Diabetic encephalopathy:

a cerebrovascular disorder?

Diabetische encefalopathie:
een cerebrovasculaire aandoening?

(met een samenvatting in het Nederlands)

Proefschrift

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Sanne Manschot

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Cover: Els Nijhof: vrij naar `section through the cerebrum and pons, also showing the laminar structure of the hippocampus´ by Felix Vicq d`Azyr, French Anatomist (1748-1794), eerder verschenen op de kaft van het proefschrift van dr. G.J. Biessels.

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ZOET

Wat een zoete baby,
wat speelt hij zoet,
Zal je zoet zijn als ik weg ben?
Zoet op school, honingzoet.
Zoet is lekker, zoet is goed, snoepgoed.
Zoetekauw, tanden poetsen moet!
Zoethoudertje, zoete broodjes bakken.
Zoethout, zoetstof, wat zoetsappig
Zoet is de liefde, dat is prachtig, zoetelief
en ik ben al bijna tachtig .
Waarom is zoet nu niet goed voor mij?
Au, die prik doet pijn!
Zuster, ik ben zoet hoor, zuster.

Aan mijn ouders

Contents

General introduction

1

Diabetes mellitus is a metabolic disease characterised by hyperglycaemia resulting from defects in insulin secretion, insulin action, or both [1]. The most common form is type 2 diabetes, which is characterised by a combination of resistance to insulin action and an inadequate compensatory insulin secretion.

The prevalence of diabetes in adults world-wide was estimated to be 4.0% in 1995 and is expected to rise to 5.4% by the year 2025 [2]. In the Netherlands, the prevalence of diabetes (type 1 and 2) is estimated at 474.000 patients, 216.00 males and 258.000 females. The prevalence for males between 65-85 years is estimated 16% and for females 12% (source: RIVM). An increase of 35% in the next 20 years is estimated. About 85% of these diabetic patients suffer from type 2 diabetes.

Diabetes may lead to several severe complications such as retinopathy, nephropathy, angiopathy and neuropathy. The development of these complications is dependent on the duration of diabetes and the quality of metabolic control [3], and can be only partially prevented by intensive (insulin) treatment [4]. In type 2 diabetes, hyperglycaemia may cause pathologic and functional changes in various target tissues (without clinical symptoms) a long time before diabetes is diagnosed.

When we started this project in 1999 it had become increasingly clear that the brain was another site for diabetic end-organ damage, a condition referred to as diabetic encephalopathy [5]. Cognitive deficits had been reported, which were generally modest in young adult patients [6], but could be more pronounced in elderly type 2 diabetic patients [7]. Neuropsychological studies in type 2 diabetes showed moderate impairments in cognitive function, particularly involving verbal memory or complex information processing [7]. Recent studies reported an association between type 2 diabetes and the development of dementia (e.g. [7-9]). Notably, this association was evident for both vascular dementia and Alzheimer's disease [10]. Radiological studies provided further evidence that diabetes may affect the brain. Modest cortical and subcortical atrophy had been reported [11], as well as a tendency for an increased occurrence of white matter hyperintensities [11-13]. The number of radiological studies, however, was still small and the link between radiological changes and cognitive deficits had not yet been investigated systematically.

Behavioural studies in diabetic rodents also demonstrated cognitive deficits. In streptozotocin (STZ)-diabetic mice, complex tasks, such as active avoidance in a shuttle box or in a T-maze, were impaired in one study [14] and spatial learning as tested in the Morris water maze was affected in STZ diabetic rats in another study [15]. In aged rats (24 months) the diabetic deficit in water maze performance was larger than expected in young-adult rats, suggesting an interaction between ageing and diabetic cerebral dysfunction [16].

The pathogenesis of diabetic encephalopathy is likely to be to be a multifactorial process involving the adverse effects of chronic hyperglycaemia, and probably also the effects of recurrent hypoglycaemia,

on the brain [17]. In type 2 diabetes, however, hypoglycaemia does not appear to be the key factor, as cognitive deficits also develop in subjects that are not treated with insulin or oral hypoglycaemic drugs [18]. Analogous to the pathogenesis of peripheral diabetic neuropathy, chronic hyperglycaemia may lead to both metabolic and vascular disturbances in the brain. Indeed, cerebrovascular changes had been found in both diabetic rodents and patients [17;19]. In diabetic rodents, structural changes, such as thickening of capillary basement membranes and shortening of the length of the capillary network in the neocortex, had been noted. There remained, however, controversy concerning the effects of experimental diabetes on cerebral blood flow, as different studies show conflicting results. Studies of diabetic patients reported regional decreases in cerebral blood flow and impaired cerebrovascular reactivity, as well as structural alterations in the cerebral vasculature, including thickening of capillary basement membranes [19]. Although it is reasonable to assume that these vascular changes are involved in the development of diabetic encephalopathy, this involvement had yet to be examined in detail.

Given the prevalence of type 2 diabetes among the elderly and the impact of cognitive impairment and dementia on both the quality of life of patients and on health care resources, the development of additional therapeutic interventions is in demand. Vascular risk factors play a central role in the pathogenesis of diabetic complications. For the cerebral complications of diabetes, the relationship between vascular risk factors and cognitive dysfunction had not yet been investigated systematically. Because vascular deficits are a potential target for new treatment strategies aimed at preventing cognitive dysfunction and dementia in type 2 diabetic patients, this relationship is investigated in this thesis, that includes two interrelated research projects:

The first project addressed the role of vascular disturbances in the development of experimental diabetic encephalopathy. In **chapter 2** we tested the effect of the Angiotensin Converting Enzym (ACE-) inhibitor enalapril on cerebral blood flow and spatial learning in STZ-diabetic rats. We selected this vasoactive compound because ACE-inhibitors are known to slow the development of other diabetic complications including nephropathy, retinopathy and neuropathy [20]. In **chapter 3**, we looked at the effect of prevention and intervention treatment with enalapril on peripheral nerve conduction velocity and central evoked potentials in STZ-diabetic rats. In **chapter 4** we describe the effects of different dosages enalapril in long-term treatment with enalapril on nerve conduction velocity and evoked potential latencies in STZ-diabetic rats.

In the second project we examined associations between vascular risk factors, cognitive functioning and structural changes in the brain in patients with type 2 diabetes mellitus. We hypothesised that micro- and macrovascular dysfunction are major determinants for the development of diabetic encephalopathy in patients with type 2 diabetes. For this aim we initiated the Utrecht Diabetes Encephalopathy Study (UDES), a large cross-sectional study involving 125 patients with type 2 diabetes and 64 matched non-diabetic controls.
The primary objectives of the UDES were:

- To assess cognitive dysfunction in type 2 diabetic patients and quantify structural cerebral changes with magnetic resonance imaging.
- To relate these structural changes to cognitive dysfunction.
- To assess the relationship between measures of micro- and macrovascular function on the one hand and cognitive dysfunction and structural cerebral changes on the other in type 2 diabetic patients.

All participants underwent an extensive research protocol on two consecutive days which, among others, consisted of brain MRI, a neurological and neuropsychological examination, retinal photography of both eyes and measurement of the common carotid intima-media thickness (IMT). Also, a standardised questionnaire concerning medical history and medication use was taken, fasting blood samples were collected, blood pressure was recorded at home to measure blood pressure at standardised hours and urine was collected overnight.
Chapter 5 describes the cognitive performance and structural changes on brain MRI found in this group of patients, and relates structural changes to cognitive functioning. In **chapter 6**, we related microvascular and macrovascular complications, risk factors and hyperglycaemia related factors to cerebral deficits. In **chapter 7**, we examined the association between abnormalities in the peripheral nervous system and in the central nervous system, based on the hypothesis that the pathogenesis of diabetic encephalopathy might share features with that of peripheral neuropathy. In **chapter 8**, we examined cerebrovascular reserve capacity in the UDES population, and related this to cognition and other disease variables.

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Angiotensin converting enzyme inhibition partially prevents deficits in water maze performance, hippocampal synaptic plasticity and cerebral blood flow in streptozotocin diabetic rats

2

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Introduction

Diabetes mellitus is a metabolic disorder characterised by hyperglycaemia resulting from defective insulin secretion, resistance to insulin action, or both. With the current standards of medical care for diabetic patients blood glucose can be maintained within acceptable limits. Despite this treatment, the development of diabetic complications remains a serious concern. It has become increasingly clear that diabetic complications can affect the central nervous system, a condition that may be referred to as diabetic encephalopathy. Diabetic patients may express cognitive deficits, as well as neurophysiological, neurochemical and neuroradiological cerebral changes [1]. Cognitive deficits are generally modest in young adult diabetic patients but tend to be more pronounced in the elderly [2]. Recent epidemiological studies even report an association between diabetes and dementia [3;4].

Cerebral deficits have also been demonstrated in experimental diabetes [5]. The latencies of auditory and visual evoked potentials, for example, are increased in streptozotocin (STZ)-diabetic rats [6;7]. Behavioural studies with the Morris water maze show learning impairments [8] that can be prevented, but only partially reversed by insulin treatment [9]. In rats, spatial learning and the use of contextual clues in behavioural paradigms are dependent on the integrity of the hippocampus [10]. On the cellular level information is thought to be stored in the brain by plastic changes in synaptic strength. Long-term potentiation (LTP) is an activity dependent form of synaptic plasticity, that can be elicited in the hippocampus in vivo or in vitro by brief high-frequency stimulation of excitatory afferents [11;12]. Hippocampal LTP has been linked to water maze performance [12] and this form of synaptic plasticity is widely used as a model to study the cellular mechanisms of learning and memory [11;12]. In diabetic rats disturbances in water maze learning and hippocampal LTP consistently occur in parallel[9;13], in agreement with findings in non-diabetic animals [11].

The pathogenesis of cerebral dysfunction in diabetes appears to be a multifactorial process [5]. Vascular disease may play an important role, analogous to the pathogenesis of peripheral diabetic complications [1]. Both functional (e.g. altered cerebrovascular reactivity) and structural (e.g. capillary basement membrane thickening) alterations have been demonstrated in the cerebral vasculature of diabetic patients and in experimentally diabetic animals [14;15]. There remains, however, some controversy concerning the effects of diabetes on cerebral blood flow. Previous studies in diabetic rats reported cerebral blood flow to be decreased [16-18], unchanged [19] or increased [20]. A similar controversy initially existed for studies on nerve blood flow in diabetic rats, where differences in the techniques used to assess blood flow ultimately proved to be the major source of the variable results [21;22]. By measuring blood flow at tissue level, with quantitative techniques such as autoradiography or the hydrogen clearance method [21], it has now been firmly established that diabetes is associated with a decrease in sciatic nerve blood flow, leading to endoneurial hypoxia, thus playing a key role in the pathogenesis of diabetic

neuropathy [22]. Likewise, differences in the techniques used to assess blood flow may explain the variable results of previous studies on cerebral blood flow in diabetic rats. The present study therefore applied the hydrogen clearance method. This technique is known, from studies outside the field of diabetes research, to provide reliable, quantitative measurements of cerebral blood flow at tissue level, and is also well accepted in studies of nerve blood flow in experimental diabetes [23;24].

The potential role of vascular disorders in the pathogenesis of cerebral dysfunction in diabetes may provide a target for therapy, as treatment aimed at the vasculature is known to have an effect on other diabetic complications. ACE-inhibitors, in particular, slow the development of diabetic nephropathy, retinopathy and neuropathy through effects on the vessel wall, probably partially independent from their antihypertensive action [25]. In STZ-diabetic rats ACE-inhibition prevents the development of muscle and nerve dysfunction, improves nerve blood flow and stimulates angiogenesis [26]. Given the beneficial effect of ACE-inhibitors on microangiopathy in peripheral complications of diabetes, we chose to treat diabetic rats with the ACE-inhibitor enalapril in this experiment.

The aim of the present study was to examine i) whether experimental diabetes is associated with reduced cerebral blood flow, measured with hydrogen clearance, and ii) whether treatment with ACE-inhibitors improves cerebral blood flow and functional cerebral deficits. Therefore, the effects of diabetes and preventive treatment with enalapril were examined on cerebral blood flow, water maze performance and hippocampal synaptic plasticity in STZ-diabetic rats.

Materials and Methods

Animals

Male Wistar rats (starting weight ~340g, approximately 10 weeks of age) UWU-CPD, Harlan, Utrecht, The Netherlands) were housed on sawdust, and maintained on a 12h-12h light-dark cycle, lights out at 7:00 am. Behavioural testing was performed after 9:00 am. Rats were given food and water *ad libitum* and were weighed weekly. Diabetes was induced by a single intravenous injection of STZ (Serva Feinbiochemica GMBH, Heidelberg, Germany) at a dose of 33 mg/kg body weight dissolved in saline. Four days after the STZ injection, blood glucose was determined in blood samples, obtained by tail prick, by a strip-operated blood glucose sensor (Companion2, Medisense Ltd, Birmingham, United Kingdom). Blood glucose levels were >15.0 mmol/L in all STZ-injected animals. All experiments were conducted according to the guidelines of the Utrecht University Committee for Welfare of Experimental Animals.

Drugs

Enalapril was added to the drinking water at a concentration adapted to obtain a daily intake of approximately 24 mg/kg. This dose was based on

previous studies, on the effect of lisinopril on experimental peripheral diabetic neuropathy in the STZ-diabetic rat [26] and the effect of enalapril treatment on brain edema in spontaneously hypertensive rats [27] .

Experimental Design

Three groups of rats were used: a nondiabetic control group (con; *n*=12), an untreated diabetic group (DMu; *n*=12) and an enalapril-treated group (DMt; *n*=12). Enalapril treatment was initiated directly after confirmation of diabetes and continued throughout the experiment. The water maze test was performed after 14 weeks of diabetes. Cerebral blood flow was measured after 16 weeks of diabetes. A separate study was performed to examine the effect of enalapril on synaptic plasticity using different animals. Eighteen rats were used: 6 nondiabetic control, 6 untreated and 6 enalapril-treated diabetic rats. Enalapril treatment was initiated directly after diabetes induction. LTP was measured after 26 weeks of diabetes.

Morris water maze test

The Morris water maze is widely used to test the rats abilities to learn, remember and go to a place in space defined only by its position relative to extramaze cues [10]. The water maze consists of a large circular black pool (210 cm diameter, 50 cm height, filled to a depth of 30 cm with water (28±1°C)), which is placed in a darkened room, illuminated by sparse red light. Within the pool a submerged platform (black, round, 8 cm diameter, 1cm below surface) is hidden on a fixed location, 55 cm from the edge of the pool. The rat can climb on the platform to escape the necessity of swimming. During a series of trials, the rat learns to locate the platform. The experimenter was blind to the treatment of the animals.

On 5 consecutive days rats are given 3 acquisition trials per day. Rats are allowed a maximum of 120 s to find the hidden platform and are permitted to stay on it for 30 s. Rats that failed to locate the platform after 120 s are placed on it by the experimenter. The position of the rat in the pool is automatically monitored by a video computer system. Measurements comprised latency to reach the platform, distance swum and the percentage of time the rat spent in the border zone of the pool (within 20 cm of the edge). The time in the border zone of the pool provides a measure of task comprehension; naive rats try to escape by the borders of the pool, whereas trained rats search for the more centrally oriented platform and consequently spent less time in the border zone.

Hydrogen clearance

Cerebral blood flow was measured by hydrogen clearance microelectrode polarography adapted from a protocol for measuring nerve blood flow [28]. The experimenters were blind to the treatment of the animals. In final experiments rats were anaesthetised by intraperitoneal injection of urethane 10% (1-1.5 ml/100 gram bodyweight), followed by intubation and mechanical ventilation with O_2/N_2 (20/80%) throughout the hydrogen

clearance protocol, which lasted approximately 2 hours. Body temperature was continuously measured and kept within the physiological range, using a heating pad. In all rats a femoral artery was cannulated for pressure monitoring and to obtain samples for blood gas analysis. A craniotomy was performed on the right side of the skull, near the midline, with a length of 8mm and a width of 4 mm. Next, the rat was placed in a stereotactic apparatus. The skin around the craniotomy was sutured to a metal ring and used to form a pool which was filled with liquid paraffin at 37°C to a depth of at least 0.5 cm to minimise diffusion of gasses directly to or from the brain. A glass-insulated platinum microelectrode (tip diameter 2-8 µm) was inserted into the CA1 region of the hippocampus (coordinate range A-3.3 to -4.3; L2.5 to 3.0; D3.0, reference point bregma) and after that into the ventral posteromedial thalamic nucleus of the thalamus (coordinate range A-3.3 to -4.3; L2.5 to 3.0; D6.0, reference point bregma) and polarised at 0.25 V with respect to a reference electrode inserted subcutaneously in the flank of the rat. Ten percent H_2 was added to the inspired gas, the proportions of O_2 and N_2 being adjusted to 20% and 70%, respectively. When the H_2 current recorded by the electrode had stabilised (5-15 min), indicating equilibrium with arterial blood, the H_2 supply was shut off and N_2 delivery was increased appropriately. The H_2 clearance curve was recorded until baseline (10-20 min), the latter being defined as no further decline in electrode current over 3 min. Separate readings were taken from two hippocampal and two thalamic sites. After the experiment, clearance curves were digitised and mono- or bi-exponential curves were fitted to the data by computer using appropriate non-linear regression software that employed the Marquardt algorithm and the least squares method for optimising goodness-of-fit (Inplot; Graphpad, San Diego,CA). In practice, the majority (91%) of clearance curves was mono-exponential, however, when bi-exponential curves were encountered, the slow component was taken to represent nutritive flow [29]. The averages of the two measurements were taken to represent hippocampal and thalamic blood flow. Vascular conductance was calculated by dividing blood flow by mean arterial pressure during the recording period, thus compensating for changes in blood pressure.

Hippocampal long-term potentiation

LTP was measured in the CA1-field of hippocampal slices, according to the method described by Ramakers *et al* [30]. The experimenter was blind to the treatment group.

Rats were decapitated and the brains were rapidly removed from the skull. The medial part of the hippocampus was cut in 450 µm thick transverse slices. Slices were transferred into a recording chamber containing oxygenated (95% O_2, 5% CO_2) medium. Composition (in mmol/l): 124.0 NaCl, 3.3 KCl, 1.2 KH_2PO_4, 1.3 $MgSO_4$, 10.0 glucose, 20.0 $NaHCO_3$ and 2.5 $CaCl_2$ at 30°C.

Bipolar stainless steel electrodes were placed on the afferent fibers of the

stratum radiatum of the CA1 region of the hippocampus. Glass microelectrodes were used to record field-excitatory postsynaptic potentials (f-EPSPs) in the stratum radiatum. Only those slices in which the amplitude of the f-EPSP was 1 mV or more at maximum-response level were included in the experiment. The stimulus intensity was adjusted to evoke f-EPSPs of half-maximal amplitude and this intensity was used throughout the measurements. Stimulation frequency was 0.05 Hz. The first 15 min of the recording served to obtain a baseline recording of the f-EPSP. After baseline recordings, a high frequency train of stimuli (100 Hz for 1 s) was applied and f-EPSPs were recorded for another 60 min. The average slope of the f-EPSP at baseline was set at 100%, and changes in slope were expressed as changes in percentage from baseline.

Statistical analysis

Data are presented as means ± SEM. Between group differences in body weight, blood glucose, cerebral perfusion and baseline slope of the f-EPSPs in the hippocampus were analysed by one-way analysis of variance (ANOVA) with post-hoc Duncan's multiple range tests. Between group differences in water maze performance and LTP expression were analysed by analysis of variance for repeated measures (ANOVAR).

Results

Water maze learning and cerebral blood flow

Animals (table 1)

One rat in the enalapril treated group died one week after diabetes induction. No apparent cause of death was established. Diabetic animals failed to gain weight during the course of the experiment. They had significantly reduced final body weights below original prediabetic levels, and raised levels of blood glucose, compared to control animals (Table 1). Enalapril treatment had no effect on bodyweight or glucose level.

Table 1: Bodyweight (g), blood glucose (mmol/L) at baseline and at 16 weeks of diabetes; rectal temperature (°C) and mean arterial bloodpressure (MAP; mm Hg) during hydrogen clearance measurements.

		Week 0		Week 16			
	n	bodyweight	glucose	bodyweight	glucose	temp	MAP
diabetic	12	360±3	20.1±0.7	269±10*	27.6±0.9*	38.5±0.1	102±3.4
diabetic -treated	11	363±2	18.9±0.5	268±13*	29.1±0.9*	38.4±0.3	78±3.2*
Control	12	373±3		537±10	5.2±0.2	38.0±0.2	104±2.4

Data are mean±SEM and were analysed by one way analysis of variance with post-hoc Duncan's multiple range test, *: $p<0.001$

Morris maze (figure 1a-c)

During the 5 days of training, the latency to reach the platform (Fig. 1a) and percentage of time spent in the border of the pool (Fig. 1b) in

untreated diabetic animals was significantly higher than in controls (latency, $p<0.001$; percentage of time in border, $p<0.05$). Swimming distance (Fig. 1c) was significantly increased in diabetic rats only on days 4 and 5 ($p<0.05$). Enalapril treatment improved the performance of diabetic rats, which was statistically significant for latency (DMu vs. DMt: $p<0.05$) (fig 1a). Percentage of time spent in the border was also improved, but this did not reach statistical significance. Enalapril treatment had no significant effect on swimming distance.
On examination of the eyes, all of the controls and 8 of the 12 untreated rats had no cataract, 2 rats had unilateral cataract and 2 rats had bilateral cataract. In the enalapril treated diabetic rats, 5 of the 11 rats had no cataract, 2 rats had unilateral cataract and 4 rats had bilateral cataract. There was no difference in water maze performance in rats with cataract compared to rats without cataract.

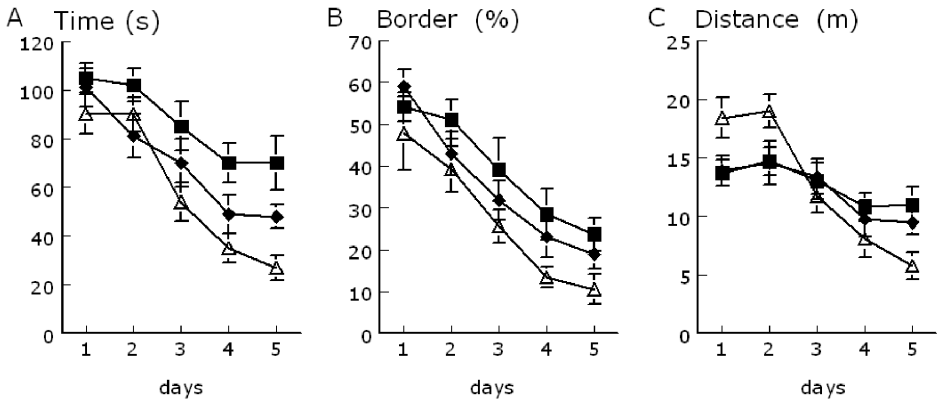

Fig. 1a,b,c: Morris water maze; mean performance per day. Diabetes duration 14 weeks. Fig. 1a: latencies to reach the platform. Fig. 1b: percentage of time spent in the border zone of the pool. Fig 1c: swimming distance in arena. All data are group means ± SEM. Control rats (Δ; n=12), diabetic rats (■; n=12), enalapril treated diabetic rats (◆; n=11). Performance of diabetic rats was impaired compared to controls. Δ vs ■: fig. 1a:$p<0.001$, fig 1b: $p<0.05$, fig 1c: day 4 and 5: $p< 0.05$ (ANOVAR). Enalapril treatment partially prevented deficits in water maze performance in diabetic rats. ◆ vs ■: fig 1a: $p<0.05$, fig 1b: not significant, fig 1c: not significant. Δ vs ◆: fig 1a: not significant, fig 1b: not significant, fig 1c: day 5 : $p<0.05$ (ANOVAR)

Cerebral blood flow (figure 2a-d)
Cerebral blood flow was measured in 9 control rats, 10 untreated diabetic rats and 10 treated diabetic rats. In a further 3 control rats, 2 untreated and 1 treated diabetic rat no reliable measurements could be obtained due to technical problems such as failed cannulation, and respiratory or cardiac arrest directly following urethane administration. Blood pressure was similar in control and diabetic rats, in line with previous studies using urethane anaesthesia [31]. Enalapril treatment resulted in a significant reduction in blood pressure in the diabetic group ($p<0.001$). In diabetic animals blood flow was reduced by 30% ($p< 0.001$) in the hippocampus

and 37% (p< 0.005) in the thalamus compared to nondiabetic controls (Fig. 2a and 2b). Enalapril treatment significantly improved blood flow in the hippocampus (DMu vs. DMt: p<0.05). There was a similar increase in thalamic blood flow with enalapril, but this did not reach statistical significance. Vascular conductance was significantly higher in enalapril treated compared to untreated diabetic groups for both the hippocampus (p<0.001) and the thalamus (p<0.05) (figs 2c and 2d).

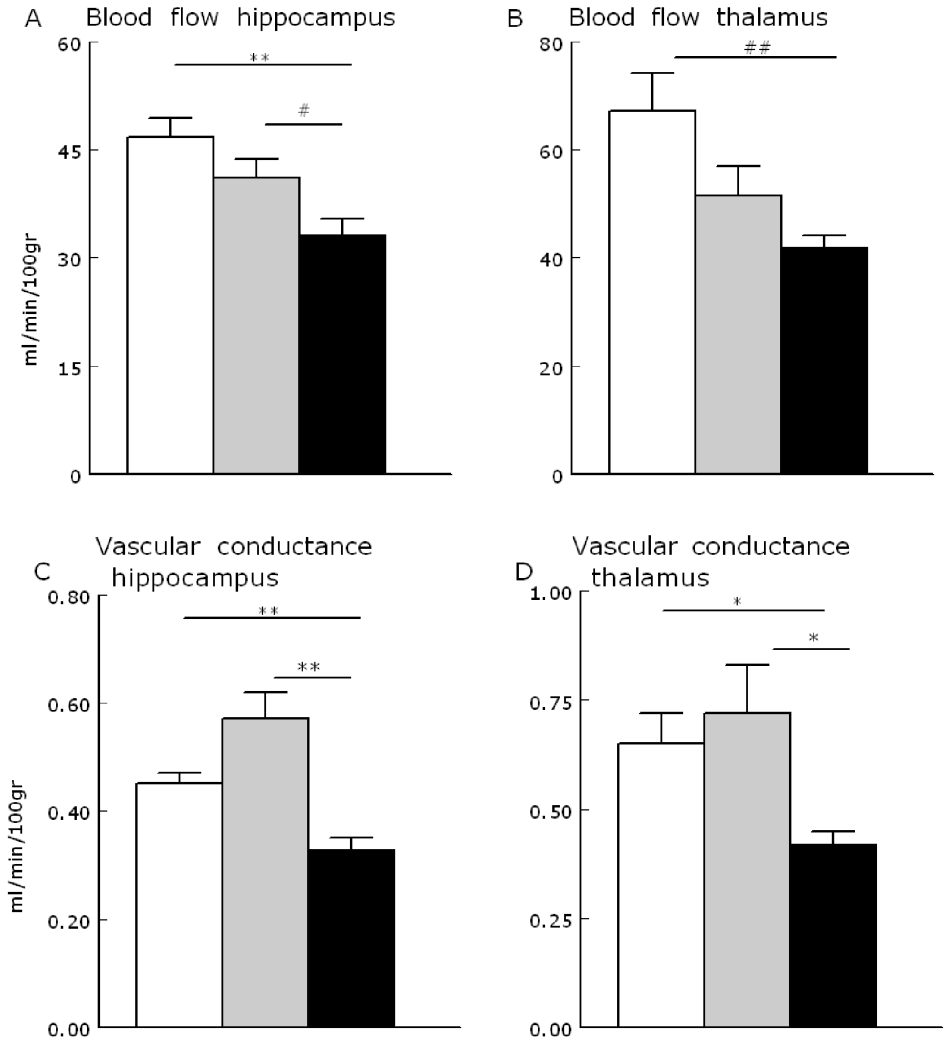

Fig. 2a, b, c, d: Blood flow hippocampus (2a) and thalamus (2b), vascular conductance hippocampus (2c) and thalamus (2d). Diabetes duration 16 weeks. All data are group means ± SEM. Control group (□; n=9), diabetic treated (■; n=10), diabetic untreated (■; n=10). All significant differences between groups are indicated in the figures. *=p<0.01; #=p<0.05; **=p<0.001; ##=p<0.005.

Hippocampal synaptic plasticity

Animals

All diabetic animals failed to gain weight during the course of the experiment and were hyperglycemic (bodyweight (g): con: 551±12; DMu: 241±14; DMt: 278±27, blood glucose (mmol/l): con: 5.2±0.1, DMu: 24±0.7; DMt 25±0.8). Enalapril treatment had no effect on bodyweight or glucose level.

Hippocampal LTP (fig 3)

High frequency stimulation of afferent fibers led to an initial increase in f-EPSP slope in all groups (Fig. 3), but this initial increase was markedly impaired in diabetic animals as compared to controls (con: 77±13%, DMu: 24±7%, DMt: 31±6%; DMu vs con: $p<0.005$; DMt vs con: $p<0.05$). Expression of LTP was significantly less in the untreated diabetic rats compared to the control group ($p<0.001$). In enalapril treated diabetic rats LTP expression was significantly improved as compared to untreated diabetic rats ($p<0.05$), but enalapril failed to fully restore the expression of LTP to nondiabetic control levels although the remaining deficit was statistically marginal ($p=0.06$).

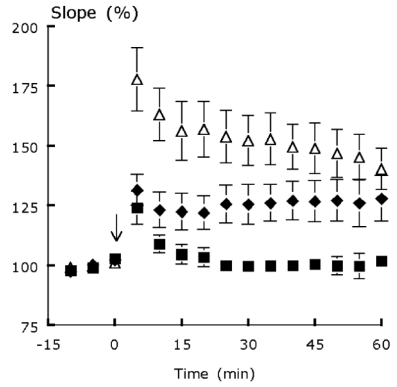

Fig. 3: Hippocampal LTP, % of f-EPSP slope. Diabetes duration 26 weeks. All data are group means ± SEM. Effect of high frequency stimulation (arrow) on the f-EPSP slope in control rats (Δ; n=6), diabetic rats (■; n=6), and enalapril treated diabetic rats (◆; n=6). The increase in slope after stimulation was impaired in ■ as compared to Δ ($p<0.005$). Enalapril improved LTP expression in diabetic rats, but not towards control levels. ◆ vs ■: $p<0.05$; ◆ vs Δ: $p=0.06$

Discussion

In diabetic rats a significant reduction in hippocampal and thalamic blood flow was observed. Furthermore, water maze performance was markedly reduced and hippocampal LTP was attenuated. Enalapril treatment partially prevented these deficits in cerebral perfusion, water maze performance and hippocampal LTP.

Vascular disease may play an important role in the pathogenesis of cerebral dysfunction in diabetes. Diabetes is a well known riskfactor for ischaemic stroke, leading to acute cerebral deficits. The slowly progressive end-organ damage in the brain that constitutes diabetic encephalopathy, however, is most probably related to more chronic functional and structural vascular alterations. Cerebrovascular reactivity, for example, is impaired, as reflected in an altered response of cerebral blood flow to increased pCO_2 in inspired air [32], and autoregulation is disturbed [33]. A number of studies have

examined cerebral blood flow. Results, however, have been inconsistent, as both decreased [34] and increased [35] cerebral blood flow has been reported. This may be attributed to the use of different techniques. Moreover, study populations have usually been relatively small and heterogeneous [14].
In experimental studies in diabetic rodents slowly progressive structural alterations in the cerebral vasculature have been noted, including arteriolar endothelial cell necrosis and thickening of capillary basement membranes [36]. Vascular reactivity was also found to be disturbed [37]. Like in diabetic patients, previous studies on cerebral blood flow in diabetic rats have provided inconsistent results. Again, differences in the techniques used for assessing blood flow are a likely source of variability. For example, studies that assessed blood flow indirectly, by measuring venous outflow [20] or red blood cell velocity in cortical arterioles [38]reported increases in cerebral blood flow. Rubin and Bohlen [38] noticed a decreased quantity of venules and enlargement of arterioles in the brains of rats with 8-10 weeks of diabetes. They found that resting cerebral blood flow as determined by red blood cell velocity in cortical arterioles in the diabetic rats was greater than in control rats, and cerebral autoregulation was intact. In contrast, studies that assessed blood flow at tissue level, by measuring the cerebral uptake of tracers like [^{14}C]iodoantipyrine in awake or anaesthetised rats, consistently report flow reductions of 10 to 15 % during the first month of diabetes [16;39] and 10 to 30 % after 4 months of diabetes [17;18], with some degree of regional variation. Our hydrogen clearance data concur with the findings of these latter studies. Each of the different methods that have been used to measure cerebral blood flow in experimental diabetes has its own merits and drawbacks. For example, methods that rely on the measurement of the movement of erythrocytes like laser Doppler flowmetry or video registration [38] can be influenced by diabetes related rheological changes or alterations in the vascular wall. Moreover, these latter techniques do not provide a true indication of the delivery of nutrients to the tissue, which may be disturbed due to changes in the blood brain barrier or the capillary basement membrane. In contrast, methods that use freely diffusible tracers, like the hydrogen clearance method or [^{14}C]iodoantipyrine autoradiography, permit measurement of the nutritive (capillary) flow component, which is most pertinent to neural function. We are therefore of the opinion that these latter techniques are the method of choice in the assessment of cerebral blood flow in experimental diabetes.
Untreated STZ-diabetic rats showed a performance deficit in the Morris maze and an impairment of LTP expression in the hippocampus, in line with previous studies [8;13]. Both the deficits in maze performance and in hippocampal LTP could be partially prevented by enalapril treatment. Although LTP was measured in a separate group of rats after 26 weeks of diabetes, these measurements are likely to be relevant for the behavioural findings at 14 weeks, as deficits in hippocampal LTP have developed fully after 10-12 weeks of diabetes, and show no progression thereafter [9;40]. Previous studies indicated that the impaired performance of diabetic rats in

the water maze is largely due to cognitive dysfunction [8;9] and that the influence of sensorimotor deficits, including the occasional occurrence of cataract, and motivational factors, if any, is limited. In the present study, the percentage of time spent in the borderzone of the pool was significantly increased in the diabetic rats, reflecting an altered search strategy. The relatively decreased swimming distances on the first days of training are likely to be secondary to this altered strategy, as previous studies with non-spatial versions of the maze demonstrated that swimming abilities were unaffected in 3 month diabetic rats [8].

ACE-inhibitors are vasoactive drugs blocking the conversion of (the biological inactive) angiotensin I (Ang I) to angiotensin II (Ang II). ACE-inhibitors are well known for their beneficial effect on cardiovascular disease in diabetic patients [41] and were previously shown to ameliorate peripheral diabetic neuropathy. Treatment with lisinopril, for example, prevented nerve conduction slowing in STZ-diabetic rats [26]. ACE-inhibitors were also shown to improve peripheral neuropathy in diabetic patients [42]. To our knowledge, no other studies on the effects of long-term ACE-inhibition on cerebral function in experimental diabetes have been published thus far. However, acute subcutaneous administration of a single dose of ramipril was previously shown to improve retention of a footshock active avoidance task in STZ-diabetic mice [43]. The beneficial effects of ACE-inhibition on peripheral diabetic neuropathy are considered to depend on lowering peripheral vascular resistance and on preventing reductions in nerve blood flow [26]. Likewise, the improvement of learning and plasticity by enalapril could well be mediated through the vasculature, as reflected in the increase in cerebral blood flow. Alternatively, the effects of enalapril on water maze performance and hippocampal synaptic plasticity in diabetic rats could, at least in part, be mediated directly through cerebral ACE and Ang II receptors. Ang II receptors and ACE are known to be present in several brain areas, and play a role in physiological functions such as drinking, sodium appetite, and pituitary releasing actions [44]. Enalapril appears to be capable of passing the blood-brain-barrier, because oral administration of 10 mg/kg/day has previously been shown to reduce ACE-activity in the cerebral cortex in rats [45], however, enalapril did not reduce ACE-activity in the hippocampus in that study. It should be noted that peripheral angiotensinogen [46] and angiotensins [47;48] do not penetrate the blood-brain-barrier. Therefore, although direct effects on cerebral ACE and Ang II receptors may be involved, improvement of cerebral blood flow appears to be a key factor in the effects of enalapril on water maze performance and hippocampal synaptic plasticity in diabetic rats.

In conclusion, experimental diabetes is associated with reduced cerebral perfusion. Treatment aimed at the vasculature can partially improve cerebral blood flow, deficits in Morris maze performance and synaptic plasticity, indicating that angiopathy could play a role in the development of cerebral dysfunction in diabetic rats and provides a potential target for treatment.

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Nerve conduction velocity and evoked potential latencies in streptozotocin diabetic rats: effects of treatment with an angiotensin converting enzyme inhibitor

3

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Introduction

The association between diabetes mellitus and acute cerebrovascular complications like stroke is well-recognised [1]. Epidemiological studies have shown a two- to three- fold increased incidence of stroke in patients with diabetes. Furthermore, stroke patients with diabetes have a higher mortality, worse neurological outcome, and more severe disability than those without diabetes (review[2]).

Diabetes mellitus also leads to more slowly progressive cerebral dysfunction and leads to a lesser performance on cognitive tests [3 4-6]. Cognitive impairments are generally modest in young and middle aged patients. In elderly patients with type 2 diabetes, however, cognitive function is associated with a poorer ability in diabetes self-care and greater dependency[4]. Furthermore, recent epidemiological studies demonstrate an association between diabetes and vascular dementia as well as Alzheimer's disease [3;5].

The pathogenesis of cerebral disorders in diabetes is a multifactorial process involving both metabolic and vascular factors[7]. In diabetic patients, as well as in animal models, diabetes is associated with structural alterations in both small and large cerebral blood vessels (review[2]) and functional vascular changes such as disturbed cerebral blood flow (review[2]) [8 9] and altered vascular reactivity [10;11].

In streptozotocin (STZ) diabetic rats, deficits in cerebral function develop gradually in the course of months. [12] Learning deficits have been noted, which develop in association with deficits in synaptic plasticity. [13] In addition, deficits in impulse conduction velocity develop in the brain, as reflected in increased evoked potential latencies [12].

Angiotensin converting enzyme inhibitors (ACE-inhibitors) are a class of drugs that are known to have beneficial effects on several diabetic complications. For example, lisinopril inhibits the development of human diabetic nephropathy and retinopathy, whereas trandolapril slows the development of neuropathy in diabetic patients [14-16]. Moreover, ramipril lowered the risk of acute cerebrovascular complications, like stroke by 33% in diabetic patients (the HOPE-study [17]). These effects appeared to be partially independent of the antihypertensive action of ACE-inhibitors. Furthermore, we have recently shown that treatment with enalapril partially prevents deficits in cerebral blood flow, spatial learning and synaptic plasticity in streptozotocin diabetic rats [9].

The aim of the present study was to examine whether treatment aimed at the vasculature could prevent or reverse peripheral and central neurophysiological deficits in experimental diabetes. Therefore, the prevention and intervention effects of treatment with the ACE-inhibitor enalapril were examined on nerve conduction velocity and evoked potential latencies in STZ-diabetic rats.

Materials and Methods

Animals

Male Wistar rats (starting weight ~325g, approximately 10 weeks of age, UWU-CPD, Harlan, Utrecht, The Netherlands) were housed on sawdust, maintained on a 12h-12h light-dark cycle. Rats were given food and water *ad libitum* and were weighed weekly. Diabetes was induced by a single intravenous injection of streptozotocin (STZ) (Serva Feinbiochemica GMBH, Heidelberg, Germany) at a dose of 33 mg/kg body weight dissolved in saline. Four days after the STZ injection, blood glucose was determined in blood samples, obtained by tail prick, by a strip-operated blood glucose sensor (Companion2, Medisense Ltd, Birmingham, United Kingdom). Blood glucose levels were >15.0 mmol/L in all STZ-injected animals. All experiments were conducted according to the guidelines of the Utrecht University Committee for welfare of experimental animals.

Drugs

Enalapril was added to the drinking water at a concentration adapted to obtain a daily intake of approximately 24 mg/kg. This dose was based on previous studies, on the effect of lisinopril on experimental peripheral diabetic neuropathy in the STZ-diabetic rat [18] and the effect of enalapril treatment on brain edema in spontaneously hypertensive rats [19] .

Experimental design:

Enalapril prevention study

To examine the prevention effect of enalapril treatment on neurophysiological changes in the peripheral and central nervous system three groups of rats were used: a non-diabetic control group (con; n=10), an untreated diabetic group (DMu; n=13) and an enalapril-preventive treated group (DMp; n=13). Enalapril treatment was initiated directly after confirmation of diabetes and continued throughout the experiment. Motor nerve conduction velocity (MNCV) and sensory nerve conduction velocity (SNCV) in the sciatic nerve were used to monitor the effects on the peripheral nervous system. Brain stem auditory evoked potentials (BAEP's) and visual evoked potentials (VEP's) were used to monitor the effects on the central nervous system. Nerve conduction velocity and evoked potential latencies were measured in separate weeks. All measurements were performed within 5 minutes after anaesthesia in order to prevent cooling down of the animal. MNCV and SNCV were measured before diabetes induction and again every three weeks for a period of 15 weeks. BAEP and VEP were measured from 10 weeks of diabetes duration and again every 3 weeks until 16 weeks of diabetes duration. We started the measurement of the evoked potential latencies in both experiments at 10 weeks of diabetes duration, because previous studies show that increases in evoked potential latencies are noticeable after 2 to 3 months of diabetes [12].

In a pilot study we examined the effect of enalapril prevention treatment on nerve conduction velocity and evoked potential latencies in non-diabetic

rats. There were no statistically significant differences between the treated and non-treated animals (data not shown).

Enalapril intervention study
To examine the intervention effect of enalapril treatment on neurophysiological changes in the peripheral and central nervous system three groups of rats were used: a non-diabetic control group (con; n=10), an untreated diabetic group (DMu; n=14) and an enalapril-intervention treated group (DMi; n=14). Enalapril treatment was initiated at 15 weeks of diabetes and continued until 25 weeks of diabetes. MNCV and SNCV were measured before diabetes induction and were repeated every 3 weeks until 24 weeks of diabetes. BAEP and VEP were measured from 10 weeks of diabetes duration and were repeated every three weeks until 25 weeks of diabetes.

Sciatic nerve conduction velocity:
MNCV and SNCV were measured in the sciatic nerve according to the method described by De Koning & Gispen [20]. In short, the sciatic and tibial nerves were stimulated at the sciatic notch and ankle, respectively. The latencies of the responses of the musculature of the foot were measured. The MNCV and SNCV were calculated by dividing the distance between the two stimulation points by the differences in latencies after proximal and distal stimulation.

Evoked potential latencies:
Placement of recording electrodes: after 9 weeks of diabetes, the rats were anaesthetised with Hypnorm® (Janssen Pharmaceutica BV, Tilburg, the Netherlands; containing fluanisone 10 mg/ml and fentanylcitrate 0.315 mg/ml; dose 0.1 ml/100g intramuscularly). One stainless steel screw was implanted permanently into the skull as a reference point (co-ordinates A-3.0, L 3.0; reference point Bregma). [21] Care was taken not to penetrate the dura. The skin was closed over the screw. All animals were allowed to recover for one week. For measurement of the evoked potential latencies rats were slightly sedated with a low dose of diluted (with 0.9%NaCl; 1:2) Hypnorm® (0.1 ml/100g subcutaneously), in order to prevent them from moving. BAEP's were recorded with subcutaneously placed stainless steel needle electrodes, insulated except for the tip. The recording electrode was placed at A-8.0 L2.0, reference point bregma, using the subcutaneously placed screw as a reference point. The reference electrode was placed subcutaneously on the nose of the animal. An earth electrode was connected to the front paw. The stimulation protocol for BEAP and VEP was according to the method described by Biessels. [12]
Stimulation, recording and analysis BAEP: The rat was placed in a soundproof, darkened room. For the recording of auditory evoked potential latencies a speaker was placed at 15 cm above the head of the rat. Acoustic stimuli were presented as clicks (unfiltered square waves of 100

µs duration with constant polarity, applied at a frequency of 10 Hz). The threshold of the BAEP was determined, defined as the minimal sound pressure level to evoke a response of at least 0.5 µV at a latency of ~5 ms. Next, BAEP's were recorded at a sound pressure level of 60 dB above threshold. BAEP's were amplified, filtered (band pass 216-3400 Hz) and stored in a computer. For analysis, 512 traces (sweep length 40 ms) were analysed. The latencies of waves I, III and V were determined. Although some uncertainty remains as to the generators of these waves, wave I is generally assumed to be generated in the auditory nerve, wave III in the superior olivary complex and wave V in the lateral lemniscus or the inferior colliculus [22-24]. The latency of peak I and the interpeak latency I-III are known to be under the influence of maturation and growth effects, which hampers their interpretation in diabetic animals. [12] Therefore, in this experiment we only used the interpeak latency III-V as a measure of the function of the rostral pontine and midbrain region.
Stimulation, recording and analysis VEP's: VEP's were evoked with flash stimuli (Mecablitz 40 MZ-3i flashbulb, Metz Werke GmbH, Fürth, Germany; flash duration 70 ms, output per flash 3J) delivered at an upward angle of 90° at 25 cm from the eyes at a frequency of 0.67 Hz. The ears of the animals were occluded. VEP's were amplified, filtered (band pass 1-586 Hz) and stored in a computer. For analysis, 128 traces (sweep length 450 ms) were averaged. Four waves could be identified, which were designated n1, p1, n2 and n3. The latencies of these waves were ~30, 38, 63, 108 ms respectively. These latencies and the general appearance of the VEP in the present study were comparable to VEP's in the rat in previous studies [12;25]. Peak n1 and p1 have been suggested to be generated in the primary and secondary visual cortex, whereas n2 and n3 may be generated in associative cortical areas beyond the classically defined visual cortex [25]. Peak p1 could be identified most reliably in this study and therefor the latency of this peak was used to monitor the effects of diabetes and the effects of treatment on the VEP.

Blood pressure:
At the end of the experiment, rats were anaesthetised by intraperitoneal injection of urethane 10% (1.3 ml/100 gram bodyweight for diabetic animals, 1.4 ml/100 gram for control animals), followed by intubation and mechanical ventilation with O_2/NO_2 (20/80%). Body temperature was continuously measured and kept within the physiological range, using a heating pad. In all rats a femoral artery was cannulated for arterial pressure monitoring. The pressure was monitored for a period of time that was needed to have a stable pressure reading during 5 minutes.

Statistical analysis*:*
Data are presented as mean ± standard error of the mean (SEM). Between group differences in body weight and blood glucose were analysed by one way analysis of variance (ANOVA) with post-hoc Duncan's multiple range

tests. An analysis of variance for repeated measurements (ANOVAR) was used to study between group differences in MNCV, SNCV and BAEP and VEP latencies. First we performed a single ANOVAR comparing the three groups. If this ANOVAR gave a significant p-value ($p<0.05$) pairwise post hoc ANOVAR were performed between the different experimental groups.

Results:

Animals (prevention and intervention)(table 1)

Diabetic animals failed to gain weight in the course of the experiment. They had significantly reduced final body weights, as well as raised levels of blood glucose, compared to control animals (table 1). Enalapril treatment had no effect on bodyweight or glucose level.

In the prevention experiment, three rats in the enalapril treated group died, one during anaesthesia, two following progressive weight loss. In the intervention experiment, before the start of treatment, one control and one diabetic rat died during anaesthesia. After the start of treatment, two enalapril treated and three untreated diabetic rats died, after progressive weight loss or without an apparent cause of death.

In the datasets presented in the graphs and in the statistical analysis only data from animals that completed the experiment were included.

Table 1: Starting bodyweight and blood glucose and bodyweight, blood glucose and mean arterial blood pressure (MAP) at 16/25 weeks of diabetes.

		Week 0		Week 16/25		
	n	bodyweight	blood glucose	bodyweight	blood glucose	MAP
DMu	13	339±2	19.0±0.4	263±12*	25.2±0.8*	111±4
DMp	10	339±3	18.2±0.5	269±17*	26.3±0.9*	78±3*
Con	10	334±5		522±9	5.3±0.1	104±2
DMu	11	345±6	21.1±0.7	300±16*	24.7±1.1*	102±3
DMi	11	342±7	22.2±1.2	286±14*	26.1±1.0*	83±4#
con	9	349±10		559±18	5.2±0.1	

Data are mean ± SEM and were analysed by one way analysis of variance with post-hoc Duncan's multiple range test, *: $p<0.001$ vs. control, #: $p<0.01$ vs control.

Enalapril prevention study

Sciatic nerve conduction velocity (figure 1A,B):

MNCV and SNCV in non-diabetic control rats increased gradually during the first 9 to 12 weeks of the experiment. The increase in MNCV and SNCV in the non-diabetic control rats is commonly seen in longitudinal studies in young adult rats and is related to maturation of the nerve[12]. In untreated diabetic rats, the MNCV and SNCV increased only slightly compared to controls and reached a plateau after 6 weeks of diabetes (DMu vs con: $p<0.001$). Enalapril treatment partially prevented MNCV and SNCV deficits in diabetic animals (DMu vs DMp: $p<0.001$). MNCV and SNCV in enalapril treated animals did not reach control levels (con vs DMp: $p<0.005$).

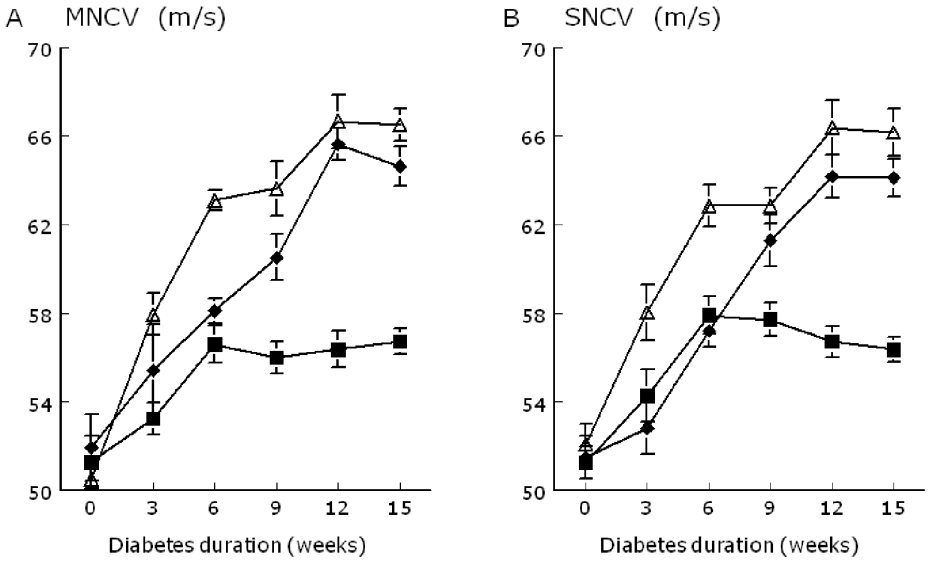

Fig. 1A,B: Enalapril prevention study: motor and sensory nerve conduction velocities (MNCV and SNCV), in control (△; n=10), untreated diabetic (■; n=13) and treated diabetic rats (◆; n=10).
Enalapril treatment prevented deficits in MNCV and SNCV (■ vs ◆:$p<0.001$). In untreated diabetic rats MNCV and SNCV were significantly decreased compared to controls (■ vs △: $p<0.001$). MNCV and SNCV in enalapril treated animals did not reach control levels (△ vs ◆: $p<0.005$).

***Evoked potential latencies*:**

BAEP (Fig 2A):

The minimal sound pressure level to evoke a detectable response was similar in diabetic and control animals and did not change during the course of the study (data not shown). Peak III and V could be identified in all rats. The interpeak latency III-V was relatively stable in control rats (±2.59 ms). The interpeak latency III-V of the untreated diabetic group was increased compared to the control group throughout the experiment (DMu vs con $p<0.001$). Enalapril treatment prevented the increase in interpeak latency III-V (DMu vs DMp $p<0.01$). There was no statistically significant difference between prevention treated diabetic animals and controls.

VEP (Fig 2B):

Peak p1 of the VEP could be identified in all but one untreated diabetic rat. In non-diabetic control animals the latency of peak p1 was relatively stable in time at a latency of 37 ms. The p1 latency in the untreated diabetic group was increased compared to the control group (DMu vs con $p<0.001$). Enalapril treatment partially prevented the increased p1 latencies in

diabetic rats (DMu vs DMp p<0.005). The p1 latency in enalapril treated animals did not reach control levels (DMp vs con p<0.001).
On examination of the eyes, 9 of the 12 untreated rats had no evidence for cataract, 1 rat had unilateral cataract and 2 rats had bilateral cataract. In the enalapril treated diabetic rats prevention group, 4 of the 10 rats had no evidence for cataract, 3 rats had unilateral cataract and 3 rats had bilateral cataract. There was no statistically significant difference in VEP latency in rats with cataract compared to rats without cataract.

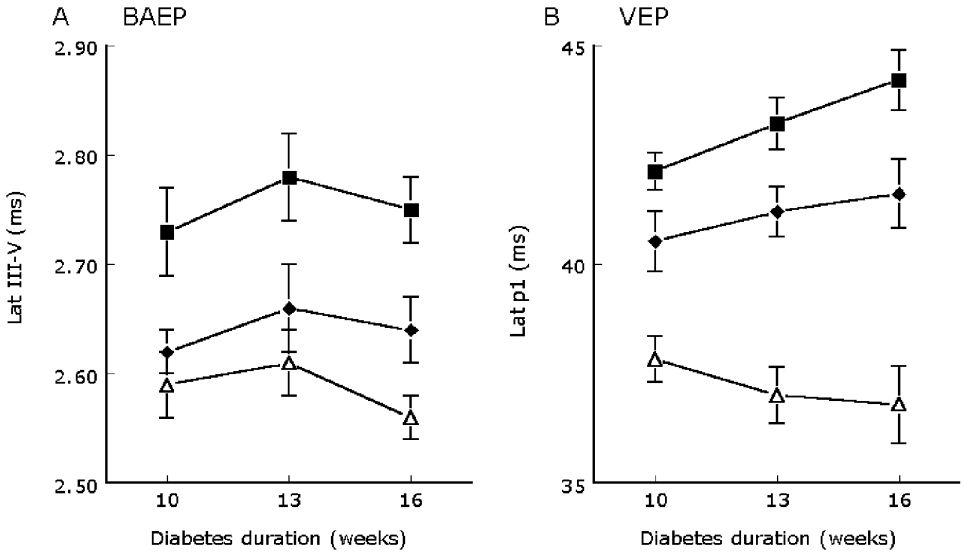

Fig. 2A: Enalapril prevention study: brainstem auditory evoked potential interpeak latencies III-V in control (△; n=10), untreated diabetic (■; n=13) and treated diabetic rats (◆; n=10). Enalapril treatment prevented deficits in BAEP III-V interpeak latencies (■ vs ◆: p<0.01). In untreated diabetic rats interpeak latencies were significantly increased compared to controls (■ vs △: p<0.001). Enalapril treatment improved the interpeak latency III-V towards control levels (◆ vs △ : not significant).
Fig. 2B: Enalapril prevention study: latencies of the p1 wave of the visual evoked potential in control (△; n=10), untreated diabetic (■; n=12) and treated diabetic rats (◆; n=10). Enalapril treatment partially prevented deficits in the p1 latencies (■ vs ◆: p<0.005). In both groups of diabetic rats p1 latencies were significantly increased compared to controls (■ vs △: p<0.001; ◆ vs △: p<0.001).

Blood pressure (table 1):

Mean arterial pressure (MAP) was measured in 10 untreated diabetic rats, 10 prevention treated diabetic rats and 9 control rats. In 3 untreated diabetic rats and 1 control rat no reliable measurements could be obtained due to technical problems such as failed cannulation, and respiratory or cardiac arrest directly following urethane administration. MAP was significantly reduced in prevention treated diabetic rats compared to untreated diabetic rats (DMu vs DMp: p<0.001). There was no statistically significant difference between untreated diabetic rats and control rats (DMu vs con: ns).

Enalapril intervention study

Sciatic nerve conduction velocity (Fig 3A,B):

MNCV and SNCV in non-diabetic control rats and diabetic rats showed the same development as in the first experiment. Intervention treatment started at 15 weeks of diabetes. There was no difference in nerve conduction velocity between the two diabetic groups at that point. There was no significant effect of intervention treatment on nerve conduction velocity in diabetic rats.

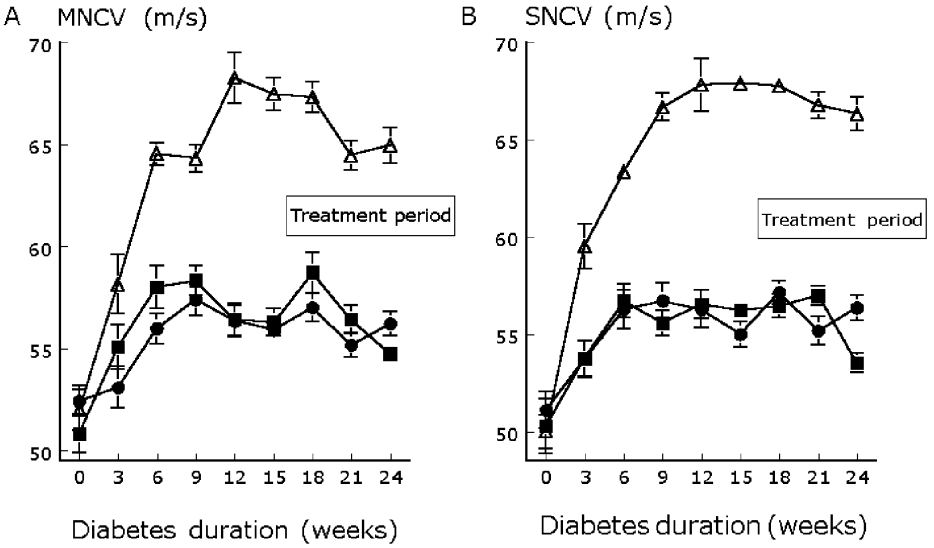

Fig. 3A,B: Enalapril intervention study: motor and sensory nerve conduction velocities (MNCV and SNCV), in control (△; n=9), untreated diabetic (■; n=11) and treated diabetic rats (●; n=11). Intervention treatment started at 15 weeks of diabetes. There was no difference in nerve conduction velocity between the two diabetic groups at that point. There was no significant effect of intervention treatment on nerve conduction velocity in diabetic rats.

Evoked potential latencies:

BAEP (Fig 4A):

Peak III and V could be identified in all rats. The interpeak latency III-V was relatively stable in control rats (±2.60 ms). Before intervention treatment started, there was no statistically significant difference between the two diabetic groups. Intervention treatment significantly improved the interpeak latency III-V in diabetic rats (DMu vs DMi $p<0.05$). Enalapril treatment improved the interpeak latency III-V towards control levels (DMi vs control $p<0.08$).

VEP (Fig 4B):

Peak p1 of the VEP could be identified in all rats. In non-diabetic control animals the latency of peak p1 was relatively stable in time at a latency of ±37 ms. Before intervention treatment started, there was no statistically significant difference between the two diabetic groups. Enalapril treatment led to some improvement of the p1 latency, but this effect was not statistically significant.

In diabetic animals cataracts were observed from week 13 onwards. In week 25, on examination of the eyes, 2 of the 11 untreated rats had no evidence for cataract, 1 rat had unilateral cataract and 8 rats had bilateral cataract. In the enalapril intervention group, 4 of the 11 rats had no evidence for cataract, 2 rats had unilateral cataract and 5 rats had bilateral cataract. There was no statistically significant difference in VEP latency in rats with cataract compared to rats without cataract.

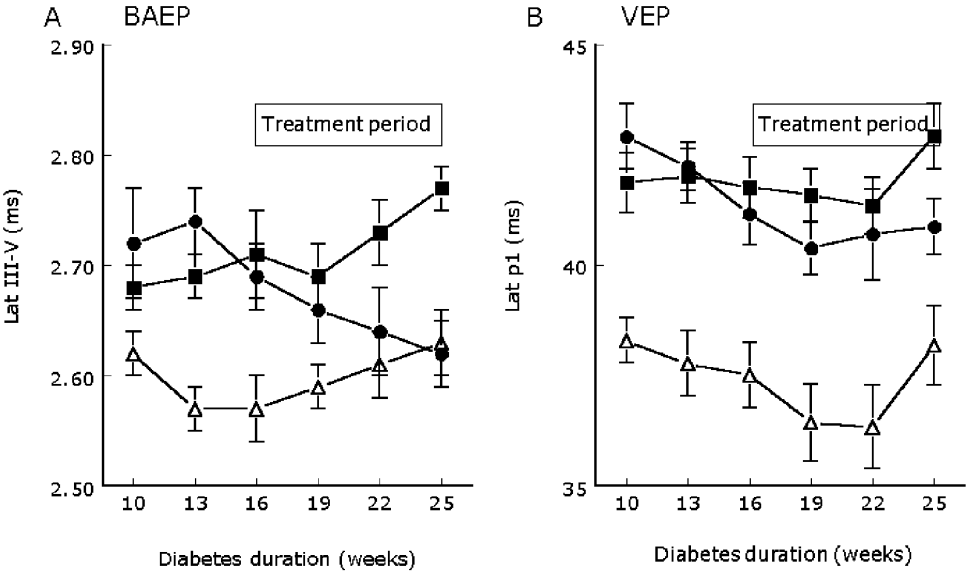

Fig. 5A: Enalapril intervention study: brainstem auditory evoked potential latencies in control (△; n=9), untreated diabetic (■; n=11) and treated diabetic rats (●; n=11). Enalapril treatment significantly improved BAEP III-V interpeak latencies (■ vs ●: $p<0.05$). In untreated diabetic rats interpeak latencies were significantly increased compared to controls (■ vs △: $p<0.001$). Enalapril treatment improved the interpeak latency III-V towards control levels (● vs △ $p<0.08$).

Fig. 5b: Enalapril intervention study: visual evoked potential latencies in control (△; n=9), untreated diabetic (■; n=11) and treated diabetic rats (●; n=11). Enalapril treatment did not significantly improve p1 latencies (■ vs ●: not significant). In both groups of diabetic rats p1 latencies were significantly increased compared to controls (■ vs △: $p<0.001$; △ vs ●: $p<0.001$).

Blood pressure (table 1):

MAP was measured in 9 untreated diabetic rats and 10 intervention treated diabetic rats. In 2 untreated and 1 intervention treated diabetic rat no reliable measurements could be obtained due to technical problems such as failed cannulation, and respiratory or cardiac arrest directly following urethane administration. There was a statistically significant difference between the MAP of untreated diabetic rats (MAP: 102±3) and intervention treated rats (MAP: 83±4) (DMu vs. DMi: $p<0.01$). Compared to the control rats in the diabetic prevention experiment, there was no statistically significant difference between the MAP of untreated diabetic rats and control rats.

Discussion:

In diabetic rats a significant deficit in nerve conduction velocity and evoked potential latencies was observed. Enalapril prevention treatment partially prevented these deficits. Enalapril intervention treatment had no effect on nerve conduction velocity and on visual evoked potential latencies, but improved brain stem auditory evoked potential latencies after 10 weeks of treatment.

In this experiment, the time course of the development of impairments in sciatic nerve conduction velocity in STZ-diabetic rats is compatible with previous studies [12;26]. The finding that MNCV and SNCV in diabetic rats only increased marginally throughout the experiment does not just reflect a disturbed maturation: if diabetes is induced in full-grown rats, deficits in MNCV and SNCV develop like in young adult rats, although the rate of development appears to be slightly slower [27;28]. The increased interpeak latency III-V in the BAEP and VEP p1 latency found in diabetic rats is in agreement with earlier studies [12;29;30]. In line with previous observations, the latencies of the flash evoked VEP were not affected by cataract formation in diabetic rats [12].

Enalapril significantly prevented the deficit in nerve conduction velocity in diabetic rats. Earlier prevention studies in this model also demonstrated a beneficial effect of an ACE-inhibitor on nerve conduction velocity [18 31] and nerve blood flow [18]. However, in contrast to previous intervention studies in STZ-diabetic rats [32-34], enalapril intervention did not improve nerve conduction velocity. This could be related to the timing of intervention. In previous studies, lisinopril [32;33] or cilazapril [34] were given after only 5 weeks [33], 6 weeks [32] or 1 month [34] of untreated STZ-diabetes. The apparent differential effects of early and late intervention may be related to the nature of the nerve conduction velocity deficits. Early deficits in nerve conduction velocity have been attributed to metabolic changes in the nerve that are mostly rapidly reversible [35]. After two to three months of experimental diabetes structural changes develop that may be more difficult to reverse [35 12;36]. Still, studies with insulin treatment, through subcutaneous implants or islet cell grafts, show that deficits in nerve conduction velocity are potentially reversible up to 6 months after diabetes induction [12;37]. In these latter studies, treatment led to a complete reversal of the diabetic state. Treatment with enalapril alone, without correction of blood glucose and insulin deficiency, is apparently insufficient to reverse late deficits in nerve conduction velocity. This is in line with observations on other pharmacological compounds, where the effects of intervention therapy were limited compared to prevention therapy [38;39].

Enalapril partially prevented deficits in BAEP and VEP latencies, but intervention treatment significantly improved BAEP latencies only. The potential reversibility of increased evoked potential latencies in experimental diabetes has thus far been addressed in a limited number of studies. In one study pancreatic islet transplantation, performed at a diabetes duration of 4 months, did not improve BAEP, VEP and

somatosensory evoked potential latencies at 8 months of diabetes [37]. In another study, insulin treatment through subcutaneous implants, initiated after 6 months of diabetes, led to a partial restoration of both BAEP and VEP latencies [12]. As these previous studies showed equivocal results and the mechanisms underlying increases in evoked potential latencies in STZ-diabetes are still largely unknown it is difficult to explain the apparent dissimilar effect of enalapril on BAEP and VEP latencies in the intervention study.

Studies into the mechanism of action of ACE-inhibitors in experimental diabetic neuropathy have identified improvement of nerve blood flow as a key factor [32;40]. Angiotensin II (Ang II) is a powerful vasoconstrictor. ACE-inhibitors block the conversion of (the biological inactive) Ang I to Ang II and reduce the degradation of the vasodilator bradykinin, which in turn affects endothelial nitric oxide synthetase [41]. In diabetes, the former mechanism appears to be most important, as the beneficial effect of ACE-inhibition on nerve conduction velocity and nerve blood flow can be mimicked by angiotensin AT_1 receptor antagonists [32;40]. In diabetic rats circulating ACE-levels are elevated [42;43], which may lead to an increased synthesis of Ang II{988}. We have recently shown that treatment with enalapril partially prevents deficits in cerebral blood flow, spatial learning and synaptic plasticity in STZ-diabetic rats [9], suggesting that the improvement of central neurological deficits may also be mediated through vascular effects. In this previous study, improvement of cerebral blood flow was associated with a reduction of mean arterial blood pressure comparable to the present findings. Apparently, the enalapril-induced reduction of blood pressure does not impede the blood supply to the brain. Ang II receptors and ACE are known to be present in several brain areas, and play a role in physiological functions such as drinking, sodium appetite, and pituitary releasing actions [44]. This could provide an additional mode of action for enalapril in our model. Peripheral angiotensinogen [45] and angiotensins [46;47] do not penetrate the blood-brain-barrier, thus precluding direct effects of systemic ACE inhibition on cerebral Ang II receptors. Still, oral administration of 10 mg enalapril/kg/day has previously been shown to reduce ACE-activity in the cerebral cortex in rats [48].

In conclusion, experimental diabetes is associated with deficits in peripheral nerve conduction velocity and increased evoked potential latencies. Treatment aimed at the vasculature can partially prevent or reverse these impairments, indicating that angiopathy could play a role in the development of peripheral and cerebral dysfunction in diabetic rats. ACE-inhibitors are a promising lead in the treatment of peripheral and central neurological complications of diabetes.

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Long-term angiotensin converting enzyme inhibition dose-dependently improves nerve conduction velocity and evoked potential latencies in streptozotocin diabetic rats

4

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Introduction

Diabetes is associated with slowly progressive deficits in the central nervous system, a condition that may be referred to as diabetic encephalopathy. The pathogenesis of cerebral dysfunction in diabetes probably is a multifactorial process [1], in which vascular disease appears to play an important role, analogous to the pathogenesis of peripheral diabetic complications [2;3]. We therefore hypothesised that treatment aimed at the vasculature might protect the brain against some of the adverse effects of diabetes. In previous studies we have shown that treatment with the ACE-inhibitor enalapril indeed prevented both reductions in cerebral blood flow and deficits in peripheral nerve conduction, evoked potential latencies, cognition and hippocampal synaptic plasticity in streptozotocin (STZ-) diabetic rats, up to 16 weeks after induction of diabetes. However, these functional improvements were associated with a marked reduction (25 %) of systemic mean arterial blood pressure [4;5].

The aim of the present study was to examine whether the effect of the ACE-inhibitor on functional outcome could be maintained over a longer period of time and if similar functional effects could be achieved at a lower dose, without an accompanying reduction of blood pressure.

Materials and Methods

Animals

Male Wistar rats (starting weight ~340g, approximately 10 weeks of age, UWU-CPD, Harlan, Utrecht, The Netherlands) were housed on sawdust, maintained on a 12h-12h light-dark cycle. Rats were given food and water *ad libitum* and were weighed weekly. Diabetes was induced by a single intravenous injection of streptozotocin (STZ) (Serva Feinbiochemica GMBH, Heidelberg, Germany), dissolved in saline, at a dose of 33 mg/kg body weight. Four days after the STZ injection, blood glucose was determined in blood samples, obtained by tail prick, by a strip-operated blood glucose sensor (Companion2, Medisense Ltd, Birmingham, United Kingdom). Blood glucose levels were >15.0 mmol/L in all STZ-injected animals. All experiments were conducted to the guidelines of the Utrecht University Committee for welfare of experimental animals.

Drugs

Enalapril was added to the drinking water at a concentration adapted to obtain a daily intake of approximately 12 or 24 mg/kg. The 24 mg/kg dose was used in our previous studies [4;5], and was based on earlier studies, on the effect of lisinopril on experimental peripheral diabetic neuropathy in the STZ-diabetic rat [6] and the effect of enalapril treatment on brain oedema in stroke-prone spontaneously hypertensive rats [7]. The 12 mg/kg dose was chosen to see if a lower dose could give the same improvements in peripheral and central nerve conduction velocity, without associated hypotension.

Experimental design:
To examine the long term effect of enalapril treatment on neurophysiological changes in the peripheral and central nervous system 4 groups of rats were used: a non-diabetic control group (con; n=10), an untreated diabetic group (DMu; n=12), an enalapril 12 mg/kg treated group (DM+12; n=14) and an enalapril 24 mg/kg treated group (DM+24; n=13). Enalapril treatment was initiated directly after confirmation of diabetes and continued throughout the experiment. Motor nerve conduction velocity (MNCV) and sensory nerve conduction velocity (SNCV) in the sciatic nerve were used to monitor the effects on the peripheral nervous system. Brainstem auditory evoked potentials (BAEP) and visual evoked potentials (VEP) were used to monitor the effects on the central nervous system. MNCV and SNCV were measured every 3 weeks for a period of 24 weeks, starting 3 weeks after diabetes induction. BAEP and VEP were measured at 10 weeks of diabetes duration and again every 3 weeks until 25 weeks of diabetes duration. We started the measurement of the evoked potentials at 10 weeks of diabetes duration, because previous studies show that the diabetic effect on evoked potentials is noticeable after 2 to 3 months of diabetes [8]. The investigator who performed the neurophysiological measurements was blinded to the treatment of the animals.

Sciatic nerve conduction velocity:
MNCV and SNCV were measured in the sciatic nerve according to the method described by De Koning & Gispen [9]. In short, the sciatic and tibial nerves were stimulated at the sciatic notch and ankle, respectively. The latencies of the responses of the musculature of the foot were measured. The MNCV and SNCV were calculated by dividing the distance between the two stimulation points by the differences in latencies after proximal and distal stimulation.

Evoked potentials:
Placement of recording electrodes: This procedure was performed as described before [4]. In short, after nine weeks of diabetes one stainless steel screw was implanted permanently into the skull (co-ordinates A-3.0, L-3.0; reference point bregma) [10]. The skin was closed over the screw, which served as a permanent reference point for placement of recording electrodes during the course of the experiment. All animals were allowed to recover for one week. For measurement of the evoked potential latencies, rats were slightly sedated with a low dose of diluted (with 0.9% NaCl; 1:2) Hypnorm® (0.1 ml/100 g subcutaneously), in order to prevent them from moving. EPs were recorded with subcutaneous stainless steel needle electrodes, insulated except for the tip, which was placed over the left occipital cortex (co-ordinates) A-8.0 L2.0. The reference electrode was placed subcutaneously on the nose of the animal. An earth electrode was connected to the front paw.
Stimulation, recording and analysis of BAEP: The stimulation protocol for BAEP and VEP was performed according to the method described by

Biessels [8]. In short, the rat was placed in a soundproof, darkened room. Acoustic stimuli were presented as clicks (unfiltered square waves of 100 µs duration with constant polarity, applied at a frequency of 10 Hz, at 60 dB above threshold level). For analysis, 512 traces (sweep length 40 ms) were averaged. The latencies of waves I, III and V were determined.
Stimulation, recording and analysis of VEP's: VEP's were evoked with flash stimuli (Mecablitz 40 MZ-3i flashbulb, Metz Werke GmbH, Fürth, Germany; flash duration 70 ms, output per flash 3J) delivered at an upward angle of 90° at 25 cm from the eyes at a frequency of 0.67 Hz. The ears of the animals were occluded. For analysis, 128 traces (sweep length 450 ms) were averaged. Four waves could be identified, which were designated n1, p1, n2 and n3. Peak p1 can be identified most reliably and, therefore, the latency of this peak was used to monitor the effects of diabetes and the effects of treatment on the VEP.

Metabolic analysis/ Blood pressure:
Because there was a high mortality in the DM+24 group, we decided to perform renal and metabolic function tests in the surviving animals, to determine what might have caused this high mortality. We performed these tests in the DMu, DM+24 and non-diabetic control groups. The rats were weighed and placed in metabolic cages for 24h, with free access to food and water. Urine was collected for determination of urinary creatinine excretion. Plasma and urinary creatinine levels were determined colorimetrically (Sigma Diagnostics Inc., St Louis, MO). After urine collection, the blood pressure measurements took place, for which rats were anaesthetised by intraperitoneal injection of urethane 10% (1.3 ml/100 gram bodyweight for diabetic animals, 1.4 ml/100 gram for control animals), followed by intubation and mechanical ventilation with O_2/NO_2 (20/80%). Body temperature was continuously measured and kept within the physiological range, using a heating pad. In all rats a femoral artery was cannulated for arterial pressure monitoring. The pressure was monitored for a period of time that was needed to have a stable pressure reading during 5 minutes. This was followed by collection of blood from the vena cava for determination of plasma creatinine and lipids. Plasma cholesterol and triglycerides were determined enzymatically (Roche diagnostics GmbH, Mannheim, Germany).

Statistical analysis*:*
Data are presented as mean ± standard error of the mean (SEM). Between group differences in body weight, blood glucose and blood pressure were analysed by one way analysis of variance (ANOVA) with post-hoc Duncan's multiple range tests. An analysis of variance for repeated measurements (ANOVAR) was used to study differences between the groups on MNCV, SNCV and BAEP and VEP latencies. The difference at individual timepoints was analysed by one-way analysis of variance (ANOVA) and Duncan's multiple range test.

Results

Animals (table 1)

Diabetic animals failed to gain weight in the course of the experiment. They had significantly reduced final body weights as well as raised levels of blood glucose compared to control animals. Enalapril treatment had no effect on body weight or blood glucose. In the DM+12 group, two rats died during the course of the experiment, in week 19 and 25, both following progressive weight loss. In the DM+24 group six rats died during the course of the experiment, either after progressive weight loss, or without an apparent cause of death. In general, the animals that died appeared to have the lowest bodyweight among their group. The rats died in week 9, 14, 15, 21 (two rats) and 23. Because of these deaths we performed two statistical analyses, one analysis only includes data from the animals that were alive at the end of the experiment, the second analysis includes the data from all the animals that were alive at 10 weeks of diabetes duration (the start of the evoked potential measurements).

Table 1: Starting body weight and blood glucose and body weight, blood glucose and mean arterial blood pressure (MAP) at 25 weeks of diabetes duration.

		Week 0			Week 25		
	n	Bodyweight (gr)	Blood glucose (mmol/L)	n	Bodyweight (gr)	Bloodglucose (mmol/L)	MAP (mm Hg)
Con	10	350 ± 4	6.3 ± 0.3	10	557 ± 20	5.1 ± 0.1	114 ± 8
DMu	12	343 ± 2	18.0 ± 0.9*	12	250 ± 11*	22.0 ± 0.9*	122 ± 5
DM+12	14	347 ± 3	18.7 ± 0.9*	12	263 ± 15*	24.8 ± 1.1*	112 ± 4
DM+24	13	339 ± 3	17.6 ± 0.3*	7	270 ± 24*	25.0 ± 0.7*	75 ± 4*

Data are mean ± SEM and were analysed by one-way analysis of variance with *post hoc* Duncan's multiple range test. *p<0.05 vs control

Sciatic nerve conduction velocity (figures 1a.1, 1b.1 and 1a.2, 1b.2)

MNCV and SNCV in non-diabetic control rats increased gradually during the first 9 to 12 weeks of the experiment. The increase in MNCV and SNCV in non-diabetic control rats is commonly seen in longitudinal studies in young adult rats and is related to maturation of the nerve [8]. In untreated diabetic rats, the MNCV and SNCV increased only slightly compared to controls and reached a plateau after 6 weeks of diabetes duration (DMU vs con: $p<0.001$). In the DM+12 group no effect of enalapril was seen on MNCV and SNCV (DMu vs DM+12: ns). Enalapril 24 mg/kg partially prevented MNCV and SNCV deficits in diabetic animals up to 21 weeks of diabetes (DMu vs DM+24: $p<0.001$). MNCV and SNCV in DM+24 did not reach control levels (DM+24 vs con: $p<0.05$). At 24 weeks of diabetes duration, the beneficial effect of enalapril treatment on MNCV and SNCV had disappeared in the DM+24 group, and reached DMu levels. The second analysis, in which all animals alive at 10 weeks of diabetes were included, provided similar results.

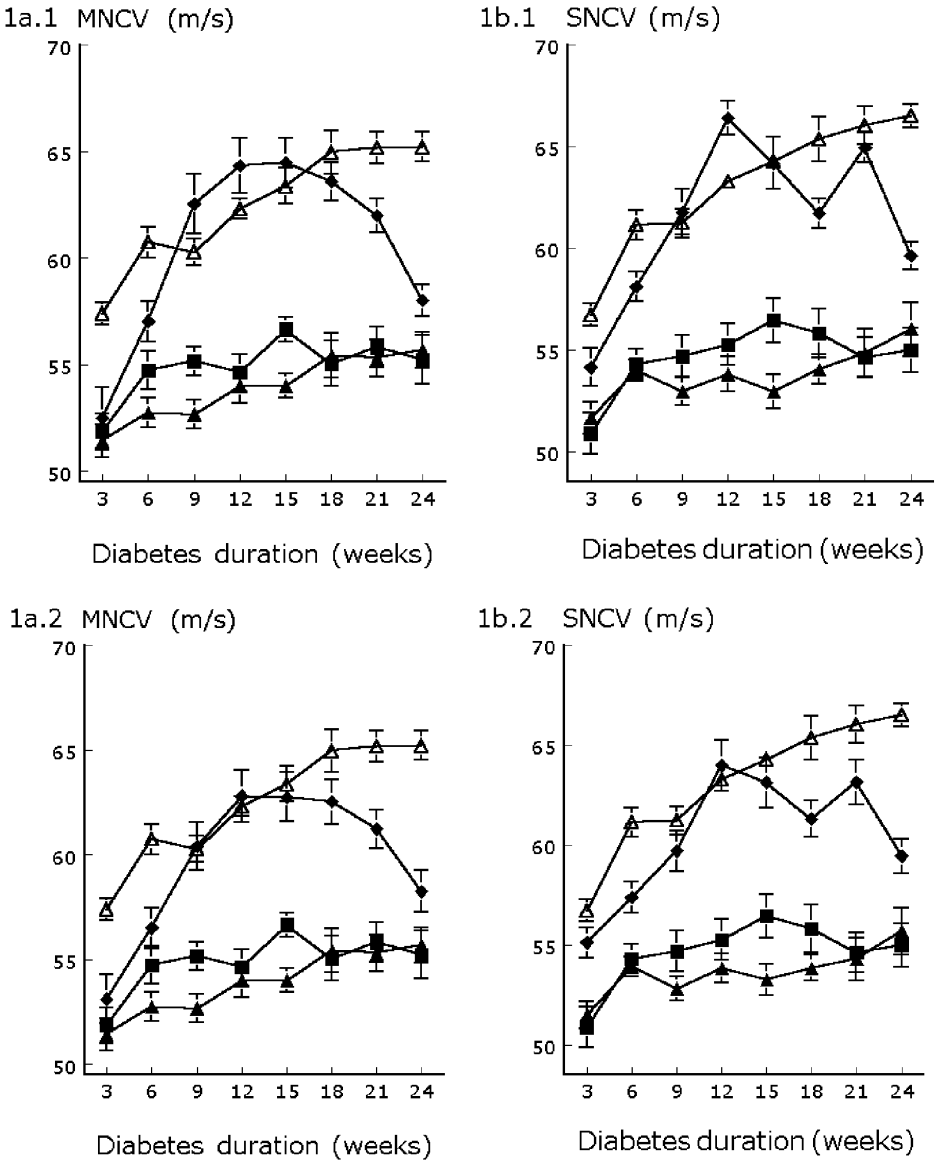

Figure 1a.1 and 1b.1: Motor and sensory nerve conduction velocities (MNCV and SNCV), in control (△, N=10), untreated diabetic (■, n=12), diabetic treated with 12 mg/kg enalapril (▲, n=12) and diabetic treated with 24 mg/kg enalapril (◆, n=7). Enalapril 24 mg/kg treatment prevented deficits in MNCV and SNCV (■ vs ◆: $p<0.001$), MNCV and SNCV did not reach control levels (△ vs ◆: $p<0.05$). There was no significant effect of enalapril 12 mg/kg treatment on nerve conduction velocity in diabetic rats. Only data from animals that were alive at the end of the experiment were included in this analysis.
Figure 1a.2 and 1b.2: Animals that died after week 10 are also included, using a last observation carried forward approach. △, N=10; ■, n=12; ▲, n=14; ◆, n=12. ■ vs ◆: $p<0.001$; △ vs ◆: $p<0.01$

Evoked potential latencies (figure 2A,B)

BAEP (figure 2a): The minimal sound pressure level to evoke a detectable response was similar in diabetic and control animals and did not change during the course of the experiment (data not shown). Peak III and V could be identified in all but 2 rats from the 12 mg/kg group and 1 rat from the 24 mg/kg group, because no usable traces could be acquired. The interpeak latency III-V was relatively stable in time in control rats (±2.59 ms). The interpeak latency III-V of the untreated diabetic group was increased compared to the control group throughout the experiment (DMu vs con: $p<0.001$). Enalapril 12 mg/kg partially prevented the increase in interpeak latency III-V (DM+12 vs DMu: $p<0.05$), but not towards control levels (DM+12 vs con: $p< 0.001$). Enalapril 24 mg/kg prevented the increase in interpeak latency III-V (DM+24 vs DMu: $p< 0.01$), and improved it towards control levels (DM+24 vs con: ns). The second analysis, in which all animals alive at 10 weeks of diabetes were included, provided similar results (figure 2b).

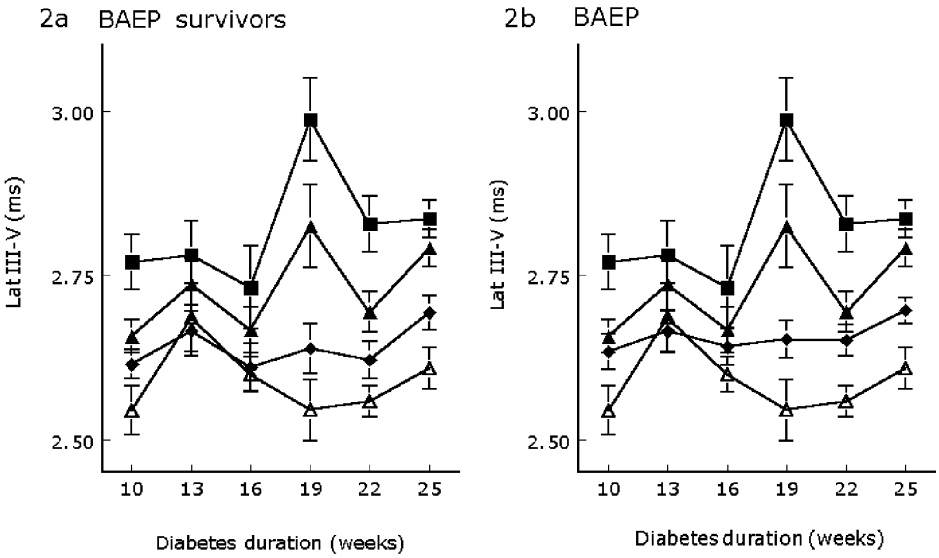

Figure 2a: Brain stem auditory evoked potentials (BAEP), in control (△, N=10), untreated diabetic (■, n=12), diabetic treated with 12 mg/kg enalapril (▲, n=12) and diabetic treated with 24 mg/kg enalapril (◆, n=7). Peak III and V could be reliably identified in all but 2 rats from the 12 mg/kg group and 1 rat from the 24 mg/kg group. Enalapril 24 mg/kg treatment prevented deficits in BAEP peak III-V interpeak latencies (■ vs ◆: $p<0.01$), and improved the interpeak latency towards control levels (△ vs ◆: not significant). Enalapril 12 mg/kg treatment prevented deficits in BAEP peak III-V interpeak latencies (■ vs ▲: $p<0.05$), but not towards control levels (△ vs ▲: $p<0.001$). Only data from animals that were alive at the end of the experiment were included in this analysis.

Figure 2b: Animals that died after week 10 are also included, using a last observation carried forward approach. △, N=10; ■, n=12; ▲, n=12; ◆, n=11. ■ vs ◆: $p<0.001$; △ vs ◆: $p<0.05$; ■ vs ▲: $p<0.05$; △ vs ▲: $p<0.001$.

VEP (figure 3a): Peak p1 of the VEP could be identified in all but 1 untreated diabetic rat, 2 DM+12 mg/kg rats and 2 DM+24 mg/kg rats. In non-diabetic control animals, the latency of peak p1 was relatively stable in time at a latency of 37 ms. The p1 latency in the untreated diabetic group was increased compared to the control group throughout the experiment (DMu vs con: $p<0.001$). Enalapril 12 mg/kg showed a trend towards improvement, but this was not statistically significant (DM+12 vs DMu: 0.3). Enalapril 24 mg/kg partially prevented the increased p1 latencies in diabetic rats (DM+24 vs DMu: $p< 0.05$). The p1 latency in DM+24 did not reach control levels (DM+24 vs con: $p<0.01$). After 24 weeks of diabetes duration, the beneficial effect of enalapril treatment on VEP had disappeared in the DM+24 group, and reached DMu levels. The second analysis, in which all animals alive at 10 weeks of diabetes were included, provided similar results (figure 3b).
On examination of the eyes, in week 25 of the experiment, 7 of the 11 untreated rats had bilateral cataract, 2 unilateral cataract and 2 no evidence for cataract. In the DM+12 group, 11 of the remaining 12 treated rats had bilateral cataract and one had unilateral cataract. In the DM+24 group, 1 of the 6 remaining treated rats had bilateral cataract, 2 had unilateral cataract and 3 had no evidence for cataract. In this study and previous studies we did not find an effect of cataract on VEP latencies [4].

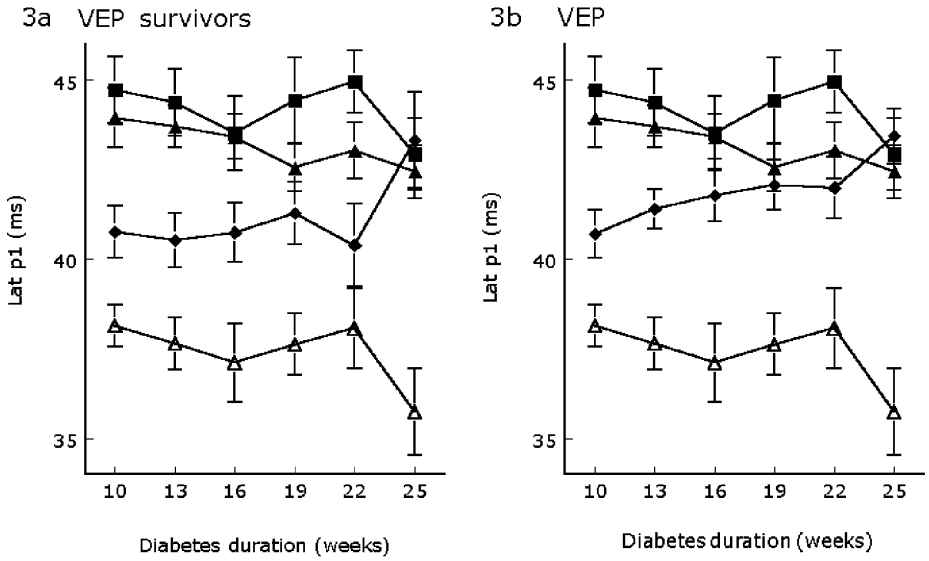

Figure 3a: Visual auditory evoked potentials (VEP), in control (△, N=10), untreated diabetic (■, n=11), diabetic treated with 12 mg/kg enalapril (▲, n=12) and diabetic treated with 24 mg/kg enalapril (◆, n=6). Peak p1 of the VEP could be identified in all but 1 untreated diabetic rat, 2 DM+12 mg/kg rats and 2 DM+24 mg/kg rats. Enalapril 24 mg/kg treatment partially prevented deficits in the p1 latencies (■ vs ◆: $p<0.05$), but not towards control levels (△ vs ◆: $p<0.01$). Enalapril 12 mg/kg treatment did not significantly prevented deficits in the p1 latencies (■ vs ▲: not significant). Only data from animals that were alive at the end of the experiment were included in this analysis.

Figure 3b: Animals that died after week 10 are also included, using a last observation carried forward approach. △, N=10; ■, n=11; ▲, n=12; ◆, n=11. ■ vs ◆: $p<0.05$; △ vs ◆: $p<0.001$; ■ vs ▲: not significant.

Renal and metabolic function tests and blood pressure (table 1,2)

Urinary glucose was significantly higher in diabetic animals compared to controls (table 2). There was no significant difference in plasma creatinine levels between diabetic animals and control animals, although there was a trend towards higher plasma creatinine levels in DMu vs controls. Creatinine clearance was significantly lower in DMu compared to controls (table 2). There was no difference in creatinine clearance between DM+24 compared to controls (table 2). No difference in plasma cholesterol was observed among the different groups but DMu rats had significantly higher plasma triglycerides compared to controls. There was no significant difference between plasma triglycerides between DM+24 and controls.

Mean arterial blood pressure (MAP) was measured in 11 untreated diabetic rats, 10 DM+12 rats, 7 DM+24 rats and 9 control rats. In 1 untreated diabetic rat, 2 DM+12 rats and 1 control rats, no reliable measurements could be obtained owing to technical problems such as failed cannulation, and respiratory or cardiac arrest directly following urethane administration. MAP was significantly reduced in DM+24 rats compared to untreated diabetic rats (DM+24 vs DMu: $p<0.001$) (table 1). There was no statistically significant difference between DMu, DM+12 rats and control rats (DMu vs con: ns, DM+12 vs con: ns).

Table 2: Urinary glucose, plasma creatinine levels, Creatinine clearance and plasma lipids at 25 weeks of diabetes duration.

	n	Urinary glucose (mmol/day)	Plasma creatinine levels (umol/L)	Creatinine clearance (ml/min)	Plasma cholesterol (mmol/L)	Plasma triglycerides (mmol/L)
Con	7	2.7 ± 0.4	63 ± 2	2.9 ± 0.2	1.7 ± 0.2	0.8 ± 0.1
DMu	6	416 ± 15*	90 ± 21	1.0 ± 0.2#	2.7 ± 0.3	2.6 ± 0.4*
DM+24	7	401 ± 18*	62 ± 14	2.7 ± 0.6	2.4 ± 0.3	2.1 ± 0.5

Data are mean ± SEM and were analyzed by one way analysis of variance with *post hoc* Duncan's multiple range test. *$p<0.05$ vs control; #$p<0.05$ vs control and DM+24

Discussion

In diabetic rats a significant deficit in nerve conduction velocity and evoked potential latencies was observed. Enalapril 12 mg/kg did not prevent nerve conduction velocity deficits, but partially prevented deficits in BAEP latencies, and showed a trend towards prevention of deficits in VEP latencies. Enalapril 24 mg/kg prevented deficits in nerve conduction velocity, and partially prevented deficits in BAEP and VEP latencies. However, in the enalapril 24 mg/kg group, mortality was high compared to the other groups, and the beneficial effect of enalapril on nerve conduction

velocity and VEP latencies disappeared at 24 weeks after diabetes induction. Furthermore, in the enalapril 24 mg/kg group, the rats had a marked reduction in blood pressure, which did not occur at 12 mg/kg.
Angiotensin II is a powerful vasoconstrictor. ACE-inhibitors are vasoactive drugs blocking the conversion of (the biological inactive) angiotensin I to angiotensin II. ACE-inhibitors are well known for their beneficial effect on cardiovascular disease in diabetic patients [11] and we previously showed improvement of peripheral nerve conduction velocity, evoked potential latencies, learning, hippocampal synaptic plasticity and cerebral blood flow in diabetic rats [4;5]. ACE-inhibitors were also shown to improve peripheral neuropathy in diabetic patients [12]. The beneficial effects of ACE-inhibition on peripheral diabetic neuropathy are considered to depend on improvement of nerve blood flow [6]. Likewise, the improvement of diabetic encephalopathy in STZ-diabetic rats by enalapril could well be mediated through the vasculature, as reflected in an increase in cerebral blood flow [5]. This study confirms our previous findings on peripheral neuropathy and evoked potential latencies in STZ-diabetic rats, but, unfortunately, the beneficial effect of 24 mg/kg enalapril in long term treatment could not be maintained. Furthermore, in the 12 mg/kg group, we showed that there is no effect on blood pressure, but also there is no beneficial effect on nerve conduction velocity and only a trend towards improvement of the VEP.
The mortality among the 24 mg/kg diabetic rats described in this study was an unexpected finding, which was not anticipated. ACE inhibitors are widely used in clinical practice, and numerous large randomised clinical trials with ACE-inhibitors, including enalapril, show that ACE inhibition decreases, rather than increases mortality [13-15]. When comparing the casualties with the survivors in the 12 and 24 mg/kg group, nerve conduction velocities and evoked potential latencies prior to their death were similar. Laboratory tests even showed an improvement of renal function by enalapril. Hence, apart from the marked hypotension, we could detect no other adverse effects of enalapril that could account for the increased mortality. In the STZ-diabetic model, animals are polyphagic, polyuric and polydipsic, and tend to loose 10-20% of their initial weight in the months after STZ-injection. The combination of cachexia and the high fluid turnover may make them extra vulnerable to the effects of hypotension due to high-dose enalapril, particularly after prolonged diabetes, leading in 6 cases to sudden death. This suggestion is supported by the observation that the casualty rate was highest among the animals with the lowest bodyweights. The finding that the beneficial effects of enalapril on nerve conduction and evoked potential latencies could not be sustained may also be due to the severe and prolonged hyperglycaemia. Of course this raises questions on the usefulness and validity of this model, and the significance of the treatment results. In the present and previous studies we have clearly shown that untreated STZ-diabetic rats develop features of cerebral dysfunction that can also be encountered in patients, such as impaired cognition and increased evoked potential latencies [1]. The fact that

impairments of cerebral function are also reported in other, mostly genetic, models of type 1 and type 2 diabetes, in which glucose levels are usually less elevated [1;16], supports the notion that the impairments that are observed in the STZ-model do not just reflect the severity of the diabetic state, or STZ toxicity. The model can therefore make a contribution to our understanding of the effects of (severe) hyperglycaemia on the brain, despite its obvious limitations. It should also be noted that the experiments with high dose enalapril treatment were not primarily initiated to develop a therapy that could be readily applied in clinical practice. The main aim of our previous and present experiments was to provide "proof of principle" that a therapy directed at the vasculature can prevent impairments of cerebral dysfunction in experimental diabetes. The fact that we now have repeatedly shown that enalapril prevents cerebral dysfunction and reductions in cerebral blood flow in STZ-diabetic rats, despite sustained hyperglycaemia, clearly emphasises the importance of the role of the vasculature in the pathogenesis of cerebral complications of diabetes.

In conclusion, enalapril treatment prevents deficits in peripheral nerve conduction an evoked potential latencies in STZ-diabetic rats, thus providing provide proof of principle that a therapy directed at the vasculature can prevent impairments of cerebral dysfunction in experimental diabetes. The increased mortality in the enalapril treated animals clearly shows the limitations of the STZ-diabetes model, particularly in long-term studies.

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Brain MRI correlates of impaired cognition in patients with type 2 diabetes mellitus

5

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On behalf of the Utrecht Diabetic Encephalopathy Study Group*

Diabetes, in press

Introduction

Type 2 diabetes mellitus (DM2) can affect the central nervous system [1]. Neuropsychological studies reported moderate degrees of cognitive impairment, particularly in tasks involving verbal memory or complex information processing [2;3]. Epidemiological studies demonstrated an association between diabetes and dementia [4;5]. It is not clear which factors mediate accelerated cognitive decline in patients with DM2. Both co-morbid conditions (e.g. hypertension and depression) and diabetes-specific factors (e.g. glycemic control) have been implicated [2;6]. Some investigators have suggested that hypertension is an important mediator [2;7;8], but this was not supported by others [9;10].

The structural correlates and pathophysiological mechanisms underlying these cognitive impairments are still uncertain. Previous studies report that modest cortical and subcortical atrophy and symptomatic or asymptomatic infarcts are more common in DM2 patients than in controls [11-15]. Findings from studies on so-called white matter lesions are less consistent, some reporting an association with DM2 [16], whereas others report no statistically significant effects [11;17]. To the best of our knowledge, there are no published studies that combine detailed assessment of cognitive functioning and magnetic resonance imaging of the brain MRI in patients with DM2.

The present study aimed to compare cognition and brain MRI in DM2 patients to non-diabetic controls, and to relate cognitive functioning in the DM2 patients to MRI findings, and to blood pressure and metabolic control.

Research design and methods

Patients

The Utrecht Diabetic Encephalopathy Study (UDES) is a cross-sectional, population based study on determinants of impaired cognition in DM2. Because the study aimed to identify potential risk factors for cognitive impairment in DM2, patients were not selected for the presence or absence of diabetic complications, co morbid conditions (e.g. hypertension), or exposure to other risk factors (e.g. smoking). For inclusion DM2 patients had to be 55 to 80 years of age, with a minimal diabetes duration of 1 year, and had to be functionally independent, and Dutch speaking. Exclusion criteria for all participants were a psychiatric or neurological disorder (unrelated to diabetes) that could influence cognitive functioning, a history of alcohol or substance abuse and dementia, and a fasting blood glucose ≥ 7.0 for controls [18]. Subjects with a history of non-invalidating stroke could be included. Twice as many patients as controls were included, to increase statistical power for within group analyses in the DM2 group.

In the UDES 122 patients with DM2 (age 56-80 yrs), 40 patients with DM1 (52-77 yrs), and 61 controls (53-78 yrs) were included between September 2002 and November 2004. General practitioners in the area were asked to participate in the project (see acknowledgements), and to

invite all eligible DM2 patients from their practice. Controls were recruited among the spouses or acquaintances of the patients. The study was approved by the medical ethics committee of the University Medical Center Utrecht and each participant signed an informed consent form. Participants attended the clinic on two consecutive days and underwent MRI of the brain, and neuropsychological and neurological examinations. Medical history and medication use was recorded. Fasting blood samples were collected, blood pressure was recorded and urine was collected overnight. The study protocol for the DM1 patients was slightly different, and will be reported separately.
In 9 patients with DM2 no MRI could be obtained, due to MRI contraindications, such as claustrophobia or a pacemaker. The present study includes all DM2 patients with an MRI (n=113, age 56-80 yrs), and all control subjects with an MRI that were at least 56 years of age (n=51, 57-78 yrs).

MRI scanning protocol

The MRI investigation (1.5 Tesla, Philips Medical systems, Best, the Netherlands) consisted of an axial T1-weighted and an axial T2 and T2 fluid attenuating inverse recovery (FLAIR) scan (TR/TE/TI: 6000/100/2000, FOV 230 mm, matrix 180 x 256, slice thickness 4.0 mm, contiguous slices, 38 slices). The images were printed on hard copy with a reduction factor of 2.9. White matter lesions (WMLs), atrophy and number and location of infarcts were rated on hard copies, or on digital images on a personal computer.

White matter lesions (WMLs)

WMLs were considered present if these were hyperintense on FLAIR images and not hypointense on T1 weighted images. WMLs were distinguished into periventricular and deep (subcortical) lesions and rated according to the Scheltens rating scale [19]. Periventricular WMLs (PWMLs) were rated semi-quantitatively per region, adjacent to the frontal horns (frontal capping), adjacent to the lateral wall of the lateral ventricles (bands) and adjacent to the occipital horns (occipital capping), on a scale ranging from 0 (no PWMLs), 1 (PWMLs ≤ 5mm (real size)) and 2 (PWMLs > 5 mm). The overall degree of PWMLs was calculated by adding up the scores for the three separate categories on the left and right (range 0-12). This is slight modification of the original scale, which only counts the side with the highest score (range 0-6).
For the rating of deep WMLs (DWMLs) the brain was divided into 6 regions: frontal, parietal, occipital, temporal, basal ganglia and infratentorial. This is a slight modification of the original scale, which divides the basal ganglia and infratentorial regions into 5 and 4 different smaller subregions [19]. The different brain regions were determined according to anatomical

landmarks, namely the central sulcus, the Sylvian fissure and the parieto-occipital sulcus and were shown on templates during the rating. Per region the size and number (n) of the DWMLs were rated, on a scale ranging from 0 (no DWML), 1 (≤3 mm (real size); n≤5), 2 (≤3 mm; n≥6), 3 (4-10 mm; n≤5), 4 (4-10 mm; n≥6), 5 (> 11 mm; n≥ 1) and 6 (confluent). The overall degree of DWMLs was calculated by adding the scores of all regions (range 0-36).
Furthermore, brain infarcts were scored, by location (cortical and subcortical), type (lacunar or large) and number. A lesion was considered a lacunar infarct if its score was hypo-intense on T1 and FLAIR images and if its appearance was unlike a perivascular space.

Atrophy rating scales

Cortical atrophy was evaluated quantitatively by the frontal interhemispheric fissure ratio (FFR): the maximal width of the interhemispheric fissure from any of the cuts demonstrating the frontal lobes divided by the trans-pineal coronal inner table diameter [20] and by the Sylvian fissure ratio (SFR): the average of the maximal Sylvian fissure widths taken from the cut showing the widest Sylvian fissure divided by the trans-pineal coronal inner table diameter [20]. Subcortical atrophy was evaluated by the bicaudate ratio (BCR) on the cut best showing the caudate nuclei and by the bifrontal ratio (BFR) measured on the same cut as the BCR. BCR and BFR are defined respectively as the minimal distance between the caudate indentations of the frontal horn [20] or the distance between the tips of the frontal horns divided by the distance between the inner tables of the skull along the same line [20]. In order to relate cerebral atrophy to cognitive functioning, the raw data were converted into a cortical atrophy *z*-score (mean (*z*-FFR and *z*-SFR)) and subcortical atrophy *z*-score (*z*-BCR and *z*-BFR), based on the pooled mean of the whole study population.
All MRI scans were independently rated by two raters blinded for diabetic status (S.M.M. and G.J.B.). In case of disagreement of more than 1 point on the WML scales in a particular region or more than 5 mm (actual size) on any of the atrophy measurements (2 mm for fissure widths), a consensus reading was held (this involved 0% of PWML, 4% of DWML, and 4% of atrophy ratio readings). In all other cases the readings of both readers were averaged.

Neuropsychological tests

All participants performed an extensive neuropsychological examination tapping the major cognitive domains in both a verbal and a non-verbal way. Eleven tasks were administered in a fixed order which took about 90 minutes to complete. These tasks were divided into five cognitive domains in order to reduce the amount of neuropsychological variables and for

clinical clarity. This division was made a priori, according to standard neuropsychological practice and cognitive theory, as described in detail in Lezak's "Neuropsychological Assessment" [21]. The domain "abstract reasoning" was assessed by the Raven Advanced Progressive Matrices (12-item short form). The domain "memory", included four sub domains: "working memory" assessed by the forward & backward Digit Span of the Wechsler Adult Intelligence Scale-III (WAIS-III) and the Corsi Block-Tapping Task; "immediate memory and learning rate" including verbal memory assessed by the Rey Auditory Verbal Learning Test and visual memory assessed by the Location Learning Test; "forgetting rate" assessed by the delayed task of the Rey Auditory Verbal Learning Test and of the Location Learning Test and "incidental memory" assessed by the delayed trial of the Rey-Osterrieth Complex Figure. The domain "information processing speed" was assessed by the Trail Making Test Part A, the Stroop Color-Word Test (Part I and II) and the subtest Digit Symbol of the WAIS-III. The domain "attention and executive function" was assessed by the Trail Making Test Part B, the Stroop Color-Word Test (Part III), the Brixton Spatial Anticipation Test and a Verbal Fluency Test, using the N and A, and Category Fluency using animal names. The domain of visuoconstruction was assessed by the Rey-Osterrieth Complex Figure (copy trial). A premorbid IQ was tested with the Dutch version of the NART.
Since depression is more common in patients with DM2 than in controls [22], and depression may influence cognitive functioning, mood was assessed with a Beck Depression Inventory [23]. Both the total score on this self-rated depressive symptoms inventory and the percentage of people scoring above the cut-off criterion of 15 were recorded.
In order to compare the five different cognitive domains between the two groups, and to perform regression analysis within the DM2 group, the raw-scores were standardised into *z*-scores per domain. These *z*-scores were calculated on the pooled mean of the whole study population.

Medical history, blood pressure, blood samples and vascular disease

In a standardised interview, participants were asked about diabetes duration, length and weight, history of hypertension, stroke or cardiovascular disease and smoking. Furthermore, all participants measured their blood pressure at home at 9 different time points during the day (Omron MX3, Mannheim, Germany). Fasting glucose, HbA_{1c}, and fasting triglycerides and cholesterol were determined. Body Mass Index (BMI) was calculated as weight divided by height square. Hypertension was defined as an average systolic blood pressure ≥ 160 mm Hg or diastolic blood pressure ≥ 95 mm Hg or self reported use of blood pressure lowering drugs. Hypercholesterolemia was defined as a fasting cholesterol > 6.2 mmol/l or self reported use of cholesterol lowering drugs [24].

Microvascular and macrovascular complications were also assessed. Fundus photographs were rated according to the Wisconsin Epidemiologic Study of Diabetic Retinopathy scale [25]. A score ≥1.5 was defined as retinopathy. Albuminuria was defined as microalbuminuria (albumin 30-250mg/l) or macroalbuminuria (albumin 250mg/l or positive protein dipsticktest) in the overnight urine sample. Neuropathy was defined as a score ≥6 on the Toronto Clinical Neuropathy Scoring System [26]. "Any microvascular disease" was defined as retinopathy, or albuminuria, or neuropathy. "Any macrovascular event" was defined has a history of myocardial infarction, stroke, or surgery or endovascular treatment for coronary, carotid or peripheral (legs, abdominal aorta) artery disease. More detailed data on these complications in relation to cognition and brain MRI will be reported separately.

Statistical analysis

For the population characteristics, cognition and brain MRI findings between-group differences were analyzed with *t*-test for means, Mann-Whitney U for non-parametric data and chi-square test for proportions. Between group differences on cognition and brain MRI were also assessed by regression analyses and expressed as estimated between group difference with 95% confidence intervals (CI). The primary analyses were adjusted for age, sex, and estimated IQ, and in additional analyses also for blood pressure, or the depression inventory score.

Within the DM2 population associations between cognition, brain MRI findings, and disease variables were assessed by linear or logistic regression analysis, adjusting for age, sex and estimated IQ. For the between group comparisons $p<0.05$ was considered statistically significant. For the within group analyses in the DM2 patients a significance level of $p<0.01$ was used, to accommodate for the effects of repeated testing.

Results

Clinical data

The groups were well balanced for age, sex, level of education (seven categories) [27], and estimated IQ (Table 1). Of the 113 DM2 participants, 11 (10%) subjects had no treatment or only dietary treatment, 68 (60%) received oral antidiabetic drugs and 34 (30%) received insulin. Patients with DM2 had more hypertension, whereas their lipid profile was better than that of controls.

Table 1: Participant characteristics:

characteristic	Patients with DM2	controls
Number of subjects(male/female)	113(56/57)	51(22/29)
Mean age	66.1±5.6	65.1±5.3
Education level	4(3-5)	4(3-5)
Estimated IQ	99±15	100±14
Depressive symptoms[&]	7%	2%
Diabetes duration (years)	8.8±6.2	-
History severe hypoglycemia	6%	-
HbA_{1c}	6.9±1.2**	5.5±0.3
Fasting serum glucose (mmol/l)	8.6±3.0**	5.5±0.6
Use of insulin	30%	-
Body Mass Index (kg/m^2)	28.0±4.3	27.2±4.9
Hypercholesterolemia (%)	63	49
Fasting serum cholesterol (mmol/l)	5.0±0.9**	5.7±0.9
Fasting triglycerides (mmol/l)	1.9±1.0*	1.5±0.9
Use of lipid lowering drugs	53%**	22%
Hypertension (%)	73%**	33%
Use of antihypertensive drugs	70%**	33%
Systolic BP (mm Hg)	147±19**	137±19
Diastolic BP (mm Hg)	81±10	78±9
Any microvascular disease	58%**	18%
Any macrovascular event	29%**	6%

Data are given as means ± SD, median (IQR) or percentage. *p<0.05, **p<0.01
& Beck Depression Inventory score ≥16

Differences between patients with DM2 and controls on brain MRI and cognitive domains

Patients with DM2 had more cortical and subcortical atrophy compared with controls (FFR: p<0.001; SFR: p<0.001; BCR: p=0.003; BFR: p=0.14.) (Table 2). Patients with DM2 had a higher DWML score compared with controls (p=0.02) (Figure 1), but PWMLs were not different between the groups (p=0.13). Furthermore, patients with DM2 had more (silent) cerebral infarcts compared with controls (DM2 22/113; controls 4/54; p=0.06). From the 22 patients with infarcts, 12 had lacunar infarcts, 6 had other infarcts (e.g. cortical or large subcortical), and 4 had both lacunar and other infarcts. Six of the 22 patients with a visible infarct on their MRI reported a history of stroke. One control patient had a lacunar infarct and 3 controls showed other infarcts on their MRI, but none of them reported a

history of stroke. The effect of diabetes on cortical atrophy persisted after adjusting for the presence of WML and infarcts.
Performance of patients with DM2 on all five cognitive domains was worse compared with controls, but statistically significant changes were observed only for the domains attention and executive functioning (p=0.01), information processing speed (p=0.01) and memory (p=0.01) (Figure 2). Effect sizes were in the small to moderate range (0.2-0.4).
Adjustment for the possible effects of blood pressure (mean arterial pressure) did not affect the difference between the DM2 and control group on the MRI measures or cognition (Table 3). Adjustment for the presence or absence of hypertension gave similar results (data not shown). Moreover, if the findings in hypertensive and non-hypertensive subjects were analyzed separately, the magnitude of the effect of diabetes remained essentially the same (Table 3). The exclusion of controls (n=10) with impaired fasting glucose (>6.0 mmol/l) [28] did not have an apparent effect on between group differences on cognition or brain MRI.
One control and 8 DM2 patients scored above the above the cut-off criterion of 15 for the depression inventory score. Exclusion of these participants from the analyses, or adjustment for the depression score, did not affect the between group differences on cognition (data not shown).

Table 2: Cerebral atrophy: sulci and ventricle to brain Ratios

	Patients with DM2	controls
Frontal fissure ratio (FFR)*10^2	4.3±1.5**	3.3±1.3
Sylvian fissure ratio (FFR)*10^2	4.1±1.3**	3.3±0.8
Bicaudate ratio (BCR)*10^2	14.9±3.6*	13.1±3.0
Bifrontal ratio (BFR)*10^2	33.0±4.8	31.9±4.4

Data are given as means ± SD. *p<0.01, **p<0.001

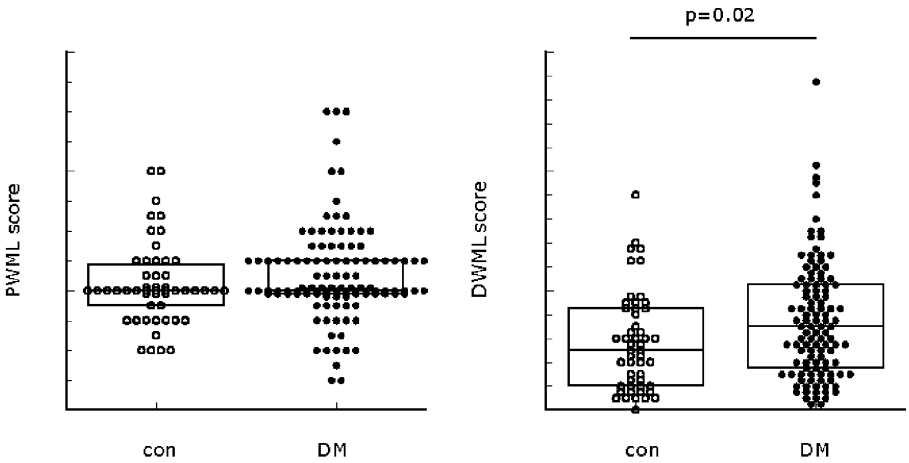

Figure 1: MRI findings in controls and patients with DM2: PWML and DWML (box represents median with interquartile range) (PWMLs: Scheltens scale 0-12; DWMLs: Scheltens scale: 0-36) [19]

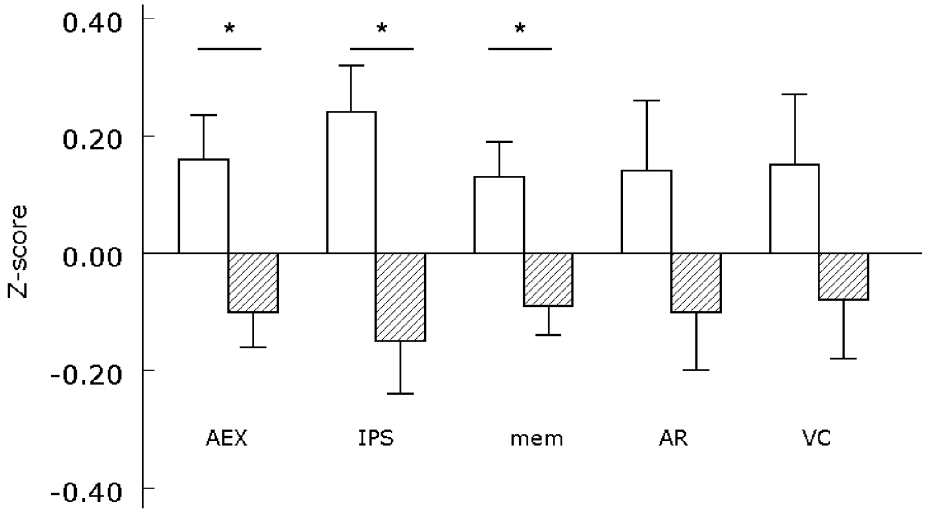

Figure 2:
Cognitive domains in controls (open bars) and patients with DM2 (hatched bars), raw-scores were standardised into *z*-scores per domain (mean ± SEM).

Table 3: Adjusted between group differences on MRI and cognition.
Data are presented separately for the whole population and for subgroups with or without hypertension.

	Whole population		No hypertension	Hypertension
	Model 1[#]	Model 2		
n (DM2/con)	113/51	113/51	30/33	83/18
PWMLs	0.5 (0; 0.5)	0 (-0.5; 0.5)	0.5 (-0.5; 1)	0 (-1; 0.5)
DWMLs	1.5 (0; 3)*	1.5 (0; 3)*	1.5 (-0.5; 4)	1.5 (-1; 4)
FFR*10^3	7.9 (3.6; 12.3)**	7.7 (3.2; 12.1)**	9.6 (3.0; 16.2)**	7.7 (0.7; 14.7)*
SFR*10^3	6.4 (2.8; 10.0)**	5.9 (2.2; 9.5)**	5.5 (1.0; 9.9)*	6.3 (0; 12.6)
BCR*10^3	13.9 (3.9; 24.1)**	14.8 (4.6; 25.0)**	12.1 (-0.1; 24.2)	9.7 (-7.8; 27.3)
BFR*10^3	7.0 (-6.8; 20.9)	8.1 (-6.0; 22.2)	7.4 (-7.7; 22.6)	10.0 (-15.1; 35.1)
Executive function	-0.19 (-0.36; -0.02)*	-0.19 (-0.37; -0.02)*	-0.10 (-0.321; 0.13)	-0.23 (-0.52; 0.06)
Information processing	-0.30 (-0.55; -0.04)*	-0.29 (-0.55; -0.03)*	-0.17 (-0.52; 0.17)	-0.41 (-0.83; 0.02)
Memory	-0.16 (-0.30; -0.02)*	-0.18 (-0.32; -0.03)*	-0.30 (-0.53; -0.08)**	-0.13 (-0.34; 0.09)
Abstract reasoning	-0.13 (-0.41; 0.14)	-0.10 (-0.38; 0.18)	0.04 (-0.31; 0.38)	-0.14 (-0.61; 0.34)
Visuoconstruction	-0.17 (-0.48; 0.14)	-0.16 (-0.48; 0.16)	0.04 (-0.34; 0.41)	-0.08 (-0.60; 0.45)

In model 1 estimated mean differences (95% CI) between the control and DM2 group are adjusted for age, sex and IQ. Model 2 includes additional adjustment for the mean arterial pressure. In the final two columns comparisons between subgroups of DM2 patients and controls are presented separated based on the absence or presence of hypertension, and adjusted for age, sex and IQ. Estimated mean differences >0 for MR, and <0 for cognition reflect worse scores in the diabetic group relative to controls. *: $p<0.05$; ** $p<0.01$

Relation between cognition, MRI and disease variables in the patients with DM2

Within the DM2 group statistically significant associations between MRI abnormalities and cognition were noted, after adjustment for age, sex and IQ (Table 4). DWMLs, cortical atrophy and infarcts were related to information processing speed ($p<0.01$). PWMLs and subcortical atrophy also tended to be related to information processing speed ($p<0.05$). Subcortical atrophy was related to attention and executive function ($p<0.01$), and tended to be related to 2 other cognitive domains. In a multivariate model, with age, sex, IQ and the different MRI measures as the independent variables, and information processing speed as the dependent variable, only the associations with age ($p=0.001$), IQ ($p<0.001$), DWMLs ($p<0.001$) and infarcts ($p<0.001$) were statistically significant.

Age was evidently related to atrophy and WML severity (Table 5). After adjusting age for diabetes duration, these results did not change notably. No significant (at the $p<0.01$ level) relations between MRI parameters and blood pressure, HbA_{1c} and diabetes duration were found, although mean arterial blood pressure tended to be related to PWMLs ($p<0.05$). Age was also significantly related to cognition in 3 of the 5 cognitive domains (Table 5). Adjustment for diabetes duration did not notably change these results. Mean arterial blood pressure tended to be related to improved memory function ($p<0.05$), whereas hypertension was associated with lower performance on the other 4 cognitive domains, albeit not statistically significant. HbA_{1c} levels and diabetes duration tended to be related to information processing speed ($p<0.05$). A history of macrovascular events tended to be related to impaired cognition ($p<0.05$) and more severe DWMLs ($p<0.05$). Although microvasular disease was not related to brain MRI findings, there was an association with lower performance on the 5 cognitive domains, albeit not statistically significant. There was no relation between sex and cognition, but subcortical atrophy was more pronounced in men. For the domains of information processing speed and memory the association with age appeared to be stronger in the DM2 than in the control group (B values per 5 years: information processing speed: Con: -0.09 ($p=0.19$); DM2: -0.32 ($p<0.001$); memory: Con: -0.06 ($p=0.25$); DM2: -0.21 ($p<0.001$)). For memory, this interaction between age and group was statistically significant ($p=0.01$).

Table 4: Relations between brain MRI abnormalities and cognitive function in patients with DM2 expressed as regression coefficients B(CI).

	Attention and executive function	Information processing speed	Memory	Abstract reasoning	Visuoconstruction
PWMLs	-0.08*(-0.15;-0.02)	-0.13* (-0.24;-0.03)	-0.03 (-0.08;0.03)	-0.04 (-0.16;0.07)	0.04 (-0.08;0.17)
DWMLs	-0.01 (-0.04;0.01)	-0.06** (-0.09;-0.03)	-0.01 (-0.03;0.01)	-0.02 (-0.05;0.02)	0.02 (-0.02;0.06)
Cortical atrophy	-0.09 (-0.21;0.03)	-0.27** (-0.44;-0.10)	-0.03 (-0.12;0.07)	-0.17 (-0.36;0.02)	-0.01 (-0.25;0.23)
Subcortical atrophy	-0.17**(-0.29;-0.05)	-0.19 *(-0.38;0.00)	-0.06 (-0.16;0.04)	-0.25* (-0.45;-0.06)	-0.29* (-0.52;-0.06)
infarcts	-0.02 (-0.29;0.25)	-0.77** (-1.14;-0.39)	-0.10 (-0.31;0.10)	-0.41 (-0.82;0.01)	0.02 (-0.47;0.51)

Adjusted for age, estimated IQ and sex. *: $p<0.05$; **$p<0.01$

Table 5: Relations between disease variables and MRI abnormalities and cognition in patients with DM2 expressed as regression coefficients B(CI).

	PWMLs	DWMLs	Cortical atrophy	Subcortical atrophy	Infarct#
Age (per 5yrs)	0.5** (0.2; 0.7)	1.0* (0.2; 1.8)	0.33** (0.18; 0.48)	0.35** (0.21; 0.49)	1.5 (1.0; 2.4)
Sex[##]	-0.4 (-0.2; 1.0)	1.1 (-0.7; 2.9)	-0.20 (-0.53; 0.14)	-0.58** (-0.90; -0.27)	0.6 (0.2; 1.6)
MAP (per 10 mmHg)	0.3* (0.0; 0.5)	0.2 (-0.6; 1.0)	0.06 (-0.09; 0.21)	-0.05 (-0.19; 0.10)	1.1 (0.7; 1.7)
Hypertension	0.2 (-0.5; 0.8)	0.5 (-1.5; 2.6)	0.03 (-0.35; 0.40)	0.07 (-0.29; 0.42)	1.4 (0.5; 4.4)
Microvascular disease	-0.1 (-0.6; 0.5)	-1.0 (-2.9; 0.9)	0.19 (-0.16; 0.53)	-0.10 (-0.42; 0.22)	0.9 (0.3; 2.4)
Macrovascular events	0.1 (-0.6; 0.7)	2.0 (0.0; 4.1)*	0.11 (-0.27; 0.50)	0.20 (-0.16; 0.56)	2.8 (1.1; 7.9)*
DM duration (per 5yrs)	-0.2 (-0.4; 0.0)	-0.5 (-1.3; 0.3)	0.10 (-0.05; 0.24)	-0.00 (-0.04; 0.03)	1.2 (0.8; 1.7)
HbA_{1c} (per %)	-0.1 (-0.3; 0.2)	0.4 (-0.3; 1.2)	-0.03 (-0.11; 0.16)	-0.03 (-0.15; 0.10)	1.1 (0.7; 1.6)
	Attention and executive function	Information processing speed	Memory	Abstract reasoning	Visuoconstruction
Age (per 5yrs)	-0.13** (-0.23; -0.04)	-0.32** (-0.46; -0.17)	-0.21** (-0.28; -0.14)	-0.13 (-0.28; 0.02)	-0.03 (-0.20; 0.14)
Sex	-0.09 (-0.31; 0.11)	-0.07 (-0.38; 0.25)	0.12 (-0.04; 0.28)	-0.10 (-0.43; 0.23)	-0.14 (-0.52; 0.23)
MAP (per 10 mmHg)	0.01 (-0.09; 0.10)	-0. 03 (-0.17; 0.12)	0.08* (0.01; 0.15)	-0.16 (-0.21; 0.09)	-0.01 (-0.16; 0.18)
Hypertension	-0.15 (-0.38; 0.09)	-0.21 (-0.57; 0.14)	0.18 (0.00; - 0.36)	-0.31 (-0.68; 0.06)	-0.39 (-0.79; 0.03)
Microvascular disease	-0.09 (-.030; 0.13)	-0.09 (-0.42; 0.24)	-0.13 (-0.29; 0.04)	-0.32 (-0.66; 0.01)	-0.29 (-0.67; 0.09)
Macrovascular events	-0.12 (-0.36; 0.11)	-0.43 (-0.78; -0.08)*	-0.21 (-0.40; -0.03)*	-0.21 (-0.59; 0.17)	0.04 (-0.39; 0.46)
DM duration (per 5yrs)	0.00 (-0.09; 0.09)	-0. 15* (-0.28; -0.02)	-0.07 (-0.13; 0.00)	-0.02 (-0.18; 0.14)	0.15 (-0.02; 0.31)
HbA_{1c} (per %)	-0.05 (-0.14; 0.03)	-0.17* (-0.30; -0.04)	-0.04 (-0.10; 0.03)	-0.13 (-0.27; 0.01)	0.07 (-0.08; 0.21)

An increase in MR scores reflects more severe MRI abnormalities, whereas a decrease in z-values for cognition reflects more pronounced performance impairments.

Discussion

On brain MRI patients with DM2 had more cortical and subcortical atrophy and more DWMLs and infarcts than controls. The performance of the patients with DM2 on the neuropsychological examination was worse, particularly affecting the domains attention and executive functioning, information processing speed and memory. Adjustment for hypertension did not affect the results. Within the DM2 group cognitive function was inversely related with WMLs, atrophy and the presence of infarcts, and there was a modest association between impaired cognition and HbA_{1c} and diabetes duration. This association was strongest for age, even more so than in the control group.

Cognitive function of patients with DM2 has been the subject of several studies [review [2;3]], generally reporting deficits in verbal memory, information processing speed and less consistently, in executive functioning and non-verbal memory. Our results are in line with these findings. Studies which examined relations between different disease variables and cognitive functioning showed that patients with worse glycemic control were more likely to show cognitive deficits [29]. Most of these studies used smaller sample sizes than we did in our study. Moreover, although most studies did not use age as an independent predictor, the largest effect of DM2 on cognitive function was observed in studies in which patients were older [30].

When addressing the relation between hypertension and cognition, the results of previous studies are less consistent. One population based study found that hypertensive patients with DM2 were at the greatest risk for poor cognitive performance [31], but other longitudinal studies did not observe an evident relation between hypertension and cognitive performance [9;32;33], in line with the present observations.

Thus far, relatively few studies have specifically addressed brain MRI abnormalities in patients with DM2. In line with the present observations, these studies indicate that modest cortical and subcortical atrophy and symptomatic or asymptomatic infarcts are more common in DM2 patients than in controls [11-15]. The relation between cerebral atrophy and hypertension in DM2 patients, however, is less clear, one study reporting no effects of adjustment for hypertension [12], consistent with our findings, whereas another study indicated that hypertension appeared to be a major determinant of cerebral atrophy in DM2 patients [11]. Results of previous studies on the association between DM2 and WMLs are inconsistent. The majority of these studies involved selected subgroups of patients with, for example, clinically manifest cardiovascular disease or stroke [16;17], and used relatively insensitive measures to rate WMLs. Some of these studies in patients with vascular disease reported relatively more severe WML in patients with DM2, [16], whereas others reported no statistically significant relation between DM2 and WMLs [17]. The study on WMLs in elderly subjects with DM that involved the largest cohort and the most detailed rating method thus far reported no effect of DM on PWML, although the volume of DWML tended to be higher in the DM group [11].

There are, to our best knowledge, no previous studies that specifically addressed the relation between brain MRI abnormalities and cognitive functioning in patients with DM2. However, previous studies in general populations of elderly subjects that looked at brain MRI abnormalities and cognitive function show results that are comparable with ours. A study of 139 healthy adults (50-81 years old), for example, observed an association between atrophy of the prefrontal cortex and the volume of WMLs in the prefrontal region and age-related impairments of executive functioning, but not with memory [34]. Another study, involving 68 healthy, non-demented individuals, tested at ages 50, 60, 70 and 80, reported that PWMLs were related to decline in information processing speed and visuoconstruction and DWMLs were related to visuoconstruction [35].

The present study may provide some clues regarding the causes of cognitive deficits in patients with DM2, as cognitive impairments were associated with subcortical ischemic MRI abnormalities (silent infarcts and WMLs). However, non-vascular mechanisms could also be involved, because more diffuse cerebral changes, like cortical atrophy, were also related to impaired cognition. Blood pressure showed a relation with PWMLs, and might thus to some extent be involved in the cognitive deficits. However, as is clearly indicated in table 3 adjustment for hypertension had no obvious effect on the differences in cognition and MRI ratings between controls and patients with DM2. Chronic hyperglycemia could also be involved, although the relation between HbA_{1c} and diabetes duration and changes in cognition was only modest. In the present study the strongest determinant of changes in cognition and on MRI was age. Age was strongly related to all MRI parameters and to 3 of the 5 cognitive domains in patients with DM2, and the interaction term of age and group was significant for the domain of memory. This points to an interaction between DM2 and ageing. In fact, several processes that have been implicated in brain ageing, including oxidative stress, accumulation of so-called advanced glycosilation end-products, microvascular dysfunction, and alterations in cerebral glucose and insulin metabolism may be accelerated by DM [36], which may explain part of this interaction.

A strength of our study is that we combined a detailed analysis of both cognitive function and brain imaging by means of MR thus allowing an accurate assessment of the relation between these parameters. A possible limitation of our study could be selection bias. In general, persons who participate in research projects that include a detailed work-up at a hospital tend to be less affected than persons who refuse participation. Hence, we do not think this selection bias has a large impact on our results, if any, the strength of the associations would be underestimated, because of a healthier study population. We specifically decided not to exclude participants with co-morbidity such as hypertension or macrovascular events, as this co-morbidity is an integral part of the diabetic condition. If we had excluded subjects with these disorders a priori our findings would not be generalizable to the general population of DM2 subjects. The

characteristics (e.g. HbA_{1c}, total cholesterol, triglycerides, blood pressure and body mass index) of the patients with DM2 that were included in the present study are similar to those reported in large population based surveys of DM2 subjects in the Netherlands [37;38]. Mean HbA_{1c} was 6.9% in our DM2 population, which indicates a moderately good controlled diabetes. Another limitation of the present study might be its cross-sectional design. This can clearly affect the interpretation of the data on the relation between impaired cognition and potential mediators such as blood pressure and glycemic control. In elderly individuals many different factors can affect the outcome measures that were assessed in the present study, of which only a proportion are directly related to DM2. A follow-up project involving the present study population has been initiated, which may provide more detailed information on the potential role of different metabolic and vascular risk factors.

We conclude that cognitive impairments in patients with DM2 are related to structural changes in the brain. These changes are indicative of a vascular etiology, although the increased cortical brain atrophy and the relation with age are also suggestive of other mechanisms such as accelerated brain ageing.

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Metabolic and vascular determinants of changes in cognition and on brain MRI in patients with type 2 diabetes

6

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Introduction

Diabetes mellitus is associated with slowly progressive changes in the brain, a complication that is referred to as diabetic encephalopathy [1]. A number of previous neuropsychological studies show that patients with type 2 diabetes mellitus (DM2) have mild to moderate impairments in attention and executive functioning, information processing speed and memory [review: [2;3]]. Patients with DM2 also show changes on brain MRI, such as cortical and hippocampal atrophy [4;5]. We have recently shown that cognitive dysfunction in patients with DM2 was associated with white matter lesions (WML), with (silent) brain infarcts, and to a lesser extent with atrophy [Manschot et al; Diabetes, in press].

The determinants of changes in cognition and abnormalities on brain MRI in DM2 have not yet been examined in sufficient detail, and are subject to debate [2]. Some studies report associations with hypertension [3;4;6;7], but this was not supported by other studies [5;8;9], one of which showed an association between impaired cognition and chronic hyperglycaemia [8].

Clues on potential determinants of impaired cognition in DM2 may be provided by studies on other diabetic complications. Complications like nephropathy, retinopathy and neuropathy are thought to be caused by hyperglycaemia-induced microangiopathy [10;11], with additional involvement of hypertension and macrovascular disease [11-13]. Since atherosclerosis and hypertension have also been identified as risk factors for age-related cognitive decline and brain MRI changes in the general population [14-16], we hypothesised that the combined effects of atherosclerotic macrovascular disease, chronic hyperglycaemia and hypertension are involved in the development of cognitive impairments in patients with DM2.

The aim of the present study was to identify possible metabolic and vascular determinants of cognitive dysfunction and changes on brain MRI in patients with DM2. Given the uncertainty about these determinants, an exploratory design was chosen, in which a detailed neuropsychological examination and brain MRI were obtained from a large cross-sectional sample of DM2 patients and related to different measures of glucose metabolism, vascular risk factors, microvascular complications and macrovascular disease.

Patients and Methods

Patients

The Utrecht Diabetic Encephalopathy Study aims to identify determinants of cognitive impairment in patients with diabetes [Manschot et al; Diabetes, in press]. Therefore, patients were not selected for the presence or absence of diabetic complications, co-morbid conditions (e.g. hypertension), or exposure to other risk factors (e.g. smoking). For inclusion patients had to be 55 to 80 years of age, with a minimal diabetes duration of 1 year, and had to be functionally independent, and Dutch speaking. Exclusion criteria for all participants were a psychiatric or neurological disorder that could influence cognitive functioning, a history of alcohol or substance abuse and

dementia, and a fasting blood glucose ≥ 7.0 for controls [17]. Subjects with a history of non-invalidating stroke could be included. Twice as many patients as controls were included, to increase statistical power for within group analyses in the DM2 group.
122 patients with DM2 (age 56-80 yrs), and 56 controls (57-78 yrs) were included in the present study. General practitioners in the area were asked to participate in the project (see acknowledgements), and to invite all eligible DM2 patients from their practice. Controls were recruited among the spouses or acquaintances of the patients. Controls were chosen with the aim to obtain groups that were comparable on age, sex and educational level. The study was approved by the medical ethics committee of the University Medical Center Utrecht and each participant signed an informed consent form. All participants underwent a two-day protocol which included brain MRI, a neurological and neuropsychological examination, retinal photography and ultrasonography of the carotid arteries. Also, a standardised questionnaire concerning medical history and medication use was taken, fasting blood and urine samples were collected, and blood pressure was recorded. No neuropsychological examination could be obtained in 1 control and 2 DM2 patients. An MRI was not obtained in 5 controls and 9 DM2 patients, mostly due to MRI contraindications (claustrophobia, pacemaker).

Neuropsychological examination

The neuropsychological examination tapped the major cognitive domains in both a verbal and a non-verbal way. Eleven tasks were administered in a fixed order and took about 90 minutes to complete. These tasks were divided into five cognitive domains, as described previously [Manschot et al; Diabetes, in press]: attention and executive functioning, information processing speed, memory, abstract reasoning and visuoconstruction. For analysis the test scores were standardised into z-scores for each of the 5 domains, based on the means of the whole group.
Premorbid IQ was assessed with the Dutch version of the National Adult Reading Test. To control for possible effects of mood disturbances or affective disorders a Beck depression inventory [18] was performed.

Brain MRI

The MRI investigation (1.5 Tesla, Philips Medical systems, Best, the Netherlands) consisted of an axial T1-weighted and an axial T2 and T2 fluid attenuating inverse recovery (FLAIR) scan (TR/TE/TI: 6000/100/2000, field of view 230 mm, matrix 180 x 256, slice thickness 4.0 mm, contiguous slices, 38 slices).
White matter lesions (WML) were rated on hard copies or digital images according to the Scheltens scale [19]. Periventricular WML (PWML) were rated on a severity scale (0-2) at the frontal and occipital horns and the body of the lateral ventricle on both sides. These 6 ratings were summed (range 0-12). This is a slight modification of the original scale, which only counts the

side with the highest score (range 0-6) [19]. For the rating of deep (subcortical) WML (DWML) the brain was divided into six regions: frontal, parietal, occipital, temporal, basal ganglia and infra-tentorial. This is a slight modification of the original scale [19], which divides the basal ganglia and infra-tentorial regions into smaller subregions. Per region the size and number (n) of the WML were rated, on a scale ranging from 0 to 6, with the total DWML score being the sum of these 6 scores (range 0-36).
Cortical atrophy was evaluated by the frontal interhemispheric fissure ratio and the Sylvian fissure ratio [20]. Subcortical atrophy was evaluated by the bifrontal ratio and by the bicaudate ratio [20]. These ratios were converted to z-scores: a cortical atrophy z-score (mean of z- frontal fissure ratio and z-Sylvian fissure ratio) and a subcortical atrophy z-score (mean of z-bicaudate ratio and z-bifrontal ratio).
All MRI scans were rated by two investigators (SMM and GJB) blinded for presence or absence of diabetes or other characteristics. In case of disagreement of more than 1 point on the WML scales in a particular region or more than 5 mm (actual size) on any of the atrophy measurements (2 mm for fissure widths), a consensus reading was held (this involved 0% of PWML, 4% of DWML, and 4% of atrophy ratio readings). In all other cases the readings of both raters were averaged.

Diabetes characteristics and glucose metabolism
Participants were asked about medication use, diabetes duration and the life-time occurrence of severe hypoglycaemic episodes (defined as episode of hypoglycaemia that was severe enough to require the assistance of another person, hospitalisation or emergency room visit). Body Mass Index (BMI) was calculated as weight divided by height square.
Blood was drawn by venepuncture to assess HbA_{1c}, fasting glucose level and insulin levels. Insulin resistance was estimated with the "homeostasis model assessment" (HOMA-IR), which is calculated as: (fasting glucose*fasting insulin)/22.5[21].

Vascular risk factors
Blood pressure was measured at home at 9 fixed time points during the day with an Omron 705CP (GmbH Germany) automatic blood pressure machine. These 9 measurements were averaged. In the primary analysis hypertension was defined as a mean systolic blood pressure above 160 mmHg or a mean diastolic pressure above 95 mmHg or the use of antihypertensive medication. In a second analysis cut-of values for systolic an diastolic blood pressure of 140 and 90 mmHg were used.
Smoking habits were classified into "current", "past" or never". Total cholesterol, high- and low-density lipoprotein (HDL and LDL)-cholesterol and triglycerides were assessed in a fasting venous blood sample.

Microvascular disease
Following mydriasis with phenylephrine and tropicamide, single-field

photographs were taken with a 50-degree retinal camera (Zeiss FF 450) of both eyes, centered on the macula. Retinopathy was rated on slides, according to the diabetic retinopathy severity scale (grades 1-7) as used in the Wisconsin Epidemiologic Study of Diabetic Retinopathy [22]. Photocoagulated eyes were rated at grade 5 or higher, corresponding with severe non proliferative diabetic retinopathy. Ratings were performed by two investigators, blinded for patient characteristics. In case of disagreement, a third investigator was involved and a consensus made. Retinopathy was defined as a grade of 1.5 or higher.
Neuropathy was rated with the Toronto Clinical Neuropathy Scoring System (neuropathy scale) [23], with a slight modification. A sensory test for temperature was not performed, so that the maximum score is 18 points (severe polyneuropathy) instead of 19. A score from 0-5 indicated no neuropathy, 6-8 mild neuropathy, 9-11 moderate neuropathy and ≥ 12 severe neuropathy. Neuropathy was defined as a score of 6 and higher.
An urine sample was collected overnight. Albuminuria was defined as microalbuminuria (albumin 30 to 250mg/l) or macroalbuminuria (albumin 250mg/l or positive protein dipsticktest).

Macrovascular disease
Several composite measures of macrovascular disease were defined. "Any peripheral arterial disease" was defined as current complaints of intermittent claudication (assessed with the Rose questionnaire [24]) or a history of surgery or endovascular treatment for arterial disease of the legs or the abdominal aorta. "Ischaemic heart disease" was defined as a history of myocardial infarction or surgery or endovascular treatment for coronary artery disease. "Any vascular event" was defined as a history of myocardial infarction or stroke, or a history of operative or endovascular treatment for coronary, carotid or peripheral (legs, abdominal aorta) artery disease.
Brain infarcts were rated on brain MRI, by location (cortical and subcortical), size (lacunar (<1.5 cm) or large) and number. A lesion was considered an infarct if it was hypo-intense on T1 and FLAIR images and if its appearance was unlike a perivascular space.
Carotid Intima/Media thickness (CIMT) was measured in both common carotid arteries as described previously [25] with an ATL Ultramark 9 (Advanced Technology Laboratories) equipped with a 10-MHz linear-array transducer. Scanning was performed at three different longitudinal projections (anterior-oblique, lateral and posterior-oblique). The CIMT was measured in a 1-cm section proximal to the beginning of the dilatation of the carotid bulb in all three projections, in both carotid arteries. CIMT was calculated as the average of these six measurements.

Statistical analysis
The differences between DM2 and controls were examined with t-test for means, Mann-Whitney U for non-parametric data, and chi-square test for

proportions. In the text and tables, data are shown mean±SD, or proportions unless stated otherwise.
Within the DM2 population associations between cognition (5 domains), brain MRI findings (cortical and subcortical atrophy z-scores, PWML, DMWL and infarcts) were related to the different measures of glucose, insulin and lipid metabolism, microvascular complications and macrovascular disease, by linear or logistic regression analysis, adjusting for age, sex and estimated IQ. In order to limit the number of regression analyses the cognitive data were pooled in a "composite cognitive z-score", which was the mean of the z scores on the 5 cognitive domains. If a variable was significantly associated with this pooled z-score, post-hoc tests were performed per domain.
For the between and within group analyses $p<0.05$ was considered statistically significant.

Results

Patients with DM2 and controls were comparable with regard to age, sex, level of education (seven categories) [26] and estimated premorbid IQ (see table 1). Of the 122 DM2 participants, 12 (10%) subjects had no specific treatment or only dietary treatment, 75 (61%) received only oral anti-diabetic drugs and 35 (29%) received insulin (alone or in combination with oral drugs).

Neuropsychological and MRI data

Detailed neuropsychological and MRI data have been reported previously [Manschot et al; Diabetes, in press]. In short, performance of patients with DM2 was worse than controls across all five cognitive domains, with statistically significant differences on the domains attention and executive functioning (difference mean z-scores 0.23 (95%CI 0.03; 0.43); p=0.02), information processing speed (0.40 (0.17; 0.63); p=0.001) and memory (0.20 (0.05; 0.36); p=0.01).
Patients with DM2 had more pronounced cortical atrophy (difference in mean z-scores 0.62 (95%CI 0.33; 0.91); p0<0.001) and subcortical atrophy (0.38 (0.07; 0.68); p=0.01) than controls. Furthermore, patients with DM2 had more severe DWML (controls (median, range): 5 (0-18); DM2: 7 (0.5-27.5); p=0.02), but PWML severity in the two groups was similar (con: 6(4; 10); DM2: 6(3; 12); p=0.13). Patients with DM2 also had more (silent) cerebral infarcts than controls (DM2 22/113, controls 4/54; p=0.06; DM2 12 lacunar, 6 other (i.e. cortical or large subcortical), 4 both lacunar and other; controls 1 lacunar, 3 other).

Table 1: Subject characteristics, diabetes treatment, glucose metabolism

Characteristic	DM2	controls
Number of patients	122	56
Sex (male/female)	62/60	25/31
Age (yrs)	66.0±5.8	con 65.1±5.2
Level of education	4 (3-5)	4 (3-5)
Estimated premorbid IQ	99±15	101±14
Diabetes duration (yrs) x	8.7±6.1	
Diabetes treatment		
Diet	10%	
Oral medication alone	61%	
Insulin x	29%	
HbA_{1c} (%) x	6.9±1.2** ▽	5.5±0.3
Fasting glucose levels (mmol/l) x	8.6±2.9**	5.5±0.6
Fasting insulin levels mU/l # x	17.3±15.8** (n=82) ▼	10.9±7.2 (n=54)
HOMA-IR # x	6.6±6.4** ▼	2.6±1.8
BMI (kg/m^2) x	28.1±4.4	27.3±5.3

Data are given as number (percentage) or mean±SD; **p<0.01 DM2 versus controls
only from subjects who were not treated with insulin and did not have antibodies against insulin.
x: entered as explanatory variable in the regression analyses within the DM2 group: statistically significant association with composite cognitive z-score: ▽: p<0.05; association with MRI abnormalities: ▼ p<0.01; ▼: p<0.05. Details in text.

Glucose metabolism: relation to cognition and brain MRI (Table 1)
HbA_{1c}, fasting glucose and insulin levels were higher (all p<0.01) in patients with DM2 than in with controls. BMI was similar in the two groups. Mean HbA_{1c} was 6.9% in our DM2 population, which indicates adequate glycaemic control. Mean diabetes duration was 8.7±0.6 years. Only a small proportion (6%) of DM2 patients ever experienced a severe hypoglycaemic event (Table 1).
In the regression analyses within the DM2 group HbA_{1c} levels were significantly related to cognition (composite z score: B(per % HbA_{1c}): -0.07 (-0.14; 0) p=0.047; post-hoc per domain: information processing speed: B(per % HbA_{1c}): -0.15 (95% CI: -0.27; -0.2), p=0.02; abstract reasoning: B: -0.15 (-0.29; -0.01), p=0.04). Elevated fasting insulin levels and HOMA-IR were related to increased DWML severity (B(per mU/l insulin): 0.10 (0.03; 0.18), p=0.009; B(HOMA-IR): 0.21 (0.04; 0.39), p=0.02).

Vascular risk factors: relation to cognition and brain MRI (Table 2)
Patients with DM2 had higher systolic blood pressure (p<0.01), pulse pressure (p<0.05) than controls, and more often had hypertension (p<0.01). Total cholesterol was lower in the DM2 group (p<0.01), but the

proportion of subjects taking lipid lowering drugs was higher in that group (p<0.01). There were no significant differences in the proportion of subjects that smoked, or had dyslipidemia between patients with DM2 and controls (Table 2).
In the regression analyses within the DM2 group there were no statistically significant associations with the composite cognitive z-score, only marginally for hypertension and current smoking (hypertension B: -0.19 (-0.38; 0), p=0.053, smoking B: -0.21 (-0.43; 0), p=0.051). Mean arterial pressure was associated with more severe PWML (B (per 10 mm Hg): 0.28 (0.03; 0.53), p=0.03). Reanalysis with cut-of values for hypertension of 140/90 mmHg made the association with the composite cognitive z-score less strong. The use of lipid lowering drugs (statins in all but one patient) was associated with less severe MRI abnormalities (PWML: B: -0.68 (-1.25; -0.12), p=0.02; cortical atrophy: B: -.0.36 (-0.69; -0.03): p=0.03). Moreover the use of these drugs tended to be associated with a higher composite cognitive z-score (B: 0.15 (-0.03; 0.32), p=0.10). These effects were not affected by additional adjustment for the actual cholesterol levels.

Table 2: Vascular risk factors

Characteristic	DM2 (n=122)	Controls (n=56)
Mean arterial pressure [x]	103±11* ▼	98±10
Pulse pressure [x]	65±15*	59±16
Hypertension [x]	73%**	34%
Antihypertensive drugs	70%**	32%
Current smoking [x]	22%	14%
Total cholesterol [x]	5.0±0.9**	5.8±1.1
Cholesterol/HDL [x]	4.3±1.2	4.4±1.5
Triglycerides [x]	1.9±1.0	1.6±1.1
Lipid lowering drugs [x]	54%** ▲	21%

Data are given as number (percentage) or mean±SD; *p<0.05, **p<0.01 DM2 versus controls
x: entered as explanatory variable in the regression analyses within the DM2 group: statistically significant association with more severe MRI abnormalities: ▼: p<0.05; upward triangle indicates reverse association. Details in text.

Microvascular disease: relation to cognition and brain MRI (Table 3)

In 20 patients with DM2 and 8 controls, no retinal photograph could be performed, and in 21 patients and 13 controls no overnight urine sample could be obtained, because of logistical reasons. Patients with DM2 had more retinopathy and neuropathy than controls (both p<0.01). Although albuminuria was more common in patients with DM2 than in controls, this difference was not statistically significant (Table 3).

In the regression analyses within the DM2 group there were no statistically significant associations with the composite cognitive z-score. Retinopathy was associated with more pronounced cortical atrophy (B: 0.48 (0.11; 0.85), p=0.01).

Table 3: Microvascular disease

Characteristic	DM2 (n=122)	Controls (n=56)
Retinopathy (DM2 n=112; con n=48) x	37 (33%)** ▼	1 (2%)
Background	33	1
Severe non proliferative	4	
Neuropathy x	47 (39%)**	7 (13%)
Mild neuropathy	25	7
Moderate neuropathy	18	0
Severe neuropathy	4	0
Albuminuria (DM2 n=101; con n=43) x	16 (17%)	3 (7%)
Microalbuminuria	9	3
Macroalbuminuria	7	0

Data are given as number (percentage); **p<0.01 DM2 versus controls
x: entered as explanatory variable in the regression analyses within the DM2 group: statistically significant association with MRI abnormalities: ▼: p<0.05 . Details in text.

Macrovascular disease: relation to cognition and brain MRI (Table 4)

Patients with DM2 more often had intermittent claudication (p<0.01) or a history of ischaemic heart disease than controls (p<0.01). There was no difference between the two groups in the CIMT (table 4).
In the regression analyses within the DM2 group a history of "any vascular event" and the presence of brain infarcts on MRI were associated with an impaired composite cognitive z-score (vascular event: composite z score B: -0.25 (-0.44; -0.05), p=0.01; post-hoc per domain: information processing speed (B: -0.46 (-0.80; -0.12), p=0.008), and memory (B: -0.23 (-0.41; -0.06), p=0.01; infarct on MRI: composite z score B: -0.28 (-0.50; -0.06), p=0.01; post-hoc per domain: information processing speed (B: -0.77 (-1.14; -0.39), p<0.001), and abstract reasoning (B: -0.41 (-0.82; 0.01), p=0.06). On MRI a history of "any vascular event" was associated with more pronounced DWML (B: 2.0 (0; 4.1), p=0.05), and with an increased occurrence of infarcts (OR: 2.9 (1.1; 7.9), p=0.04). Patients with a (silent) infarct on MRI had more pronounced PWML (B: 0.7 (0; 1.4), p=0.06), and cortical atrophy (B: 0.51 (0.10; 0.92), p=0.02) relative to DM2 patients without infarcts on MRI.

Table 4: Macrovascular disease

Characteristic	DM2 (n=122)	Controls (n=56)
Any peripheral arterial disease	18 (15%)**	0
Claudicatio intermittens	14 (11%)**	0
Vascular surgery femoral artery	4 (3%)	0
Vascular surgery AAA	3 (3%)	0
Ischaemic heart disease	23 (19%)** ▼	3%
Myocardial infarction	15 (12%)*	1 (2%)
CABG	13 (11%)*	1 (2%)
Brain infarct on MRI	22 (20%)* ▽/▼	4 (8%)
History of brain infarct	7 (6%)	2 (4%)
Carotid surgery	2 (2%)	1 (2%)
Any vascular event	33 (27%)** ▽/▼	4 (7%)
CIMT	0.093±0.018	0.093±0.030

Data are given as number (percentage) or mean±SD; *p<0.05, **p<0.01 DM2 versus controls
x: entered as explanatory variable in the regression analyses within the DM2 group: statistically significant association with impaired cognition: ▽: p<0.05; association with MRI abnormalities: ▼: p<0.05. Details in text.

Discussion

Patients with DM2 had more cortical and subcortical atrophy and more DWML than controls and their overall performance on the five cognitive domains was worse. As expected, patients with DM2 had more microvascular complications, more macrovascular (atherosclerotic) disease and more hypertension than controls. Within the DM2 group impaired cognitive functioning was associated with higher HbA_{1c} levels, brain infarcts on MRI and a history of vascular events. DM2 patients with retinopathy or with infarcts on MRI had more severe cortical atrophy, whereas insulin levels, mean arterial pressure, and macrovascular disease were associated with WML. DM2 patients who used statins had less pronounced cortical atrophy and PWML.

Cognitive function in patients with DM2 has been the subject of several studies [review [2;3]], which generally report mild to moderate deficits in verbal memory, information processing speed and less consistently, in executive functioning and non-verbal memory. Our results are in keeping with these findings. Thus far, relatively few studies have specifically addressed brain MRI abnormalities in patients with DM2. In agreement with our observations, modest cortical and subcortical atrophy and symptomatic or asymptomatic infarcts have been found more often in DM2 patients than in controls [4;5;27-29]. Results of previous studies on the association between DM2 and WMLs are less consistent [4;5;30-32]. Several of these studies, however, involved selected subgroups of patients with, for example,

clinically manifest cardiovascular disease or stroke [30;31]. Moreover, the scales that were used to rate WML were often relatively insensitive [30-32]. Two large population-based studies on the relation between WML and DM2 reported no statistically significant associations [4;32], although in one of these studies the volume of DWML tended to be higher in the DM2 patients [4].

Macrovascular atherosclerotic disease appeared to be the most consistent determinant of both impaired cognition and brain MRI abnormalities in the DM2 patients in the present study. The association with cognition was most evident for the domain of information processing speed, whereas the association with brain MRI involved WML, infarcts as well as cortical atrophy. We did not find previous studies that presented detailed data on the relation between macrovascular disease and cerebral changes in DM2. In the general population however, several studies showed that macrovascular atherosclerotic disease is associated with age-related cognitive impairment and changes on brain MRI. In a large cross-sectional study previous vascular events, presence of plaques in the carotid arteries, and presence of peripheral arterial atherosclerotic disease were negatively associated with cognitive performance [33]. In a prospective study, both cognitive impairment at baseline and further decline in cognition during follow-up were associated with asymptomatic stenosis of the internal carotid arteries [34]. Moreover, the association between the number of cardiovascular disease conditions and cognitive impairment appeared to show a dose-response relation [35]. With regard to brain MRI changes, a history of stroke or myocardial infarction has been associated with the presence of WML [36], and plaques in the carotid artery with PWML [15;37]. Previous studies in the general population indicate that risk factors for vascular disease, such as hypertension, dyslipidemia, increased BMI and smoking are associated with an increased risk of cognitive decline and dementia and with brain MRI changes, including WML [e.g. [38-41]]. Previous studies on the modulating effect of vascular risk factors on cognitive function in DM2 have mainly addressed hypertension, with conflicting result [6;8;42;43]. In the present study, hypertension was only marginally related with a lesser cognitive performance and mean arterial pressure was related with more severe PWML. Nevertheless, adjustment for the effects of hypertension did not affect the differences between the DM2 and the control group [Manschot et al, Diabetes, in press].

Chronic hyperglycaemia might be another determinant of cerebral changes in DM2. In the present study HbA_{1c} levels were related to the composite cognitive z-score. Moreover, retinopathy, which is generally considered to be a consequence of chronic exposure to hyperglycaemia [10], was related to cortical atrophy. Previous studies on cognition in patients with DM2 have also provided evidence for an association with glycaemic control, as reflected in HbA_{1c} levels [2;8;44]. However, no consistent relations between cognitive performance and fasting blood glucose [44;45] or duration of diabetes [44;45] have been found. There are no previous studies that have

provided detailed data on the association between glycaemic control and MRI changes in DM2. A recent meta-analysis showed that microvascular complications appeared to be a determinant of these changes in cognitive functioning [46], but also indicated that further studies are needed to establish the relation with chronic hypergylcaemia more firmly.

The reverse association between the use of statins and the severity PWML and cortical atrophy is intriguing. There has been a previous report from a small randomised placebo controlled trial on the effects of atorvastatin treatment on cognition in dyslipidaemic patients with DM2, showing that improvement of the lipid profile was associated with improvement of memory during follow-up [47]. The association between statin use, cognition and age-related brain MRI changes in the general population is still debated [48]. In a nested case control study the use of statins was associated with a decreased risk of dementia [49], but this association has not been replicated in prospective observational studies [Review [48]]. Moreover, a prospective randomised placebo-controlled trial with pravastatin treatment in elderly subjects with vascular disease or with vascular risk factors did not show effects on progression of WML or cortical atrophy [50;51]. Therefore, the present findings in DM2 patients will also need to be confirmed by prospective studies.

The strength of our study is that we combined detailed data on cognitive function and brain MRI with detailed data on metabolic and vascular risk factor clusters, thus allowing an accurate assessment of the relation between these factors. Possible limitations include patient selection, the cross-sectional design and the large number of explanatory variables that were entered in the regression analyses within the DM2 group. With regard to patient selection, we aimed to obtain a representative sample of functionally independent patients with DM2 from the general population. Still, it must be acknowledged that the rather demanding testing protocol may have deterred patients with relatively severe mental or physical limitations. Still, the prevalence of microvascular and macrovascular disease in our study sample is comparable to other population based studies in The Netherlands [52;53], as were the presence of hypertension and smoking habits[53] and the level of metabolic control [52-55]. The cross-sectional design of our study precludes inferences about causal relationships. Moreover, the cognitive and MRI outcome measures that were assessed are probably influenced by a large number of factors, some of which are specific to DM (e.g. chronic hyperglycaemia, diabetes treatment) and some not (e.g. age, hypertension, atherosclerosis). In this exploratory analysis we examined a large number of explanatory variables, which has certain drawbacks. Firstly, different explanatory variables might be interrelated, and given the relatively small regression coefficients and effect sizes the confounding effects of this interrelation cannot be reliably assessed with the current data set. Secondly, the large number of regression analyses can lead to type I errors. Nevertheless, we feel that this first detailed study of cognition and brain MRI in DM2 patients in relation to metabolic and

vascular risk factors does provide important leads that can be further evaluated in future studies. These studies should preferably have a longitudinal design, should include assessment of cognition and brain MRI in relation to chronic hyperglycaemia and atherosclerotic vascular disease, and should allow the assessment of potential confounders (e.g. hypertension).

We conclude that diabetic encephalopathy in patients with DM2 is a multifactorial condition, for which, in this cross-sectional study, atherosclerotic ('macrovascular') disease is the most important determinant with additional effects of chronic hyperglycaemia, and possibly hypertension. Longitudinal studies are required to determine which of these risk factors are predictors for accelerated cognitive decline.

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Peripheral and central neurologic complications in type 2 diabetes mellitus: no obvious association in individual patients

7

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Introduction

Diabetes mellitus is a common metabolic disorder, and is known for its peripheral complications, such as nephropathy, neuropathy and retinopathy. Diabetes also leads to slowly progressive changes in the brain, a complication referred to as diabetic encephalopathy [1]. The characteristics of this diabetic encephalopathy are becoming increasingly clear, and include changes in cognition, neurophysiological changes and abnormalities on brain imaging.

The pathogenesis of diabetic encephalopathy has not been clarified yet, it but may show similarities to that of peripheral diabetic neuropathy. In fact, some of the processes that have been implicated in the pathogenesis of diabetic neuropathy, such as enhanced flux through the polyol pathway, accelerated formation of advanced glycation end products, oxidative damage and microvascular changes are also detected in the brain [1]. If peripheral and central neurological complications are indeed at least partially due to the same mechanisms and to exposure to the same risk factors, one would expect these complications to occur together in individual patients. Indeed, such an association has been demonstrated in patients with DM1, in whom cognitive dysfunction and peripheral neuropathy appear to co-occur [9;10]. To our knowledge, only two previous studies have related cognitive performance to peripheral neuropathy in patients with DM2 [11;12]. One reported that increases in vibration threshold were associated with a decline in memory [11]. The other study showed a relation between memory dysfunction and autonomic neuropathy, but not peripheral neuropathy [12].

The aim of the present study was to examine if there is an association between peripheral neuropathy and diabetic encephalopathy, as expressed in changes in cognitive function and changes on brain MRI, in patients with DM2.

Patients and methods

Patients

The Utrecht Diabetic Encephalopathy Study aims to identify potential risk factors for cognitive impairment in patients with diabetes [Manschot et al; Diabetes, in press]. Therefore, patients were not selected for the presence or absence of diabetic complications, co-morbid conditions (e.g. hypertension), or exposure to other risk factors (e.g. smoking). For inclusion patients had to be 55 to 80 years of age, with a minimal diabetes duration of 1 year, and had to be functionally independent, and Dutch speaking. Exclusion criteria for all participants were a psychiatric or neurological disorder that could influence cognitive functioning, a history of alcohol or substance abuse and dementia, and a fasting blood glucose ≥ 7.0 for controls [17]. Subjects with a history of non-invalidating stroke could be included. Twice as many patients as controls were included, to increase statistical power for within group analyses in the DM2 group.

122 patients with DM2 (age 56-80 yrs), and 56 controls (56-78 yrs) were included in the present study. General practitioners in the area were asked to participate in the project (see acknowledgements), and to invite all eligible DM2 patients from their practice. Controls were recruited among the spouses or acquaintances of the patients. The study was approved by the medical ethics committee of the University Medical Center Utrecht and each participant signed an informed consent form. All participants underwent a two-day protocol which included brain MRI, a neurological and neuropsychological examination, retinal photography and ultrasonography of the carotid arteries. Also, a standardised questionnaire concerning medical history and medication use was taken, fasting blood and urine samples were collected, and blood pressure was recorded. No neuropsychological examination could be obtained in 1 control and 2 DM2 patients. An MRI was not obtained in 5 controls and 9 DM2 patients, mostly due to MRI contraindications (claustrophobia, pacemaker).

Neurological examination

A standardised questionnaire especially aimed at complaints of neuropathy was taken from every participant. All participants underwent a neurological examination, with special attention for signs of neuropathy. The following sensory modalities were measured: touch, pin prick, vibration sense and joint position sense. The sensory system was graded as follows [15]: touch and pin prick sense normal=4, distal to wrist/ankle abnormal=3, distal half forearm/leg abnormal=2, distal to elbow/knee abnormal =1, distal to axilla/groin abnormal=0. Vibration sense: tuning fork perception (128 Hz) on the middle finger/hallux (big toe) for 10 s=4, decreased on middle finger/hallux=3, ulnar styloid/medial malleolus=2, elbow/knee=1, clavicula/crista or higher=0. Joint position sense of middle finger/hallux normal=2, diminished=1, absent=0. Summation of all sensory modalities could lead to a maximum total sensory sum score of 28 in the arms and 28 in the legs [15]. Vibration sense was assessed quantitatively by means of an electromagnetic vibrator (100 Hz, Vibrameter Type 3, Somedic AB, Stockholm, Sweden)[16]. Thresholds were measured at the dorsum of the second metacarpal bone of the left hand and at the first metatarsal bone of the left foot. The stimulus strength was increased until vibrations were perceived (vibration perception threshold), next the stimulus strength was decreased until the sensation disappeared (vibration disappearance threshold). The average of the vibration perception and the vibration disappearance threshold was taken as the vibration threshold (VT).

Neuropathy was rated with the Toronto Clinical Neuropathy Scoring System (neuropathy scale) [17], with a slight modification, in that we did not perform a sensory test for temperature, so that the maximum score is 18 points (severe polyneuropathy) instead of 19. A score from 0-5 indicated no neuropathy, 6-8 indicated mild neuropathy, 9-11 indicated moderate neuropathy and ≥ 12 indicated severe neuropathy.

Ten standardised questions concerning complaints of autonomic neuropathy were included addressing dry eyes or mouth, palpitations, orthostatic hypotension, changes in sweating, heat intolerance, impotence in men, bladder problems, nausea, diarrhoea and constipation. The number of positively answered questions (0-10, in females 0-9) was used as an indication of the presence of autonomic neuropathy (autonomic symptom score).

Brain MRI

The MRI investigation (1.5 Tesla, Philips Medical systems, Best, the Netherlands) consisted of an axial T1-weighted and an axial T2 and T2 fluid attenuating inverse recovery (FLAIR) scan (TR/TE/TI: 6000/100/2000, FOV 230 mm, matrix 180 x 256, slice thickness 4.0 mm, contiguous slices, 38 slices). The images were printed on hard copy with a reduction factor of 2.9. Ischaemic white matter lesions and atrophy were rated on hard copies, or on digital images on a personal computer.

White matter lesions were rated as described in detail before [Manschot et al; Diabetes, in press], in short, they were distinguished into periventricular lesions and deep (subcortical) lesions and rated according to the Scheltens rating scale [18]. Periventricular white matter lesions (PWML) were rated on a severity scale (0-2) at the frontal and occipital horns and the body of the lateral ventricle, with the total periventricular white matter lesion score being the sum of these three scores (range 0-12).

For the rating of deep white matter lesions (DWML) the brain was divided into six regions: frontal, parietal, occipital, temporal, basal ganglia and infra-tentorial. This is a slight modification of the Scheltens rating scale [18], which divides the basal ganglia an infra-tentorial regions into 5 and 4 different smaller regions. Per region the size and number (n) of the white matter lesions were rated, on a scale ranging from 0 to 6, with the total deep white matter lesion score being the sum of these six scores (range 0-36).

Cortical and subcortical atrophy were rated by measuring different ratios, as described in detail before [Manschot et al; Diabetes, in press]. Cortical atrophy was evaluated by the frontal interhemispheric fissure ratio and the Sylvian fissure ratio [19]. Subcortical atrophy was evaluated by the bifrontal ratio and by the bicaudate ratio)[19].

For analyses these ratios were converted to z-scores: a cortical atrophy z-score (mean of z- frontal fissure ratio and z- Sylvian fissure ratio) and a subcortical atrophy z-score (mean of z-bicaudate ratio and z-bifrontal ratio).

All MRI scans were rated by two raters blinded for diabetic status (S.M.M. and G.J.B.). In case of disagreement of more than 1 point on the white matter lesions scale and the template atrophy scale or more than 2mm on the atrophy ratios, a consensus reading was held. In all other cases the readings of both readers were averaged.

Neuropsychological tests

All participants underwent an extensive neuropsychological examination tapping the major cognitive domains in both a verbal and a non-verbal way. Eleven tasks were administered in a fixed order and took about 90 minutes to complete. These tasks were divided into five cognitive domains and are described previously [Manschot et al; Diabetes, in press]. The first domain is attention and executive function; the second domain is information processing speed; the third domain is memory, which can be divided into four smaller domains: "working memory"; "immediate memory and learning rate" which includes verbal memory and visual memory; "forgetting rate" and "incidental memory"; the fourth domain is abstract reasoning. The fifth domain is visuoconstruction. An estimate of overall premorbid cognitive functioning was determined with the Dutch version of the NART. To control for possible effects of mood disturbances or affective disorders the Beck Depression Inventory [20] was performed and the Symptom Checklist-Revised (SCL-90-R), a self-administered inventory of psychiatric syndromes [21], was completed .

In order to compare the five different cognitive domains the raw-scores were converted into *z*-scores per domain per group. These *z*-scores were calculated based on the mean of the whole group taken together (patients and controls).

Medical history, blood pressure and blood samples

We asked participants about diabetes duration, length and weight and history of hypertension and smoking. Furthermore, all participants measured their blood pressure at home at 10 different time points during the day and of every participant fasting glucose, glycosylated haemoglobin (HbA_{1c}) and fasting triglycerides and cholesterol were determined. Body Mass Index (BMI) was calculated as weight divided by height square. Hypertension was defined as an average systolic blood pressure ≥ 160 mm Hg or diastolic blood pressure ≥ 95 mm Hg or self reported use of blood pressure lowering drugs. Mean Arterial Pressure (MAP) was calculated as follows: ((2 x diastolic blood pressure) + systolic blood pressure)/3. Hypercholesterolemia was defined as a fasting cholesterol > 6.2 mmol/l or self reported use of cholesterol lowering drugs [22].

Statistical analysis

The differences between DM2 and controls were examined with *t*-test for parametric data, Mann-Whitney U for non-parametric data and chi-square test for proportions. All data are shown as mean±SEM, percentage or median (interquartile range (IQR)), unless reported otherwise.

Within the DM2 population, relations between neuropathy scores (including autonomic neuropathy), deficits on brain MRI and deficits in cognitive functioning were calculated by linear regression analysis, adjusted for age, sex and estimated IQ. Because of multiple testing, relations were considered statistically significant when p-values were <0.01.

Results

Clinical data (Table 1)

Patients with DM2 and controls were comparable on age, sex, educational level and estimated IQ (see table 1). In 2 controls and 9 patients with DM2, no MRI could be obtained, due to MRI contraindications, such as claustrophobia or a pacemaker. In 1 control and 2 patients with DM2, no neuropsychological examination could be obtained. Of the 122 DM2 participants, 12 (10%) subjects had only dietary treatment, 75 (61%) received oral antidiabetic drugs and 35 (29%) received insulin with or without oral antidiabetic drugs. Patients with DM2 had more hypertension and used more statins than controls.

Table 1: Characteristics

characteristic	Patients with DM2	controls
Number of subjects(male/female)	122(62/60)	56(25/31)
Mean age	66.0±6	65.1±5
Education level	4(3-5)	4(3-5)
Estimated IQ	99±15	101±14
Diabetes duration (years)	8.7±6	-
HbA1c	6.9±1.2**	5.5±0.3
Fasting plasma glucose (mmol/l)	8.6±2.9**	5.5±0.6
Use of insulin	29%	-
Body Mass Index (kg/m^2)	28.1±4.4	27.3±5.3
Hypercholesterolaemia[#] (%)	60	47
Fasting serum cholesterol (mmol/l)	5.0±0.9**	5.8±1.1
Use of statines	53%**	18%
Fasting triglycerides (mmol/l)	1.9±1.0	1.6±1.1
Hypertension[##] (%)	73%**	34%
Systolic BP (mm Hg)	146±19**	138±19
Diastolic BP (mm Hg)	81±10	78±9

Data are given as means ± SD, median (IQR) or percentage. *p<0.05, **p<0.01
[#] Hypercholesterolemia is defined as a fasting cholesterol > 6.2 mmol/l and / or self reported use of cholesterol lowering drugs.
[##] Hypertension is defined as an average systolic blood pressure ≥ 160 mm Hg and/or diastolic blood pressure ≥ 95 mm Hg and/or self reported use of blood pressure lowering drugs.

Peripheral nervous system

Patients with DM2 had a significant lower sensory sum score of the arms (con: median 28 (range 26-28); DM2: median 28 (range 18-28); p<0.01) and legs (con: median 26 (range 12-28); DM2: median 25 (6-28); p<0.001). Furthermore in patients with DM2 VT was significantly higher compared with controls on the left metacarpal 2 (con: median 0.36 (IQR:

0.26-0.51) DM2 0.56 (IQR: 0.34-1.0); p<0.001) and left metatarsal 1 (con: median 3.2 (0.9-5.8); DM2: median 7.3 (2.5-21.6; p<0.001). Also patients with DM2 had higher scores on the neuropathy scale compared with controls (con: median 1 (0-2); DM2: median 4.5 (2-7); p<0.001). Within the DM2 group, 75 patients had a score from 0-5 indicating no neuropathy, 25 patients had a score from 6-8 indicating mild neuropathy, 18 patients had a score from 9-11 indicating moderate neuropathy, and 4 patients had a score from ≥ 12 indicating severe neuropathy. In short, 39% of the patients with DM2 had neuropathy. In the control group, 49 patients had a score from 0-5 and 7 participants had a score from 6-8.
Concerning autonomic neuropathy, patients with DM2 had significantly more complaints on the standardised questionnaire compared with controls (con: median 1 (0-2); DM2: median 2 (1-3); p<0.001).
Neuropathy was significantly associated with diabetes duration (sensory sum score: p<0.001; VT: p<0.03; neuropathy scale: p<0.001) and HbA_{1c} (sensory sum score: p<0.05; VT: p=0.28; neuropathy scale: p<0.05). A relation with the presence of hypertension (sensory sum score: p=0.07; VT: p=0.9; neuropathy scale: p=0.5) or MAP (sensory sum score: p=0.06; VT: p=0.9; neuropathy scale: p=0.1) was much less obvious.

Central nervous system

Neuropsychological and MRI data

Detailed neuropsychological and MRI data have been reported previously [Manschot et al; Diabetes, in press]. In short, performance of patients with DM2 was worse than controls across all five cognitive domains, with statistically significant differences on the domains attention and executive functioning (difference mean z-scores 0.23 (95%CI 0.03; 0.43); p=0.02), information processing speed (0.40 (0.17; 0.63); p=0.001) and memory (0.20 (0.05; 0.36); p=0.01).
Patients with DM2 had more pronounced cortical atrophy (Difference mean z-scores 0.62 (95%CI 0.33; 0.91); p0<0.001) and subcortical atrophy (0.38 (0.07; 0.68); p=0.01) than controls. Furthermore, patients with DM2 had more severe DWML (controls (median, range): 5 (0-18); DM2: 7 (0.5-27.5); p=0.02), but PWML severity in the two groups was similar (con: 6(4; 10); DM2: 6(3; 12); p=0.13). Patients with DM2 also had more (silent) cerebral infarcts than controls (DM2 22/113, controls 4/54; p=0.06; DM2 12 lacunar, 6 other (i.e. cortical or large subcortical), 4 both lacunar and other; controls 1 lacunar, 3 other).

Relation between peripheral and central neurological complications (table 2)

In the patients with DM2 the sensory sum score of the legs, the VT of the metatarsal 1 and the neuropathy scale were related to the four MRI parameters and five cognitive domains, using linear regression analysis, adjusted for age, sex and estimated IQ. We found no relation between the severity of peripheral neuropathy and the severity of cerebral deficits.

Furthermore, when we related the autonomic symptom score to MRI parameters and the five cognitive domains, no statistically significant relations were seen.

Discussion

Patients with DM2 had a significant lower sensory sum score of the arms and legs, VT was significantly higher compared with controls and patients with DM2 had higher scores on the neuropathy scale compared with controls. Overall, 39% of the patients with DM2 had neuropathy according to the neuropathy scale. Patients with DM2 had more cortical and subcortical atrophy and more deep white matter lesions than controls and the overall performance of the patients with DM2 on the five cognitive domains was worse. However, the severity of peripheral neuropathy or the autonomic symptom score and the severity of cerebral deficits in patients with DM2 were unrelated.

The presence of neuropathy in this population of patients with DM2 was comparable to large population based studies in the Netherlands and other developed countries [23-25]. Since there is no "gold standard" available for the assessment of diabetic neuropathy, three different methods were used in combination to determine the (severity of) diabetic neuropathy. Neurological examination was performed according to a standardised protocol, especially aimed at sensory signs of neuropathy [15]. The VT is a well accepted standardised quantitative sensory test that is often used in the assessment of diabetic neuropathy [26]. The Toronto Clinical Neuropathy Scoring System is a validated clinical rating scale that reflects the presence and severity of diabetic neuropathy and correlates with quantitative measures such as sural nerve morphology and electrophysiology [17].

Only a few studies have reported on the possible relation between peripheral neuropathy and cognitive deficits in patients with DM2. One study compared 140 patients with DM2 with 38 controls on three cognitive tests (memory-related and reaction time)[11]. There was no relation between diabetes duration, fasting glucose and autonomic neuropathy on the one side and cognitive performance on the other, but both age and increases in VT were associated with a decline in memory. Possibly, age
was a confounder in the observed relation between vibration threshold and cognition, because both cognition and vibration sense are both age-dependent.

Table 2: Relations between peripheral neuropathy and MRI abnormalities, cognitive domains and autonomic neuropathy, adjusted for age, estimated IQ and sex, expressed as B(CI).

	Sumscore legs	VT legs	Neuropathy score	Autonomic neuropathy
PWML	-0.008 (-0.08/0.07)	0.008 (-0.02/0.03)	-0.02 (-0.11/0.07)	-0.03 (-0.21/0.15)
DWML	0.19 (-0.02/0.40)	0.02 (-0.06/0.10)	-0.2 (-0.5/0.03)	-0.3 (-0.9/0.2)
Cort atrophy	0.01 (-0.02/0.05)	-0.003 (-0.015/0.010)	-0.02 (-0.07/0.02)	0.01 (-0.08/0.10)
Subcort atrophy	0.006 (-0.03/0.05)	0.002 (-0.01/0.02)	-0.02 (-0.06/0.03)	0.004 (-0.09/0.10)
AEX	0.02 (-0.007/0.04)	-0.004 (-0.01/0.004)	0.0004 (-0.03/0.03)	-0.05 (-0.11/0.009)
IPS	0.009 (-0.03/0.05)	0.0004 (-0.01/0.01)	0.007 (-0.04/0.05)	-0.03 (-0.12/0.07)
Memory	0.01 (-0.006/0.03)	-0.006 (-0.01/0.001)	-0.007 (-0.03/0.02)	-0.01 (-0.06/0.04)
AR	0.04 (-0.002/0.08	-0.002 (-0.02/0.01)	-0.01 (-0.06/0.04)	-0.10 (-0.20/-0.005)
VC	0.004 (-0.04/0.05)	-0.0007 (-0.02/0.01)	-0.01 (-0.07/0.04)	-0.08 (-0.19/0.03)

For both white matter lesions and atrophy, positive B values indicate that abnormalities on a measure of neuropathy are related with more severe MRI abnormalities.
For the five cognitive domains, negative B values indicate that abnormalities on a measure of neuropathy are related with more severe deficits in cognition.
AEX= attention and executive function; IPS= information processing speed; AR= abstract reasoning;
VC= visuoconstruction.

When we related measures of peripheral neuropathy to the five cognitive domains without correction for age in our study, the severity of peripheral neuropathy was related to a lesser performance on the domain memory (results not shown). Another study found an association (after correction for age, education, diabetes duration and fasting plasma glucose) between autonomic neuropathy (evaluated by the Ewing tests [27]) and visual memory; 20 diabetic patients with autonomic neuropathy achieved significantly lower scores on visual memory tests than 29 patients with DM2 without autonomic neuropathy and 34 non-diabetic controls[12]. Approximately 15% of the diminished performance in visual memory tests was explained by the presence of autonomic neuropathy[12]. The presence of peripheral neuropathy, evaluated by clinical criteria, was not associated with cognitive performance in patients with DM2[12]. The characteristics (e.g. HbA_{1c}, total cholesterol, triglycerides, blood pressure and body mass index) of the patients with DM2 that were included in the present study are similar to those reported in large population based surveys of DM2 subjects in the Netherlands[30;31]. Mean HbA_{1c} was 6.9% in our DM2 population, which indicates a moderately well controlled diabetes. Our patients differ from the patients with DM2 in the study by Zaslavsky et al [12] in mean age (60 years compared to 66 years in our study) and HbA1c (8.5% compared to 6,9% in our study). Besides differences in the assessment of autonomic neuropathy between Zaslavsky's study and ours, these differences in age and HbA_{1c} might also explain why we found no relation between autonomic neuropathy and cognitive performance in our study.

An association between peripheral neuropathy and cognitive performance has been demonstrated more clearly in patients with DM1, in whom cognitive dysfunction and peripheral neuropathy usually occur together. Adult patients with DM1 usually show modest performance deficits on a wide range of neuropsychological tests compared with non-diabetic controls. A recent meta-analysis of studies on cognition in DM1 showed modest but significant reductions of overall performance, including a mild to moderate impairment of overall intelligence, speed of information processing, psychomotor efficiency, visual and sustained attention, mental flexibility and visual perception in patients with DM1 [2]. Ryan et al showed that clinically significant distal symmetrical polyneuropathy was strongly associated with psychomotor slowing [9]. Furthermore, in patients with DM1 with one or more complications (distal symmetrical polyneuropathy; advanced background or proliferative retinopathy; overt nephropathy; severe hypoglycaemia) regression analysis indicated that a diagnosis of polyneuropathy was the best biomedical predictor of cognitive test performance [10]. Autonomic neuropathy has also been related to a decline in psychomotor speed in patients with DM1 [28]. In the prospective part of that study, incident autonomic neuropathy, measured by heart rate variability, showed a relation with psychomotor slowing. There was a trend towards a relation between incident distal symmetric polyneuropathy and psychomotor slowing in the same group. However, Lawson et al [29] did not

find a relation between peripheral neuropathy or autonomic neuropathy and cognitive performance in a group of 45 patients with DM1.

In DM1, hyperglycaemia-related microvascular complications, such as polyneuropathy, seem to be a strong predictor of a decline in cognitive performance [9;10], and peripheral and central neurological complications thus seem to share pathogenetic mechanisms. The present observations indicate that the mechanisms underlying peripheral and central neurological complications may differ in DM2. Whereas neuropathy is known to be associated with exposure to hyperglycemia (diabetes duration and HbA_{1c}) [review[32]], we only found a modest relation between hyperglycemia (diabetes duration and HbA1c) and cerebral complications in patients with DM2, and no relation between the presence or severity of peripheral neuropathy and cerebral complications in DM2. This suggests that other mechanisms, such as vascular co-morbidity associated with DM2, might play an important role in the development of these cerebral complication. This might be an important subject in future studies.

In conclusion, we did not find a relation between peripheral neuropathy and cerebral deficits in patients with DM2, in contrast to previous studies in DM1 patients. This indicates that central and peripheral neurological complications of DM2 may not share the same mechanisms and risk factors. In peripheral complications hyperglycemia appears to be the driving factor. In cerebral complications the link with hyperglycemia is less evident and other, possibly vascular, mechanisms may be involved.

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Cerebrovascular reserve capacity is preserved in a population-based sample of patients with type 2 diabetes mellitus

8

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Cerebrovascular Diseases, in press

Introduction

The risk of ischemic stroke is increased in patients with diabetes mellitus (DM) and outcome after stroke is worse compared to non-diabetic subjects[1]. In addition, DM is associated with more insidious changes in the brain, which are reflected in cognitive impairments, particularly in elderly type 2 DM (DM2) patients [2].

Both structural and functional vascular disturbances can affect the integrity of the brain in diabetic patients [1]. Structural changes can be found both in large (e.g. atherosclerosis of carotid and intracranial arteries) and in small (e.g. basement membrane thickening of cerebral capillaries) vessels [1]. Functional disturbances include regional changes in cerebral perfusion and impaired cerebrovascular reserve capacity (CVR). In patients with DM1, CVR has been shown to be decreased in patients with a long (>10-15years) disease duration [3] and in patients with autonomic neuropathy [4;5]. Previous studies in DM2 patients have shown similar results, mostly reporting impaired CVR only in selected of patients with advanced complications or long disease duration [6-9]. These findings may not be representative for the general DM2 population. Therefore, the aim of the current study was to evaluate CVR in a population based sample of DM2 patients, and to relate CVR to cognitive impairments and factors that potentially affect CVR, such as hypertension, the use of vasoactive drugs, use of statines, diabetes duration, metabolic control, atherosclerosis of the major supplying arteries of the brain (Carotid Intima/Media Thickness (CIMT)) and small vessel disease (retinopathy and micro-and macroalbuminuria).

Methods

Patients and Controls

Patients were recruited from the Utrecht Diabetic Encephalopathy Study (UDES), which is a cross-sectional, population based study on determinants of impaired cognition in patients with DM. The study protocol includes a neurological and neuropsychological examination, laboratory investigations, brain MRI, and a transcranial Doppler (TCD) examination. For the UDES 122 patients with DM2 (age 56-80 yrs) and 61 controls (53-78 yrs) were included between September 2002 and November 2004. For inclusion DM2 patients had to be 55 to 80 years of age, with a minimal diabetes duration of 1 year, and had to be functionally independent, and Dutch speaking. Exclusion criteria for all participants were a psychiatric or neurological disorder (unrelated to diabetes) that could influence cognitive functioning, a history of alcohol or substance abuse and dementia, and a fasting blood glucose ≥ 7.0 for controls. Twice as many patients as controls were included, to increase statistical power for within group analyses in the DM2 group. Patients were recruited through their general practitioner. Controls were recruited among the spouses or acquaintances of the patients. The study was approved by the medical ethics committee of the University Medical Center Utrecht and each participant signed an informed consent

form.
102 DM2 patients underwent a TCD examination. In 21 of these patients no reliable CVR reading could be obtained, mostly because of lack of a penetrable window for the ultrasound through the temporal bone. The present study includes all DM2 patients (n=81, age 55-79 yrs), and all control subjects (n=38, 55-78 yrs) with a reliable CVR reading that were at least 55 years of age.

Subject characteristics
Weight and height were recorded and expressed as body mass index in kg/m². Hypertension was defined as a mean systolic blood pressure above 160mmHg or a mean diastolic pressure above 95mmHg, or the use of antihypertensive medication. Cholesterol, LDL-cholesterol, HDL-cholesterol, triglycerides, HbA1c, and fasting glucose level were assessed. Hypercholesterolemia was defined as a cholesterol/HDL-cholesterol-ratio higher than 5[10] or the use of cholesterol-lowering drugs. An overnight urine sample was collected to assess urinary albumin or protein excretion. Albuminuria was defined as a urine albumin level above 30 mg/l.

Fundoscopy
Single-field fundus photography was performed with a 50-degree retinal camera (Zeiss FF 450) [11]. Two graders, who were blinded for patient characteristics, independently evaluated the slides for the presence and severity of diabetic retinopathy. Retinopathy was defined present at or above grade 1.5 on the Wisconsin scale [12] or when patients had been treated with photocoagulation.

Transcranial Doppler (TCD) examination
TCD examination including CO2 reactivity was performed as described previously [13]. Both mean blood flow velocities (mBFV) and Gosling's Pulsatility index (PI) ($BFV_{systolic}$ – $BFV_{diastolic}$)/BFV_{mean}) [14] were assessed bilaterally in the major cerebral arteries. During the CO2 reactivity test, the CO2 content of the expired air was monitored continuously. CO_2-reactivity, as a measure of CVR, was determined as the relative change in blood flow velocity in the MCA after 1.5 minutes of carbogene inhalation.

Neuropsychological evaluation
As described previously, subjects underwent an extensive neuropsychological evaluation [15]. On the basis of the scores of 11 neuropsychological tests addressing different cognitive domains, patients were classified as having CIND (cognitive impairment, no dementia), or as having no cognitive impairment [15].

CIMT measurement
The CIMT was measured in both carotid arteries in a 1-cm trajectum proximal to the beginning of the dilatation of the carotid bulb in three

different longitudinal projections and was calculated as the mean of these six measurements as described previously [16].

Statistical analysis

All analyses were performed with the Statistical Package for the Social Sciences (SPSS). After testing for equality of variance, the student *t* test was used to assess patient characteristics if these were evenly distributed. Binominal distributed parameters were analysed using the chi-square test or the Fisher's exact test. Between group differences for TCD measurements were analysed with a Univariate General Linear Model adjusting for age and sex and additionally for potential mediating factors such as hypertension and use of anti-hypertensive drugs. A $p<0.05$ was regarded as statistically significant.

Results

Clinical data (Table 1)

DM2 patients were slightly older than the controls, and as expected, hypertension was more common among the patients. The excluded subjects, from whom no reliable TCD signal could be obtained, were comparable in age and disease duration, though consisted of more women than the study group.

Transcranial Doppler (Table 2 and 3)

mBFV and mean PI, adjusted for age and sex, were similar in all arteries in patients and controls.

The percentage increase in CO_2 content in the exhaled air after carbogene administration did not differ between the groups, indicating a similar vasodilatory stimulus. CVR did not differ between patients and controls (51.3 vs. 47.8%, mean difference 3.5%, confidence interval -4.8:11.8%). IMT was similar in patients and controls (0.093 vs. 0.098cm, mean difference -0.06(-0.015:0.003)).

Within the DM2 group there was no association between CVR and vascular risk factors, diabetes characteristics or CIND. DM2 patients with retinopathy tended to have lower CVR values than patients without retinopathy (46.3 vs. 54.5%, mean difference -7.9(-18.0:2.2)) (Table 3).

Table 1: Clinical characteristics

Parameter	**Patients** (n = 81)	***Controls*** (n = 38)
Age(y)	66.7±6.0	63.4±5.2
Sex (%male)	56	42
Body Mass Index (kg/m^2)	28.3±4.5	28.0±5.6
Hypertension(%)	78	29
Systolic blood pressure(mmHg)	149±18	138±16
Diastolic blood pressure(mmHg)	81±9.9	79±8.6
Antihypertensive drugs(%)	76	29
Hypercholesterolaemia(%)	57	41
Cholesterol-lowering drugs(%)	51	16
Diabetes duration(y)	9.4±6.2	
Retinopathy(%)	33	0
CIND (%)	46	21
Laboratory		
Fasting blood glucose(mmol/l)	8.6±2.8	5.5±0.58
HbA_{1c}(%)	6.9±1.0	5.5±0.37
Triglyceride(mmol/l)	1.9±1.0	1.7±1.1
LDL-cholesterol	2.9±0.91	3.8±0.92
Total cholesterol/HDL	4.4±1.3	4.3±1.4
Albuminuria(%)	19	8

Values are mean ± SD.

Table 2: Mean Blood Flow Velocity and Gosling's Pulsatility index

Artery	Patients		Controls		Difference	
	mBFV	PI	mBFV	PI	mBFV	PI
MCA_{left}	55.0	1.05	51.5	0.99	3.5(-2.2:9.3)	0.06(-0.02:0.14)
MCA_{right}	52.7	1.12	53.3	1.03	-0.57(-5.6:4.4)	0.09(-0.02:0.19)
ACA_{left}	45.4	1.13	42.3	1.05	3.1(-1.8:8.0)	0.07(-0.02:0.16)
ACA_{right}	46.3	1.10	47.8	1.04	-1.5(-7.0:3.9)	0.06(-0.03:0.14)
PCA_{left}	32.6	1.15	31.5	1.11	1.0(-3.0:5.1)	0.04(-0.06:0.15)
PCA_{right}	33.7	1.12	32.0	1.07	1.7(-2.2:5.5)	0.06(-0.04:0.16)
BA	35.2	1.11	35.1	1.10	0.06(-4.3:4.4)	-0.01(-0.09:0.12)

mBFV in cm/s, Gosling's pulsatility index (PI) and mean between group differences for mBFV and PI, adjusted for age and gender, are given with 95% confidence intervals.

Table 3: Mean CVR in DM2 in relation to disease variables

Variable	Present	Absent	Mean difference and 95% confidence interval
HbA1c>10	53.2±15.2	50.5±19.7	3.2 (-5.2:11.7)
Disease duration>10y	55.8±17.0	48.3±18.5	5.8 (-2.9:14.4)
Albuminuria	46.3±8.3	50.4±19.6	-3.7 (-15.5:8.0)
Retinopathy	46.3±17.0	54.5±18.9	-7.9 (-18.0:2.2)
CIMT>1.10	53.3±17.2	50.7±19.0	2.5 (-8.5:13.6)
Hypertension	49.9±17.9	56.0±18.6	-4.8 (-14.6:5.1)
Diastolic pressure>95mmHg	50.0±17.4	51.5±18.4	-0.01 (-11.6:11.6)
Systolic pressure >160mmHg	52.9±15.2	50.8±18.9	0.9 (-9.2:11.0)
Use of vasoactive drugs	49.9±18.4	54.3±17.2	-3.5 (-13.1:6.0)
CIND	52.4±18.6	49.1±16.9	3.2 (-4.9:11.2)
Use of insulin	52.4±18.6	49.1±16.9	3.9 (-4.8:12.6)

The effect of disease variables on CVR was assessed by dichotomising the DM2 group according to the above specified cut-off points. Mean CVR in the subgroups and mean difference with 95% confidence interval, adjusted for age and gender are indicated (%).

Discussion

This study shows that CVR in a population-based sample of patients with DM2 is similar to that in controls. CVR was not affected by diabetes duration, metabolic control, the presence of hypertension, CIMT, or albuminuria. CVR tended to be lower in DM2 subjects with retinopathy, but this effect was not statistically significant.

A strength of the present study is that patients were recruited from the general population. Patients were not selected for the presence or absence of diabetic complications, co-morbid conditions (e.g. hypertension), or exposure to other risk factors (e.g. smoking). Indeed, our DM2 patients were comparable to patients of previous population-based studies in the Netherlands with respect to mean blood pressures, metabolic control (HbA1c), BMI, smoking habits, lipid profiles, and the prevalence of microvascular complications, such as retinopathy and albuminuria [17;18]. A possible limitation of our design is that despite the relatively large sample size, the actual number of patients with advanced DM associated complications is small, as might be expected from a population-based sample. Therefore, the present data give a good indication of CVR in patients with relatively uncomplicated DM2, but our findings may not be applicable to patients with advanced complications, such as for example severe autonomic neuropathy. This may explain some of the discrepancies with previous studies of CVR in DM2, that mostly involved selected patient populations, recruited in a hospital setting, and had relatively small (<50) sample sizes. These studies showed disturbed CVR in selected patients, such as patients with a prolonged disease duration (more than 10-15years) [6;7], patients with advanced retinopathy [8], and insulin-dependent patients assessed during anaesthesia for major surgery [9].

Despite the observation that CVR was preserved in our study population, the patients in our sample did exhibit impairments of cognition relative to controls [19]. Moreover, on MRI more pronounced cortical and subcortical atrophy and white matter hyperintensities were noted [Manschot et al; Diabetes, in press].

We conclude that CO_2 reactivity of the MCA is not impaired in an unselected population-based sample of DM2 patients and that disturbances of CVR are probably not a prime determinant of cognitive impairments in these patients.

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General discussion

9

This thesis describes two interrelated research projects on the effects of diabetes on the brain. The first project addressed the role of vascular disturbances in the development of experimental diabetic encephalopathy in rats. In the second project we examined associations between vascular risk factors, cognitive functioning and structural changes in the brain in patients with type 2 diabetes mellitus (DM2).

Animal study

The aim of the first part of this thesis was to investigate the role of vascular disturbances in the development of experimental diabetic encephalopathy. In **chapter 2**, we found that experimental diabetes is associated with reduced cerebral perfusion, and treatment with enalapril partially improved cerebral blood flow, deficits in Morris maze learning and hippocampal synaptic plasticity. In **chapter 3** we found that STZ-diabetes is associated with deficits in peripheral nerve conduction velocity and increased evoked potential latencies and that treatment with enalapril could partially prevent or reverse these impairments. In **chapter 4** we found that long term treatment with enalapril prevented deficits in nerve conduction velocity, and partially prevented deficits in BAEP and VEP latencies, comparable to the previous study in **chapter 3**. However, we observed an increased mortality in the long-term enalapril treated animals, probably due to hypotension in combination with the effects of the STZ-model

We thus showed that impaired cerebral perfusion probably plays an important role in the development of diabetic encephalopathy in experimental diabetes, and showed for the first time that treatment aimed at improvement of perfusion of the brain can partially prevent deficits in cerebral function.

Advantages of the STZ-model

The STZ-diabetic rat model has been used extensively in studies on the pathophysiology of diabetes and its complications. STZ-rats are hypoinsulinemic and hyperglycaemic (blood glucose levels are 25-30 mmol/l (normal 5 mmol/l), but do not require insulin treatment to survive. Like diabetic patients, STZ-diabetic rats develop end-organ damage affecting the eyes, kidneys, heart, blood vessels and nervous system. In this thesis it is clearly shown that untreated STZ-diabetic rats develop features of cerebral dysfunction that can be encountered also in patients, such as impaired cognition and increased evoked potential latencies, in line with previous studies with this model[1]. The fact that impairments of cerebral function have been reported also in models of type 1 and type 2 diabetes, in which glucose levels are usually less elevated [1;2], supports the notion that the impairments that are observed in the STZ-model do not just reflect the severity of the diabetic state, or STZ toxicity. The main advantage of the STZ-model is that it is well established and that investigators in our laboratory had extensive experience with this model. Taken together, when we started this project, the STZ model was the most

appropriate model to test our hypothesis that vascular insufficiency plays a major role in the pathogenesis of diabetic encephalopathy.

Disadvantages of the STZ-model

The injection of STZ causes damage to the insulin producing β cells of the islets of Langerhans, and causes an actual insulin shortage. Therefore the endocrinological features of the model are different from patients with DM2 in whom there is mainly an insulin resistance that results in hyperglycaemia. Moreover, insulin resistance is a gradually progressing disorder in contrast to the STZ injection, which causes hyperglycaemia within a few days. Also, DM2 in humans is associated with vascular co-morbidity, whereas the STZ-model lacks this co-morbidity. These differences in the aetiology of diabetes makes the STZ-model less comparable to the patient with DM2.

In **chapter 4** we encountered another drawback of this model. In the STZ-diabetic model, animals are polyphagic, polyuric and polydipsic, and tend to loose 10-20% of their initial weight in the months after STZ-injection. The combination of cachexia and the high fluid turnover may have made these animals extra vulnerable to the effects of hypotension due to high-dose enalapril, particularly after prolonged diabetes. Therefore, the increased mortality in the enalapril treated animals clearly shows the limitations of the STZ-diabetes model, particularly in studies on the long-term outcome.

There are several other, mostly genetic, type 1 and 2 diabetic models. The Zucker rat is a hyperphagic, hyperlipidaemic and obese rat, which also expresses peripheral insulin resistance, hyperinsulinemia and an impaired glucose tolerance [1;3]. Hyperglycaemia is usually mild. The OLETF rat is characterised by mild obesity and an impaired glucose tolerance, followed by hypertriglyceridemia followed by hyperinsulinemia, hyperglycaemia and finally hypoinsulinemia [2;3]. Both models certainly mimic DM2 to some extent, but the genetic defect that underlies each of them is not the primary defect encountered in humans with DM2. Observations concerning impairments in cognition and synaptic plasticity in these models are much more variable compared to observations in STZ-models. The genetic profile of these models appears to be an important source of this variation, and is thought to have a direct impact on the brain.

Conclusion and implications for further research

In conclusion, the STZ-diabetic model should be the preferred model to study the effects of diabetes on the brain, but has some important disadvantages. For further research into the pathogenesis and treatment of diabetic encephalopathy, there is a need for a mixed animal model of vascular disease, hypertension and diabetes. Such a model should serve to study the causes of cognitive impairments by unravelling the mechanisms by which diabetes interacts with chronic or acute ischemic disease.

Clinical study

The aim of the second part of this thesis was to examine associations between vascular risk factors, cognitive functioning and structural changes in the brain in patients with DM2. To do so, we initiated the Utrecht Diabetes Encephalopathy Study (UDES), a large cross-sectional study involving 125 patients with DM2 and 64 matched non-diabetic controls.
The primary objectives of the UDES were:

- To assess cognitive dysfunction in patients with DM2 and quantify structural cerebral changes with magnetic resonance imaging.
- To relate these structural changes to cognitive dysfunction.
- To assess the relationship between measures of micro- and macrovascular function on the one hand and cognitive dysfunction and structural cerebral changes on the other in patients with DM2.

In **chapter 5**, we found that patients with DM2 had more cortical and subcortical atrophy and more deep white matter lesions and infarcts on brain MRI than controls. The overall performance of patients with DM2 on the neuropsychological examination was worse, particularly affecting the domains attention and executive functioning, information processing speed and memory. Within the DM2 group cognitive function was inversely related with white matter lesions, atrophy and the presence of infarcts.
In **chapter 6**, we found that patients with DM2 had more microvascular complications, more macrovascular (atherosclerotic) disease and more hypertension than controls. Within the DM2 group impaired cognitive functioning was associated with higher HbA_{1c} levels, brain infarcts on MRI and a history of vascular events. DM2 patients with retinopathy or with infarcts on MRI had more severe cortical atrophy, whereas insulin levels, mean arterial pressure, and macrovascular disease were associated with white matter lesions. DM2 patients that used statins had less pronounced cortical atrophy and periventricular white matter lesions. In **chapter 7** we did not find a relation between peripheral neuropathy and cerebral deficits in patients with DM2, in contrast to previous findings in DM1 patients. This suggests that peripheral and central neurological complications in DM2 develop independently form each other, suggesting that the underlying mechanisms of these complications are not identical.
In **chapter 8** we showed that cerebrovascular reserve capacity (CVR) in the patients with DM2 from the UDES population was similar to that in controls. CVR was not affected by diabetes duration, metabolic control, the presence of hypertension, intima/media thickness, or albuminuria. Hence, disturbed CVR does not appear to be an important determinant of impaired cognitive functioning and changes on brain MRI in patients with DM2.
In conclusion, diabetic encephalopathy is a multifactorial condition, for which a history of macrovascular atherosclerotic disease seems to be the most important risk factor. Hyperglycaemia, vascular risk factors like hypertension and microvascular complications seem to be of lesser importance.

Risk factors for diabetic encephalopathy: results from previous studies

Previous studies have reported on possible determinants of impaired cognition and increased risk of dementia in patients with DM2. The following section provides a brief overview of these studies. There are no published studies that provide detailed data on determinants of MRI changes.

Hypertension:

Several previous studies have provided exploratory analysis into the modulating effect of hypertension on cognitive function in DM2. In one study, the underlying mechanisms of diabetes and hypertension (systolic blood pressure>180 mmHg) interacted to affect the relative risk of any dementia, Alzheimer's disease and in particular vascular dementia [4]. Two other studies, however, reported that adjusting for hypertension and other vascular risk factors had no effect on the relative risk of dementia in diabetic patients [5;6]. In the relation between diabetes and hypertension in studies on cognitive changes short of dementia there is similar controversy. Some studies suggest that hypertension is an important mediator. The Framingham study, for example, found that history and duration of DM2 and high blood pressure were significant risk factors for poor cognitive performance[7], in particular on tests measuring visual organisation and memory. Another study found the greatest cognitive decline (measured with the Mini Mental State Examination) among persons with co-morbid diabetes and hypertension [8]. Other studies showed that the effect of diabetes on cognition was independent of blood pressure [9-11].

Dyslipidaemia:

Different previous studies have shown that dyslipidaemia is associated with cognitive decline in patients with DM2. In one study, serum total and very-low-density lipoprotein triglycerides were negatively related to verbal fluency in patients with DM2 [21]. Another study showed that patients with DM2 and high triglyceride levels showed reduced memory and attention and were significantly slower on a simple reaction time measure compared to patients with DM2 with low triglyceride levels [22]. This finding was also replicated by a cross-sectional study, which reported a negative correlation between fasting triglycerides and reaction time measures as well as between fasting cholesterol and auditory and visual attention in patients with DM2 [16].

Diabetes related risk factors

Diabetes duration:

In some previous studies, a longer duration of diabetes was associated with poorer cognitive performance [7;11]. Two studies showed that screening identified cases of diabetes (which presumably have a shorter or less severe exposure to hyperglycemia) had a lower risk of dementia than people with a known history of diabetes [5;12]. Another study showed no

relation between diabetes duration and the risk of dementia [13].

Glycemic control:

Previous studies showed that chronic (modest) hyperglycaemia, as reflected in elevated HbA1c levels are associated with relative impairments of cognition in untreated patients with DM2, as well as non-diabetic subjects with impaired glucose tolerance. Also, in treated patients with DM2, patients with higher HbA1c levels have worse performance on cognitive tests compared with patients with lower HbA1c levels. [14-16]. Other studies found no relation between HbA1c and cognitive function [17-19].

Treatment of diabetes:

The Rotterdam study found that patients with DM2 that received insulin had the highest risk of developing dementia [12]. The Framingham study and the Washington Heights-Inwood Columbia Ageing Project also found an increased risk of severe cognitive impairment associated with insulin treatment [7;20]. Whether these results show the severity of diabetes, or an effect of insulin treatment itself is unknown.

Microvascular complications:

In one study, a decline in memory in patients with DM2 was associated with both age and increases in vibration threshold, but not with autonomic neuropathy [15]. A second study found an association between autonomic neuropathy and visual memory, but not with peripheral neuropathy [19]. Another study found no relation between microvascular disease (retinopathy and nephropathy) and cognitive decline in patients with DM2 [10].

Conclusions and implications for future research

In conclusion, as shown in the section above, several different studies show conflicting results concerning risk factors for the development of diabetic encephalopathy. Most of these studies are large cross-sectional studies, which, unlike the UDES, were not specifically designed to assess the effects of diabetes on the risk of cognitive deficits, but rather aimed to identify risk factors for cognitive dysfunction or dementia in the general elderly population. Also, the methods used to assess cognitive function in several of these studies were often not sensitive enough to detect the relatively mild cognitive deficits found in patients with DM2. In the UDES we combined for the first time an extensive neuropsychological examination with brain MRI in patients with DM2 to characterise diabetic encephalopathy and specifically tried to identify potential risk factors for the development of this encephalopathy. Despite the fact that we compared a relatively large population of DM2 patients with controls, it remains difficult to determine the relative importance of each of the potential determinants of diabetic encephalopathy separately. There is probably an interaction between age and diabetes on cognition and brain MRI changes. In order to eliminate strong confounding intra-individual factors (like age) it is necessary to perform a longitudinal study, in which the participants of this cross-sectional study will be re-investigated 4 years

after their initial participation. This longitudinal study has been started recently. We expect this study to give an even better insight in the relation between changes in cognition and on brain MRI and relevant disease variables in DM2 patients.

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Summary

9

Diabetes mellitus is associated with slowly progressive changes in the brain, a complication that is referred to as diabetic encephalopathy. Previous studies show that patients with type 2 diabetes mellitus (DM2) have mild to moderate impairments in attention and executive functioning, information processing speed and memory. Patients with DM2 also show changes on brain MRI, such as cortical and hippocampal atrophy.
The pathogenesis of diabetic encephalopathy is likely to be to be a multifactorial process involving adverse effects of chronic hyperglycaemia, on the brain. Vascular disturbances are also likely to play an important role, but up to now, this has not been investigated systematically.

This thesis includes two interrelated research projects. The first project addressed the role of vascular disturbances in the development of experimental diabetic encephalopathy in rats. In the second project we examined associations between vascular risk factors, cognitive functioning and structural changes in the brain in patients with type 2 diabetes mellitus.

In **chapter 2**, we examined whether experimental diabetes is associated with reduced cerebral blood flow and whether treatment with enalapril can improve cerebral perfusion and function (blood flow and functional cerebral deficits). Streptozotocin-diabetic rats were treated with the Angiotensin Converting Enzyme (ACE)-inhibitor enalapril (24 mg/kg) from onset of diabetes. After 14 weeks of diabetes, 12 enalapril treated and 12 untreated diabetic rats, and 12 nondiabetic age-matched control rats were tested in a spatial version of the Morris water maze. After 16 weeks of diabetes, in the same groups, blood flow in the hippocampus and thalamus was measured by hydrogen clearance microelectrode polarography. In a separate study, hippocampal long-term potentiation was measured after 26 weeks of diabetes. Water maze performance and hippocampal long-term potentiation were impaired in diabetic rats. Furthermore, blood flow in diabetic rats was reduced by 30% ($p < 0.001$) in the hippocampus and by 37% ($p < 0.005$) in the thalamus compared to nondiabetic controls. Enalapril treatment significantly improved water maze performance ($p<0.05$), hippocampal long term potentiation ($p<0.05$) and hippocampal blood flow ($p<0.05$).
We conclude that cerebral perfusion is reduced in diabetic rats compared to controls. Treatment aimed at the vasculature can improve cerebral blood flow, deficits in Morris maze performance and long term potentiation. These findings suggest that vasculopathy plays a role in the development of cerebral dysfunction in diabetic rats.

In **chapter 3**, we examined if prevention and intervention treatment with enalapril could improve peripheral and central neurophysiological deficits in streptozotocin diabetic rats.
Sciatic nerve conduction velocities were measured prior to diabetes induction and again every 3 weeks. In the prevention study the final nerve

conduction measurements were performed at 15 weeks, in the intervention study at 24 weeks. Brainstem auditory and visual evoked potential latencies were measured every 3 weeks from 10 weeks after diabetes induction onwards. In the prevention study the final evoked potential measurements were performed at 16 weeks, in the intervention study at 25 weeks. Treatment with enalapril was started directly after diabetes induction (prevention treatment) and after 15 weeks of diabetes (intervention treatment).
Nerve conduction velocity, brain stem auditory evoked potential latencies and visual evoked potential latencies were impaired in diabetic rats. Enalapril prevented deficits in nerve conduction velocity ($p<0.001$), brain stem auditory evoked potential latencies ($p<0.01$) and visual evoked potential latencies ($p<0.005$). Enalapril intervention treatment had no effect on nerve conduction velocity and on visual evoked potential latencies, but improved brain stem auditory evoked potential latencies ($p<0.05$) after 10 weeks of treatment.
In conclusion, enalapril partially prevents the development of neurophysiological alterations in the peripheral and central nervous system and partially reverses deficits in brain stem auditory evoked potential latencies in STZ-diabetic rats.

In **chapters 2 and 3** it was shown that treatment with enalapril (24 mg/kg) prevented neurophysiological and cognitive deficits in streptozotocin diabetic rats, and improved cerebral blood flow, despite a reduction in systemic mean arterial blood pressure. In **chapter 4**, we examined if these effects could be sustained with long-term treatment, and if treatment with a lower dose (12 mg/kg) could prevent peripheral and central neurophysiological deficits without causing hypotension. Sciatic nerve conduction velocities were measured every 3 weeks after diabetes induction, until 24 weeks. Brain stem auditory (BAEP) and visual evoked potentials (VEP) were measured every three weeks from 10 weeks after diabetes induction, until 25 weeks. Nerve conduction velocity was decreased, and BAEP and VEP latencies increased in untreated diabetic rats. At the end of follow-up 12 mg/kg enalapril partially prevented evoked potential abnormalities, but not nerve conduction deficits, whereas 24 mg/kg enalapril largely prevented deficits in nerve conduction velocity ($p<0.001$), as well as BAEP ($p<0.01$) and VEP latencies ($p<0.05$). Mean arterial blood pressure was 122 mmHg in the untreated diabetic group, 75 mmHg in the 24 mg/kg group and 112 mmHg in the 12 mg/kg group. Sustained treatment with enalapril at 24 mg/kg was associated with increased mortality, which may be related to the marked hypotension at this dosage. We conclude that long-term treatment with enalapril at a dose of 24 mg/kg can prevent peripheral and central neurophysiological deficits in streptozotocin diabetic rats, but that adverse effects preclude sustained treatment.

The second part of this thesis examined associations between vascular risk factors, cognitive functioning and structural changes in the brain in patients with DM2. Therefor, we initiated the Utrecht Diabetes Encephalopathy Study (UDES), a large cross-sectional study involving 125 patients with DM2 and 64 matched non-diabetic controls.

In **chapter 5**, we compared cognition and brain MRI in DM2 patients to non-diabetic controls. In addition, the relation between cognition and MRI findings, blood pressure and metabolic control was assessed. Brain MRI scans were obtained from 113 patients and 51 matched controls and rated for white matter lesions, cortical and subcortical atrophy and infarcts. Neuropsychological test scores were divided into 5 cognitive domains and expressed as standardised *z*-values.
DM2 was associated with deep white matter lesions ($p=0.02$), cortical ($p<0.001$) and subcortical atrophy ($p<0.05$) and (silent) infarcts ($p=0.06$), and impaired cognitive performance (attention and executive function, information processing speed and memory; all $p<0.05$). Adjustment for hypertension did not affect the results. Within the DM2 group cognitive function was inversely related with white matter lesions, atrophy and the presence of infarcts (adjusted for age, sex, and estimated IQ), and there was a modest association with HbA_{1c} and diabetes duration. This association was strongest for age, even more so than in controls.
We conclude that cognitive impairments in patients with DM2 are associated with subcortical ischemic changes in the brain, but also with increased brain atrophy.

In **chapter 6**, we aimed to identify metabolic and vascular factors that are associated with cognitive dysfunction and changes on brain MRI in type 2 diabetic (DM2) patients.
The study included 122 patients and 56 controls. In addition to the neuropsychological testing and the performance of a brain MRI scan, detailed information on diabetes characteristics and glucose metabolism, vascular risk factors and micro- and macrovascular disease was obtained. In regression analyses within the DM2 group impaired cognitive functioning was associated with higher HbA_{1c} levels ($p<0.05$), brain infarcts on MRI ($p<0.01$) and a history of macrovascular events ($p<0.01$). Retinopathy ($p<0.01$) and infarcts on MRI ($p=0.02$) were associated with cortical atrophy. Insulin levels ($p=0.02$), mean arterial pressure ($p=0.03$), and macrovascular disease ($p<0.05$) were associated with WML. Statin use was associated with less pronounced cortical atrophy ($p=0.03$) and WML ($p=0.02$). These findings indicate that atherosclerotic vascular disease is an important determinant for diabetic encephalopathy in patients with DM2, with additional effects of chronic hyperglycemia and possibly hypertension.

In **chapter 7**, we hypothesized that end-organ complications in the peripheral and to the central nervous system in diabetes mellitus might share a common etiology, and as such may co-occur in the same patient. The aim of this chapter was to relate different measures of peripheral neuropathy in type 2 diabetic patients to cognition and brain MRI.
A standardised neurological examination and questionnaire, neuropsychological examination and brain MRI were performed in 122 type 2 diabetic patients and 56 matched controls. Measures of peripheral neuropathy were vibration threshold, a sumscore of sensory symptoms and the Toronto Clinical Neuropathy Scoring System. Differences between patients with type 2 diabetes mellitus and controls were studied and among patients with type 2 diabetes mellitus neuropathy scores were correlated to cognitive functioning and MRI findings
Diabetes was associated with the presence of peripheral neuropathy ($p<0.001$), 39% of patients with diabetes had polyneuropathy. Within the diabetic group peripheral neuropathy was not related to MRI abnormalities or cognitive dysfunction (linear regression analysis, adjusted for age, education, sex).
We conclude that peripheral neuropathy in type 2 diabetic patients is not related to cognitive dysfunction and brain abnormalities. This suggests that different pathogenic mechanisms are involved in peripheral and central neurological complications in patients with type 2 diabetes mellitus.

In type DM2 vascular dysfunction, such as impaired cerebrovascular reactivity (CVR), may be a determinant of increased risk of stroke and cognitive impairments. Previous studies on CVR in DM2 have provided variable results, in selected populations of patients. In **chapter 8**, we aimed to examine CVR in the population-based sample of DM2 patients from the UDES.
CO_2 reactivity (CVR) of the middle cerebral artery was examined using transcranial Doppler ultrasonography (TCD) in 81 DM2 patients and 38 controls. In DM2 patients CVR was correlated with diabetic parameters, vascular risk factors and cognitive functioning.
CVR was similar in patients and controls (51 vs. 49%). Within the DM2 group, there was no statistically significant relationship between CVR and DM-duration, $HbA_{1c,}$ albuminuria, blood pressure, intima/media thickness and cognition. CVR tended to be lower in diabetic patients with retinopathy (46% vs.55%, mean difference:-7.9 (-18.0:2.2)).
We conclude that CVR is not impaired in unselected patients with DM2 and therefore probably does not play a major role in the etiology of cognitive impairment.

In conclusion, the studies presented in this thesis provide new inside in the pathophysiology and clinical features of diabetic encephalopathy. In a rodent model cognitive impairments are associated with reduced cerebral blood flow, and improvement of blood flow by treatment with the ACE-

inhibitor enalapril is associated with improved cognition and improvement of evoked potential latencies. We have performed the first detailed study on the relation between cognitive dysfunction and brain MRI changes in patients with DM2. In patients with DM2 we found more deep white matter lesions, cortical and subcortical atrophy and (silent) infarcts. Furthermore, we found an impaired overall cognitive performance, especially on the domains attention and executive function, information processing speed and memory. Within the DM2 group cognitive function was inversely related with white matter lesions, atrophy and the presence of infarcts. Risk factors for the development of diabetic encephalopathy are atherosclerotic vascular disease and to a lesser extent chronic hyperglycemia and hypertension. Longitudinal studies are needed to determine the importance of these risk factors more exactly.

Samenvatting

9

Diabetes mellitus is geassocieerd met langzaam progressieve veranderingen in het brein, een complicatie die diabetische encefalopathie genoemd wordt. Eerdere studies laten zien dat patiënten met type 2 diabetes mellitus (DM2) milde tot matige stoornissen hebben in aandacht, executieve functies, snelheid van informatie verwerken en geheugen. Op een MRI-scan van de hersenen in patiënten met DM2 worden ook veranderingen gezien, zoals meer corticale en hippocampale atrofie.
De pathogenese van diabetische encefalopathie is waarschijnlijk een multifactorieel proces, waarbij chronische hyperglycemie een schadelijk effect heeft op de hersenen. Vasculaire veranderingen spelen waarschijnlijk ook een belangrijke rol, maar tot nu toe is dit nog niet systematisch onderzocht.

Dit proefschrift omvat twee aan elkaar gerelateerde onderzoeksprojecten. In het eerste project wordt de rol van vasculaire veranderingen in het ontstaan van diabetische encefalopathie bij proefdieren onderzocht.
In het tweede project hebben we gekeken naar de associaties tussen vasculaire risico factoren, cognitief functioneren en structurele afwijkingen in de hersenen van patiënten met DM2.

In **hoofdstuk 2**, is onderzocht of diabetes bij proefdieren geassocieerd is met een verminderde cerebrale bloed flow en of behandeling met enalapril deze cerebrale perfusie en daarbij cerebrale functie kon verbeteren. Streptozotocine (STZ)-diabetische ratten werden behandeld met de Angiotensine Converting Enzyme (ACE)-inhibitor enalapril (24 mg/kg) vanaf het debuut van de diabetes. Na 14 weken diabetes duur werden 12 diabetische ratten behandeld met enalapril en 12 onbehandelde diabetische ratten, en 12 niet-diabetische leeftijd-gematchde controle ratten getest in een spatiële versie van de Morris water maze. Na 16 weken diabetes duur werd in dezelfde groepen bloed flow in de hippocampus en thalamus gemeten door middel van waterstof klaring microelectrode polarography. In een aparte studie werd leren en geheugen op cellulair niveau gemeten, namelijk hippocampale long-term potentiation (LTP) na 26 weken diabetes duur.
Diabetische ratten presteerden minder goed in de water maze en ook de hippocampale LTP was minder. Daarnaast was de bloed flow in diabetische ratten verminderd met 30% ($p<0.001$) in de hippocampus en 37% ($p<0.005$) in de thalamus in vergelijking met niet-diabetische controles. Behandeling met enalapril verbeterde de prestatie op de water maze ($p<0.05$), hippocampale LTP ($p<0.05$) en hippocampale bloed flow ($p<0.05$).
Concluderend is cerebrale perfusie verminderd in diabetische ratten in vergelijking met controles. Behandeling gericht op de bloedvaten verbetert cerebrale bloed flow, prestatie in de Morris water maze en hippocampale LTP. Deze bevindingen suggereren dat schade aan de bloedvaten een rol spelen in het ontstaan van cerebrale disfunctie in diabetische ratten

In **hoofdstuk 3** hebben we onderzocht of een preventie dan wel interventie behandeling met enalapril perifere en centrale zenuwschade in STZ-diabetische ratten kon verbeteren.
Snelheid van zenuwgeleiding in de n. ischiadicus werd gemeten voorafgaand aan de diabetes inductie en vervolgens iedere 3 weken. In de preventie studie werden de laatste metingen verricht in week 15, in de interventie studie in week 24. Brainstem auditory en visual evoked potential latenties (BAEP en VEP) werden ook iedere 3 weken gemeten vanaf de 10e week na diabetes inductie. In de preventie studie werden de laatste metingen verricht in week 16, in de interventie studie in week 25. Behandeling met enalapril werd direct na diabetes inductie gestart (preventie behandeling) of na 15 weken diabetes duur (interventie behandeling).
Snelheid van zenuwgeleiding was vertraagd en BAEP en VEP latenties waren verhoogd in diabetische ratten. Behandeling met enalapril voorkwam een afwijkende zenuwgeleidings snelheid ($p<0.001$), BAEP latenties ($p<0.01$) en VEP latenties ($p<0.005$). Enalapril interventie behandeling had geen effect op snelheid van zenuwgeleiding of op de VEP latenties, maar verbeterde BAEP latenties ($p<0.05$) na een behandelingsduur van 10 weken.
Concluderend voorkomt behandeling met enalapril (gedeeltelijk) het ontstaan van neurofysiologische veranderingen in het perifere en centrale zenuwstelsel van de STZ-diabetische rat en kan het reeds ontstane schade in de BAEP latenties gedeeltelijk ongedaan maken.

In de **hoofdstukken 2 en 3** is aangetoond dat behandeling met enalapril (24 mg/kg) neurofysiologische en cognitieve schade in STZ- diabetische ratten kon voorkomen, en dat het cerebrale bloed flow verbeterde, ondanks een verlaging van de gemiddelde arteriële bloeddruk. In **hoofdstuk 4** is onderzocht of deze effecten behouden werden met lange termijn behandeling en of behandeling met een lagere dosering (12 mg/kg) ook perifere en centrale neurofysiologische schade kon voorkomen zonder het veroorzaken van hypotensie.
Snelheid van zenuwgeleiding in de n. ischiadicus werd iedere 3 weken na diabetes inductie gemeten, tot 24 weken. BAEP en VEP latenties werden vanaf de 10e week na diabetes inductie iedere 3 weken gemeten tot 25 weken. De snelheid van zenuwgeleiding was vertraagd en BAEP en VEP latenties waren verhoogd in onbehandelde diabetische ratten. De 12 mg/kg enalapril behandeling voorkwam gedeeltelijk de afwijkingen van de evoked potential latenties, maar niet de schade van de snelheid van zenuwgeleiding. 24 mg/kg enalapril behandeling voorkwam zowel grotendeels de schade van de snelheid van zenuwgeleiding ($p<0.001$), als schade van de BAEP ($p<0.01$) and VEP latenties ($p<0.05$). De gemiddelde arteriële bloeddruk was 122 mmHg in de onbehandelde diabetische groep, 75 mmHg in de 24 mg/kg groep en 112 mmHg in de 12 mg/kg groep.

Langdurige behandeling met 24 mg/kg enalapril was geassocieerd met een verhoogde mortaliteit, welke gerelateerd kan zijn aan de ernstig verlaagde bloeddruk bij deze dosering.
Concluderend kan lange-termijn behandeling met enalapril in een dosering van 24 mg/kg perifere en centrale neurofysiologische schade in STZ-diabetische ratten voorkomen, maar dat bijwerkingen een langdurige behandeling tegenwerken.

In het tweede gedeelte van dit proefschrift zijn associaties tussen vasculaire risicofactoren, cognitief functioneren en structurele veranderingen in de hersenen van patiënten met type 2 diabetes (DM2) bestudeerd. Daarvoor is de Utrechtse Diabetische Encefalopathie Studie (UDES) opgezet, dit is een grote, cross-sectionele studie waarin 125 patiënten met DM2 en 64 gematchte controles zonder diabetes zijn onderzocht.
In **hoofdstuk 5** worden cognitie en afwijkingen op MRI hersenen vergeleken tussen patiënten met DM2 en controles. Daarbij is gekeken naar de relatie tussen cognitie en de bevindingen op MRI hersenen, bloeddruk en metabole controle.
Van 113 patiënten met DM2 en 51 gematchte controles is een MRI hersenen gemaakt en deze is beoordeeld op voorkomen van witte stof afwijkingen, corticale en subcorticale atrofie en infarcten. De neuropsychologische tests zijn verdeeld in 5 cognitieve domeinen en worden weergegeven als gestandaardiseerde z-waarden.
DM2 was geassocieerd met het voorkomen van diepe witte stof afwijkingen ($p=0.02$), corticale ($p<0.001$) en subcorticale atrofie ($p<0.05$) en (stille) infarcten ($p=0.06$), en met een verminderde cognitieve functie (met name in de domeinen aandacht en executieve functie, snelheid van informatie verwerken en geheugen; allen $p<0.05$). Correctie voor hypertensie veranderde de resultaten niet. Binnen de DM2 groep was een verminderde cognitieve functie gerelateerd aan het voorkomen van witte stof afwijkingen, atrofie en de aanwezigheid van infarcten (geadjusteerd voor leeftijd, geslacht en opleidingsniveau), verder was er een matige relatie met HbA_{1c} en diabetesduur. Deze relatie was het sterkst voor de factor leeftijd, en zelfs nog meer in controles.
Concluderend is een verminderd cognitief functioneren in patiënten met DM2 geassocieerd met subcorticale ischemische veranderingen in de hersenen, maar ook met een toegenomen atrofie van de hersenen.

In **hoofdstuk 6** was het doel om metabole en vasculaire factoren te identificeren die geassocieerd zijn met cognitieve dysfunctie en veranderingen op de MRI van de hersenen in patiënten met DM2.
Deze studie bevatte 122 patiënten en 56 controles. Toegevoegd aan de neuropsychologische tests en de MRI scan van de hersenen werd gedetailleerde informatie verzameld over diabetes karakteristieken en glucose metabolisme, vasculaire risicofactoren en micro- en

macrovasculaire ziekten verzameld.
In regressie analyses binnen de DM2 groep was een verminderd cognitief functioneren geassocieerd met een hoger HbA_{1c} ($p<0.05$), het voorkomen van infarcten op de MRI hersenen ($p<0.01$) en een voorgeschiedenis met macrovasculaire gebeurtenissen ($p<0.01$). Retinopathie ($p<0.01$) en infarcten op de MRI ($p=0.02$) waren geassocieerd met corticale atrofie. Insuline spiegels ($p=0.02$), gemiddelde arteriële bloeddruk ($p=0.03$) en macrovasculaire ziekte ($p<0.05$) waren geassocieerd met het voorkomen van witte stof afwijkingen. Het gebruik van statines was geassocieerd met een minder uitgesproken corticale atrofie ($p=0.03$) en witte stof afwijkingen ($p=0.02$).
Deze bevindingen suggereren dat atherosclerotische ziekte een belangrijke determinant is in het ontstaan van diabetische encefalopathie in patiënten met DM2, waarbij in mindere mate chronische hyperglycemie en mogelijk ook hypertensie een rol lijken te spelen.

Een volgende hypothese die onderzocht is, is of eindorgaan schade in het perifere en centrale zenuwstelsel mogelijk een gezamenlijke etiologie hebben en dus in dezelfde patiënt tegelijk voorkomen. In **hoofdstuk 7** hebben verschillende maten voor perifere neuropathie in patiënten met DM2 gerelateerd aan afwijkingen in cognitie en MRI hersenen.
In 122 patiënten met DM2 en 56 controles werd een gestandaardiseerd neurologisch onderzoek verricht, samen met het neuropsychologisch onderzoek en de MRI hersenen. Als maten voor perifere neuropathie werden de "vibration threshold", een somscore van sensibele verschijnselen en de "Toronto Clinical Neuropathy Scoring System" bepaald. Er werd gekeken naar de verschillen tussen patiënten met DM2 en controles en binnen de DM2 groep werden de neuropathie scores gecorreleerd aan cognitief functioneren en afwijkingen op de MRI hersenen.
Het hebben van diabetes was geassocieerd met het hebben van een perifere neuropathie ($p<0.001$), 39% van de patiënten met DM2 had een polyneuropathie. Binnen de groep diabetes patiënten was het hebben van perifere neuropathie niet gerelateerd aan afwijkingen op de MRI hersenen of verminderd cognitief functioneren.
In conclusie is perifere neuropathie in patiënten met DM2 niet gerelateerd aan een verminderd cognitief functioneren of structurele afwijkingen in de hersenen. Dit suggereert dat er verschillende pathologische mechanismen ten grondslag liggen aan het ontstaan van perifere of centrale neuropathie in patiënten met DM2.

In patiënten met DM2 kan vasculaire dysfunctie (uitgedrukt in Cerebrovasculaire Reserve Capacitieit (CVR)) een maat zijn voor een verhoogd risico op een beroerte en cognitieve schade. Eerdere studie die CVR onderzocht hebben laten verschillende resultaten zien in geselecteerde patiënten populaties. In **hoofdstuk 8** hebben we CVR onderzocht in de populatie-based patiënten groep van de UDES.

CO_2 reactiviteit (CVR) van de arteria cerebri media werd gemeten door middel van transcraniële Doppler ultrasonografie (TCD) in 81 patiënten met DM2 en 38 controles. In de DM2 groep was de CVR gecorreleerd aan diabetische parameters, vasculaire risicofactoren en cognitief functioneren. CVR was niet verschillend tussen patiënten met DM2 en controles (51 vs. 49%). Binnen de DM2 groep was er geen statistisch significante relatie tussen CVR en diabetes duur, $HbA_{1c,}$ albuminurie, bloed druk, intima/media dikte en cognitie. CVR neigde naar lagere waarden in patiënten met diabetische retinopathie (46% vs.55%, mean difference:-7.9 (-18.0:2.2)). Concluderend is CVR niet verminderd in een ongeselecteerde groep diabetes patiënten, en speelt CVR daarom waarschijnlijk geen grote rol in het ontstaan van diabetische encefalopathie.

Concluderend laten de studies in dit proefschrift een nieuw licht schijnen op de pathofysiologie en klinische uitingen van diabetische encefalopathie. In een rat model zijn cognitieve verschijnselen geassocieerd met een verminderde cerebrale bloed flow, en verbetering van deze bloed flow door middel van behandeling met de ACE-remmer enalapril is geassocieerd met een verbeterd cognitief functioneren en verbetering van de evoked potential latenties.
Dit is de eerste gedetailleerde studie naar de relatie tussen cognitief functioneren en veranderingen op MRI hersenen in patiënten met DM2. Patiënten met DM2 hebben meer diepe witte stof afwijkingen, meer corticale en subcorticale atrofie en (stille) infarcten. Daarbij vonden we een algemeen verminderd cognitief functioneren, met name op de domeinen aandacht en executieve functies, snelheid van informatie verwerken en geheugen. Binnen de groep diabetes patiënten was een verminderd cognitief functioneren gerelateerd aan het hebben van witte stof afwijkingen, atrofie en infarcten. Risicofactoren voor het ontstaan van diabetische encefalopathie zijn atherosclerotische vasculaire ziekten en in mindere mate hyperglycaemie en hypertensie. Om het belang van deze risicofactoren beter te duiden zijn nog longitudinale studies nodig.

Samenvatting voor niet-medici

9

Diabetes mellitus (suikerziekte) kan op den duur leiden tot beschadiging van verschillende organen, zoals de ogen, de nieren en het hart- en vaatstelsel. Ook neuropathie, beschadiging van het perifere zenuwstelsel, is een bekende complicatie van diabetes. In de afgelopen jaren is duidelijk geworden dat diabetes ook kan leiden tot beschadiging van het centrale zenuwstelsel, de hersenen. Deze "hersenschade" kan onder andere leiden tot geheugen problemen. Gelukkig lijken deze problemen bij de meeste patiënten mee te vallen. Bij oudere diabetes patiënten, echter, zijn de geheugenproblemen meer uitgesproken. Ook lijkt voor oudere diabetespatiënten het risico op het ontwikkelen van dementie verhoogd. Over de oorzaak van de schadelijke effecten van diabetes op de hersenen was nog onvoldoende bekend. Het doel van het in dit proefschrift beschreven onderzoek was om beter in kaart te brengen waar de schade aan de hersenen uit bestaat en om risicofactoren voor het ontstaan van schade aan de hersenen bij diabetes op te sporen.
Dit proefschrift bestaat uit twee delen. Eerst is gekeken naar de rol van stoornissen van de bloedvaten bij het ontstaan van hersenschade in diabetische ratten. Deze ratten werden behandeld met een medicijn (enalapril) wat veel gebruikt wordt in diabetes patiënten als beschermer van de bloedvatwand en als bloeddrukverlager. In deze diabetische ratten verbeterde leren en geheugen, de doorbloeding van de hersenen, synaptische plasticiteit (een cellulaire vorm van leren) en de snelheid van het doorgeven van signalen in de hersenen (evoked potentials). Wij concluderen hieruit dat stoornissen van de bloedvaten en de doorbloeding van de hersenen een belangrijke rol spelen bij het ontstaan van hersenschade in diabetische ratten, en dat de bloedvaten een mogelijk aanknopingspunt voor behandeling bieden.
In het tweede deel van dit proefschrift wordt een onderzoek beschreven waarin we gekeken hebben naar hoe "hersenschade" zich uit in diabetische patiënten en wat de risicofactoren zijn voor het ontstaan hiervan. In dit onderzoek (Utrecht Diabetic Encephalopathy Study UDES) werden patiënten met ouderdomsdiabetes (diabetes type 2) vergeleken met leeftijdgenoten. Er werden neuropsychologische tests afgenomen, om een beeld te krijgen van onder andere het leervermogen en het geheugen, de zogenaamde cognitieve functies. Daarnaast werden de hersenen onderzocht met een hersenscan (MRI). Het doel van het onderzoek was vast te stellen of de ernst van geheugen en leer problemen en de ernst van eventuele afwijkingen op de MRI-scan bij diabetes patiënten samenhangt met de aan- of afwezigheid van vaatstoornissen. Daarom werd bij alle patiënten ook uitgebreid onderzoek gedaan naar de bloedvaten in het hoofd, maar ook naar die in bijvoorbeeld het netvlies. Daarnaast werden mogelijke andere complicaties van diabetes (nieren, zenuwen) bij alle deelnemers in kaart gebracht.
Er werden bij de diabetes patiënten inderdaad veranderingen van de cognitieve functies gevonden. Zo was de prestaties op tests voor de snelheid van de verwerking van complexe informatie en voor het geheugen

licht verminderd. Ook op de MRI scan werden veranderingen gezien, waaronder een lichte toename van vaatschade en een lichte toename van veranderingen die bij veroudering van de hersenen worden gezien. Er leek een samenhang te bestaan tussen het voorkomen van atherosclerose (aderverkalking) elders in het lichaam en de ernst van de cognitieve functiestoornissen en MRI veranderingen. De relatie met ander factoren, zoals bijvoorbeeld de bloedsuikers of andere diabetische complicaties, was minder sterk. Dit onderzoek is een belangrijke stap voorwaarts, omdat het voor de eerste maal de aard van de MRI veranderingen die samenhangen met veranderingen in de cognitie beschrijft. Ook de aanwijzingen voor een rol van de vaten zijn van belang, omdat eventuele preventieve maatregelen zich op de vaten kunnen richten. Met steun van het diabetes fonds is inmiddels een vervolgonderzoek opgestart, waarbij alle deelnemers van het UDES onderzoek na 4 jaar opnieuw wordt verzocht zich te laten onderzoeken. Met dit vervolgonderzoek kunnen nauwkeurig de veranderingen in cognitie en de MRI hersenen bij iedere individuele patiënt worden vastgelegd. Hiermee kunnen we nog beter vaststellen welke factoren verantwoordelijk zijn voor versnelde achteruitgang bij de individuele patiënt.

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9

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Members of the UDES

*The Utrecht Diabetic Encephalopathy Study Group consists of (in alphabetical order): department of Clinical Neurophysiology: A.C. van Huffelen; department of Internal Medicine: H.W. de Valk; Julius Center for Health Sciences and Primary Care: A. Algra, G.E.H.M. Rutten; department of Medical Pharmacology: W.H. Gispen; department of Neurology: A. Algra, G.J. Biessels, L.J. Kappelle, S.M. Manschot, J. van Gijn; department of Neuropsychology and Helmholtz Research Institute: A.M.A. Brands (currently: Zuwe Hofpoort /Regionaal Psychiatrisch Centrum, Woerden, The Netherlands), E.H.F. de Haan, R.P.C. Kessels, E. van den Berg; department of Radiology: J. van der Grond, all part of the University Medical Center, Utrecht, The Netherlands.
All patients are recruited through the community based "Utrecht Diabetes Programma (UDP)" and the "IJsselstein Diabetes Project" (mentor: Ph.L. Salomé). The UDP is a long-running collaborative project between general practitioners (GP's) and a hospital-based endocrinologist, with the aim to improve the level of care for patients with type 2 diabetes in the community without referral to the hospital.

Curriculum vitae

Sanne M. Manschot werd geboren op 25 mei 1973 te Utrecht. Op de leeftijd van 1,5 jaar verhuisde zij naar Winschoten, waar haar beide zusjes werden geboren. In december 1980 verhuisde de familie naar Venlo, waar ze in 1991 haar VWO-B diploma behaalde aan het St. Thomas college te Venlo. Hierna begon zij aan haar studie geneeskunde aan de Universiteit Utrecht. Tijdens deze studie deed zij, onder supervisie van prof. Dr. J. van Gijn en dr. E. Buskens, onderzoek naar het gebruik van verschillende schalen voor het beoordelen van de reflexen en de reproduceerbaarheid daarvan. Verder heeft ze tijdens de studie verschillende student-assistentschappen gedaan (Neurowetenschappen, hoofd-hals-zintuigen en klinische epidemiologie en genetica). In 1996 behaalde zij haar doctoraalexamen geneeskunde. Tijdens haar co-schappen deed zij onderzoek naar het familiair voorkomen van polyneuropathie en monoclonale gammopathie onder supervisie van dr. N.C. Notermans en prof. dr. L. van den Berg. In 1998 behaalde zij het arts-examen. In 1998 werd zij AGNIO op de afdeling Neurologie van het UMCU en in 1999 startte zij met het dierexperimentele gedeelte van het promotie-onderzoek. In 2000 begon zij met de opleiding tot neuroloog (opleider prof. dr. J. van Gijn) en in 2001 kon zij met behulp van een subsidie van het Diabetes Fonds starten met het klinische gedeelte van het promotie-onderzoek. Momenteel volgt zij gedurende een jaar de opleiding klinische neurofysiologie in het UMCU (opleider prof. dr. A.C. van Huffelen). In juni 2001 trouwde zij met Freddy Lipke, in juli 2003 werd hun zoon Tijmen geboren en in mei 2005 hun dochter Nina. In februari 2009 hoopt zij de opleiding neurologie af te ronden.

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