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CHARACTERIZATION OF THE CENTRAL NERVOUS SYSTEM IN DIABETIC PERIPHERAL NEUROPATHY

ASPECTS OF COGNITIVE, STRUCTURAL, AND FUNCTIONAL BRAIN ALTERATIONS IN TYPE 1 DIABETES

BY
SUGANTHIYA SANTHIAPILLAI CROOSU

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DENMARK

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Publications

1. **Croosu SS,** Hansen TM, Brock B, Drewes AM, Brock C, Frøkjær JB. Altered functional connectivity between brain structures in adults with type 1 diabetes and polyneuropathy. Brain Res. 2022: Epub ahead of print

2. Røikjer J, **Croosu SS,** Hansen TM, Frøkjær JB, Andersen HH, Arendt-Nielsen L, Mørch, CD, Ejskjaer N. The Histamine-Induced Axon-Reflex Response in

People With Type 1 Diabetes With and Without Peripheral Neuropathy and Pain: A Clinical, Observational Study. J Pain. 2022. Epub ahead of print

3. **Croosu SS,** Frøkjær JB, Drewes AM, Hansen TM. Tapentadol and oxycodone affect resting-state functional brain connectivity: A randomized, placebo-controlled trial. J Neuroimaging. 2021: Epub ahead of print.

4. **Croosu SS,** Hansen TM, Røikjer J, Mørch, CD, Ejskjaer N, Frøkjær JB. Gray matter brain alterations in type 1 diabetes – findings based on detailed phenotyping of neuropathy status. Experimental and Clinical Endocrinology & Diabetes. *Accepted. 2022*

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6. Røikjer J, **Croosu SS,** Frøkjær JB, Hansen TM, Ejskjaer N, Mørch, CD. Perception Threshold Tracking: A novel method for assessing the function of large and small nerve fibers in people with diabetes. *Diabetes. Invited for revision. 2022*

7. **Croosu SS,** Røikjer J, Mørch, CD, Ejskjaer N, Frøkjær JB, Hansen TM. Alterations in functional connectivity of thalamus and primary somatosensory cortex in diabetic peripheral neuropathy and neuropathic pain. *Under review at Diabetes Care. 2022*

8. Røikjer J, **Croosu SS,** Hansen TM, Frøkjær JB, Brock C, Mørch, CD, Ejskjaer N. The co-existence of peripheral and autonomic neuropathy in type 1 diabetes with and without pain. *Under review at Diabetic Medicine. 2022*

LIST OF PAPERS

This PhD thesis is based on the following papers:

I. **Croosu SS[*],** Gjela M[*], Røikjer J, Hansen TM, Mørch, CD, Frøkjær JB, Ejskjaer N. The influence of diabetic polyneuropathy and neuropathic pain on cognitive function in type 1 diabetes. *BMJ open diabetes research and care. Invited for revision. 2022*

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ABBREVIATIONS

ACE-III:	Addenbrooke's Cognitive Examination-III
BOLD:	Blood-oxygen-level-dependent
CNS:	Central nervous system
DN4:	Douleur Neuropathique en 4
DPN:	Diabetic peripheral neuropathy
DTI:	Diffusion tensor imaging
fMRI:	Functional magnetic resonance imaging
GMV:	Gray matter volume
HbA1c:	Hemoglobin A1c
MEDON:	Methods for Early Detection Of diabetic peripheral Neuropathy
MR:	Magnetic resonance
MRI:	Magnetic resonance imaging
NP:	Neuropathic pain
PNS:	Peripheral nervous system
T1DM:	Type 1 diabetes mellitus
VBM:	Voxel-based morphometry

ENGLISH SUMMARY

Type 1 diabetes mellitus is a chronic autoimmune disease characterized by insulin deficiency and clinically manifests with hyperglycemia. One of the major complications of diabetes mellitus is diabetic peripheral neuropathy (DPN) characterized by dysfunction of the peripheral nerves that starts distally in the toes but progresses proximally to the upper limbs. DPN manifests with sensory symptoms like tingling, prickling, and neuropathic pain. Both DPN with and without neuropathic pain are associated with high health care costs, lower quality of life, and increased morbidity and mortality.

Unfortunately, early diagnosis and proper treatment of neuropathic complications remain complex and challenging. This is mainly due to the limited knowledge about the pathogenesis of DPN and neuropathic pain. Most studies have focused on understanding the mechanisms in the peripheral nerves. However, growing evidence has now suggested that the central nervous system (CNS) may also be involved in the sensory abnormalities experienced in individuals with DPN and neuropathic pain. However, the full extent of the CNS involvement remains largely unknown and needs further investigation. This PhD thesis aims to provide a deeper understanding of CNS alterations in individuals with type 1 diabetes and neuropathic complications using cognitive tests and non-invasive structural and functional magnetic resonance imaging (MRI) of the brain.

Three papers compile this PhD thesis based on a cross-sectional study named MEDON (Methods of Early Detection of diabetic peripheral Neuropathy). The study included four groups of participants: 1) type 1 diabetes with neuropathic pain, 2) type 1 diabetes with DPN, 3) type 1 diabetes without DPN, and 4) healthy controls. Paper I investigated cognitive alterations in the four groups using a cognitive questionnaire and cognitive test. The main finding of the study showed poorer memory in the DPN group compared to healthy controls, and no differences were observed between the three diabetes groups. The cognitive alteration was not associated with peripheral nerve functions or diabetes-related clinical parameters.

Paper II aimed to investigate structural brain changes in the four groups included. Compared to the healthy controls total gray matter volume was lower in the diabetes group with neuropathic pain and in the diabetes group without DPN. Regional GMV loss, including regions relevant for sensory processing, was found in all three diabetes groups compared to healthy controls. No associations were found to peripheral nerve

functions or diabetes-related clinical parameters. Overall, gray matter volume alterations were observed in individuals with diabetes regardless of the presence of DPN and neuropathic pain.

Finally, Paper III investigated functional connectivity at rest in brain regions (thalamus, primary somatosensory cortex, and insula) involved in pain processing. It showed hyper-connectivity between thalamus/primary somatosensory cortex and cortical motor areas in diabetes without DPN compared to diabetes with neuropathic pain and healthy controls. Most remarkably, higher connectivity patterns were associated with poorer peripheral nerve function and higher pain intensity.

Overall, based on the three papers, this PhD thesis demonstrated that CNS alterations occur regardless of the presence of DPN and neuropathic pain. The method used to assess different CNS parameters is of essential value in connecting the CNS alterations to underlying neuropathic complications. Especially, functional parameters of brain regions involved in sensory processing have shown great potential in the pathogenesis of DPN and neuropathic pain and in developing early biomarkers for risk stratification.

DANSK RESUME

Type 1 diabetes mellitus er en kronisk autoimmun sygdom, som er karakteriseret ved insulinmangel og som manifesterer sig klinisk med hyperglykæmi. En af de mest almindelige komplikationer ved diabetes mellitus er diabetisk perifer neuropati (DPN), som er karakteriseret ved dysfunktion af de perifere nerver. Symptomerne starter distalt i tæerne, men udvikler sig efterhånden proksimalt til at involvere fødder og ben. DPN manifesterer sig med sensoriske symptomer som prikken, stikken og neuropatiske smerter. DPN både med og uden neuropatiske smerter er forbundet med høje sundhedsomkostninger samt dårligere livskvalitet, øget sygelighed og dødelighed.

Desværre er tidligt diagnose og passende behandling af neuropatiske komplikationer både svære og udfordrende. Dette skyldes primært den begrænsede viden der er omkring patogenesen af DPN og neuropatiske smerter. De fleste undersøgelser har fokuseret på at forstå mekanismerne i det perifere nervesystem. Dog er der nu voksende evidens, der tyder på, at det centrale nervesystem (CNS) også er involveret i den sensoriske anormaliteter, som opleves af personer med DPN og neuropatiske smerter. Det fulde omfang af viden omkring CNS involvering er dog stadig uvist og yderligere studier på dette er påkrævet. Denne ph.d.-afhandling har til formål at give en dybere forståelse af CNS-ændringer hos personer med type 1-diabetes og neuropatiske komplikationer ved brug af kognitive test og ved brug af det non-invasive strukturel og funktionel magnetisk resonans (MR) teknik af hjernen.

Denne ph.d.-afhandling er baseret på tre artikler. Data for de tre artikler kommer fra et tværsnitsstudie kaldet MEDON (Methods of Early Detection of diabetic peripheral Neuropathy). Studiet inkluderede fire grupper af deltagere: 1) type 1 diabetes med neuropatiske smerter, 2) type 1 diabetes med DPN, 3) type 1 diabetes uden DPN og 4) raske kontroller. Artikel I undersøgte kognitive ændringer i de fire grupper ved hjælp af et kognitivt spørgeskema og en kognitiv test. Studiets hoved fund viste at den gruppe med DPN have dårligere hukommelse sammenlignet med de raske kontroller, og der var ingen kognitive forskelle mellem de tre diabetes grupper. Yderligere var de kognitive ændringer ikke associeret med perifere nervefunktion eller diabetesrelaterede kliniske parametre.

Artikel II havde til formål at undersøge strukturelle hjerneforandringer i de fire inkluderede grupper. Sammenlignet med de raske kontroller var det totale volumen af grå substans lavere i diabetesgruppen med neuropatiske smerter og i diabetesgruppen uden DPN. Sammenlignet med raske kontroller, var grå substans reduceret i alle tre

diabetes grupper i regionale hjerneområder, som blandt andet inkluderede områder relevant for sensorisk processering. Der blev ikke fundet nogen sammenhænge til perifere nervefunktioner eller diabetesrelaterede kliniske parametre. Reduceret grå substans forekom i personer med diabetes uanset tilstedeværelsen af DPN og neuropatisk smerter.

Artikel III undersøgte funktionel konnektivitet under hvile i hjerneområder (thalamus, primær somatosensorisk cortex og insula) som er involveret i smerteprocessering. Det viste en hyper-konnektivitet mellem thalamus/primær somatosensorisk cortex til kortikale motor områder i diabetesgruppen uden DPN sammenlignet med diabetesgruppen med neuropatiske smerter. Mest bemærkelsesværdigt var at højere konnektivitet var associeret med dårligere perifer nervefunktion og højere smerteintensitet.

Baseret på de tre artikler, viste denne ph.d.-afhandling samlet set, at CNS-forandringer forekommer uanset tilstedeværelsen af DPN og neuropatisk smerte. Metoden, der bruges til at vurdere forskellige CNS-parametre, er væsentlig for at forbinde CNS-ændringerne med underliggende neuropatiske komplikationer. Især funktionelle parametre for hjerneområder involveret i sensorisk processering har vist at have et stort potentiale i patogenesen af DPN og neuropatisk smerte og i udvikling af tidlige biomarkører for at risikostratificere.

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Suganthiya Santhiapillai Croosu, May 2022

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CHAPTER 1.
INTRODUCTION

Diabetes mellitus is associated with several complications, and among these, diabetic peripheral neuropathy (DPN) is the most common one, which affects up to half of the individuals with diabetes.(1) DPN is characterized by dysfunction of the peripheral nerves and typically presented with sensory abnormalities, including neuropathic pain, starting distally in the toes but progressing proximally to the upper limbs.(2–4) Neuropathic pain, also known as painful DPN, affects approximately one-third of all individuals with DPN.(5,6) Both DPN and neuropathic pain are associated with significantly lower quality of life, high health care costs, and increased morbidity and mortality.(7)

The presence of DPN is often diagnosed relatively late in the process based on peripheral nerve fiber functions. Also, effective and curative treatments that target the pathophysiologic mechanisms of DPN and neuropathic pain are not established and remain the highest unmet needs.(8) Early detection and better treatment are limited by the inadequate understanding of the underlying mechanisms of DPN and neuropathic pain, which also contribute to the limited knowledge of why some people develop DPN with neuropathic pain and others develop DPN without neuropathic pain. The previously suggested pathophysiology was primarily restricted to the peripheral nervous system (PNS). However, recently growing evidence has also proposed the involvement of the central nervous system (CNS) in the development of DPN (9–15) and neuropathic pain.(10,15–18) Notably, individuals with DPN and neuropathic pain have shown brain abnormalities, including in brain regions involved in sensory processing.(10,15,18,19) However, the full extent of the involvement of CNS in DPN and neuropathic pain remains unfulfilled.

The vast majority of studies investigating CNS alterations in diabetes are limited by the lack of phenotyping of DPN and neuropathic pain. Hence, studies investigating CNS involvement in well-phenotyped DPN groups are limited, and those existing have focused on neuropathic pain, while studies of CNS changes in DPN are more sparse.(8) There is still a high need to establish a deeper understanding of the CNS changes that may potentially result in or contribute to the pathogenesis of DPN and neuropathic pain.

The performance of CNS can be evaluated using cognitive tests Another way to non-invasively and directly investigate the brain is to use magnetic resonance imaging (MRI). MRI has a high spatial resolution and can provide essential information on structural volume loss, including gray matter alterations.(20,21) Also, the functional activity of the brain can be obtained using functional MRI (fMRI), which utilizes a blood-oxygen-level-dependent (BOLD) signal. This approach may give valuable information about the infrastructure of the brain that otherwise may not be "visible".(22) Characterization of the cognitive function, structure of the brain, and function of the brain in clinically well-phenotyped diabetes groups with DPN with and without neuropathic pain may significantly improve our understanding of the CNS involvement in DPN and its complications. This will bring us closer to early detection and help identify specific targets for potential treatments to prevent the development of DPN and neuropathic pain and/or normalize function in individuals suffering from DPN with and without neuropathic pain.(8)

CHAPTER 2.
BACKGROUND

2.1. TYPE 1 DIABETES MELLITUS

Diabetes mellitus is a chronic heterogeneous metabolic disorder, which in 2019 was globally estimated by the International Diabetes Federation to affect 462 million people.(23) The incidence and prevalence are globally increasing, and the number is furthermore projected to increase by 25% in 2030 and 51% in 2045. (23,24). The most common forms of diabetes are type 1 diabetes mellitus (T1DM) and type 2 diabetes mellitus, where the latter is the most prevalent of the two accounting for around 90% of all diabetes cases. It is characterized by a combination of insulin resistance and inadequate insulin secretory response.(23,25,26) T1DM accounts for approximately 5-10% of all cases of diabetes worldwide (23,26) and will be the focus of this PhD thesis.

T1DM is characterized by autoimmune destruction of the pancreatic beta cells, which leads to insulin deficiency and clinically manifests with hyperglycemia.(25) An immediate need for exogenous insulin replacement is also a hallmark of T1DM, for which lifetime treatment is needed. T1DM can occur at any age but frequently develops during childhood with the onset of symptoms related to hyperglycemia, typically including polyuria, polydipsia, polyphagia, and ketoacidosis.(27,28) The symptoms in adult-onset are more variable.(27,28)

The concern about the increasing prevalence of diabetes is typically related to the complications followed, which are often associated with higher health expenditure, lower quality of life, and increased morbidity and mortality.(7,29) The complications are typically categorized into macrovascular complications, including cardiovascular disease, cerebrovascular accidents, and peripheral vascular disease, and microvascular complications, including retinopathy, nephropathy, and diabetic neuropathy.(30) Among all the diabetes-related complications, the DPN is the most common and most costly of them all.(1,31,32) DPN goes under the category of diabetic neuropathy, which encompasses a broad spectrum of neuropathic conditions. Another common form of diabetic neuropathy is autonomic neuropathy.(3,29,33) However, DPN will be the focus of this PhD thesis.

2.2. DIABETIC PERIPHERAL NEUROPATHY

DPN affects approximately 50% of all individuals with diabetes.(1,34) The Toronto Consensus defines DPN as *"a symmetrical, length dependent sensorimotor polyneuropathy attributable to metabolic and microvessel alterations as a result of chronic hyperglycemia exposure and cardiovascular risk factors"*.(2)
Although DPN involves both sensory and motor nerves, the symptoms presented are primarily of sensory character where loss of sensation in the feet is pronounced. Other symptoms include numbness, tingling, prickling, and neuropathic pain.(3,30,35) The symptoms typically start distally in the toes but slowly progress proximally and then involve the upper limbs in a gloving and stocking distribution.(35) The loss of sensation and insensitivity to trauma is often the cause of foot ulceration, resulting in amputation in the worst case.(36) Some of the risk factors that have been related to DPN are hyperglycemia, diabetes duration, and age. Cardiovascular-related risk factors have also been suggested, including raised triglycerides, obesity, hypertension, and smoking.(35,37)

DPN affects both the small nerve fibers (Aδ and C) and the large nerve fibers (Aα and Aβ).(35) It has been suggested small nerve fibers are affected in the early stages of DPN and large fibers injury usually in the later stages, but this is not always the scenario.(35,38) The understanding of underlying pathophysiological mechanisms of DPN are incomplete and debated. However, various pathogenesis processes have been proposed to explain the development.

The impaired function and neuronal damage of the peripheral nerves, especially the terminal sensory axons in the periphery, have, among others, been coupled with hyperglycemia and hyperlipidemia.(33,35) Also, reduction of microvessels has been related to the dysfunctions of the peripheral nerves.(35) The metabolic changes that typically occur in individuals with diabetes are suggested to cause an imbalance in different pathways that lead to several pathological alterations in the neuronal cells and, as a result of this, cause nerve dysfunction. (33,35) Some of the pathways included are the polyol pathway, hexosamine pathway, and glycolysis pathway. Increased glucose and lipid cause an overload of energy resulting in mitochondrial dysfunction in the form of bioenergetic failure, loss of normal mitochondrial membrane ssfunction (mitochondrial depolarization), insufficient production of energy (decreased adenosine triphosphatase), and increased reactive oxygen species. Overall, these processes lead to the mitochondria losing their ability to normally traffic down axons, inflammation, endoplasmic reticulum stress, DNA damage, apoptosis of the neurons, and axonal failure, and all these together induce nervous system dysfunction. (33,35) An overview of the process is illustrated in Figure 2.1. Even though the vast

majority of the existing studies have restricted the investigation of the underlying mechanisms of DPN to the PNS, a growing evidence have also suggested the involvement of the CNS in DPN. This will be presented in section 2.5.

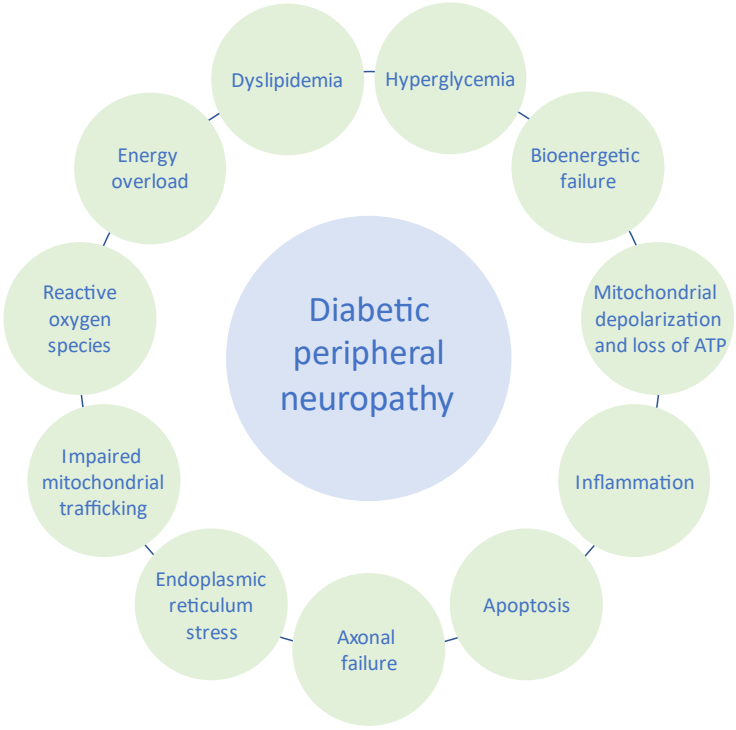

Figure 2.1. Overview of the suggested chain of events underlying the pathophysiology of diabetic peripheral neuropathy. Excess glucose and lipids contribute to energy overload that causes mitochondrial bioenergetic failure with mitochondrial depolarization, loss of adenosine triphosphatase, and accumulation of reactive oxygen species. This, in turn, leads to impaired mitochondrial trafficking from the cell body and down the axons, endoplasmic reticulum stress, apoptosis of neurons, and axonal failure. Adapted from (33) and modified. Abbreviations: ATP: adenosine triphosphatase.

2.2.1. NEUROPATHIC PAIN

While the sensory loss in DPN may go undetected in individuals with diabetes, the neuropathic pain or painful DPN, which is the most disturbing symptom of DPN, is one of the main reasons for the individual to seek medical advice for symptom re-

lief.(36,39) Neuropathic pain is defined as *"pain as a direct consequence of abnormalities in the peripheral somatosensory system in people with diabetes"*(6) and affects up to 30% of those with DPN.(5,6) The pain is often experienced as burning, electric shock, and sharp pains.(6) The painful DPN is one of the most distressing complications and results in a profound limitation in activities of daily living and lower quality of life. Whereas the risk factors for DPN are more or less well-known, the evidence of risk factors of neuropathic pain is debated and remains sparse.(36,37,40) However, some studies have suggested age, duration of diabetes, and gender, where women are at higher risk of developing neuropathic pain than men as risk factors.(6)

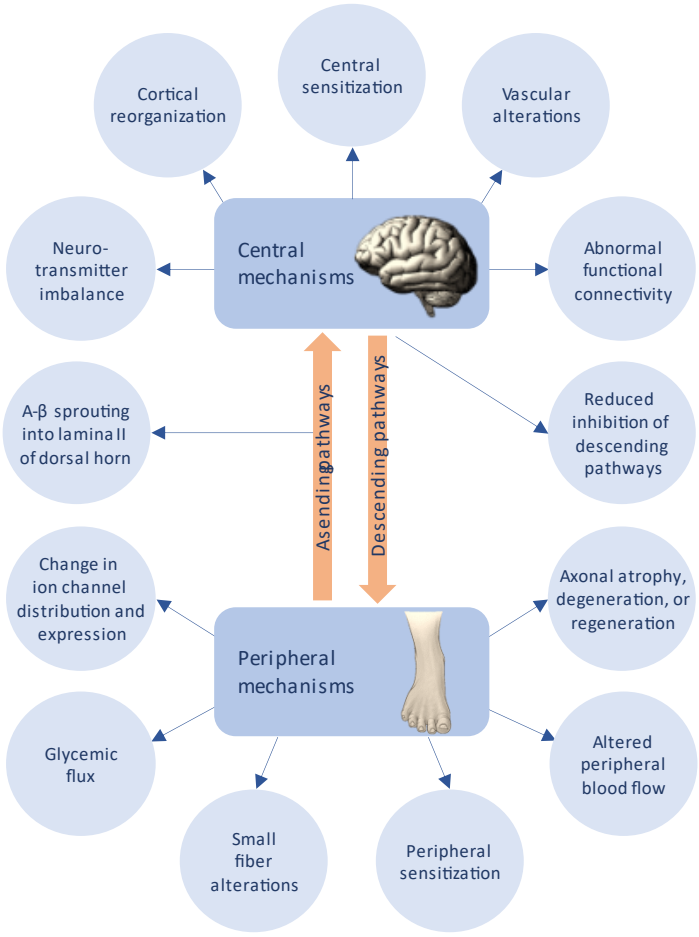

Figure 2.2. An overview of the current suggested involvement of peripheral and central mechanisms in the pathogenesis of diabetic neuropathic pain. Adapted from (6) and modified.

Similar to the underlying mechanisms of DPN, the mechanisms of neuropathic pain is not fully understood. Still, several underlying pathogeneses have been suggested. This includes changes in both the PNS and CNS mechanisms related to sensory transmission and perception. The peripheral mechanisms suggested for neuropathic pain are somehow similar to the proposed mechanisms of DPN and include changes in the ion channel distribution and expression, axonal atrophy, altered peripheral blood flow, and damage to the small nerve fibers.(6,7) The central mechanisms include central sensitization, abnormal functional connectivity, and reduced inhibition of descending pathway.(6,7) Also, the involvement of the spinothalamic tract has been proposed. Changes in the ion channel and the abnormal activity of the periphery have been proposed to lead to higher synaptic transmission within the dorsal horn of the spinal cord and higher nociceptive inputs (central sensitization).(8,35,41) Central sensitization, together with an affected balance between inhibitory interneurons and the descending pain modulatory system, may facilitate or inhibit the transmission of the nociceptive information at spinal levels.(8,35,42) The affected balance in these systems is believed to contribute to the development of neuropathic pain.(8) However, a huge gap still exists in the involvement of CNS in neuropathic pain to fully understand the development. Figure 2.2 illustrates the suggested interplay between the PNS and CNS in the development of neuropathic pain.

2.3. DIAGNOSIS

The diagnosis of DPN in clinical practice is recommended to be based on the presence of symptoms and signs of peripheral nerve dysfunction after other etiologies have been excluded.(2,3) Typically, the presence of more symptoms or signs of nerve dysfunction gives higher accuracy of the diagnosis. The diagnostic criteria recommended by the Toronto Consensus divided the accuracy of the DPN diagnosis into “possible”, “probable”, and “confirmed” DPN depending on the number of symptoms and signs and tests used (2):

“<u>Possible DPN:</u> The presence of symptoms or signs of DPN may include the following: symptoms – decreased sensation, positive neuropathic sensory symptoms (e.g., “asleep numbness,” prickling or stabbing, burning or aching pain) predominantly in the toes, feet, or legs; or signs – symmetric decrease of distal sensation or unequivocally decreased or absent ankle reflexes.
<u>Probable DPN:</u> The presence of a combination of symptoms and signs of neuropathy include any two or more of the following: neuropathic symptoms, decreased distal sensation, or unequivocally decreased or absent ankle reflexes.

Confirmed DPN: The presence of an abnormality of nerve conduction and a symptom or symptoms or a sign or signs of neuropathy confirm DPN. If nerve conduction is normal, a validated measure of small fiber neuropathy may be used." (2)

Despite the recommendations, the screening to confirm or exclude DPN is in most clinics limited to bedside examinations targeting the large nerve fiber function by testing the loss of sensation using subjective tests. The tests typically include vibration perception and 10-g monofilament.(3,43,44) Objective tests like the recommended nerve conduction studies and small nerve fiber function tests, such as heat and cold sensation tests, are not routinely used in clinical practice and are more commonly used in research settings.(35,45) Also, the diagnosis of neuropathic pain is primarily based on careful history taking after pain caused by other etiologies has been excluded. Screening tools like the validated Douleur Neuropathique en 4 (DN4) questionnaire for diabetic neuropathic pain can be used (ref), but it is also mostly used in research settings.(31,46) DPN and neuropathic pain are often detected when the presence of impairment and/or pain is permanent. Early detection of DPN and neuropathic pain remains a major challenge in the clinic.(45)

2.4. TREATMENT

Management of DPN includes efforts to alter the natural history and symptomatic treatments.(47) There are currently no disease-modifying treatments available for DPN.(36) Hence, the current approach to "treat" DPN is limited in the improving of the glycemic control, as it will partly prevent the progression of DPN.(48)
The treatment of neuropathic pain aims solely to relieve the symptoms.(3) The first-line treatments include drug classes of anticonvulsants, serotonin and noradrenaline reuptake inhibitors, and tricyclic antidepressants. The treatment of neuropathic pain is challenging since only one-third of the individuals with diabetic neuropathic pain experience 50% pain relief (49). Unfortunately, almost all the pharmacological agents are associated with side effects and low levels of satisfaction.(50,51) Treatment for DPN and more efficient and safer treatment for neuropathic pain are of great need. However, the limited understanding of the underlying mechanism and interactions between DPN and neuropathic pain also limits the development of treatments based on the mechanisms.

2.5. ROLE OF THE CENTRAL NERVOUS SYSTEM IN DIABETIC PERIPHERAL NEUROPATHY AND NEUROPATHIC PAIN

For more than 100 years, it has been known that the CNS is affected in individuals with diabetes.(52) The metabolic changes that cause neuronal damage to the PNS and the macro and microvascular disease in diabetes have also been suggested to be implicated in the CNS. The vast majority of the studies investigating CNS changes in diabetes have, for a long period, focused on individuals with diabetes in general without the specification or phenotyping of the presence of DPN or neuropathic pain.(53,54) Some of these studies have provided evidence that CNS alterations occur in diabetes *per se.* However, in the last decades, growing evidence has suggested the involvement of CNS in both DPN and neuropathic pain.(12,13,55,56) Though, such studies are still very limited.

The cognitive function reflects the performance of the CNS and may contribute to understanding the clinical implications of CNS alterations.(8) Until now, it has been suggested that cognitive function is affected in diabetes in cognitive domains such as information processing speed, executive functions, and memory.(57–60) Few studies investigated the association between cognitive function and DPN and found that individuals with diabetes and cognitive impairment had a higher prevalence of DPN.(61) Also, studies have demonstrated an association between peripheral nerve function and cognitive function.(62) Furthermore, pain-related cognitive impairment has been proposed, which may also be attributable to those with neuropathic pain.(8,63) However, the association and possible mechanisms of DPN and neuropathic pain on cognitive decline remain to be understood.

Recently, an increasing amount of brain imaging literature has provided insights into structural and functional changes of the brain in individuals with neuropathic complications. Still, the involvement of CNS in DPN and neuropathic pain continue to be far from fully understood. The existing literature has been focused on the role of CNS in neuropathic pain (covered in section 2.2.1), while studies investigating the involvement of CNS in DPN are even more sparse. The majority of the studies investigate brain alterations with different MRI modalities. Reduction in the spinal cord area measured with MRI was reported in individuals with DPN compared to healthy controls.(9,64) Interestingly, spinal cord atrophy was also observed in individuals with early subclinical DPN.(16) This important finding raise the question of whether the CNS is involved in the early stages of the pathogenesis of DPN.
Macrostructural changes in brain regions involved in sensory processing have been reported in individuals with DPN and individuals with neuropathic pain compared to

individuals with diabetes without DPN and healthy controls.(15,17) Thalamic neuronal dysfunction and increased vascularity have also been suggested using MR (magnetic resonance) spectroscopy, fMRI, and MR perfusion imaging both in individuals with DPN and neuropathic pain. (8,9,17,18) Additionally, abnormalities in the somatosensory cortex and other regions relevant to pain processing have reported to be altered in individuals with neuropathic pain.(10,65,66)

Previously, it has been suggested that CNS changes in individuals with DPN are secondary to PNS alterations. However, the detection of alterations in the CNS in subclinical and early DPN stages indicates that changes may occur before the clinical manifestation of PNS alterations.(16) Another alternative is that the CNS and PNS alterations arise simultaneously. A clear answer to this is still urged to be investigated by disentangling the unknown fact of the involvement of CNS in the pathophysiology of DPN and neuropathic pain.

2.6. ASSESSING BRAIN CHANGES USING MAGNETIC RESONANCE IMAGING

As previously mentioned, cognitive tests are useful to investigate CNS performance. To obtain information about structural and functional alterations of the brain, non-invasive neuroimaging techniques like computed tomography (CT), MRI, electroencephalogram (EEG), and position emission tomography (PET) exist.(67) Recent knowledge about changes in the brain of individuals with diabetes has increasingly been recognized using MRI. MRI has high spatial resolution and is without known risks even though the individuals are exposed to high-intensity magnetic fields. (67) The current PhD thesis will only focus on structural and functional MRI used in the thesis.

Obtaining structural MRI enables studying the brain's soft tissue as gray matter and white matter. The most commonly used MRI sequence to study gray matter volume (GMV) is the 3D T1-weighted image, a method used in this PhD thesis. Broadly, the MRI signal varies across tissue types. Thus, the T1-weighted scans, which show tissues with high fat content as bright, provide good contrast between gray matter, white matter, and cerebrospinal fluid. The gray matter tissue that contains neuronal cell bodies will appear as dark gray, white matter enriched with fat due to its myelinated axons will appear as lighter gray, and cerebrospinal fluid will appear dark or black.(67,68) Using diffusion-based MRI (diffusion tensor imaging), it is possible to reveal detailed information about brain neural integrity, microstructure, and white matter nerve fiber

connections. Diffusion tensor imaging will not be covered further in this PhD thesis.(67)

Resting-state fMRI can be used to map spontaneously activated brain regions without external stimulation or task.(67,69) The most common approach in fMRI uses the BOLD signal, which measures the ratio of oxygenated to deoxygenated hemoglobin in the blood across time.(67,69) Hence, the signal does not measure neuronal activity directly but measures the consumption of oxygen induced by neuronal activity. (69) Hemoglobin exists in two different states, the oxyhemoglobin, and the deoxyhemoglobin, where each of them has different magnetic properties producing different magnetic fields. In the oxyhemoglobin state, the hemoglobin carries oxygen and is diamagnetic, while in the deoxyhemoglobin state, the hemoglobin is not carrying oxygen, which makes it paramagnetic. The deoxyhemoglobin suppresses the MRI signal. Hence, when a region of the brain becomes active or the neuronal activity increases, the metabolic demand for oxygen also increases (oxygen consumption), and there will, in turn, be an increased blood flow. This leads to decreased deoxyhemoglobin levels and increased cerebral blood volume. Together this leads to increased fMRI signal in the active brain areas, which is attributed to the changes in the ratio of oxygenated and deoxygenated hemoglobin.(22,70)

2.7. CRITICAL ISSUES WITH DIABETIC PERIPHERAL NEUROPATHY AND NEUROPATHIC PAIN

DPN and neuropathic pain are usually diagnosed when the progression of nerve damage is well-established. Effective and curative treatments that target the pathogenesis of DPN and neuropathic pain are not established and unsatisfactory and remain the highest unmet needs.(8) Although different underlying mechanisms have been proposed, these do not seem to give a complete and clear picture of the development of DPN and neuropathic pain. Even less is understood of why some people develop DPN without pain and others develop DPN with neuropathic pain. Insufficient understanding of the underlying pathophysiology and interactions between DPN and neuropathic pain are major factors that prohibit early detection and development of disease-modifying treatments. There is thus an urgent need to establish a deeper understanding of the mechanistic changes that lead to DPN and neuropathic pain for early detection and new targets for treatments.

The DPN and neuropathic pain were classically considered a disease of the PNS. However, recently increasing evidence has suggested that CNS is involved in devel-

oping DPN and neuropathic pain.(6,9–18,39) Still the full extent of the CNS involvement remains unfulfilled. This is partly caused by the current limitations of most diabetes studies, which investigate CNS alterations without defining the presence or phenotyping of DPN or neuropathic pain. Approaches to establishing a deeper understanding of the CNS changes in diabetes groups phenotyped based on DPN with and without neuropathic pain still need to be met. This will contribute to distinguishing the groups based on CNS changes and may indicate the underlying mechanism of CNS in the development of DPN with and without neuropathic pain.

Evidence has also stated CNS alterations in diabetes *per se*.(12,13,55,56) Hence, there is also a need to distinguish the CNS alterations specific for diabetes *per se* and CNS alterations specific for DPN and neuropathic pain. A deeper understanding of the underlying pathogenesis will improve the risk stratification and management of DPN and neuropathic pain, but above all, it will help the person who is heavily suffering from DPN complications with foot ulcerations, amputations, and lower quality of life.(8,36)

CHAPTER 3.
AIMS AND HYPOTHESES

The overall aim of this PhD thesis was to provide a deeper understanding of CNS alterations in individuals with T1DM and neuropathic complications. The thesis explores the cognitive function by using cognitive tests, reflecting the performance of CNS, and explores the structural and functional properties of CNS using non-invasive brain MRI in T1DM with neuropathic pain, T1DM with DPN, T1DM without DPN, and healthy controls. These insights may contribute to a better understanding and description of the underlying mechanisms leading to neuropathic complications. The ultimate goal is provision of robust clinical endpoints for early detection, prevention, and hopefully therapeutic interventions in order to prevent and slow the progression of DPN and neuropathic pain.

The thesis is based on one published peer-reviewed original papers and two original papers submitted for publication. The three papers are based on a study named MEDON (Methods for Early Detection Of diabetic peripheral Neuropathy). The thesis consists of three aims that correspond to each paper (See Figure 3.1).

Aim 1: To compare cognitive function in T1DM with neuropathic pain, T1DM with DPN, T1DM without DPN, and healthy controls and explore the association to peripheral nerve function and clinical measurements.

The hypothesis was that individuals with T1DM and neuropathic complications have more pronounced cognitive alterations compared to T1DM without neuropathic complications, and these alterations would associate with peripheral nerve function.

Aim 2: To compare structural brain alterations in T1DM with neuropathic pain, T1DM with DPN, T1DM without DPN, and healthy controls and explore the association to cognitive parameters, peripheral nerve functions, and clinical measurements.

The hypothesis was that structural brain alterations would be more pronounced in T1DM with neuropathic complications compared to T1DM without neuropathic complications, and the alterations would associate with peripheral nerve function.

Aim 3: To compare functional resting-state brain alterations in regions related to sensory processing (thalamus, primary somatosensory cortex, and insula) in T1DM with

neuropathic pain, T1DM with DPN, T1DM without DPN, and healthy controls and explore the association to peripheral nerve function and pain intensity.

The hypothesis was that individuals with T1DM and neuropathic complications have functional alterations in the specified brain regions, and the findings would correlate to peripheral nerve function and pain intensity.

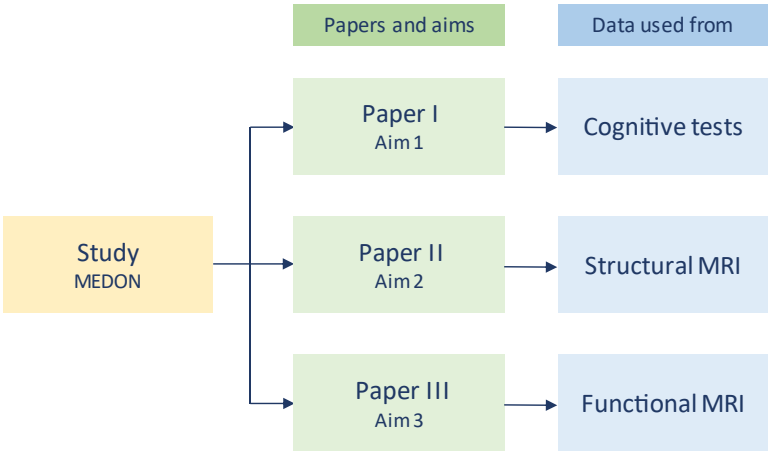

Figure 3.1. Overview of study, papers, aims, and data used in each paper. Abbreviations: MRI: magnetic resonance imaging

CHAPTER 4.
MATERIALS AND METHODS

The results of this PhD thesis were based on data collected from a larger clinical study, MEDON.(71) The study was conducted according to the Declaration of Helsinki. The North Denmark Region Committee on Health Research Ethics granted the ethical approval (N-20190003), and registered with clinicaltrials.gov (NCT04078516). Before enrollment in the study, informed consent was obtained from participants after oral and written information was given. The MEDON study will be presented below, and only methods used in this PhD thesis will be presented in detail.

4.1. MEDON STUDY

The MEDON study was a collaboration study conducted at the Department of Endocrinology, Steno Diabetes Center North Denmark and Department of Radiology at Aalborg University Hospital, Denmark. In this cross-sectional, observational, case-control study, T1DM participants with and without neuropathic complications and healthy controls went through several sessions to prospectively investigate the CNS and PNS using different methods. An overview of the methods is illustrated in figure 4.1. The MEDON study included four groups of participants between August 2019 and April 2021. The groups were 1) 20 individuals with T1DM and neuropathic pain, 2) 20 individuals with T1DM screened for probable DPN, 3) 20 individuals with T1DM without DPN, and 4) 20 healthy controls. Each participant in each group was age and sex matched to a participant in the other three groups. This was ensured by initially recruiting the least prevalent group of participants, those with neuropathic pain. Then the rest of the participants for the other three groups were recruited based on age (+/- 2 years) and sex of each participant in the neuropathic pain group. The individuals with diabetes were recruited through the outpatient clinic at the Department of Endocrinology, Steno Diabetes Center North Denmark, Aalborg University Hospital, and the healthy controls were recruited through a local database.

The individuals with T1DM were pre-screened using their medical records, and at the screening session, they were assigned to their respective groups. Participants aged between 18 and 70 years were included in the study. Exclusion criteria included previous or current alcohol and/or drug abuse, vitamin- or immune deficiencies, presence of severe or chronic viral infections or other diseases known to cause neural damage,

abnormalities in the thyroid- or parathyroid metabolism, impaired liver- or kidney function, known ischemia of the lower extremities, severe skin disease; pregnancy, hematologic diseases including cancer, active cancer disease and previous chemotherapy or consumption of experimental medicine. The exclusion criteria ensured that no other causes of neuropathy were present in the cohort. Data for this PhD thesis were obtained in session 3 (see. Figure 4.1).

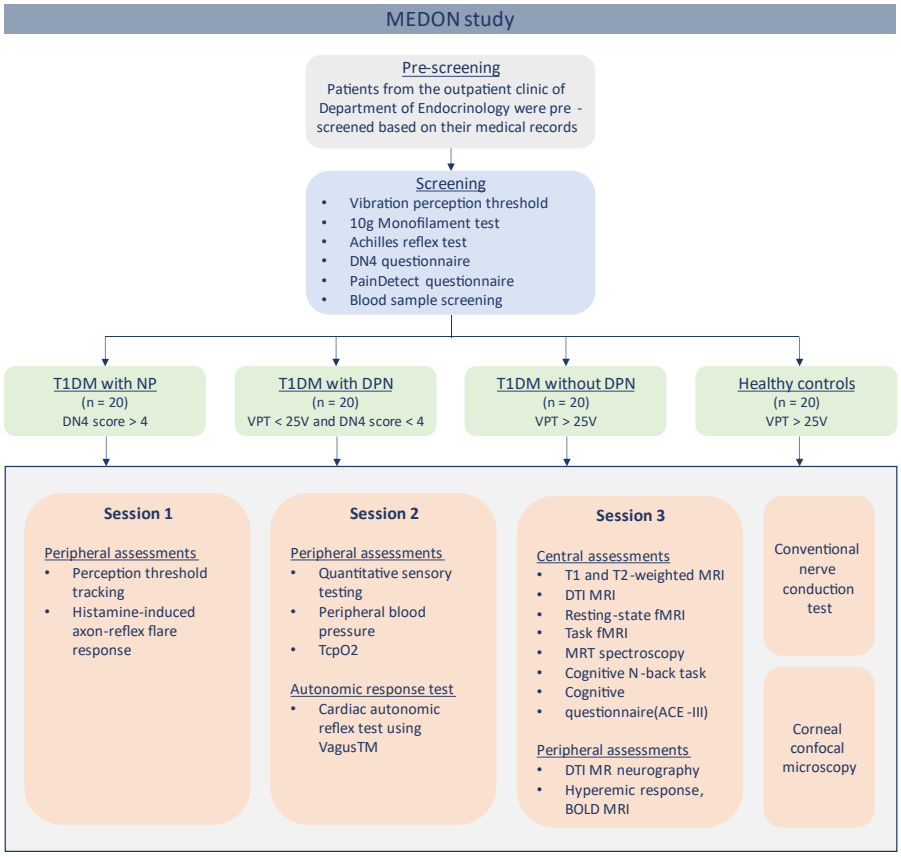

Figure 4.1. Overview of the methods used in MEDON study. Session 1: Assessment of peripheral small nerve fiber function using perception threshold tracking and axon reflex flare response provoked by epidermal histamine. Session 2: Assessment of peripheral small and large nerve fiber functions using quantitative sensory testing, assessments of peripheral blood pressure to ensure pain was of neuropathic origin and not caused by ischemia, and assessment of transcutaneous oxygen pressure. Session 3: Besides the cognitive tests and MRI modalities used in this PhD thesis, several other MRI modalities were obtained to assess central and peripheral nerve alterations. Furthermore, two additional smaller sessions were performed using conven-

tional nerve conduction study to test the peripheral large fiber nerve functions and corneal confocal microscopy to assess corneal small nerve fibers. Abbreviations: ACE-III: Addenbrooke's Cognitive Examination III, DN4: Douleur Neuropathique en 4, DPN: diabetic peripheral neuropathy, DTI: diffusion tensor imaging, fMRI: functional magnetic resonance imaging, MR: magnetic resonance, MRI: magnetic resonance imaging, NP: neuropathic pain, T1DM: type 1 diabetes mellitus, TcPO2: transcutaneous oxygen pressure, VPT: vibration perception threshold.

Figure 4.2. An illustration of a participant A) in the MR scanner and B) performing the N-back cognitive task.

4.2. PHENOTYPING THE PARTICIPANTS

The participants in the diabetes group with neuropathic pain were phenotyped based on clinical confirmation by two independent medical doctors and further supported by the DN4 questionnaire, a validated screening tool to identify diabetic neuropathic pain.(46) The 10 items questionnaire includes seven items related to pain description and abnormal sensations and three items of neurological examination in the painful area.(46,72) Participants with a score equal to or above 4 were classified as having neuropathic pain.

In the MEDON study, the absence of DPN in healthy controls and in the T1DM group without DPN was ensured by a normal vibration perception threshold (lower than 25 V) and absence of neuropathic symptoms. The phenotyping of the DPN group was based on an abnormal vibration perception threshold (above 25 V). The test was performed using a biothesiometry on the participant's first toe. However, in the studies investigating CNS alterations (Paper I-III), the presence and absence of DPN were additionally confirmed based on the nerve conduction study according to the Toronto

consensus.(2) Thus, those T1DM participants without DPN having normal vibration but abnormal nerve conduction measurements and those T1DM participants with DPN and absence of abnormal nerve conduction measurements were excluded from the data analysis.

4.3. ASSESSMENTS OF LARGE AND SMALL NERVE FIBER FUNCTION

The nerve conduction study tested the peripheral large nerve fiber function and was performed on the right leg by trained neurophysiologists at the Department of Neurophysiology, Aalborg University Hospital. The nerve conduction velocities, amplitudes, and F-waves of the motor nerves (peroneal, tibial, and ulnar) and sensory nerves (sural, radial, and median nerves) were assessed following the clinical standard.(73) Warming measures were used to ensure standardized skin temperature at 32°C. For recording digital sensory nerve action potential, spring-ring electrodes were used. For all other nerves, Nutab Diagnostic Tab Electrodes were used. The results were processed according to the local clinical reference values. Measurements not detectable due to severe nerve damage were denoted with zero.

4.4. ASSESSMENTS OF SMALL NERVE FIBER FUNCTION

The peripheral small nerve fiber function was assessed using warm and cold detection thresholds.(74,75) The test was performed using a thermal sensory testing device (Thermal Sensory Analyzer (TSA), Medoc, Israel). The thermode was placed 2-3 centimeters proximal to the second toe, and the detection thresholds were obtained by continuously increasing or decreasing the thermal stimuli. The examinations were conducted in a standardized room and skin temperature (Figure 4.1, Session 2).

4.5. ASSESSMENTS OF COGNITIVE FUNCTION

The cognitive assessments were implicated in session 3 (Figure 4.1, session 3). More technical details of the tasks are described in Paper I.

4.5.1. ADDENBROOKE'S COGNITIVE EXAMINATION III

The participants completed Addenbrooke's Cognitive Examination-III (ACE-III), which is a screening tool to differentiating individuals with and without cognitive impairment.(76) The cognitive questionnaire test five cognitive domains: attention, memory, verbal fluency, language, and visuospatial abilities. The highest score of the test is 100 points, which is allocated in the five domains as follows: attention 18 points, memory 26 points, verbal fluency 14 points, language 26 points, and visuospatial abilities 16 points. Higher scores indicate better cognitive performance.(76)

4.5.2. VISUAL N-BACK TASK

The visual N-back task assesses the cognitive domains of working memory and psychomotor speed.(77) The task consisted of presenting a series of visual stimuli, which in this case was letters. The participants had to respond to a letter presented *N* trials previously. 0-back, 1-back, and 2-back tests were used. The load of the task is defined by the number of *N*, where a higher number of *N* makes the task more difficult. Thus, the 2-back task means that the participants have to respond to letters presented two trials back. In the 0-back task, one has to respond to a predetermined letter. See Figure 4.2 B.

4.6. ASSESSMENTS OF BRAIN STRUCTURE AND FUNCTION

MRI was used to assess structural and functional alterations of the brain in session 3 (figure 4.1). The following sections describe the two MRI techniques used in Paper II and Paper III. Detailed descriptions of MRI sequence settings of each method can be found in the respective papers. See Figure 4.2 A.

4.6.1. STRUCTURAL BRAIN MRI

One of the most commonly used MRI sequences to assess gray matter alterations is the T1-weighted.(78) The images make it possible to perform voxel-based morphometry (VBM) analyses, which allows the investigation of GMV between two or more groups of subjects.(79) The VBM analysis involves a relative voxel-wise comparison of regional gray matter "density", and hereby the presence of possible gray matter atrophy can be assessed.(80) Before the statistical analysis, the VBM procedure includes some preprocessing steps of the images, which is an essential part of MRI analysis. The preprocessing steps ensure to 1) minimize the influence of data acquisition and physiological artifacts, 2) check statistical assumptions and transform the data to meet these assumptions, and 3) standardize the location of brain regions across different subjects to achieve validity and sensitivity in group analysis.(22,80)

The preprocessing steps for VBM analysis include a) segmentation of the gray matter, white matter, and cerebrospinal fluid, b) normalization, which is a form of image registration to a standard brain anatomical reference space using DARTEL, which allows comparisons of subjects with different brain morphologies, and c) smoothing, to partly overcome limitations in the normalization step by blurring any residual anatomical differences and partly due to the Gaussian random field theory, which is a multiple-comparisons correction procedure, that requires a certain degree of smoothness. (22,80) The statistical analyses were performed after these steps, also briefly described in section 4.7. See figure 4.3 for an overview of the data analysis.

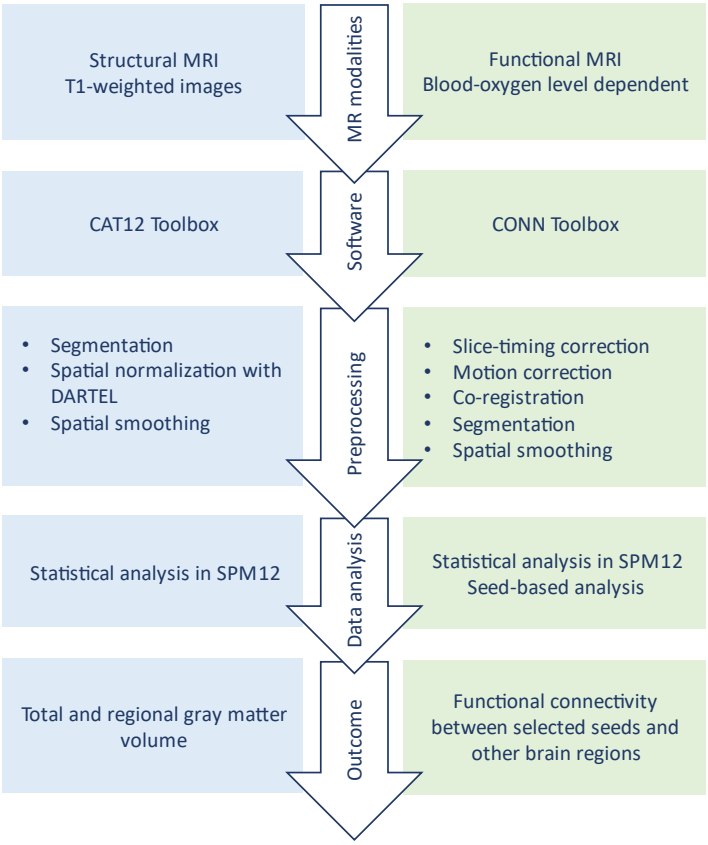

Figure 4.3. Overview of the MRI data analysis. Two MRI modalities were used in the current PhD thesis. Structural MRI was used to assess alterations in the gray matter volume, and functional MRI was used to assess brain connectivity of regions involved in sensory processing to other brain areas. Abbreviations: CAT12: Computational Anatomy Toolbox, MRI: magnetic resonance imaging, SPM12: Statistical Parametric Mapping.

4.6.2. FUNCTIONAL BRAIN MRI

Functional MRI was obtained using gradient echo-planar imaging. As well as the structural images, the fMRI data also undergoes a series of preprocessing steps before the statistical analysis and include slice timing correction, motion correction, segmentation, normalization, and smoothing.(22) Slice timing: The fMRI is obtained slice-wise, which causes some slices to be obtained later than others. In the statistical analysis, it is assumed that every voxel in an image is obtained simultaneously. Thus, the slice time correction shifts each voxel's time series so that the voxels appear to have been obtained simultaneously.(22) Motion correction: It is assumed that a particular voxel depicts the same region of the brain at every time point during the time series. Thus, if the head moves between the acquisitions, the voxel's signal intensity will be "contaminated" by the signal from the neighbor voxels. The motion correction eliminates these "contaminations" by rotating and translating each subject's image.(22) Co-registration: In this step, the 3D T1-weighted structural image is aligned with the functional images and allows the transformation of fMRI images to a standard coordinate system.(22) The final preprocessing steps are segmentation, normalization, and smoothing, performed similarly to the structural images described in section 4.6.1. After these preprocessing steps, statistical analyses were performed, which are briefly described in section 4.7. See figure 4.3 for an overview of the data analysis.

4.7. CLINICAL PARAMETERS

Clinical and demographic data, including sex, age, body mass index, disease duration, age of diabetes onset, retinopathy, and nephropathy, were obtained during the screening session. Also, blood samples were obtained to analyze Hemoglobin A1c (HbA1c). Furthermore, blood glucose levels were assessed in session 3 to avoid high levels when MRI and cognitive data were obtained. High glucose levels may affect these data.

4.8. STATISTICAL ANALYSES

Different statistical models were used in the three papers depending on the data and aims. Details on the statistical analyses are reported in the three papers. Briefly, one-way analysis of variance (ANOVA) or Kruskal Wallis test was used for the comparisons between the groups of the clinical data. Chi-squared or Fisher's exact test was used for the binary data. One-way ANOVA or Analysis of Covariance (ANCOVA)

was used to investigate group differences in the MRI data. Pearson or Spearman correlation tests were used for the correlation analyses. *P*-value less than 0.05 was considered statistically significant.

CHAPTER 5.
KEY RESULTS

The overall aim of this PhD thesis was to provide a deeper understanding of CNS alterations in individuals with T1DM and neuropathic complications. To address this, the key findings from the three papers underlying this thesis and how they are employed to answer the three aims of the thesis are presented in the following chapter presents. Detailed descriptions of all the results are reported in Paper I-III. Figure 5.1 illustrates an overview of the key results.

	T1DM without DPN	T1DM with DPN	T1DM with NP
Cognitive alterations	No cognitive alterations	No cognitive alterations	Lower memory score compared to HC
Structural brain alterations	• Reduced total GMV compared to HC • Reduced volume of insula compared to HC • Reduced volume of primary somatosensory cortex compared to HC	• Reduced volume of insula compared to HC • Reduced volume of cerebellum compared to HC	• Reduced total GMV compared to HC • Reduced volume of insula compared to HC • Reduced volume of thalamus compared to HC • Reduced volume of hippocampus compared to HC
Functional brain connectivity alterations	• Higher connectivity between thalamus and cortical regions including motor areas compared to T1DM with NP and HC • Higher connectivity between primary somatosensory cortex and other cortical regions including motor areas compared to T1DM with NP	No functional alterations	• Lower connectivity between thalamus and cortical regions including motor areas compared to T1DM without DPN • Lower connectivity between thalamus and caudate compared to HC • Lower connectivity between primary somatosensory cortex and other cortical regions including motor areas compared to T1DM without DPN

Figure 5.1. Overview of the results across methods and phenotyped T1DM groups. Abbreviations: DPN: diabetic peripheral neuropathy, GMV: gray matter volume, HC: healthy controls, NP: neuropathic pain, T1DM: type 1 diabetes mellitus.

5.1. AIM 1

Aim: To compare cognitive function in T1DM with neuropathic pain, T1DM with DPN, T1DM without DPN, and healthy controls and explore the association to peripheral nerve function and clinical measurements (Paper I).

Key results:

- T1DM with DPN showed a lower memory score compared to healthy controls (p=0.013)
- No differences were observed in the other groups (p>0.050).
- The cognitive measurements were not significantly related to clinical or peripheral nerve conduction measurements (all p>0.050).

Interpretation: Individuals with T1DM and DPN may be more vulnerable to memory decline than the other two diabetes groups. The cognitive alterations were not associated with clinical or peripheral nerve measurements suggesting that other factors not investigated in the current study may contribute to the cognitive alterations observed. However, it is essential to pay attention to the small sample size of the phenotyped groups, and caution must be applied in the interpretation.

5.2. AIM 2

Aim: To compare structural brain alterations in T1DM with neuropathic pain, T1DM with DPN, T1DM without DPN, and healthy controls and explore the association to peripheral nerve functions, cognitive parameters, and clinical measurements (Paper II).

Key results:

- Lower total GMV was observed in T1DM without DPN (P=0.019), and T1DM with NP (p=0.024) compared to healthy controls.
- The volume of insula was lower in T1DM without DPN (p≤0.004), T1DM with DPN (p=0.006), and T1DM with NP (p=0.050) compared to healthy controls.
- Lower volume of primary somatosensory cortex was observed in T1DM without DPN compared to healthy controls (p=0.010). Lower volume of cerebellum was observed in T1DM with DPN compared to healthy controls (p≤0.018). Lower thalamus and hippocampus volume were observed in T1DM with NP compared to healthy controls (p≤0.018).

- Lower memory score was associated with lower total GMV in the group with T1DM and DPN (p=0.003).
- The total GMV was not significantly associated with peripheral nerve conduction and clinical measurements (all $p \geq 0.050$).

Interpretation: Total GMV and insula volume seem to be affected in T1DM regardless of the underlying neuropathic complications. This was further supported by the lack of association between total GMV and peripheral nerve function measurements. However, lower volume of brain regions specific to the three phenotyped groups was observed, potentially providing information on the underlying brain pathophysiology behind neuropathic complications. No association between total GMV and clinical measurements like diabetes duration and HbA1c was observed, suggesting that these factors were not the main reason for GMV alterations.

5.3. AIM 3

Aim: To compare functional resting-state brain alterations in regions related to sensory processing (thalamus, primary somatosensory cortex, and insula) in T1DM with neuropathic pain, T1DM with DPN, T1DM without DPN, and healthy controls and explore the association to peripheral nerve function and pain intensity (Paper III).

Key results:

- Higher thalamic connectivity to supplementary motor cortex and superior frontal cortex were observed in T1DM without DPN compared to T1DM with NP and healthy controls (all $p \leq 0.011$). The other way around, the T1DM group with neuropathic pain showed lower thalamic connectivity compared to T1DM without DPN.
- Lower thalamic connectivity to caudate was demonstrated in T1DM with NP compared to healthy controls (p=0.017).
- Lower primary somatosensory cortex connectivities to other cortical areas, including the precentral gyrus, were observed in T1DM with neuropathic pain compared to T1DM without DPN and healthy controls (all $p \leq 0.029$).
- No connectivity alterations were found between insula and other brain regions ($p > 0.050$).
- The thalamic and primary somatosensory cortex connectivities to motor areas were associated with most peripheral nerve function measurements and pain intensity (all $p \leq 0.043$).

Interpretation: Overall, the T1DM group without neuropathic complications showed increased thalamic and primary somatosensory cortex connectivity to other cortical regions, while those with neuropathic pain had decreased connectivity. This suggests that higher connectivity of the thalamus and primary somatosensory cortex may contribute as a compensatory mechanism to preserve normal sensory processing. This was further supported by the associations between the connectivity parameters and peripheral nerve measurements and pain intensity; lower connectivities were associated with poorer nerve functions and higher pain scores. The associations were more profound for the connectivity parameters of thalamus and may propose this for further investigation and as a potential indicator for DPN complications.

CHAPTER 6.
DISCUSSION

Cognitive tests and structural and functional MRI techniques were used to get aspects of CNS changes in individuals with T1DM without DPN, T1DM with DPN, and T1DM with neuropathic pain. The findings included in this PhD thesis were based on the MEDON study. This discussion is divided into several parts. First, the cognitive manifestations of potential changes in the CNS will be discussed. Secondly, the brain changes, including structural and functional brain changes, will be addressed. Thirdly, methodological considerations will be outlined together with future perspectives.

6.1. COGNITIVE ALTERATIONS

There is a vague indication in the literature that cognitive alterations are associated with the presence of DPN.(61,62) This is mainly due to the lack of studies directly investigating the cognitive impairment in well-phenotyped groups of diabetes with neuropathic complications. Paper I investigated several cognitive domains in T1DM groups with and without neuropathic complications.(81) The diabetes group with DPN showed a significantly lower memory score compared to healthy controls. This difference was not observed when comparing the three phenotyped diabetes groups.

Cognitive alterations have been associated with CNS alterations especially structural brain atrophy. Paper II demonstrated lower volume in cerebellum of the DPN group compared to healthy controls.(82) While the cerebellum is essential for controlling movement, it has also been recognized to be highly involved in cognitive processing.(83) It is hence questionable if the lower volume of cerebellum observed in the DPN group may contribute to the lower memory score followed in the same group. Although Paper I suggested that the DPN group has impaired memory, it is also important to mention other factors that may have contributed to the memory alterations observed. There is evidence that hypoglycemia and hyperglycemia impact cognitive function.(84–87) In the current study, HbA1c was highest in the group with DPN, and could therefore have influenced the cognitive alterations observed in the current group.

Paper I also investigated the cognitive features when merging all three diabetes groups, where the common denominator was T1DM. Interestingly, when comparing

this combined group to the healthy controls, several cognitive domains were observed to be altered. This included lower total cognitive score, which summed up scores from several cognitive domains, lower memory score, and poorer reaction time (psychomotor speed) in the N-back task. Since the common denominator for the merged group was T1DM, it may suggest that having diabetes itself contribute to cognitive alterations. This may partly explain the lack of cognitive differences between the three phenotyped diabetes groups. The theory is further supported by several studies, which have demonstrated cognitive decrements in children and adolescents with T1DM, who may not have developed DPN or neuropathic pain.(84) As early as two years after diabetes onset in children, cognitive function is observed to be affected.(88)

The results of Paper I may be interpreted with caution since the sample size of the three diabetes groups is a limitation. Other studies investigating cognitive impairment in diabetes have included larger sample.(89) This may also partly explain the lack of cognitive differences observed between the three diabetes groups. Also, the finding that only one cognitive domain was altered in the DPN group compared to healthy controls questions the validity of the results. However, the memory domain is one of the common cognitive domains commonly reported to be altered in T1DM.(84,89) Hence, finding alterations in this particular domain indicates that memory in the DPN group is attenuated to a detectable degree even with small sample size. The majority of studies investigating the involvement of DPN in cognitive function have been conducted on type 2 diabetes.(90,91) These studies have indeed found an association between DPN and cognitive alterations. Even though the DPN group was more likely to present with impaired memory in Paper 1, caution to the results may be applied, and larger sample size in the phenotyped groups is required to make any conclusions.

6.2. STRUCTURAL BRAIN ALTERATIONS

Paper II showed GMV alterations in all three diabetes groups. Compared to healthy controls, the total GMV was lower in the diabetes group with neuropathic pain and in the T1DM group without DPN. No significant differences in total GMV were observed between the three diabetes groups.(82) Overall, these data provide the knowledge that GMV is altered in T1DM regardless of the presence of DPN or neuropathic pain. This was further supported by the lack of association between total GMV and peripheral nerve function.

Limited studies have investigated structural brain changes in groups phenotyped based on DPN and neuropathic pain. These have primarily reported gray matter alter-

ations in T1DM group with DPN and T1DM group with neuropathic pain, while alterations in T1DM group without DPN were not observed.(10,17) Latter was in contrast to Paper II. Nevertheless, the mentioned studies were based on groups of small sample sizes.(10,17) However, other studies investigating GMV alterations in T1DM without other diabetic complications, including DPN, have reported total or regional GMV loss.(12,92,93) These are in line with Paper II, indicating structural brain alterations regardless of underlying DPN and neuropathic pain.

In paper II regional GMV alterations specific for each diabetes group were found. Volume loss of the primary somatosensory cortex was identified in the T1DM group without DPN compared to healthy controls. The primary somatosensory cortex is highly involved in sensory processing, but similar results were not observed in the T1DM group with DPN nor the T1DM group with NP, both experience sensory abnormalities. Other studies have reported the somatosensory cortex volume alterations in diabetes with neuropathic complications.(10,17) However, the findings were based on cortical thickness measures rather than local GMV changes used in Paper II. Another possible explanation for the somatosensory alterations in the T1DM group without DPN may be the general central plasticity experienced in individuals with diabetes. Especially structural alterations have been reported not only in those experiencing neuropathic complications but also in diabetes *per se* as outlined previously.(10,12,13,17,92,93)

The thalamus is a core brain structure in the spinothalamic tract and the sensory/pain processing.(94) In Paper II lower volume of thalamus was observed in the diabetes individuals experiencing neuropathic pain.(82) The structural changes may contribute to the development of neuropathic pain but also to the functional thalamic connectivity alterations observed in the same group in Paper III. This will be discussed later in section 6.3. Other studies have also found volume alterations of the thalamus in diabetes groups with neuropathic complications, primarily with DPN, but also in diabetes groups without DPN.(13–15,17,18)

Paper II suggested no association between GMV and peripheral nerve function and diabetes-related clinical assessments, contradicting other studies.(15,17,18) However, Paper II was limited by using only the total GMV parameter for the correlation analysis. A direct association to regional GMV, for instance, the thalamus volume or any of the other regions altered, may contribute to understanding the alterations that occurred in specific brain regions of each diabetes group. Furthermore, the paper only included VBM analyses. Studies have reported alterations in cortical thickness of somatosensory cortex in individuals with diabetes and neuropathic complications.(10)

Hence, including other analysis methods may also contribute to other relevant findings.

The absence of DPN in the diabetes group without DPN was solely based on normal peripheral large nerve fiber function assessed with nerve conduction studies. Thus, individuals with small nerve fiber abnormalities detected with thermal thresholds were not excluded. Small nerve fiber abnormalities may indicate early stages or subclinical DPN. Hence, it can not entirely be excluded that the structural alterations in the T1DM group without DPN are not a result of small nerve fiber neuropathy.

Although the results from Paper II propose that GMV is altered in T1DM regardless of the presence of neuropathic complications, it also raises the question of whether the structural brain alterations in the T1DM group without DPN are an indicator for subclinical DPN or just related to diabetes. Furthermore, whether the structural brain alterations observed may contribute to abnormal sensory perceptions in later stages is still urged to be investigated. One way to address this is to perform longitudinal studies.

6.3. FUNCTIONAL BRAIN ALTERATIONS

In Paper III increased functional resting-state connectivity of both thalamus and primary somatosensory cortex to other brain regions in the cortex was observed in T1DM without DPN compared to T1DM with neuropathic pain and healthy controls. In turn, this also means that the connectivity of the thalamus and primary somatosensory cortex was lower in those with neuropathic pain compared to those without DPN. No connectivity difference was observed in the T1DM group with DPN compared to the other groups.(95)

As mentioned in the previous sections thalamus and primary somatosensory cortex are regions of the spinothalamic tract and are highly involved in sensory processing.(94,96,97) Hence, the higher connectivity of the two regions in the T1DM group without neuropathic complications, together with the insight in the existing literature, raises several speculations. It is speculated whether the higher connectivity may work as a compensatory mechanism to preserve normal sensory perception. This was further supported by the fact that lower connectivity of the thalamus and of the primary somatosensory cortex was associated with poorer nerve functions and higher pain intensity scores. In turn, those with higher connectivity had more preserved peripheral nerve functions and lower pain scores. A functional brain compensatory mechanism has been suggested in another study investigating children with

T1DM.(98) This study demonstrated that greater brain functional modulation was associated with better cognitive performance and contributed to preserving the normal ability to cognitive function.(98) The fact that a compensatory functional brain mechanism is observed in children raises additional speculations: whether the increased connectivities of the thalamus and primary somatosensory cortex reported in Paper III are innate to all T1DM individuals and whether the loss of this higher connectivity will contribute to the development of neuropathic complications. Alternative speculation is that increased connectivity will be developed in some people with diabetes and those not developing are more vulnerable to developing neuropathic complications. Nevertheless, this connectivity pattern is an interesting finding that may play a key role in sensory dysfunction and the speculations may be addressed in longitudinal studies.

In Paper III, the thalamic connectivity was stronger associated with the peripheral nerve function assessments than the primary somatosensory cortex connectivity, a finding that the anatomy of the spinothalamic tract may explain. The peripheral sensory inputs are initially received to the thalamus and transmitted through the third-order neurons to the primary somatosensory cortex.(94) The more “direct” input of peripheral sensory input to the thalamus may indicate a better association with peripheral nerve measurements. Also, a recent study using resting-state fMRI reported a similar association between thalamic connectivity and peripheral nerve functions in a mixed group of T1DM and type 2 diabetes with neuropathic complications. They reported that lower connectivity between the thalamus and cortical areas as the somatosensory cortex was associated with more severe nerve function deficits.(99)
In Paper III it was possible to roughly distinguish between those T1DM with DPN/neuropathic pain and those T1DM without DPN based on the thalamic connectivity parameters,. This indicates a potential biomarker in the future for individuals with T1DM who are at risk of developing peripheral neuropathic complications. Another study has proposed the possibility of classifying different phenotypes of diabetic neuropathic pain by using resting-state fMRI.(99)

Most fMRI studies in T1DM are performed without the phenotyping of neuropathic complications or performed with peripheral stimuli.(10) Resting-state fMRI studies in individuals with T1DM phenotyped based on their neuropathic complications are limited. However, the few existing have reported promising results, including the results from Paper III.(19,95,99) Collectively, these findings suggest that the quick 6-minutes resting-state fMRI, without relying on external stimulation tasks, as a promising method in the study of understanding CNS involvement and study of biomarkers for early detection of neuropathic complications.(8)

One major drawback of Paper III was that the individuals with diabetes were not free of analgesics which may affect the resting-state fMRI results. Especially the group with neuropathic pain, who were more vulnerable to intake treatment for their underlying pain, are at greater risk of affected data. However, alterations in the thalamus and primary somatosensory cortex have also been reported in other studies in T1DM with neuropathic complications.(10,15,18,19) Hence the results of Paper III are suggested to be reliable. Furthermore, Paper III investigated only three brain regions relevant for sensory and pain processing. Other sensory or pain-related regions, such as the anterior cingulate cortex, which have been reported to have an increased cerebral blood flow in individuals with diabetic neuropathic pain (65), may also be included in the resting-state fMRI analysis.

Paper II and Paper III reported common brain regions (thalamus and primary somatosensory cortex) both structurally and functionally altered but not consequently in the same phenotyped diabetes group. Hence, structural and functional MRI findings seem not to be directly associated with each other. This may be attributable to the fact that structural brain changes seem to be a more generalized finding of T1DM, while functional brain alterations, especially in the thalamus and primary somatosensory cortex, seem to be more related to peripheral sensory dysfunction.

6.4. LIMITATIONS

Limitations of Paper I, II, and III are summarized below and addressed more thoroughly in each paper.

Paper I investigated the cognitive alterations in diabetes individuals. ACE-III was an abbreviated cognitive questionnaire, which is not validated in diabetes. However, to our best knowledge, there are no validated cognitive questionnaires for individuals with diabetes. This may also be a possible explanation that only one cognitive domain from ACE-III was reported to be altered in the T1DM group with DPN. A more comprehensive neuropsychological assessment will be needed to detect more subtle cognitive dysfunction. Paper I did not provide any information about the confounding demographic variables like educational level and intelligence quotient since the study did not obtain this information. These factors may have an impact on the cognitive results.(76)

Paper I, II, and III were all based on the MEDON study. Hence, they share some common strengths and study limitations. As MEDON study is a larger collaboration project including several methods, the sample size of the phenotyped groups was not

based on the cognitive alterations nor on the structural and functional brain alterations. More exact, the sample size calculations were based on the measurement of sensory nerve fibers. The sample size not based on CNS alterations may contribute to undetected changes between the diabetes groups. However, previous studies have demonstrated that a sample size less than 20 was sufficient to detect CNS alterations, especially structural and functional brain alterations in phenotyped DPN groups.(10,17) Moreover, to our knowledge, this is the first study investigating CNS alterations comparing several T1DM groups phenotyped based on DPN and neuropathic pain with larger sample size.

The included diabetes groups were phenotyped based on peripheral large nerve fiber nerve functions. However, studies have suggested that diabetes individuals not diagnosed with DPN still may be presented with small nerve fiber dysfunction.(36,38) This also applied to the T1DM group without DPN in the MEDON study, where abnormal thermal detection thresholds represented small fiber alterations. Small nerve fiber dysfunction represents the early stages of DPN, which precedes large nerve fiber dysfunction. Hence, it may not be excluded that early alterations of the CNS also occur in the T1DM group without DPN. Additional analyses that regroup the T1DM individuals based on their small nerve fiber alterations may add valuable indication about early CNS alterations in early DPN stages.
The T1DM group with neuropathic pain was heterogeneous, with different levels of severity and duration of pain and differences in the presence of large and small nerve fiber dysfunction. This nature of neuropathic pain suggests that neuropathic pain is not one group but can be divided into several phenotypes also indicated by other studies.(100,101) Deep phenotyping of the individuals with DPN and neuropathic pain will provide additional essential pathophysiological insights.

Another limitation was that the included participants were not free of medications. Hence, central acting medications like analgesics, mainly used by the group with neuropathic pain, may influence the data collected, especially the cognitive function (Paper I) and the functional brain alterations (Paper III). At last, the study was a cross-sectional design. Hence, it is not possible to conclude the true causal relationship between CNS abnormalities and the underlying neuropathic complications.

6.5. CLINICAL IMPLICATIONS AND FUTURE PERSPECTIVES

Giving a deeper understanding of the underlying mechanisms of DPN and neuropathic pain may contribute to the early detection and identification of mechanism-based treatments. The first step of this strategy was done in this PhD thesis by giving a

deeper understanding of the CNS alterations and potential involvement in T1DM groups phenotyped based on with and without DPN and neuropathic pain. The PhD thesis suggests that the type of CNS parameters used, like the cognitive, structural brain, and functional brain parameters, is essential in connecting the CNS alterations to neuropathic complications. This knowledge contributes to improving insight into understanding the involvement of CNS in DPN and neuropathic pain but also general in T1DM. Especially, functional brain parameters like thalamic connectivity have shown great potential to serve as biomarkers for the early detection of neuropathic complications, especially neuropathic pain. However, as the study was of cross-sectional design, the following step to fulfilling the strategy for understanding and developing central biomarkers for early detection is to perform longitudinal studies.

Studies have revealed that DPN and neuropathic pain groups can additionally be phenotyped based on the individuals' sensory profiles.(102,103) Hence, as also briefly mentioned in section 6.5, future studies may focus on deep-sensory phenotyping to further understand the involvement of CNS in DPN and neuropathic pain.

Regarding the MRI-related future perspectives, several other promising MRI modalities assessing CNS may be used. Even though this PhD thesis only included volumetric and resting-state fMRI data, the overall MEDON study also obtained other relevant MRI modalities of the CNS that need further investigation. The modalities include MR spectroscopy of pain-related regions, including the thalamus and anterior cingulate cortex. This method allows the study of metabolites in the selected regions. Furthermore, diffusion tensor imaging was obtained to assess microstructural white matter changes and structural connectivity. fMRI was also obtained when performing a cognitive task, which is relevant to investigating functional connectivity and activity of the brain in other stages than rest. Combining several MRI modalities of CNS may provide valuable insight into whether the different parameters may interact and contribute to DPN and neuropathic pain and may also be a better predictor for early detection.

Future research should also address the interaction between the CNS and PNS in DPN and neuropathic pain. Most CNS studies including Paper I-III investigate CNS alterations to clinical assessments of the PNS. These tests are either limited by their subjective approach (vibration perception threshold, thermal perception threshold ect.) or the more objective measurements like nerve conduction study are not able to detect severe nerve damages. Hence, using MR neurography of the peripheral nerves may provide anatomical pathological information.(104) Thus, using a multi-level MRI approach of the CNS and PNS may provide a direct association and provide potential

information about the interaction between the nervous systems. The mentioned peripheral modality was also obtained in the MEDON study, and will in the future be analyzed and investigated.

CHAPTER 7.
CONCLUSION

This PhD thesis had three aims to provide a deeper understanding of CNS alterations in individuals with T1DM and neuropathic complications.

In summary, it was demonstrated that individuals with DPN had poorer cognitive memory than healthy controls. No differences in cognitive function between T1DM with neuropathic pain, T1DM with DPN, and T1DM without DPN were observed, and no association to peripheral nerve function or diabetes-related clinical parameters were found. When combining the three diabetes groups into one group, poorer cognitive performance was observed in several domains compared to healthy controls. This indicates that cognitive alterations also occur to some degree in all individuals with T1DM (Aim 1).

Using structural MRI, it was observed that compared to healthy controls, reduced total GMV occurred in T1DM with neuropathic pain and in T1DM without DPN. Total GMV was not associated with peripheral nerve functions or diabetes-related clinical parameters. Regional GMV loss, including regions relevant for sensory processing, was found in all three diabetes groups compared to healthy controls. While some regions were commonly altered in all three diabetes groups, other regions were more specific for each group. Thus, reduced gray matter volume occurred in T1DM regardless of the presence of DPN and neuropathic pain (Aim 2).

Finally, a functional reorganization of thalamic and primary somatosensory cortex connectivity was observed using the resting-state fMRI. In particular, hyper-connectivity was observed between these brain regions and cortical motor areas in T1DM without DPN compared to T1DM with neuropathic pain and healthy controls. Most importantly, these connectivity parameters were associated with large and small nerve fiber function and pain intensity. Thus, poorer peripheral nerve function and higher pain intensity were associated with lower connectivity. Hence, based on the connectivity parameters, it was possible to distinguish between T1DM with neuropathic complications and T1DM without DPN based. The hyper-connectivity is suggested to act as a mechanism to prevent developing neuropathic complications (Aim 3).

Overall, this PhD thesis contributes with three major points: 1) It has demonstrated that CNS alterations occur in T1DM regardless of the presence of DPN or neuropathic

pain. However, some changes in the CNS were associated with DPN and neuropathic pain, while others were more general for T1DM. 2) Hence, the underlying method used to assess different CNS parameters is of essential value in connecting the CNS alterations to underlying neuropathic complications. 3) Especially resting-state functional parameters of the thalamus, and primary somatosensory cortex have shown great potential in the pathogenesis of DPN and neuropathic pain and in the development of early biomarkers for risk stratification.

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