotransmitter; thus, these neurons are referred to as cholinergic. In sympathetic postganglionic neurons, norepinephrine is the primary neurotransmitter, and these neurons are referred to as adrenergic. Norepinephrine affects β_1 , β_2 , α_1 , and α_2 receptors located in the cardiovascular system, and whereas α -receptors stimulate vasoconstriction, β -receptors stimulate vasodilatation allowing for increases in blood perfusion, as a result of sympathetic stimulation, heart rate, contractility, and conductivity increases¹⁰¹. In addition to autonomic modulation, heart rate is influenced by other mechanisms, including neurohumoral influence, stretching of the sinoatrial node, local temperature changes, and ionic changes within the sinoatrial node¹⁰².

A dynamic autonomic relationship exists in healthy hearts. As a result of sympathetic and parasympathetic modulation, the beat-to-beat interval changes, and the autonomic modulation is primarily responsible for the heart rate variability (HRV)¹⁰². Parasympathetic nerves exert their effects more rapidly (<1 second) than sympathetic nerves (> 5 seconds), and the parasympathetic nerves have the ability to slow the heart rate down to 20-30 bpm¹⁰⁰. During rest and sleep, the parasympathetic tone is the dominant influence on the sinoatrial node¹⁰³.

Short-term HRV measurements are also influenced by sinoatrial node stretching in resting conditions. In healthy individuals at rest, sinoatrial node stretching has a small influence on HRV of about 2-4%¹⁰⁴ however, in denervated hearts, such stretching may account for up to 100%¹⁰⁵.

The heart's activity is regulated through a combination of afferent and efferent neurons. Afferent neurons convey sensory information from receptors located within the cardiovascular system, including baroreceptors and chemoreceptors. Afferent information is primarily transmitted through the vagal nerve, although some information is transmitted via sympathetic spinal afferents¹⁰⁶. Within the brainstem, the nucleus tractus solitarius (NTS) receives most afferent information from the heart and serves as a relay point for cardiovascular reflexes. In contrast, input from spinal afferents is received by centers in the thalamus, brainstem, and hypothalamus¹⁰⁷. Through interaction between the NTS and other brainstem nuclei, reflexive autonomic motor responses are generated¹⁰⁸. Furthermore, cortical control centers, such as the amygdala, and prefrontal, cingulate, and insular cortices, which are related to pain perception and emotion, are also involved in the modulation of cardiovascular responses¹⁰⁶.

The efferent part of the sympathetic innervation arises from the rostral ventrolateral medulla (RVLM) and caudal ventrolateral medulla (CVLM), located in the medulla oblongata, which exerts sympatoexcitatory and sympatoinhibitory effects respectively¹⁰⁹. These connect to preganglionic neurons in the intermediolateral nucleus (ILM), which leaves the spinal cord through the white rami at the T1-T4 level. After leaving the spinal cord, preganglionic neurons enter the sympathetic ganglia. From here, postganglionic neurons innervate the heart through deep and superficial cardiac plexuses, providing autonomic control of the sinoatrial node, atria, and ventricles¹⁰¹.

The efferent part of the parasympathetic innervation arises primarily from the nucleus ambiguus (NA), which interacts with the vagal dorsal motor nucleus (VDMN) located in the medulla oblongata. Preganglionic neurons, primarily located in the vagal nerve (but also in part by the recurrent pharyngeal nerve¹¹⁰), synapse with postganglionic neurons in intracardiac ganglia before converging into the cardiac plexuses. For a more comprehensive overview, see Fig. 2.

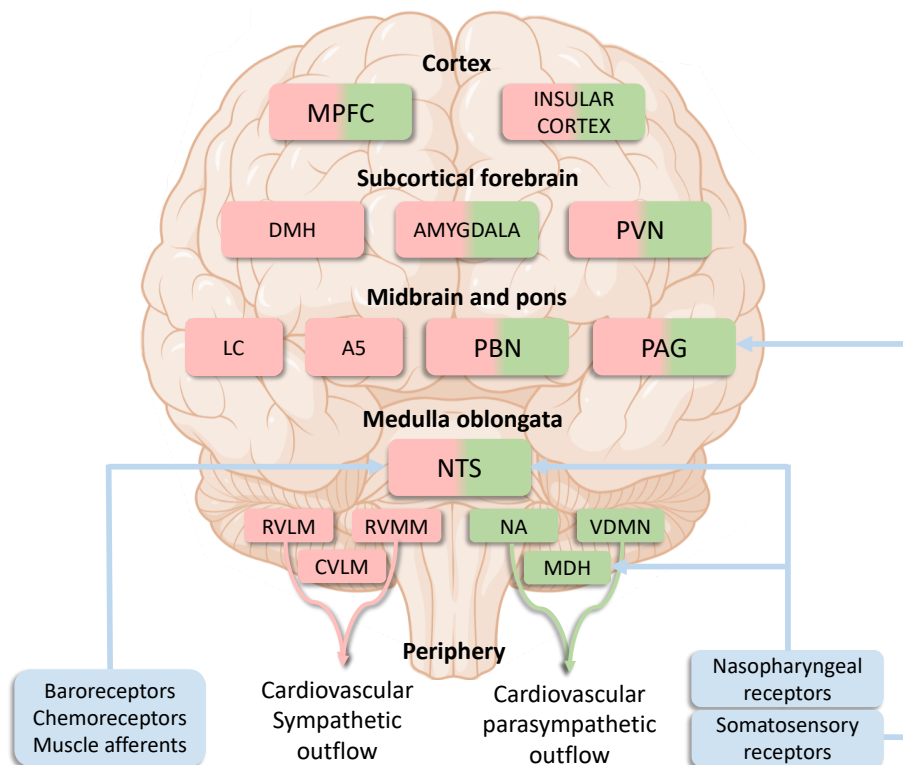


Figure 2. Schematic overview of interconnected structures involved in central autonomic cardiovascular regulation. Pink refers to areas involved in the sympathetic response, and green to areas involved in the parasympathetic response. Blue refers to peripheral afferent pathways. MPFC; medial prefrontal cortex, DMH; dorsomedial hypothalamus, PVN; hypothalamic paraventricular nucleus, LC; locus coeruleus, A5; A5 noradrenergic cell group in central pons, PBN; parabrachial nuclei, PAG; periaqueductal gray, NTS; nucleus tractus solitarius, RVLM; rostral ventrolateral medulla, RVMM; rostral ventromedial medulla, CVLM; caudal ventrolateral medulla, NA; nucleus ambiguus, VDMN; vagal dorsal motor nucleus, and MDH; medullary horn of the trigeminal nucleus. Modified from *Ruffazini et al.*¹⁰⁷. Graphics supplied, in part, by Servier Medical Art, licensed under a Creative Commons Attribution 3.0 unported license.

2.2.2 PHARMACOLOGIC AND PHYSIOLOGIC MODULATION OF THE AUTONOMIC NERVOUS SYSTEM

Phenylephrine

Phenylephrine is a sympathomimetic drug commonly used topically in ophthalmology to induce dilatation of the pupil, allowing for examination of the

fundus and posterior chamber. The mechanism is through α -adrenergic stimulation of the iris dilator muscle, contraction of radial fibers occurs, resulting in dilatation of the pupil.

In addition to stimulating the iris dilator muscle, phenylephrine also stimulates the sympathetically innervated superior and inferior tarsal muscle¹¹¹, by activating α - and β -receptors¹¹², resulting in increased palpebral fissure height (PFH)^{113–115}. Thus, phenylephrine can be used to assess the suitability of Müllers muscle-conjunctival resection surgery in patients with mild to moderate upper eyelid ptosis¹¹⁶. This test is known as the phenylephrine test¹¹⁷.

In study I, we hypothesized that long-term type 1 diabetes mellitus causes neuropathy to the peripheral sympathetic nerve fibers that innervate the superior and inferior tarsal muscles. Thus, phenylephrine hydrochloride 10% was used to stimulate the superior and inferior tarsal muscles in the right eye. Measurements of PFH were performed in both the phenylephrine-stimulated and the unstimulated eye, and the difference between the two eyes (Δ PFH) was calculated and used for further analyses.

Cold Pressor Test

The cold pressor test is a validated and simple test that can be used to evaluate the cardiovascular autonomic function¹¹⁸ or serve as an experimental pain stimulus used to evaluate pain treatments¹¹⁹. The cold pressor test typically involves the immersion of the hand into circulating ice water, between 1-5 °C, for a period of 1-6 minutes¹²⁰. In healthy subjects, the cold pressor test elicits cardiac sympathetic activation, resulting in increased heart rate and blood pressure^{118,121–123}, possibly co-mediated by increases in neurohormones¹²⁴ such as epinephrine and norepinephrine^{125–127}. Whether or not the cold pressor test also results in parasympathetic withdrawal remains controversial^{118,122}. The effect of the cold pressor test, or cold tonic pain, on heart rate, is complex, and high individual variety has been reported; both cold pressor-induced increases and decreases in heart rate have been shown^{120,128}. The heart rate responses have not been shown to differ between individuals with diabetes and healthy controls^{127,129,130}, although one study failed to show an increase in heart rate in individuals with T1DM¹³¹. The autonomic response may vary in case of efferent or afferent neuronal pathway damage. In individuals with DAN, one study found that the diastolic blood pressure response was lower, and heart rates were higher than in healthy controls¹³⁰. In regards to the effect of the cold pressor test on HRV measures, both increases¹³², decreases¹¹⁸, and no changes¹²⁵ have been shown in low-frequency (LF) power, and high-frequency (HF) power has been shown to remain unchanged¹²⁵ or to decrease¹¹⁸.

Exposure to tonic cold pain causes activation of peripheral thermo- and nociceptors, which enter the spinal cord through the dorsal root ganglion. Following contralateral crossing, they form the spinothalamic tract before traveling to the thalamus. At the level of the thalamus, contralateral is sent to the reticular formation, from which these may stimulate the RVLM and cause sympathetic stimulation of the cardiovascular system^{124,133}. Through other projections, cortical and subcortical structures in the hypothalamus may be affected, causing neuroendocrine responses¹³⁴.

In studies III and IV, cold tonic pain exposure was used as a provocative challenge test, equivalent to that of the treadmill test in the assessment of cardiac performance. Participants were placed in a supine position and instructed to breathe spontaneously. The left hand was then immersed in ice-chilled water ($\sim 2.0^{\circ}\text{C}$) for 2 minutes. In case of intolerable pain, the hand was withdrawn from the water.

2.3 GLUCAGON-LIKE PEPTIDE 1 RECEPTOR AGONISTS

Glucagon-like peptide-1 (GLP-1) is an incretin hormone secreted centrally or by enteroendocrine L-cells in the intestinal mucosa¹³⁵. It is released in response to nutritional components, such as carbohydrates, protein, amino acids, bile acids, and fatty acids in the small intestine^{136,137}. It exerts its effects by stimulating insulin secretion from pancreatic β -cells and inhibiting glucagon secretion in a glucose-dependent manner, thereby improving β -cell glucose sensitivity and consequently decreasing blood glucose levels and hemoglobin A1c levels^{138–140}. Following a meal intake, a rapid increase in GLP-1 secretion occurs from the beforementioned intestinal L-cells¹⁴¹, resulting in stimulation and regulation of pancreatic islet function, accounting for most of the postprandial insulin secretion^{27,142}. In addition to insulinotropic effects, GLP-1 has been shown to protect against β -cell apoptosis, stimulate β -cell proliferation and preserve β -cell mass¹⁴¹ as well as delay gastric emptying, influence regulation of appetite, and induce weight loss¹⁴³.

GLP-1 receptors (GLP-1 R) are widely distributed throughout the body. GLP-1 R's are highly expressed in the pancreas but have also been reported in the lung, stomach, intestine, kidney, heart, brain, retina, and immune system^{144–147}. GLP-1 receptors have been found within the heart in the myocytes of the sinoatrial node, endothelium, coronary arteries, and smooth muscle cells of the heart^{145,146,148}. Furthermore, GLP-1 R's have been found on the vagal nodose ganglia neurons¹⁴⁹.

Several GLP-1 receptor agonists (GLP-1 RA) have been developed in the last decades, including short- and long-acting formulations. Liraglutide is a long-acting GLP-1 RA, sharing 97% amino acid sequence identity with native GLP-1¹⁵⁰. In contrast to native GLP-1, liraglutide is resistant to metabolism by dipeptidyl peptidase 4, and thus the half-life of liraglutide is about 13 hours¹⁵¹.

GLP-1 RA's have shown both cardioprotective^{152,153,154}, renoprotective^{154,155}, neuroprotective^{156–159} and anti-inflammatory effects¹⁶⁰. In addition, GLP-1 RA's been associated with neurite outgrowth in sensory neurons, and in type 1 diabetic mice, the treatment improved epidermal sensory conduction, thermal sensation, and restored mechanical sensitivity¹⁶¹. In humans, however, GLP-1 RA's did not change intra-epidermal nerve fiber density¹⁶², latencies of evoked potentials (nerve conduction)¹⁶⁰, or CAN measures^{162,163}.

2.3.1 OCULAR EFFECTS OF GLP-1 RA'S

In healthy eyes, GLP-1 R's have been shown in cells within the human retinal ganglion layer but not in eyes with PDR¹⁴⁴. Other studies found GLP-Rs in microvascular endothelial cells within the human retina¹⁶⁴, as well as in the retinal pigment epithelium¹⁶⁵. Although sparse, GLP-1 R's have also been found in the inner and outer nuclear layers¹⁶⁶, and GLP-1 has even been found to be locally produced within the retina¹⁶⁶.

Several outcome studies have found that treatment with GLP-1 was associated with a progression in DR, although these changes could potentially be attributed to rapid decreases in blood glucose levels^{143,167,168}. In animal studies, topically applied liraglutide has been shown to prevent diabetic retinal neurodegeneration and vascular leakage through inhibition of endoplasmic reticulum- and oxidative stress as well as through anti-apoptotic, anti-inflammatory, and anti-VEGF effects^{31,166,169,170}. Similar effects have been found for semaglutide¹⁷¹. Furthermore, GLP-1 RA's have been shown to protect the blood-retinal barrier against diabetes-induced damage^{172,173}. In addition, even liraglutide-induced retinal neuro regenerative effects were found³¹.

Hernandez et al. investigated both topical and systemic treatment with liraglutide, and systemic treatment was shown to prevent retinal neurodegeneration in diabetic mice¹⁶⁶. Furthermore, both topical and systemic treatment prevented abnormalities in electroretinography recordings¹⁶⁶. In addition, GLP-1 RA's may also have potentially beneficial neuroprotective effects in individuals with glaucoma¹⁷⁴.

To the best of our knowledge, it remains unknown if such effects also apply to humans with long-term type 1 diabetes.

In study II, we investigated the effects of 26 of weeks treatment with liraglutide on the retinal nerve fiber layer thickness in individuals with long-term type 1 diabetes and DSPN.

2.3.2 GLP-1 RA'S AND CARDIOVASCULAR AUTONOMIC FUNCTION

GLP-1 RA's has been shown to reduce major cardiovascular events^{152,175}, arteriosclerosis¹⁷⁶, endothelial dysfunction, inflammation, oxidative stress¹⁷⁷, improve lipid profiles^{178,179}, as well as decrease body weight and blood-pressure^{153,176,180}. A recent meta-analysis of eight cardiovascular outcome trials showed that treatment with GLP-1 RA's was associated with a 13% reduction in risk of cardiovascular death,

a 16% reduction in nonfatal stroke, and a 10% reduction in heart failure hospitalization as well as a 12% reduction in all-cause mortality¹⁵⁴. Such improvement can be obtained by reducing the above-mentioned risk-factors, however, it could also have a direct effect on the heart.

GLP-1 RA's have consistently been shown to increase heart rate in type 1-¹⁸¹ and type-2 diabetes^{152,182–189}, and in a recent meta-analysis, treatment with liraglutide increased heart rate by 2.7 bpm¹⁹⁰, although increases up 6.4 bpm has been reported¹⁹¹. The underlying mechanism(s) are undetermined, but several mechanisms have been proposed. These include GLP-1 RA-induced activation of the sinoatrial node¹⁸⁴, GLP-1 RA-induced increases in sympathetic tone¹⁹², GLP-1 RA-induced decreases in parasympathetic tone¹⁹³ or through activation of baroreceptors in response to a reduction in systemic vascular resistance¹⁹⁴. Furthermore, central mechanisms may be involved, as GLP-1 RA's has been shown to activate sympathetic preganglionic neurons in the brainstem^{195–197}.

Although a recent meta-analysis concluded that GLP-1 RA's does not influence HRV measures¹⁹⁸, several studies have found GLP-1 RA-induced alteration in HRV measures (see Table 1).

In study IV, we investigated the effect of 26 weeks of treatment with liraglutide on heart rate and HRV in individuals with long-term type 1 diabetes and DSPN.

2.3.3 CENTRAL EFFECTS OF GLP-1 RA'S

In humans and rats, GLP-1 R's have been found in the cerebral cortex, thalamus, hypothalamus, medulla oblongata, hippocampus, globus pallidum, and caudate-putamen^{197,199,200}. GLP-1 has been shown to be synthesized by neurons in the nucleus tractus solitarius, and GLP-1 R's have been found in both nucleus tractus solitarius and the paraventricular nucleus, both involved in the regulation of autonomic control^{197,201,202}. In mice, GLP-1 RA has been found to inhibit neurotransmission to cardiac vagal neurons in the nucleus ambiguus¹⁹³.

Neuroprotective effects of GLP-1 RA's have been shown in diseases such as diabetes, Alzheimer's, and Parkinson's disease^{159,203}, primarily through inhibition of oxidative stress, inflammation, and apoptosis^{156,204–208}. In addition, GLP-1 RA's (e.g., liraglutide) reduces systemic inflammation, increases synaptic plasticity, and stimulates neurogenesis^{158,204,209,210}. Furthermore, GLP-1 is also involved in memory, learning as well as reward behavior²⁰¹

Moreover, GLP-1 RA's has been shown to reduce the risk of stroke¹⁷⁵, and in animal stroke models, to reduce the volume of infarction, oxidative stress, neuroinflammation, excitotoxicity, apoptosis, blood-brain barrier leakage as well as increase neurogenesis, neuroplasticity, angiogenesis, and brain perfusion²¹¹.

Study	Type	Subjects	Treatment	Dosage	Duration	HR	SBP	DBP	SDNN	SDANN	SDNNI	RMSSD	TP	VLF	LF	HF	LF/HF
Jaiswal et al. 2015 ¹⁵⁴	OL. RCT	T2DM	Exenatide (n=22)	10 µg once daily	18 M	→			→			→					→
Sivalingam et al. 2022 ⁷⁰	DB. RCT	T1DM	Liraglutide (n=43)	1.8 mg once daily	26 W	↑			→			→		→	→		
Brock et al 2019. ¹⁵²	DB. RCT	T1DM	Liraglutide (n=19)	1.8 mg once daily	26 W	→	→	→	→	→	→	→	→	→	→	→	→
Hansen et al. 2019 ¹⁵⁵	DB. RCT	T1DM	Liraglutide (n=50)	1.8 mg once daily	24 W	↑			→					→	→	→	→
Cacciatori et al. 2018 ¹⁸⁰	OBS.	T2DM	Exenatide ER (n=28)	2 mg once weekly	6 M	↑	↓	↓					→		↓	→	↓
Hara et al. 2015 ¹⁶⁶	OBS.	T2DM	Liraglutide (n=7)	0.9 mg once daily	24 W	↑	↓	→	↓					↓	↓	↓	→
Nystrom et al. 2019 ¹⁸¹	OL. RCT	T2DM	Liraglutide (n=33)	1.8 mg one daily	18 W	↑			→	→	→	→	→	→	→	→	→
van Ruiten et al. 2022 ¹⁸²	DB. RCT	T2DM	Exenatide (n=17)	10 µg twice daily	16 W	→	→	→	↓			→					→
Kumarathurai et al. 2017 ¹⁸³	DB. RCT	T2DM	Liraglutide (n=30)	1.8 mg one daily	12 W	↑	→	→	↓	↓	↓	↓	↓	↓	↓	↓	→
Smits et al. 2017 ¹⁸⁴	DB. RCT	T2DM	Liraglutide (n=19)	1.8 mg once daily	12 W	↑	↓	→	→			→					→
Nakatani et al. 2017 ¹⁶⁵	OL. RCT	T2DM	Liraglutide (n=30?)	0.9 mg once daily	4 W?	↑											↑
Nakatani et al. 2016 ¹⁶⁵	OL. RCT	T2DM	Lixisenatide (n=30?)	20 µg once daily	4 W?	↑											→

Table 1: Overview of studies investigating the effect of GLP-1 RA on HRV parameters in individuals with T1DM and T2DM. DB; double-blinded, RCT; randomized (placebo) controlled trial, OL; open-label, OBS; observational study, ER; extended release, HR; heart rate, SBP; systolic blood pressure, DBP; diastolic blood pressure, SDNN; standard deviation of NN intervals, SDANN; standard deviation of all NN intervals for each 5-minute segment of a 24 hour-recording, SDNNI; mean of the standard deviation of all NN intervals for each 5-minute segment of a 24 hour-recording, RMSSD; root mean square of successive differences in NN intervals, TP; total power, VLF; very low-frequency, LF; low-frequency, HF; high-frequency, LF/HF; ratio.

3 CLINICAL ASSESSMENTS

3.1 ASSESSING DIABETIC RETINOPATHY

Diabetic retinopathy can be assessed visually, either by funduscopy or by assessing fundus photographs.

Characteristics of diabetic retinopathy include intraretinal hemorrhages (dot-blot or flame-shaped), hard exudates, cotton wool spots, venous beading, and the presence of intraretinal microvascular abnormalities or neovascularization⁴⁵. Furthermore, diabetic macular edema may become present as the disease progresses.

Based on the number and/or presence of these characteristics, DR can be staged as NPDR (mild, moderate, or severe, based on the number of characteristics) or PDR (presence of neovascularization)⁴⁵.

For studies I and II, evaluations of DR stage were performed by the same ophthalmologist, and photos were taken by the same photographer to avoid inter-observational bias. PDR was defined by the presence of neovascularization(s), previous or actual, assessed by visually inspecting fundus photos and/or OCT scans. Based on the absence or presence (actual or previous) of neovascularization, participants were divided into an NPDR or PDR group, respectively.

3.2 ASSESSING PALPEBRAL FISSURE HEIGHT

Palpebral fissure height (PFH) is defined as the height between the eyelid margins (upper and lower) in the pupillary plane in a primary gaze position (see Fig. 3) and is, in part, determined by the functionality of the sympathetically innervated tarsal muscles.

In healthy individuals, one study found the average PFH to be 9.9 mm²¹², and phenylephrine has been shown cause increases in PFH between 1.2-1.6 mm¹¹³⁻¹¹⁵. In insulin-dependent diabetes, the average PFH was 8.3 mm, and the decrease was associated with the disease duration and severity of DR²¹². As such, a reduction in PFH in diabetes may, amongst other causes of ptosis, be a very visual manifestation of diabetic autonomic neuropathy. Thus, the PFH response to phenylephrine could potentially act as a surrogate measure of sympathetic nerve function.

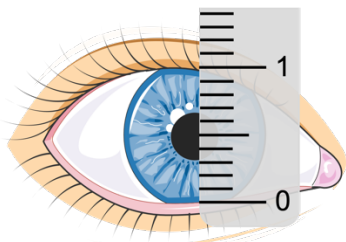


Figure 3. Assessment of palpebral fissure height (PFH).

In study I, measurements of PFH were performed on both the unstimulated left eye and phenylephrine-stimulated right eye. Measurements for both eyes were obtained from a single standardized frontal photo 15-minutes after topical application of phenylephrine hydrochloride 10% to the right eye. The difference between the two eyes (Δ PFH) was used for further analysis.

3.3 ASSESSING THE RETINAL NERVE FIBER LAYER THICKNESS

Optical coherence tomography (OCT) is a non-invasive imaging technique that allows topologic and visualization of the microstructure of individual retinal layers as well as the microvasculature (OCT-Angio). OCT is used to diagnose and manage diseases such as diabetes and glaucoma. OCT allows for the assessment of retinal nerve fiber loss, and thinning of the retinal nerve fiber layer has been associated with the severity of DPN⁶⁵.

In study II, assessments of peripapillary retinal nerve fiber layers thickness were obtained using Topcon 3D OCT-200 (see Fig. 4). Scans were fixated in the center of the optic disc, and 1024 scans were taken in a circle of 3.4 mm in diameter. RNFL thickness of the inferior, superior, nasal, and temporal quadrants was obtained at baseline and after 26 weeks of treatment with liraglutide.

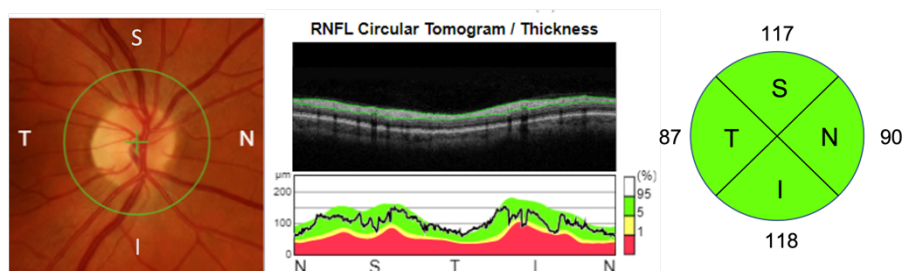


Figure 4: Assessment of peripapillary retinal nerve fiber layer (RNFL) thickness using Topcon 3D OCT. T = temporal quadrant, S = superior quadrant, I = inferior quadrant, and N = nasal quadrant.

3.4 ASSESSING DISTAL SYMMETRIC POLYNEUROPATHY

According to the Toronto Diabetic Neuropathy group³, DPN can be classified as 1) possible DPN (presence of symptoms, such as decreased sensation or signs, such as decreased ankle reflexes), 2) probable DPN (two or more symptoms or signs), and 3) confirmed DPN (abnormal nerve conduction and a symptom or sign of DPN),

Furthermore, the quality and severity of possible neuropathic pain can be assessed by the validated Michigan neuropathy screening instrument^{213,214}. Several other self-reported questionnaires, e.g., the McGill Pain Questionnaire exist⁷⁵.

Several experimental methods of assessing DSPN exist, including quantitative sensory testing, vibration threshold, electrophysiological peripheral nerve testing, and intra-epidermal nerve density in skin biopsies⁷⁵. Ocular measures, such as small fiber nerve lengths assessed with corneal confocal microscopy, have also been used, and reduced retinal nerve fiber layer thickness can be used as surrogate markers of DSPN⁷⁵. Whereas quantitative sensory testing, skin biopsies, and corneal confocal microscopy can be used to assess the presence of small-fiber pathology^{215,216}, investigations of peripheral nerve testing include nerve conduction velocity and amplitudes, and, thus, assess the presence of neuropathy in large myelinated nerve fibers²¹⁷.

In studies III and IV, peripheral nerve testing with standardized electrophysiological measures were obtained by the Department of Neurophysiology, Aalborg University Hospital.

3.5 ASSESSING CARDIOVASCULAR AUTONOMIC NEUROPATHY

The function of the ANS can be assessed by several methods, including investigation of e.g., cardiovascular, sudomotor, and pupillomotor¹⁶.

Cardiovascular autonomic reflex tests (CARTs) represent the gold standard for CAN testing. The CAN Subcommittee of the Toronto Consensus recommends measuring changes in heart rate and blood pressure to different provocative physiological maneuvers. These maneuvers include the deep breathing test, lying-to-standing test, the Valsalva maneuver, and the blood pressure response to standing²¹⁸. The Valsalva maneuver is, however, not advisable in patients with proliferative retinopathy due to an increased risk of retinal hemorrhage.

According to the extent of neuropathy, CAN can (☺) be classified into subclinical-, early- and advanced stages. The subclinical stage is characterized by abnormal sympathovagal balance and decreased baroreflex sensitivity but with no symptoms hereof. In the early stage, by the presence of tachycardia at rest and one abnormal cardiovascular autonomic reflex test (CARTs), and in advanced stages by exercise intolerance, orthostatic hypotension, two or more abnormal CARTs, cardiomyopathy, and asymptomatic myocardial ischemia^{9,218}.

Several methods for the investigation of cardiac autonomic dysfunction have been proposed in clinical research. The most sensitive and specific tests are heart rate variability (HRV), cardiac sensitivity to the baroreflex (CBS), muscle sympathetic nerve activity, measurements of plasma catecholamines, and heart sympathetic imaging¹⁰². HRV is widely used in clinical research and is a non-invasive method for providing information on autonomic modulation of the cardiovascular system, evaluated using time- and frequency domain indices of heart rate variability¹⁹. Recently HRV as shown to be a reliable measure of CAN in type 1 diabetes²¹⁹.

3.5.1 HEART RATE VARIABILITY

Whereas heart rate is a measure of heart beats per minute, HRV is defined by the fluctuations in time between consecutive heartbeats¹⁹ and reflects the complex coupling between the brain and the heart. A healthy heart rate is non-chronometrical (high HRV), allowing for adaption to internal and external stimuli to maintain homeostasis. In contrast, a chronometrical, non-adaptive heart rate (low HRV) that barely responds to stress, exercise, sleep, etc., indicates cardiac denervation^{7,10} and has been shown to predict cardiovascular events²²⁰. When compared to healthy, individuals with T1DM are known to have reduced HRV parameters^{84,221,222}.

HRV is widely used as a non-invasive assessment to characterize autonomic modulation of the heart²²³. In addition to the autonomic modulation, short-term HRV recordings (~5 min.) are influenced by respiratory-driven regulation of the heart rate, CBS, and rhythmic variations in vascular tone¹⁰⁰. However, long-term HRV recordings (24-hour) are influenced by, e.g., circadian rhythms, body temperature, the renin-angiotensin system, and by metabolism²²⁴.

HRV can be analyzed using both *time-domain-derived* HRV parameters and *frequency-derived* HRV parameters¹⁹ (see Table 2).

HRV parameter	Units	Definition
<i>Time-domain</i>		
Mean RR	ms	Mean of all RR-intervals
SDNN	ms	Standard deviation of all NN-intervals (normal RR-intervals)
SDANN	ms	Standard deviation of the average all NN-intervals for each 5 minute segment of a 24 hr HRV recording
SDNNI	ms	Mean of the standard deviation of the average all NN-intervals for each 5 minute segment of a 24 hr HRV recording
RMSSD	ms	Root mean square of successive RR interval differences
<i>Frequency-domain</i>		
VLF	ms ²	Absolute power of very-low-frequency band (0.0033-0.04 Hz)
LF	ms ²	Absolute power of low-frequency band (0.04-0.15 Hz)
HF	ms ²	Absolute power of high-frequency band (0.15-0.4 Hz)
LF/HF	%	LF/HF ratio

Table 2: Overview of heart rate variability parameters used in this thesis.

Time-domain derived HRV parameters represent the total beat-to-beat variability over time. SDNN is considered an estimate of the overall adaptability of the heart, reflecting both sympathetic and parasympathetic tone^{100,224}, and individuals with lower SDNN values have a higher risk of mortality than those with higher values²²⁵. RMSSD is generally believed to reflect parasympathetic tone¹⁹.

Frequency-domain derived parameters, however, reflect the distribution of power (signal energy) across different frequency bands (see Fig. 5) and can be assessed using power spectral analysis¹⁹; VLF is believed to be intrinsically generated by the heart, and the amplitude and frequency are modulated by efferent sympathetic activity²²⁴. LF content is believed to reflect both sympathetic and parasympathetic activity^{19,100}, although it has also been proposed to reflect baroreflex function. Some authors use a LF cut-off value of 0.1 Hz, as the sympathetic influence may not produce rhythms above 0.1 Hz however, parasympathetic influence may influence rhythms down to 0.05 Hz¹⁰⁰. HF content is considered to reflect the parasympathetic activity and corresponds to respiratory-driven influence on heart rate¹⁰⁰. LF/HF is generally believed to represent the sympathovagal balance¹⁹, and a low LF/HF ratio reflects parasympathetic dominance¹⁰⁰. The underlying influence(s) of both VLF, LF, and LF/HF ratio are, however, controversial and subject to great scientific debate^{226–229}.

In addition, time-domain parameters decrease with age, and HRV differences related to sex and respiration pattern have been shown^{230,231}.

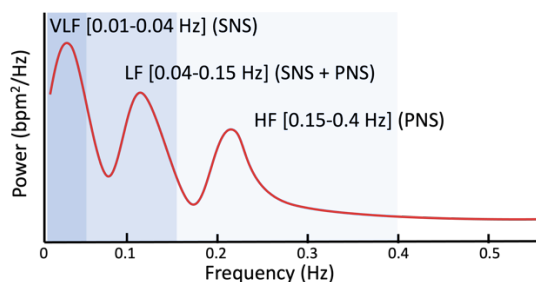


Figure 5: Visualizations of frequency-derived HRV parameters used in this thesis. SNS = sympathetic nervous system influence, PNS = parasympathetic nervous system influence.

Although 24-hour HRV recordings are considered the “gold standard” for HRV recordings, shorter, more clinically applicable HRV recordings, such as short-term (~ 5 min) and ultra-short-term (< 5 min), can be used to assess HRV¹⁰⁰. Although no standardization of ultra-short-term measurement protocols exists, several authors have investigated the reliability and validity of ultra-short-term measurements. 10 seconds seems to be enough for valid heart rate measures; however, proposed minimum recording lengths for other parameters differ; SDNN (60-240 s.), RMSSD (10-120 s.), VLF (50-270 s.), LF (50-90 s.), HF (20-120 s.) and LF/HF (20-180 s.)²³²⁻²³⁶.

According to the Task Force of The European Society of Cardiology and The North American Society of Pacing and Electrophysiology, a minimum of 1 and 2 minutes are required to address the LF and HF components respectively¹⁹.

In studies III and IV, measurements of HRV were obtained using *Lifecard® CF* (Del Mar Reynolds Medical, Spacelabs Healthcare Inc.). Electrocardiographic recordings were obtained in accordance with internationally recommended standards¹⁹, and visually inspected for artifacts, arrhythmias, ectopic beats, etc. Time-domain analysis was performed using *Pathfinder®* (Del Mar Reynolds Medical, Spacelabs Healthcare Inc.), and frequency-domain analyses was performed using *HRV Tools* (Del Mar Reynolds Medical, Spacelabs Healthcare Inc.) using Fast Fourier transformation. The derived time-domain parameters were mean RR, SDNN, SDANN, SDNNI, and RMSSD, and the following frequency-domain derived parameters were VLF, LF, HF, and the LF/HF ratio, derived with cut-off values in accordance with *Shaffer et al.*¹⁰⁰. The assessment of dynamic changes in HRV in response to cold tonic pain included the division of electrocardiographic measures into two-minute segments. These segments were further grouped into pre-test (4 min. before exposure), initial response (2 min. during, and 2 min. post-exposure), sustained response (4-10 min. post-

exposure), and recovery phase (10-16 min. post-exposure). Differences between baseline and follow-up (26 weeks) were denoted as Δ .

3.5.2 CARDIAC SENSITIVITY TO THE BAROREFLEX

CBS refers to the ability of baroreceptors to respond to changes in blood pressure and maintain blood pressure homeostasis. Baroreceptors, primarily located in the aortic arch and carotid sinus, are specialized nerve cells that sense changes in arterial blood pressure and work as a negative feedback system. This information is transmitted to the brainstem, which initiates an autonomic response.

Assessing CBS requires continuous and synchronized measures of heart rate and blood pressure, and CBS is widely used to quantify the parasympathetic response to changes in blood pressure¹⁰². In case of a sudden increase in blood pressure, an increase in parasympathetic activity and a decrease in sympathetic activity occurs, resulting in a reduction in heart rate and blood pressure¹⁰². A sudden decrease in blood pressure will result in the opposite response. For example, standing increases sympathetic activity and reduces CBS²³⁷, whereas, e.g., deep-breathing increases parasympathetic activity and CBS²³⁸.

CBS is known to be reduced in individuals with type 1 diabetes²³⁹, even in individuals without clinical symptoms²⁴⁰. Thus measurements of CBS may provide early detection of autonomic dysfunction in individuals with diabetes²⁴¹.

In study III, CBS was assessed using Neuroscope® (Medifit Instruments, Enfield, Essex, UK)

3.5.3 CARDIAC VAGAL TONE

Cardiac vagal tone (CVT) is a validated, non-invasive, real-time measure of the brainstem's efferent parasympathetic influence on the heart²⁴². Whereas CBS represents the afferent influence on the heart, CVT represents the efferent parasympathetic influence²⁴³.

Following an increase in blood pressure, baroreceptors terminating in the nucleus tractus solitarius are activated, resulting in a decrease in sympathetic output in the RVLM and an increase in parasympathetic output from the nucleus ambiguus and VDMN, consequently decreasing heart rate²⁴⁴. As previously mentioned, the parasympathetic response is much quicker than that of the sympathetic, and thus short-term changes in heart rate are primarily attributed to changes in parasympathetic activity¹⁰⁰. The relationship between baroreceptor-driven changes in heart rate (RR

intervals) and parasympathetic output can be measured on a linear vagal scale, with values above zero representing efferent vagal tone²⁴². The higher the values, the healthier the parasympathetic function^{242,243}.

In individuals with type 1 diabetes, CVT has been shown to be decreased and to correlate with 24-hour HRV measures²⁴⁵. Furthermore, CVT has been proposed as a screening tool for CAN in type 1 diabetes²⁴⁶.

In study III, CVT was assessed using Neuroscope® (Medifit Instruments, Enfield, Essex, UK)

4 HYPOTHESES AND AIMS

The overall aims of this Ph.D. thesis were to investigate the effect of long-term type 1 diabetes on autonomic nerve function and the effect of liraglutide on the neurocardiac regulation and on the retinal nerve fiber layer thickness.

The hypothesis and aims for study I-IV were as follows:

STUDY I

Hypothesis:

- Long-term type 1 diabetes causes neuropathy to the peripheral sympathetic nerve fibers innervating the superior and inferior tarsal muscles³².

Aims:

- 1) To measure the sympathetic paresis by assessing eye-to-eye differences in palpebral fissure height (Δ PFH) in response to topical application of 10% phenylephrine into one eye in individuals with long-term type 1 diabetes and distal symmetrical polyneuropathy³².
- 2) To investigate possible associations between changes in palpebral fissure height (Δ PFH) and the severity of diabetic retinopathy, disease duration, and autonomic function³².
- 3) To investigate the association between autonomic function and the severity of diabetic retinopathy³².

STUDY II

Hypothesis:

- Treatment with liraglutide increases peripapillary retinal nerve fiber layer thickness by inducing nerve regeneration and restoring vascularization³³.

Aim:

- 1) To assess differences in retinal nerve fiber layer thickness between individuals with long-term type 1 diabetes, randomized to treatment with liraglutide or placebo³³.
- 2) To assess differences in retinal nerve fiber layer thickness between individuals with proliferative- and non-proliferative diabetic retinopathy³³.

STUDY III

Hypothesis:

- Exposure to tonic cold pain causes enhanced sympathetic drive and attenuates the dynamic sympathico-vagal response in participants with long-term type 1 diabetes when compared to healthy controls.

Aims:

- 1) To assess the dynamic heart rate variability response following tonic cold pain exposure in healthy individuals and in individuals with type 1 diabetes and distal symmetrical polyneuropathy.
- 2) To assess the differences in 24-hour heart rate variability measures, cardiac sensitivity to the baroreflex, and cardiac vagal tone between the two groups.

STUDY IV

Hypothesis:

- Treatment with liraglutide lead to increased heart rate and sympathetic drive in situational dynamic heart rate variability measures, evident as increased low-frequency and very low-frequency content

Aims:

- 1) To assess the heart rate variability as a proxy for neurocardiac regulation in response to tonic cold pain exposure following 26 weeks of treatment with liraglutide and placebo in individuals with long-term type 1 diabetes
- 2) To investigate dynamic differences in heart rate variability responses between the liraglutide-treated and placebo-treated group, based on ultra-short epochs before, during, and after exposure to tonic cold pain in order to investigate the autonomic-regulated mechanisms underlying increased heart rate during liraglutide treatment.

5 MATERIALS AND METHODS

This thesis is based on data collected at Mech-Sense, Department of Gastroenterology and Hepatology, Aalborg University Hospital. Data was collected as part of the TODINELI study (clinicaltrials.gov: NCT02138045), approved by The Scientific Ethical Committee of The North Denmark Region (N-20130077). The study was performed in accordance with the International Council for Harmonization guideline for Good Clinical Practice and the Declaration of Helsinki.

A total of 48 participants (10 female/38 male, mean age 50 years) with long-term type 1 diabetes (mean duration 32 years) and confirmed distal symmetrical polyneuropathy were enrolled in the main study. The studies were conducted in individuals with long-term type 1 diabetes to assess the anti-inflammatory and neuroprotective effects, independently of anti-hyperglycemic effects. The studies included in this thesis are based on secondary analysis of data collected as part of the TODINELI study. Inclusion and exclusion criteria can be found elsewhere¹⁶⁰.

Table 3 and Figure 6 provide an overview of the studies included in this thesis. Descriptions of the different clinical assessments used for these studies can be found in chapter 2: *Clinical assessments*. Description of cold tonic pain, under “*Cold pressor test*” in the “*Pharmacologic and physiologic modulation of the autonomic nervous system*”-section.

Study	Design	Subjects	Intervention	Dosage	Duration	Stimulation	Assessments
I	EX. C-S.	37 T1DM + DSPN				Ogtt. Phenylephrine 10%	PFH DR stage HRV BP
II	DB. RCT	37 T1DM + DSPN: Liraglutide (n=17) Placebo (n=20)	SC. Liraglutide	1.8 mg/day	26 weeks		RNFL DR stage HbA1c
III	EX. C-S.	48 T1DM + DSPN 21 healthy controls				Cold tonic pain exposure	HRV CBS CVT NCV Pain ratings MNSI BP Blood samples
IV	DB. RCT	32 T1DM + DSPN: Liraglutide (n=17) Placebo (n=15)	SC. Liraglutide	1.8 mg/day	26 weeks	Cold tonic pain exposure	HRV NCV Pain ratings MNSI BP Blood samples

Table 3: Overview of study design, subjects, treatment, stimulations, and assessments in the studies included in this thesis: EX. = explorative study, C-S. = cross-sectional study, DB = double blinded, RCT = randomized controlled trial, T1DM = type 1 diabetes, DPSN = distal symmetrical polyneuropathy, Ogtt. = oculogutta (eye drops), SC. = subcutaneous, PFH = palpebral fissure height, DR = diabetic retinopathy stage, HRV = heart rate variability, CBS = cardiac sensitivity to the baroreflex, CVT = cardiac vagal tone, HbA1c = glycosylated hemoglobin, NCV = nerve conduction velocity, MNSI = Michigan neuropathy screening instrument, BP = blood pressure, pain ratings; eleven point modified Likert scale, with 0 = no pain and 10 = worst imaginable pain.

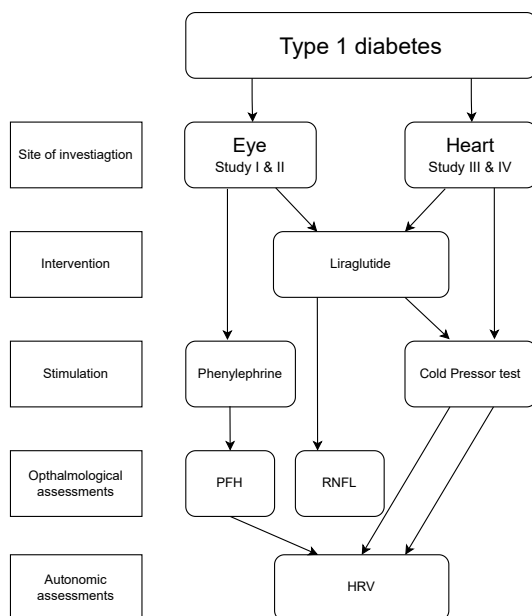


Figure 6: Graphical overview of studies included in this thesis. PFH = palpebral fissure height, RNFL = retinal nerve fiber layer, HRV = heart rate variability.

STATISTICAL ANALYSIS

For all studies, Epidata software® (The Epidata Association, Odense, Denmark) was used to manage data. In studies I and II, statistical analysis was performed using SPSS® v. 25.0.0 (IBM, Armonk, NY, and for studies III and IV, Stata® v. 17.0 (StataCorp LLC, Texas, USA) was used.

All data was tested for normality by inspecting QQ-plots, histograms, and by Shapiro-Wilk testing. Eligible data was compared using t-tests and categorical data by Spearman's rank correlation. $P < 0.05$ was considered statistically significant.

STUDY I

Differences in the palpebral fissure between the phenylephrine-stimulated and non-stimulated eye (Δ PFH) were compared using independent samples t-test. Pearson's correlation coefficient was used to test for associations between Δ PFH and heart rate variability parameters. One-way analysis of variance (ANOVA) was used to test for associations between Δ PFH and the presence of NPDR or PDR³².

STUDY II

Differences in RNFL between baseline and follow-up (Δ) were compared between groups using a two-sample t-test. Linear mixed model analysis with treatment and DR stage as independent factors were used to calculate fixed effect estimates in Δ -values. Differences in Δ HbA1c between groups were compared using independent samples t-test³³.

STUDY III

Differences in situational HRV parameters between baseline visit and follow-up visit (Δ) between groups were compared using repeated measures-ANOVA, followed by linear comparison analysis, and crude data were subsequently adjusted for BMI and age. To accommodate for violations in normality, bootstrapping with 1000 replications was performed.

STUDY IV

Differences in mean situational heart rate between baseline and follow-up for each group were compared using t-tests. Differences in heart rate and HRV between groups in response to tonic cold pain were analyzed using repeated measures-ANOVA followed by linear comparison, and crude data were adjusted for BMI and age, as well as changes in body weight between baseline and follow-up (Δ kg). Correlations between mean RR/HR and HRV parameters were performed using the Pearson correlation coefficient. Pain ratings were compared using independent t-tests.

6 MAIN FINDINGS

STUDY I

Main results:

- Following the topical application of phenylephrine, the mean difference in palpebral fissure height (Δ PFH) was 1.02 ± 0.29 mm.³².
- Δ PFH showed 1) associations with the severity of diabetic retinopathy, 2) associations with disease duration, and 3) positive associations with the low-frequency HRV content³².
- Low Δ PFH was associated with lower HRV parameters (total power, VLF, and LF)³².

STUDY II

Main results:

- Liraglutide treatment did not alter mean peripapillary, inferior, superior, nasal or temporal retinal nerve fiber layer thickness³³.
- Furthermore, no differences were found between the PDR or NPDR groups or associations with changes in HbA1c³³.

STUDY III

Main results:

- Following exposure to tonic cold pain, dynamic heart rate variability responses revealed adynamic patterns in the T1DM group, indicating impaired neurocardiac flexibility.
- 24-hour time- and frequency domain parameters of heart rate variability and cardiac sensitivity to the baroreflex were lower in the T1DM group, indicating diabetes-induced dysautonomia. No significant differences between groups were found for cardiac vagal tone.

STUDY IV

Main results:

- 26-week treatment with liraglutide increased overall situational heart rate by 3.6 beats per minute.
- Exposure to tonic cold pain revealed liraglutide-induced increases in SDNN, as well as decreases in VLF and LF when compared to placebo. No differences were found in parasympathetic measures of RMSSD and HF.

7 DISCUSSION

THE USE OF PALPEBRAL FISSURE HEIGHT AS A MARKER OF AUTONOMIC FUNCTION (STUDY I)

In summary, we found that stimulation with phenylephrine increased palpebral fissure height by an average of 1.02 mm compared to the untreated eye. Furthermore, we showed that Δ PFH was lower with increasing duration of diabetes, was lower in individuals with PDR, and that lower Δ PFH was associated with lower total power, VLF, and LF frequency-domain parameters of HRV. Moreover, we found that almost all HRV parameters were lower in the PDR group when compared to the NPDR group. To our knowledge, this is the first time this simple measure has been utilized in individuals with T1DM and DSPN.

A significant difference in PFH between the treated and untreated eyes (Δ PFH) was found, similar to the results reported by *Bastiaensen et al.*²¹². Compared to the PFH response found in our study, healthy individuals exhibit a response of up to 70% higher^{113–115}. This shows that within our cohort, a significantly decreased excitability of the tarsal muscles to phenylephrine was present, suggesting dysfunction of the sympathetic nerves innervating these muscles. Additionally, we observed a more pronounced decrease in sympathetic capacity in the PDR group compared to the NPDR group, indicating severe microvascular complications associated with the duration of diabetes.

Moreover, our study found a positive correlation between Δ PFH and heart rate variability frequency-domain parameters VLF and LF, indicative of systemic sympathetic dysfunction. However, the interpretation of LF heart rate variability is controversial, as it may reflect both sympathetic and parasympathetic content. The absence of associations between Δ PFH and RMSSD, HF, and the LF/HF ratio, which reflect parasympathetic modulation, further support this finding. Thus, our results suggest that PFH could be a simple, easily applicable marker of sympathetic dysfunction.

Furthermore, we found that HRV measures were decreased in the PDR group compared to the NPDR group, which is in line with findings in other studies^{247,248}.

THE EFFECT OF LIRAGLUTIDE ON THE RETINAL NERVE FIBER LAYER THICKNESS (STUDY II)

Although GLP-1 RA's have been found to prevent thinning of RNFL and even promote neuro-regenerative effects of retinal cells^{31,166,170}, we found that 26-week treatment with liraglutide did not change the RNFL thickness in individuals with long-term T1DM and DSPN. Nor did it change RNFL thickness in the NPDR or PDR groups. This may, to some degree, be attributed to the loss of GLP-1 R-positive retinal cells seen in more advanced stages of DR^{166,249}. Moreover, the changes in RNFL may not accurately represent the neuroregeneration, as increases in cells within the retinal ganglion layer may not necessarily reflect changes in the RNFL thickness. The same applies to GLP-1 RA-included prevention of apoptosis in inner- and outer nuclear layers³¹. Increases in RNFL may also occur due to edema within this layer and could mistakenly be interpreted as nerve regeneration. Thus, the assessment of RNFL using OCT is associated with several pitfalls. Therefore, measurements of other retinal layers, including the retinal ganglion cell layer, or even electrophysiologic tests, such as electroretinogram or visually evoked potentials, may allow for a more optimal evaluation of the possible beneficial effects of GLP-1 RA on the retina.

Although *Hernandez et al.* investigated the effects of systemically and topically administered GLP-1 RA and found no differences, most other studies have investigated the effects of topical or intravitreal administration of GLP-RA^{31,166,171,250–252}. To our knowledge, differences in retinal drug concentration between systemically and topically administrated GLP-1RA have not been compared. Thus, it remains unknown if different administration methods result in different local drug concentrations.

Moreover, in our cohort with long-term T1DM, the retinal neurodegeneration, and microvascular manifestations may be too pronounced to be reversed. Thus, it would be of great interest to investigate the effect of GLP-1 RA's in earlier stages of DR or with a more extended treatment duration.

THE EFFECT OF LONG-TERM TYPE 1 DIABETES ON HEART RATE VARIABILITY PARAMETERS (STUDY III)

In study III, we investigated the effect of long-term type 1 diabetes on neurocardiac regulation during 24-hour HRV and in response to tonic cold pain exposure. We found that, compared to healthy, and when adjusting for BMI and age, the T1DM group had lower 24-hour HRV parameters, except for RMSSD and LF/HF. Moreover, CBS was lower in the T1DM group. These results indicate T1DM-induced dysautonomia, primarily related to sympathetic modulation, as parasympathetic measures (RMSSD and LF/HF) were not significantly different from healthy. The results are similar to the results found in other studies^{84,221,222,253}.

UTILIZING COLD TONIC PAIN EXPOSURE IN THE ASSESSMENT OF AUTONOMIC NEUROPATHY (STUDY III)

Cold tonic pain exposure was used in studies III and IV as an external stimulus, allowing for interpreting dynamic physiological stress-related responses in HRV. The concept of such challenge testing is similar to stress tests used in assessing asthma and to the treadmill test used in evaluating stress-related cardiac performance. By utilization of 2 min. ultra-short-term HRV measures, we investigated HRV in the initial, sustained, and recovery phases and compared these to pre-test measures. This allowed for assessing dynamic adaptive capacity in response to tonic cold pain exposure. In neither study III nor IV, differences were found in experienced pain between the T1DM group and healthy participants or between individuals with T1DM treated with liraglutide vs. placebo. In study III, the diabetes group experienced less unpleasantness, suggesting sensory deficits were present to some degree.

In study III, tonic cold pain exposure resulted in a decreased mean RR and an increased SDNN and RMSSD in healthy controls. Moreover, both LF and HF parameters increased, indicating increased autonomic modulation. Other studies have found both increases²⁵⁴ and decreases²⁵⁵ in RMSSD in healthy participants, as well as increases in both LF and HF^{120,254,256}. One study found that neither SDNN nor RMSSD changed²⁵⁷, and LF²⁵⁴ and HF²⁵⁸ have also been shown to decrease. Parasympathetic modulation is known to decrease with increasing age²³¹, and as both age and methodologies differ between these studies, direct comparisons are difficult.

In the T1DM group, mean RR and RMSSD increased in the initial response, indicating an increase in sympathetic modulation. Moreover, LF and LF/HF decreased, indicating a simultaneous decrease in parasympathetic modulation. Compared to healthy controls, the T1DM group exhibited adynamic responses following tonic cold pain exposure, indicating an impaired stress-related dynamic autonomic adaptability. Both mean RR, SDNN, RMSSD, total power, and LF/HF were less adaptable than in healthy. When adjusting for BMI and age, LF/HF was no longer significant, indicating that these factors confounded this measure.

THE EFFECT OF LIRAGLUTIDE ON HEART RATE AND HEART RATE VARIABILITY IN RESPONSE TO CHALLENGES TESTING (STUDY IV)

In study IV, we aimed to investigate the effect of liraglutide on HRV parameters in response to cold tonic pain exposure in patients with T1DM and DSPN. *Brock et al.* investigated the effect of liraglutide within the same cohort and found that 24-hour HRV recordings did not differ significantly compared to placebo¹⁶⁰. These findings are in line with a recent meta-analysis¹⁹⁸, although some authors have found that GLP-1 RA's cause decreases in SDNN, RMSSD, LF, and HF in individuals with T2DM^{182,185}.

Following 26 weeks of treatment with liraglutide, cold tonic exposure revealed consistent increases in HR and SDNN, as well as decreases in VLF and LF compared to placebo. These findings indicate that GLP-1 RA-induced alterations in autonomic cardiac modulation may be unmasked in response to physiological stress. Moreover, we found that when compared to placebo, liraglutide increased HR by 3.6 bpm, which is in line with previous findings in T1DM²⁵⁹. As previously mentioned, the underlying mechanism(s) are not fully understood but may involve direct activation of GLP-1 R's in the sinoatrial node, decreased parasympathetic activity, activation of baroreceptors in response to a reduction in systemic vascular resistance¹⁹⁴, increased sympathetic activity or even via direct activation of sympathetic neurons in the brainstem.

The increase in SDNN indicates that heart rate was not chronometric, suggesting that, although direct activation of GLP-1 R's in the sinoatrial node may, in part, influence the increase in HR, some degree of preserved autonomic modulatory capacity is present. This is in line with *Baggio et al.*, who found that, in *ex vivo* studies in mice, GLP-1 RA's did not increase heart rate by itself but required neuronal inputs to do so²⁶⁰. Furthermore, we found no liraglutide-induced alterations in RMSSD and HF, indicating that parasympathetic modulation was not altered by 26 weeks of treatment with liraglutide. These findings are in line with previous findings in 24-hour recordings^{160,189,261}, although decreases have also been found¹⁸².

Moreover, treatment with liraglutide decreased the dynamic VLF and LF in response to cold tonic pain. As previously mentioned, the VLF is believed to be intrinsically generated by the heart, and the amplitude and frequency are modulated by efferent sympathetic activity²²⁴. VLF has also been proposed to reflect thermoregulatory and sympathetic content^{7,14}. LF reflects both sympathetic and parasympathetic content, and as parasympathetic measures RMSSD and HF did not change, these changes are believed to be caused by alterations in sympathetic modulation. *Mendis et al.* found that the increase in HR following GLP-1 RA's could be explained by sympathetic activation in response to vasodilatation¹⁹⁴, and others have found both increases in cardiac output, epinephrine levels, and blood pressure further indicative of a sympathetic response²⁶². This contrasts the findings of others, who found that HR increases were not attributed to changes in sympathetic modulation²⁶³, and *Skov et al.*, who found that a single dose of liraglutide was not accompanied by increases in the release of catecholamines²⁶⁴.

In rodents, liraglutide has been shown to be able to cross the blood-brain-barrier²⁶⁵, and as GLP-1 R's are widespread within the central nervous system^{197,199,200}, central mechanisms may also be involved in GLP-1 RA-induced HR increases. In mice, Exendin-4 (GLP-1 RA) has been shown to depress inhibitory and excitatory vagal neurons in the nucleus ambiguus¹⁹³. Moreover, GLP-1 R's have been

found in both the nucleus tractus solitarius and the paraventricular nucleus, which are involved in autonomic regulation^{197,201,202}. Additionally, GLP-1 release within the spinal cord has been shown to increase the sympathetic modulation of the heart²⁶⁶.

Taken together, the underlying mechanisms of GLP-1 RA-induced HR increases are complex, and more studies are needed to further elucidate the mechanism involved.

7.1 STRENGTHS AND LIMITATIONS

The participants enrolled in these studies all had long-term type 1 diabetes, confirmed DSPN, and many had orthostatic hypotension indicative of autonomic neuropathy. As such, we know that diabetic neuropathy was present. The studies were conducted in individuals with T1DM to assess proposed liraglutide-induced anti-inflammatory and neuroprotective effects independent of glycemic modulation.

STUDY I

Strengths:

- The primary advantage of this study is its capability to link a novel measure with established validated autonomic measures of heart rate variability³².

Limitations:

- Firstly, we did not take a frontal photograph before the phenylephrine test to assess any pre-existing asymmetry. In primary gaze, PFH asymmetry has previously not been shown to be more frequent in one eye over the other²⁶⁷. Thus, we assume that any resulting asymmetry would be equally increased or decreased and would cancel out³².
- Secondly, the external generalizability or validity of our findings to other types or stages of diabetes remains unclear and requires further investigation³².
- Thirdly, our cohort with severe and longstanding neuropathy may not be representative of future cohorts with prolonged duration of diabetes, as guidelines now advocate for more strict glycemic control³².
- Finally, validating the use of PFH against sympathetic content in HRV highlights the need to examine whether reductions in PFH are associated with cardiac autonomic reflex testing, most commonly used to diagnose CAN³².

STUDY II

Strengths:

- To our knowledge, we are the first to examine the effect of 26 weeks of treatment with liraglutide on RNFL thickness in individuals with T1DM and confirmed DSPN³³.

Limitations:

- Firstly, the intervention period in this study lasted only 26 weeks, which is relatively short compared to the typical disease duration for DR. As a result, possible anti-inflammatory and neurotrophic effects of the intervention may not have been fully reflected in the RNFL thickness measurements. Given the nonlinear nature of DR progression, a follow-up period of at least five years would have been preferable to assess the changes between the treatment and control groups³³.
- Secondly, changes in RNFL thickness should be interpreted cautiously as several factors may result in changes in RNFL thickness³³.
- Thirdly, this study only included a small number of participants, and type 2 errors cannot be excluded. Therefore, the results of this study should be considered exploratory, aimed at assessing the effect of GLP-1 on retinal nerve fibers. Further studies are necessary to fully understand if long-term treatment with GLP-1 agonists can induce anti-inflammatory and neurotrophic effects within the human retina³³.

STUDY III

Strengths:

- To our knowledge, this is the first time that the dynamic alterations in HRV, in response to tonic cold pain exposure, have been investigated in a cohort of individuals with T1DM and DSPN and compared to healthy controls.

Limitations:

- Firstly, this study was conducted on individuals with long-term type 1 diabetes and verified DSPN. 90% of the participants had orthostatic hypotension, indicative of CAN, but CAN was not evaluated using cardiovascular autonomic reflex tests. Including individuals with T1DM with no or early signs of CAN could potentially reveal discrepancies in the autonomic response to tonic cold pain exposure in early disease stages.
- Secondly, assessments of the dynamic responses were based on 2-minute ultra-short-term HRV measurements, which are known to be vulnerable to physiological noise caused by movement, coughing, or the stress response

caused by the tonic cold pain exposure. However, data was calculated within predefined intervals, so we believed this might only represent a minor issue.

- Thirdly, the inclusion of Poincaré plot may have provided additional information on changes in heart rate variability measures however, this was not included in the study.
- Fourthly, cold tonic pain exposure has been shown to have a high inter-individual variability, and conflicting results have been reported in healthy. We did, however show a consistent adynamic pattern in the T1DM group.
- Lastly, including periodic dynamic depolarization of the T-wave and deceleration capacity, which are novel parameters of sympathetic and parasympathetic activation, could potentially improve our understanding of the complex sympatico-vagal neurocardiac regulation.

STUDY IV

Strengths:

- To our knowledge, this is the first time the dynamic alterations in situational electrocardiographic HRV recordings, in response to cold tonic pain exposure, have been investigated in individuals with type 1 diabetes receiving either liraglutide or a placebo.

Limitations:

- Firstly, the HRV measures were only measured once by the same individual using validated software. However, the predefined 2-minute ultra-short-term HRV measurements likely minimized inter- and intra-observer differences.
- Secondly, the 2-minute epochs are susceptible to physiological noise, such as stress response, coughing, and movements, which may have resulted in missing data. As the conducted ECG recordings were closely inspected for artifacts, arrhythmia, etc., this problem is likely of minor significance.
- Thirdly, we used classical HRV measures, including mean RR, SDNN, RMSSD, total power, VLF, LF, HF, and LF/HF, provided by the software solution.
- Finally, the lack of measures of catecholamines may have limited the investigation of potential underlying mechanisms of GLP-1-induced neurocardiac modulation. Moreover, including periodic dynamic depolarization of the T-wave and deceleration capacity may have improved our understanding of the complex sympatico-vagal neurocardiac regulation. Further studies are needed to address these limitations and provide a more comprehensive understanding of this topic.

7.2 FUTURE STUDIES AND PERSPECTIVES

STUDY I

It would be of great interest to validate changes in PFH in response to instillation against traditional gold-standard cardiac autonomic reflex tests used for diagnosing CAN. Moreover, it would be interesting to investigate whether diminished PFH can be seen in the early stages of diabetic retinopathy and diabetic neuropathy. One way to do so would be to design a follow-up study in which cardiac autonomic reflex tests, autonomic neuropathy tests, such as electroneurographic recordings, and evaluation of diabetic retinopathy stages were assessed with meaningful intervals.

STUDY II

The assessment of retinal nerve fiber layer thickness or ganglion cell layer thickness may enable the evaluation of systemic neurodegeneration. Therefore, establishing a longitudinal database to assess neurodegenerative changes in both peripheral, autonomic, and retinal nerves would be of great interest. This would enable the evaluations of associations between RNFL or ganglion cell layer thickness and autonomic and peripheral measures and possibly allow the stratification of high risk for developing neuropathy based on changes in RNFL or ganglion cell layer changes. Furthermore, it would be interesting to evaluate the effect of liraglutide, or other potential neuroprotective agents, in earlier stages of diabetes or diabetic retinopathy.

STUDY III

It would be highly intriguing to evaluate the dynamic HRV responses, in response to cold tonic pain exposure, in earlier stages of diabetes, as this could potentially reveal early signs of alterations in neurocardiac responses in individuals with early cardiovascular autonomic neuropathy. Moreover, it would be interesting to evaluate the use of other interventions, such as vagal nerve stimulation, lifestyle changes, or cognitive therapy of the dynamic responses. Furthermore, and as previously mentioned, the inclusion of periodic dynamic depolarization of the T-wave and

deceleration capacity could potentially improve our understanding of the sympatico-vagal neurocardiac regulation.

STUDY IV

It would be of great interest to assess the effect of GLP-1 RA' on the autonomic neurocardiac regulation in earlier stages of diabetes. Moreover, it would be interesting to assess other potential neuroprotective drugs, such as Finerenone and SGLT-, or supplements²⁶⁸, such as Omega-3, in a similar randomized controlled trial design in individuals with T1DM or T2DM.

8 CONCLUSION

STUDY I

We showed that the effect of phenylephrine on palpebral fissure height was associated with the severity of DR and disease duration and validated heart rate variability parameters, total power, VLF, and LF. Hence, the effects of phenylephrine on palpebral fissure height could potentially be a simple clinical indicator of autonomic dysfunction in individuals with type 1 diabetes³².

STUDY II

We did not find measurable changes in peripapillary RNFL thickness measures following 26 weeks of treatment with liraglutide. Not did we find differences between the NPDR and PDR groups. RNFL thickness measurements using OCT may not be sensitive enough to detect possible GLP-1 RA-induced neuro regenerative effects. The assessment of the ganglion cell layer thickness may potentially be more sensitive³³.

STUDY III

We showed that ultra-short-term HRV measures revealed complicated and dynamic centrally regulated neurocardiac responses in response to challenge testing using cold tonic pain exposure. These changes may not otherwise be present in resting conditions. We found that individuals with long-term-type 1 diabetes and DSPN showed an adynamic response to tonic cold pain exposure compared to healthy controls. Whereas tonic cold pain exposure has traditionally been considered to result in sympathetic activation, we found a more complex response, with both linear and non-linear co-activation.

STUDY IV

Challenge testing, utilizing tonic cold pain exposure, revealed alterations in dynamic HRV responses following 26 weeks of treatment with liraglutide. We showed that HR and SDNN increased, whereas VLF and LF decreased, indicating withdrawal of sympathetic activity. We found no changes in parasympathetic measures of RMSSD and HF. Moreover, we found no correlations between mean RR or HR and HRV parameters. Although our data suggest a more complex regulation than the classical

reciprocal sympathovagal balance, we cannot draw any firm conclusions about the exact underlying mechanisms of increased HR in individuals treated with liraglutide.

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