Sandra Elise Olsen

**Hypoglycemia in adults with Type 1 Diabetes Mellitus**

**Investigating symptoms and awareness of hypoglycemia and the association between impaired awareness of hypoglycemia, autonomic neuropathy and cognition**

Thesis for the degree of Philosophiae Doctor

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Norwegian University of Science and Technology
Faculty of Medicine
Department of Cancer Research and Molecular Medicine
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1 PREFACE

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1.2 Summary

Hypoglycemia is still the main limitation for the management of Type 1 diabetes mellitus (T1DM). If it were not for hypoglycemia, treatment of T1DM would be easy. Subjects with T1DM have impaired defense mechanisms against hypoglycemia; this makes them dependent on symptoms in order to detect a hypoglycemic episode and take appropriate action to restore blood glucose levels. Impaired awareness of hypoglycemia (IAH) is a reduced ability to perceive the onset of hypoglycemia. Subjects with IAH thereby have increased risk for development of severe hypoglycemia.

We wanted to investigate the symptomatology of hypoglycemia in relation to diabetes duration. In addition, we wanted to investigate if symptom intensity is associated with IAH. We found that lower autonomic symptom intensity and a predominance neuroglycopenic symptoms, is associated with longer diabetes duration. We found IAH to be associated with higher overall- and higher neuroglycopenic symptom intensity, but not with lower autonomic symptom intensity.

Activation of the autonomic nervous system is important for counterregulation during hypoglycemia. Although results of previous studies have not been convincing of such an association, it has almost been regarded as an unwritten truth that autonomic neuropathy contributes to the development of IAH. We therefore found it appropriate to investigate this hypothesis. We did not find any association between IAH and autonomic neuropathy.

Cognitive impairment is getting increasing recognition as a complication of T1DM. It is likely that cognitive impairment affects diabetes self-management. We wanted to investigate if cognitive impairment is associated with IAH. We found IAH to be associated with impaired memory, learning, pattern separation and possibly also executive function.
1.3 **Abbreviations and definitions**

CASS: Composite Autonomic Scoring Scale

CGM: Continuous Glucose Monitoring

CNS: Central nervous system

CSS: Cross sectional study/studies

DAN: Diabetic autonomic neuropathy

DCCT study: The Diabetes Control and Complication Trial, a large prospective study that investigated if intensive blood glucose control reduces long-term complication of diabetes

DSFN: Diabetic Distal Small-Fiber neuropathy

DSPN: Diabetic Symmetrical Peripheral Neuropathy. The most typical manifestation of diabetic peripheral neuropathy

EDIC: Epidemiology of Diabetes Interventions and Complications study, the follow up study after DCCT

HAAF: Hypoglycemia-Associated Autonomic Failure

IAH: Impaired awareness of hypoglycemia

NAH: Normal awareness of hypoglycemia

SH: Severe hypoglycemia; in this thesis defined as episodes with seizure or coma, or episodes in which external assistance was required to restore the blood sugar level.

T1DM: Type 1 Diabetes Mellitus

QSART: Quantitative Sudomotor Axon Reflex Test
1.4 List of papers

Paper I

Hypoglycaemia symptoms and impaired awareness of hypoglycaemia in adults with Type 1 diabetes: the association with diabetes duration

Olsen SE, Åsvold BO, Frier BM, Aune SE, Hansen LI, Bjørgaas MR

Diabetic Medicine 31, 1210–1217 (2014)

Paper II

Impaired Awareness of Hypoglycemia in Adults With Type 1 Diabetes Is Not Associated With Autonomic Dysfunction or Peripheral Neuropathy

Olsen SE, Bjørgaas MR, Åsvold BO, Sand T, Stjern M, Frier BM, Nilsen KB

Diabetes Care 2016; 39:426–433

Paper III

Impaired awareness of hypoglycemia in adults with type 1 diabetes is associated with specific cognitive deficits

Hansen TI, Olsen SE, Haferstrøm EC, Sand T, Frier BM, Haaberg AK*, Bjørgaas MR*

*Contributed equally

Manuscript ready for submission
2 INTRODUCTION AND BACKGROUND

The Diabetes Control and Complications Trial (DCCT) was a landmark study which demonstrated that intensive glucose-lowering therapy reduced the risk of long term complications of type 1 diabetes mellitus (T1DM) [1]. However, the DCCT also underlined that hypoglycemia is an adverse effect of intensive diabetes management, with severe episodes occurring three times more frequently in the intensively treated group [2]. This increased risk of severe hypoglycemia (SH) with intensified insulin therapy was confirmed in a smaller Scandinavian study [3]. Thus, hypoglycemia is still the main limitation in the management of T1DM [4,5]. Hypoglycemia may cause physical and psychological morbidity and sometimes death [5-7]. Knowledge about mechanisms that increase the risk of severe hypoglycemic episodes is therefore of utmost importance, and is essential in order to optimize diabetes control.

This thesis focuses on hypoglycemia in adult subjects with T1DM. In three cross sectional studies we have explored symptoms of hypoglycemia, and the syndrome of impaired awareness of hypoglycemia (IAH), which is defined as a decreased ability to perceive the onset of hypoglycemia [8]. Further, we have investigated if autonomic neuropathy and impaired cognitive function is associated with IAH.

2.1 Hypoglycemia

The brain cannot store, nor synthesize glucose, and is dependent on a continuous supply of glucose via the bloodstream. When blood glucose is low, the brain can make use of other fuel sources such as ketones, lactate and amino acids, but this is not sufficient to maintain cerebral function [9]. Prolonged glucose deprivation of the brain can ultimately lead to cerebral damage and death [10]. Glucose homeostasis is therefore crucial for survival. Insulin and glucagon are the most important hormones for regulating blood glucose levels. In subjects with T1DM insulin production is negligible due to beta cell destruction, and glucagon secretion in response to hypoglycemia is also severely attenuated [9]. Hypoglycemia in subjects with T1DM is the result of insulin excess and impaired defense mechanisms against falling blood glucose level [5].

2.1.1 Definition

The American Diabetes Association Workgroup of Hypoglycemia has defined hypoglycemia as all episodes of an abnormally low plasma glucose concentration that expose the individual to potential harm [5], but this is a descriptive definition and not useful in a clinical setting. Intuitively, it would be sensible to decide on a specific blood glucose level; a glycemic threshold, for the definition of hypoglycemia. However, the glycemic thresholds for the generation of physiological responses and symptoms of hypoglycemia are individual, and also vary within the subject with T1DM [11]. Instead, clinical definitions are used. However, as a guiding alert value for clinicians and patients, patients at risk of hypoglycemia are often
instructed to keep blood glucose above 3.9 mmol/l, and such a cutoff value is sometimes included in definitions [5,12].

A hypoglycemic episode is most convincingly confirmed if it fulfills the criteria of Whipple’s triad: 1) typical symptoms, 2) measurement of low blood glucose levels and 3) relief of the typical symptoms when blood glucose levels are restored [5]. However, if the assessment of hypoglycemic episodes were based on these criteria, the occurrence of hypoglycemic episodes would be greatly underestimated. In the following are some useful clinical definitions of hypoglycemia (Box 1).

**Box 1: Definitions of hypoglycemia**

<table>
<thead>
<tr>
<th>Definitions of hypoglycemia</th>
</tr>
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<tbody>
<tr>
<td>• Asymptomatic hypoglycemia: low blood glucose on a routine blood test without symptoms</td>
</tr>
<tr>
<td>• Mild symptomatic hypoglycemia: symptoms of hypoglycemia (reversed by appropriate treatment); episode corrected by patient alone</td>
</tr>
<tr>
<td>o Documented symptomatic hypoglycemia: typical symptoms and measured low blood glucose level</td>
</tr>
<tr>
<td>o Probable symptomatic hypoglycemia: typical symptoms, but not accompanied by blood glucose determination, but presumably caused by low blood glucose level</td>
</tr>
<tr>
<td>• Severe hypoglycemia: assistance is required to take corrective actions</td>
</tr>
<tr>
<td>• Profound hypoglycemia: causes CNS damage or death</td>
</tr>
<tr>
<td>• Pseudo-hypoglycemia: typical symptoms, but measurement does not reveal low blood glucose level</td>
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</table>


### 2.1.2 Counterregulation in non-diabetic subjects

When blood sugar level decreases, it triggers counterregulatory mechanisms (Figure 1). In non-diabetic subjects, the first mechanism is the decrease of insulin secretion, and the second is the secretion of glucagon, the most important counterregulatory hormone [9]. Insulin lowers blood glucose levels by stimulating glucose uptake by the liver, muscle and fat, inhibits gluconeogenesis (production of glucose from precursors), and inhibits lipolysis (mobilization of free fatty acids as alternative fuel source) [9]. Glucagon mobilizes glucose by stimulating glycogenolysis (breakdown of glycogen in the liver), and gluconeogenesis [9]. Further decrease in blood sugar levels triggers a catecholamine response; the release of adrenaline and noradrenaline. The catecholamines suppress insulin secretion, increase glucagon secretion, decrease peripheral glucose utilization and increase lipolysis, making more glucose available for the CNS [9,13]. Falling blood sugar levels also trigger growth
hormone and cortisol release, but these responses are very slow, so they do not play a major part in the acute counter-regulation, but may play a role when hypoglycemia is prolonged, or as an adaptive response [9].

Figure 1: Hierarchy of responses to hypoglycemia

![Hierarchy of responses to hypoglycemia](image)


2.1.3 Counterregulation in subjects with type 1 diabetes mellitus
In subjects with T1DM, hypoglycemia is iatrogenic, caused by insulin replacement therapy, so the first hormonal defense against hypoglycemia, a decrease in insulin secretion, is not possible. Also, possibly due to the low intra islet concentration of insulin [4,14], the glucagon secretion in response to hypoglycemia is attenuated. Subsequently, the first counterregulatory response in subjects with T1DM is catecholamine secretion, which is often also attenuated [5]. When referring to counterregulation in subjects with diabetes, it is mainly the catecholamine responses that are implied [5], since insulin and glucagon responses are negligible. In addition the growth hormone and cortisol responses are present, but play a minor role during acute hypoglycemia.

2.1.4 Body glucose sensing
Several organs in the body contain specialized cells for glucose sensing, and although these mechanisms are not well understood, they probably work together in a network [15]. Such cells are found in the pancreas (β-cells), intestine, liver, carotid sinus and in several areas of the brain [15]. Current views is that glucose sensing in the brain, and especially by cells in
the ventromedial thalamus and the brainstem, are most important for the detection of falling blood glucose levels and the activation of counterregulation [15,16]. These glucose sensing mechanisms in the central nervous system are probably affected by antecedent hypoglycemia (further described below), and in turn contribute to impaired counterregulation, including IAH [15]. Two principal theories have been described for the effect of antecedent hypoglycemia on counterregulation, one concerning glucose transporters, and one concerning upregulation of stress responses [15].

2.1.5 Activation of the autonomic nervous system
Among those counterregulatory responses activated by low blood glucose, is stimulation of hypothalamic autonomic centers within the brain [15]. The sympathetic, parasympathetic and sympatho-adrenal division of the autonomic nervous system are thereby activated [9]. This leads to cardiovascular changes: Heart rate and cardiac output increase, total peripheral resistance decreases, resulting in an increase in systolic blood pressure. Regional changes in blood pressure also occur; increased cerebral flow and splanchnic blood flow contribute to increased substrate delivery [9]. Epinephrine is secreted and autonomic symptoms are generated [16].

2.1.6 Symptoms of hypoglycemia
As discussed above, subjects with T1DM have few intact counterregulatory mechanisms that can restore blood sugar levels in case of hypoglycemia. Release of catecholamines is present, but this is not as effective as glucagon, and catecholamine release is blunted with antecedent hypoglycemic episodes [14]. Symptom generation, and the detection and interpretation of these symptoms as symptoms of hypoglycemia followed by corrective action are therefore crucial to restore blood glucose levels.

Due to the importance in patient education, symptoms perceived during acute hypoglycemia have been investigated in several studies, both retrospective studies, field studies, and by induced hypoglycemia [17-20]. Factor analyses and induced hypoglycemia in combination with pharmacological agents that block neurotransmission in the autonomic nervous system [21], have contributed to a subdivision of key symptoms into three groups based on how they are generated: 1) *neuroglycopenic symptoms*, generated by direct effects of glucose deprivation on the brain; 2) *autonomic symptoms*, generated through activation of the autonomic nervous system; and 3) *symptoms of general malaise* with uncertain origin [17,21-23]. Symptoms vary between subjects and between episodes in the same subject [11]. Subjects with diabetes therefore have to be aware of their own typical symptoms in order to avoid SH, but they should also be aware that these symptoms may vary from one hypoglycemic episode to another. The symptom profile may also change with progressing disease duration [24], and the blood sugar level at which symptoms are generated may also vary [23]. Based on previous studies, both field studies and physiological studies, a list of common and reliable symptoms was developed, the Edinburgh hypoglycemia scale [17,23]. This instrument will be further discussed in the method section of this thesis.
The blood sugar level at which hypoglycemic symptoms are generated is denoted “the glycemic threshold” for symptom development [23]. These symptoms are generated within a narrow range of blood glucose levels, and it is of great importance that the symptoms are generated before the effect of low blood glucose on the brain becomes disabling [23]. Autonomic symptoms have historically been viewed as especially important for early recognition of a hypoglycemic episode [14], and have in non-diabetic subjects, using stepped glucose clamp, been found to be generated at a higher blood glucose level than neuroglycopenic symptoms [25,26]. The difference is estimated to be in the range of 0.5 mmol/L [25], and is probably smaller in subjects with T1DM, in whom antecedent hypoglycemic episodes may result in autonomic symptoms to be generated at lower blood glucose levels [14]. In a study by Cox et al subjects with T1DM reported that they experience autonomic and neuroglycopenic symptoms with equal frequency as initial warning symptoms [22], indicating that these symptoms have similar importance for the recognition of a hypoglycemic episode. However, autonomic symptoms are often prominent in the early years of the disease [9], and might therefore feel more familiar and reliable for people with T1DM.

One previous study has indicated that changes in symptoms are more frequently reported in subjects with longer diabetes duration [24]. Knowledge about how the hypoglycemic symptom profile might change with increasing diabetes duration is important in the continuous education of subjects with T1DM. Because of limited research in this field, we found it important to investigate how diabetes duration may affect symptoms of hypoglycemia, and if changes in symptomatology during the diabetes course may be associated with changes in awareness of hypoglycemia.

2.1.7 Recognition of hypoglycemia

In addition to physiological reactions and symptoms that are generated in response to hypoglycemia, behavioral and psychological aspects affect the recognition and management of hypoglycemic episodes [9,22,27]. An overview of the different steps of hypoglycemia recognition and prevention and possible affecting factors summarizes this (Table 1). Steps 1-4 are the steps in recognition of hypoglycemia, but once a hypoglycemic episode is recognized it has to be followed by corrective action in order to prevent SH (step 5 and 6).
### Table 1: Stages in recognition and prevention of severe hypoglycemia and possible affecting factors

<table>
<thead>
<tr>
<th>Stage in recognition and prevention of severe hypoglycemia</th>
<th>Affecting factors</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) Generation of physiological response</td>
<td>• Degree of hypoglycemia</td>
</tr>
<tr>
<td>• Counterregulation (mainly catecholamines)</td>
<td>• Metabolic control</td>
</tr>
<tr>
<td>• Dysfunction of the central nervous system</td>
<td>• Antecedent hypoglycemia</td>
</tr>
<tr>
<td></td>
<td>• Integrity of counterregulation</td>
</tr>
<tr>
<td>2) Generation of symptoms</td>
<td>• Amount of adrenaline</td>
</tr>
<tr>
<td>• Autonomic</td>
<td>• Brain glucose level</td>
</tr>
<tr>
<td>• Neuroglycopenic</td>
<td>• Medication</td>
</tr>
<tr>
<td>• General malaise</td>
<td>• Caffeine</td>
</tr>
<tr>
<td>3) Detection of symptoms</td>
<td>• Degree of neuroglycopenia</td>
</tr>
<tr>
<td></td>
<td>• Symptom intensity</td>
</tr>
<tr>
<td></td>
<td>• Alcohol</td>
</tr>
<tr>
<td></td>
<td>• Attention</td>
</tr>
<tr>
<td></td>
<td>• Distraction</td>
</tr>
<tr>
<td></td>
<td>• Salience</td>
</tr>
<tr>
<td></td>
<td>• Relevant activity</td>
</tr>
<tr>
<td>4) Interpretation of symptoms as symptoms of hypoglycemia</td>
<td>• Knowledge</td>
</tr>
<tr>
<td></td>
<td>• Symptom beliefs</td>
</tr>
<tr>
<td></td>
<td>• Competing explanation (i.e. exercise)</td>
</tr>
<tr>
<td></td>
<td>• Denial</td>
</tr>
<tr>
<td></td>
<td>• Impaired consciousness</td>
</tr>
<tr>
<td>5) Decision to treat hypoglycemia</td>
<td>• Perceived risk</td>
</tr>
<tr>
<td></td>
<td>• Cost-benefit analysis</td>
</tr>
<tr>
<td></td>
<td>• Tolerance</td>
</tr>
<tr>
<td></td>
<td>• Personality, coping</td>
</tr>
<tr>
<td>6) Self-treatment</td>
<td>• Availability of carbohydrate</td>
</tr>
<tr>
<td></td>
<td>• Degree of neuroglycopenia</td>
</tr>
</tbody>
</table>


### 2.2 Impaired awareness of hypoglycemia, IAH

To date, no comprehensive definition of IAH has been agreed on, but the most extensively used definition is “a reduced ability to perceive the onset of hypoglycemia” [28,29], and this is the definition we have used in our papers. It accounts for all the different factors that may influence awareness; that is a change in any of the steps that constitute recognition of a hypoglycemic episode (Table 1). Another similar definition is “a reduced ability to recognize hypoglycemia at the plasma glucose concentration at which warning symptoms normally occur” [30]. Impaired awareness is associated with a six fold risk of SH [31,32], and is estimated to affect 19-25% of subjects with T1DM [8,29].

Previously the term hypoglycemia unawareness was used, but the term IAH is preferred, because a total loss of awareness is seldom the case [28,33]. Awareness is not an all- or-nothing phenomenon, it is a continuum both regarding hypoglycemic episodes that are correctly identified at onset, and as to the intensity of symptoms experienced from episode
to episode [9]. A loss of, or blunted, autonomic symptoms, or for autonomic symptoms to be generated the after onset of neuroglycopenic symptoms, is sometimes defined as IAH [33,34], this is further discussed in the discussion section of this thesis.

2.2.1 Assessment of IAH
Different definitions are in use and this is reflected by the different assessment methods. This complicates the research field and the comparison of studies. Some researchers use the definition of delayed generation of symptoms, especially autonomic symptoms [34]. This is only identifiable in clamp studies, in which symptom generation is evaluated in response to induced hypoglycemia, and this assessment method is therefore not useful in large-scale studies. Other assessment methods include repeated low blood glucose measurement in an asymptomatic subject (which may indicate IAH), evaluation of the clinical consequences of IAH (mainly SH), or self-reporting of the state of awareness [31]. Self-report methods agree reasonably well with clamp methods [35], and is most widely used since they are simple and manageable in a clinical setting [35]. Subjects who report that they have reduced hypoglycemia awareness are generally correct [36], and questionnaires to assess the patients’ subjective estimation of awareness status have been validated [32,36,37], but in adults no consensus has been reached concerning which method should be preferred [29,31,38]. There has been found a clear association between IAH and increased frequency of SH using all three self-report methods [29].

2.2.2 Pathogenesis of IAH
The mechanisms that contribute to IAH are not fully understood, and since changes in all of the steps that constitute recognition of a hypoglycemic episode could affect awareness, several mechanisms probably contribute to the same syndrome. This is supported by the fact that IAH is reversible in some subjects, but not in all, and so IAH can be chronic condition in some subjects and a dynamic condition in other subjects [32,39]. Suggested mechanisms that contribute to IAH are: 1) CNS adaption, in which antecedent hypoglycemia may induce changes in glucose transport or stress responses [9,15], this might in turn affect counterregulation. 2) CNS counterregulation deficiencies due to other mechanisms that affect catecholamine responses and symptom generation [9]. 3) Peripheral nervous system dysfunction such as autonomic neuropathy or changes in peripheral receptor sensitivity that affect presentation and interpretation of symptoms [9]. Changes related to antecedent hypoglycemic episodes are the best documented mechanisms and will be further discussed below.

2.2.2.1 Hypoglycemia-Associated Autonomic Failure
Antecedent hypoglycemia changes the way the body responds to subsequent episodes of hypoglycemia. It leads to reduced glucose counterregulation through reduced secretion of protective hormones (mainly epinephrine in subjects with T1DM) and a change in blood glucose threshold for epinephrine secretion and development of symptoms of hypoglycemia [14,40-42]; lower blood glucose concentrations are required to trigger such responses. This
mechanism is supported by the fact that prevention of hypoglycemic episodes restores counterregulatory hormone secretion and the threshold for development of symptoms and hormonal secretory responses [43,44]. The changes in counterregulation and awareness of hypoglycemia constitute the components of the syndrome known as Hypoglycemia-Associated Autonomic Failure (HAAF) [12,45]. This means that subjects with IAH not only have increased risk of SH due to changes in symptom generation, but also because of associated attenuated glucose counterregulation [34], and the result is a vicious circle of hypoglycemic episodes; previous episodes increase the risk of new and more profound episodes.

2.2.3 Management of IAH
Small research studies have demonstrated that meticulous avoidance of hypoglycemia can improve hypoglycemia awareness [46,47], and recent recommendations for the management of IAH include avoidance of hypoglycemia and for these subjects to raise their glycemic targets for a period of time [48,49]. However, avoidance of hypoglycemic episodes is hard to maintain, does not restore awareness in all subjects, and long term relaxation of glycemic targets will increase the risk of diabetic late complications.

In addition, a recent review evaluated other interventions that have been developed in order to restore awareness [50]. This review included educational, technological and pharmacological interventions and concluded that structured education programs that include psychotherapeutic and behavioral techniques and blood glucose awareness training can improve awareness [50]. Technological interventions such as insulin pump, CGM or sensor augmented pump could contribute to improved awareness when combined with follow-up and structured education [50].

2.3 Diabetic peripheral neuropathy
Diabetic peripheral neuropathy is defined as the presence of symptoms and/or signs of peripheral nerve dysfunction in people with diabetes after exclusion of other causes [51]. It is often asymptomatic, and the overall prevalence is around 26% in T1DM [52]. Nerve fibers can be categorized based on function or on their diameter and conduction velocity (dependent on myelination) [53]. Based on function, fibers are classified as somatic and autonomic fibers, and somatic fibers can further be classified as somatic motor and somatic sensory fibers. Based on diameter and conduction velocity fibers are classified as large diameter myelinated A-alfa and A-beta, medium size myelinated A-gamma, and small diameter myelinated A-delta and unmyelinated C-fibers [53]. All fibers can be involved in diabetic neuropathy, and can thereby affect almost every system in the body. Risk factors for diabetic neuropathy include poor glycemic control, high age and diabetes duration [51]. Stringent glycemic control is the most important measure for preventing diabetic neuropathy [51].
2.3.1 Pathogenesis of diabetic neuropathy
The exact processes for the development of diabetic neuropathy are not well understood [51,52,54]. We do however know that hyperglycemia is an important mechanism for the development of neuropathy. Through multiple mechanisms it causes oxidative stress that may cause direct neuronal damage or endothelial dysfunction and reduced blood flow in the small capillaries that supply the nerves, and thereby nerve damage and impaired regeneration [52,54].

2.3.2 Manifestation of diabetic neuropathy
Typical manifestation of diabetic peripheral neuropathy is a chronic, symmetric, length-dependent, sensory and motoric neuropathy, with a stocking-, and sometimes also glove-, distribution. Sensory changes, symptoms and signs start distally in the toes and progress upwards [55]. This is often denoted distal symmetric length-dependent sensorimotor polyneuropathy (DSPN) [56]. The most common change is sensory loss, and numbness in the affected area, but pain and muscle weakness may occur. Autonomic neuropathy is also common, but is often subclinical and undiagnosed [57-59]. Diabetic neuropathies can also have numerous other presentations, but they are not as well characterized or studied [56].

2.3.3 Diagnosis of diabetic neuropathy
A clinical assessment including thorough anamnesis and an examination is the first step in the diagnosis of peripheral neuropathy. Targeted neurological examination usually reveals sensory loss of the lower limbs and reflexes are usually diminished [51]. The foot should be examined for deformities and ulcers. Nerve conduction studies are the gold standard for detection of neuropathy, but these tests investigate large fiber neuropathy and are not sensitive for small fiber changes [52]. A similar gold standard for detection of small fiber neuropathy does not exist [53,60], but Quantitative Sensory Testing is becoming increasingly available as a clinical diagnostic tool, and is recommended in the evaluation of diabetic neuropathy [61]. In addition, several tests have been developed for the diagnosis of autonomic neuropathy, as further discussed below. Other methods do also exist but the availability is limited or these tests are mostly used as research tools [53,60].

2.4 Autonomic peripheral neuropathy
Autonomic neuropathy has been given increasing interest in recent years due to an association with increased mortality [51,58,62]. The autonomic nervous system is the part of the peripheral nervous system that controls all inner organs, and autonomic neuropathy can thereby have a number of manifestations [57]. Affection of the cardiovascular system is often asymptomatic, but in severe cases it leads to tachycardia, exercise intolerance and postural hypotension, and silent myocardial ischemia can also occur. Numerous gastrointestinal symptoms, erectile dysfunction, bladder problems, and changes in sweat pattern (increased or decreased) can be caused by autonomic dysfunction [51,57,63].
In diabetes, autonomic neuropathy can often be present in subjects with distal symmetric neuropathy [64], thus presence of somatic peripheral neuropathies substantiates the presence of autonomic neuropathies. Diabetic Distal Small-Fiber neuropathy (DSFN) is another variation of diabetic neuropathy that is more restricted to small fibers, and affects both somatic small fibers mediating temperature and pain, and autonomic fibers [58]. Alterations of the autonomic nervous system may occur early in the diabetes course [48,58].

To investigate if autonomic neuropathy is present, several tests, investigating different organ systems have been developed; cardiovascular reflex tests [65] and sudomotor function tests are commonly used [53,66]. In addition pupillary function tests are common in studies of people with diabetes [67-69]. Tests evaluating other organ systems are often invasive, resource demanding require expensive, specialized equipment and trained personnel [53]. A combination of several tests is preferred [64]. Also, tests should evaluate different parts of the autonomic systems, so that an adequate distribution of autonomic dysfunction is quantified [70]. A composite score, the Composite Autonomic Scoring Scale (CASS score), based on several parameters from cardiovascular reflex tests and the Quantitative Sudomotor Axon Reflex Test (QSART), has been developed [64]. If the full battery of tests is not available, then a combination of tests examining cardiovagal function should be combined with other tests of adrenergic function [64]. An overview of the autonomic function tests used in the present study is presented in the method section of this thesis.

2.4.1 Autonomic peripheral neuropathy and IAH

As mentioned above, the most important defense mechanisms against hypoglycemia in subjects with T1DM are the catecholamine response and the generation of symptoms. Both epinephrine secretion and the generation of autonomic symptoms, by some considered especially important for hypoglycemia awareness, are dependent on the activation of the autonomic nervous system, and so traditionally autonomic neuropathy has been considered to be a cause of IAH [9,71]. Autonomic neuropathy can lead to reduced epinephrine secretion [72-77], but although epinephrine can augment the intensity of some autonomic symptoms, it is not essential for symptom generation [76,78]. Some studies have demonstrated that subjects with autonomic neuropathies have intact autonomic symptom responses [79-81], while others suggest that autonomic neuropathies are associated with blunted autonomic symptom responses [40,74,82]. A modest association has been found between SH and autonomic neuropathy, and IAH is one possible mechanism for such an association [71,83]. In addition, the prevalence of IAH increases with diabetes duration [8,24], and diabetic complications, such as neuropathy, have been a suggested explanation.

Three previous studies have investigated the association between self-reported awareness and autonomic neuropathy [67,84,85]. The study by Hepburn et al. reported a possible association [84], but the two other studies demonstrated no association [67,85]. However these studies were small, the assessment of awareness was not validated, and later
developments in tests and assessment of autonomic neuropathies allow more subtle
differences to be detected.

We therefore found it appropriate to design a study to investigate this association
thoroughly, by controlling for possible confounders and using a novel approach to detect
subtle impairment in autonomic peripheral neuropathy and somatic peripheral neuropathy.

2.5 T1DM and cognition

Conditions such as retinopathy, nephropathy, neuropathy and cardiovascular disease are
acknowledged late complications in diabetes mellitus. Although addressed as early as in
1922, less focus has been given to cognitive impairment as a probable complication of
diabetes [86,87]. Several studies have been performed to investigate cognitive impairments
in T1DM, and several cognitive domains have been found to be negatively affected by T1DM
[86,88,89]. The pathogenesis is poorly understood, but hypoglycemia, hyperglycemia,
vascular disease and C-peptide/insulin deficiencies are hypothesized to be possible
mechanisms [89].

2.5.1 Cognition and IAH

Since cognitive impairment is a recognized complication in subjects with T1DM, it is likely
that cognitive dysfunction could contribute to impaired diabetes self-management.
Awareness of hypoglycemia is dependent on the generation of symptoms and their
interpretation as symptoms of hypoglycemia. With progressing disease, the symptom
composition often changes [24] and although most subjects retain some symptoms of
hypoglycemia, they have to be aware of these changes to detect hypoglycemic episodes. It is
likely that both detection of a hypoglycemic episode and the planning involved in timely
corrective action might be influenced by cognitive impairment. In a study by Howorka et al.,
subjects with a history of SH were found to have reduced vigilance compared to subjects
without a history of SH, and Howorka et al. hypothesized that the reduced vigilance leads to
IAH and in turn repeated episodes of SH [90]. Also indicative of impaired cognitive function,
IAH seems to be associated with reduced adherence to therapeutic decisions [91] and a
reduced ability to modify behavior in order to avoid hypoglycemia [32].

Three early studies found a possible association with between IAH and cognitive
impairments in euglycemic state [92-94], but this was not subject for further research, and
the issue remained unresolved. If such an association exists, this would further underline the
need for routine assessment of awareness to provide adequate patient education to
subjects with IAH. We therefore found it important to conduct a study to investigate if
subjects with IAH have impairment in aspects of cognition.

Previous studies hypothesized that increased frequency of SH in subjects with IAH
predispose for cognitive impairment [92-94]. However, whether SH leads to persistent
cognitive dysfunction is debated [95]. Data from 1144 adults investigated by the prospective
DCCT/EDIC study group, did not find any association between the occurrence of SH and
impairment in several cognitive domains [96], a finding further supported by a meta-analysis [88]. However, the studies included in this meta-analyses had conflicting results, and in a more recent meta-analysis, reduced executive function and memory were found to be associated with SH [89]. In our present study, in addition to investigate the association between cognitive function and awareness, we wanted to assess if this potential association could be partly attributed to SH burden, and if SH burden in itself is associated with reduced cognitive functioning.

3 AIMS

3.1 Overall aims:

In order to optimize diabetes treatment, factors that increase the risk for SH should be identified. IAH is a known risk factor for SH. The aim of the present thesis is to investigate some potential risk factors for IAH, and by this gain knowledge that might be useful in the field of patient education and diabetes management.

3.2 Specific aims:

Paper I

To investigate if there is an association between the duration of T1DM and the intensity of symptoms that are experienced during hypoglycemia. Moreover, to examine the prevalence of IAH in adults with T1DM attending the diabetes outpatient clinic at St. Olavs Hospital, and to examine potential risk factors for IAH.

Paper II

To examine if there is an association between IAH and the presence of autonomic peripheral neuropathy or somatic peripheral neuropathy.

Paper III

To investigate if there is an association between impaired cognitive function and IAH, and if such an association can be linked to the history of SH.
4 POPULATION AND STUDY DESIGN

4.1 The cross sectional study in 2011, study population of paper I

Data collection for this thesis started with an cross sectional study. In 2011, a study questionnaire was mailed to adults with type 1 diabetes mellitus attending the outpatient clinic at St. Olavs Hospital, Trondheim, Norway.

To be included in the study, participants had to fulfil the following criteria:

- T1DM diagnosis, defined as requirement for continuous treatment with insulin < 6 months after the onset of diabetes and elevated anti-glutamic acid decarboxylase antibody or islet cell antibody levels, low insulin C-peptide concentration or being aged < 20 years at onset
- Age between 18-75 years
- Diabetes duration ≥ 2 years
- Attended the outpatient clinic at St. Olavs hospital at the time of data collection (that is, had been to an outpatient appointment within the two preceding years)
- Understood the language well enough to complete the questionnaire.

The outpatient diabetes register included 1163 subjects at the time of data collection. Six hundred and forty-eight subjects with type 1 diabetes received the questionnaire, and 445 responded. An overview of this study population is seen in Figure 2.
Figure 2: The cross sectional study in 2011, study population of paper I

All patients with diabetes in the outpatient register: 1163

- Wrongly did not receive questionnaire: 58
- Did not meet inclusion criteria: 457
- Received questionnaire: 648
  - Correctly received questionnaire: 636
  - Wrongly received questionnaire: 12

  - Responders: 445
    - Did not report awareness status: 5
      - IAH: 74
        - Recruited to the CSS in 2012/2013: 33
      - NAH: 366
        - Recruited to the CSS in 2012/2013: 35
  - Non responders included in analyses: 179
  - Actively refused participation: 12

- Did not meet inclusion criteria:
  - Age ≠ 18-75: 13
  - Diabetes duration < 2 years: 19
  - Not been to the outpatient clinic the last two years: 346
  - Did not understand Norwegian: 8
  - Did not meet criteria for type 1 diabetes: 67
  - Other reasons for not being able to respond: 4
- Did not report awareness status: 5
- Wrongly did not receive questionnaire: 58

IAH: Impaired awareness of hypoglycemia
NAH: Normal awareness of hypoglycemia
CSS: Cross sectional study/studies
4.2 The cross sectional studies in 2012/2013, study population of paper II and III

From the cross sectional study in 2011, we recruited participants to the clinical cross sectional studies in 2012/2013. Based on awareness status in 2011, we recruited subjects with IAH (Gold score ≥ 4) or normal awareness of hypoglycemia (NAH) (Gold score 1-2) [32]. We did not include NAH subjects with Gold score 3, due to the uncertain awareness status related to this score.

Inclusion criteria were as follows:

- Age between 19 and 65 years
- Not pregnant or breast-feeding
- No history of addiction to alcohol or other substances
- No mental, neurological, or systemic illness
- Not substantially reduced vision or hearing
- No routine use of medication that could influence the test results for either of the studies (adrenoceptor b- and a-blockers, tricyclic antidepressants, anticonvulsants, antihistamines, and analgesics).
- For participants with T1DM: Gold score in the cross sectional study (2011) of 1-2 or 4-7

Of the 56 people with IAH who met the inclusion criteria, 33 (59 %) agreed to participate. For each person with IAH, one NAH participant was randomly drawn from subjects of the same sex, similar age, and diabetes duration (±5 years) and eligible subjects were requested to participate. If the selected subject did not fulfil inclusion criteria, or declined, the process was repeated. Two NAH subjects with Gold score 3 in 2011 were included by misunderstanding, and therefore 35 NAH subjects participated in the study. In paper II, which had a matched design as to data analyses, 33 NAH subjects were included, whereas in paper III, 35 NAH participants were included in the data analyses. The two subjects with a Gold score of 3 in 2011 had normal awareness when this was assessed in 2012/13. An overview of these study populations is seen in Figure 3.

4.2.1 Non-diabetic controls

Thirty-eight non-diabetic control subjects were recruited through advertisement at the intranets of St. Olavs Hospital and the Norwegian University of Science and Technology. They had to fulfil the same criteria as the subjects with type 1 diabetes, but, in addition, could not have any history of impaired glucose regulation. For both paper II and III, the non-diabetic controls where recruited to supplement the normal material for the different tests. In paper II, three subjects were later excluded because of comorbidity, which was not remarked at inclusion, but this comorbidity was not relevant for paper III. One non-diabetic control did not perform the cognitive tests, and thus 37 controls were included in paper III.
Figure 3: Cross sectional studies in 2012/2013, study population of paper II and III

IAH subjects from CSS in 2011: 74
- Did not meet inclusion criteria: 18
  - Age > 65: 7
  - Medication: 5
  - Comorbidity: 6
- Requested to participate: 56
  - Participated in clinical CSS: 33
    - Did not meet inclusion criteria: 16
      - Age > 65: 4
    - Included in analyses paper II and III: 33

NAH subjects from CSS in 2011: 366
- Randomly drawn subjects: 59
  - Declined: 8
  - Participated in clinical CSS: 35
    - Comorbidity: 12
    - Included in analyses paper II/III: 33/35

Participating healthy controls paper II/III: 38/37
- Comorbidity relevant for paper II: 3
  - Included in analyses paper II/III: 35/37

IAH: Impaired awareness of hypoglycemia
NAH: Normal awareness of hypoglycemia
CSS: Cross sectional study/studies
4.2.2 Subpopulations

The classification of IAH and NAH participants was based on hypoglycemia awareness status in 2011, but nine participants changed their awareness status from 2011 to 2012/2013. Thus, nine IAH-NAH pairs were excluded in sub-analyses in paper II. Between paper II and III, a review of hospital records revealed that four of these participants had started with CGM, which explained their change in perceived awareness status. However, although the use of CGM reduces the risk of SH, it does not seem to restore awareness [97]. Therefore, only five subjects were excluded in sub-analyses of paper III.

5 METHODS

The methods have been described in detail in the individual papers, but the most important methods will be discussed below.

5.1 Evaluation of awareness status (paper I, II and III)

In order to evaluate awareness status, we used the method by Gold et al. [32]. This method poses the question “do you know when your hypos are commencing?”, and respondents answer by selecting a number on a Likert scale, with 1 representing “always aware” and 7 representing “never aware”, and a score of ≥ 4 representing IAH [31]. Awareness status from the cross-sectional study in 2011 was used to estimate the prevalence of IAH in the outpatient population, and was the basis for recruitment to the cross-sectional studies in 2012/2013. Awareness status was evaluated anew on the day of testing, using the same method by Gold et al., but, in addition, participants responded to eight questions regarding their exposure to mild and severe hypoglycemic episodes, their symptoms of hypoglycemia and glycemic thresholds for symptom generation that constitute the method by Clarke et al. [36]. Based on the Gold score, we identified individuals that had changed their awareness status, but if these participants had IAH based on the method by Clarke et al. (score of ≥ 4), they were considered to have persistent IAH. In paper II, nine subjects were considered to have changed awareness status vs five subjects in paper III; this has been elaborated in the population section of this thesis. Both the Gold and the Clarke methods are based on self-estimation of the state of awareness. Classification of IAH is further discussed in the discussion section of the thesis.

5.2 Evaluation of symptoms (paper I)

Based on previous studies, both field studies and physiological studies, a list of common and reliable symptoms has been developed; the Edinburgh Hypoglycemia scale [17]. This Hypoglycemia scale has later been revised, and it is this revised version we used in paper I [23]. The subjects are presented with a list of symptoms, and are asked to score the symptoms that they usually experience during a typical daytime episode of hypoglycemia using a Likert scale from 1 (“not present”) to 7 (“present a great deal”). In that way, the scale
evaluates the presence and intensity of these symptoms, which both are important for awareness of hypoglycemia. Since its publication, this scale has been used extensively [9]. Our list included autonomic symptoms (sweating, pounding heart, hunger, anxiety and trembling), neuroglycopenic symptoms (confusion, drowsiness, weakness, dizziness, warmth, difficulty speaking, inability to concentrate, blurred vision and tiredness) and general malaise symptoms (nausea and headache), and these symptoms were presented in a mixed order.

5.3 Evaluation of autonomic neuropathy (paper II)

5.3.1 Cardiovascular autonomic tests
Cardiovascular autonomic tests are considered the gold standard for testing the autonomic nervous system [55]. They are valid, safe, non-invasive and can easily be performed by technicians after a short training period. Several tests are used, and several parameters can be obtained from these tests. As recommended for investigation of diabetic autonomic neuropathy [59], we performed Heart rate variability tests, Valsalva maneuver and Tilt test. All tests were performed in a temperature and humidity regulated room, while participants were supine, strapped to a tilt table. They underwent standard electrocardiography, respiratory monitoring, and continuous blood pressure monitoring using the Finapres technique. Each participant started the test between 08:30 and 09:00 AM.

5.3.1.1 Heart rate variability
Since conduction in the vagus nerve cannot be measured directly, indirect measures have been developed [58]. Heart rate variability is a sensitive parasympathetic test that indirectly measures cardio-vagal function [58,66]. Several stimuli exist to measure heart rate variability, but cyclic deep breathing is the best validated stimulus [66,98], with maximal effects with 5-6 breaths per minute and continuous measurement of heart rate; thereby calculation of the R-R interval or sequential heart rate data [66,98].

In our study we collected one parameter from this test, denoted Heart rate deep breathing, for which we calculated inspiration-to-expiration heart rate difference, using the mean of the heart rate difference from five consecutive cycles during paced breathing of six breaths per minute. The breathing frequency was ascertained by a metronome with visual feedback (a movie of a rectangle that increased and shrunk in size), and breathing pattern was monitored continuously with a thermistor covering both nostrils and upper lip.
5.3.1.2 **Valsalva maneuver**

The Valsalva maneuver is a forceful exhalation against resistance which causes increased intrathoracic pressure, and it tests both the parasympathetic and the sympathetic nervous system [66]. In our study, this was performed by letting the participant blow in to a mouthpiece with a pressure of 40 mmHg for 15 seconds [98]. This mouthpiece was connected to a manual sphygmomanometer, which allowed the participant to see that correct pressure was obtained and sustained. A small leakage had to be present to prevent the glottis from closing [98]. Heart rate, ECG and blood pressure were recorded, and allowed us to calculate the heart rate response and identify the four phases of the blood pressure response of the Valsalva maneuver [66].

In phase I, there is a brief increase in blood pressure due to compression of great vessels when the intrathoracic pressure suddenly increases when the subject starts blowing. In phase II early, the blood pressure declines due to decreased venous return (Frank-Starling effect), and vagal release. In phase II late, the heart rate and total peripheral pressure, and thereby the blood pressure increases due to sympathetic activation and adrenaline release. In phase III, the intrathoracic pressure falls because the subject stops blowing and the blood pressure briefly falls. In phase IV, there is a blood pressure overshoot due to a normalized cardiac output, but still high total peripheral pressure due to sympathetic activity [58,66]. The heart rate normally increases during the Valsalva maneuver, and undershoots below baseline values after the maneuver [66].

In our study, we collected several parameters from the Valsalva maneuver, eight of these parameters were selected (elaborated later in the thesis) and included in our analyses. We calculated the mean systolic blood pressure for all phases (denoted **Valsalva maneuver – phase I, -phase II early, -phase II late, -phase III and phase IV**). We calculated the **Valsalva Ratio**, the ratio between the highest and lowest heart rate recorded in relation to the Valsalva maneuver. We calculated the time from the lowest value of the systolic blood pressure of phase III to systolic blood pressure returned to baseline value, denoted **Recovery**
time. Finally, we recorded the lowest heart rate value after the Valsalva maneuver, denoted Heart rate phase IV.

5.3.1.3 Standing and tilting

Postural hypotension can be a sign of severe autonomic neuropathy. Testing can be done from the lying to standing position, but passive tilting is used in most laboratories to evaluate heart rate and blood pressure responses to a change in posture. Tilt angles vary between laboratories; angles between 60 and 90° are commonly used [66].

Subjects should be in supine rest for 15-30 minutes before the tilt. ECG is measured continuously throughout the test. This is especially important due to the risk of bradycardia and transient asystole during the procedure [66]. Blood pressure is measured at regular intervals using a manual brachial cuff, and/or with continuous monitoring. Baseline values are recorded before tilt. Blood pressure should return to normal within 1-2 minutes of standing [66]. During the procedure, the subjects should be asked if symptoms occur.

Not all subjects with postural hypotension have autonomic dysfunction. An early fall in blood pressure is more likely to be caused by autonomic failure, but a late fall in blood pressure is likely due to vasovagal mechanisms. However some may have a delayed decline in blood pressure due to a milder adrenergic insufficiency [99]. An evaluation of postural hypotension should therefore include blood pressure measurements for 10 minutes after tilt, but the result should be evaluated together with results from other autonomic tests [66].

In our study, we recorded blood pressure and heart rate with one minute interval using a manual brachial cuff. First, we recorded 5 minutes during rest in a lying position, before the subject was tilted to 60 degrees for 10 minutes, and laid back down for 2 minutes. From these recordings, three parameters were selected (elaborated later in thesis) and included in the analyses. We calculated the difference between the blood pressure values at 3 minutes after tilt and the mean values of the three last measurements before tilt, denoted Tilt.
systolic blood pressure 3 min and Tilt diastolic blood pressure 3 min. We also calculated the difference between systolic blood pressure at 1 minute and the mean values of the three last measurements before tilt, denoted Tilt systolic blood pressure 1 min.

5.3.2 Pupillometry
A combination of several parameters from cardiovascular reflex tests and the QSART into a composite score (CASS) for evaluation of autonomic neuropathy is recommended [64]. Our laboratory did not have the specialized, expensive equipment or the trained technicians that were necessary to implement QSART in our study [63,66]. Instead we combined results from the cardiovascular tests with results from the pupillary response test. This test has traditionally been incorporated into the evaluation of autonomic function in diabetes [67,100-104], might identify subclinical autonomic dysfunction earlier than the cardiovascular tests, and thus complement these tests in the evaluation of diabetic autonomic neuropathy [67,101,105]. Instead of using the CASS, we developed a new composite score, described in detail below.

Autonomic testing of the pupil can be done in a noninvasive, nonpharmacological way using infrared pupillometry [106]. This is a sensitive test of both sympathetic and parasympathetic function [58]. In our study, a custom-built infrared pupilometer was used to assess resting diameter and light reflex. Infrared light-emitting diodes illuminated the eye, and allowed for a video recording of the eye by cameras sensitive to infrared light. Every recording lasted for 15 seconds, using a frame rate of 50 Hz. A light stimulus with white light started 1 second into the recording. Markers in the computer program detected the contrast between the pupil and the iris, allowed for a continuous measurement of the horizontal diameter, and generated direct and indirect response curves. We used one stimulus intensity of 50 lux, and two different stimulus durations (0.2 seconds ad 1 second). The reflex tests were repeated twice for both stimuli durations.

From these recordings, 28 parameters were selected (elaborated later in thesis). From both direct and indirect response curves (right and left eye) and for both stimulus durations, the following parameters of the pupil reaction were retrieved: Basal diameter (diameter at stimuli onset), Latency until stimuli onset (time from stimuli onset until the pupil starts to contract), Latency until max contraction (time from stimuli onset until maximum contraction of the pupil), Contraction velocity; (difference in diameter from start of contraction to maximum contraction divided by time from start of contraction until maximum contraction), Pupil contraction response (change from basal diameter to maximum contraction diameter), Early and late redilatation time (a summation of early and late redilatation time, that is time from maximum contraction until diameter has reached 50% of basal diameter and time from diameter has 50% of basal diameter to pupil has reached 75% of basal diameter), Diameter at 75% redilatation (diameter at 75% of basal diameter).
5.4 Evaluation of somatic peripheral neuropathy (paper II)

5.4.1 Thermal Quantitative Sensory Testing (QST)

Autonomic function, temperature and pain are mediated by small unmyelinated C fibers or small myelinated A-delta fibers, so in the presence of small fiber neuropathy, it is likely that both somatic and autonomic function are affected [53]. In our Quantitative sensory tests, a handheld thermode was placed on the skin and used to mediate ascending (warm), and descending (cold) temperatures [53]. This enabled us to establish heat detection threshold (WDT), cold detection threshold (CDT), heat pain threshold (HPT) and cold pain threshold (CPT) [107,108]. Stimulation started at a baseline of 32 °C, with a change of 1 °C/s, upper limit of 50°C, and a lower limit of 10°C. Participants were instructed to report the first perception of any temperature change or sensation of pain by pushing a button, which caused the thermode immediately to return to baseline temperature. Thresholds were measured on the left thenar and distal to the left and right medial malleolus.

From these recordings, six parameters were selected (elaborated later in thesis). **Warm threshold thenar, Cold threshold thenar, Warm threshold left malleol, Warm threshold right malleol, Cold threshold left malleol, Cold threshold right malleol.**

5.4.2 Nerve conduction studies

Nerve conduction studies (NCS) primarily test large myelinated somatic fiber function, and should be included as part of evaluation of diabetic polyneuropathy in clinical research [109]. Nerve conduction tests are the most objective and quantitative indicators of diabetic peripheral neuropathy, provided that they are performed and interpreted proficiently [56]. Suitable criteria based on comparison with reference values should be applied [56], and this is thoroughly elaborated later in the thesis.

Nerves are activated by brief 0.1 – 0.5 ms supramaximal electrical impulses, and resultant responses are recorded from muscles or from the nerve itself [110]. The action potential conduction time from the stimulation site until a response is recorded from the recording electrodes is denoted latency. When the latency between two electrodes with a known
distance is measured, one can calculate the (maximal) **conduction velocity**. Both parameters are abnormal when the nerve is demyelinated [110]. When referring to a motor nerve, the response is the result of the action potential of the activated muscle fibers, when referring to a sensory nerve it is the electrical recording from the action potential of the axons within the nerve itself. For this reason the recorded electrical potential is much smaller in sensory than in motor NCS (µV vs mV) [110]. The difference between the measured basal electrical potential and the maximum response is denoted **amplitude of the compound action potential**. When amplitude is reduced it is usually an expression of axonal loss or CV-dispersion in severe demyelination [110].

In addition, more proximal segments of the nerve can be evaluated using late responses. The **F-wave** is a response that can be recorded with a stronger electrical impulse (supramaximal) [110]. This is in order to activate all the nerves and associated fibers. In addition to an initial distal response (**M-wave**) action potentials also travel proximally up to the spinal cord were they reactivates a small proportion of motor axons, causing a smaller second activation of muscle fibers (**F-wave**) [110].

In the present study, standard nerve conduction studies were performed. From the median, ulnar, peroneal and posterior tibial motor nerves, motor amplitude, distal latency, conduction velocity and F-responses were measured. From the median (finger III), ulnar (finger V), radial, sural, superficial peroneal and plantar medial sensory nerves in the left arm and both legs, sensory amplitude and conduction velocity were measured. Fractionated conduction velocity and amplitude from proximal stimulation were obtained from the elbow (ulnar nerve) and knee (peroneal nerve) region, with stimulation above the elbow and above the knee. Recordings were performed on both legs, but only recordings from the left leg were later used in analyses. Studies were conducted in humidity and temperature regulated room, the temperature was kept between 22-24°C, and skin temperature was kept ≥ 33° C by heat packs and an infrared lamp. Experienced technicians performed the tests, which were evaluated by a senior consultant neurophysiologist (Trond Sand).

From these recordings, eight variables were selected (elaborated later in thesis): **Motor nerve conduction velocity** Peroneal nerve, **Amplitude abductor hallucis tibial nerve**, **Mean F-wave latency** ulnar nerve, **Mean F-wave latency** tibial nerve, **Sensory nerve action potential amplitude ulnar nerve dig V (fifth finger)**, **Sensory nerve action potential amplitude sural nerve**, **Sensory conduction velocity** peroneal nerve, **Sensory conduction velocity** plantar medial nerve.

### 5.5 Evaluation of cognition (paper III)

The cognitive tests were administered via the self-administered web-based neuropsychological test battery, Memoro [Trondheim fMRI group: Norway] [111,112]. These tests are based on acknowledged pencil-and-paper tests, and adapted to fit the web-based format. Since the development of the web-based test battery by the fMRI group, this test
battery has been used in several studies, and a validation article has been published, in which the web-based tests were compared to their traditional pencil-and-paper test analogues [111].

In our study, the participants followed a standardized auditory and written instruction including pretrial tests provided by the test platform. The following tests of cognitive function were applied.

5.5.1 Verbal Memory Test
In this test, the participants were instructed to pay attention to a list of 16 Norwegian words (target list), and to type as many of these words as they could remember afterwards. This procedure was repeated four times (denoted recall 1-4). Thereafter, the participants were presented with a list of new words (distraction list), and instructed to type these (denoted distraction recall). Afterwards, they were instructed to type as many words from the original list as they could remember (denoted immediate recall). After the digit span backwards test and the tower test (see below), the participants were once again instructed to type as many words as they could remember from the original list (denoted delayed recall). The web based test is an adaption of The California Verbal Learning Test Version 2 [113,114]. It measures verbal learning and memory [113]. Performance was scored as number of correctly recalled words in each trial. Three scores were extracted from this test, denoted Distraction recall, Immediate recall and Delayed recall, and in addition we evaluated the overall trends in recall/learning curves.

5.5.2 Digit Span Backwards
In this test, the participants were instructed to memorize a series of digits presented consecutively, and thereafter type these digits backwards. The test consisted of 18 trials, with number of digits increasing by one digit every second trial. The test ended if a participant made three consecutive erroneous responses. This web-based test is an adaptation of The Digit Span Backwards subtest of the Wechsler Memory Scale 3rd edition [113,115], assessing working memory [113]. Performance was scored as number of correct trials, the parameter is denoted Digit Span Backwards.
5.5.3 Tower Test

In this test, the participants were presented with a picture of three pegs with discs in different colors; the target position. Underneath were a set of three pegs with discs on them, and the participants were then instructed to recreate the target position by moving one disc at a time between the pegs (picture above). The discs could only be moved following certain rules. The goal was to reach the target position using as few moves as possible. This web-based test is an adaption of The Tower of London test, a subtest of The Cambridge Neuropsychological Test Automated Batteries (CANTAB) [113,116,117]. It measures executive function, which includes planning ability [113,116]. Performance was scored as total number of moves used to solve all trials, and total number of illegal moves, respective parameters is denoted Total moves and Illegal moves.

5.5.4 Objects in Grid

In this test, the participants were instructed to remember the location of drawings of various objects presented in a grid. After 90 seconds, the drawings disappeared from the grid and appeared in rows beneath the grid. The participants were then instructed to drag and drop the object back to its former position in the grid. This web-based test is an adaptation of the Location Learning Test [118], and tests Object-location memory [118]. Performance was scored as number of correctly placed objects, the parameter is denoted Objects in Grid.

5.5.5 Pattern Separation

In this test, the participants were presented with a sequence of pictures. For every picture, the participants were presented with 3 alternatives; 1) new picture 2) a repetition of a previously shown picture, or 3) a slightly different version of a previously shown picture (a so-called lure). The pattern separation task exists in several versions, and has been refined since its initial versions [119]. It tests pattern separation, “the ability to learn and remember distinct non-overlapping representations of highly similar pictures of everyday objects” [120,121]. Performance was scored as number of correct decisions; the parameter is denoted Pattern separation.
5.5.6 Coding
In this test, the participants were presented with a reference key connecting geometrical symbols with single digit numbers. Below they were presented with a row of symbols and empty cells beneath these symbols, and they were then instructed to connect as many of the symbols with numbers as possible, in a predefined period. The web-based test is an adaptation of the Symbol–Digit Modalities Test [122]. It assesses information processing speed [123]. Performance was scored as number of correct responses minus the erroneous responses, the parameter is denoted Coding.

5.6 Ethics:
Approval for these studies was obtained from The Regional Ethical Committee for Medical research in Mid-Norway, one approval for paper I and a joint approval for paper II and III (REK midt 2011/244/ and 2012/439). In addition, an amendment to obtain information about non-responders from hospital records was accepted. Written informed consent was obtained from the participants by a separate form for paper II and III, and by returning the questionnaire for paper I.

6 STATISTICAL ANALYSES
Statistical analyses were performed using The Statistical Package for the Social Sciences (SPSS) version 19.0 and 22.0. Two sided p values < 0.05 (alfa level), and a CI that did not include zero, were considered statistically significant.

6.1 Z scores (paper II and III) and composite Z scores (paper II)
When several parameters are obtained from several tests, as in this study, abnormality may defined either by counting the number of abnormal parameters with results outside of defined reference values (e.g. ≤2,5th/≥97,5th based on normative data), or by calculating a combined score based on the summation or averaging of Z scores (normal deviates) of parameters obtained. In recent years the criteria for diagnosis of diabetic peripheral nephropathy have been evaluated, and the use of composite Z scores is advocated [124,125].

When a parameter is defined as abnormal or normal by the use of cutoff values, one can lose information about borderline values. A better way to express results from each parameter is to use a Z score, the number of standard deviations (SD) from the mean expected value (based on normative data) [125]. Such data are easier to interpret since prior knowledge of normative values is not required, and Z scores also allow for grading of abnormality. The parameter has to have a normal distribution in the control subjects in order to calculate Z scores, if this is not the case the data need to be transformed in order to fit a normal distribution. Due to the independence from the normative values of the different parameters, Z scores can be combined and averaged. This combined Z score is then
analyzed in the normal controls, to establish its distribution, and can then be used to compare groups. The use of combined Z scores evens out random errors and can enhance both sensitivity and specificity of tests, resulting in higher diagnostic accuracy [109,125].

The use of combined Z scores is established in the field of nerve conduction studies [125], but there is no agreement regarding the number of nerves and parameters to be included in such combined Z scores. This method has not been used as to autonomic neuropathy, but the CASS score has been considered the most sensitive measurement [64]. This composite score is based on cutoff values, and therefore sensitivity can be lost due to the neglect of borderline values. The present study did not provide sudomotor data required for this score. Instead, we applied the method of composite Z scores to combine several parameters from cardiovascular reflex tests and pupillometry.

In our study, five composite Z scores (cZ scores) were selected: 1) **CAN score** with a combination of 12 Z scores from cardiovascular autonomic tests. 2) **Pupillometry score** with a combination of 28 Z scores from pupillometry tests. 3) **Autonomic score** with a combination of 40 Z scores from both cardiovascular autonomic tests and pupillometry. 4) **Thermal detection score** with a combination of seven Z scores from thermal tests and 5) **Nerve conduction score** with a combination of eight Z scores from nerve conduction studies (Paper II, table 4 and supplementary table paper II). The parameters that are included in the composite Z scores are described in detail above. How the parameters and combination of parameters into composite Z scores were selected, are described below.

6.2 **Paper I**

To evaluate if there were differences between responders and non-responders, we used the two-sample t test for the normally distributed variables, the Mann-Whitney U test for the non-normally distributed variable and Chi-squared test for the categorical variable.

Linear regression analyses were used to investigate if there were an association between diabetes duration and mean symptom intensity in the different symptom groups or between diabetes duration and awareness score. The assumption of linearity, variance and normal distribution of residuals was fulfilled for the mean autonomic symptom score, the mean neuroglycopenic symptom score, and the awareness score. The neuroglycopenic/autonomic symptom ratio had to be log transformed because of a non-normal distribution of residuals. The statistical significance for the associations was expressed as p for trend. Separate trend analyses were performed for the individual autonomic symptoms; we used linear regression or Spearman’s rank-order correlation if assumptions for linear regression could not be fulfilled despite of transformation.

Log-binomial regression analyses were used to assess the association between the prevalence of IAH and categories of potential risk factors for IAH. The statistical significance for the associations was expressed as p for trend across the categories. Log-binomial regression analysis was also used to assess if these associations changed after multivariable
adjustment. In order to examine whether low hypoglycemia symptom intensity was associated with IAH, we categorized symptom intensity scores into quartiles and used log binomial regression analysis to calculate $p$ for trend across the categories.

6.3 Power analysis (paper II and III)

A pre-test power analyses was performed before inclusion for paper II and III. We estimated that we could include 40 subjects in each group. A difference of 1 SD was considered clinically relevant. The power of a two sample $t$ test with $n = 40 \times 2$ is 95 % for an effect size of 0.8 SD. The use of post hoc analyses of power is debated [126], but we found it appropriate as we did not manage to include the estimated number of participants. With the actual number of included participants $n = 33 \times 2$, power for the predefined effect-size (0.8 SD) was 90 %. The matched design with paired $t$ tests used in paper II may even yield slightly higher power. Thus, our sample size is sufficient to detect clinically relevant differences between IAH and NAH subjects.

6.4 Paper II

Data from healthy participants from previous studies in our laboratory, supplemented with data from non-diabetic controls in the present study, were used to calculate reference ranges. Available subjects in the control database varied between tests; 378 for large-fiber nerve conduction study assessment, $n = 118$ for thermal threshold tests, $n = 28$ for pupillometry, $n = 37$ for tilt and deep breathing and $n = 33$ for Valsalva.

Based on these normal data, $Z$ scores were calculated for the isolated parameters. Data were assessed for normality and transformed with power or logarithmic functions when necessary to fit a normal distribution before calculation of $Z$ scores. If reference data correlated significantly with age and/or height, $Z$ scores were adjusted accordingly by linear regression. The $Z$ scores were adjusted to ensure that abnormality always produced high positive values. Missing variables were imputed with $Z = 0$, while non-recordable sensory and motor amplitudes in the nerve conduction study were scored as 0 µV.

$Z$ scores from isolated variables were averaged into composite $Z$ scores ($cZ$ scores). Variables to be included in $cZ$ scores were selected based on recommendations in the literature combined with $p$ values for the comparison between all participants with diabetes and the non-diabetic controls. The $cZ$ scores that best distinguished between control subjects without diabetes and participants with diabetes were selected, this was determined both by size of SD and by $p$ values for the comparison of $cZ$ scores between all participants with diabetes and healthy control subjects using paired two-sample $t$ test. We used weighted averaging in order to adjust for variables that could be given similar physiological interpretation, and to even out the contribution of the sympathetic and parasympathetic autonomic nervous system in the autonomic $cZ$ scores.
Paired two-sample t tests were used to compare the different cZ scores between the IAH and the NAH group, and also the isolated variables included in the cZ scores. Fisher exact test were used for comparison of neuropathic pain.

We performed the same analyses after excluding pairs in which one of the subjects had changed awareness status.

6.5 Paper III

For group characteristics two sample t tests were used to investigate differences between the NAH and IAH groups in which data variables were normally distributed and Mann-Whitney U-tests were applied for non-normally distributed variables. Chi-squared or Fischer’s exact tests were used on categorical variables as appropriate.

Normal data from healthy controls included in study III were used to calculate reference ranges. Based on these normal data, Z scores were calculated for the isolated scores. Data were assessed for normality and transformed with logarithmic functions when necessary to fit a normal distribution before calculation of Z scores.

Unpaired two-sample t tests were used to investigate statistical differences between the NAH and IAH groups and Cohen’s d was calculated as an estimate of effect size for this difference of means.

Repeated measures ANOVA were employed to investigate if there was a difference in performance throughout trials in the verbal learning test between the IAH and IAH participants, and to investigate if there were an interaction between group and trials. Spearman’s rank-order correlation analyses were performed to investigate possible relationships between test scores and SH in all participants with diabetes, and separately in IAH and NAH groups.

We performed the same analyses after excluding participants that had changed their awareness status.

7 SUMMARY OF RESULTS:

7.1 Paper I

In this study, we aimed to investigate the association between duration of T1DM and intensity of symptoms that are experienced during hypoglycemia. Moreover, we examined the prevalence of IAH, and we examined potential risk factors for IAH.

We found longer duration of T1DM to be associated with lower mean autonomic symptom score and higher neuroglycopenic/autonomic symptom ratio, thus a predominance of neuroglycopenic symptoms. For the autonomic symptoms of trembling, hunger and
sweating, we also found an association between long diabetes duration and low symptom intensity.

The prevalence of IAH was found to be 17% in our population. IAH was associated with SH, age and diabetes duration, with prevalence of IAH increasing with higher age, longer diabetes duration and number of SH episodes experienced in the preceding year. The association with age did not reach statistical significance after adjustment for diabetes duration.

We found no association between autonomic symptom score and the prevalence of IAH, but we found an association with mean neuroglycopenic symptom score, overall mean symptom score and neuroglycopenic/autonomic symptom ratio; higher prevalence of IAH was associated with higher scores.

7.2 Paper II

In this study, we aimed to examine if there is an association between IAH and the presence of autonomic peripheral neuropathy or somatic peripheral neuropathy. We compared the neurophysiological test results between closely matched participants with IAH and NAH.

We found no difference between the participants with IAH and NAH in the three composite scores that evaluated autonomic neuropathy, nor did we find a difference in the two composite scores that evaluated somatic peripheral neuropathy. This was confirmed in post hoc analyses showing no difference between the IAH and the NAH participants for all isolated parameters that constituted these composite scores.

We performed the same analyses after excluding pairs in which one of the subjects had changed his/her awareness status. The results remained unaltered.

In conclusion, we did not find IAH to be associated with autonomic neuropathy or somatic peripheral neuropathy.

7.3 Paper III

In this study we wanted to investigate if there is an association between impaired cognitive function and IAH, and if such an association could be linked to previous SH burden.

In the Verbal Memory Test we found that IAH group scored significantly lower on Distraction recall and Delayed recall compared to the NAH group. There was significantly lower total performance across the trials for the IAH group compared with the NAH group. This suggests an association between impaired verbal memory, impaired verbal learning ability and IAH.

The IAH subjects also scored significantly lower on Objects in Grid test compared to the subjects with NAH, suggesting impaired Object-location memory and immediate recall. IAH subjects also scored lower in the Pattern Separation tests compared to IAH subjects.
We did not find an association between a history of SH and the cognitive test scores. However, in the IAH group a significant correlation was found between number of invalid tests and number of SH episodes since diagnosis.

When we performed the same analyses after excluding subjects that had changed their awareness status, the differences between the groups became more prominent. In addition, a significant difference between the IAH and NAH subjects emerged for Tower Test illegal moves, which had only been a tendency in original analyses, suggestive of impaired executive function which includes planning abilities.

In conclusion, we found IAH to be associated with impairments in memory, learning and pattern separation.

8 DISCUSSION

8.1 Study design and study population

Which research questions that can be answered from a study, are dependent on study design. All of our studies are non-experimental, cross sectional studies. Cross sectional studies are carried out at one point of time, or over a short period of time [127]. They are carried out to evaluate prevalence of an outcome of interest in a given population, or in subgroups within the population [127]. Often data on individual characteristics are collected as well, and used to investigate if there is an association between possible risk factors and the outcome of interest [127]. Since such investigations are carried out at one time point, we do not know about sequence of events, and thus a cross-sectional study cannot be used to evaluate causation [127]. In all our papers we have distinguished clearly between correlation and causation, our results indicate an association, but our design does not allow us to claim causality. We used validated methods thoroughly explained above, appropriate statistical methods, and controlled for confounding factors through statistical methods and/or study design.

In paper I, we included subjects from the outpatient clinic at St. Olavs hospital in order to find the prevalence of IAH and determine how diabetes duration affects symptom profile. This is an appropriate design to evaluate prevalence, but in order to evaluate long term changes in hypoglycemia symptom profile, a prospective study, in which symptoms are evaluated prospectively at intervals over several years, would be a more appropriate design. However, our results convincingly indicate that there is an association between diabetes duration and changes in symptom profile. Major strengths are the response rate of 70%, and the large study population. These factors are also important for paper II and III.

From the study population of paper I, using a matched inclusion process, we recruited subjects with IAH and NAH to the cross sectional studies that formed the basis for paper II and III. We wanted to compare if there was a difference in autonomic and somatic nerve
function or in cognition between these two groups. That is, we wanted to investigate differences in an outcome between subgroups of the population as can be done using a cross sectional design.

8.2 Methods

Our choices of methods have been discussed in the method section and in the statistics section of this thesis. Some aspects will also be further discussed in the sections below.

8.3 Internal validity

It is always important to consider if results in a study can be held true, or if there are alternative explanations for the results of a study.

*Internal validity* reflects whether results from the study population (sample) can be held true for the source population, *external validity* reflects whether results can apply for subjects outside the source population [128]. Violations of internal validity are processes or effects that lead to a nonrandom deviation of results away from the “truth” and can be categorized into three categories: selection bias, information bias and confounding [128].

8.3.1 Selection bias:

A consequence of selection bias is a difference between exposure and outcome between those selected for analyses and other eligible participants in the population, meaning that inclusion of these other eligible subjects could have changed the results [129]. Factors in the recruitment and factors that influence study participation can result in selection bias.

Our source population was the outpatient population of St. Olavs hospital. The 70% response rate in paper I is a major strength of this study; it decreases the possibility of selection bias, and strengthens internal validity. The study population of paper II and III were recruited from the study population of paper I, and the validity of these results is thus dependent on response rate in paper I and the recruitment to study II and III. We did, however, find that non-responders of paper I were younger, had shorter diabetes duration, and higher HbA1c than the responders, and a larger percentage were men. This could be indicative of some degree of selection bias. It is likely that the prevalence of IAH would be slightly lower if the non-responders had been included, as we know that increased age/diabetes duration is associated with a higher prevalence of IAH. The higher HbA1c in the non-responders could indicate that these subjects are less compliant, and therefore perhaps less likely to participate in studies. In paper II and III 59% of the IAH subjects agreed to participate, resulting in a moderate sample size and possibly selection bias. However, it is unlikely that the hypothesized associations between autonomic neuropathy and IAH or cognition and IAH would differ between those who participated in the study and those who declined.

Selection bias was considered thoroughly when we designed the clinical cross sectional studies of paper II and III. The investigations for these two studies were conducted on the
same day, so considerations and precautions had to be taken for both studies. We know that recent hypoglycemic episodes can affect cardiovascular reflexes [130] and affect cognition for some time after the event [131]. However, if we had excluded those who reported mild hypoglycemic episodes in the day preceding the tests, we would have introduced a selection bias because subjects with IAH would to a lesser extent be able to report such an episode compared to those with NAH. We dealt with this issue by asking the participants to report severe episodes of hypoglycemia, but not to report mild episodes.

It was important for the strength of the studies to include as many subjects as possible; we therefore strived for the tests to be conducted on one day only and to be minimally invasive. It could, for instance, be argued that we should have included induced hypoglycemic clamp to complement subjective report of awareness. But it would not have been possible to conduct this procedure on the same day as the rest of the investigations. Furthermore, including a hypoglycemic clamp could have hampered recruitment and is not without risk in people with longstanding T1DM. Skin biopsy was also considered in order to evaluate neuropathy, but was not included because of its invasive character.

For every subject with IAH that agreed to participle, one subject with NAH (matched on gender, age and diabetes duration) was randomly drawn from NAH-subjects. This procedure will also have contributed to minimized risk of selection bias.

As to paper III, self-selection bias might be appropriate to consider, since subjects that believed that they might not perform well on neuropsychological tests might have been less likely to participate. It is however unlikely that awareness status affected this decision to decline and will therefore not have contributed to a difference in associations between cognition and IAH between participants and non-participants.

8.3.2 Information bias
Bias in estimating an effect, information bias, can be introduced if there are measurement errors in essential information on the participants. Such measurement errors are usually referred to as misclassification [128]. When it comes to misclassification, it is relevant to consider if the proportion of subjects misclassified depends on the subjects’ status in other variables included in analyses. If it does not, it is referred to as nondifferential misclassification. If it does, it is referred to as differential misclassification [128]. Nondifferential misclassification is preferred, as it is predictable and will lead to a bias towards the null, and minimize associations, while differential misclassification can lead to bias in either direction [128].

The possibility of misclassification of awareness status has been discussed in the three present studies. There is no consensus as to which method should be used to assess awareness [28]. To reduce the risk of misclassification, we excluded subjects with Gold score 3 when recruiting subjects to the cross sectional studies in 2012/2013. Nine subjects had changed their awareness status from that reported by the Gold method in 2011. This could
be because of misclassification, and could thus represent a weakness of the Gold method, or it could be due to a real change in awareness status. In order to reduce the risk of misclassification, awareness was evaluated both with the Gold score and the Clarke score in the subjects that had altered their awareness status from 2011 to 2012/13. They were included in the analyses if the Clarke score confirmed the Gold awareness score of 2011. It is also a possibility that the translation of the Clarke and Gold methods introduced errors [28], but in order to reduce the risk of such errors, we used professional translators and forward-backward translation [132]. Misclassification of awareness is likely to be independent of symptom intensity and peripheral neuropathy, but cognition could affect the subjects’ ability to evaluate awareness status, and could possibly lead to differential misclassification.

It is known that the investigators’ knowledge about disease or exposure can lead to differential probing and recording [133]. To reduce the risk of such differential misclassification, investigators performing the clinical assessments were blinded as to the participants’ awareness status during the investigations in paper II. For the cardiovascular autonomic tests, pupillometry tests and QST, investigators were also blinded as to diabetes status. Concerning the cognitive tests in paper III, the participants followed standardized auditory and written instructions and performed self-administered tests. This reduced the risk of investigators influence on performance as can be the case for their traditional pencil-and-paper test analogues. All analyses of tests in paper II and III were blinded. In order to achieve this level of blinding, the investigators performing the clinical assessments could not take part in the inclusion process, and the participants had to be recoded twice due to a potential risk of recognition of serial numbers.

Regarding history of SH, the risk of recall bias is well-known [37]. However, one study has compared rate of SH reported prospectively and retrospectively and found that recall is acceptable at least up to one year [37], but that the risk of recall bias is greater in those with a higher number of events. In addition, severe hypoglycemic episodes are possibly more susceptible to affect cognition if they occur in young age [134-138], and these early episodes is likely not to be remembered by the subjects. Recall bias therefore has to be taken in to consideration when we did not find an association between retrospectively reported SH burden and cognitive function. In Norway, loss of consciousness, as might be experienced with SH, may lead to withdrawal of the driver’s license. Subjects could therefore have been withholding information about SH, because of fear of losing their driver’s license [139].

Due to the risk of hypoglycemia during tests, the participants were requested to raise their glycemic targets during the test day. It is known that acute hyperglycemia can impair cognitive performance [140-143], and so our lack of upper limit for blood glucose level prior to tests might have contributed to measurement error. However, blood glucose levels before and after test was similar in both groups (~10.0 mmol/l; 180 mg/dl). Since we found an association between higher blood glucose levels and better performance on the digit span backwards, but no association between blood glucose levels and performance for any of the
other tests, it is not likely that blood glucose variations among participants in our study have influenced our results.

The Z scores used in paper II and III will reduce the risk of measurement errors since information on borderline values is not lost. The compound Z scores used in paper II will even out effect of measurements errors in the separate parameters.

### 8.3.3 Confounding

A confounder is a factor that is related to the exposure, but not affected by it, and is related to the disease, but not affected by it. In its most evident form, it is a common cause for both the exposure and the disease [128]. In paper I, we adjusted for possible confounding factors when evaluating the association between prevalence of IAH and possible risk factors. Due to the substantial correlation between diabetes duration and age, the separate contribution of these variables could not be examined when we assessed risk factors for IAH or changes in symptom profile.

Regarding the association between IAH and neuropathy and IAH and cognition, we know that age and diabetes duration are potential confounders, possibly also gender [51,144]. This is the reason for the matched design of paper II and III. Although educational level is a likely confounder for the association between awareness and cognitive function, it was not possible to match participants for education level at inclusion. However, education level turned out to be comparable between the IAH and NAH groups in paper III. In paper III it might seem sensible to adjust for SH to evaluate the association between IAH and cognition. However, SH is affected by awareness status, and can possibly affect cognition; it is thus a possible mediator for the association between IAH and cognition. By adjusting for severe hypoglycemia we could have introduced bias by removing the effect that is possibly mediated trough hypoglycemia.

Finally, there might always be unknown confounders that have affected our results.

### 8.4 Generalizability (external validity)

Generalizability, or external validity, refers to whether our results are generalizable to subjects and populations outside the source population [128]. The adult outpatient population with type 1 diabetes at St.Olavs hospital was the source for our study populations. In Norway, all subjects with T1DM are supposed to attend an outpatient clinic in a hospital, and subsequently our sample is likely to be representative of adult subjects with T1DM I in Norway. A study is most generalizable to subjects that are very similar to the ones included in the study. The generalizability of our study to diabetic subjects outside our source population was supported by data from the Norwegian Diabetes Registry, in which the subjects had similar mean age, mean diabetes duration and HbA1c values as in the participants in paper I [145]. It is also probable that our results can be generalizable to other T1DM populations in countries that use modern insulin treatment and have updated recommendations for diabetes management. In addition, it is biologically plausible that
autonomic neuropathy and cognitive impairments in subjects with T1DM have similar correlation to IAH regardless of population.

In the future, further developments in diabetes management, with improvements in education, blood glucose measurement techniques and continuous glucose monitoring (CGM) systems may lead to more stable blood glucose levels and hence improved awareness and perhaps result in different symptom profiles than we have demonstrated in this study. It is, however, interesting that in spite of the improvement in diabetes treatment and management, the prevalence of IAH has not changed substantially during the last two decades [8].

For the three publications in this thesis there was a reasonably short time between data collection and publication, which increases generalizability.

As to generalizability, it is relevant to mention other methods of evaluating awareness. We know that the clamp methods for awareness evaluation agrees reasonably well with self-report methods [35], and that a strong correlation have been found between the self-report methods of Gold and Clarke [31]. The same correlation was not found with the method of Pedersen-Bjergaard, but a dichotomous version of the method was assessed, and it is possible that the current version with three categories would demonstrate better correlations with the Clarke and Gold methods [38]. However, we know that subjects identified as having IAH with these three methods have different characteristics [29], and the prevalence of IAH varies within the same population between these methods [29]. It is therefore possible that a study investigating the same associations as in our papers, but by using another method for the assessment of IAH, would gain different results.

8.5 Random error /chance

As discussed above, we want results in our study population to reflect the results we could get if the whole population was included. Because of chance, different samples from the same population can produce different results, even without bias. Since such factors are random, they can affect the results in any direction and thereby lead to greater variation, and reduce precision [128]. Some degree of random error will always be present, but high precision, as expressed by narrow confidence intervals, indicate that random error has not influenced results substantially.

A large study size is a common way to reduce random error. In paper I, the high number of participants resulted in relatively narrow confidence interval around the mean for our results, even when we estimated awareness score and symptom intensities by 5-year categories for diabetes duration. There is a moderate sample size in paper II and III. In paper II we did not find an association between peripheral neuropathy and IAH. An alternative explanation for our results could be that we did not have enough participants to find statistically significant differences. However confidence intervals for the mean differences in composite Z scores between IAH- and NAH participants were narrow, with lower limits in the
range (-0.51) – (-0.31) and upper limits in the range 0.16-0.61. In pretest power analyses we defined 1 SD as clinically relevant, and thus our confidence intervals do not include values considered clinically significant. In addition, our pre- and posttest power analyses supported that we should be able to detect clinically relevant differences between IAH and NAH subjects in paper II and III.

It is also important to remember that a test can be statistically significant due to chance alone. When using an alfa level of 0.05, there is a 5% probability of rejecting the null hypothesis (no difference between groups) given that the null hypothesis is true. The chance of false positive findings increase with number of tests performed. How to deal with multiple comparisons is debated [146,147]. However there is a difference between fishing for possible associations and investigating associations based on prior beliefs and biological plausibility [146].

In paper I, we investigated associations between IAH and suspected risk factors, based on findings in previous studies or biological plausibility. For specific symptoms, we only performed individual analyses on the autonomic symptoms, since overall autonomic symptom score, but not neuroglycopenic symptom score, had demonstrated a clear association with diabetes duration. The probability that our findings emerged because of chance is substantially reduced by the high significance levels of our results. P for trend was in the range of 0.001-0.007 for the associations we demonstrated, except for the association between neuroglycopenic/autonomic symptom ratio and IAH, for which p for trend were 0.02.

In paper II, we found no association between IAH and autonomic or somatic peripheral neuropathy. In this paper the number of tests, and thus the risk of chance findings, were substantially reduced by the use of compound Z scores. In the post hoc analyses of the 55 isolated parameters that constitutes the Z scores, a small but significant difference emerged for one parameter; latency until pupillary contraction for the pupillary reflex test. This finding was in the opposite direction as the hypothesis, and was therefore considered to be a chance finding.

In paper III, the probability of our findings to emerge because of chance is also reduced by the high significance levels of our results. P values were in the range of 0.007-0.018, except for the association between performance in Objects in Grid and awareness, for which the p value was 0.043. In addition, effects size varied from 0.55-0.70; effects sizes between medium to large. For the Tower Test illegal moves and Coding there were borderline significant findings of p 0.057 and 0.057 respectively, but with moderate effect sizes (d = 0.57 and d = 0.52). This might indicate a difference, but that the sample size is too little to find significant difference.
8.6 Discussion of main findings

8.6.1 The association between duration of T1DM and intensity of symptoms that are experienced during hypoglycemia (Paper I)

In paper I, we found an association between longer duration of T1DM and lower intensity of autonomic symptoms, but not with a lower intensity of neuroglycopenic symptoms. The neuroglycopenic/autonomic symptom ratio suggested that autonomic symptoms dominate in the early years with T1DM, but that this dominance declines with progressing disease. Further analysis revealed an association between reduced intensity of the individual symptoms trembling, hunger and sweating and longer duration of diabetes. The natural progression of the symptomatology of hypoglycemia during the diabetes course had not been given much focus before our study. One study by Fanelli et al. indicated that diabetes duration influenced the recovery of symptom generation after avoidance of hypoglycemia [34]. A study by Pramming et al. [24] demonstrated that changes in symptoms were more common with increasing diabetes duration. Sweating and tremor were the most commonly reported symptoms, but their occurrence was negatively associated with diabetes duration. Hence, our findings are in concordance with those of Pramming et al.

After the submission of paper I, another relevant paper was published [148]. In children and adults with T1DM this study by Amin et al. compared retrospectively reported symptom intensity with four weeks of prospectively reported symptoms; that is symptom intensity reported right after a hypoglycemic episode experienced during these weeks. Amin et al. used a questionnaire developed specifically for children and slightly different from our questionnaire [149]. For almost every symptom, retrospectively reported symptom intensity was higher than intensity reported immediately after a hypoglycemic episode [148]. It is therefore likely that the retrospectively reported symptom intensities in our study would be lower if reported immediately after a hypoglycemic episode. However, this applied to autonomic and neuroglycopenic symptoms alike, [148] and it is thus likely that our results would have been the same if symptoms were reported prospectively. The study also reported that the most common symptoms of hypoglycemia among adults were hunger, sweating, trembling and weakness [148]. This is in accordance with previous findings [11,24,150], and underlines the importance of our study, since we found an association between diminished symptom intensity of sweating, hunger and trembling and diabetes duration.

8.6.2 The association between symptom intensity and IAH (Paper I)

In paper I, we found no association between autonomic symptom intensity and the prevalence of IAH. This is perhaps a bit surprising, since autonomic symptoms traditionally have been considered especially important for awareness; failure to recognize autonomic symptoms because they are attenuated or generated at a lower blood glucose level, is sometimes used to define IAH [33,34,92]. This brings us back to the lack of consensus concerning the definition of IAH. The method by Gold et al. poses the question, “Do you
know when your hypos are commencing?”, and is directly linked to the definition “a reduced ability to perceive the onset of hypoglycemia”, without implying the reason for this reduced awareness. Changes in autonomic symptoms could lead to IAH in some subjects, especially if they rely on these symptoms alone. However, neuroglycopenic symptoms may be equally common as initial warning symptoms [22,151], and may therefore be just as important for awareness. However, our findings are not in accordance with two previous studies that used the same definition of IAH and were performed in northern Europe a few years before our study [8,29]. It is not plausible that the association between IAH and symptom intensity should be different between Norwegian subjects with T1DM and other subjects with T1DM in Northern Europe, so this discrepancy is likely to be due to systematic or random error.

We also found that higher prevalence of IAH was associated with higher overall symptom intensity, higher neuroglycopenic symptom intensity and higher neuroglycopenic/autonomic symptom ratio; implying that a predominance of neuroglycopenic symptoms is associated with IAH. This may also seem contradictory, as one would suspect that IAH is associated with lack of symptoms. In the questionnaire, participants were asked to score the symptoms that they experience during a typical daytime episode of hypoglycemia, and not only their initial symptoms. The dominance of neuroglycopenic symptoms found in subjects with IAH, can perhaps be due to emergence of autonomic symptoms at a lower blood glucose level [92] causing neuroglycopenic symptoms to develop before or simultaneously as autonomic symptoms. Many neuroglycopenic symptoms are indicative of cognitive dysfunction, and may delay corrective action [92]. This might explain why subjects with IAH report that they develop many symptoms, both autonomic and neuroglycopenic, during an episode of typical daytime hypoglycemia before corrective action restores the blood glucose level. Previous studies have also demonstrated that IAH is not associated with a total loss of symptoms, and have found equal intensity of neuroglycopenic and general malaise symptoms in subjects with impaired and normal awareness [8,29]. However, they have not demonstrated the association with higher total or neuroglycopenic symptom intensity [8,29]. Although it was not formally tested, these studies also implied that a predominance of neuroglycopenic symptoms is associated with IAH [8,29]. One previous study found that neuroglycopenic symptoms were reported more commonly in subjects with reduced awareness compared to subjects with normal awareness of hypoglycemia [18], this is accordance with our findings.

8.6.3 The association between autonomic neuropathy and IAH (paper II)
In paper II, we found no association between IAH and autonomic neuropathy. This is in accordance with the lack of association between autonomic symptom intensity and IAH that we found in paper I. Previous studies have found an association between autonomic neuropathy and reduced intensity of autonomic symptoms [40,74,82]. However, it is the initial symptoms that is important for awareness [9]; when awareness is classified using induced hypoglycemia, symptom generation at lower blood glucose levels is defined as IAH. Previous studies have found no association between autonomic neuropathy and the threshold for generation of autonomic symptoms [74,76,82]. IAH has been diagnosed in
subjects without autonomic neuropathy, and this has previously been interpreted as an argument for a lack of causation [33]. However, it is likely that several mechanisms can lead to the syndrome of impaired awareness, so this does not rule out an association between diabetic autonomy neuropathy and IAH.

In our study, we investigated if there are any difference in autonomic neuropathy between subjects with IAH and subjects with NAH. We have used validated methods for the classification of awareness, matched inclusion of participants to control for potential confounding, a large battery of neurophysiological test and a novel statistical approach to achieve a very high sensitivity for the detection of between-group differences. If autonomic neuropathy is a risk factor for the development of IAH, we would have expected to find a difference. Our findings support and extend the findings in two previous studies [67,85], whereas a third study found a possible association between self-reported awareness and autonomic neuropathy [84]. These studies did not use validated methods for the classification of awareness, and investigated few IAH subjects (n =12, n = 7, n = 14, respectively), which renders their results vulnerable to type I error. This may explain why the traditional view that autonomic neuropathy leads to IAH has been maintained. Our findings are also in accordance with a recent study that found restoration of awareness following pancreas transplantation not to be influenced by the presence of autonomic neuropathy [152].

8.6.4 The association between impaired cognitive function and IAH
In paper III, we found an association with memory (verbal memory, object-location memory), learning and pattern separation and IAH. In sub-analyses (after excluding subjects with changed awareness status), an association between executive function, which includes planning abilities, and IAH emerged. We did not find an association between SH burden and impaired test scores.

Our findings confirm and expand upon the tentative findings of three studies from the early 1990s, which demonstrated an association between IAH and selective attention (trail-making part B), verbal memory and reduced IQ [92-94]. The authors of these previous studies hypothesized that the cognitive impairment in IAH subjects might be due to recurrent SH episodes [92-94]. Since then, cognitive impairment and IAH has not been subject to much research, instead focus has been on other risk factors for IAH, mainly antecedent hypoglycemia, and how awareness can be restored after hypoglycemia avoidance [34,44,46,153-155]. However, as IAH can be a chronic condition in some subjects and a dynamic condition in other subjects, multiple mechanisms probably contribute to the development of IAH [39]. If reduced cognition contribute to the development of IAH, it is likely that this is a more chronic form of IAH. This could explain why differences became more evident, and new associations emerged, when we excluded five subjects that had changed their awareness status and thus possibly had a dynamic form of IAH.
In a study by Smith et al. IAH was found to be associated with reduced adherence to agreed changes in diabetes management [91]. Several of these non-compliant IAH subjects had previously undertaken an education program to improve awareness, without the desired effect [91]. It might seem that IAH sometimes is quite difficult to manage; some subjects with IAH have a hard time modifying their behavior and comply with the agreed diabetes management, and cognitive impairment or personality traits might contribute to these challenges [32,156]. Cognitive impairment is getting increasing recognition as a probable complication of diabetes [86,88,89], and it is likely that cognitive dysfunction could contribute to impaired diabetes self-management, which may lead to IAH. In our study we found impairments in memory, learning, pattern separation and planning abilities in IAH subjects, which may affect these subjects’ ability to recognize hypoglycemic episodes, take timely corrective action, learn from previous hypoglycemic episodes and in turn modify their behavior.

In a cross sectional study, it is not possible to establish the sequence of events. We can therefore not establish if premorbid cognitive impairment predisposes subjects to develop IAH, if the associated SH burden contributes to the development of cognitive impairments in IAH subjects, or if another common factor leads to both cognitive impairments and IAH.

We did not find SH burden to be associated with impaired cognition overall or in the IAH or NAH group. We did, however, find SH to be associated with the number of invalid tests in the IAH group. In addition, learning, memory and pattern separation are especially dependent on the hippocampus [157-160]; a structure susceptible to damage during hypoglycemia [161-164]. Impairments in several hippocampal dependent cognitive abilities, as we found in subjects with IAH, might suggest that SH plays a role in the development of cognitive impairment in these subjects.

9 CLINICAL IMPLICATIONS, IMPLICATION FOR FURTHER RESEARCH AND CONCLUDING REMARKS

9.1 Clinical implications

Our findings have implications for diabetes management and patient education. In recent years, much research has been done on structured education for subjects with type 1 diabetes mellitus, and educational interventions have been found to improve awareness of hypoglycemia and reduce the risk of SH [50]. Knowledge about symptoms of hypoglycemia and suspected risk factors for IAH may contribute to improvements in such educational interventions and thereby prevent SH.

Our findings suggest that the autonomic symptom intensity is attenuated with progressing disease, and that this applies to the cardinal symptoms of hunger, sweating and trembling. It is known that subjects may develop beliefs concerning their cardinal symptoms of
hypoglycemia based on symptoms that they are familiar with [165]. Our results suggest that autonomic symptoms dominate early in the early diabetes course, but that this dominance declines, and is succeeded by neuroglycopenic dominance. The association between IAH and higher total and neuroglycopenic symptom intensity, and a lack of association with autonomic symptom intensity, underlines that a total loss of symptoms is seldom the case in subjects with IAH. This substantiates why subjects with IAH may benefit from blood glucose awareness training [50] and supports the need for repeated patient education. The patients need to know that their symptomatology may change with disease progression, and they should be aided to identify symptoms that are unfamiliar, since such symptoms may become important warning symptoms.

We did not find an association between autonomic neuropathy or somatic peripheral neuropathy and IAH. This suggests that autonomic neuropathy is not a risk factor for the development of IAH, and it is appropriate to apply continuous stringent glycemic goals to reduce progression of neuropathy unless these subjects have other factors that lead to less stringent goals to be recommended [48].

The association between cognitive impairment and IAH is also important. We found impairments in learning, memory, pattern separation and planning abilities in subjects with IAH compared to the aware subjects. Our results confirm, and may partly explain, the suggestions that IAH subjects are less compliant and do not modify their behavior in order to avoid hypoglycemia [91,156,166,167]. It has been hypothesized that cognitive barriers may exist in IAH subjects that remain resistant to interventions to restore hypoglycemia awareness. This notion is supported by the finding that some subjects with persistent IAH may benefit from psychotherapeutic techniques in addition to more traditional structured diabetes education [50,168,169].

9.2 Implications for further research

In paper I, we found that diabetes duration is associated with changes in symptom intensity. A prospective study to evaluate symptoms at specific time intervals would be appropriate to further investigate this association.

In paper II, we used a relatively new instrument to evaluate symptoms of autonomic neuropathy, The Survey of Autonomic Symptoms (SAS) [170]. Furthermore, we performed extensive testing of the autonomic nervous system and employed a novel approach for data analysis. Our data may be used for further validation of the SAS.

The use of Compound Z scores for the investigation of polyneuropathy is recommended and was used in paper II [124]. A paper is in progress elaborating on the method used for evaluation of nerve conduction studies in the present paper II. It investigates the best subset of nerve conduction parameters to be included in a combined Z score for evaluation diabetic neuropathy [171]. In paper II, compound Z scores were also used for the evaluation of autonomic neuropathy. This is a novel approach, and in further studies it would be
interesting to compare this method to the CASS score [64], the currently recommended tool for investigating the autonomic nervous system.

The association between cognition and IAH should be further explored, and cognitive tests should be included in studies that evaluate the effectiveness of treatment strategies for restoring awareness, in order to see if cognition affects recovery of awareness. In addition, the association between cognition and IAH should be further explored in prospective studies in order to establish the sequence of events.

9.3 Concluding remarks

- Longer diabetes duration is associated with lower intensity of autonomic symptoms and lower intensity of the autonomic symptoms of trembling, hunger and sweating. This suggests that symptoms experienced during an everyday hypoglycemic episode change with progressing disease.
- Low autonomic symptom scores were not associated with IAH. High total symptom score and high neuroglycopenic symptom scores were associated with IAH. This suggests that IAH is not mediated through attenuated autonomic symptom intensity and underlines that a complete loss of symptoms is seldom the case in subjects with IAH.
- We did not find an association between autonomic neuropathy and IAH. This suggests that autonomic neuropathy is not likely to cause IAH
- We found an association between impaired learning, memory, pattern separation, planning abilities and IAH. This suggests that subjects with IAH have cognitive impairments that may affect diabetes management.
- We did not find an association between SH burden and cognitive test performance. We did, however, find SH to be associated with the number of invalid tests in the IAH group. In addition, IAH subjects had impairments in several cognitive abilities dependent on structures susceptible to damage during severe hypoglycemia. This might suggest that SH plays a role in the development of cognitive impairment in these subjects.
REFERENCES


11 PAPER I-III