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Early autonomic dysfunction in type 1 diabetes: Insights into its significance and mechanisms

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ACADEMIC DISSERTATION

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"Breath is the bridge which connects life to consciousness, which unites your body to your thoughts."
Thich Nhat Hanh (1926-)

"To learn how to treat a disease, one must learn how to recognize it. The diagnosis is the best trump in the scheme of treatment."
Jean Martin Charcot (1825-1893)

To Niklas, Julia, and Marc

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications:

I. Rosengård-Bärlund M, Bernardi L, Fagerudd J, Mäntysaari M, af Björkesten CG, Lindholm H, Forsblom C, Wadén J, Groop P-H; FinnDiane Study Group. Early autonomic dysfunction in type 1 diabetes: a reversible disorder? Diabetologia 52(6):1164-72, 2009

II. Rosengård-Bärlund M, Bernardi L, Holmqvist J, De Barbieri G, Mäntysaari M, af Björkesten CG, Forsblom C, Groop P-H; FinnDiane Study Group. Deep breathing improves blunted baroreflex sensitivity even after 30 years of type 1 diabetes. Diabetologia 54(7):1862-70, 2011

III. *Bernardi L, *Rosengård-Bärlund M, Sandelin A, Mäkinen VP, Forsblom C, Groop P-H; the FinnDiane Study Group. Short-term oxygen administration restores blunted baroreflex sensitivity in patients with type 1 diabetes. Diabetologia 54(8):2164-73, 2011

IV. Rosengård-Bärlund M, Bernardi L, Sandelin A, Forsblom C, Groop P-H; FinnDiane Study Group. Baroreflex sensitivity and its response to deep breathing predict increase in blood pressure in type 1 diabetes in a 5-year follow-up. Diabetes Care 34(11):2424-30, 2011

*equal contribution

ABBREVIATIONS

ABPM	ambulatory blood pressure monitoring
AER	albumin excretion rate in urine
AGE	advanced glycation end products
AHT	antihypertensive treatment
ANS	autonomic nervous system
BMI	body mass index
BP	blood pressure
BPV	blood pressure variability
BRS	baroreflex sensitivity
CAD	coronary artery disease
CAN	cardiovascular autonomic neuropathy
CNS	central nervous system
CVD	cardiovascular disease
DBP	diastolic blood pressure
DCCT	Diabetes Control and Complications Trial
DPN	diabetic peripheral neuropathy
DSPN	distal symmetric polyneuropathy
ECG	electrocardiogram
EDIC	Epidemiology of Diabetes Interventions and Complications trial
E/I ratio	expiration: inspiration ratio
FFT	Fast Fourier Transformation
GLUT	glucose transporter
HbA_{1c}	glycosylated haemoglobin
HDL	high-density lipoprotein
HF	high frequency
HR	heart rate
HRV	heart rate variability
LDL	low-density lipoprotein
LF	low frequency
MSNA	muscle sympathetic nerve activity
NO	nitric oxide
PARP	poly-ADP-ribose polymerase
PP	pulse pressure
RAGE	receptor for advanced glycation end products
RMSSD	root mean square of the differences of successive RR intervals
ROS	reactive oxygen species
RRI	time interval between two successive R-peaks on the ECG
SBP	systolic blood pressure
SD	standard deviation
SDNN	standard deviation of the normal-to-normal RR intervals
SEM	standard error of mean
TP	total power
WHR	waist-to-hip ratio

ABSTRACT

Background and aims

Diabetic autonomic neuropathy is a serious complication, associated with increased risk of morbidity and mortality, but it is perhaps the least understood of the diabetic complications. The challenge lies in the early diagnosis of this often subclinical condition, in the time window when it would still be treatable. Notably, when detected with the current diagnostic tools, diabetic autonomic neuropathy has been considered as the result of irreversible nerve damage. Reduced baroreflex sensitivity (BRS) is a sensitive marker of autonomic dysfunction, and importantly, also a prognostic marker in cardiovascular medicine. Abnormalities in the BRS occur in conditions characterized by functional autonomic abnormalities such as myocardial infarction, heart failure, and hypertension.

Accordingly, we hypothesized that early autonomic dysfunction in type 1 diabetes, as demonstrated by reduced BRS, is functional. The aim of this thesis was to elucidate the early markers of autonomic dysfunction in patients with type 1 diabetes of various durations. We reasoned that if the BRS in patients with type 1 diabetes responds to slow, deep breathing, a manoeuvre shown to reduce sympathetic activity, or responds to oxygen administration, such a finding would support a functional aetiology. We also studied whether autonomic dysfunction, as established by reduced BRS, progresses alongside increasing diabetes duration to a stage where it is no longer improved by a functional manoeuvre. Moreover, we aimed to elucidate the role of BRS as potentially a predictor of increased blood pressure (BP) level during a 5-year follow-up.

Subjects and methods

We studied 117 patients with short (8.9 $\pm$ 0.1 years) and 37 patients with long duration (33.7 $\pm$ 0.5 years) of type 1 diabetes, and a total of 73 age- and sex-matched, healthy control participants. Twelve heart-transplanted patients served as a model of cardiac denervation. An autonomic score was calculated from autonomic function tests. Spectral analysis of heart rate variability (HRV), blood pressure variability (BPV), and BRS came from recordings during normal (15/min) and slow, deep (6/min) controlled breathing. Of those with short-duration type 1 diabetes, 96 subjects were studied during a prospective visit by similar autonomic assessment as at baseline but in addition, with BRS assessed during inhalation of 100% oxygen at a flow rate of 5L/min. In a total of 80 patients with complete data available, we compared autonomic indices and ambulatory BP at baseline and follow-up.

Results

BRS was already reduced in patients with short-duration type 1 diabetes, but even more reduced in those with long duration or with increasing autonomic involvement. Slow breathing elevated the BRS to the level of control subjects at a normal breathing

rate (15/min) in all patients except in those with an abnormal autonomic score. BRS also increased with oxygen during spontaneous breathing in diabetic but not in control participants, and with oxygen the difference in BRS was no longer significant. Slow breathing in normoxia restored the BRS to a similar extent as did oxygen. In the follow-up study, spontaneous BRS declined over time, but the change was not significant when the deterioration due to ageing was taken into account. Low BRS at baseline did not advance to cardiovascular autonomic neuropathy (CAN) but predicted an increase in night-time systolic blood pressure. Furthermore, the BRS response to deep breathing at baseline predicted the increase found in 24-hour ambulatory BP.

Conclusions

These results indicate that even in long-duration diabetes, any abnormal BRS is at least in part of functional origin. The increased baroreflex response to oxygen supports the hypothesis of a functional reduction in parasympathetic activity occurring in patients with type 1 diabetes. The follow-up study showed that the decline in spontaneous BRS over time in patients with type 1 diabetes seems to be mainly due to normal ageing. Although early autonomic dysfunction seems functional and does not necessarily develop into autonomic neuropathy during a 5-year follow-up, the BRS and the response to deep breathing at baseline are associated with a future increase in BP. More research and a longer follow-up time will be required to fully clarify the prognostic significance of BRS in type 1 diabetes.

1 INTRODUCTION

In Finland and worldwide, the prevalence of type 2 diabetes has shown a dramatic increase, and in addition, a large number of patients are living with still-undiagnosed diabetes (1). In parallel, despite its being totally divergent in pathophysiology, incidence of type 1 diabetes has also shown a rapid increase during the last few decades (2, 3), demonstrating the highest incidence in the world in Finland. During the 10 years of the Finnish National Diabetes Prevention Program (DEHKO), treatment of type 2 diabetes has resulted in clear improvement in terms of glycaemic control, and in the management of the cardiovascular risk factors (4). However, despite modern insulin treatment, including insulin pumps and even the possibility of continuous glucose monitoring, results from treatment of type 1 diabetes in Finland are not as encouraging. Less than 10% of the Finnish patients with type 1 diabetes reach all the treatment goals comprising glycaemic control (HbA_{1c} <7.5 %), blood pressure (BP) <135/85 mmHg, and lipid levels (LDL-cholesterol < 2.6 mmol/l) (4, 5).

Diabetes is the leading cause of autonomic neuropathy in the developed countries. Its prevalence ranges from 1% to 90%, depending on diagnostic method, on characteristics of the patient cohort, and on the type of diabetes studied (6). Although autonomic neuropathy is often subclinical, it is associated with an increased risk for other diabetic complications and mortality (7), and might even precede or predispose to those complications. Notably, the American Diabetes Association states in its most recent recommendation: Standards of Medical Care in Diabetes—2013, that "screening for signs and symptoms of cardiovascular autonomic neuropathy (CAN) should be instituted at diagnosis of type 2 diabetes and 5 years after the diagnosis of type 1 diabetes. Special testing is **rarely needed** and **may not even affect the management or outcomes**" (8). The last sentence summarizes the picture. Autonomic neuropathy is still a true challenge for diabetologists due to the lack of easily available, sensitive, diagnostic methods; as a consequence, the possibility is lacking to start interventions at a stage when the disorder is still reversible. Despite advances in the understanding of the pathophysiology of diabetic neuropathy, the treatments aimed at reversing this process have been unsuccessful.

Baroreflex sensitivity (BRS) integrates information from heart rate variability (HRV) and blood pressure variability (BPV) to provide a robust and sensitive measure that enables earlier detection of autonomic dysfunction than do the conventional autonomic function tests (9-12). Importantly, reduced BRS carries a risk of worse prognosis in all individuals with hypertension, renal insufficiency, post-myocardial infarction, heart failure, or cerebral stroke (13-17), conditions associated with functional alterations in cardiovascular autonomic regulation, not with organic neuropathy. Defining the threshold between functional versus organic, refractory autonomic dysfunction in patients with type 1 diabetes would mean a totally new approach in terms of treatment. Notably, in cardiovascular medicine, interventions based on the concept of functional autonomic abnormalities have improved prognosis (18-20), in contrast to vain attempts to treat diabetic autonomic neuropathy.

However, many questions still remain unanswered. To the best of our knowledge, no longitudinal studies explore the prognostic significance of BRS in type 1 diabetes. Thus, whether low BRS predicts CAN, or whether it inevitably progresses to CAN, or even both, is unclear. Moreover, whether it is possible to correct early autonomic abnormalities such as reduced BRS in patients with type 1 diabetes by any intervention, remains unknown. Autonomic imbalance is a key player in the aetiology of hypertension, but whether low BRS is the cause of and also predicts future elevated BP in type 1 diabetes is not evident. The aim of this thesis was therefore to characterize autonomic function in well-defined cohorts of patients with type 1 diabetes and to study the possible reversibility of early diabetic autonomic dysfunction by testing patients' response to functional interventions.

2 REVIEW OF THE LITERATURE

2.1 Types of diabetes mellitus

2.1.1 Type 1 diabetes

Diabetes mellitus comprises a heterogeneous group of metabolic disorders with chronic hyperglycaemia as the consequence of either absolute or relative insulin deficiency. Under normal conditions the blood glucose level is tightly controlled by insulin secreted from the pancreatic beta cells. An elevated concentration of blood glucose stimulates the release of insulin, which in turn facilitates glucose-uptake into the cells of insulin-sensitive tissues (muscle, liver, fat). In type 1 diabetes, previously termed insulin-dependent or juvenile diabetes, the insulin-producing beta cells are gradually destroyed, resulting in complete insulin deficiency and subsequent hyperglycemia. This T-cell-mediated autoimmune cascade targeted at the beta cells is triggered by some infectious or other environmental factor (21) in genetically susceptible individuals (22, 23).

Type 1 diabetes is primarily diagnosed in children or young adults (peak age at diagnosis is 5–9 years), although it can occur at any age. Symptoms usually develop rapidly over a short period of time, although beta cell destruction can begin years earlier. The classic triad of clinical signs of diabetes includes polydipsia, polyuria, and fatigue, but often also with a history of weight loss, and in more serious cases, diabetic ketoacidosis.

Before the discovery of insulin in 1921, attempts were made to treat diabetes with diet alone, but within a few years of diagnosis, the patients died. The goal of modern insulin treatment is to mimic the physiological secretion of insulin by the pancreas either by multiple daily injection therapy with long-acting basal insulin and short-acting insulin at every meal, or by insulin-pump therapy with continuous, adjustable administration of insulin. In addition to insulin replacement therapy, lifestyle management and cardiovascular risk factor intervention are essential. During recent decades, islet cell or pancreas transplantation have emerged as a potential way to achieve independence of exogeneous insulin. These therapies still face a number of challenges, because recipients would need to adhere to life-long immunosuppression, with only a portion of the recipients remaining insulin independent during follow-up (24).

2.1.2 Other types of diabetes mellitus

In addition to type 1 diabetes, the American Diabetes Association classifies the other types of diabetes into type 2 diabetes, types of diabetes due to other causes, and gestational diabetes (8). The other major form of diabetes, type 2, is characterized by hyperinsulinemia and hyperglycaemia due to insulin resistance and relative insulin deficiency, and a slow, progressive loss of beta-cell function. Other types of diabetes result from genetic defects in beta cell function (monogenic forms of diabetes such as various forms of maturity-onset diabetes of the young [MODY], genetic defects in mitochondrial DNA, as well as neonatal diabetes), genetic defects in insulin action, diseases of the exocrine pancreas, and drug- or chemical-induced diabetes. Gestational diabetes is hyperglycaemia diagnosed during pregnancy and shares a pathogenetic similarity with type 2 diabetes because of its being characterized by a combination of insulin resistance and inadequate insulin secretion. Although gestational diabetes is not yet overt diabetes, up to half the individuals with this disorder will develop type 2 diabetes later in life (25).

2.1.3 Epidemiology

Worldwide, the prevalence of diabetes is showing a dramatic increase and has reached epidemic proportions, with a current estimation of 382 million people living with diabetes, half of them still undiagnosed (1). The major part of this increase is due to the rapidly rising prevalence of type 2 diabetes, which accounts for 90% of the cases in developed countries (1). Despite the fact that among all patients with diabetes, the proportion with type 1 diabetes is small, incidence of type 1 diabetes has been steadily increasing during the past two decades (2) with an annual average increase of 2.5% to 3.0% worldwide (3).

The incidence of type 1 diabetes shows large geographic variation, with Finland and Sardinia demonstrating the highest rates, and China along with Venezuela the lowest in the world (3, 26). In addition to the increase in overall incidence, the steepest increase has been for the youngest children. It is likely that genetic variation explains to some extent the differing incidence and prevalence rates among people with varying ethnicity. However, the rapidly increasing incidence over the last decades cannot be explained solely by genetic factors, implying that environmental factors also contribute. Notably, according to the most recent observations, it seems that in these high-incidence-rate countries, rates may have reached a "plateau" (27, 28).

Despite the improved prevention and treatment of diabetic complications, expectation is that the prevalence of microvascular complications will increase and also shift towards the younger age groups, because typically a prevalence peak in microvascular complications occurs during the second decade of diabetes (29, 30).

During the last two decades, type 2 diabetes has become a growing problem also among children and adolescents (31), most likely due to the obesity epidemic and low level of physical activity among young people. What is of note is that obesity and features of type 2 diabetes are nowadays frequent findings also in type 1 diabetes (32). Thus, strategies for delaying or preventing the clinical onset of type 1 diabetes are essential, but in addition common strategies to prevent obesity and to promote physically active lifestyle are of utmost importance.

Types of diabetes, other than type 1 and type 2, account for only a few percent of all patients.

2.2 Long-term complications in type 1 diabetes

The purpose of lifelong insulin replacement therapy in treatment of type 1 diabetes is to maintain near-to-normal blood glucose in order to prevent acute diabetic complications, i.e. ketoacidosis with a possible fatal outcome. Beyond this, the main goal is primary prevention of diabetic long-term complications. Such complications have a substantial impact on quality of life (33) and also account for the major part of all costs of diabetes treatment (34). Although modern treatment allows a normal life, long-term complications can result in loss of working ability, shortened life expectancy, and reduced quality of life (33, 35-37).

Long-term complications comprise micro- and macrovascular complications. The former comprise diabetic nephropathy, retinopathy, and neuropathy. Macrovascular disease denotes cardiovascular, cerebrovascular, and peripheral arterial disease.

2.2.1 Macrovascular disease

Macrovascular complications of diabetes refer to accelerated atherosclerosis of large blood vessels, manifested mainly as coronary artery disease (CAD), peripheral arterial disease, and cerebrovascular disease. Atherosclerosis is the major cause of premature morbidity and mortality in patients with type 1 diabetes (38, 39). Although the key role of hyperglycaemia in the development of diabetic microvascular complications is convincingly established, defining the role of hyperglycaemia beyond the standard cardiovascular risk factors in the pathogenesis of the macrovascular complications has not been straightforward.

Despite the notion of a clearly increased risk for macrovascular disease in type 1 diabetes, large epidemiologic studies have failed to demonstrate an association between glycaemic control and CAD events. Importantly, the DCCT/EDIC finally confirmed the significance of early, intensive glycaemic management by showing a clear reduction in long-term risk for CAD after patients had undergone 6 years of

intensive treatment (40). It seems that hyperglycaemia plays a significant role in early atherogenic development of the lesions, but later the effect of glycaemic control becomes more multifaceted. The complex role of hyperglycaemia is supported by a recent study in which HbA_{1c} variability, but not mean HbA_{1c}, predicted incident CAD events in patients with type 1 diabetes (41). Risk for CAD is further modified by standard cardiovascular risk factors such as smoking, hypertension, dyslipidemia, obesity, insulin resistance (42). A major part of the CAD in type 1 diabetes is associated with diabetic kidney disease, so insulin resistance may prove to be a common pathogenetic pathway of these two conditions (43).

Hypertension, and also arterial stiffness and its surrogate marker, pulse pressure (PP), are even earlier markers of premature arterial ageing than is manifested CAD. The independent relationship between BP and cardiovascular disease (CVD) is a well-established phenomenon, noticeable even in the prehypertensive range, at BP levels as low as 115/75 mmHg (44). BP determined by 24-hour ambulatory blood pressure monitoring (ABPM) is considered a better predictor of target organ damage (45, 46) than is isolated office BP measurement. Consequently, several studies have suggested that lack of the physiological dipping in nocturnal systolic blood pressure (SBP) is a sensitive marker of incipient diabetic nephropathy (47, 48). Importantly, PP increases prematurely in patients with type 1 diabetes (49) and predicts mortality (42), and predicts the first-ever CVD event in type 1 diabetes independently from renal disease (50). Accordingly, in addition to treatment of hypertension and other standard CVD risk factors, prevention of diabetic kidney disease is essential in the management and prevention of diabetic macrovascular complications.

2.2.2 Diabetic nephropathy

Diabetic nephropathy manifests histologically as glomerulosclerosis of the kidney, characterized by a progressively increasing leakage of albumin into the urine, elevated BP, and a gradual decline in renal function. Microalbuminuria is considered a marker of endothelial dysfunction and more generalized damage to the cardiovascular system (51, 52). Occurrence of proteinuria carries a 37-fold increased risk for cardiovascular mortality (35). Conversely, according to a study by the Finnish Nephropathy Study Group (FinnDiane), patients with type 1 diabetes and without chronic kidney disease showed similar mortality rates as the general population, regardless of diabetes duration (37). Furthermore, the survival of patients with type 1 diabetes and end-stage renal disease has improved during the last few decades (53). Previously, diabetic nephropathy developed in approximately one-third of patients with type 1 diabetes, but according to more recent studies the incidence seems to be decreasing.

Established risk factors for diabetic nephropathy are poor glycaemic control, smoking, male gender, hypertension, dyslipidemia, and predisposing genes (54-56). Early detection of microalbuminuria by regular screening is essential, because treatment with renoprotective agents such as angiotensin-converting enzyme inhibitors and

angiotensin receptor-blockers, along with management of the other modifiable risk factors, has effectively retarded progression of the disease (57-59).

2.2.3 Diabetic retinopathy

Diabetic retinopathy is the leading cause of blindness in developed countries, despite advances in diabetes treatment (60). Diabetic retinopathy is rare during the first five years after disease onset, but after two decades of diabetes nearly all patients with type 1 diabetes show some signs of retinopathy (61). Its earliest manifestations are microaneurysms and increased vascular permeability, followed by moderate to severe changes with intravascular clotting, defined as nonproliferative diabetic retinopathy (62). The most severe form of diabetic retinopathy is proliferative diabetic retinopathy, characterized by pathologic growth of new blood vessels in the retina and vitreous. Macular oedema is a sight-threatening complication caused by retinal thickening due to leaky blood vessels, and it can occur at any stage of diabetic retinopathy (62).

Diabetes duration, poor glycaemic control, hypertension, microalbuminuria, and dyslipidaemia are recognized risk factors (63-66). Furthermore, pregnancy (67, 68) and puberty (69, 70) are associated with the presence and risk for diabetic retinopathy progression. Prevention and treatment of diabetic retinopathy includes strict control of the risk factors and regular screening to detect early and treatable changes. Laser photocoagulation and vitrectomy have proved effective in preventing vision loss in proliferative diabetic retinopathy. Consequently, findings emerge of reduction in the cumulative incidence of severe diabetic retinopathy in patients with type 1 diabetes over the recent decades (71).

2.2.4 Diabetic neuropathy

Diabetic neuropathy is defined by the American Diabetes Association as "the presence of symptoms and/or signs of peripheral nerve dysfunction in people with diabetes after the exclusion of other causes" (8). Despite the fact that diabetic neuropathies contribute significantly to mortality and morbidity, their management is still far from optimal and is challenging. Neurological complications associated with diabetes had already been recognized over 150 years ago. As early as in 1798, John Rollo, a surgeon in the British Royal Artillery who pioneered in treatment of diabetes, described symptoms indicative of diabetic autonomic neuropathy (72). The direct link between the blood sugar disorder and the nerve symptoms, however, was not evident until Marchal de Calvi detected it in 1864. Contemporarily, in 1968, Jean-Martin Charcot, a French neurologist, described neuropathic arthropathies in (non-diabetic) patients with tabes dorsalis (demyelination of neurons secondary to an untreated syphilis infection). Tertiary syphilis was considered the most common cause of Charcot's joint until William Jordan linked this condition to diabetes-induced neuropathy in 1936. Wayne Rundles from the University of Michigan was the first to give a clear

description of diabetic autonomic neuropathy in his study in 125 patients with diabetic neuropathy (73). Following the observation by Wheeler and Watkins in 1973 of the absence of respiratory sinus arrhythmia in diabetic patients with symptoms of autonomic neuropathy (74), screening methods have developed for CAN.

2.2.4.1 Classification of diabetic neuropathies

The diabetic neuropathies involve different parts of the nervous system and are heterogeneous regarding their clinical manifestations, course, risk factors, and underlying mechanisms. Classification of the diabetic peripheral neuropathies has proved challenging and a number of classifications have been introduced to assist in clinical evaluation. In 1986 Boulton and Ward proposed a classification based on various clinical presentations (75), and more recently a classification based on the natural history of the syndromes was introduced by Watkins (76). Currently the classification most used, originally suggested by Bruyn and Garland, is based on the location of clinical involvement and whether it is symmetrical or asymmetrical. This classification has been modified by others (77, 78) and recently updated by an international expert panel (79) (Table 1).

Typical diabetic peripheral neuropathy (DPN) is a chronic, nerve-length-dependent, **symmetrical sensorimotor polyneuropathy (DSPN)** and is regarded as the most common form of the diabetic neuropathies (80). DPN is, together with peripheral arterial disease, the major underlying cause of diabetic foot ulcers, which are both disabling for the patient and costly to the health care system (81). In serious cases, the loss of protective sensation, often triggered by a minor traumatic event, may lead to progressive joint destruction and diabetic neuropathic osteo-arthropathy (Charcot's joint disease). Approximately one-third of all individuals with diabetic neuropathy suffer from unpleasant sensory symptoms ranging from mild paresthesias to **painful DPN**, which have a substantial impact on the quality of life (82). **Diabetic autonomic neuropathy** may affect cardiovascular, gastrointestinal, and urogenital systems and sudomotor function (function of the nerves that stimulate the sweat glands) and is often associated with the other generalized neuropathies.

The **atypical DPNs** differ from the typical neuropathies regarding onset, course and manifestation, and possibly also pathomechanisms. More research is necessary prior to further classification and characterization (83). What has been debated is whether cognitive decline in type 1 diabetes reflects a central equivalent to the peripheral neuropathy. Degeneration of the central nervous system (CNS) manifested as cognitive decline, brain atrophy, and white matter lesions is associated with long term type 1 diabetes (84-86). This was further supported by studies demonstrating that cognitive decline is associated with peripheral neuropathy (87). It is of note however, that cognitive decline may also be influenced by other factors like depression or cerebrovascular disease, which are also frequent findings in long term diabetes (88, 89). A body of evidence is also increasing that central neuropathy may modulate pain perception in diabetic peripheral neuropathy (90). Until now data are scarce on the incidence of central diabetic neuropathies, in part due to the lack of a classification.

Table 1 Classification of the diabetic neuropathies according to Tesfaye et. al (79)

Generalized diabetic neuropathies ▪ typical DPN (distal symmetrical polyneuropathy) ▪ atypical DPN ▪ autonomic neuropathy
Focal and multifocal neuropathies ▪ mononeuropathy ▪▪ limb neuropathies ▪▪ cranial neuropathy ▪ multifocal neuropathy ▪▪ mononeuritis multiplex ▪ proximal motor neuropathy (amyotrophy) ▪ compression and entrapment neuropathies

2.3 Cardiovascular autonomic neuropathy (CAN)

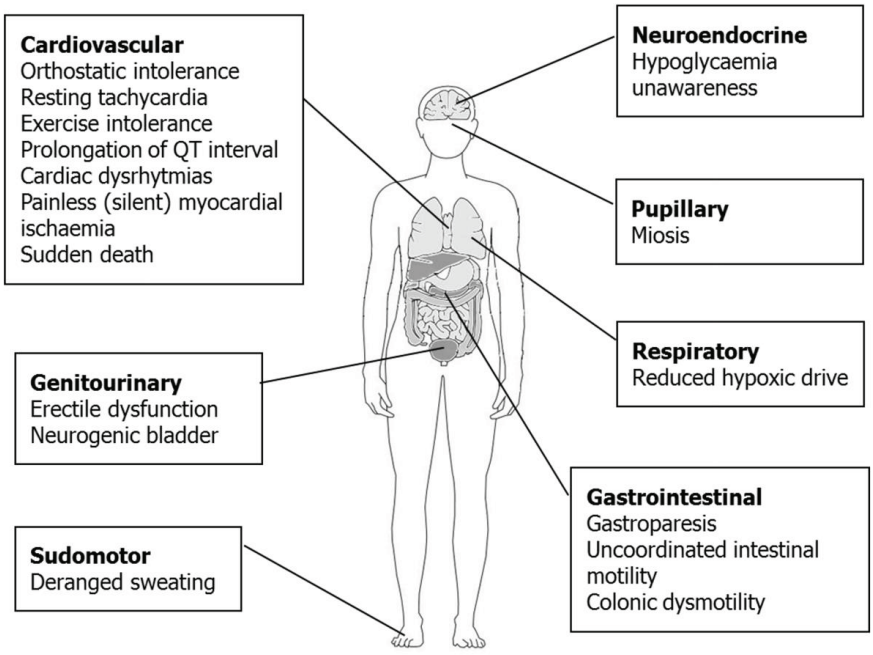
In developed countries, diabetes is the most common cause of autonomic neuropathy. CAN, the most studied form of diabetic autonomic neuropathy, is defined as impairment of autonomic control of the cardiovascular system in the setting of diabetes and after exclusion of other possible causes. Diagnostic criteria and staging are still under debate but according to a recent recommendation (79) the presence of one abnormal cardiovagal test identifies possible or early CAN; at least two abnormal heart rate-based tests are required for a definite or confirmed diagnosis of CAN; and orthostatic hypotension (asymptomatic or symptomatic), in addition to any heart rate-based test abnormalities, identifies a condition of severe or advanced CAN. More advanced stages of CAN carry an increasingly worse prognosis (91).

2.3.1 Clinical manifestations of autonomic dysfunction

The most typical clinical manifestations of CAN are presented in Figure 1. Due to the widespread distribution of the ANS, practically all organs are potentially susceptible to autonomic dysfunction. Autonomic dysfunction tends to be subclinical for years after the onset of type 1 diabetes and becomes symptomatic only at an advanced stage of the disorder. Thus, screening tests are essential to detect the condition at an earlier stage, as is also the case regarding the other diabetic complications.

Screening for CAN is recommended 5 years after the diagnosis of type 1 diabetes, particularly in patients at greater risk for CAN due to a history of poor glycaemic control or clustering of other diabetic micro- or macrovascular complications, or if there are signs of DPN, unexplained tachycardia, orthostatic hypotension, or poor exercise tolerance (79).

Figure 1 Clinical manifestations of autonomic neuropathy



2.3.2 Prevalence

The reported prevalence of CAN varies widely depending on the methodology, the diagnostic criteria used, and the population studied. Assessment of prevalence is in addition affected by the inconsistency of diagnostic methodology, but also by type of diabetes studied. Particularly earlier studies have often included both patients with type 1 and with 2 diabetes and in some cases the type of diabetes has not even been defined. While abnormal test results indicative of CAN may be present already at the diagnosis of type 2 diabetes or even in impaired glucose tolerance (92), probably due to a long preceding period of disturbed glucose metabolism, CAN manifested in type 1 diabetes is considered a late finding. Nonetheless, CAN has been reported also in newly diagnosed type 1 diabetic patients (93, 94).

In unselected, clinic-based populations including both type 1 and type 2 diabetic patients the reported prevalence of confirmed CAN has ranged from 16 to 20% (95, 96). However, when different cohorts of type 1 diabetic patients have been studied, the prevalence for CAN has shown a wider range from 1.6% to 90%, which can be attributed to the tests used, to the stage of the disease, or to the presence of other diabetic complications (80, 95, 97-104). Overall, the reported prevalence rates increase with age and increasing diabetes duration. It is of note that most of the prevalence studies were performed decades ago; during recent years there have been reports that the prevalence of the other diabetic complications decreases (53, 105-107). A similar improvement has not yet been confirmed regarding the prevalence of CAN.

2.3.3 Pathophysiology

2.3.3.1 Risk factors

Exposure to hyperglycaemia is undoubtedly established as the primary cause in development of diabetic neuropathy (108, 109), but this process is further modified by genetic and environmental factors. In the DCCT Study intensive glycaemic control, over a mean follow-up period of 6.5 years, reduced the CAN incidence in patients with type 1 diabetes by 50% (101). As observed for retinopathy and nephropathy, the beneficial effect of previous intensive treatment on neuropathy persisted up to 14 years after the end of the DCCT/EDIC Study, despite later deterioration of glycaemic control (104). Thus, it seems that there exists a "metabolic memory" effect also for measures of CAN, i.e. the earlier glycaemic exposure is remembered by the nervous tissue (40). It is of note that according to the DCCT, only 11% of the incidence of diabetic microvascular complications can be explained by hyperglycaemia measured by HbA_{1c} (110), but the remaining 89% was explained by other factors. HbA_{1c} does not capture all the effects of hyperglycaemia, as it is insensitive to fluctuations, moreover, the risk for neuropathy is further modified by other risk factors.

Age, diabetes duration, hypertension, and presence of diabetic polyneuropathy, diabetic retinopathy, microalbuminuria or diabetic nephropathy are risk factors for development of CAN in type 1 diabetes (102, 109, 111, 112).

2.3.3.2 Mechanisms of glucose neurotoxicity

Neurons have a high glucose demand, and unlike muscle or adipose cells, they are not normally able to use free fatty acids as an energy source. Thus, in order to ensure a continuous energy supply, the glucose uptake of neurons and Schwann cells has evolved to be independent of insulin (113, 114). Unlike most other cell types, these cells are not able to adjust the glucose transport into the cell when exposed to hyperglycaemia. Consequently, the extracellular hyperglycaemia is reflected as a direct increase in intracellular glucose level and results in perturbed intracellular metabolism (115).

Although not controlled by insulin, glucose transport across the blood-brain barrier, and into the neurons is carrier-mediated by the glucose transporters GLUT1 and GLUT3 (116). The blood-brain barrier offers some protection against hyperglycaemia in the CNS. Although the peripheral nerves have a corresponding blood-nerve barrier formed by endoneurial microvessels and the perineurium, data suggest that the peripheral nerves might be even more vulnerable to hyperglycaemia than is the CNS (117).

It is of note that most studies on the pathological pathways of glucose neurotoxicity have been performed in models of diabetic somatic neuropathy. While typical DPN initially affects the longest axons and is characterized as distal axonopathy in both myelinated and unmyelinated fibres, the pathogenesis of autonomic dysfunction is not yet fully established. Autonomic axons may be lost together with somatic nerves as part of DSPN. Other possible mechanisms may be degenerative changes in the autonomic ganglia or reduced blood flow through loss of autonomic nerve function that may contribute to the progression of diabetic peripheral neuropathy (118).

The hyperglycaemic milieu and metabolic derangements result in overproduction of reactive oxygen species (ROS) in the mitochondrial electron transport chain of the endothelial cells. Hyperglycaemia-induced tissue damage is thought to arise through four major mechanisms: the polyol pathway, accumulation of advanced glycation end products (AGEs) and increased expression of the AGE receptor (RAGE) and its activating ligands, activation of protein kinase C isoforms, and increased activity of the hexosamine pathway (115). Oxidative stress plays a crucial role in the development of diabetes complications, since all these pathways are activated by ROS. This unifying hypothesis of intracellular hyperglycaemia-induced tissue damage was originally suggested in regards to development of diabetic microvascular complications but has later also been linked to the pathogenesis of macrovascular disease. However, in diabetic macrovascular disease and cardiomyopathy the overproduction of ROS appears to be a consequence of increased oxidation of fatty acids, at least in part due to pathway-specific insulin resistance. The increased oxidation of free fatty acids

causes mitochondrial overproduction of ROS and ultimately activates the same detrimental pathways as does hyperglycaemia (119).

When the normal glycolytic pathway is saturated, the glucose overload is shunted into **the polyol (sorbitol/aldose reductase) pathway**, where it is converted to sorbitol by the enzyme aldose reductase, and further oxidized to fructose. This process consumes nicotinamide adenine dinucleotide phosphate (NADPH), and results in decreased levels of glutathione and nitric oxide (NO), thus contributing both to intracellular oxidative stress and to inhibition of vascular relaxation. The accumulation of sorbitol may also damage the cell either by a direct toxic effect or by tissue swelling through an increased osmotic effect. Because Schwann cells are especially rich in aldose reductase, the saturation of this pathway may play a key role in these cells (120).

In addition to haemoglobin, other proteins in the human body are also subject to glycation. Hyperglycaemia results in the formation of **advanced glycation end products (AGEs)** through non-enzymatic, irreversible reactions between carbohydrates and free amino groups of proteins. The glycolytic metabolite methylglyoxal is a major precursor of the AGEs, which are now widely accepted as key players in the pathophysiological process of diabetic complications. The intracellular glycation of macromolecules injures the nervous tissue through several mechanisms, including uncontrolled activation of cellular metabolism and signalling pathways. The AGE-modified myelin is susceptible to phagocytosis by macrophages, a process that may thus contribute to segmental demyelination of the peripheral nerves. Moreover, the AGEs modify major axonal cytoskeletal proteins, which results in axonal degeneration and impairment of axonal transport and regenerative activity (121). **The receptor for advanced glycation end products (RAGE)** is a multiligand member of the immunoglobulin superfamily of cell surface receptors. It is able to interact with a broad range of endogenous ligands in addition to AGEs. In conditions such as diabetes with increased AGE formation, the activation of RAGE induces production of ROS and a subsequent increase in the expression of proinflammatory cytokines. The activation of nuclear factor KB signalling in neurons is a potential mediator of neuronal dysfunction. Notably, diabetes-induced loss of pain perception is prevented in RAGE-deficient mice and also prevented by treatment with soluble RAGE (122).

Intracellular hyperglycaemia and overproduction of ROS cause increased synthesis of diacylglycerol, which results in **activation of the protein kinase C isoforms**. Activation of these phosphorylating enzymes affects several signal transduction cascades and may in addition lead to vasoconstriction and reduction of neuronal blood flow through enhanced production of the vasoconstrictor endothelin-1 and a reduced level of endothelial NO synthase (123). Ultimately, this pathway results in accumulation of free radicals which activate poly-ADP-ribose polymerase (PARP). In diabetes, it seems that this relationship is bidirectional, and it also seems that PARP activation leads to free-radical and oxidant generation (124). Overactivity of PARP results in cell death by energy depletion. Several studies indicate that PARP activation

is a potential mechanism of neuronal cell death in ischaemic stroke and in neurodegenerative disorders (125, 126).

The **hexosamine pathway** is activated when excessive amounts of glycolytic metabolites accumulate. Some of the fructose-6-phosphate is shunted into a diverging signaling pathway where it is converted by the enzyme glutamine fructose-6 phosphate amidotransferase, through glucosamine-6-phosphate to the major end product uridine diphosphate-N-acetyl-glucosamine. The metabolic effects of this pathway are thought to be mediated by increased *O*-GlcNAcylation of the transcription factor Sp1, which results in enhanced expression of plasminogen activator inhibitor-1 promoter in vascular smooth muscle cells and of transforming growth factor-β1 in arterial endothelial cells (127).

2.3.3.3 Other potential mechanisms of neural damage

According to current opinion, diabetic neuropathy develops through complex interactions between metabolic and vascular factors (128). Reduced nerve perfusion is a crucial factor in pathogenesis of diabetic nerve damage but whether it is a cause or a consequence is still under debate. Notably, blood vessels depend on neural regulation in order to maintain their normal function, and on the other hand neurons depend on nutrient supply through the capillaries. Irrespective of whether neural damage is caused by direct neuronal injury or through an impairment of the neuronal blood supply, the neural damage is usually seen as irreversible: intervention studies have failed to reverse the process. Microvascular dysfunction seems to occur early in diabetes, in parallel with neural dysfunction, and differing roles have been suggested for the microvascular dysfunction in development of neuropathy between type 1 and type 2 diabetes. Importantly, studies in streptozotocin-induced diabetic rats show that vasodilating drugs improve both nerve conduction velocity and blood flow deficits (129).

Nerve growth factor and other members of the neurotrophin family of peptides are essential in the maintenance of nerve structure, function, and neuronal blood supply, and studies suggest their potential role also in the pathogenesis of diabetic neuropathy (130, 131). Insulin also has neurotrophic effects, and insulin-deficiency in type 1 diabetes may contribute to development of neuropathy (132). Another possible mechanism of insulin deficiency-induced neuropathy is the absence of proinsulin C-peptide. Replacement of C-peptide has beneficial effects on nerve conduction velocity and on nerve structural changes in type 1 diabetic animal models, probably through NO-mediated vasodilation (133).

Deficiency in essential fatty acids on the one hand (134), and accumulation of free fatty acids on the other both have a direct toxic effect on the nerves and may therefore contribute to the pathogenesis of diabetic neuropathy. In addition, autoimmune reactions against nervous tissues (135), chronic low-grade inflammation (136, 137), and frequent hyperinsulinemia-induced hypoglycaemic episodes may also be potential mediators of diabetic nerve damage (138). Overall, it has been suggested that some

pathways are more involved than are others in the pathogenesis of diabetic neuropathy, depending on stage of the disorder (139).

2.4 Cardiovascular autonomic function and its assessment

2.4.1 The autonomic nervous system (ANS)

The autonomic nervous system (ANS) is a complex network of neurons widespread throughout all organs of the body. The ANS operates mainly at the subconscious level to maintain homeostasis, to adapt the body to physiological changes, and to evoke the "fight-or-flight" response (if needed). John Langley, Professor of Physiology at the University of Cambridge from 1903 until his death, is renowned for his studies on this specific part of the nervous system earlier designated as the organic, vegetative, sympathetic, visceral, or the involuntary nervous system. In 1898, at the suggestion of Richard Jebb, Professor of Greek at the University of Cambridge, Langley coined the term "autonomic nervous system", since "the word implies a certain degree of independent action, but exercised under control of a higher power" (140). He identified the separate components of this nervous system, with the term "sympathetic" confined to the thoracic outflow of the autonomic system; he introduced the term "parasympathetic" to designate its cranial and sacral outflows (141).

Today, ANS is still considered anatomically and functionally divided into two parts, the sympathetic and the parasympathetic system, also called the vagal (142). In addition, a third subsystem of neurons (NANC, for non-adrenergic, non-cholinergic neurons) are integrated into the ANS, primarily in the gut and lungs, and these use NO as a neurotransmitter (143). The two main systems work in a coordinated fashion, generally acting in opposition to one another. The target organs are not equally innervated by these two systems, however, and in some situations, effects of the sympathetic and parasympathetic nervous systems are complementary.

Functionally the ANS is based on a reflex arch containing a visceral receptor, an afferent pathway, the CNS, an efferent pathway, and finally, the target organ. The efferent autonomic pathways consist of two-neuron chains, where a preganglionic neuron, originating in the CNS, synapses in the autonomic ganglia with a postganglionic neuron which innervates the effector organ. All parasympathetic and preganglionic sympathetic neurons are cholinergic, meaning that they release the neurotransmitter acetylcholine, whereas the majority of the postganglionic sympathetic neurons are adrenergic and release norepinephrine (144).

2.4.2 Cardiovascular autonomic regulation

The primary task of the cardiovascular autonomic system is to maintain tissue perfusion. To achieve this, the control mechanism is based on complex interactions between cardiovascular reflex mechanisms and humoral factors (144). Importantly, the heart is able to function even in the absence of autonomic control, as is the case in a transplanted heart, but heart rate (HR) is essentially under the control of the ANS. Although the heart at rest is mainly under vagal modulation (145), the prevailing HR reflects the balance of influences both from the sympathetic and parasympathetic nerves. While the sympathetic innervation supplies all regions of the heart, the vagus provides a rich innervation to the sino-atrial node, to atrioventricular conducting pathways, and to the atrial myocardium (146).

The arterial baroreceptor reflex plays the key role in short-term BP control (144) by modulating the HR and peripheral resistance. This maintains BP within the normal range, and buffers BP fluctuations. These baroreceptors are stretch receptors located in the arterial wall of the aortic arch and carotid sinuses, at the gateway to the brain, to ensure its sufficient blood circulation. The afferent fibers originating from these receptors meet at the brainstem, and their stimulation (by increased BP) induces a dual response, both vagal activation and sympathetic withdrawal, resulting in a rapid reduction in HR. The subsequent decline in BP level is due to an initial bradycardia-induced reduction in cardiac output, followed by a slower vasodilating response that is secondary to the sympathetic withdrawal. Conversely, a drop in BP reduces baroreceptor stimulation and elicits adaptive mechanisms that counteract the BP change through increased sympathetic activation and vagal withdrawal (144).

2.4.3 Cardiovascular reflex tests

The function of the ANS can indirectly be examined by cardiovascular reflex tests. The basis for performing a cardiovascular reflex test is to induce a disturbance in the system and to monitor the cardiovascular response. Despite the development of more sensitive methods, the cardiovascular reflex tests ("Ewing tests"), as proposed by Ewing and Clarke in 1982 (147), are still considered the gold standard for clinical autonomic testing. It is recommended to use a set of cardiovascular reflex tests to avoid false-positive results. A key factor in autonomic assessment is standardization of confounding conditions such as time of day, room temperature, breathing pattern, and food intake.

Antecedent hypoglycaemia may blunt cardiovascular autonomic function (148), and thus it is recommended to exclude hypoglycaemias prior to the autonomic testing if possible. Moreover, drugs with adrenergic and anticholinergic properties may have substantial effects on the tests and before testing should be discontinued when possible. Since autonomic parameters decline with age, age-specific reference values are necessary (149-151). Due to their reproducibility, tests measuring RR variation

(151-153) have also been widely useful in research. During a Valsalva maneuver, the intraocular pressure rises, leading to a theoretical risk for intraocular haemorrhage (154). What is thus recommended is avoiding the Valsalva maneuver in patients with proliferative retinopathy or with unknown retinal status, although large-scale studies have shown that the procedure of cardiovascular autonomic reflex testing is safe and practically never causes complications (155).

The tests most widely used to evaluate cardiac parasympathetic and sympathetic function are presented in Table 2. Usually, the result of two abnormal tests is considered a clinically abnormal finding. However, a clear decrease in BP, or fainting as a sole finding in an active or passive orthostatic test suggests a diagnosis of CAN (156).

Table 2 Description of sympathetic and parasympathetic cardiovascular reflex tests

Cardiovascular autonomic reflex tests	Procedure	Measurement
Parasympathetic tests		
HR variation during deep breathing	Subject breathes in and out at six breaths per minute during ECG monitoring	E/I (expiration:inspiration) ratio: Ratio between longest RRI during expiration and shortest RRI during inspiration
HR response to Valsalva manoeuvre	Subject exhales forcibly into a mouth-piece against fixed resistance (40 mmHg) for 15 seconds during ECG monitoring	Valsalva ratio: ratio of longest RRI during the maneuver to shortest RRI following it
HR response lying-to standing	After supine rest, subject stands up during continuous ECG monitoring	30:15 ratio: Ratio between longest RRI around the 30^{th} beat and shortest RRI around the 15^{th} beat after standing
Sympathetic tests		
BP response lying-to-standing	After supine rest the subject stands up, and systolic blood pressure is measured after 2 min	Difference in blood pressure values measured at rest and 2 minutes after standing up
BP response to Valsalva manoeuvre	Subject exhales forcibly into a mouth-piece against fixed resistance (40 mmHg) for 15 seconds during beat-to-beat BP monitoring	Beat-to-beat blood pressure response is evaluated during different phases of the manoeuver
BP response to isometric handgrip	Subject squeezes a handgrip dynamometer to establish a maximum. Grip is then squeezed at 30% of maximum for 5 min	Diastolic blood pressure response to isometric exercise

HR, heart rate; BP, blood pressure; RRI, time interval between two successive R-peaks on the ECG

2.4.4 Heart rate variability (HRV)

HRV refers to the phenomenon of continuous oscillation in the intervals between consecutive heartbeats. Quantification of HRV from short- and long-term ECG recordings is a non-invasive method widely used in the assessment of cardiovascular autonomic regulation. Short-term recordings are usually 1- to 10-min ECG recordings obtained in stationary laboratory conditions, whereas long-term recordings can be obtained for example from 24-hour ECG recordings. Standardized conditions, stationarity, and high-quality ECG acquisition are essential to enable precise detection of the R-waves and are necessary for the correct assessment of HRV (157). Importantly, unless controlled, respiration is a major source of HRV and may bias the assessment. What is of note is that the Task Force did not consider the effect of respiration (157), which is now acknowledged in a more recent recommendation (158). Accordingly, the respiratory frequency of the subject should either be recorded or the breathing carefully controlled. Studies have shown that paced breathing (15/min), if properly performed, induces no major modifications of the autonomic tone, yet it allows correct analysis of HRV by removing artefact effects of irregular respiration into the low-frequency (LF) band. With spontaneous breathing, subjects should be instructed to maintain regular breathing and to avoid deep breaths.

Thorough visual inspection of the raw signals is a requirement for identification of any technical artefacts and ectopic beats, which requires editing before analysis. Continuous ECG signals are digitized with a minimum sampling rate of 200 Hz and analyzed with specific software to obtain a tachogram (time series of RRI, the time-interval between two successive R-peaks on the ECG). Quantification of HR fluctuations over time is usually performed by statistical (time domain) or by spectral (frequency domain) analysis of the RRI. In addition to the conventional methods, HRV can also be analyzed with non-linear methods based on the hypothesis that the components involved in cardiovascular regulation may interact with each other in a non-linear way.

2.4.4.1 Time-domain analysis of HRV

Time-domain analysis provides simple indices of overall HRV, reflecting mainly parasympathetic activity, but it does not allow separation of the sympathetic and parasympathetic components of the variability (157). The most common measures derived from time domain analysis is SDNN, which is mathematically equal to the square root of the total power (TP) of HRV and reflects global RR variability, and RMSSD, the square root of the mean squared differences of successive normal-to-normal RRIs. All these measures mainly explore HR parasympathetic regulation (158, 159).

2.4.4.2 Frequency domain analysis of HRV

Frequency domain analysis, also called power spectral analysis of RRI, quantifies the amplitude of HR fluctuations at specific oscillation frequencies. This method provides information on both sympathetic and parasympathetic influence on the HR (160, 161). Estimates of the RRI power spectra can be obtained by different algorithms, of which the non-parametric fast Fourier transformation (FFT) and parametric autoregressive modeling are the most frequently used. One advantage of the autoregressive method is its ability to detect the oscillatory components even from short recordings. Stationarity of the data is a prerequisite for both approaches; linear detrending and high-pass filtering are therefore usually applied to improve the quality of the data before spectral analysis. Spectral analysis divides the RR variation into four principal spectral components; HF (high-frequency, 0.15-0.40 Hz, average 0.25 Hz), LF (low-frequency, 0.04-0.15 Hz), VLF (very-low-frequency, 0.003-0.04 Hz), and ULF (ultra-low-frequency, below 0.003 Hz) power. The HF and LF spectral components are usually both given as absolute powers, and as normalized units by dividing the absolute power of a given component by the LF power plus the HF power multiplied by 100 (157).

The influence of the sympathetic and of the parasympathetic systems on the HRV markedly differ. Parasympathetic modulation raises the TP of the spectrum (total variability) whereas the sympathetic system modulates the variability of the LF band. The power of HF oscillations is related to respiration. This phenomenon, respiratory sinus arrhythmia, because it is abolished by atropine or vagotomy, so HF is considered a marker of parasympathetic activity (162).

The respiratory component of HR variation is mainly influenced by central impulses from the respiratory centre but also by BP changes secondary to respiratory movements mediated through arterial baroreceptors and by the reflex response to lung inflation, mediated through thoracic stretch receptors. The LF oscillations of the HRV are mediated by both sympathetic and parasympathetic activity and originate from baroreflex mechanisms and activity of an endogenous oscillator in the brainstem or the spinal cord. Fluctuations in the other lower frequencies (VLF, ULF) are believed to relate to other factors such as changes in activity and posture and probably reflect parasympathetic modulation. LF as absolute power does not reflect the sympathetic activity, whereas LF as a proportion of the TP provides an estimate of sympathetic influence on the HRV (158). During sympathetic activation such as during tilting, the power in all frequency components is reduced due to parasympathetic withdrawal and increased HR (163). The influence of respiration on HRV becomes progressively smaller, and as a result LF predominates over HF during sympathetic activation. The ratio between LF and HF often serves as an indicator of sympathovagal balance (164).

2.4.5 Short-term blood pressure variability

Similar to HRV, BP is also characterized by continuous fluctuations. In 1876 Sigmund Mayer observed periodic fluctuations in the BP with a cycle of once per 10 seconds (Mayer waves*)*. In addition to invasive, intra-arterial methods, continuous arterial BP can be monitored non-invasively by the beat-to-beat finger photoplethysmographic volume-clamp method (165). BP recordings obtained with a noninvasive volume-clamp-based monitor are considered equivalent to those measured by intra-arterial methods (166). Although this method shows poor correlation with the absolute BP values obtained invasively, it gives a good description of BP fluctuations and enables spectral analysis of the oscillations (165).

Short-term BPV can be quantified from continuous BP recordings by power spectral analysis and further dissected into the same spectral components as described for HRV. HF oscillations of the BPV are related to respiratory activity and reflect mechanical effects of stroke volume due to changes in venous return and respiration (157). The LF oscillations, also known as vasomotor or Mayer waves, are mainly under sympathetic control (167-169), but they are also modulated by arterial baroreflexes through changes in vascular tone and peripheral resistance (170, 171). The other spectral components are still poorly understood.

2.4.6 Baroreflex sensitivity (BRS)

BRS integrates information derived from both HR and BP. An increase in BP sensed by the baroreceptors reduces the firing of the sympathetic cardiac and vascular efferents and enhances the firing of the vagal efferents, resulting in a rapid decrease in HR and BP. The BP decrease is a consequence of decreased cardiac output through lower HR, and of vasodilation due to sympathetic withdrawal. Hence, in order to correctly assess the components of BRS, the sympathetic efferent activity also should be considered. Sympathetic BRS can be measured with simultaneous recordings of muscle sympathetic nerve activity (MSNA), but this technique is invasive and requires special equipment. Accordingly, the term baroreflex sensitivity usually denotes cardio-vagal control of BP regulation. Several methods allow study of the baroreflex, and in general, all these methods estimate the response of the HR to either spontaneous BP fluctuations or changes induced by vasoactive agents (phenylephrine, sodium nitroprusside, nitroglycerine) or other interventions (Valsalva maneuver, deep breathing, external neck suction) (172-174).

The invasive, pharmacological-based, modified Oxford method (phenylephrine method) is considered the gold standard of BRS estimation (175, 176). The phenylephrine method is based on an open-loop estimation of BRS, with the assumption that RRI changes are linearly related to changes in SBP. However, in recent years, non-invasive techniques have emerged to estimate BRS from spontaneous fluctuations in arterial pressure and RRIs, either during spontaneous or

timed breathing, or by induction of changes through slow, deep breathing or a Valsalva maneuver. What is noteworthy is that the non-invasive BRS measurements are measured under closed-loop control. The arterial baroreflex (control) senses the change in BP level (input), and then the activation of the baroreceptors aims to adjust for blood-pressure change to maintain the preferred BP level, i.e. the system output. The new BP level is in turn looped back to alter the control, i.e. the BRS. In a closed-loop model it is impossible to quantify the input to the baroreflex system (the BP change) separately from the output of the same system (the new BP level) (177).

2.4.6.1 Methods to estimate spontaneous BRS

Time-domain methods

The sequence method is the most frequently used time domain technique for assessment of BRS. It is based on the assumption that changes in RRIs result from linear, independent changes in SBP, mediated by the baroreflex arc (178). Valid sequences, i.e. sequences of three or more beats, in which the SBP spontaneously either increases or decreases followed by parallel changes in the RRI (at the minimum of 1 mmHg or 5 ms) are identified and their slopes determined. Usually, a correlation coefficient > 0.85 is required. The BRS estimate is obtained by averaging the regression slopes by averaging the negative and positive regression slopes separately.

Spectral methods

The spectral methods of BRS estimation are based on the assumption that a given change in BP is detected by the baroreceptors, followed by a reflectory change in RR variability of the corresponding frequency (179). Accordingly, the spectral estimates of BRS can be calculated as the ratio of the fluctuation in RRIs over the ratio of the fluctuations in BP at the same frequency.

The alpha indices, BRS-αLF and BRS-αHF, are computed as the square root of the ratio between the spectral powers of the RRI and the SBP series in the LF (0.04-0.15 Hz) or HF (0.15-0.4 Hz) range, respectively. The average of these two measures is termed **the alpha coefficient.** To ascertain that the RRI changes are related to the BP changes usually requires a coherence >0.5 between the signals. In addition, analysis is performed only of sequences with a negative phase value, which implies that the RRI changes are preceded by changes in SBP, reflecting a baroreceptor-mediated mechanism.

Transfer function gain is based on the assumption that the baroreceptor-heart rate reflex is a simple linear single-input single-output system, where BRS is the change in RRI (output signal), caused directly by a unit change in SBP (input signal). The transfer function gives an estimate of the gain at the given frequency. Commonly, the cross-spectral transfer function BRS is computed by averaging the transfer function gain between the SBP and the RRI time series (SBP-RR cross-spectrum divided by the

SBP spectrum) across the LF range (0.04-0.15 Hz), where the coherence exceeds 0.5 (180, 181).

BRS estimates derived from the sequence and cross-spectral methods show variable correlation with those of the pharmacological phenylephrine method (182-185). Studies have, however, also shown a poor correlation between the different indices of spontaneous BRS, and none of the methods has shown a clearly superior performance over the other (186). In sum, the biological meaning and the possible prognostic information provided by the various estimates is unclear, and it is possible that the estimates reflect different aspects of cardiac baroreflex. Thus far, most of the BRS estimates have integrated signal requirements to ensure that the estimate is based on a baroreflex-mediated mechanism. However, in collaboration with a group of scientists from Italy, we have recently introduced a method that deviates from this approach. We demonstrated that the ratio between the SD of the RRI and SD of SBP gives a robust estimate of BRS (187), which is also associated with worse prognosis in patients with systolic heart failure (188). In addition, our results showed that high-pass filtering improved the consistency of the different BRS estimates in general.

2.4.7 Other methods to evaluate autonomic cardiovascular function

Autonomic cardiovascular function can be evaluated by a number of other indirect methods, the simplest being resting HR. Other ECG-derived methods are HR recovery after exercise (189, 190), HR turbulence, QT-interval dispersion, and T-wave alternans that also serve as markers of autonomic imbalance and risk for sudden cardiac death in conditions other than diabetes (191). The non-dipping phenomenon in 24-hour ABPM is regarded as a marker of autonomic dysfunction (192).

Direct assessment of myocardial sympathetic innervation is possible through imaging with radiotracers such as MIBG (iodine-131-meta-iodobenzylguanidide). This method provides a sensitive and highly reproducible tool to detect early sympathetic dysfunction in type 1 diabetic patients (193, 194).

The most direct measure of sympathetic activity is assessment of MSNA, meaning measurement of bursts of efferent sympathetic activity in skeletal muscle (176). It is the only method to directly assess the sympathetic vascular arm of the arterial baroreflex, but on the other hand, is both invasive and time-consuming and thus used only in research.

Corneal confocal microscopy is a promising new non-invasive technique to assess structural morphology of the small nerve fibres in the cornea. Corneal nerve fiber damage correlates with intra-epidermal nerve fibre density and severity of diabetic neuropathy in patients with diabetes; it and might also provide a measurement of subclinical small-fibre injury (195-197).

Postganglionic sympathetic cholinergic sudomotor function can be evaluated with a quantitative sudomotor axon reflex test. Sudomotor dysfunction is one of the earliest detectable abnormalities in distal small fibre neuropathy (198, 199).

Finally, the degree of sympathetic activity can be evaluated by measuring the plasma concentration of plasma catecholamines (200). Plasma level depends, however, not only on amount of secretion but also on the rate at which the catecholamines are removed from the circulation. The norepinephrine spillover technique gives an estimate of tissue clearance of norepinephrine by assessing the degree of dilution of a small amount of intravenously administered radiolabeled norepinephrine. The spillover rate is thought to reflect norepinephrine release from sympathetic nerve endings and thus, more specifically, the sympathetic activity (201). Although measurement of circulating catecholamines has contributed to the understanding of pathogenetic mechanisms, the accuracy of this method does not provide any additional power to the diagnosis and staging of diabetic autonomic neuropathy (158).

2.4.8 Association between autonomic dysfunction, morbidity, and mortality

Back in 1976, Ewing showed the survival disadvantage in patients with diabetes and CAN based on cardiovascular autonomic reflex tests (202). Thereafter, longitudinal studies have consistently reported an increased risk of mortality in patients with diabetes and autonomic neuropathy (42, 203-206). It is noteworthy that these associations could be in part explained by other comorbid complications. However, a more recent meta-analysis of published data demonstrated that impaired cardiovascular autonomic function, as measured by HRV, doubled the relative risk for silent myocardial ischaemia and mortality independently of other diabetic complications (7). This meta-analysis comprised studies including both type 1 and type 2 diabetic individuals. What is, however, evident is that autonomic dysfunction is associated with standard cardiovascular risk factors (109, 111, 112, 207), and with markers for cardiovascular morbidity such as attenuation or loss of the nocturnal fall in 24-hour ABPM (non-dipping) (47, 192, 208), silent myocardial ischemia (7, 209), increased coronary artery calcification (210, 211), arterial stiffness (212-214), QT prolongation (215), left ventricular abnormalities (216-218), and markers of chronic low-grade inflammation (136, 137, 219).

The association between autonomic dysfunction and other diabetic micro- and macrovascular complications is well established, although the temporal and causal relationships are still unclear. Indirect evidence indicates, however, that autonomic dysfunction—particularly when assessed with more sensitive methodology—occurs at an early stage of type 1 diabetes, and that it precedes and even predisposes to other diabetic late complications. In a follow-up study, autonomic dysfunction, assessed with pupillometry and cardiovascular function tests, was associated with a 4- to 5-fold risk for developing microalbuminuria or retinopathy (220). Moreover, several studies

show that autonomic abnormalities to independently predict progression of diabetic nephropathy (204, 221, 222). In addition, some studies (47, 48)—although with some controversy (223)—have suggested that the lack of nocturnal SBP dipping is a sensitive marker of incipient diabetic nephropathy.

2.4.9 Prognostic significance of abnormal HRV and BRS in cardiovascular medicine

Autonomic abnormalities are usually attributed to diabetic neuropathy, although these abnormalities are also reported in a number of other conditions associated with a functional sympatho-vagal imbalance. A depressed HRV predicts mortality in the general population (224, 225), in post-myocardial-infarction patients (14, 226-228), in heart failure (229, 230), and in chronic kidney disease (231, 232). A reduced BRS is an independent marker of risk of mortality and major adverse cardiovascular events in hypertensive patients (17). Notably, also in hypertensive patients with chronic renal failure and dialysis treatment, reduced BRS is an independent predictor of sudden death (16). Impaired BRS is a strong independent prognostic marker of survival in heart failure (13, 14), but also in patients having survived a myocardial infarction, even with preserved left ventricular function (233). Reduced BRS is furthermore associated with worsened short- and long-term prognosis after acute ischaemic stroke (15, 234).

2.4.10 Role of autonomic function in pathogenesis of hypertension

Although numerous pathophysiological factors have been implicated in development of essential hypertension, increased sympathetic activity (and the subsequent autonomic imbalance) also seems to play a key role. The sympathetic nervous system operates not only in short-term BP control, but also in the pathogenesis of hypertension and in maintenance of elevated BP level. Mechanisms behind the increased sympathetic activity are complex and involve alterations in baroreflex and chemoreflex pathways. HR, the simplest indicator of sympatho-vagal imbalance, is a an independent predictor of diastolic blood pressure (DBP) in young adults (235). The association between sympathetic over-activity and BP has been demonstrated also with other methods, such as MSNA (236) and plasma epinephrine level (237).

Reduced HRV is associated with a higher risk for hypertension (238, 239). However, decreased overall HRV, increased sympathetic, and reduced vagal indices of the HRV are already present at an early stage of hypertension (240, 241). The close relationship between reduced BRS and high BP is evident (242-245), but whether low BRS is the cause or the consequence of elevated BP is still unclear. However, similar to HRV, BRS is already reduced at high-normal BP. Thus, it is possible that a blunted BRS (due to sympathetic activation) is one of the mechanisms allowing increased fluctuations in short-term BPV, hence progressively leading to resetting of the system. It is of note

that increased variability in SBP, assessed by visit-to-visit BPV (246, 247), or by ABPM (248, 249) is associated with increased risk for end-organ damage. Whether BRS or other autonomic indices predict hypertension in type 1 diabetes is as yet unknown.

2.4.11 Effect of respiration and respiration rate on HRV and BRS

By 1733, Hales had already observed that HR increases during inspiration and decreases during expiration. One potential role of respiratory sinus arrhythmia is to improve respiratory gas exchange efficiency through better matching of the alveolar ventilation and capillary perfusion (250). Importantly, respiration is the main source of fluctuations in RRI and in SBP (251). HR oscillations in the HF band (0.15-0.4 Hz), the typical frequency range of normal adult respiration, are vagally mediated. The respiratory oscillations of the HR may arise through several possible mechanisms: through BRS-mediated responses to fluctuations in BP, through responses to respiration-synchronized fluctuations in pulmonary and thoracic stretch receptors (252), through central cardiorespiratory coupling, and through chemoreflexes. In the intact heart, the mechanical stretch on the sinoatrial node seems to play only a minor role (174). Intra-thoracic pressure changes during the respiratory cycle modify left ventricular stroke volume and BP through changes in cardiac venous return, which results in parallel BRS-mediated changes in HR (253, 254).

Slow breathing at 6 cycles/minute causes RR fluctuations to merge at the respiratory rate and increases the amplitude of the fluctuations. Studies have shown that slow, deep breathing increases BRS and reduces sympathetic tone in healthy individuals (255), as well as in conditions like heart failure (256, 257), hypertension (258), and chronic obstructive pulmonary disease (259). Slowing of the respiratory rate reduces dyspnea and improves both resting pulmonary gas exchange and exercise performance in patients with heart failure (260). In essential hypertension, MSNA falls during short-term slow breathing (261). One additional mechanism behind these findings is that slow breathing and the increased tidal volume stimulate the Hering-Breuer reflex. This is an inhibitory reflex triggered by stretch receptors in the lungs that serves to prevent over-inflation of the lungs, elevates vagal activity and may reduce sympathetic activity. In sum, it seems that changes in sympathetic activity and in BRS are closely interrelated. Although the exact mechanisms are unclear, slow breathing seems to induce a generalized decrease in the excitatory pathways regulating respiratory and cardiovascular systems.

2.4.12 Effect of oxygen on HRV and BRS

The respiratory and cardiovascular systems are tightly coupled and therefore a modification of the respiratory component may induce changes in cardiovascular regulation and vice versa. The chemoreflexes are important modulators of autonomic activation. The peripheral chemoreceptors situated in the carotid bodies respond

mainly to hypoxaemia, whereas the central chemoreceptors located in the brainstem respond to hypercapnia. Activation of either chemoreflex normally induces sympathetic activation, an increase in ventilation, and depression of the BRS (262). Normally, baroreflex activation has the opposite effect on ventilation. Moreover, the arterial baroreflexes have a strong inhibitory effect on the chemoreflexes, especially on the peripheral chemoreflex (263).

Inhaled oxygen reduces HR and raises those HRV indices that are related to parasympathetic activation (264-267), but not in serious autonomic neuropathies such as familial dysautonomia (268). Treatment with hyperbaric hyperoxia enhances parasympathetic modulation of HRV in healthy individuals (264), and in patients with type 2 diabetes (267). In addition, oxygen administration has a dose-dependent effect on the parasympathetic lung afferents, resulting in increased ventilation (269, 270). Exposure to hypoxia at high altitude induces vasodilation and reflectory sympathetic activation followed by BRS reduction (271). Conversely, oxygen supplementation raises BRS in healthy individuals (266) and in patients with CHF and COPD (265, 272). In patients with severe obstructive sleep apnea syndrome, BRS is depressed probably due to sympathetic activation through hypoxia-induced chemoreceptor stimulation (273). Notably, inhaled oxygen has been part of the standard treatment in patients with acute coronary syndrome, despite the absence of conclusive evidence as to any beneficial (or harmful) effects of oxygen in normoxic patients with acute coronary syndrome (274).

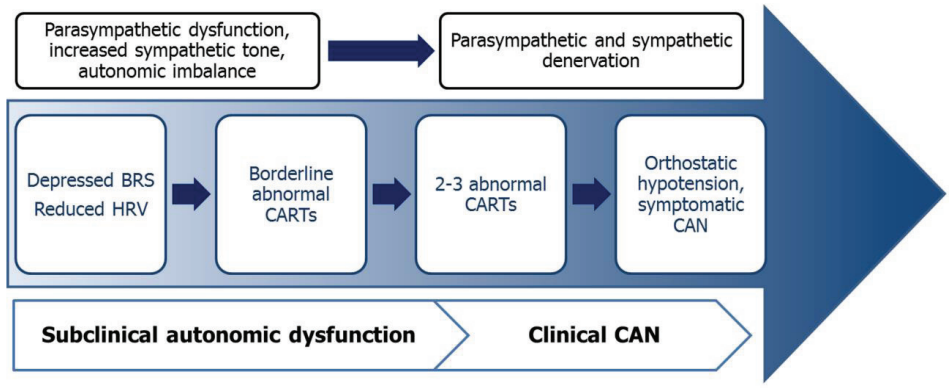
2.4.13 Natural history of autonomic dysfunction in type 1 diabetes

Nerve function is dependent on axonal transport: a sufficient supply of metabolic substrates, enzymes, and structural proteins mainly originating from the cell body itself. This is the probable explanation for the fact that the longest nerve fibres are especially susceptible to neuropathy. The vagus nerve, the longest autonomic nerve, mediates approximately 75% of all parasympathetic activity. Thus, the earliest manifestation of autonomic neuropathy in patients with diabetes tends to be associated with parasympathetic denervation (275). One hypothesis is that diabetic autonomic neuropathy begins as vagal denervation that leads to autonomic imbalance. However, another suggestion is that a compensatory increase may already occur in cardiac sympathetic tone in response to subclinical peripheral denervation early in the course of autonomic dysfunction (276), one that augments the autonomic imbalance. Over time, this disorder may advance to denervation of both the parasympathetic and the sympathetic nervous systems (Fig. 2). Similar to vagal and peripheral neuropathy, cardiac sympathetic denervation also begins distally, at the apex of the ventricles, and progresses towards the base (277).

Notably, a discrepancy seems to exist between the high prevalence of subclinical autonomic abnormality or of autonomic imbalance and, as yet, the fairly small proportion of patients with severe, symptomatic autonomic neuropathy. BRS is considered a more sensitive measure of autonomic function than are conventional

autonomic function tests (9, 10), but whether low BRS predicts a progression to CAN is as yet unknown; also unknown is whether there exists a point of no return, after which the autonomic dysfunction progresses to a stage refractory to all interventions. This would indicate that autonomic dysfunction could, in its earliest phase, be functional and does not necessarily progress to CAN in all patients. The concept of a functional autonomic disorder does not diminish the importance of early diagnosis, given that reduced BRS is an established marker of poor prognosis in hypertension, renal failure, post-myocardial infarction, heart failure, and cerebral stroke, conditions associated with functional alterations in the ANS (13-17). Moreover, even were the disorder functional, it could still predispose to or predict future diabetic complications. Studies have demonstrated the beneficial effect of physical activity in management of functional autonomic abnormalities, but what is unknown is whether it is possible to correct these abnormalities in patients with type 1 diabetes either by short or more longstanding interventions. Tissue hypoxia is accepted as a potential factor in the pathogenesis of diabetic complications, but no studies concern its possible role in autonomic dysfunction in patients with type 1 diabetes.

Figure 2 Progression of autonomic dysfunction in diabetes and its relation to clinical abnormalities. At the earliest, subclinical stage of diabetic autonomic neuropathy, the autonomic imbalance is a result of either augmentation of sympathetic tone, parasympathetic denervation, or both. Over time, the disorder may advance to denervation of both the parasympathetic and the sympathetic nervous systems, thus often manifested as symptomatic autonomic neuropathy.



BRS, baroreflex sensitivity; HRV, heart rate variability; CART, cardiovascular autonomic reflex test; CAN, cardiovascular autonomic neuropathy. Adapted by permission from Macmillan Publishers Ltd: Nature Reviews Endocrinology. Michael Kuehl & Martin J. Stevens. "Cardiovascular autonomic neuropathies as complications of diabetes mellitus", Copyright 2012 (278).

3 AIMS OF THE STUDY

The specific aims of the present study were:

1. To characterize the autonomic function (HRV and BRS) in well-defined cohorts of patients with short- (I-II) and long-duration (II) type 1 diabetes

2. To learn whether early autonomic dysfunction as evidenced by low baroreflex sensitivity (BRS) is correctable by slow, deep breathing in short- (I) and long-duration (II) type 1 diabetes

3. To determine the BRS response to slow, deep breathing in patients with type 1 diabetes with different levels of autonomic involvement (II)

4. To determine how oxygen administration affects BRS and ventilation in patients with type 1 diabetes as compared to healthy controls (III)

5. To determine whether autonomic indices decline more in patients with type 1 diabetes than in healthy subjects, and whether baseline BRS predicts progression to CAN during a 5-year follow-up (IV)

6. To elucidate the role of BRS as a potential predictor of increased blood pressure during a 5-year follow-up (IV)

4 SUBJECTS AND STUDY DESIGN

The IDEAL Study (IDentification of EArly mechanisms in the pathogenesis of diabetic Late complications) is part of the nationwide Finnish Diabetic Nephropathy (FinnDiane) Study and it was carried out at the Department of Diabetes Genetics of Folkhälsan Research Center, University of Helsinki and at the Department of Medicine, Division of Nephrology, Helsinki University Central Hospital, during 2003-2009. Type 1 diabetes was defined as C-peptide deficiency (<0.03 nmol/l) and initiation of permanent insulin treatment within 1 year after the diabetes diagnosis. All patients and control subjects gave their informed consent before their inclusion. The study protocol was approved by the Ethics Committee of Helsinki University Hospital and by the Ethics Committee of the University of Pavia, Italy (for heart-transplanted participants), and the study was carried out in accordance with the principles of the Declaration of Helsinki as revised in 2000. Clinical characteristics of the patients with type 1 diabetes and healthy control subjects are shown in Table 3.

4.1 Study populations

4.1.1 Study I

The participants were recruited through the register of the Social Insurance Institution that comprises all patients entitled to special reimbursement for insulin or glucose-lowering medication in Finland. This register covers approximately 98% of all Finnish patients with type 1 diabetes (279). Selection criteria were: diabetes diagnosed before age 35, diabetes duration of 6 to 12 years, and age at inclusion between 18 and 35 years. A total of 400 individuals residing in the Helsinki metropolitan area fulfilled these selection criteria and received invitations. Of the 165 who responded, 25 were excluded since they had types of diabetes other than type 1, were currently pregnant, or failed to attend. Consequently, 140 participants were enrolled and 125 participated in all examinations at baseline (I). None of these patients showed clinical signs nor had any history of CVD. Their diabetes duration was 8.9±0.1 years. The 36 age- and gender-matched healthy control participants were recruited by email advertisements among university students and staff. Included were only those with normal fasting glucose and without first degree relatives with diabetes mellitus. Patients with type 1 diabetes had significantly higher SBP than did the control subjects. Of the patients, 6% were on antihypertensive treatment (AHT). Their serum lipid concentrations were equivalent except for LDL cholesterol, which in the diabetic patients was higher.

Data from a third group of heart-transplanted participants were analyzed retrospectively. This group, studied in Italy at the University of Pavia, comprised 12

participants (5 men, 7 women, aged 54.7±2.4 years) who had undergone orthotopic heart transplantation 34.7±4.9 months earlier. They had taken part in a study documenting absent or only rudimentary re-innervation of the heart after heart transplantation (174).

4.1.2 Study II

For this study, we examined a group of Finnish patients with long-duration (33.7± 0.5 years) type 1 diabetes (N=37). These patients had participated initially in another study in Finland and were recruited to the present study at a follow-up visit (207). They had been diagnosed with type 1 diabetes between 1968 and 1978 and their age at onset was <15 years. An age- and sex-matched control group originated in an earlier study in Italy of healthy individuals (N=37). Data on the patients with long-duration type 1 diabetes and control subjects were compared and pooled with the data from patients with short-duration type 1 diabetes and control subjects from Study I. The group of 12 heart-transplanted subjects served also in this study as a model of definitive cardiac denervation.

4.1.3 Study III

At the prospective visit of the Ideal Study, we restudied 96 patients (of the original 140) with type 1 diabetes and 40 age-matched healthy controls. None of the patients showed clinical signs nor had any history of CVD. However, six patients had undergone laser treatment for diabetic retinopathy. Unfortunately, we were unable to restudy the original control group (I), but instead recruited new healthy volunteers. The healthy control subjects were recruited by email advertisements among university students and staff. Included were only individuals with normal fasting glucose and without first-degree relatives with diabetes mellitus.

4.1.4 Study IV

A total of 125 patients participated in the examinations at baseline in 2003-2004; 96 of these were restudied at the follow-up visit in 2008-2009. Overall, 80 patients had complete data (autonomic testing and ABPM) from both the baseline and follow-up visits. Data from 425 healthy controls of an age range of 16 to 60 years served as controls (187). Duration at follow-up was 13.8±0.2 years, with a mean follow-up time of 5.0±0.0 years. A total of 21 patients were using AHT (17 new patients) at the time of the follow-up visit, whereas none used β-blockers. Office SBP remained unchanged over time, but the patients with AHT had higher BP both at baseline and follow-up. The patients who started AHT were older (35.6±0.9 vs. 30.8±0.7 years; $p<0.001$) with a higher age at onset of diabetes (21.3±1.0 vs. 17.2±0.7 years; $p<0.05$). These patients

also had a higher total cholesterol level ($p<0.05$). Four patients were laser treated due to retinopathy during follow-up. Urinary albumin excretion rate increased marginally ($p<0.05$). No major cardiovascular events were reported during follow-up.

Table 3 Clinical characteristics of patients with type 1 diabetes and healthy control subjects in the various studies (I-IV)

Study	I-II		II		III		IV	
	Short-duration type 1 diabetes	Healthy control subjects	Long-duration type 1 diabetes	Healthy control subjects	Patients with type 1 diabetes	Healthy control subjects	Type 1 diabetes at baseline	Type 1 diabetes follow-up
Sex (male/female)	62/55	17/19	18/19	37	54/42	19/21	46/34	46/34
Age (years)	26.3±0.5	27.6±0.7	42.7±0.8	40.4±0.7	31.5±0.6	31.0±1.1	27.0±0.6	32.0±0.6[c]
Duration of diabetes (years)	8.9±0.1	-	33.7±0.5	-	13.5±0.4	-	8.8±0.2	13.8±0.2[c]
Age at onset (years)	17.4±0.6	-	9.0±0.5	-	17.9±0.6	-	18.2±0.6	-
BMI (kg/m^2)	24.7±0.4	24.4±0.6	25.1±0.5	23.3±0.8	25.7±0.4[b]	23.8±0.6	24.8±0.4	25.7±0.5[c]
WHR	0.86±0.01	0.85±0.01	0.90±0.01	†	0.87±0.01	0.86±0.01	0.86±0.02	0.87±0.01
Current smokers (%)	21.6	16.7	25.0	†	16.7	7.5	16.0	13.8
Antihypertensive treatment (%)	5.1	0	40.5	†	22.9	0	6.3	26.3[c]
Laser-treated retinopathy (%)	0.9	-	35.1	†	6.3	-	1.3	6.3
HbA_{1c} (%)	7.6±0.1[a]	5.2±0.0	8.2±0.2	†	8.1±0.1[b]	5.3±0.0	7.5±0.1	8.0±0.1[c]
HbA_{1c} (mmol/mol)	59.4±1.1[a]	33.7±0.4	66.2±1.9	†	65.4±1.3[b]	33.8±0.5	58.1±1.4	64.4±1.3[c]
Total cholesterol (mmol/l)	4.6±0.1	4.4±0.2	4.8±0.1	†	4.6±0.1	4.4±0.2	4.7±0.1	4.5±0.0
HDL-cholesterol (mmol/l)	1.7±0.0	1.8±0.1	1.9±0.1	†	1.6±0.1	1.6±0.1	1.7±0.1	1.6±0.1[c]
LDL-cholesterol (mmol/l)	2.4±0.1[a]	2.0±0.1	2.3±0.1	†	2.4±0.1	2.3±0.1	2.4±0.1	2.4±0.1
Triglycerides (mmol/l)	0.95 (0.41-5.17)	0.87 (0.49-10.68)	0.90 (0.20-13.90)	†	0.99[b] (0.40-4.59)	0.85 (0.39-1.68)	0.97 (0.47-5.17)	0.99 (0.40-4.59)
Urinary albumin excretion rate (mg/24 h)	3 (0-774)	4 (1-33)	7 (0-898)	†	8 (1-147)	-	3 (1-113)	4 (0-458)[c]
Office systolic BP (mmHg)	132±1[a]	122±2	130±2	†	130±1[b]	121±3	134±2	132±1
Office diastolic BP (mmHg)	78±1	75±1	73±1	†	77±1	75±1	78±1	78±1

Data are mean ± SEM or median (range). a: p<0.05 short-duration type 1 diabetes vs. healthy controls (Study I); b: p<0.05 type 1 diabetes vs. healthy controls (Study III); c: p<0.05 type 1 diabetes baseline vs. follow-up (Study IV); †: no data available; BMI, body mass index; WHR, waist-to-hip ratio; BP, blood pressure

5 METHODS

5.1 Study protocol

Baseline and prospective visits were carried out according to the same protocol. During these two visits, the patients underwent a clinical examination either by a physician or a trained research assistant including resting ECG, laboratory testing, one 24-hour and two overnight urine collections, ABPM, standard autonomic function evaluation by cardiovascular reflex tests, and assessment of HRV and BRS. Data on medication, cardiovascular status, and diabetic complications were obtained and verified from the medical files. Each participant completed a detailed questionnaire on life style, smoking habits, and family history.

5.2 Autonomic testing

Testing of autonomic function was performed under standardized conditions. Before the testing, the participants received both oral and written instructions. They were asked to abstain from alcohol for 36 h, and from caffeinated beverages and cigarettes for 12 h before the examination. A light meal was permitted 2 h before testing. The participants were investigated in a quiet room, at a temperature between 19°C and 23°C, between 08:00 and 14:00 hours. Blood glucose was measured to exclude hypoglycaemia.

Participants underwent a set of four cardiovascular autonomic function tests:
[1] expiration: inspiration ratio of the RRI during slow deep-breathing [2] maximum: minimum 30:15 ratio of the RRI during active standing [3] SBP response to standing [4] maximum: minimum ratio of the RRI during a Valsalva manoeuver. Individual test results were graded according to Finnish age-specific reference values (150). In line with current recommendations, borderline CAN was defined as the presence of one, and definite CAN as the presence of two or more abnormal tests (79).

5.2.1 Signal acquisition

Autonomic testing began after a 10-minute supine rest. ECG was recorded with a bipolar precordial lead. Continuous BP was monitored by a plethysmographic finger-cuff method (Finapres 2300; Ohmeda, Louisville, CO, USA). With the cuff around the middle finger of the right hand, the right arm was kept motionless at heart level. The self-adjustment procedure of the Finapres was performed immediately before the recordings and then turned off. At the baseline visit, timed breathing was visually

controlled by a trained nurse. At the follow-up visit, in addition, two respiratory signals were obtained by inductive plethysmography (Z-rip®, Pro-Tech, Mukilteo, WA, USA), from belts positioned around the chest and the abdomen. At the follow-up visit, other values obtained were for pulse oximetry and expired carbon dioxide (CO_2) partial pressure (Cosmo, Novametrix, Wallingford, CT, USA).

These signals were simultaneously recorded in the supine position during 5 min of spontaneous breathing and 2 to 5 minutes of controlled breathing at a frequency similar to that of spontaneous breathing (15 breaths per minute), and during 1 to 2 minutes of slow deep breathing at the rate of 6 breaths per minute. At the follow-up visit, each subject repeated the entire protocol while breathing 5 L/min oxygen delivered through a nasal cannula. Signal recordings started after the first 5 min of oxygen administration to allow stabilization of oxygen saturation and ventilation. The sequence of breathing rate (spontaneous, 15/min and 6/min) was randomized within each session (normoxia and hyperoxia). Signal recordings were also taken during an active orthostatic test (5 min in the supine and 7 min in standing position) and a Valsalva test (6).

Recorded signals were simultaneously digitized at 12-bit resolution at a sampling rate of 200 Hz with a data acquisition system (WinAcq; Absolute Aliens, Turku, Finland) and transferred onto a computer and analyzed with a menu-driven software package. Only signals free from ectopic beats and artefacts were acceptable. Some recordings were excluded due to a technical artefact, or a large number of ectopic beats during recording, or due to an anamnestic hypoglycaemic episode during the preceding 24 hours. Analysis of the HRV, BPV, and BRS was performed as described in Section 2.4. In short, total variability, SDNN, and RMSSD served as time-domain measures of RRI variability. FFT provided spectral components of the HRV (I, II, IV) and autoregressive model in Study III. Within the follow-up data, we performed a correlation between FFT and autoregressive model estimates; no difference was detectable. We did this to justify the use of 1- to 2-minute recordings in Study IV. Power spectral analysis of SBP was performed with FFT to obtain systolic BPV in the LF band (0.04-0.15 Hz).

5.2.2 Assessment of BRS

Spontaneous BRS came from the same recordings of ECG and continuous BP during spontaneous breathing, controlled breathing, and slow deep breathing. BRS was determined from spontaneous fluctuations in RRI and SBP by sequence methods (BRS+/+ and BRS-/-), the alpha indices (BRS-αLF and BRS-αHF), and transfer function (BRS-TF) as described in Section 2.4.6. Furthermore, we used a new method (BRS-SD), based on calculation of the ratio between SD of the RRI divided by SD of the SBP as a measure of BRS (187). Finally, we calculated an average of all the methods used ($BRS_{average}$), since none of the methods has proved superior to the others (186). It has been suggested that the procedure of controlled breathing by forcing the patient to breathe 15 breaths per minute may induce sympathetic

activation; this, however, is still unclear (259, 280). Thus, in our studies, BRS during spontaneous breathing served to represent the resting level. Deep breathing reduces sympathetic activity (257), so we calculated an increase in BRS as a response to deep breathing.

5.2.3 Analysis of respiration (III)

Signals from the inductive plethysmographic belt signals were analyzed with interactive software to identify the positive and negative respiratory peaks, the respiratory period, and the end-expiratory (end-tidal) value in the CO_2 signal. The sum of the signals from the two belts represented the relative index of tidal volume. Inductive belt data allowed a semi-quantitative intra-subject analysis of ventilation, by comparison of relative changes in tidal volume and minute ventilation induced by oxygen inhalation or differing breathing patterns. The strong linear relationship between tidal volume and inductive belt signals produced an estimate of the ventilation in relative units (281). The minute ventilation obtained during spontaneous breathing of room air was set as 100% for each subject, and the minute ventilation or tidal volume in changes from that value was calculated for each recording.

5.3 Laboratory tests

Venous blood samples were drawn after a light breakfast and were analyzed for HbA_{1c}, lipids and serum creatinine. HbA_{1c} concentrations were determined by immunoturbidometry. Serum lipids (cholesterol, triacylglycerol, HDL-cholesterol) and creatinine were measured by enzymatic methods. Serum LDL cholesterol was calculated by Friedewald's formula. In addition, blood glucose was measured bedside with a Beta-glucose analyzer (HemoCue Glucose 201+; HemoCue, Ängelholm, Sweden). Urinary albumin concentration was measured by immunoturbidometry from three consecutive timed urine collections, one 24-h and two overnight collections. Normal albumin excretion rate (AER) was defined as an AER persistently <20 μg/min or <30 mg/24 h, microalbuminuria as an AER≥20<200 μg/min or ≥30<300 mg/24 h, and macroalbuminuria as an AER≥200 μg/min or ≥300 mg/ 24 h in at least two of three urine collections.

5.4 Ambulatory blood pressure monitoring (ABPM)

The 24-hour ambulatory blood pressure was monitored at baseline in a subset of the participants (99 patients, 29 healthy control participants) with a monitoring device (SpaceLab 90207; Spacelabs, Redmond, WA, USA), that uses an oscillometric method. The recording day was a typical weekday, and the subjects were allowed to perform their normal daily activities except for rigorous physical exercise. Subjects were asked to relax the arm at their side when the cuff was inflated. Measurements were performed automatically for the non-dominant arm every 20 min during the day (07:00-23:00 hours) and every 60 min at night (23:00-07:00 hours). Monitor accuracy was checked by performance of three simultaneous readings with a standard BP monitor at the beginning of the monitoring session. Patients kept detailed diaries of their daily activities and sleeping periods. For analysis, day- and night-time periods were defined according to individual sleeping schedule. Participants with a nocturnal decrease in SBP or in DBP of less than 10% of the corresponding daytime value were defined as non-dippers. In the follow-up study (IV), ABPM was available from both visits in a subset of 71 patients. A total of 21 patients initiated AHT during follow-up. Analysis of the association between baseline indices and the BP change during follow-up was therefore only performed with data from the 50 patients without AHT.

5.5 Statistical analyses

Data were analyzed by the statistical programs SPSS (versions 16-17) for Windows (SPSS, Chicago, IL, USA) or PASW Statistics 17 (PASW, Chicago, IL, USA). Data in tables are mean ± SEM or median (range) when appropriate. The data were tested for normal distribution, and variables with skewed distribution, such as power variables, served for statistical analysis only after logarithmic transformation. Group differences between type 1 diabetic patients and control participants were analyzed with the $\chi 2$ test (for qualitative variables), and unpaired Student's t test by ANOVA (for quantitative variables). Post hoc analysis was done with Tukey's test. Analysis of covariance allowed adjustment for age and sex. Pearson's correlation coefficients were used to evaluate associations. P-values of 0.05 or less were considered significant. Due to the markedly differing values seen in the heart-transplanted participants as compared with the other groups, only differences between 15 and 6 breaths per minute were tested (paired t test).

Statistical differences in BRS response to intervention (6 breaths per minute vs. 15 breaths per minute controlled breathing; supine vs. standing: breathing normal air vs. oxygen) between patients with type 1 diabetes and healthy control subjects were tested by mixed-design two- or three-level analysis of variance (factorial design to test differences between groups, and repeated measures to test for the effect of intervention). In Study II, the impact of autonomic involvement and duration was analyzed separately by grouping the diabetic patients according to duration of diabetes (short or long) or severity of CAN (CAN-0, CAN-1 or CAN-2). Furthermore, due to diverging ages between the two groups with diabetes of differing durations, the ANOVA models were reanalyzed after adjusting BRS data for age. Whenever the main effect or interaction was significant, a subsequent post hoc multiple comparison was performed with Tukey's test. Because of the small number (5) of patients in the CAN-2 group, no statistical analyses were performed with this group.

In addition, because of more complex interactions between interventions, in Study III the statistical differences between groups and interventions (6/min vs. 15/min controlled breathing and breathing normal air vs. oxygen) were tested by means of a normal linear model. The continuous variables, BRS and BP, were separately modelled as outcome; conditions (normoxia/hyperoxia), breathing patterns (spontaneous, controlled at 15 breaths/min and 6 breaths/min), and participant group (healthy control/diabetic) were included as categorical covariates (282). Sheffe's was the test for significances between breathing rates. To test the simple effect of oxygen in diabetic and control subjects during spontaneous breathing, we used a similar mixed-design two-level ANOVA (repeated measures to test for the effect of oxygen, and factorial design to test between diabetic and control subjects) as in Studies I and II.

In Study IV, for a separate group of 425 healthy controls from Italy, ages 16 to 60 years, we calculated age regression curves for each BRS index, SDNN, and mean RRI. For the autonomic function tests (E/I ratio, 30/15 ratio, Valsalva ratio, and lying-to-

standing change in SBP), we obtained the age regression slopes from an earlier published study that had involved 120 healthy subjects aged 22 to 92 years (149). Equations for the linear regressions are in Table 4. Figure 3 demonstrates a scatterplot (age vs. BRS-SD) and regression formula obtained from our 425 healthy controls. Using the equations obtained in healthy subjects, we calculated the physiologic age-dependent deterioration for each subject over the individual follow-up period (regression coefficient multiplied by follow-up time). To obtain age-adjusted values, we added the age-dependent BRS deterioration value to the value of the follow-up result. Thus, the difference between baseline and age-adjusted follow-up value reflected changes not due to aging. To evaluate the change in BRS over time, we performed a paired t test between baseline BRS and both the measured BRS and the age-adjusted BRS at follow-up.

Figure 3 The baroreflex (BRS-SD), as related to age and the equation for the regression line in 425 control subjects aged between 16 and 60 years.

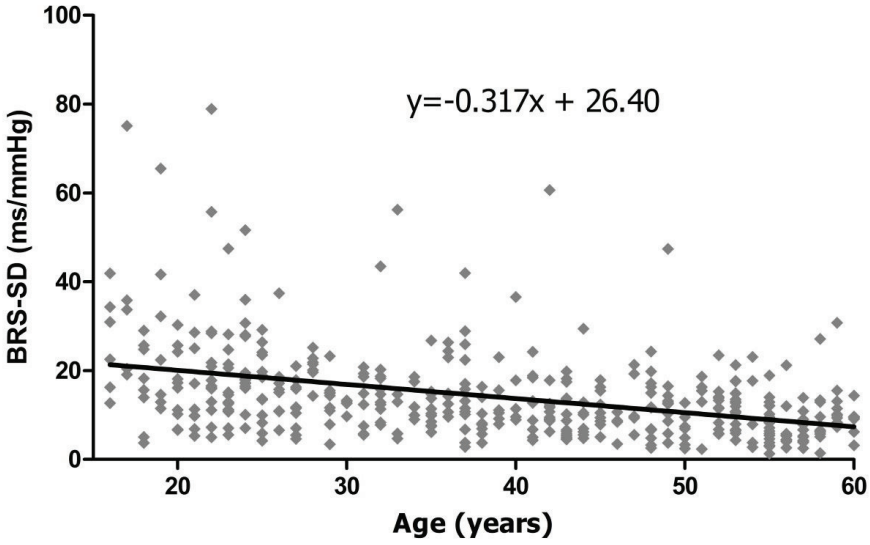


Table 4 Linear regression equations for autonomic indices used in Study IV in prediction of age-dependent deterioration in autonomic function. The equations are obtained from our own database with 425 healthy control subjects from Italy, except for those marked with asterisks (*), that came from an earlier published study (149).

Autonomic index	Equation
Mean RRI	y = 0.6716x + 861.58
E/I ratio	ln y = -0.00658x + 0.614 *
30/15 ratio	ln y = -0.00352x + 0.308 *
Valsalva´s ratio	ln y = -0.0064x + 0.700 *
SBP response to orthostatic test	y = -0.14x + 1.5 *
SDNN	y = -0.7495x + 64.679
BRS-αLF	y = -0.2917x + 24.488
BRS-αHF	y = -0.4964x + 40.264
BRS-TF	y = -0.3725x + 30.245
BRS+/+	y = -0.2909x + 35.471
BRS -/-	y = -0.5732x + 47.377
BRS-SD	y = -0.3175x + 26.408
$BRS_{average}$	y = -0.4216x + 35.075 (6 methods) y = -0.4258x + 36.735 (4 methods)

RRI, time interval between two successive R-peaks on the ECG; E/I ratio, expiration:inspiration ratio; 30/15 ratio, ratio between longest and shortest RRI in orthostatic test; SBP, systolic blood pressure; SDNN, standard deviation of the normal-to-normal RR intervals

As described in the methods section, we used a set of different BRS test methods and calculated an average value (I-IV). To justify this approach, we performed a test of consistency of the BRS prior to Study I, but using a larger database. It is of note that no general agreement exists regarding methodology for comparing more than two methods. Firstly, we analyzed the agreement between methods using an intraclass correlation coefficient (283) and found that when comparing all methods, the overall coefficient was 0.929. This result was in agreement with another approach, Cronbach's alpha, as suggested by Bland and Altman, which again gave 0.929 for all methods (284). A coefficient >0.90 we regarded as evidence of satisfactory agreement between methods.

Consistency between methods was further analyzed by repeats of the previous tests and by removing the methods from the analysis one by one. Results indicate that a very high degree of consistency resulted from all these indices. Secondly, we compared

each BRS estimate against the median of the other estimates to identify any deviations between methods using repeated measures ANOVA, as a multidimensional extension of the two-method comparison of Bland-Altman plots (285). Some methods gave systematically higher values, but all methods provided comparable data after removing the systematic differences. Finally, we assessed the correlation coefficients between each method and the median of the other methods. Although not a measure of agreement, this is nevertheless commonly reported ($p<0.001$ for all comparisons). These independent statistical approaches showed that different tests gave similar directional results, but with systematically different values amongst the different methods. Moreover, this justifies the averaging of different methods but also underlines the fact that all measures are acceptable when applied alone, provided that systematic differences in different methods are taken into account.

6 RESULTS

6.1 Autonomic function tests, HRV, and BPV in patients with type 1 diabetes and healthy controls (I, II, IV)

6.1.1 Autonomic function tests (I, II, IV)

Data from the conventional autonomic function tests are depicted in Table 5. In Study I with patients with short duration of type 1 diabetes, results of the four cardiovascular reflex tests did not significantly differ when compared with those of healthy control participants. The only exception was Valsalva´s test, which in fact showed higher values in the patients with short-duration type 1 diabetes, compared to these patients' controls. One participant in each group (both patients and controls) fulfilled the diagnostic criteria for CAN (CAN-2). Due to an updated classification for CAN, in Study II, a total of three patients (instead of only one in Study I) with short-duration diabetes fulfilled the diagnostic criteria for CAN. In Study II, when the two groups with type 1 diabetes (short and long duration) were pooled, the autonomic score was normal in 126 (CAN-0), borderline in 23 (CAN-1), and abnormal in 5 patients with diabetes (CAN-2). The numbers of patients with short/long duration in the CAN-0 were 101/25, in CAN-1 13/10, and in CAN-2 groups 3/2.

In Study IV, of the 80 patients with type 1 diabetes, at baseline, 68 patients had no signs of CAN (CAN-0), whereas 11 patients had borderline (CAN-1), and one had evident CAN (CAN-2). At follow-up, 63 patients presented with CAN-0, 16 with CAN-1, and one with CAN-2. One of the patients progressed to CAN-2, and in 12 patients the CAN score advanced. The only patient with CAN-2 at baseline reversed to CAN-1, and seven patients improved their autonomic scores. Thus, a total of 61 patients had an unchanged CAN score over time. When all patients were analyzed together (Study IV), only the E/I ratio of the autonomic function tests declined significantly during follow-up, but after age adjustment the change was no longer significant. However, when the patients with AHT were studied separately, a significant decline with time occurred in both E/I and 30/15 ratios, one that persisted also after age adjustment (data not shown).

Table 5 Results of autonomic function tests in patients with type 1 diabetes and healthy control subjects (I, II, IV). Data from Study IV apply only to patients with type 1 diabetes at baseline and at follow-up and at follow-up with age-dependent deterioration removed.

	Study II		Study I		Study IV		
	Long-duration type 1 diabetes	**Healthy controls**	**Short-duration type 1 diabetes**	**Healthy controls**	**Baseline**	**Follow-up**	**Follow-up adjusted**
n	**37**	**37**	**117**	**36**	**80**	**80**	**80**
E/I ratio	1.22±0.02[a]	no data	1.40±0.01	1.40±0.03	1.38±0.02	1.31±0.02[c]	1.36±0.02
30/15 ratio	1.33±0.04[a]	no data	1.67±0.03	1.65±0.03	1.64±0.03	1.59±0.04	1.61±0.04
Valsalva ratio	1.61±0.06[a]	no data	2.02±0.04[b]	1.87±0.06	2.03±0.05	1.97±0.05	2.02±0.05
SBP change as response to standing	2.2±1.7	no data	5.0±0.7	3.0±1.4	+0.8±1.1	+2.1±1.0	+2.8±1.0
CAN (0/1/2)	25/10/2[a]	no data	101/13/3	-	68/11/1	63/16/1	-

Data are mean ± SEM. a: p<0.05 long vs. short duration of type 1 diabetes (adjusted for age and gender), b: p<0.05 short duration of type 1 diabetes vs. healthy controls, c: p<0.05 for change over time, baseline vs. follow-up; RRI, time interval between two successive R-peaks on the ECG; E/I ratio, expiration:inspiration ratio; 30/15 ratio, ratio between longest and shortest RRI in orthostatic test; SBP, systolic blood pressure; CAN-0: normal autonomic score; CAN-1: borderline autonomic dysfunction; CAN-2: autonomic neuropathy

6.1.2 Time-domain and frequency domain (spectral) analysis of HRV and BPV (I-II)

Results from the time-domain and spectral analyses of HRV and BPV, obtained during controlled breathing (15 breaths/min) are in Table 6 (I-II). It should be pointed out that some of the marginally statistically significant differences in HRV values between patients with short-duration type 1 diabetes and their matched controls in Study I no longer remained significant in Study II, when more groups were compared by post hoc tests. During controlled breathing at this near-to-normal breathing rate, patients with short-duration type 1 diabetes showed, in time-domain analysis of HRV, non-significantly lower global HRV, as evidenced by reduced SDNN and RMSSD, than did healthy control subjects. In spectral analysis, patients with short-duration type 1 diabetes had significantly reduced power in the HF band, and increased power in the LF band as expressed as normalized units (I). In Study II, the patients with long-duration diabetes had lower values for all absolute HRV indices than did patients with short duration (Table 6). Moreover, power in the LF band of the SBP tended to be higher in diabetic patients, particularly in those with the shorter duration, although the difference did not reach statistical significance.

Table 6 Time-domain and spectral analyses of HRV and BPV during controlled breathing in healthy control participants and in patients with type 1 diabetes of different durations (I-II)

Variable	Short-duration diabetes (8.9 years)		Long-duration diabetes (33.7 years)	
	Type 1 diabetes	Healthy controls	Type 1 diabetes	Healthy controls
n	**117**	**36**	**37**	**37**
Mean RRI (ms)	897±12	939±25	911±18[b]	816±22
RMSSD	42.3±2.2	55.6±1.1	24.8±3.0	no data
SDNN	42.6±1.7	49.7±4.5	31.4±2.9[c]	30.9±2.2
Ln RRI LF (ms^2)	5.55±0.09	5.47±0.17	4.67±0.22[d]	4.83±0.15
Ln RRI HF (ms^2)	6.12±0.10	6.54±0.20	4.71±0.24[b,d]	5.86±0.16
RRI LF/HF	0.93±0.12	0.47±0.06	1.63±0.35[a,c]	0.67±0.13
RRI nLF (%)	37.2±1.8[a]	28.0±2.6	47.9±3.4[b]	30.0±3.5
RRI nHF (%)	61.0±1.8[a]	70.7±2.6	50.2±3.5[b,c]	67.1±3.5
Ln SBP LF ($mmHg^2$)	0.97±0.10	0.61±0.13	0.74±0.14	0.70±0.08

Data are mean ± SEM. a: p<0.05, b: p<0.01 type 1 diabetes vs. its age-matched healthy control group; c: p<0.05, d: p<0.01 long vs. short-duration diabetes; RRI, time interval between two successive R-peaks on the ECG; RMSSD, root mean square of the differences of successive RR intervals; SDNN, standard deviation of the normal-to-normal RR intervals; LF, low-frequency; HF, high-frequency; nLF, normalized LF; nHF, normalized HF; SBP, systolic blood pressure

6.1.3 Effect on HRV and BPV of autonomic involvement (II)

Table 7 demonstrates time-domain and spectral analyses of HRV and BPV during controlled breathing in healthy control participants and in patients with type 1 diabetes grouped by autonomic score. Due to the small number of patients (5) in the CAN-2 group, no statistical analyses were performed regarding this group, but mean and SEM are given. With deteriorating autonomic score, SDNN, as well as HRV in both the LF and the HF bands decreased. The LF/HF ratio showed a trend toward higher values (compared to those of healthy controls) in those with no or mild CAN (CAN 0-1) and seemed to decrease again in evident CAN, but without reaching statistical significance. Power in the LF band of SBP also showed a trend toward increased values in those diabetic patients with a normal autonomic score, and declining power in patients with a higher autonomic score.

Table 7 Time domain and spectral analyses of HRV and BPV during controlled breathing in healthy control participants and in patients with type 1 diabetes grouped by autonomic score (II)

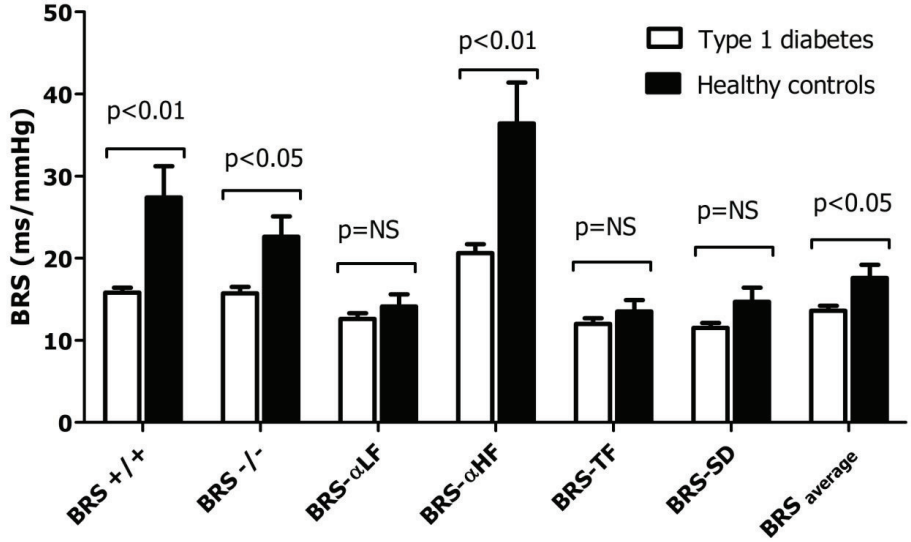
Variable	All healthy control subjects pooled	p-value adjusted for age and sex		Patients with type 1 diabetes grouped by autonomic score		
		Healthy controls vs. CAN-0	Healthy controls vs. CAN-1	CAN-0	CAN-1	CAN-2
n	**73**			**126**	**23**	**5**
mean RRI	880±19	ns	ns	905±11	916±25	730±26
SDNN	40.3±2.8	ns	ns	42.3±1.6	32.7±3.4*	11.8±1.6
Ln RRI LF (ms^2)	5.17±0.12	ns	ns	5.55±0.08	4.74±0.25*	2.76±0.28
Ln RRI HF (ms^2)	6.22±0.13	ns	<0.01	5.99±0.10	5.15±0.33*	3.15±0.57
RRI LF/HF	0.56±0.08	<0.01	ns	1.06±0.12	1.35±0.51	0.93±0.36
RRI nLF (%)	28.6±2.2	<0.01	<0.01	39.9±1.8	39.8±4.4	39.0±8.7
RRI nHF (%)	69.1±2.2	<0.01	<0.05	58.4±1.8	58.5±4.4	55.0±8.3
Ln SBP LF ($mmHg^2$)	0.66±0.07	<0.05	ns	1.04±0.08	0.57±0.20	-0.63±0.25

*Data are mean ± SEM. *:p<0.05 CAN-0 vs. CAN-1. No statistical comparisons were performed with CAN-2 due to the small number of subjects (n=5). RRI, time interval between two successive R-peaks on the ECG; RMSSD, root mean square of the differences of successive RR intervals; SDNN, standard deviation of the normal-to-normal RR intervals; LF, low-frequency; HF, high-frequency; nLF, normalized LF; nHF, normalized HF; SBP, systolic blood pressure*

6.2 Resting BRS (I-III)

Resting BRS was estimated by four to six methods during controlled breathing. As explained in the Methods section, we included a new BRS variable calculated as the average of four (I-II) or six (III-IV) BRS indices. Moreover, in Studies II to IV we also used a new index of BRS (BRS-SD), mathematically expressed as the ratio between the SD of RRI divided by SD of SBP as a measure of BRS (187). Figure 4 shows resting BRS values in patients with short-duration type 1 diabetes and healthy controls (Study I), estimated by all our available methods. Some of the individual measures of resting BRS (with most of the methods) and the $BRS_{average}$ all showed significantly lower values in patients with type 1 diabetes than in control participants (Fig. 4).

Figure 4 Resting BRS estimated by various methods in patients with short-duration type 1 diabetes and healthy control subjects obtained during controlled breathing (15 breaths/minute). Data are mean and SEM, adjusted for age and gender (I).



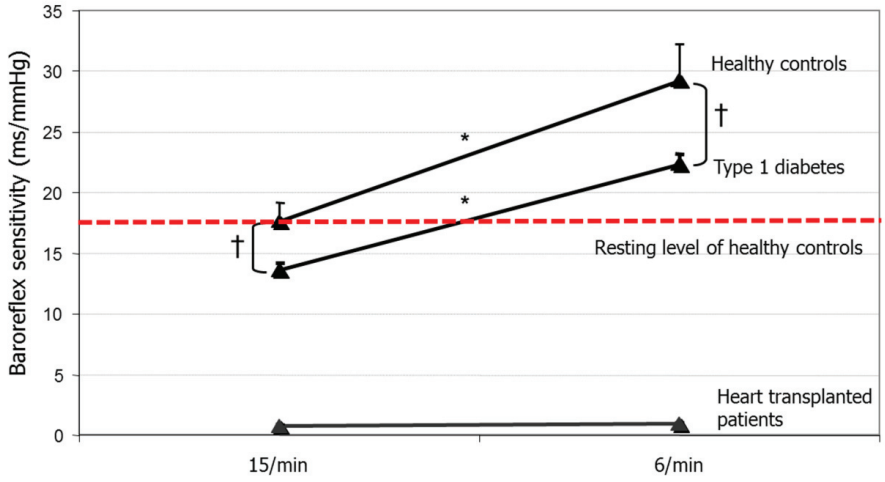
6.3 BRS response to interventions (I-IV)

To study the potential functional and reversible impairment of BRS in patients with type 1 diabetes, we studied the change in BRS induced by two functional manoeuvres: deep breathing and oxygen administration.

6.3.1 BRS response to deep breathing (I-III)

Figure 5 demonstrates changes in the average BRS induced by the respiratory maneuver in patients with short-duration diabetes, in healthy control subjects, and in heart-transplanted subjects (I). Slow, deep breathing induced a general increase in BRS in both type 1 diabetic patients and healthy control subjects (p within group <0.005), although the magnitude of the increase was lower in those with type 1 diabetes. Levels in diabetic patients were not statistically different from those in the control subjects at a normal breathing rate (15 breaths/minute), in contrast to those with definite cardiac denervation, who showed very low levels of resting BRS during controlled breathing, levels which remained unchanged with slow breathing.

Figure 5 Effect of breathing rate on average BRS in patients with type 1 diabetes, in healthy control subjects, and in patients surgically denervated by heart transplation (I).



*Values are mean and SEM. †p < 0.005 for difference between groups, *p < 0.005 for difference within group*

6.3.1.1 Effect of diabetes duration and age (II)

In Study II a group of patients with long-duration type 1 diabetes was incorporated and analyzed together with the group from Study 1 with short-duration type 1 diabetes. Figure 6a demonstrates the change in average BRS in response to slow, deep breathing in patients grouped by diabetes duration, in healthy control subjects, and in heart-transplanted subjects. The resting level of average BRS (15 breaths/min) was reduced in patients with type 1 diabetes and even lower in those with a long duration ($p<0.001$). Slow breathing raised BRS to a level not statistically different from the level obtained in the control subjects at resting level irrespective of the duration of type 1 diabetes, in contrast to the heart-transplanted participants. Tested by mixed-design ANOVA, the main effect of the intervention (change in breathing rate), as well as the effect of group were highly significant ($p<0.001$). Interactions were also significant ($p<0.001$) between intervention and group (controls vs. short duration, $p<0.05$; controls vs. long duration, $p<0.001$; short duration vs. long duration, $p<0.001$). After correction for age, the results of the groups with diabetes of long or short duration were superimposed (Fig. 6b). The interactions between healthy controls and patients with long ($p<0.001$) and short duration ($p<0.001$) remained significant, but the interaction between duration groups disappeared. The intervention induced in all groups a significant increase in BRS ($p<0.001$), even after adjustment (Fig. 6b).

6.3.1.2 Effect of autonomic impairment (II)

Figure 7a. shows change in average BRS induced by slow, deep breathing in type 1 diabetic patients stratified by CAN score (CAN -0, -1, and -2), in healthy control subjects and in heart-transplanted subjects. The main effects of the intervention and of the levels of autonomic involvement were significant ($p<0.001$) as was the interaction between intervention and groups ($p<0.05$). Deep breathing induced in all groups, except in CAN-2 and in heart-transplanted subjects a significant increase in BRS ($p<0.001$). After adjustment for age, these results remained unchanged, and interactions between control subjects and CAN-0- and CAN-1-groups ($p<0.001$) remained significant (Fig. 7b).

Figure 6 Effect of breathing rate on average BRS in healthy subjects, in patients with type 1 diabetes of long and short duration, and in heart-transplanted subjects. a: No adjustments; b: Adjustment for age; *: p<0.05, **: p<0.001

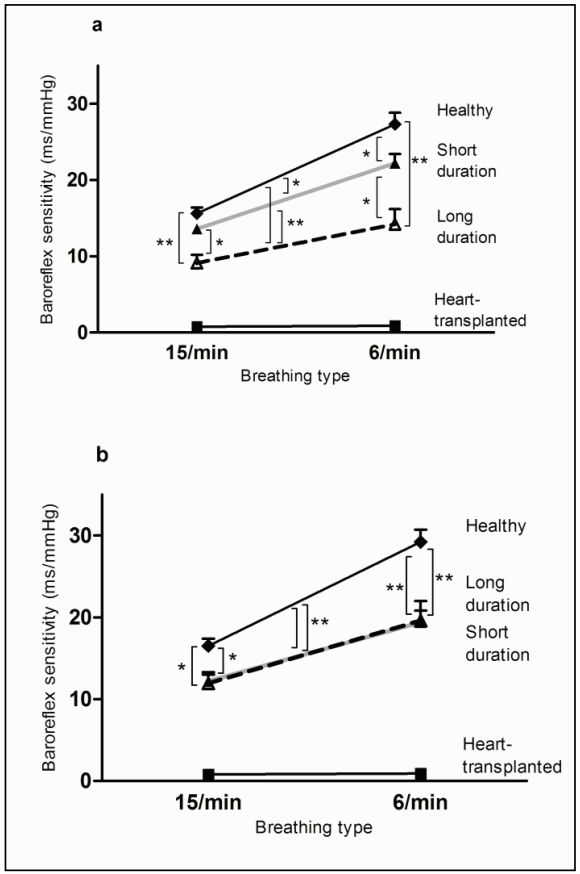
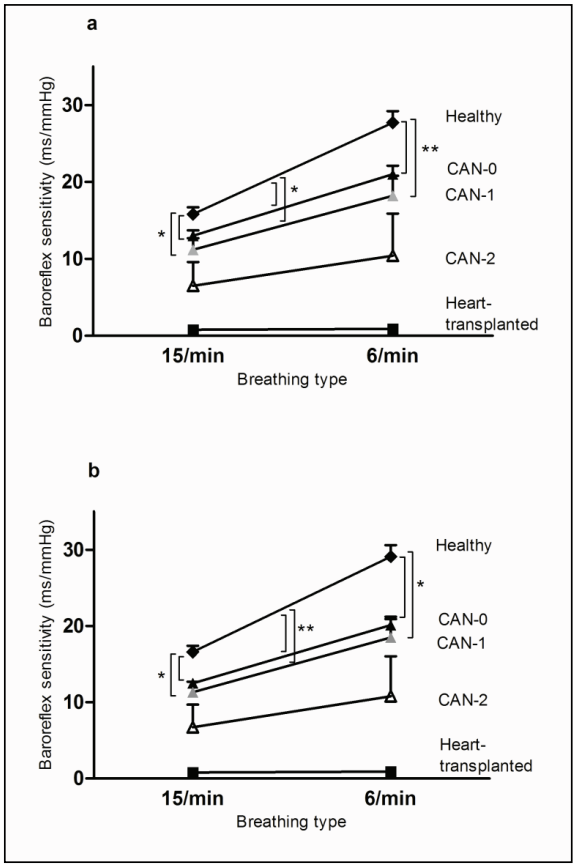


Figure 7 Effect of breathing rate on average BRS in healthy subjects, in patients with type 1 diabetes grouped by autonomic score, and in heart-transplanted subjects. a: No adjustments; b: Adjustment for age; *: p<0.05, **: p<0.001



6.3.2 Effect of oxygen on BRS, BP and HRV (III)

The cardio-respiratory response to oxygen was studied by comparing values obtained while breathing normal room air (normoxia) with those obtained during oxygen administration 5 L per minute (hyperoxia), and in addition, separately during spontaneous breathing, controlled breathing (15 breaths/min), and slow, deep breathing (6 breaths/min).

Table 8 shows changes in BP, HRV, and tidal volume during normoxia vs. hyperoxia and the different respiratory manoeuvres. Inhalation of oxygen 5L/min raised oxygen saturation in both groups to nearly 99% ($p<0.001$) on average. During controlled and slow, deep breathing oxygen saturation still was raised still further by oxygen administration, although the effect was less evident. During spontaneous breathing, oxygen induced a reduction in HR, and an increase in SDNN and in BP. The trend was similar for both groups, but the change was significant only for patients with type 1 diabetes. Consequently, the difference in BP, already present at normoxia, increased during oxygen administration. Moreover, oxygen administration induced a significant increase in minute ventilation during spontaneous breathing only in patients with type 1 diabetes. Breathing frequency remained unchanged, whereas patients with type 1 diabetes showed an increase in tidal volume. The relative increase in ventilation was also confirmed by a reduction in end-tidal CO_2.

Controlled breathing at 15 breaths/min resulted in a significant increase in HR and a reduction in SDNN. During this controlled breathing oxygen administration induced only minor changes in respiratory variables. Both the controlled breathing and the slow, deep breathing, raised oxygen saturation ($p<0.001$) in normoxia. However, whereas controlled breathing induced a large increase in ventilation at normoxia in both groups ($p<0.001$), slow breathing induced only a weak, non-significant increase.

Table 8 Effect of oxygen and respiratory patterns on cardiorespiratory variables

	Variable	Spontaneous breathing		Controlled breathing 15 breaths/min		Slow, deep breathing 6 breaths/minute	
		Normoxia	**Hyperoxia**	**Normoxia**	**Hyperoxia**	**Normoxia**	**Hyperoxia**
RRI ms	Type 1 diabetes	944±14	1019±17[c]	907±14[f]	958±15[c,f]	921±13[e]	960±15[c,f]
	Control	971±27	1029±25[c]	935±23[e]	982±23[c,f]	946±23	986±20[c,f]
	p for T1D vs healthy	ns	ns	ns	ns	ns	ns
SBP (mmHg)	Type 1 diabetes	130.0±1.4	137.0±1.4[c]	132.3±1.4[e]	135.2±1.5[a,i]	132.3±1.4[d]	135.3±1.6[a,h]
	Control	123.1±2.2	123.1±2.6	124.6±2.5	124.9±2.3	124.5±2.7	126.4±2.7
	p for T1D vs healthy	<0.01	<0.0001	<0.01	<0.001	<0.01	<0.005
DBP (mmHg)	Type 1 diabetes	63.3±0.9	67.4±0.9[c]	63.1±0.9	65.3±0.9[b,f,g]	62.6±0.9	65.0±1.0[b,f]
	Control	58.0±1.2	57.1±1.5	56.4±1.6	55.7±1.5	56.3±1.7	55.5±1.4 [g]
	p for T1D vs healthy	<0.001	<0.0001	<0.0001	<0.0001	<0.001	<0.0001
SDNN (ms)	Type 1 diabetes	31.5±1.7	36.0±2.5[a]	26.1±1.7[f]	29.7±2.2[b,f]	60.2±2.8[f]	62.7±3.2[f,i]
	Control	39.4±3.9	43.4±4.3	34.1±3.2[d]	36.2±3.3[e]	68.6±4.9[f]	72.7±5.3[f,i]
	p for T1D vs healthy	ns	ns	ns	ns	ns	ns
Oxygen saturation (%)	Type 1 diabetes	97.2±0.1	98.6±0.1[c]	97.8±0.1[f]	98.7±0.1[c,i]	97.8±0.1[f]	98.7±0.1[c,i]
	Control	97.4±0.2	98.7±0.1[c]	97.9±0.2[f]	98.8±0.1[c,i]	97.9±0.2[f]	98.8±0.1[c,d,i]
	p for T1D vs healthy	ns	ns	ns	ns	ns	ns
Respiratory rate (breaths/min)	Type 1 diabetes	13.0±0.3	12.9±0.3	14.9±0.0	15.0±0.0	6.0±0.0	6.0±0.0
	Control	13.0±0.6	12.5±0.6	15.0±0.0	15.0±0.0	6.0±0.0	6.0±0.0
	p for T1D vs healthy	ns	ns	ns	ns	ns	ns
Tidal volume (% change from baseline)	Type 1 diabetes	100	122.6±13.7	182.4±26.1	185.6±28.1[f,h]	228.4±29.2[f]	219.8±29.4[b,f,i]
	Control	100	115.7±13.0	138.1±9.6[f]	149.9±17.4[e,h]	220.5±17.1	214.6±19.9[f,i]
	p for T1D vs healthy	ns	ns	ns	ns	ns	ns
Minute ventilation (% change from baseline)	Type 1 diabetes	100	126.0±14.9	227.1±29.8	233.3±31.8[f,i]	115.4±13.6	111.8±13.6 [b,e]
	Control	100	112.3±13.9	174.9±14.7	197.6±31.5[f,h]	116.3±12.4	115.8±15.0
	p for T1D vs healthy	ns	ns	ns	ns	ns	ns
End-tidal $C0_2$ (mmHg)	Type 1	45.1±0.5	39.1±0.8[c]	38.5±0.5 [f]	36.0±0.6[c,f,i]	40.0±0.6[f]	36.6±0.6[c,f,i]
	Control	43.7±0.8	37.5±1.1[c]	37.1±1.0 [f]	35.4±1.0[b,e,i]	40.9±1.1[f]	37.7±1.0[c,i]
	p for T1D vs healthy	ns	ns	ns	ns	ns	ns

Data are mean ± SEM. a: p<0.05, b: p<0.01, c: p<0.001 for hyperoxia vs normoxia, d: p<0.05, e: p<0.01, f: p<0.001, vs spontaneous breathing (normoxia vs normoxia or hyperoxia vs hyperoxia), g: p<0.05, h: p<0.01, i: p<0.001, vs spontaneous breathing in normoxia
T1D, type 1 diabetes; RRI, time interval between two successive R-peaks on the ECG; SBP, systolic blood pressure; DBP, diastolic blood pressure; RMSSD, root mean square of the differences of successive RR intervals; SDNN, standard deviation of the normal-to-normal RR intervals

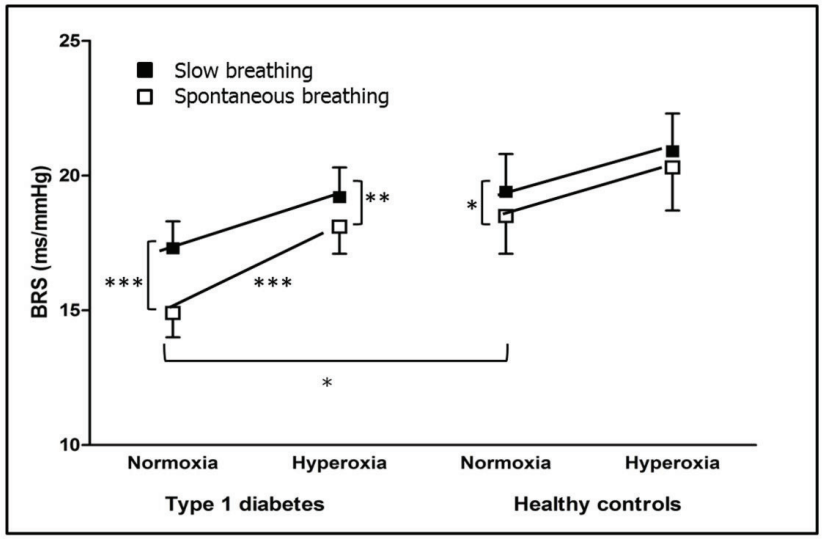
6.3.3 Interaction of oxygen and respiratory pattern: effects on the BRS (III)

Confirming our results from Studies I-II, patients with type 1 diabetes showed at normoxia significantly lower resting BRS ($p<0.05$), and both higher SBP and DBP ($p< 0.05$) than did the healthy control subjects. During slow, deep breathing, the induced change in BRS was significant ($p<0.01$) only in patients with diabetes. Thereby, the difference observed during spontaneous breathing in normoxia disappeared. Controlled breathing at 15 breaths/minute induced a significant reduction in the BRS both in patients and in healthy control subjects.

BRS values obtained during spontaneous breathing correlated with BRS increase induced by oxygen administration ($r=-0.216$ $p<0.05$), and indicated a reverse effect for the higher BRS values. Addition of oxygen could not further elevate the BRS during slow, deep breathing, as it seems that the BRS had already reached its highest values during slow breathing at normoxia. During controlled breathing (15 breaths/min) and hyperoxia, the BRS increased, but that change failed to reach statistical significance.

Figure 8 demonstrates interactions between the effects of oxygen on one hand, and slow, deep breathing on the other, for BRS in patients with type 1 diabetes and for healthy control subjects. During spontaneous breathing and normoxia, patients with type 1 diabetes showed a blunted BRS. However, in hyperoxia, the BRS increased more than in the healthy control subjects, and consequently the difference observed at baseline disappeared. Slow breathing raised BRS during normoxia in both groups to an extent similar to that in hyperoxia, and thus eliminated the difference between diabetic and control participants. Hyperoxia during deep breathing did not further increase the BRS in healthy controls, suggesting that these effects are related.

Figure 8 Effect of oxygen and slow breathing on baroreflex sensitivity. The figure demonstrates BRS in patients with type 1 diabetes and in healthy control subjects while breathing normal room air (normoxia) vs. inhaled oxygen 5 L per minute (hyperoxia), and during spontaneous breathing vs. slow breathing (6 breaths/min).



Data are mean±SEM. White squares: Spontaneous breathing, black squares: Slow, deep breathing
**p<0.05, **p<0.01, ***p<0.001*

6.4 Progression of HRV and BRS over 5 years and effect of age (IV)

Table 9 shows the autonomic and BRS indices at baseline and at the follow-up visit 5 years later in 80 patients with type 1 diabetes, grouped by initiation of AHT during follow-up. In addition, we calculated the expected value for each parameter at follow-up, when the physiological ageing was taken into account. The adjustment for time (or the physiological aging) is explained in detail in the statistics section. Patients who started AHT showed a significant decline with time in E/I ratio, 30/15 ratio, SDNN, BRS-αHF, BRS-/-, and $BRS_{average}$, but only the changes in E/I ratio, 30/15 ratio, and BRS-αHF remained significant even after age adjustment ($p<0.01$). In those without AHT, their E/I ratio and four of six BRS indices declined significantly during follow-up, but after age adjustment, none of these changes was any longer significant.

Table 9 Autonomic function tests and spontaneous BRS at baseline and follow-up. Follow-up values are adjusted for follow-up time.

	AHT + at follow-up (n=21)			**AHT - at follow-up (n=59)**		
	Baseline	**Follow-up**	**Follow-up adjusted**	**Baseline**	**Follow-up**	**Follow-up adjusted**
Mean RRI (ms)	922±24	910±28	913±28	917±16	945±18	949±18*
E/I ratio	1.38±0.04	1.24±0.03**	1.29±0.03††	1.39±0.02	1.33±0.02**	1.38±0.02
30/15 ratio	1.66±0.06	1.40±0.05**	1.43±0.05††	1.64±0.04	1.65±0.04	1.68±0.04
Valsalva ratio	2.10±0.10	1.98±0.09	2.03±0.10	2.10±0.06	1.98±0.06	2.03±0.10
SBP response to standing (mmHg)	0.6±1.6	3.8±2.1	3.8±2.1	0.9±1.3	1.7±1.1	2.4±1.1
SDNN (ms)	52±5	45±5*	49±5	57±3	53±3	57±3
BRS-αLF (ms/mmHg)	11.1±1.0	9.9±1.8	12.1±1.8	13.8±1.0	11.7±0.8	13.9±0.8
BRS-αHF (ms/mmHg)	21.0±3.0	13.7±2.9**	16.2±2.9††	24.5±1.2	19.2±1.6**	21.6±1.6
BRS-TF (ms/mmHg)	10.3±0.9	8.9±1.4	10.7±1.4	12.9±0.9	11.1±0.7	12.9±0.7
BRS +/+ (ms/mmHg)	17.0±1.7	13.3±2.2*	14.8±2.2	20.5±1.8	16.0±1.2*	17.4±1.2
BRS -/- (ms/mmHg)	17.7±2.0	15.9±3.2	18.7±3.2	19.6±1.1	16.0±1.1**	18.8±1.0
BRS-SD (ms/mmHg)	8.9±0.8	8.0±1.2	9.6±1.2	10.2±0.6	9.3±0.6	11.0±0.6
$BRS_{average}$ (ms/mmHg)	14.1±1.3	11.3±1.9*	13.4±1.9	16.9±0.9	13.9±0.8**	15.9±0.8

*Data are mean ± SEM. * p<0.05, ** p<0.01 for baseline vs. follow-up , † p<0.05, †† p<0.01 for baseline vs. follow-up adjusted for follow-up time; AHT+, antihypertensive treatment at follow-up; AHT-, no antihypertensive treatment at follow-up; RRI, time interval between two successive R-peaks on the ECG; E/I ratio, expiration:inspiration ratio; 30/15 ratio, ratio between longest and shortest RRI in orthostatic test; SBP, systolic blood pressure; SDNN, standard deviation of the normal-to-normal RR intervals;*

6.5 BRS and BP (IV)

Various associations among spontaneous BRS and 24-hour ABPM and office BP at baseline were analyzed in all 71 patients with ABPM data available (Table 10). The BRS showed in general an inverse correlation with the ambulatory 24h and daytime BP, mainly with DBP and MAP. All BRS variables except BRS+/+ correlated with 24-hour DBP (r -0.238 to -0.331, $p<0.05$) and all but BRS+/+ and BRS-/- with daytime DBP (r -0.295 to -0.400, $p<0.05$), whereas the correlations with night-time BP were non-significant. Moreover, office BPs (SBP, DBP, and MAP) correlated significantly with BRS-αLF (r -0.234 to -0.275, $p<0.05$), and BRS-TF (r -0.251 to -0.281, $p<0.05$).

Table 10 Correlations between baseline BRS indices obtained during spontaneous breathing and blood pressure (24-hour ambulatory blood pressure and office blood pressure).

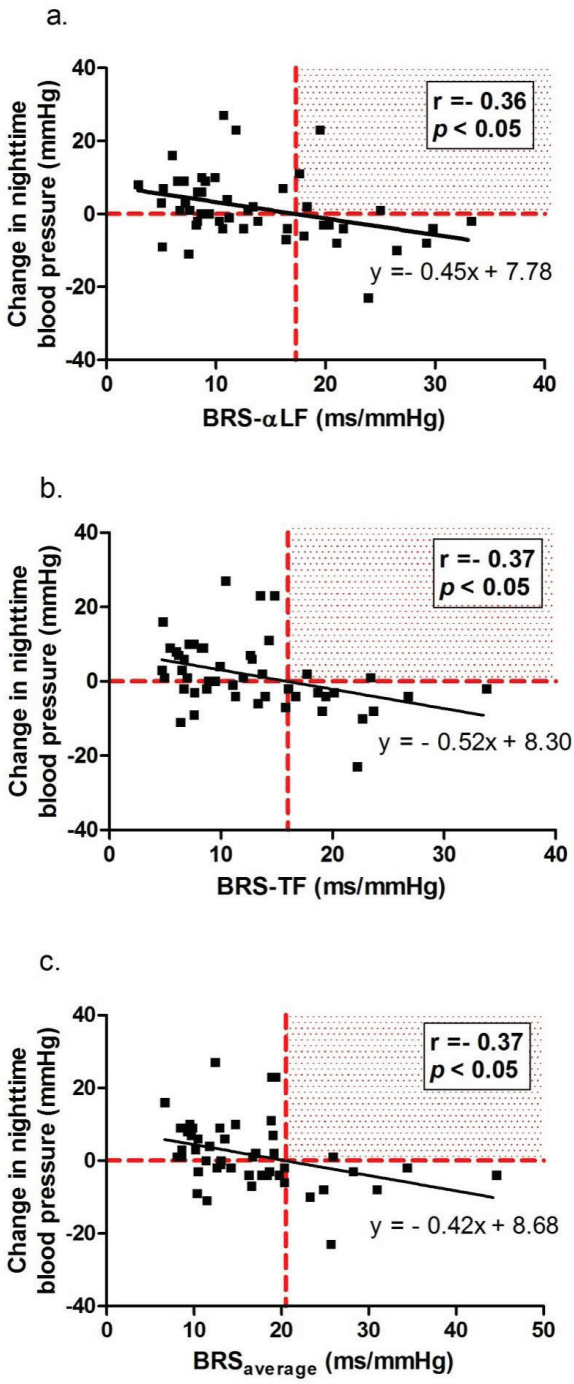
	BRS during spontaneous breathing						
	BRS+/+	**BRS -/-**	**BRS-αLF**	**BRS-αHF**	**BRS-TF**	**BRS-average**	**BRS-SD**
24 h							
SBP (mmHg)	-0.071	-0.071	-0.145	-0.158	-0.157	-0.123	-0.015
DBP (mmHg)	-0.184	**-0.262**	**-0.319**	**-0.361**	**-0.353**	**-0.316**	**-0.238**
MAP (mmHg)	-0.156	-0.224	**-0.278**	**-0.331**	**-0.308**	**-0.274**	-0.173
Daytime							
SBP (mmHg)	-0.127	-0.129	**-0.251**	-0.180	**-0.255**	-0.209	-0.090
DBP (mmHg)	-0.215	**-0.295**	**-0.366**	**-0.373**	**-0.400**	**-0.359**	**-0.304**
MAP (mmHg)	-0.205	**-0.270**	**-0.359**	**-0.338**	**-0.375**	**-0.339**	**-0.245**
Night-time							
SBP (mmHg)	0.034	0.057	0.006	-0.036	-0.016	0.023	0.055
DBP (mmHg)	-0.100	-0.142	-0.177	-0.202	-0.205	-0.184	-0.090
MAP (mmHg)	-0.037	-0.059	-0.096	-0.148	-0.127	-0.096	-0.028
Office blood pressure							
SBP (mmHg)	-0.183	-0.149	**-0.234**	-0.109	**-0.251**	**-0.234**	-0.159
DBP (mmHg)	-0.116	-0.162	**-0.250**	-0.150	**-0.254**	-0.215	-0.177
MAP (mmHg)	-0.167	-0.177	**-0.275**	-0.148	**-0.285**	**-0.253**	-0.193
PP (mmHg)	-0.139	-0.063	-0.098	-0.022	-0.114	-0.126	-0.062

Data are Pearson's r, correlations significant at 0.05 level are bolded. SBP: systolic blood pressure; DBP: diastolic blood pressure; MAP: mean arterial pressure; PP: pulse pressure.

Correlations occurred between baseline spontaneous BRS variables (BRS-αLF, BRS-TF and $BRS_{average}$) and the change in night-time SBP over 5 years (Fig. 9). Only patients without AHT were included (n=50). The lower was the BRS at baseline, the more the night-time SBP increased over time (r-0.348 to -0.381, $p<0.05$). Figure 9b shows that except for two subjects, none with BRS-TF >15 to 16 ms/mmHg

increased their night-time SBP during the 5-year follow-up. In addition to resting BRS, we also calculated the increase in BRS induced by deep breathing at baseline (data not shown). Two of the BRS methods showed a significant correlation with the increase in BP over time, mainly with 24-hour (BRS-αLF: $r=0.323$-0.346, $p<0.05$; BRS-SD: $r=0.352$-0.454, $p<0.05$) and night-time indices (BRS-αLF: $r=0.303$-0.434, $p<0.05$; BRS-SD: $r=0.333$-0.481, $p<0.05$), and with daytime MAP (BRS-αLF: $r=0.302$, $p<0.05$; BRS-SD: $r=0.339$, $p<0.05$). With the other BRS methods correlations between the response in BRS and BP variables were non-significant.

Figure 9 Correlations between baseline spontaneous BRS and the change in night-time SBP during 5-year follow-up.



7 DISCUSSION

The findings presented in this thesis have demonstrated a marked reduction in spontaneous BRS in patients with type 1 diabetes irrespective of diabetes duration, even in the absence of any clinically detectable diabetic complications. The reduction in BRS co-segregated with signs of sympathetic predominance. The novel findings of this thesis are that diabetic patients were able to elevate their BRS in response to slow deep breathing, except for those with definite CAN. Thus, the vast majority of our patients might well have retained a component of reversible autonomic involvement despite their long-duration diabetes. In addition, it seems that the effect of diabetes duration on BRS is mainly an effect of age because, after adjustment for age, differences in BRS between duration groups vanished. BRS showed an inverse correlation with BP and in addition, during the 5-year follow-up, baseline BRS predicted an increase in night-time BP. Although reduced BRS does not necessarily advance to CAN, our results suggest that in patients with type 1 diabetes, BRS may play a role in development of hypertension.

7.1 Limitations of the study

This study examined the autonomic function in well-defined cohorts of patients with type 1 diabetes. Some limitations, however, deserve mention. Although the group of patients with shorter-duration type 1 diabetes were population-based (I, II), one major limitation is that these subjects had only mild autonomic involvement. It is possible that patients with worse glycaemic control and potential signs of diabetic complications despite the short duration did not volunteer for the study. The surprisingly low prevalence of definite CAN in patients with long-duration type 1 diabetes (II) can be at least in part explainable by the exclusion of patients on beta-blockers; one of the most important indications for beta blockers is CAD, and such patients also often suffer from other long-term complications such as neuropathy. Consequently, the low prevalence of CAN in the patients with long-term diabetes may have been due to selection or survival bias. Although the proportion of more severe diabetic complications was undoubtedly underrepresented, this setting allowed us to study the role of duration per se.

All in all, the prevalence of CAN was very low compared to rates in the literature (6), which may have been due to patient selection, but on the other hand, that may have also reflected a better level of care than in earlier studies. Results therefore may differ in patients with more advanced disease, in whom a large proportion of organic neural lesions could be expected. The small number of subjects in our CAN-2 subgroup did not allow statistical comparisons (II), but also these results indicated that slow, deep breathing was unable to normalize BRS in severe neuropathy resembling the response in patients with a surgically denervated heart.

One limitation worth mentioning is our selection of an age- and gender-matched control group for the patients with longer duration of diabetes in Study II. These data came from an earlier study in Italy with healthy individuals; some effect of genetic differences cannot therefore be ruled out. Moreover, detailed background data from those Italian individuals were unfortunately unavailable.

Methodology chosen (HRV, BRS) is both well documented and sensitive, and the measurements, after appropriate instructions and preparations, were performed carefully in laboratory conditions. Nonetheless, one limitation is our failure to assess in our laboratory the repeatability of HRV and BRS. Furthermore, at baseline (I-II) respiration could not be measured directly, although it was thoroughly visually controlled. During the follow-up visit respiration was also monitored with respiration belts (III-IV).

One issue requiring comment is the length of the HRV and BPV recordings. The European Task Force (157) recommends a minimum of 2 minutes for measures including LF—with 5 minutes being ideal, since it is thought that spectral powers are highly influenced by the length of recordings. It is of note that in this recommendation, the Task Force assumes that what took place was spontaneous breathing. However, when the breathing is thoroughly controlled at a specific pace, breathing creates a resonance in the cardiovascular system which unifies the oscillations. Consequently, the outcome is much more robust than during spontaneous breathing and this being the case, any algorithm will be appropriate to evaluate the spectrum, even during a short period of time. Conversely, under conditions involving irregular breathing or when multiple frequencies are to be expected, the FFT algorithm indeed requires a better frequency definition that can be achieved only by an increase in the number of points, i.e. the length of the recording. The length of our slow, deep breathing recordings is 1 or 2 minutes. We compared these recordings in 20 patients by calculating the RRI spectra and BRS of a 2-minute recording, and compared this with the first and second half of the same recording (data not shown). No statistical difference emerged in the spectral and BRS indices between the recordings of either 1 or 2 minutes. These data therefore support our view that 1-minute recordings, when obtained during controlled respiration (6 or 15 breaths/minute) are appropriate.

Moreover, except for Study IV, our data are mainly cross-sectional, and do not allow for temporal analyses or conclusions regarding cause-effect relations between autonomic cardiovascular function and its determinants. In these studies, reversibility in BRS was studied with acute interventions. Whether BRS can be restored for a longer time period is unknown. Although our study provides longitudinal data on BRS in a well characterized group of patients with type 1 diabetes, a major limitation is our small sample size. No significant differences emerged between those individuals who completed follow-up and those who did not, regarding clinical characteristics, BP, or autonomic measures, except for a small difference in 24-hour HR and the proportion of smokers (data not shown). Furthermore, the association between baseline BRS indices and change in BP over time was studied only in patients not treated with AHT:

the study was therefore not designed to assess such aspects as any association between BRS and albuminuria.

7.2 Early autonomic abnormalities in type 1 diabetes

By the selection of a cohort with a mean duration of less than 10 years of type 1 diabetes, Study I was designed to evaluate the autonomic function of the patients in the window before diabetic late complications appear. Studies in more heterogeneous groups of patients with type 1 diabetes have demonstrated reductions in various indices of the HRV (204, 286, 287), suggestive of a reduction in vagal activity. Those findings were replicated by showing a lower amount of global HRV (SDNN), by the relative increase in the LF components, by the relative and absolute reduction in the HF components of the HRV, and by the higher LF/HF ratio, as assessed by spectral analysis. Moreover, our findings regarding BRS also support earlier findings that reduced BRS is a sensitive marker of autonomic cardiovascular dysregulation in more heterogeneous or non-selected cohorts of patients with type 1 or type 2 diabetes (9-12, 288), but importantly even in the absence of clinical complications, or of detectable alterations in autonomic function tests. Although the cross-sectional design limits conclusions about mechanisms and temporal relations, it may be that autonomic dysfunction precedes other diabetic complications and may even play a role in their pathogenesis.

7.3 Significance of functional BRS alteration and effect of interventions

7.3.1 Response to slow, deep breathing (I-III)

In Study I we demonstrated for the first time, that the blunted BRS in type 1 diabetes can be increased to the resting level of the healthy control subjects simply by slowing the breathing rate to 6/min. This is the opposite of what happened in the transplanted heart, in which the absence of cardiac innervation resulted not only in virtually absent baroreflex values, but also caused no increase at all in deep breathing. The magnitude of the increase was highest in the healthy control subjects, slightly reduced in the diabetic patients, and totally absent from the subjects with denervated hearts.

In Study II we extended this finding to patients with long-duration diabetes. Only those with a definite autonomic neuropathy were incapable of restoring the BRS by deep breathing. This shoves that subjects totally lacking innervation are unable to elevate their BRS by means of functional manoeuvres. The ability to normalize BRS by

deep breathing, although it is a short-acting intervention, suggests that at least these early changes in patients with type 1 diabetes result mainly from functional autonomic involvement.

Conventionally, the reduced BRS and HRV associated with diabetes have been attributed to a loss of parasympathetic tone due to neural damage. However, similar findings of a reduced BRS have been documented in heart failure or hypertension. In these conditions, however, the cause of low resting BRS was higher sympathetic tone (257, 258, 289) and some functional disorder rather than neural damage. Moreover, both in healthy subjects and in ones with hypertension, with chronic heart failure, or with chronic obstructive pulmonary disease, functionally reduced BRS can be raised by slow breathing (255, 256, 258, 259).

The effect of slow, deep breathing is mediated through a relative increase in vagal activity as marked by a reduction in HR and BP, and a decline in sympathetic activity. As a consequence, slow breathing improves the BRS by raising both the HRV and the BPV, but the fluctuations that are induced increase much more in the RRI than in the BP (187). An increase in BRS by a breathing intervention would be impossible without a functioning parasympathetic system, as was the case in the heart-transplanted patients. In our studies (II), the sympathetic predominance was also supported by the increased LF/HF ratio in patients with type 1 diabetes even in the absence of autonomic dysfunction (CAN-0). However, along with higher CAN score, the LF/HF ratio was again reduced, which is suggestive of a decline in sympathetic neural function. Taken together, our results point in the direction of an elevated resting sympathetic tone in early autonomic dysfunction, rather than parasympathetic damage. This conclusion is also supported by the changes in BRS during active standing (I).

7.3.1.1 Sympathovagal imbalance

Many factors other than parasympathetic neural damage could influence the BRS by enhancing sympathetic activity in type 1 diabetic patients. Oral carbohydrate intake stimulates sympathetic activity in healthy subjects (290, 291), this effect being suggested as mediated by insulin, although findings have been conflicting (292, 293). Insulin therapy may also cause its effects by stimulating the sympathetic nervous system and thereby depressing the vagal arm of the baroreflex (294-298). The effect of exogenous insulin is analogous to that of hyperinsulinemia, which is present in type 2 diabetes as a result of insulin resistance. Importantly, a bidirectional relationship seems to exist between sympathetic over-activity that induces insulin resistance and hyperinsulinemia that produces sympathetic activation. Obesity and metabolic syndrome are also associated with chronic sympathetic overactivity (299-302). Importantly, metabolic syndrome is currently found in nearly 40% of the patients with type 1 diabetes (32). It is therefore possible that the development and pathomechanisms of diabetic neuropathy in type 1 diabetes will change in the future to resemble that of type 2 diabetes. Endothelial dysfunction (213, 303) and low-grade inflammation in patients with diabetes have also been related to autonomic imbalance (137, 219, 304).

In non-diabetic individuals, hypoglycaemia strongly activates the ANS (305). Indeed the islet cells are controlled by the ANS through parasympathetic and sympathetic nerves, and by adrenal medullary epinephrine. This activation induces glucagon secretion from the α-cells, which normalizes blood-sugar level. In type 1 diabetes, in parallel with the deficient β-cells, the glucagon response to insulin-induced hypoglycaemia is also impaired. The presence of autonomic neuropathy further reduces counterregulatory catecholamine responses to hypoglycaemia, and prior hypoglycaemic episodes attenuate the response of the ANS to subsequent hypoglycaemia, which may result in a vicious cycle of recurrent hypoglycaemia (306, 307). Hypoglycaemia-associated autonomic failure is in most patients reversible after a period of avoidance of hypoglycaemias.

In sum, the presence of most of the above factors is well established in type 1 diabetes, they can all be expected to contribute to the sympathovagal balance and setting of the resting BRS.

7.3.2 Effect of oxygen inhalation (III)

Oxygen administration induced an augmented overall response in patients with type 1 diabetes and could almost restore the blunted BRS (III). We also found an oxygen-induced increase in SDNN, in line with findings with other patients with type 1 diabetes, in whom HRV rose after 4 weeks of hyperbaric hyperoxia (267); to our knowledge, Sun et al. were the first and are thus far the only group who has studied the effect of hyperoxia on autonomic parameters in diabetes. Studies on healthy participants and patients with COPD have shown that oxygen administration reduces the HR and raises indices of the HRV related to parasympathetic activity (264), and also raises BRS (265, 266). These findings have been interpreted to mean that oxygen administration reduces chemoreflex activity, but this mechanism could not be confirmed, because neither the ventilation nor chemoreflex were assessed (265, 266).

Activation of the chemoreflexes normally results in sympathetic activation, raises ventilation and results in depression of the BRS, whereas baroreflex activation is associated with parasympathetic stimulation and has the opposite effect on ventilation. Here we measured neither of these two variables in absolute values, but obtained an estimate of the ventilation in relative units from inductive belt signals. Hyperoxia showed a definite trend towards increased ventilation when oxygen was administered during spontaneous breathing. This increase in the ventilation occurs after a momentary reduction mediated by a vagal reflex (269, 270, 308, 309). Signs appeared of a more pronounced effect in diabetic participants, probably as a consequence of greater vagal stimulation induced by oxygen. Although resting ventilation was not directly measured, lower end-tidal carbon dioxide confirmed that the increase in ventilation was at least as large as in the control subjects.

So the question arises, why did the patients with type 1 diabetes show a pronounced response to oxygen? Based on our findings, a logical explanation could be that

preexisting tissue hypoxia in connection with endothelium-related vascular changes may have been responsible for the early autonomic dysfunction in patients with type 1 diabetes. Hypoxia is, in fact, a potent stimulator of the sympathetic nervous system and the ventilation, and acts to correct the hypoxia. Moreover, tissue hypoxia is recognized as a key factor in the origin and development of diabetic complications (310, 311).

Another new finding of the present study is the marked oxygen-induced increase in BP seen in diabetic individuals, possibly mediated by a direct local effect. This increase in BP may have stimulated BRS and parasympathetic activity. Such a response is directly opposite to the documented effect of hypoxia resulting in direct vasodilation, a reflectory increase in sympathetic activity, and following that, a reduction in BRS. The stronger effect of oxygen seen in type 1 diabetes could therefore be due to resting tissue hypoxia. Alternatively, it may be an effect of ROS on a dysfunctional endothelium typical of diabetes. This seems however unlikely, as hypoxia also promotes free-radicals but causes arterial vasodilation.

Slow breathing in uncomplicated type 1 diabetes raised BRS and improved oxygen saturation, even under normoxic conditions similar to what has become apparent in heart failure (256) or hypoxia-dependent diseases (259). This effect could be mediated by an increase in arterial oxygen pressure, as evidenced by the increased oxygen saturation. Accordingly, our increase in BRS evident during slow breathing showed no further augmentation by oxygen (Fig. 8). Hence, it is possible that BRS cannot be further increased after a maximal value is reached. What is also likely is that high BRS observed during slow breathing prevents a further increase in BP caused by oxygen in the control participants, whereas slow breathing failed to fully inhibit this (oxygen-induced) increase in the diabetic patients. The response to oxygen was much less evident when the breathing was controlled at a faster respiratory rate (15/min). This is probably explained by the increase in ventilation induced by faster-paced breathing, in parallel with increased sympathetic activity. Increased ventilation due to voluntary control per se, may have blunted the effects of oxygen administration.

All these findings in patients with type 1 diabetes of cardiorespiratory responses related to breathing pattern and inhaled oxygen, contrast with the concept of irreversible neural damage. Our findings indicate that hyperoxia not only modifies the chemoreflex stimulus, but also provides an additional parasympathetic stimulus, which in turn enhances ventilation. Autonomic dysfunction may be part of a more general modification of autonomic reflexes, one also involving altered control of ventilation. Respiratory and cardiovascular control are tightly intertwined. Accordingly, any modification of respiratory control will influence cardiovascular control, and vice versa, assuming that the autonomic abnormalities are reversible. Conversely, if neural damage is present, this interaction is minimal or nonexistent, as was shown in patients with severe autonomic neuropathies (268).

In short, improvement shown in BRS with oxygen in patients with type 1 diabetes supports our findings of a mainly functional disorder in patients without

complications. These findings show that the autonomic imbalance in and particularly the reduction in BRS of patients with type 1 diabetes can be in part be reversed by oxygen, suggesting a possible role played by pre-existing tissue hypoxia. Hypoxia seems, however, to be an important factor that restricts these patients' BRS and modifies their autonomic function.

7.4 Determinants of BRS in type 1 diabetes (I-II, IV)

In studies of healthy individuals, BRS was influenced by many factors, including age, gender, BP, HR, body mass index (BMI), smoking, and physical fitness (312-317). One study with more than 1000 healthy individuals explained approximately half the variation in BRS by age, HR, BP, BMI, smoking, and gender, which were independent predictors of the BRS in a multivariate model (315). Depression, which is a frequent finding in type 1 diabetes (318), is associated with reduced BRS (319, 320). Moreover, genetic factors seem to influence BRS (321-323). BRS has been inversely correlated with measures of arterial stiffness in patients with recent stroke or chronic haemodialysis (324, 325). Interestingly, renal transplantation normalizes BRS through improvement in central arterial stiffness (326). Unfortunately, we had no data on arterial stiffness.

Determinants of BRS in type 1 diabetes are not unravelled thus far. What can be anticipated is that the association between the BRS and anthropometric variables diminishes in type 1 diabetes, and those variables associated with cardiovascular risk become more important and also change with increasing diabetes duration. In our studies, BRS was inversely associated with age and BP, but not with BMI, gender, or smoking (data not shown). Some of the BRS indices showed a weak and mostly non-significant inverse correlation with HbA_{1c}.

7.4.1 Glycaemic control (I-II)

Long-term poor glycaemic control has been identified as a major contributor in the development and progression of diabetic CAN (101, 104, 108, 109, 222, 327, 328). In contrast to earlier studies, Study I showed no clear association between HbA_{1c} and the autonomic indices, including BRS and CAN score. This may be due to a number of factors, including fairly good metabolic control, relatively mild autonomic involvement, and a possible inherent lack of correlation between a single-measure of HbA_{1c}, that reflects relatively short-term metabolic control, and dysfunction that may need years to develop.

However, in Study II which also included patients with > 30 years of duration of type 1 diabetes, HbA_{1c} showed a significant, although weak, inverse association with autonomic indices including BRS values (data not shown). Thus, despite having only

one single measurement of HbA_{1c}, our findings support the central role of glycaemic control in development of diabetic neuropathy, since the patients with evident CAN also had higher HbA_{1c}. Importantly, a study by Larsen et al demonstrated that even after a 30-year diabetes duration, mean HbA_{1c} <8.4% over 18 years was associated with near-normal nerve conduction (328). On the other hand reduced cardiovascular autonomic reactivity has been observable even in patients newly diagnosed with type 1 diabetes (286, 329), supporting the role of factors other than long-standing hyperglycaemia in the pathogenesis of autonomic dysfunction. In patients with type 2 diabetes, different forms of diabetic neuropathy may already exist at the time of diagnosis of type 2 diabetes (330), or even in prediabetic disorders (331). This is probably explained by latent long-standing exposure to glucose when the subject is still unaware of the diabetes diagnosis. Other factors, however, such as sympathetic overactivity, which is related to obesity and insulin resistance probably also play a role. In contrast to other microvascular complications, glucose variability in type 1 diabetes does not seem to influence the development of peripheral and autonomic neuropathy (332).

7.4.2 Diabetes duration and BRS (II, IV)

In Study II, we explored the effect of diabetes duration on reversibility of BRS by comparing two groups of diabetic patients: one of short and one of long diabetes duration. During 15/min controlled breathing, BRS was clearly reduced in patients with type 1 diabetes overall as compared to the control subjects, and BRS was significantly lower in patients with diabetes of long duration. Slow breathing by patients with type 1 diabetes raised the BRS of most of them to a level similar to that obtained in the control subjects at their normal breathing rate (15/min). However, after adjustment for age, the resting-level BRS and the response to deep breathing no longer differed between duration groups. The lack of association in our cohort between long disease duration and the presence of autonomic dysfunction is in line with the results of earlier studies (108, 109, 328).

Studies in healthy subjects have demonstrated that BRS deteriorates with age (314, 333, 334), but age-associated changes in sympathetic BRS are not clear because most of the studies have focused on cardiovagal BRS. Our patients with type 1 diabetes showed a decline in most of the autonomic indices, but with age-dependent deterioration taken into account, the remaining decline, which might have been ascribed to the diabetes, was no longer significant (IV). However, patients with AHT showed a significant drop in E/I ratio, 30/15 ratio, and BRS-αHF, even after age-correction. The age-associated decline in BRS is most likely multifactorial, and changes in any segment of the cardiac baroreflex arc may play a role in the process. In addition to neural deficits, also arterial stiffening may contribute to age-associated reduction in BRS (335-337). In short, the patients with diabetes had already at baseline a lower BRS than did healthy controls, but it is of note that their more pronounced decline during follow-up was for most of the patients equivalent to a normal age-related decline.

7.4.3 BP and BRS (I, II, IV)

Although the relationship between sympathetic activation, the BRS, and BP is not yet fully understood, earlier data suggest that impaired BRS may precede the development of hypertension (242, 243, 245). Potential mechanisms for the increase in BP through the impaired BRS are increased BPV and resetting of the BP level. In healthy individuals, sympathetic activation is associated with a predominance of LF power oscillations of the SBP (167). The LF power of SBP is also shown to increase with rising BP levels in healthy subjects (240). In our study (II), those diabetic patients with a normal autonomic score showed a trend of increased power in the LF band of the SBP, compared to the patients with a higher autonomic score. This finding, along with an increased LF/HF ratio, is suggestive of sympathetic predominance in patients with type 1 diabetes even in the absence of CAN, but also a trend toward increasing sympathetic dysfunction with higher autonomic score.

In Study IV, we explored the association between spontaneous BRS and 24-h ABPM and office BP at baseline in all patients with ABPM data available (N=71). The majority of the BRS variables showed an inverse correlation with 24-hour and daytime BP (mainly DBP and MAP), in line with previous results in non-diabetic individuals (245). The associations between baseline BRS and change in BP were analyzed only in the patients without AHT (N=50). The baseline BRS predicted an increase in the night-time BP at follow-up. Moreover, the BRS response to deep breathing correlated with the increase in 24-hour BP indices overall. Importantly, according to a recent meta-analysis, night-time SBP is a stronger predictor of cardiovascular and non-cardiovascular mortality than daytime SBP both in hypertensive patients and in randomly selected populations (338).

The non-dipping status was not related to baseline BRS and neither did it predict an increase in BP or AER, in contrast to some, (48, 339) but not all (223) previous studies. This might be explained by the fact that the future microalbuminuric patients are most likely those already on AHT. These subjects were excluded from analysis since we analyzed the change in BP over time. In addition, the reproducibility of the dipping status is variable (340, 341), and moreover the use of a preset sleeping time might result in an over-interpretation of the nocturnal dipping phenomenon (341). Finally, the predictive value of the non-dipping phenomenon for microalbuminuria or especially in normotensive patients is still not clear. The majority of the studied patients were normotensive and within the normoalbuminuric range, whereas the relevance of non-dipping could be more evident in patients with diabetic nephropathy and hypertension. Regarding the AHT+-group, the association between baseline BRS and last AER before starting AHT could have been of interest, but unfortunately such data were not available.

Whereas spontaneous BRS was associated with an increase in night-time BP, the delta BRS (BRS-SD and BRS-αLF), i.e. the BRS response to deep breathing, correlated with a general increase in BP. The circadian BP varies according to the daily activity and interactions between the sympathetic nervous system and the renin-angiotensin system

(342, 343). Accordingly, night-time BP is better standardized than the daytime BP, and thus, an increase in night-time BP may also be a more sensitive marker of sympathetic activation. The fact that the BRS response predicted an increase in night-time BP underlines the importance of a functional deficiency of the autonomic regulation. The results did not change after removing the participants treated with drugs affecting angiotensin II, confirming no relevant confounding effects of these drugs on BRS.

Notably, several of the antihypertensive medications available aim at a reduction in the sympathetic overdrive that characterizes essential hypertension. Recently two novel, non-pharmacological approaches have been introduced for the management of resistant hypertension, i.e. carotid baroreceptor stimulation (344) and catheter-based sympathetic renal denervation (345). Also these interventions act on different targets that trigger sympathetic activation resulting in BP increase. Although both interventions have shown promising results in severe hypertension, both procedures are invasive. Moreover, their long-term BP lowering effects and the impact on end-organ damage and on cardiovascular events are not yet clear. Interestingly, impaired cardiac BRS could help to identify those patients with resistant hypertension who will respond to renal denervation (346)

Altogether, in our 5-year follow-up, we have to our best knowledge for the first time demonstrated that baseline BRS predicted a future increase in BP, first seen in night-time BP.

7.5 Prognostic significance of autonomic disorders in diabetes

The prognostic importance of CAN diagnosed with the traditional Ewing tests is undoubtedly established in patients with diabetes (7, 202). Until now there is only scarce data on the prognostic significance of the BRS in patients with diabetes, despite of the fact that it has proven to be a sensitive marker of cardiovascular risk. Studies in patients with heart failure have consequently shown that low BRS is associated with a worse prognosis, although the BRS-values in these studies have been markedly lower than those in our patients (13, 14). Nonetheless, there is no reason to believe that BRS would not be a useful prognostic marker in patients with diabetes. Within the limits of the follow-up period of 5 years we did show a clear deterioration of the BRS. The fact that the deterioration could be mainly attributed to normal aging does not exclude some effect of neuropathy that could become evident after a longer observation time. Thus, the follow-up time of 5 years may not be long enough to establish a possible relationship between reduced BRS and a future full-blown CAN. Moreover, to elucidate the role of BRS as a cardiovascular risk factor probably also requires a longer follow-up.

The deep breathing-induced BRS response correlated with the overall BP in patients without AHT (IV). Although the potential differences in the prognostic significance of the different BRS methods are unclear, a recent study demonstrated that reduced BRS

in hypertension was most evident using the non-invasive αLF-method (184). Nonetheless, in Study IV, the only method that showed a persistent and significant decline over time even after adjustment for age was the BRS-αHF. This decline could be explained by reduced respiratory sinus arrhythmia in the HF-region due to diabetes.

The coexistence of nephropathy and CAN is well established, although it is presently not known what the causal relationship between nephropathy and neuropathy is (12, 204, 221). In the present study BRS did not correlate with urinary albumin excretion rate, although the patients with evident CAN had higher urinary albumin excretion rate. Altogether, it is possible that the assessment of the BRS could provide a tool that that enables detection of patients at risk earlier than the Ewing tests, and thus allowing earlier interventions.

7.6 Implications of functionality and future prospects

Despite decades of research and numerous clinical trials, attempts to find effective treatment that prevents or reverses the development of diabetic neuropathy has failed thus far. This is probably explained by the multifactorial aetiology of diabetic neuropathy, but also by the fact that the clinicians recognize these patients far too late. Whereas it is possible that a combination therapy would be more efficient than blocking a single pathway, no other treatments than improvement of glycaemic control have proved effective to date (347). Consequently, today, the cornerstone of the therapy is to improve quality of life by control of the symptoms and attempts to prevent the progression of neuropathy through improved glycaemic control, and through multifactorial risk intervention.

The results of this thesis, and the notion that early autonomic neuropathy could be functional and respond to interventions or treatment, gives a new perspective in comparison to the past and current literature on this topic. The importance of this finding is further amplified by the possibility to predict increased BP and perhaps even other diabetic complications. Previous studies have established that patients with reduced BRS due to heart failure (18, 19) and hypertension (20) benefit from physical exercise in terms of improved BRS. Early autonomic abnormalities seem to be favorably influenced by physical exercise in type 2 diabetes (348, 349), and recently also in adolescents with type 1 diabetes (350). Nonetheless, longer follow-up times and intervention studies are needed in order to clarify the role of early autonomic abnormalities as predictors of other diabetic complications, and whether interventions also could prevent these.

The presence of hypoxia and signs of abnormal respiratory control and the functional component of these abnormalities highlight the possibility of correction using simple strategies like physical training. Another approach could be to train the abnormal respiratory regulation. While our interventions were probably short-acting, and we did not study for how long the improvement in BRS persists, recently it has been shown

in patients with COPD that the autonomic parameters and respiratory control were improved by interval hypoxic training (351). Interestingly, in patients with type 1 diabetes, there are obvious signs of adaptation in the ANS marked by improved respiratory reflexes already after one single session of intermittent hypoxia (352). Further studies with longer protocol and repeated intermittent hypoxic periods will be carried out in the future.

Based on the results from this thesis, the BRS is clearly associated with the BP in patients with type 1 diabetes with no signs of diabetic complications. Whether reduced BRS is a warning signal and predicts diabetic micro- and macrovascular complications and cardiovascular events is not yet known and a longer follow-up time is required. We aim to restudy our patients within 1-2 years and the data will probably give more information on the prognostic power of the BRS. Moreover, the measurement of the BRS requires in addition to standardized laboratory conditions, also special equipment. More research is also needed in order to develop appropriate equipment for bed-side assessment of the BRS. Our data suggest that when the testing is correctly performed and the respiration is thoroughly monitored, the testing is not time-consuming.

Altogether, the identification of the time point in the natural history of the disease at which the disorder transforms into more organic dysfunction could be crucial in order to be able to initiate effective preventive measures when the abnormalities are still reversible.

8 SUMMARY AND CONCLUSIONS

I In the course of type 1 diabetes, increased sympathetic activity and a reduced BRS are already present at an early stage. A blunted BRS can, however, be elevated to the same level as in healthy control subjects, as a response to slow, deep breathing. This finding suggests a mainly functional abnormality, at least at an early stage of the disease.

II Irrespective of the duration of type 1 diabetes, in the majority of patients blunted BRS was restored by slow, deep breathing. On the other hand, the response to slow, deep breathing in those with CAN was similar to the response in patients with a surgically denervated heart. Thus, it seems that the defect in BRS is functional and reversible throughout the course of diabetes until the development of definite CAN.

III Patients with type 1 diabetes demonstrated an augmented response to oxygen administration by the BRS and other cardio-respiratory measures. This is indicative of a pre-existing resting tissue hypoxia, which may be one of the possible causes of functional autonomic abnormalities in such patients, and also be a potential link between this autonomic dysfunction and their other diabetic complications.

IV Although BRS was reduced even at baseline, the decline in BRS during a 5-year follow-up was similar to that seen in healthy subjects, thus mainly reflecting physiological ageing. That low BRS at baseline did not progress to CAN supports its possible functional aetiology. However, resting BRS at baseline did predict an increase in night-time SBP. Moreover, the BRS response to deep breathing at baseline was associated with the increase an in 24-hour ambulatory BP.

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