# Ambulatory blood pressure, endothelial perturbation, and microvascular complications in type 2 diabetes

*Søren Tang Knudsen*

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Official opponents: Peter Rossing, Hans Ibsen and Hans Erik Bøtker.

Correspondence: Medical Department, Regional Hospital Silkeborg, Falkevej 1-3, 8600 Silkeborg. Denmark

E-mail: stk@dadlnet.dk

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This review is based on the following publications:

1. Knudsen ST, Poulsen PL, Hansen KW, Ebbehøj E, Bek T, Mogensen CE: Pulse pressure and diurnal blood pressure variation: Association with micro- and macrovascular complications in type 2 diabetes. Am J Hypertens 2002; 15(3):244-250. [1]
2. Knudsen ST, Bek T, Poulsen PL, Hove MN, Rehling M, Mogensen CE: Macular edema reflects generalized vascular hyperpermeability in type 2 diabetic patients with retinopathy. Diabetes Care 2002; 25(12):2328-2334. [2]
3. Knudsen ST, Bek T, Poulsen PL, Hove MN, Rehling M, Mogensen CE: Effects of losartan on diabetic maculopathy in type 2 diabetic patients. A randomized, double-masked study. J Intern Med 2003; 254(2):147-58. [3]
4. Knudsen ST, Foss CH, Poulsen PL, Bek T, Ledet T, Mogensen CE, Rasmussen LM: E-selectin inducing activity in plasma from type 2 diabetic patients with maculopathy. Am J Physiol Endocrinol Metab 2003; 284(1): E1-E6. [4]
5. Knudsen ST, Jeppesen P, Frederiksen CA, Andersen NH, Bek T, Ingerslev J, Mogensen CE, Poulsen PL: Endothelial perturbation: a link between non-dipping and retinopathy in type 2 diabetes? Journal of the American Society of Hypertension 2007; 1(3): 208–215. [5]
6. Knudsen ST, Jeppesen P, Frederiksen CA, Andersen NH, Bek T, Ingerslev J, Mogensen CE, Poulsen PL: Endothelial dysfunction, ambulatory pulse pressure and albuminuria are associated in Type 2 diabetic subjects. Diabet Med 2007; 24(8): 911-5. [6]
7. Knudsen ST, Andersen NH, Poulsen SH, Eiskjær H, Hansen KW, Helleberg K, Poulsen PL, Mogensen CE: Pulse Pressure Lowering Effect of Dual Blockade with Candesartan and Lisinopril versus High-dose ACE-inhibition in Hypertensive Type 2 Diabetic Subjects. A CALM II study post-hoc analysis. Am J Hypertens, 2008; 21(2):172-176. [7]
8. Knudsen ST, Laugesen E, Hansen KW, Bek T, Mogensen CE, Poulsen PL: Ambulatory pulse pressure, decreased nocturnal blood pressure reduction and progression of nephropathy in type 2 diabetic patients. Diabetologia, 2009; 52(4):698-704. [8]

Papers 1, 2, and 3 were included in my Ph.D. thesis “On the mechanisms of retinopathy in type 2 diabetic patients with particular reference to diabetic maculopathy.”, Aarhus University, Faculty of Health Sciences, 2002 [9].

**ABBREVIATIONS**

| | |
|---|---|
| ACE-i: | Angiotensin converting enzyme inhibitor |
| AHT: | Antihypertensive therapy |
| AMBP: | 24-hour ambulatory BP measurement |
| ARB: | Angiotensin II receptor blocking agent |
| BMI: | Body mass index |
| BP: | Blood pressure |
| CALM Trial | Candesartan and Lisinopril Microalbuminuria |
| CI: | Confidence interval |
| CV: | Cardiovascular |
| DMa: | Diabetic maculopathy |
| GFR: | Glomerular filtration rate |
| HbA1c: | Haemoglobin A1c |
| HPLC: | High performance liquid chromatography |
| HR: | Hazard ratio |
| ICAM-1: | Soluble intercellular adhesion molecule 1 |
| IGT: | Impaired glucose tolerance |
| IRMA: | Intraretinal microvascular abnormalities |
| OCT: | Optical coherence tomography scanning |
| PP: | Pulse pressure |
| RAS: | Renin-angiotensin system |
| RIA: | Radio immuno assay |
| SD: | Standard deviation |
| SE: | Standard error |
| TERalb: | Transcapillary escape rate of albumin |
| T2DM: | Type 2 diabetes mellitus |
| UAE: | Urinary albumin excretion rate |
| UACR: | Urinary albumin/creatinine ratio |

VCAM-1: Vascular cell adhesion molecule 1
vWF: von Willebrand factor

## 1. INTRODUCTION

The global incidence and prevalence of type 2 diabetes is rapidly increasing [10,11]; consequently, diabetic micro- and macrovascular complications constitute leading causes of visual impairment [12-14], end-stage renal disease [15,16], and cardiovascular morbidity and mortality [17,18] world-wide. Various pathophysiological mechanisms involved in the development of diabetic microvascular complications have been proposed, among these elevated blood pressure (BP) [19-21], endothelial dysfunction/activation [22-36], and increased capillary permeability to macromolecules [25,32,37-40].

The significance of an elevated arterial BP for the development of microvascular complications in type 2 diabetes is well established [20,21,41-48]. During the last decade, numerous studies have established pulse pressure (PP), i.e. the difference between the systolic and diastolic BP values, as a strong, independent predictor of cardiovascular morbidity and mortality in non-diabetic subjects [49-54]. In the late 1990´s when our present series of investigations were initiated, no publications on the potential association between PP and microvascular complications in diabetic subjects were available.

Several studies have proven 24-hour ambulatory BP measurement (AMBP) superior to conventional office BP measurement regarding the association with [1,55-59] and prediction of [60,61] hypertensive end-organ damage in diabetes. Moreover, as opposed to office BP measurement, AMBP provides the opportunity to study diurnal BP fluctuations. Specifically, focus has been directed towards the association between a blunted nocturnal BP decline ("non-dipping") and diabetic vascular complications [58,62-67].

While the effect of antihypertensive treatment (AHT) on the progression of diabetic nephropathy has been well-established for decades [43,44,68-71], results regarding retinopathy have as yet been promising, but less convincing [72], although data from several larger studies have shown that AHT might retard the progression of diabetic retinopathy [19,73,74]. No previous intervention study has specifically addressed the effect of AHT on pulse pressure.

Excessive activation of the vascular endothelium has been associated with diabetic vascular complications in numerous studies [22-36], possibly by promoting inflammation and atherosclerosis and increasing the tendency to blood clotting. A few studies have examined the mutual association between endothelial activation and the above mentioned haemodynamic abnormalities, blunted nocturnal BP decline and elevated PP, as well as their potential interaction in non-diabetic subjects [75,76].

A pathologically increased permeability of the retinal vasculature with consequential formation of retinal hard exudates and oedema is a hallmark of diabetic maculopathy (DMa), a common sight-threatening manifestation of retinopathy in type 2 diabetes [77-79]. Likewise, diabetic nephropathy is characterised by an inappropriate glomerular leakage of macromolecules, as demonstrated by an increased excretion of albumin in the urine (UAE) [80,81]. The enhanced permeability of the renal glomerulus in diabetic nephropathy has previously been shown to reflect a widespread vascular damage [23], resulting in a generalised hyperpermeability as signified by the transcapillary escape rate of albumin (TERalb) [25,32,38-40,82], i.e. the initial disappearance rate of intravenously injected, radioactively labelled albumin [37].

Even though diabetic retinopathy and nephropathy are statistically associated [23,32,83-88], data on the independent relationship between retinopathy and TERalb have been conflicting [32,39], possibly because a quantitative assessment of retinal oedema has not been available. However, with the advent of a novel diagnostic modality, Optical Coherence Tomography Scanning (OCT) [89,90], direct measurements of retinal thickness, and thereby quantification of retinal oedema, have become feasible [91-101]. Areas of increased retinal thickness, as evaluated by OCT, correlate strongly with a regionally increased leakiness of retinal blood vessels as assessed by vitreous fluorophotometry [102,103]; thus OCT measurements of retinal thickness can be considered a quantitative, surrogate measure of retinal vascular permeability.

## 2. AIMS

In general, the aims of our investigations in subjects with type 2 diabetes were to study pathophysiological haemodynamic and structural abnormalities potentially associated with the presence and development of microvascular complications and to evaluate the effect of intervention with antihypertensive agents on these risk factors and complications. We hypothesized that i) macular oedema was associated with an increased glomerular and microvascular permeability, ii) plasma from subjects with diabetic maculopathy contained factor(s) capable of inducing adhesive molecule activation on the surface of cultured endothelial cells, iii) elevated PP and reduced diurnal BP variation were associated with and predicted diabetic microvascular complications, iv) endothelial activation could represent a link between non-dipping, elevated PP, and microvascular complications, v) short-term angiotensin 2 receptor blockade could ameliorate macular oedema in patients with diabetic maculopathy, and vi) long-term dual blockade of the RAS with an ACE-inhibitor and an angiotensin 2 receptor antagonist could reduce PP to a higher degree than high-dose ACE-inhibition.

Therefore, we studied:

1. Associations between microvascular permeability in the retina, kidney, and the overall microvasculature
2. Associations between microvascular complications and pulse pressure as well as circadian BP variation
3. The ability of plasma from patients with and without diabetic maculopathy as well as non-diabetic subjects with normal or impaired glucose tolerance to facilitate adhesive molecule activation on the surface of cultured endothelial cells
4. Associations between microvascular complications, pulse pressure, circadian BP variation, and endothelial perturbation
5. The predictive value of pulse pressure and circadian BP variation for the progression of nephropathy
6. The effect of short-term angiotensin 2 receptor blockade on AMBP variables, markers of endothelial activation, as well as renal, retinal and generalised vascular permeability
7. The effect of long-term dual blockade of the RAS with an ACE-inhibitor and an angiotensin 2 receptor blocker compared with high-dose ACE-inhibition on pulse pressure and UAE

## 3. SUBJECTS, DESIGNS, AND METHODS

An overview of study designs and participants are given in **Table 1.**

**Table 1.** Participants and designs of the eight studies. Unless specified (articles 4, 5, 6), all participants were type 2 diabetic subjects (T2DM). m, male; f, female; NGT, subjects with normal glucose tolerance; IGT, subjects with impaired glucose tolerance.

| Article | N (m/f) | Subgroups | N | Design |
|---|---|---|---|---|
| 1 | 80 (49/31) | Retinopathy (none / grade 2 / grade 3-6) | 49 / 13 / 18 | Cross-sectional |
| | | Nephropathy (normo- / micro- / macroalbuminuria) | 45 / 19 / 15 | |
| 2 | 40 (24/16) | No retinopathy / maculopathy | 20 / 20 | Cross-sectional, matched |
| 3 | 24 (14/10) | Placebo / losartan | 12 / 12 | Randomised, double-masked trial |
| 4 | 80 (48/32) | NGT / IGT / T2DM w. no retinopathy / T2DM w. maculopathy | 20 / 20 / 20 / 20 | Cross-sectional, matched |
| 5 | 76 (60/16) | Healthy subjects / T2DM w. no retinopathy / T2DM w. minimal retinopathy / T2DM w. maculopathy | 19 / 19 / 19 / 19 | Cross-sectional, matched |
| 6 | 65 (52/13) | Healthy subjects / T2DM w. normoalbuminuria / T2DM w. micro- or macroalbuminuria | 19 / 34 / 12 | Cross-sectional |
| 7 | 51 (39/12) | Dual blockade (candesartan + lisinopril) / high-dose lisinopril | 25 / 26 | Randomised, double-masked trial |
| 8 | 112 (68/44) | No progression of nephropathy / progression of nephropathy | 77 / 35 | Observational, follow-up |

3.1 PARTICIPANTS

Participants in the studies comprised type 2 diabetic patients (T2DM), non-diabetic healthy subjects, and subjects with impaired glucose tolerance (IGT). All were of Caucasian ethnicity. The classification of subjects as having normal glucose tolerance (NGT), IGT, or T2DM was based on an oral glucose tolerance test (OGTT), which was evaluated according to World Health Organisation (WHO) criteria [104]. Diabetic patients were considered to have T2DM if they had an onset of diabetes after the age of 30 years, no need for insulin treatment for at least one year following the diagnosis of diabetes, and no history of ketoacidosis. Patients with T2DM were recruited from outpatient's clinics at Medical Dept.´s M and C, Aarhus Sygehus, and at Silkeborg and Randers Centralsygehus. Moreover, subjects included according to retinopathy status ([2-6]) were identified in the database of eye examinations from the screening clinic for diabetic retinopathy at the Ophthalmological Dept., Aarhus Sygehus. Healthy control subjects were recruited from hospital staff and relatives ([5,6]) and from the Fredericia Study [105] ([4]).

Subjects were classified as smokers or non-smokers (no daily use of tobacco for the preceding year). In two of the studies [2,3], smokers were further subdivided into moderate (<15 cigarettes/day or equivalent) or heavy smokers. Subjects were classified as having macrovascular disease if one or more of the following were present: Symptoms of angina pectoris, history of myocardial infarction (MI), coronary artery by-pass grafting (CABG), or percutaneous transluminal coronary angioplasty (PTCA), symptoms of or operation for intermittent claudication, amputations, or history of transient ischaemic attack or stroke.

3.2 STUDY DESIGNS

Associations between AMBP parameters, endothelial activation, and microvascular complications in T2DM were investigated in cross-sectional studies with matched [2,4,5] as well as unmatched [1,6] study populations and in an observational follow-up study [8]. Effects of antihypertensive therapy on AMBP parameters, microvascular complications, endothelial activation, and markers of regional as well as generalised vascular permeability in T2DM were evaluated in randomized, double-masked intervention studies [3,7].

In studies with matched design [2,4,5], patients with DMa were identified in the database of eye examinations from the screening clinic for diabetic retinopathy at the Ophthalmological Dept., Aarhus Sygehus. For each patient in the DMa group, we identified and ranked the ten subjects without retinopathy in the database that matched best with regard to age, gender, and known duration of diabetes. Subjects were then invited to participate in the studies according rank. In one of the studies [5], after including DMa subjects as described above, we proceeded to include individually matching subjects with minimal background retinopathy from the database, according to the same procedure, and, finally, we included a group of individually matching (gender, age) healthy control subjects among hospital staff and relatives. In study IV [4], we identified and included individually matching subjects (gender, age) with NGT and IGT from the Fredericia Study [105].

All studies adhered to the tenets of the declaration of Helsinki, and they were approved by the local committee for biomedical ethics. All included subjects gave their written informed consent to participate.

3.3 METHODS

***3.3.1 Office and 24-hour ambulatory blood pressure (AMBP) measurements***

Office blood pressure was measured after 15 minutes rest in the sitting position with a Hawksley random zero sphygmomanometer. Mean values of 3 consecutive measurements were calculated and used for the analyses. 24-h ambulatory blood pressure (AMBP) was measured by an oscillometric technique (Spacelabs Monitors, Redmond, WA, USA, validated according to the British Hypertension Society protocol [106]). The principle for oscillometric BP measurement is based on the identification of mean arterial BP as the lowest BP corresponding to the maximal oscillation of the cuff pressure [107,108]. Systolic and diastolic BP are subsequently calculated from a built-in algorithm which is kept as an industrial secret by the SpaceLabs company, hence not accessible for scientific evaluation [109]. In our laboratory, we compare corresponding values of oscillometric and auscultatory sphygmomanometer measurements every three months; if the difference exceeds 3 mmHg, the oscillometric apparatus is sent for calibration.

In two of the investigations [1,8], we used both Spacelabs 90202 [110] and 90207 [111], whereas only Spacelabs 90207 were used in the other investigations. Spacelabs 90202 obtained readings every 20 minutes between 6 a.m. and 12 p.m. and once hourly between 12 p.m. and 6 a.m., whereas Spacelabs 90207 measured at 20-minute intervals throughout the 24-hour period. Measurements were performed during a day with normal activities at home or at work. Individual sleeping diaries were used in the calculation of day and night BP and night/day BP ratios. This method, as opposed to fixed day and night periods (where the night period is typically defined as 11 p.m. to 6 a.m.), ensures that the diurnal BP variation is not erroneously underestimated [112]. If more than three hours were missing, the patient was excluded (four patients in studies 1 and 8, none in the other studies). The

standard deviation of the difference between two AMBP measurements in our laboratory is 5.7 mmHg for 24-h systolic and 2.5 mmHg for 24-h diastolic AMBP [56].
Nocturnal BP dipping was evaluated by systolic and diastolic night/day BP ratios. Originally, non-dipping in essentially hypertensive patients was defined as a reduction in systolic and diastolic BP of less than 10% from day to night [113]. However, in accordance with the currently most broadly accepted definition of non-dipping, we classified subjects as non-dippers if they had a less than 10% decline in mean systolic BP during night-time compared with day-time mean systolic BP [114]. Moreover, like in other comparable studies [76,115,116] we also included diastolic non-dipping in the analyses [117]. Importantly, diurnal BP variation should preferably be regarded as a continuum [114]; hence systolic and diastolic night/day BP ratios were also analysed as continuous variables.

### *3.3.2 Ophthalmological examinations*

All participants with T2DM underwent a routine ophthalmological examination for diabetic retinopathy including measurement of visual acuity (using visual acuity charts designed according to ETDRS principles [118]), slit lamp examination, and fundus photography. Moreover, in studies 2 and 4, patients were classified as having DMa or no retinopathy according to fundus photographs, fluorescein angiograms, and OCT, whereas in study 5, we divided patients as having no or minimal retinopathy or DMa on the basis of fundus photographs and OCT.
In the studies with matched designs and quantitative assessments of retinal thickness [2,3], we defined the study eye as the eye of the patient with DMa with the highest number of hard exudates, and, subsequently, we included the corresponding eye of the matched control subject in the study.

#### *3.3.2.1 Retinal photography and retinopathy grading*

The pupils were dilated with metaoxedrine 10% and tropicamide 1% eyedrops, and fundus photography was performed using a Canon 60UV fundus camera on Kodak Ectachrome 64 colour diapositive film. In each eye, one 60 degree image centered on the fovea and one nasally displaced field centred on the optic disk were taken. The number of each type of pathological lesion: Haemorrhages and/or microaneurysms, hard exudates, or cotton wool spots was counted, and the presence of vascular abnormalities such as intraretinal microvascular abnormalities (IRMA), venous beading, or neovascularizations was noted. Each photograph was evaluated independently by two experienced graders. When the two evaluations were discrepant, the photograph was re-evaluated by the two graders together. In case there was still discrepancy, the opinion of the most senior grader was followed. On the basis of the grading of all lesions on a photograph the eye was assigned an overall retinopathy grade on a scale from 1-6 according to the principles used in the Wisconsin Epidemiologic Study of Diabetic Retinopathy [119] with a modification to ensure that lesions implying the same risk of progression to proliferative diabetic retinopathy resulted in the same retinopathy level (ETDRS Report 12 [120]):

1: No retinopathy
2: a) Less than 20 haemorrhages and/or microaneurysms, or
b) Cotton wool spots alone.
3: a) More than or = 20 haemorrhages and/or microaneurysms, or
b) Hard exudates combined with any number of haemorrhages and/or microaneurysms, or
c) Less than 5 cotton wool spots combined with haemorrhages and/or microaneurysms or hard exudates.
4: More than or = 5 cotton wool spots or IRMA vessels combined with haemorrhages and/or microaneurysms with or without hard exudates.
5: Venous beading combined with haemorrhages and/or microaneurysms with or without hard exudates, IRMA vessels or cotton wool spots.
6: Proliferative diabetic retinopathy, or scars of photocoagulation known to have been directed at new vessels.

The above mentioned classification describes the orderly progression from no retinopathy (grade 1) over mild, non-proliferative abnormalities (microaneurysms, blot haemorrhages, and cotton wool spots) and moderate to severe non-proliferative diabetic retinopathy, characterized by venous caliber changes or beading, IRMA vessels, retinal ischaemia, and extensive intraretinal haemorrhages and microaneurysms, to PDR, characterized by the growth of new blood vessels on the retina and posterior surface of the vitreous (modified from Aiello et al. [121]). Importantly, the above mentioned grading system is most suitable for the evaluation of the development of PDR and not very suitable for the evaluation of DMa. Nonetheless, as this classification represents the “golden standard” of retinopathy grading of fundus photographs, it was used in studies 1, 3 and 8. In article 1 and 8, patients were grouped in categories of no (grade 1), mild (grade 2), or advanced (grade 3-6) retinopathy.

#### *3.3.2.2 Fluorescein angiography*

In studies 1-4, fluorescein angiography was performed on Ilford Delta 400 black/white film. A fast sequence was taken during the filling phase of the retinal vessels on the study eye, and late phase images were taken on both eyes 5-10 minutes after the injection of fluorescein. On the basis of the flourescein angiograms, it was verified that a) all maculopathies were of the exudative (non-ischaemic) type, and b) no patients in the group without retinopathy had any sign of this complication.

#### *3.3.2.3 Optical coherence tomography (OCT) scanning*

By means of optic (laser) waves, distance information is extracted from the time delays of reflected signals [89]. The high resolution (10 µm) provides a very precise assessment of retinal thickness [93], hence making the method very sensitive [122,123]. Furthermore, the method is highly reproducible [94,96,97,124]; in our clinic, the intraindividual coefficient of variation for OCT scans measured on the same day was 3 %. In study 2, 3, and 4, we used the Humphrey optical coherence tomography scanner, version A4.1 (Humphrey Instruments, San Leandro, CA, USA). Six radial scans, with a length of 2.83 mm, centred on the fixation point were performed with 30 degrees interval (central macular scans, **Figure 1a and 1b**). In article 3, four additional horizontal (“peripheral macular”) scans with a length of 2.83 mm were performed repeatedly at the location in the macular area with hard exudates. The analysis of OCT scans was performed by an ophthalmologist who had not participated in the clinical examination of the patients. For each scan five thickness measurements were obtained, located at 10%, 30%, 50%, 70%, and 90% of the length of the scan from its beginning, respectively

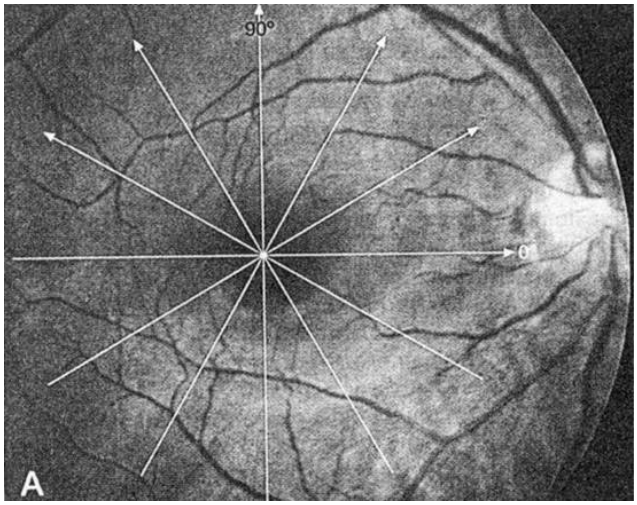


**Figure 1a.** Distribution of the six central OCT scans through the fovea.

figure 1a [96,124]. Subsequently, we calculated two different values of average retinal thickness within the macular area: Average retinal thickness in the central macular area, calculated as the average of the thirty measured thicknesses of the central scans (five measurement points on each of the six radial scans), and retinal thickness in the peripheral macula, calculated as the average of the twenty measured thicknesses of the peripheral macular scans (five measurement points on each of the four single line scans, performed in the area where hard exudates were located). In the more recent study 5, we used the Humphrey OCT-II scanner (Humphrey Instruments, San Leandro, CA, USA). On the basis of six radial scans, obtained as described above, the OCT software constructed a 2D map of the overall macular thickness and the retinal thickness in nine subareas (A2–A9) of the macular region **figure 2** [124,125]. The mean retinal thickness of areas A2–A9 was used to verify the absence of macular oedema in the groups with no or minimal retinopathy.

### *3.3.3 Nephropathy classification*

Due to the considerable intra-individual variability of the urinary albumin excretion rate (UAE), with a day to day variation of 30 - 50 % [126,127], we used three urine samples for the evaluation of UAE in all investigations. Patients received verbal as well as written information about the procedures. Women were informed to refrain from urine collection when menstruating. Urinary tract infections were excluded by dipstick testing (Multisticks 8SG, Ames, Stokes Court, UK). In articles 1 and 5-8, urinary albumin concentration was determined by an immunoturbidimetric method (Roche Diagnostics, Basel, Switzerland), and UAE was estimated as the geometric mean of albumin/creatinine ratios (UACR) from three samples of morning urine. Patients were classified as normoalbuminuric, when at least 2 out of 3 UACR were < 2.5 mg/mmol (men) or < 3.5 mg/mmol (women), microalbuminuric (between 2.5 and 25 mg/mmol (men) or between 3.5 and 35 mg/mmol (women)), or
macroalbuminuric (> 25 mg/mmol (men) or > 35 mg/mmol (women) or dip stick positive proteinuria in at least 2 out of 3 samples) [128]. In article 8, progression of nephropathy was defined as i) the development of stable micro- or macroalbuminuria in subjects with normoalbuminuria at baseline [60,129], ii) the development of stable macroalbuminuria in subjects with microalbuminuria at baseline [129], or iii) a doubling of p-creatinine or development of end-stage renal failure in subjects with macroalbuminuria at baseline [70,71].
In articles 2-4, urinary albumin concentration was measured by a radioimmunoassay method [130], and UAE was expressed as the geometric mean of three UAE´s calculated from three overnight urine samples collected within a week [81]. No preservative was added. The urine was frozen immediately. Prior to analysis aliquots were thawed once while stirred. Subjects were classified as normoalbuminuric (at least 2 out of 3 UAE´s < 20 µg/min), microalbuminuric (at least 2 out of 3 UAE´s in the range: 20 µg/min < UAE < 200 µg/min), or macroalbuminuric (at least 2 out of 3 UAE´s > 200 µg/min) [81].

### *3.3.4 Endothelial perturbation*

Endothelial perturbation was evaluated by two different methods. Firstly, the transcapillary escape rate of albumin (TERalb) was considered to reflect the overall permeability of the microvascular endothelium [23,37]. Secondly, plasma concentrations of circulating adhesion molecules, originating from the vascular endothelium were considered appropriate markers of the degree of endothelial activation [131]. In addition, we investigated whether plasma from various subjects might induce endothelial activation *in vitro* [132].

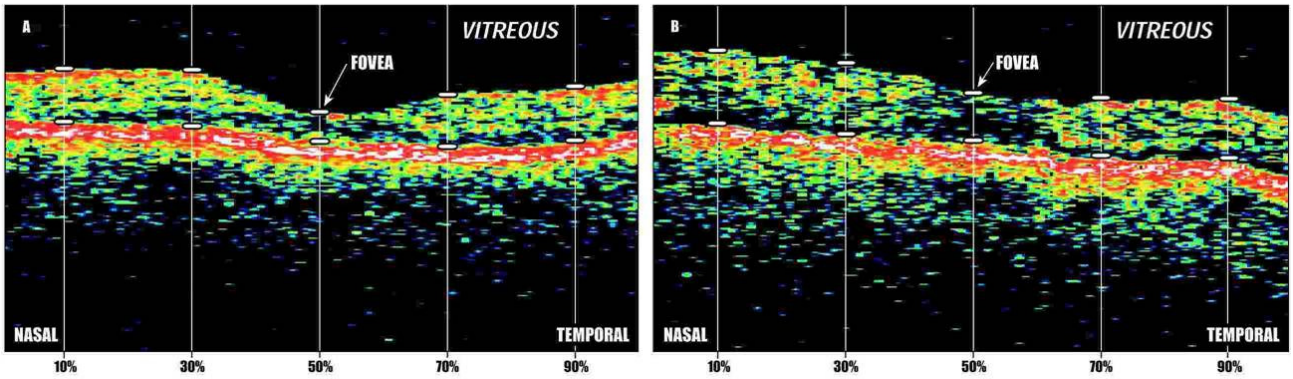


**Figure 1b.** Horizontal OCT scans from a type 2 diabetic patients A) without retinopathy and B) with maculopathy. Points used for retinal thickness measurements are indicated by the white, horizontal bars.

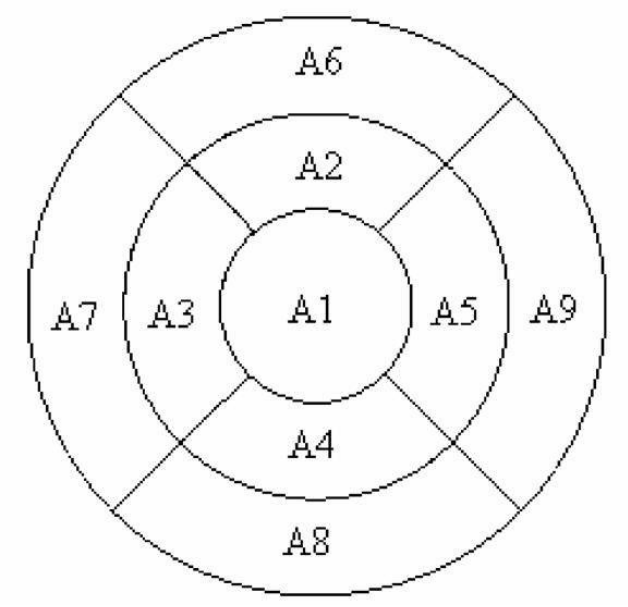


**Figure 2.** Subareas for local measurement of retinal thickness.

*3.3.4.1 Transcapillary escape rate of albumin (TERalb)*
Experiments for the assessment of the transcapillary escape rate of albumin (TERalb) were performed as described by Parving [133,134]. The examination was performed in the morning after an overnight fast. After resting for 1 hour in the supine position, the patient received an intravenous bolus injection containing 0.2 MBq 125I Human Albumin (HSA for metabolic studies, IsoPharma IT.23S). Blood samples were collected from a cubital vein in the other arm before and 10, 15, 20, 30, 40, 50, 55, and 60 minutes after the injection for counting of plasma radioactivity and measurement of total plasma protein concentration in duplicate by refractometry (Optimeter, Germany). Radioactivity was corrected for total plasma protein concentration, and the slope of the linear regression of radioactivity with time was used to calculate TERalb (i.e. the plasma tracer disappearance rate). TERalb measurements were accepted only if the correlation coefficient between the time points for blood collection and the corresponding values of a specific radioactivity exceeded 0.85. On the basis of this criterium, a total of three TERalb examinations in articles 2 and 3 were excluded from the analyses. Previously, employing the same procedure [133,134] and tracer, researchers from the Steno group have reported a mean intraindividual day-to-day coefficient of variation for TERalb of 9% in hypertensive type 2 diabetic subjects with nephropathy [135].

*3.3.4.2 Plasma markers of endothelial activation*
Venous blood samples were drawn without cuff stasis in the morning after an overnight fast (10-12 hours). Plasma samples were obtained from citrated whole blood (after discarding the first 2 ml), immediately stored in a freezer at -80°, and subsequently thawn and analyzed *en bloc*. Plasma levels of von Willebrand factor (vWF), E-selectin, P-Selectin, thrombin/antithrombin complex (TAT), prothrombin fragment 1+2 (F1+2), vascular cell adhesion molecule-1 (VCAM-1), and soluble intercellular adhesion molecule-1 (ICAM-1) were measured by enzyme-linked immunosorbent assays (vWF: Dako A/S, Copenhagen, Denmark [136]; E-selectin, P-selectin, TAT, F1+2, VCAM-1, and ICAM-1: R&D Systems, Oxon, UK). The coefficient of variation in percent (CV%) for vWF, E-Selectin, and ICAM-1 were 2.5, 5.0, and 9.2%, respectively. Plasma levels of fibrinogen and alpha-2-macroglobulin were measured by nephelometry (Dade Behring, Illinois, USA).

*3.3.4.3 Cell culture experiments*
In article 4, cell culture experiments were performed in which the effect of adding EDTA-plasma from various groups of overnight fasting (10-12 hours) diabetic and non-diabetic subjects to cultured endothelial cells was studied [4].

3.3.4.3.1 Cell cultures of human umbilical vein endothelial cells (HUVEC)
Human endothelial cells obtained from collagenase-digested umbilical veins were cultured in Dulbecco's modified Eagle Medium (glucose concentration: 5,5 mM), containing 10% foetal calf serum (FCS), 2 µg/ml ciprofloxacin, 100 µg/ml ampicillin, 1% L-glutamine, 40 µg/ml endothelial cell growth factor (ECGF), 15 U/ml heparin, and 5 mM glutamine in gelatine-coated plates (0.65 µg/cm2) and maintained at 37°C in an atmosphere of 5% CO2 and 95 % atmospheric air. The cells were subcultured after detaching with trypsin solution and replating. Cells were used between the 3' and 6' trypsination. Cell counting was performed after trypsination, using a Bürker-Türk chamber. Viability of cells was evaluated by morphology and trypan-blue exclusion.

3.3.4.3.2 ELISA procedures for cellular content of VCAM-1 and E-selectin
A total of 640 cultures were used for the analysis of adhesion molecules. A modified ELISA procedure was used to measure the cellular E-selectin and VCAM-1 content [132,137]. Cells were grown in 96-well plates, exposed to 10 % test plasma in normal medium for 6 hours, subsequently washed once with 150 µl PBS, fixed in 150 µl 100 % methanol for 10 min, air-dried, and stored at 4°C. Dried cells were rehydrated and blocked in 150 µl PBS, 0.1 % Tween-20, 0.5 % BSA (P+T+A) for 30 min and washed twice in P+T. The wells were then incubated for 2 h at room temperature with either a monoclonal antibody against human E-selectin (BBA-16, R&D Systems) diluted 1/500 in P+T+A or a polyclonal goat antibody against human VCAM-1 (BBA-19, R&D Systems) diluted 1/500 in P+T+A. After two washes in P+T, wells were incubated with horse-radish-peroxidase (HRP)-conjugated secondary antibodies diluted in P+T+A: Rabbit anti-mouse Ig-HRP (NA9310, Amersham LIFE SCIENCES) 1/4000 for E-selectin measurements and rabbit anti-goat-Ig-HRP (P0160, DAKO A/S, Denmark) 1/4000 for VCAM-1 analysis. After 1 h of incubation at room temperature, wells were washed five times in P+T, and they were subsequently coloured using 100 µl TMB-reagent (DAKO S 1600) as substrate for the bound HRP. After 5 min of incubation, the reaction was stopped by adding 100 µl 3 M H2SO4. Absorbance was read at 540 nm in an ELISA-reader. Every individual plasma sample was analysed in quadruplicate, and a mean value was calculated.

3.3.4.3.3 Proliferation studies
A total of 320 cultures were used for proliferation studies. To estimate the proliferation rate, cells were labelled with 2.5 µCi/ml 3H-thymidine during the incubation with test sera for 24 hours. Radioactive incorporation into DNA was measured in fixed (100 % methanol) and washed (3 times with 10 % ice-cold trichloricacid (TCA)) cells after solubilisation in 1 % SDS, 0.5 N NaOH. Dissolved

radioactive material was measured by liquid scintillation counting. The individual plasma sample was analysed in quadruplicate, and a mean value was calculated.

### *3.3.5 Biochemical analyses*

Blood samples were drawn after an overnight fast (10 to 12 h). HbA1c was determined by high-performance liquid chromatography (HPLC), non-diabetic range 4.4-6.4%. Blood glucose was determined by Reflolux II (Boehringer Mannheim, Frankfurt am Main, Germany). Total cholesterol, HDL cholesterol, and triglycerides were analysed using an enzymatic colorimetric method (COBAS INTEGRA 700, Roche). LDL cholesterol was calculated from the Friedewald equation: LDL cholesterol = total cholesterol - HDL cholesterol - triglycerides $\times$ 0.45.

## 4. RESULTS

### 4.1 MICROVASCULAR PERMEABILITY IN THE RETINA, KIDNEY, AND THE OVERALL MICROVASCULATURE

In 20 type 2 diabetic subjects with DMa and 20 type 2 diabetic subjects without retinopathy matched for gender, age, and known duration of diabetes, we compared retinal thickness (reflecting the degree of macular oedema, and hence the permeability of the retinal microvasculature), the glomerular leakage of albumin (as evaluated by UAE), and the general microvascular permeability (as evaluated by TERalb) [2]. Because ACE inhibitors (ACE-i) and angiotensin II receptor antagonists are known to affect UAE, and because ACE-i´s have been suggested to affect TERalb [135] and retinal vascular permeability [46], patients treated with these agents were not included. Likewise, patients previously treated with laser photocoagulation were not included.

Not surprisingly, average retinal thickness (OCT) was greater in subjects with compared to subjects without DMa (247 $\pm$ 29 vs. 227 $\pm$ 13 µm, *P*<0.01), albeit with a considerable overlap between individual values in the two groups **figure 3**. UAE was higher in the DMa group than in the group without retinopathy (9.3 $\times$/$\div$ 3.1 vs. 3.9 $\times$/$\div$ 1.9 µg/min, *P*<0.01), whereas TERalb did not differ significantly between groups (6.0 $\pm$ 1.6 vs. 6.6 $\pm$ 1.5 %, NS). AMBP values were higher in the DMa than in the control group, although differences did not reach the level of statistical significance. Finally, subjects with DMa had higher HbA1c and total cholesterol values than subjects without retinopathy.

In subjects with DMa, OCT, UAE, and TERalb, correlated significantly (OCT vs. UAE: *r*=0.58, *P*<0.01; OCT vs. TERalb: *r*=0.55, *P*<0.05; UAE vs. TERalb: *r*=0.81, *P*<0.01); conversely, in subjects without retinopathy there was no such correlation between these three permeability indicators.

In conclusion, retinal thickness in the macular area, related to the permeability of retinal blood vessels, is associated with an increased glomerular leakage as well as with a generalized microvascular hyperpermeability in type 2 diabetic subjects with diabetic maculopathy. These findings signify that hyperpermeability of small blood vessels coexists in various organ systems of diabetic patients with microvascular complications, suggesting mutual underlying pathogenic mechanisms.

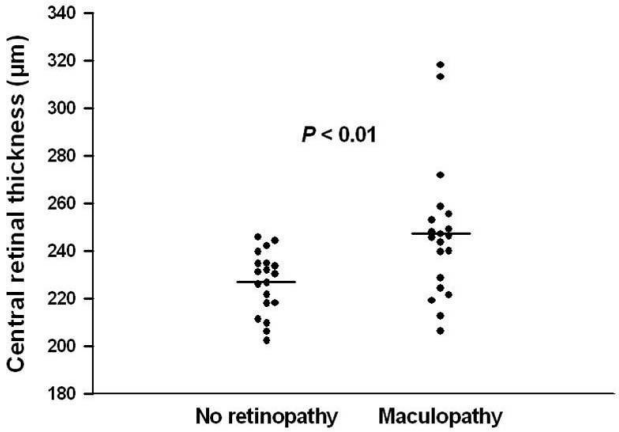


**Figure 3.** Average centrel retinal thickness in the two groups.

### 4.2 ADHESION MOLECULE EXPRESSION OF CULTURED ENDOTHELIAL CELLS AFTER ADDITION OF PLASMA FROM TYPE 2 DIABETIC PATIENTS WITH MACULOPATHY

We studied four matched (gender, age, duration of diabetes) groups of each 20 subjects: 1) control subjects with normal glucose tolerance (NGT), 2) subjects with impaired glucose tolerance (IGT), 3) type 2 diabetic subjects without retinopathy, and 4) type 2 diabetic subjects with DMa [4]. Addition of plasma from subjects in the four groups had dissimilar effects on the subsequent expression of E-selectin on the surface of cultured endothelial cells: E-selectin expression was lowest after addition of plasma from NGT subjects (198 $\pm$ 19 x 103 arbitrary absorbance units), increased in a stepwise manner through the IGT and no retinopathy groups to reach a maximum in the group with DMa (259 $\pm$ 23 x 103 arbitrary absorbance units, *P*<0.05 for the comparison between the DMa and NGT groups), **figure 4**. In contrast, the effect on VCAM-1 expression and cell proliferation did not differ between groups.

The expression of E-selectin and VCAM-1 on HUVECs did not correlate with gender, age, duration, smoking, antidiabetic or antihypertensive medication, BMI, AMBP values, blood glucose, HbA1c, lipids, or UAE; likewise, these parameters did not correlate with the proliferation rate of the HUVECs.

In conclusion, plasma from type 2 diabetic subjects contains factor(s) capable of inducing the expression of E-selectin on the surface of cultured human endothelial cells. This effect cannot directly be ascribed to hyperglycaemia, hyperlipidaemia, or other classical clinical or biochemical risk factors. An exaggerated propensity to induce E-selectin expression on the surface of vascular endothelial cells may contribute to the development of maculopathy in type 2 diabetes; however longitudinal studies are needed to confirm this hypothesis.

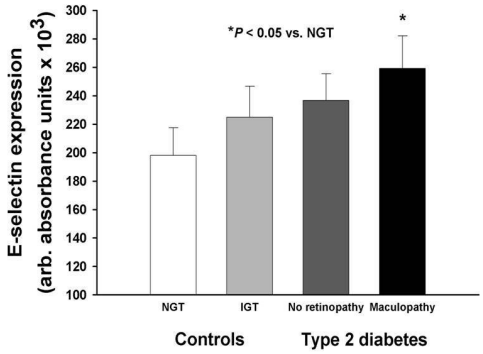


**Figure 3.** Expression of E-selectin in endothelial cells after addition of plasma from subjects with normal glucose tolerance (NGT), impaired glucose tolerance (IGT), and from type 2 diabetic patients with and without retinopathy. Error bars indicate SE. *$P$<0.05 vs. NGT.

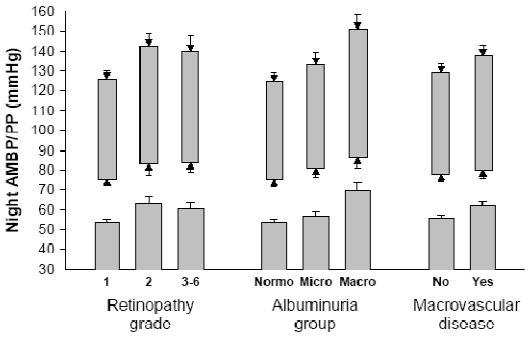


**Figure 4.** Mean values of night ambulatory blood pressure (AMBP, upper panel) and night pulse pressure (PP, lower panel) in categories of retinopathy, nephropathy, and macrovascular disease.
▼systolic BP, ▲diastolic BP. Error bars indicate SE.

4.3 MICRO- AND MACROVASCULAR COMPLICATIONS, PULSE PRESSURE, AND CIRCADIAN BLOOD PRESSURE VARIATION

80 type 2 diabetic patients were categorised according to the degree of retinopathy (none, grade 2, or grade 3-6), nephropathy (normo-, micro-, or macroalbuminuria), and macrovascular disease (no/yes), and we compared 24-hour AMBP values between these subgroups [1]. AMBP values in patients with these micro- and macrovascular complications were consistently higher than in subjects without complications. In particular, night BP values were increased to a higher extent than day BP in patients with complications, reflecting a disturbed circadian BP variation in these patients compared to patients without complications; similarly, systolic BP values were more markedly elevated than diastolic BP values, thus resulting in higher PP values in patients with compared to patients without complications (night AMBP values depicted in **figure 5**).

AMBP parameters correlated somewhat with age and duration of diabetes. Subjects with complications tended to be older and have longer diabetes duration than subjects without these complications; hence these parameters could potentially have biased the observed association between AMBP parameters and complications. When entering retinopathy level, albuminuria status, age, and duration as covariates in a multivariate analysis, there was still an independent, statistically significant effect of retinal grade on diastolic night BP ($P$=0.03), whereas the effect of retinopathy grade on the rest of the AMBP parameters was no longer statistically significant (e.g. $P$=0.06 for systolic night BP). Albuminuria status was independently and significantly associated with systolic day ($P$<0.01) and night ($P$<0.001) BP, diastolic night BP ($P$<0.01), systolic and diastolic night/day ratio ($P$<0.05 for both), and day and night PP ($P$<0.05 and $P$<0.001 respectively). After correction for differences in age and duration between groups, the effect of macrovascular disease group was still significant for systolic and diastolic night/day BP ratios, but not for the other AMBP parameters.

In conclusion, the presence of micro- and macrovascular complications is associated with blunted nocturnal BP reduction and increased PP. The cross-sectional nature of the study does not allow for firm conclusions regarding causality, but we hypothesize that these haemodynamic abnormalities are involved in the pathogenesis of micro- and macrovascular complications in type 2 diabetes.

4.4 PULSE PRESSURE, CIRCADIAN BP VARIATION, AND ENDOTHELIAL PERTURBATION

We performed two cross-sectional studies, both comprising healthy control subjects as well as type 2 diabetic subjects participating in an experiment on the autoregulation of retinal blood vessels [125]. All subjects underwent 24-hour AMBP measurement, and von Willebrand factor (vWF), fibrinogen, E-selectin, and soluble intercellular adhesion molecule 1 (ICAM-1) were measured in plasma.

In the first study [5], we examined the relation between retinopathy, non-dipping, and endothelial perturbation. We included four matched (gender, age, duration) groups, each comprising 19 subjects: Group A consisted of healthy control subjects, whereas the other three groups consisted of type 2 diabetic subjects without retinopathy (Group B), minimal background retinopathy (Group C), and diabetic maculopathy (Group D), respectively. The classification of diabetic subjects according to retinopathy status was made on the basis of a thorough clinical ophthalmological examination, fundus photography, and OCT scanning of the central retina.

Diastolic and systolic night/day BP ratios were comparable in Groups A and B, increased in group C, and were highest in Group D (e.g. for systolic night/day BP ratio 85.2 $\pm$ 5.1, 85.7 $\pm$ 5.7, 88.5 $\pm$ 6.3, and 90.5 $\pm$ 7.3 %, respectively, $P$<0.05), thus reflecting an increasingly blunted diurnal BP variation with increasing degree of retinopathy. Subjects with diabetes had higher plasma levels of endothelial markers, but these levels did not differ significantly between retinopathy groups (B-D). Among diabetic subjects, systolic and diastolic night/day BP ratio correlated significantly with plasma vWF levels ($r$=0.30, $P$<0.05 and $r$=0.43, $P$<0.01, respectively). Systolic non-dippers (n=15) had significantly higher plasma levels of vWF and fibrinogen compared to dippers (median [interquartal range (IQR)] 1.7 [1.4-2.1] vs. 1.2 [0.9-1.5] U/ml, $P$<0.01 **figure 6** and 3.6 [3.6-3.7] vs. 2.9 [2.5-3.6] g/l, $P$=0.01, respectively). Similarly, diastolic non-dippers (n=10) had higher plasma levels of vWF than had dippers (1.9 [1.7-2.2] vs. 1.2 [0.9-1.5] U/ml, $P$<0.001), whereas plasma levels of the other

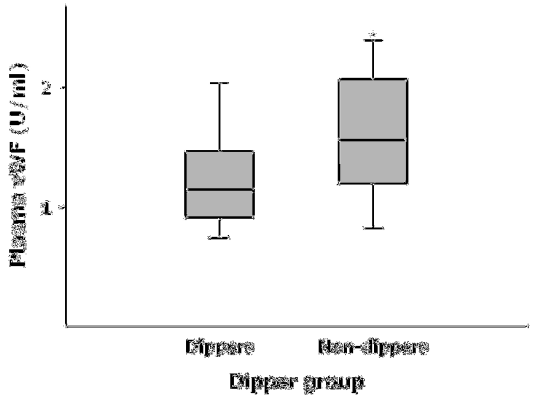


**Figure 5**. Plasma levels of von Willebrand factor (vWF) in systolic dipping and non-dipping type 2 diabetic subjects. *Bars* represent medians, and *boxes* indicate IQRs. **P*<0.01 vs. dippers.

endothelial markers did not differ significantly between dipper groups. Dippers and non-dippers had comparable mean 24-hour BP values, and they did not differ with regard to gender, age, duration of diabetes, smoking habits, HbA1c, lipids, or plasma creatinine. In contrast, night/day BP ratios increased significantly with increasing UAE (e.g. systolic night/day BP ratio 87.3 ± 7, 87.7 ± 6, and 94.9 ± 3 %, normo-, micro-, and macroalbuminuria, respectively, *P*=0.01). UAE correlated weakly with plasma vWF levels (*r*=0.34, *P*<0.05), whereas there was no significant correlation between UAE and plasma levels of the other endothelial markers. As expected, there was a preponderance of patients with more advanced nephropathy stages with increasing degree of retinopathy; thus, UAE could potentially have confounded the observed associations between non-dipping and endothelial perturbation. However, these associations persisted even after correction for UAE.

In the second study [6], we categorized 46 type 2 diabetic subjects according to their UAE. Thirty-four patients had normoalbuminuria (Group N) and 12 had micro- or macroalbuminuria (Group A). Nineteen healthy control subjects were also included in the study (Group C). The groups did not differ with regard to gender, age, smoking, antihypertensive treatment, or plasma creatinine, whereas Group A tended to have a longer duration of diabetes, poorer glycaemic control, and higher total cholesterol levels compared with Group N. PP values increased significantly from Group C to Groups N and A (e.g. night PP 43 ± 5, 48 ± 10, and 59 ± 12 mmHg, *P*<0.001).

Plasma concentrations of endothelial markers increased gradually from Group C to Groups N and A (e.g. ICAM-1, median [IQR], 191 [160-217], 213 [189-262], and 260 [218-316] ng/ml, *P*<0.01). In diabetic subjects (n=46), all PP parameters correlated significantly with plasma ICAM-1 and E-selectin levels (e.g. night PP vs. ICAM-1: *r*=0.37, *P*=0.01, **figure 7**; and E-selectin: *r*=0.38, *P*<0.01), whereas there was no significant correlation between PP values and vWF or fibrinogen. Neither PP values nor plasma levels of endothelial markers correlated with age, duration of diabetes, glycaemic control, lipids, or plasma creatinine. Moreover, the correlations between ICAM-1, E-selectin, and PP values persisted and remained statistically significant after stratification for gender,

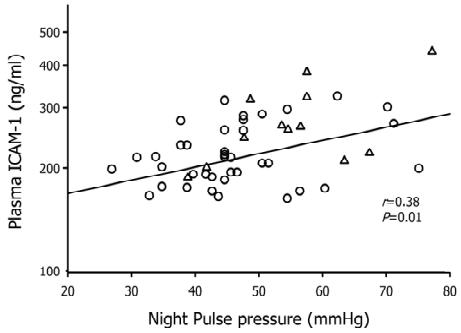


**Figure 6.** Night pulse pressure vs. plasma ICAM-1 in Type 2 diabetic subjects. Circles represent normoalbuminuric subjects (Group N). Triangles represent micro- or macroalbuminuric subjects (Group A).

antihypertensive and lipid-lowering treatment, smoking habits, and albuminuria group.

In conclusion, non-dipping and elevated pulse pressure are associated with microvascular complications as well as with elevated plasma levels of molecules signifying endothelial perturbation in type 2 diabetic subjects, suggesting that endothelial activation may represent a pathophysiological link between these haemodynamic parameters and microvascular complications in type 2 diabetic subjects.

### 4.5 PULSE PRESSURE, NON-DIPPING, AND PROGRESSION OF NEPHROPATHY

We included 118 type 2 diabetic patients in an observational, prospective study [8]. Subjects were followed for a mean of 9.5 (range 0.5-14.5) years until death or until April 1st, 2008. Four subjects were excluded due to incomplete 24-hour AMBP measurements, and two subjects were lost due to migration, thus leaving 112 subjects with available follow-up data. At baseline, all subjects underwent 24-hour AMBP. UAE was evaluated by three urinary albumin/creatinine ratios at baseline and follow-up. Progression of nephropathy was defined as progression from normo- to microalbuminuria, from micro- to macroalbuminuria, or as a doubling of plasma-creatinine or development of end stage renal failure.

During follow-up, 35 (19 of 71 patients with normo-, 7 of 26 patients with micro-, and 9 of 15 patients with macroalbuminuria) of 112 patients progressed to a more advanced stage of nephropathy. At baseline, patients who subsequently progressed tended to be older, had a slightly longer duration of diabetes, were more likely to be treated with insulin, tended to have more advanced retinopathy and macrovascular disease, and tended to have a poorer glycaemic control and a higher plasma creatinine than non-progressors (n=77); however, none of these differences were statistically significant. Strikingly, none of the office BP modalities differed significantly in progressors and non-progressors. In contrast, among progressors there was a significant preponderance of smokers and patients with a more advanced degree of nephropathy at baseline. Likewise, diastolic night/day BP ratio and 24-hour systolic BP and PP, but not 24-hour diastolic BP, were significantly higher in the group who subsequently progressed. Smokers and non-smokers had similar diastolic night/day BP ratios and 24-hour PP values (*P*>0.3 for

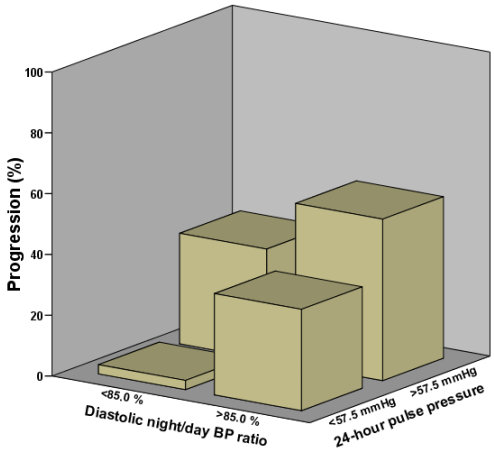


**Figure 7.** 3-D presentation of the proportion of type 2 diabetic patients with progression of nephropathy according to categories of diastolic night/day BP ratio (below or above the median value, 85.0 %) and 24-hour ambulatory pulse pressure (below or above the median value, 57.5 mmHg), *P*<0.001.

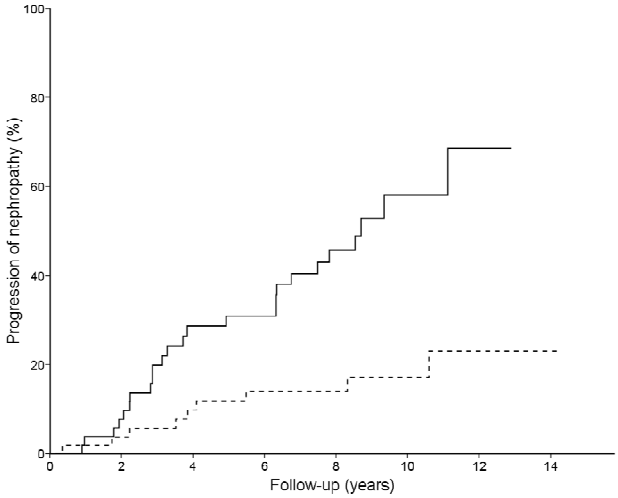


**Figure 8.** Progression of nephropathy in type 2 diabetic subjects with 24-hour ambulatory pulse pressure below (dashed line, *n*=56) or above (solid line, *n*=56) the median (57.5 mmHg). *P*<0.001 (log rank test).

both comparisons). Likewise, diastolic night/day BP ratios and 24-hour PP values did not correlate statistically (*r*=0.07, *P*=0.48). In combination, these two BP indices strongly predicted nephropathy progression: Only 1 of 33 patients (3.0 %) with diastolic night/day BP ratio and 24-hour PP below the median values progressed, whereas 17 of 32 patients (53.1%) with diastolic night/day BP ratio and 24-hour PP above the median progressed to a more advanced nephropathy stage (*P*<0.001, **figure 8**).
In unadjusted bivariate analyses, 24-hour PP was the baseline variable most strongly correlated with nephropathy progression; 46.3 % of subjects with a 24-hour pulse pressure above the median value of 57.5 mmHg exhibited progression of nephropathy, whereas only 16% of subjects with a 24-hour PP below the median value subsequently progressed; log-rank test for differences between groups, *P*<0.001, **figure 9**. In a Cox regression analysis, including gender, age, duration of diabetes, smoking, baseline UAE, HbA1c, and p-creatinine, 24-hour systolic BP, 24-hour PP, and diastolic night/day BP ratio, independent predictors of nephropathy progression were smoking (hazard ratio (HR) 2.48, 95% confidence interval (CI) 1.19-5.17, *P*=0.02), 24-hour PP (HR 1.04 (for each mmHg increase), CI 1.01-1.07, *P*<0.01), and diastolic night/day BP ratio (HR 1.05 (for 1% increase), 95% CI 1.01-1.07, *P*=0.02), whereas the other variables in the model did not have a significant, independent predictive value for nephropathy progression.
In conclusion, elevated pulse pressure, diastolic non-dipping, and smoking are strong, independent predictors of nephropathy progression in type 2 diabetic subjects. These potentially modifiable risk factors could represent important targets for pharmacological as well as non-pharmacological interventions aiming at reducing this common and serious complication of type 2 diabetes.

## 4.6 EFFECT OF SHORT-TERM ANGIOTENSIN 2 RECEPTOR BLOCKADE ON AMBP VARIABLES, MARKERS OF ENDOTHELIAL ACTIVATION, AS WELL AS RENAL, RETINAL AND GENERALISED VASCULAR PERMEABILITY

We included 24 type 2 diabetic subjects with DMa, i.e. oedema and/or hard exudates in the macular area, in a randomized, placebo-controlled, double-masked, parallel-group trial comparing the effect of 4-months treatment with either losartan 50 mg o.d. or placebo on macular oedema and hard exudates [3]. Exclusion criteria included clinically significant macular oedema (CSMO) requiring immediate macular laser photocoagulation, previous laser photocoagulation treatment, other significant ocular disease, previous or present treatment with ACE inhibitors or angiotensin 2 receptor antagonists (ARB), hypotension (office BP<110/60 mmHg), uncontrolled hypertension (office BP>175/95 mmHg), and severely reduced renal function (p-creatinine>200 µmol/l). The primary end-point was the change from baseline in central retinal thickness, as evaluated by OCT-scanning, whereas secondary end-points were changes from baseline in a) peripheral macular thickness (in the area with hard exudates), b) number of hard exudates, c) retinopathy grade, d) visual acuity, e) UAE, f) TERalb, g) markers of endothelial activation (vWF, thrombin/antithrombin complex, prothrombin fragments 1+2, P-selectin, fibrinogen, and alpha-2-macroglobulin), and h) 24-hour AMBP parameters.
Baseline values of clinical and laboratory parameters did not differ significantly when comparing the losartan and placebo group; however smoking, male gender, other AHT, and diet-only treatment of diabetes tended to be slightly more prevalent in the losartan group, whereas there was a trend towards a higher prevalence of macrovascular disease in the placebo group. Moreover, at baseline, subjects in the losartan group tended to be marginally older, have a slightly shorter duration of diabetes, be somewhat more obese, have slightly higher p-creatinine, and slightly higher clinical and 24-hour AMBP values. Mean central retinal thickness was similar in the two groups at baseline; however, at follow-up the central retinal thickness had increased in the losartan group, whereas it remained unchanged in the

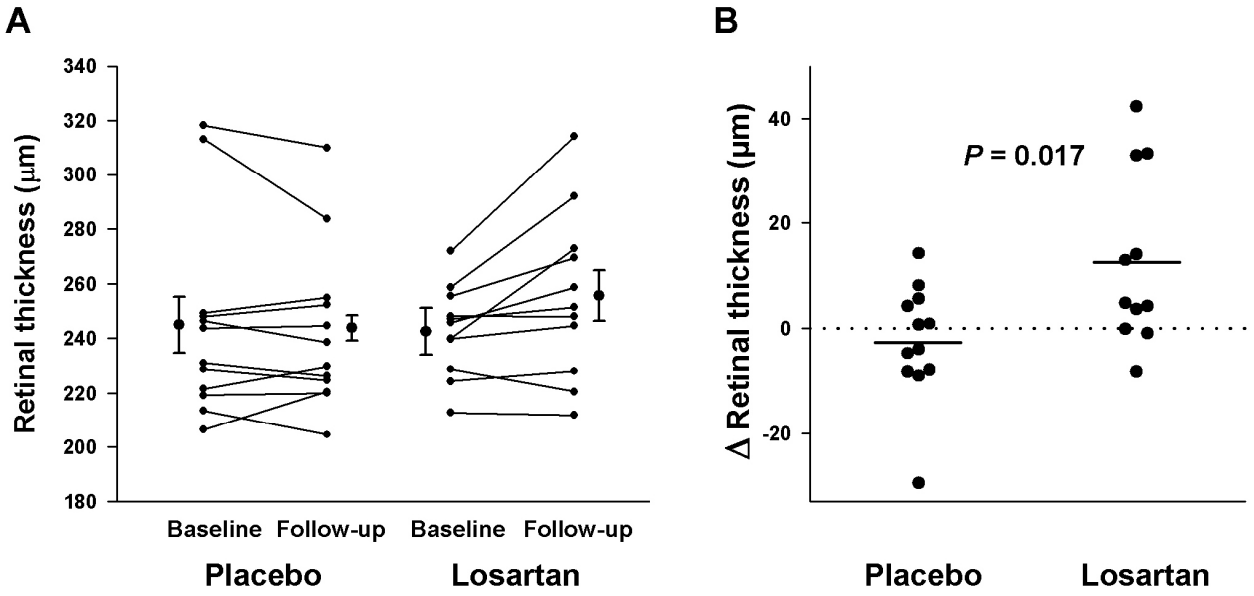


**Figure 9.** Fig. 2: Overall radial scan thickness as measured by OCT scans through the fovea. **A**: Individual values of retinal thickness at baseline and follow-up in the two groups. Error bars represent group means ± SE. **B**: Change in retinal thickness for individual patients in the two groups. Horizontal lines represent group means.

placebo group (Δ retinal thickness 13 ± 16 vs. -2 ± 11 µm, *P*=0.017, **figure 10**). Peripheral macular thickness in the area with hard exudates and macular oedema increased in both groups with a tendency towards greater increments in the losartan compared with the placebo group, but there was a large variation in retinal thickness this area, and the observed change was not statistically significant neither within nor between groups (Δ retinal thickness 18 ± 32 vs. 9 ± 24 µm, losartan and placebo groups, respectively, NS). Likewise, there were no significant changes in the number of hard exudates, visual acuity, retinopathy grade, UAE, TERalb, or markers of endothelial activation in any of the groups. At follow-up, four patients in the losartan group and one patient in the placebo group were treated with laser photocoagulation due to progression of their maculopathy. Group changes in AMBP values are depicted in **figure 11**. Day blood pressures (BP) were moderately lowered in the losartan group compared to the placebo group where BP values did not change (Δ systolic day BP -6 ± 13 mmHg, *P*=0.17; Δ diastolic day BP -5 ± 5 mmHg, *P*<0.01), whereas night BP values remained unchanged in both groups, for which reason night/day BP ratios tended to increase in the losartan group fig.ure 11. PP levels did not change significantly in either group. The observed changes in retinal thickness did not correlate with changes in glycaemic control, AMBP values, UAE, TERalb, markers of endothelial activation, or any of the other clinical or laboratory parameters in this study.

In conclusion, type 2 diabetic subjects with diabetic maculopathy do not seem to benefit from short-term (4 months) treatment with losartan, as this treatment may aggravate macular oedema. Moreover, treatment with losartan 50 mg administered in the morning only affects BP during day-time, thus tending to induce a non-dipping BP pattern.

4.7 EFFECT OF LONG-TERM DUAL BLOCKADE OF THE RAS WITH AN ACE-INHIBITOR AND AN ANGIOTENSIN 2 RECTOR BLOCKER COMPARED WITH HIGH-DOSE ACE-INHIBITION ON PULSE PRESSURE AND UAE

The CALM II study was a randomized, double-masked, parallel-group study comprising 75 type 1 and 2 diabetic subjects with hypertension, comparing the effect of treatment with lisinopril 40 mg o.d. vs. 12 months dual blockade with candesartan 16 mg o.d. plus lisinopril 20 mg o.d. on systolic office BP and AMBP values [138,139]. Secondary end-points were changes in diastolic BP values and UAE. 63 type 2 diabetic subjects were included in the study; of these, 11 did not complete the study due to persistently high SBP (8 subjects) or side effects (3 subjects), and one patient refused to undergo AMBP measurement at follow-up. We performed a post-hoc analysis in the remaining 51 type 2 diabetic subjects who completed the 12-month treatment period with available AMBP measurements at baseline and follow-up.

Baseline clinical and laboratory values were comparable in the two groups, albeit there was a trend towards a higher proportion of subjects treated with a thiazide diuretic in the dual blockade group vs. the lisinopril group (16 vs. 8, *P*=0.10). Dual blockade treatment (n=25) significantly lowered 24-hour systolic BP, whereas the effect on 24-hour diastolic BP was of minor magnitude and did not reach the level of statistical significance (-5 ± 11 mmHg, *P*=0.03 and -2 ± 7 mmHg, *P*=0.29, respectively). In the lisinopril group (n=26), the opposite outcome was observed (Δ 24-hour systolic BP -1 ± 9 mmHg, *P*=0.45 and Δ 24-hour diastolic BP -3 ± 7 mmHg, *P*=0.03). However, this qualitatively

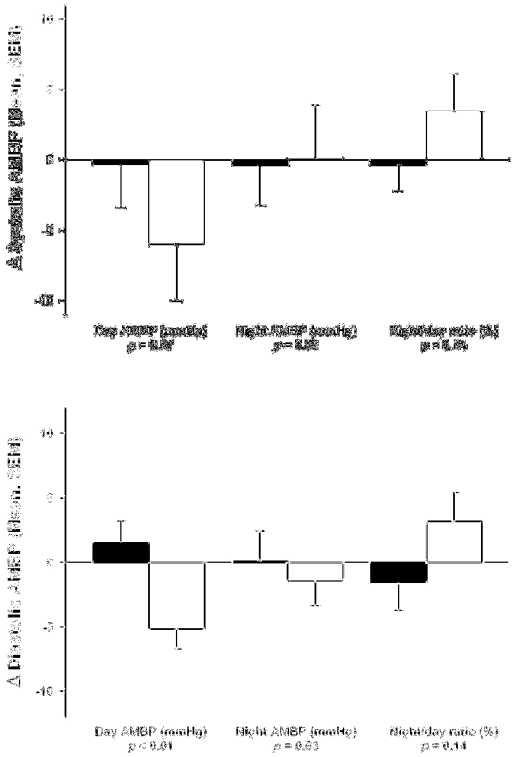

**Figure 10.** Changes over four months in day and night AMBP as well as night/day ratio in the two groups.
● Placebo group ○ Losartan group

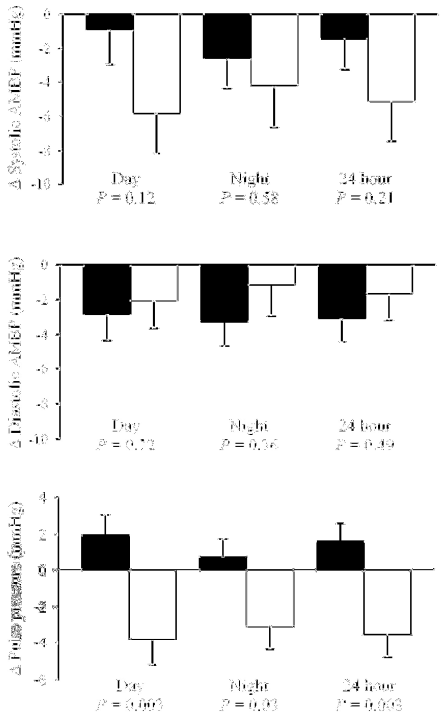

**Figure 11.** Changes in ambulatory blood pressure (AMBP) values from baseline to followup in the two groups.
● Lisinopril group ○ Dual blockade group

divergent effect between the two treatment modalities on individual BP components was not statistically significant when comparing between-group differences; intriguingly, though, the differential effect of the two AHT modalities on PP values was highly statistically significant (e.g. for Δ 24-hour PP: -5 ± 5 mmHg, *P*=0.003), **figure 12**.

Because there was a trend towards a higher proportion of subjects on concomitant thiazide diuretic treatment in the dual blockade than in the lisinopril group, we stratified the PP analysis on this parameter. Interestingly, the superiority of dual blockade over lisinopril monotherapy in lowering PP seemed more pronounced in subjects not taking a thiazide diuretic (n=27, e.g. Δ 24-hour PP: -4 ± 6 vs. 2 ± 5 mmHg , *P*<0.01) than in subjects on concomitant diuretic treatment (n=24, e.g. Δ 24-hour PP: -3 ± 7 vs. 0 ± 4 mmHg, *P*=0.29). The subjects in the present study did not have very high PP values at baseline (mean 24-hour PP was 53 ± 8 mmHg). However, 13 subjects had a very high baseline 24-hour PP (>60 mmHg). As expected, these subjects had a higher drop in PP (Δ24-hour PP, -4 ± 8 mmHg) compared with the 38 subjects with a 24-hour PP below 60 mmHg (Δ24-hour PP, 0 ± 5 mmHg), although this difference was not statistically significant *P*=0.1). Interestingly, the superiority of dual blockade over lisinopril monotherapy in lowering PP was more pronounced in the high PP group (Δ 24-hour PP -9 ± 7 vs. 1 ± 4 mmHg, respectively) than in the low PP group (Δ 24-hour PP -2 ± 5 vs. 2 ± 5 mmHg, respectively); however, the difference in the effect on PP between the two treatment modalities was statistically significant in both the high (*P*=0.02) and low (*P*=0.03) PP group.

At baseline, UAE was within the normal range in the majority of study participants, and this parameter did not change significantly from baseline to follow-up in either of the treatment groups. Likewise, creatinine clearance was unchanged during the 12-months study period.

In conclusion, in hypertensive type 2 diabetic subjects, 12 months dual blockade treatment with candesartan plus lisinopril caused a significant reduction in PP compared with high-dose monotherapy with lisinopril. Theoretically, this effect is promising; whether it will translate into a greater degree of end-organ protection must be clarified in future large-scale and long-term studies.

## 5. DISCUSSION

In my Ph.D. thesis [9], I reviewed the association between haemodynamic and structural abnormalities and microvascular complications of type 2 diabetes, with particular focus on diabetic maculopathy. The present review is an extension of my previous research, including data from an experimental *in vitro* study, two additional cross-sectional studies, an observational follow-up study, as well as a randomized, controlled clinical trial.

### 5.1 HYPERPERMEABILITY OF SMALL BLOOD VESSELS AND MICROVASCULAR COMPLICATIONS

Increased glomerular permeability to macromolecules such as albumin is a cardinal feature in diabetic nephropathy, thus giving rise to excessive albuminuria. This abnormal glomerular leakage has been shown to reflect a generalized vascular hyperpermeability in diabetic subjects with incipient and overt diabetic nephropathy [23,25,38-40,82]. Similarly, diabetic maculopathy (DMa) is characterized by an increased permeability of retinal blood

vessels, thus resulting in leakage of lipoproteins and fluid, subsequently giving rise to the formation of hard exudates and oedema in the retina. No previous studies had examined associations between retinal thickness as assessed by ocular coherence tomography and measures of glomerular and vascular permeability in diabetes. We hypothesized that hyperpermeability of retinal blood vessels in DMa was associated with a generalized vascular hyperpermeability (evaluated by TERalb) as well as with an increased glomerular leakage of albumin in type 2 diabetic subjects. In type 2 diabetic patients with diabetic maculopathy, we found strong correlations between retinal thickness, urinary albumin excretion rate, and transcapillary escape rate of albumin, whereas no such associations were present in type 2 diabetic patients without retinopathy [2]. An increased retinal thickness has previously been shown to coincide with an increased leakiness of retinal blood vessels in type 2 diabetes [102]; consequently, our findings indicate that in type 2 diabetic patients with maculopathy, the pathologically increased permeability of the retinal vessels, as reflected by increased retinal thickness, corresponds to equivalent permeability changes in kidney capillaries and in small vessels throughout the circulation of the diabetic patient with microvascular complications. Hence, like albuminuria [23], macular oedema seems to be a marker of widespread endothelial damage, as indicated by vascular and glomerular hyperpermeability.

Even though diabetic retinopathy and nephropathy are mutually statistically associated [23,32,83-88], data on the independent relationship between retinopathy and TERalb have been conflicting [32,39]. In the present study [2], TERalb did not differ significantly between the groups with and without retinopathy. A possible explanation for this negative finding is that not all subjects in the case group had advanced DMa, as the retinal changes of the included subjects varied from a single hard exudate to severe macular oedema. However, when comparing OCT measurements of retinal thickness, as a quantitative measure of macular oedema, with TERalb in these subjects, the association was statistically significant.

The above mentioned statistical association between diabetic retinopathy and nephropathy [23,32,83-88] has been based on asymmetric comparisons between semiquantitative gradings of retinopathy, as opposed to assessments of UAE as a quantitative measure for the severity of diabetic nephropathy. In the present study, we had the opportunity of comparing UAE with the degree of DMa, reflected by quantitative measurements of retinal thickness, and we demonstrated a strong correlation between these two microvascular complications in the group with DMa. Finally, in these subjects, we confirmed previous findings of an association between UAE and TERalb[23,25,38-40,82].

As previously mentioned, both DMa and diabetic nephropathy are characterized by a pathologically increased permeability of a vascular barrier [140]. In DMa, leakage of lipoproteins and water from the retinal vessels result in the formation of hard exudates and macular oedema, whereas increased permeability of glomerular capillaries in diabetic nephropathy leads to an increased UAE. A study in type 2 diabetic patients with nephropathy comparing 12 months treatment with either lisinopril or atenolol showed a reduction in albuminuria and TERalb in the ACE inhibitor treated patients, whereas this was not seen in the patients treated with atenolol, even though BP reduction was similar in the two groups [135]. This finding indicates that ACE inhibitors have direct effects on the microvasculature, thus re-establishing the barrier function of the leaky capillaries in diabetes. This theory is supported by the fact that retinal blood vessels have receptors for angiotensin II [141], and that ACE inhibition reduces the permeability of these vessels [142]. Our patients with DMa appeared to have a systemic microvascular hyperpermeability, the degree of which was reflected by retinal thickness. As treatment with ACE inhibitors is known to have beneficial effects on UAE and seems to reduce an elevated TERalb, treatment with ACE inhibitors or angiotensin II receptor antagonists could be a promising treatment modality for patients with DMa. Quantitative assessment of retinal thickness, e.g. by ocular coherence tomography, may become a useful instrument in evaluating such intervention effects.

### 5.2 *IN VITRO* ENHANCEMENT OF ADHESION MOLECULE EXPRESSION OF ENDOTHELIAL CELLS IN THE PRESENCE OF PLASMA FROM SUBJECTS WITH DIABETIC MACULOPATHY

Numerous studies have proposed excessive activation of the vascular endothelium as a mechanism underlying diabetic vascular complications [22-36,143-146]. In several of our studies, we assessed plasma levels of molecules derived from endothelial cells as markers of endothelial dysfunction/activation. In a cross-sectional study, we could not demonstrate differences in the plasma concentration of these parameters between a group of subjects with DMa and subjects without retinopathy [2]. In accordance with this finding, we found no difference in plasma concentrations of markers of endothelial activation between type 2 diabetic subjects without complications compared with matching subjects with retinopathy [5] or incipient/overt nephropathy [6], albeit we observed a positive correlation between UAE and plasma levels of von Willebrand factor [6]. However, in this context it is important to realise that none of the studies were designed to evaluate group differences in these parameters; hence the absence of significant results may well be ascribed to a lack of power (type 2 error). We hypothesized that addition of plasma from diabetic patients with maculopathy would stimulate the expression of E-selectin and VCAM-1 on the surface of cultured human umbilical vein endothelial cells (HUVECs). Furthermore, we wanted to study the effect on the proliferation rate of these cells.

Previously, addition of plasma from type 1 diabetic subjects to cultured human endothelial cells has been shown to induce a higher expression of VCAM-1 in the endothelial cells than addition of plasma from age-matched non-diabetic control subjects [132]. In a similar experimental set-up, we found that addition of plasma from diabetic patients with maculopathy stimulated the expression of E-selectin from cultured HUVECs to a significantly higher degree than addition of plasma from non-diabetic (NGT) subjects, whereas the E-selectin inducing ability of plasma from subjects with IGT and type 2 diabetic subjects without retinopathy was only moderately and non-significantly higher than in NGT subjects [4]. However, the difference in induction of E-selectin expression between the diabetic subgroups was not statistically significant. The E-selectin inducing activity did not correlate with smoking, hyperglycaemia, hyperlipidaemia, BP levels, or other classical clinical or biochemical risk factors for diabetic vascular complications. The ability of plasma to induce expression of VCAM-1 in HUVECs or to stimulate the proliferation rate of HUVECs did not differ significantly between these four groups [4]. Comparing 93 type 1 diabetic subjects with varying degrees of retinopathy with 47 healthy gender- and age-matched subjects [147], Olson et al. found that circulating plasma levels of E-selectin was highest in type 1 diabetic subjects with severe non-proliferative retinopathy, a group that is comparable to our group

with maculopathy. Levels of VCAM-1 were highest in subjects with proliferative retinopathy [147], a group that was not included in our study [4], whereas ICAM-1 levels did not differ between groups in the Olson study [147]. Moreover, Olson et al. demonstrated that sera from patients with retinopathy contained factors capable of inducing increased migratory activity of retinal capillary endothelial cells *in vitro*. Interestingly, this effect could be abolished by antibodies to E-selectin and VCAM-1, suggesting a causal role of these endothelially derived molecules in the excessive angiogenesis of proliferative diabetic retinopathy [147]. In the present study [4], we found no differences in the ability of plasma from the different groups on the proliferation rate of the endothelial cells, which is not surprising, as DMa is not a disease primarily characterised by excessive vascular proliferation. Furthermore, it should be noted that the endothelial cells in our study were derived from umbilical veins, as opposed to the retinal capillary endothelial cells used in the study by Olson et al. [147]. Finally, our study participants had type 2 diabetes [4], whereas Olson et al. studied subjects with type 1 diabetes [147].
Combining the results from the two above mentioned studies [4,147], it may be hypothesized that increased E-selectin activity is present in the earlier stages of diabetic retinopathy, whereas increased VCAM-1 activity is more important in the proliferative stages of diabetic retinopathy. Hence, an exaggerated propensity to induce E-selectin expression on the surface of vascular endothelial cells may contribute to the development of maculopathy in type 2 diabetes; moreover, increased expression of endothelial adhesion molecules on the surface of endothelial cells seems to be involved in the transition from advanced non-proliferative to proliferative retinopathy. However, caution must always be observed when such interpretations are made from *in vitro* data. Moreover, both studies were cross-sectional; hence, firm conclusions must await the results of future longitudinal studies on this subject. Finally, further studies are needed to identify the factor(s) responsible for the induction of an enhanced expression of adhesion molecules on endothelial cells in diabetes.

## 5.3 OFFICE VS. 24-HOUR AMBULATORY BP MEASUREMENTS AND MICRO- AND MACROVASCULAR COMPLICATIONS

24-hour AMBP provides a higher sensitivity and reproducibility than does conventional BP measurement [148-150]. In non-diabetic, hypertensive subjects, AMBP has been shown to be superior to conventional BP measurement in predicting (primarily cardiovascular) disease and death [54,151-156]. Numerous previous studies have established the superiority of AMBP over conventional office BP measurement for the association with [57-59] and prediction of hypertensive end-organ damage in diabetes [60,116,129]. Recent data from a longitudinal study in elderly subjects with type 2 diabetes confirm the superiority of AMBP over and above office BP measurements for the prediction of nephropathy progression [129,157].
Data from the present studies once again demonstrate the diagnostic superiority of AMBP over conventional office BP measurement **Table 2**. Importantly, these differences were observed despite the fact that in our studies, office BP measurements were performed lege artis and calculated as the mean of three random zero spygmanometric measurements, accomplished under optimal conditions. Hence, the diagnostic performance of these office BP measurements is presumably better than standard office BP measurements performed in daily clinical settings, thus emphasizing the value of employing AMBP measurements in every-day clinical practice. In addition to the increased diagnostic sensitivity and reproducibility, employment of AMBP as opposed to office BP measurement provides the opportunity to study diurnal BP fluctuations.

**Table 2.** Statistically significant differences/correlations demonstrated by employment of 24-hour AMBP measurement, although undetectable by office BP measurement (mean of three random zero sphygmanometric measurements) in subjects with type 2 diabetes.

| |
|---|
| Higher AMBP (particularly systolic night BP) in subjects with retinopathy than in subjects without retinopathy [1,5] |
| Higher AMBP in subjects with nephropathy than in subjects without nephropathy [1,6] |
| Higher systolic night AMBP, and ambulatory PP in subjects with than in subjects without macrovascular disease [1] |
| Higher 24-hour systolic AMBP, diastolic night/day BP ratio, and 24-hour ambulatory PP in subjects with subsequent progression of nephropathy than in subjects without progression of nephropathy [8] |
| Correlations between 24-hour AMBP values and markers of endothelial perturbation (E-selectin and ICAM-1) [5,6] |
| Reduction in day AMBP values induced by 4 months´ treatment with losartan 50 mg o.d. [3] |
| Reduction in ambulatory PP values with 12 months´ dual blockade treatment with candesartan and lisinopril compared with high-dose lisinopril monotherapy [7] |

## 5.4 CIRCADIAN BP VARIATION AND MICROVASCULAR COMPLICATIONS

"Non-dipping" denotes a condition where blood pressure (BP) fails to decline by at least 10% during night-time [113,114,158]. There is accumulating evidence that "non-dippers" are at an increased risk of cardiovascular events [153,159-164]. In diabetic subjects, non-dipping has been associated with both micro- [58,62-67] as well as macrovascular [165] complications and even with increased mortality [116,165]. We hypothesised that a reduced circadian BP variation was associated with micro- and macrovascular complications in type 2 diabetic subjects.
Cross-sectional data from the present studies confirm the association between non-dipping and micro- [1,5] as well as macrovascular [1] complications in type 2 diabetic subjects; furthermore, in a longitudinal design, we have demonstrated that diastolic non-dipping is a strong and independent predictor of nephropathy progression in type 2 diabetic subjects [8].
Kohner and Patel have constructed a hypothesis associating an elevated arterial blood pressure with the development of diabetic retinopathy 23;24: BP elevation leads to increased perfusion pressure of small blood vessels, subsequently leading to hyperperfusion, especially if the autoregulation of retinal blood vessels is impaired [125]. Hyperglycaemia may further exacerbate hyperperfusion [166], as an elevated blood glucose level seems to hamper autoregulation in diabetic subjects [167], a phenomenon we did not observe in young, non-diabetic subjects exposed to short-term hyperglycaemia [168]. The resulting increase in capillary shear stress leads to damage and closure of these small vessels with subsequent intensified hyperperfusion of the remaining vessels, thus establishing a true *circulus vitiosus*.
A disproportionately elevated night BP has been referred to as "the submerged portion of the iceberg" [169], as it is not recognized in routine clinic BP measurements. Thus, for a given clinic BP level, an altered diurnal BP profile with an abnormally increased night BP inflicts a higher "24-hour BP load" to the vasculature, thereby enhancing vascular wall shear stress and promot-

ing endothelial dysfunction, inflammation, and ultimately microvascular damage with subsequent development and progression of microvascular complications. Moreover, a blunted diurnal BP variation has been associated with autonomic dysfunction [57,62,170]. Although the retinal vascular system itself has no autonomic nervous supply, dysfunction of autonomic nerves to preretinal resistance vessels might adversely affect their ability to prevent the propagation of an elevated systemic BP to the retinal microcirculation, thus aggravating the capillary hypertension and hyperperfusion described above. However, autonomic neuropathy has not been the main focus of the present investigations; therefore, these assumptions remain speculative.

Considering the association between non-dipping and vascular complications, it has been discussed whether pharmacological modulation of 24-hour BP profiles, inducing a dipping BP pattern in subjects with a blunted nocturnal BP decline could confer organoprotection in these subjects [171]. Unintentionally, by administration of losartan 50 once-daily in the morning, we induced an increase in the night/day BP ratio (reflecting a more non-dipping BP pattern) in type 2 diabetic subjects with DMa [3]. This theoretically undesirable effect may explain the worsening of macular oedema observed in the losartan group of this study; however, the increase in macular oedema did not correlate with the changes in night/day BP ratios, so we have no proof of this theory. Conversely, in the HOPE study [73,172], the ACE-inhibitor, ramipril, was administered once-daily at bed-time; in a subgroup of participants (n=38) undergoing AMBP, a resulting 8 % reduction in night/day BP ratio between the placebo and ramipril groups was demonstrated after one year of follow-up [173]. The authors speculate that the improved diurnal BP rhythm was likely to account for the reduction of cardiovascular events observed in the ramipril group [173], but this theory also remains speculative as it was not possible to separate the effect of the improved 24-hour BP profile from the overall effect of BP lowering per se in this important study.

Several researchers have demonstrated that it is possible to restore the normal circadian BP pattern in non-dippers with targeted antihypertensive therapy directed towards an elevated night BP [174-177]. Hermida et al. showed that bedtime administration of valsartan was capable of restoring the physiological 24-hour BP variation in 75 % of non-dippers compared to only 24 % when valsartan was administered in the morning [175]. More importantly, the same group of researchers have demonstrated that such pharmacologically induced normalization of the diurnal BP rhythm resulted in a 41 % decrease in 24-hour urinary albumin excretion in microalbuminuric subjects with essential hypertension, and that this effect correlated strongly with the increased diurnal/night BP ratio (corresponding to a higher degree of BP dipping during night-time) [176]. These results are interesting and promising; however, further intervention studies are needed to resolve whether antihypertensive treatment should be targeted specifically at restoring the normal diurnal BP variation in non-dipping subjects.

## 5.5 PULSE PRESSURE AND MICROVASCULAR COMPLICATIONS

Until recently, major medical textbooks stated that diastolic blood pressure (BP) was the most important BP modality in relation to cardiovascular (CV) risk. Systolic BP often rises with age, while diastolic BP remains unchanged or declines [178], causing a widening of the PP. These changes, generally ascribed to the development of arteriosclerosis-induced stiffness of the arteries, were previously considered physiological and benign in nature. However, during the last decade, several studies have shown PP to be a major, independent predictor of cardiovascular events in non-diabetic subjects [49-54,179]. We hypothesised that an elevated pulse pressure was associated with micro- and macrovascular complications in this patient group.

In 2002, we were the first to describe a strong association between ambulatory PP and micro- and macrovascular complications in a group of middle-aged Danish type 2 diabetic subjects [1]. We suggested that PP could be a risk factor for the development and progression of vascular complications in this patient group, even though the cross-sectional nature of the study did not allow for any firm conclusions regarding causality. In accordance with previous observations [87,88,180,181], the presence of retinopathy and nephropathy was strongly statistically associated in several of the present studies [1,2,5,6,8]. Correcting for this statistical association may be considered an overcorrection, as the concurrent development of these microvascular complications could reflect common underlying pathophysiological mechanisms [2]. However, we chose to perform such statistical corrections where applicable [1,5,6,8], which would tend to favour the null hypothesis.

Subsequently, we and other researchers have associated an elevated PP with both micro- [6,182] and macrovascular [183-185] complications in diabetic patients. In 2006, Palmas et al. published a longitudinal study including 1040 elderly type 2 diabetic patients followed for 12-24 months [129]. After adjustment for other clinical covariates, ambulatory PP was a strong and independent predictor of albuminuria progression, above and beyond office BP. The study had considerable statistical power, due to a large number of participants and the employment of AMBP, whereas the limitations of the study included the relatively short follow-up period of only one to two years, considering the fact that diabetic nephropathy typically evolves over a decade or more [186]. Moreover, albuminuria status was evaluated on the basis of a single urine sample, even though three samples are usually recommended due to the considerable intraindividual day-to-day variability of this key parameter [128]. However, recently we confirmed these results and extrapolated them to middle-aged type 2 diabetic patients followed for 9.5 years with a more precise assessment of nephropathy status (based on three urine samples per patient at baseline as well as at follow-up) [8]. Furthermore, we found a strong and intriguing additive predictive effect of combining diastolic night/day ratio and 24-hour PP: in the group of subjects with both diastolic night/day BP ratio and 24-hour PP below the median, only 1 of 33 patients progressed, whereas 17 of 32 patients in the group with values of both BP parameters above the median progressed to a more advanced nephropathy stage [8].

Thus, a substantial number of observational studies have described the association between an elevated PP and diabetic complications. Moreover, several randomized trials have shown substantial benefits of treating isolated systolic hypertension in elderly patients [187-189], but until the beginning of 2008 no studies had specifically aimed at intervening against an elevated PP. However, Tropeano and co-workers have recently published results from a large prospective, open-label study, where 4500 general practitioners were specifically instructed to intervene against an elevated PP (>60 mmHg) in hypertensive type 2 diabetic subjects, preferentially using a fixed low-dose perindopril/indapamide combination [190]. On average, PP was lowered by 9 mmHg during the 9 months follow-up, and the authors observed a 36% reduction in the incidence of CV events in the treated subjects [190]. In a univariate analysis, baseline PP was a

strong predictor of future CV events; however, in a multivariate analysis, neither baseline PP nor PP reduction independently predicted CV events during follow-up.
In a post-hoc analysis, we compared the PP-reducing ability of 12 months dual RAS blockade with candesartan and lisinopril versus high-dose lisinopril monotherapy in 51 patients with type 2 diabetes, participating in the CALM II study [7]. The two treatment modalities had divergent effects on individual BP parameters: dual blockade treatment primarily reduced systolic AMBP, whereas the effect on diastolic AMBP was of minor magnitude and did not reach the level of statistical significance. Lisinopril treatment had the opposite effect, significantly reducing diastolic, but not systolic, AMBP values. These qualitatively contrasting effects between the two treatment modalities on individual BP components were not statistically significant; interestingly, though, the differential effect of the two AHT modalities on PP values was highly statistically significant.
A limitation of the study is the lack of AMBP measurement at follow-up in the 8 patients that were excluded due to an insufficient reduction in SBP. However, as 5 patients in the lisinopril and only 3 patients in the dual blockade group were excluded for this reason, the overall conclusion that dual blockade was more efficient than lisinopril monotherapy in reducing SBP and PP can hardly be explained by a selective drop-out in the two treatment groups.
These findings are somewhat in conflict with the results from previous short-term studies, in which dual blockade treatment caused comparable reductions in systolic and diastolic BP [191-197]. The reason for this discrepancy cannot be deduced by the prevailing data. Even so, the dual action of this regime on the RAS aims at a more pronounced vasodilating effect of smaller blood vessels than monotherapy with an ACE-inhibitor or an ARB, resulting in a decline in vascular wall shear stress that may delay or even counteract endothelial dysfunction [4,6,36] and vascular calcification, in turn opposing the advancing premature arterial stiffness seen in type 2 diabetic subjects. Conversely, the significant reduction in DBP from baseline to follow-up in the lisinopril group of the present study may be indicative of progressive arterial stiffening in these subjects over the 12 months study period, thereby reflecting the natural history of the hypertensive disease rather than a true treatment effect. Thus the reduction in PP over 12 month's dual blockade treatment may be cautiously interpreted as an indirect marker of reduced vascular stiffness, an effect that can only be detected in studies with a considerable follow-up period, which could explain the divergent results from previous short-term studies [191-197]. However, it is important to realise that the results are based on a post-hoc analysis, as the CALM II study was not originally designed to study effects of different types of AHT on PP levels [138]. Moreover, although this assumption is interesting and promising it is important to realise that it is based on theoretical considerations [198], as we did neither calculate novel indirect measures of arterial stiffness, e.g. ambulatory arterial stiffness index (AASI) [199-203], nor did we study direct measures of (central) arterial stiffness, e.g. pulse wave velocity (PVW) [204-209] and central pulse pressure [208,210,211].
Interestingly, in non-diabetic subjects, Mulvany and co-workers have demonstrated that structural changes in small arteries induced by hypertension (evaluated *in vitro* by myograph examinations of small human resistance arteries achieved by gluteal biopsies) regressed in response to AHT with vasodilatory agents [212], whereas no such effect was seen after treatment with diuretic or beta-blocking agents, despite similar BP reductions by the different treatment modalities [213,214]. In line with these findings, the same group of researchers has recently shown that the forearm vascular resistance, assessed *in vivo* by plethysmography, is reduced in response to AHT with an ARB (eprosartan), i.e. a vasodilatory agent, but not with beta-blockers [215]. These results suggest that AHT with vasodilatory agents can actually induce regression of structural artery changes caused by long-standing hypertension, hence reducing an abnormally increased arterial stiffness. Although the ACE-inhibitor, lisinopril, is itself a vasodilatory agent, results from the present study [7] suggest that addition of an ARB enhances the effect on arterial stiffness, as reflected by a reduction in PP by dual blockade treatment compared with monotherapy with an ACE-inhibitor. In this respect it is very interesting that the Mulvany group has recently demonstrated that small artery structure is an independent predictor of future cardiovascular events in subjects with essential hypertension [216].
At this time point little is known about the long-term effect of different antihypertensive treatment regimes on PP levels in diabetic subjects. The present results [7] indicate that long-term dual blockade of the RAS, in contrast to conventional AHT with an ACE-inhibitor, leads to an almost isolated reduction in systolic BP, with virtually no effect on the diastolic BP component, thereby potentially allowing for a more radical reduction of SBP, thus causing a narrowing of the PP gap.Theoretically, a pharmacologically induced reduction of PP may impede the development of micro- and macrovascular complications in diabetic subjects, thus implying that dual blockade could be particularly beneficial in this group of patients. Having said that, despite positive effects on albuminuria in some [217-219], but not all [139,220] studies, a very recent large study does not point towards particular benefits with dual blockade compared to monotherapy with an ACE-inhibitor or an ARB on renal or cardiovascular outcomes in either non-diabetic or diabetic subjects [217,221]. In fact, despite positive effects on albuminuria, the primary composite end-point of dialysis, doubling of serum creatinine, or death was significantly increased with combination therapy, compared with either monotherapy [217]. These surprising and negative results have moderated the enthusiasm regarding the dual blockade concept considerably. Very recently, the opinion that dual blockade should no longer be used in clinical practice has even been expressed [222]. However, whereas the future of dual blockade of the RAS with an ACE-inhibitor and an ARB in diabetic as well as in non-diabetic subjects seems uncertain, new combinations of AHT modulating the same important regulatory system are being investigated [223,224].

### 5.6 MICROVASCULAR COMPLICATIONS, CIRCADIAN BP VARIATION, PULSE PRESSURE, AND ENDOTHELIAL PERTURBATION

The underlying pathogenic mechanisms connecting the haemodynamic abnormalities, non-dipping and elevated PP, with vascular damage in diabetes are unknown. Numerous studies have described an association between microvascular complications and markers of endothelial perturbation in diabetic subjects [4,23,25,26,28-30,32,34,35,143-146]. We hypothesized that endothelial dysfunction could represent a link between non-dipping, elevated PP, and microvascular complications in diabetes.
In two cross-sectional studies [5,6], we found that non-dipping and elevated PP were associated with microvascular complications as well as with elevated plasma levels of molecules signifying endothelial perturbation in type 2 diabetic subjects. Three studies have examined the cross-sectional relationship between

nocturnal BP non-dipping, elevated PP, and endothelial activation in subjects without diabetes mellitus [75,76,117]. von Känel et al. [117] examined 76 younger (mean age 36 years), unmedicated normo- and hypertensive subjects. Non-dippers had significantly higher plasma levels of D-dimer, plasminogen activator inhibitor-1 (PAI-1), vWF, and soluble intercellular adhesion molecule-1 (ICAM-1) than had dippers; conversely, plasma levels of interleukin-6 did not differ between groups. However, after correction for differences in age, gender, ethnicity, smoking, hypertension status, and social class between groups, only differences in D-dimer and vWF remained statistically significant, whereas the difference in ICAM-1 between dipper groups was only borderline significant (*P*=0.055).

Lee et. al. [76] studied 73 middle-aged, predominantly non-diabetic subjects with stable coronary artery disease and preserved left ventricular ejection fraction. Non-dippers had higher plasma levels of vWF, D-dimer, fibrinogen, and soluble P-selectin than had dippers. After adjustment for differences in age, hypertensive status, history of coronary artery by-pass grafting, and 24-hour AMBP, levels of vWF, fibrinogen, and P-selectin were still significantly higher in non-dippers than in dippers. Flow-mediated, endothelium-independent vasodilation of the brachial artery (FMD), as evaluated by high-resolution ultrasound scanning before and after reactive hyperaemia and sublingual nitroglycerin administration did not differ significantly between dippers and non-dippers. Dividing participants according to mean 24-hour ambulatory PP levels (24-h PP), subjects with 24-h PP above the median (51 mmHg) had higher plasma levels of vWF, fibrinogen, soluble P-selectin, and D-dimer compared to subjects with 24-h PP below the median value. Moreover, FMD was significantly reduced in the group with high PP compared to the group with low PP. After adjustment for differences in age, dipping pattern status, white coat effects, left ventricular hypertrophy, and 24-hour ambulatory BP levels, plasma levels of vWF and D-dimer were still significantly higher, and FMD was significantly reduced in subjects with high PP compared to subjects with low PP levels.

Ceravolo et al. [75] studied 262 unmedicated subjects with essential hypertension. In a univariate linear regression analysis, a strong and highly significant inverse correlation was found between 24-h PP and acetylcholine-stimulated forearm blood flow (Ach-FBF), thus reflecting a gradually decreasing ability to dilate the brachial artery with increasing PP. In a stepwise multivariate analysis, 24-h PP was superior to all other clinical, laboratory, and BP variables as a predictor of Ach-FBF, and after adjustment for age, gender, BMI, smoking habits, serum glucose, cholesterol, triglycerides, and mean systolic and diastolic BP level, the authors estimated that each mmHg increment in 24-h PP corresponded to a decrease in Ach-FBF of 8.7%.

The results of the above-mentioned studies [75,76,117] suggest that endothelial cell activation could represent a causal link between non-dipping, elevated PP, and cardiovascular disease, possibly by promoting the process of atherosclerosis, arterial calcification, and an increased tendency of blood clotting. Our results extend these findings, suggesting that endothelial activation could also represent a link between these haemodynamic abnormalities and microvascular complications in subjects with type 2 diabetes [5,6]. Non-dipping of BP inflicts a higher "24-hour BP load" on the microvasculature [169], and this may in itself induce an inappropriate activation of the microvascular endothelium through enhanced shear stress of the vascular wall. Moreover, an augmented PP reflects a decreased elasticity of large and middle-sized arteries. Evidently, the capability of these stiffened vessels to absorb changes in BP is decreased; consequently, these vessels would be more likely to allow the propagation of an increased BP to the microcirculation. The resulting increased BP amplitude imposes a steep rise in shear stress on the microvascular wall, especially if resistance vessel innervation and autoregulation is impaired [125], theoretically resulting in capillary hypertension and hyperperfusion as well as endothelial dysfunction with subsequent development of micro- and macrovascular complications.

Importantly, the cross-sectional nature of all five studies connecting non-dipping, elevated PP, and endothelial perturbation [5,6,75,76,117] does not allow for firm conclusions regarding causality. Thus, the diabetic *milieu* of hyperglycemia, inflammation, oxidative stress etc. may promote endothelial dysfunction, causing accelerated vascular atherosclerosis and calcification, subsequently leading to arterial stiffening, widening of the PP, and, possibly, non-dipping. Hence, prospective studies are needed to further elucidate these associations.

### 5.7 INHIBITION OF THE RENIN-ANGIOTENSIN SYSTEM (RAS) IN DIABETIC RETINOPATHY

Despite the benefits of improved control of hyperglycaemia [225] and hypertension [19] as well as laser photocoagulation [226,227], diabetic retinopathy remains a leading cause of visual loss in people of working age [12-14]. However, surprisingly few randomized trials have been designed with the primary objective of evaluating effects of AHT on the course of diabetic retinopathy. In the EUCLID study [72], there was a trend towards a reduced progression of retinopathy in type 1 diabetic patients treated with the ACE-inhibitor, lisinopril. We hypothesized that short-term blockade of the angiotensin 2 receptor with losartan could reduce the severity of diabetic maculopathy (DMa) in type 2 diabetic subjects.

We found no beneficial effect of 4 months treatment with the angiotensin II receptor antagonist (ARB), losartan, in a dose of 50 mg o.d. in type 2 diabetic subjects with DMa, as this treatment actually seemed to increase central retinal thickness, indicating a deterioration of macular oedema. As previously described, this unexpected and disturbing effect could be a result of a suboptimal diurnal RAS blockade, inducing a more "non-dipping" 24-hour BP profile in losartan-treated patients, an effect which has been observed in several other studies [228-230]. Our study was designed in 1997, at a time where the maximal recommended daily dose of losartan in Denmark was 50 mg; subsequent studies have shown that the optimal dosage of losartan is considerably higher [71,231,232].

The putative role of endothelial perturbation as a pathophysiological link between a non-dipping diurnal BP profile and diabetic complications has been discussed previously [5,6]. In the present study, we specifically hypothesized that the augmentation of macular oedema in patients treated with losartan could represent a rebound phenomenon in the retina, induced by the BP reduction during day-time and successive nocturnal BP surge in these patients. However, at that time point there was limited knowledge regarding the stability of macular oedema, as evaluated by OCT scanning of central retinal thickness; therefore, the assumption of a rebound phenomenon was somewhat speculative. Subsequently, several studies have confirmed that OCT-measured central retinal thickness is highly reproducible [233,234]; however, in subjects with foveal macular oedema, retinal thickness increases overnight [235]. This overnight retinal thickening correlates strongly with the nocturnal BP change and it is accompanied by a reduction in visual acuity [235]. No such

changes were observed in healthy control subjects [235]. Furthermore, in subjects with clinically significant macular oedema (CSMO), central retinal thickness has been shown to decrease modestly but significantly over the course of the day [236,237], and, finally, this reduction in central retinal thickness during daytime is attenuated, if subjects stay in the recumbent position instead of spending day-time hours in the upright position [238]. Data from these recent studies indirectly support our original hypothesis, albeit a formal verification of this hypothesis requires a randomized, double-masked study, examining the direct effects of acute BP reduction and subsequent BP rise on central retinal thickness.

While the effect of antihypertensive treatment (AHT), particularly with agents inhibiting the RAS, on the progression of diabetic nephropathy has been well-established for decades [43,44,68-71], results regarding retinopathy have as yet been less convincing [72]. Thus, despite the statistical association between diabetic retinopathy and nephropathy [1,2,5,87,88,180,181], and the common haemodynamic and structural pathophysiological features of the two complications previously discussed [1,2], the effect of inhibiting the RAS seems less convincing in diabetic retinopathy than in diabetic nephropathy. One possible explanation for this discrepancy is the different regulation of the retinal and glomerular blood flow. In the kidney, the intraglomerular pressure can be modified by contraction or dilation of afferent and efferent arterioles, whereas such a dual regulatory system does not exist in the retina. In the kidney, inhibition of the RAS causes a dilation of the efferent arteriole, resulting in a reduced intraglomerular pressure, thus ameliorating hyperfiltration and albuminuria, whereas in the retina, the vasodilatory effect of RAS inhibition does not necessarily lead to a decreased pressure in the retinal arterioles. However, the significance of these haemodynamic differences for the divergent effects of RAS inhibition on these two microvascular complications remains speculative at the present time point.

Although some studies have reported beneficial effects of AHT on diabetic retinopathy [72-74,239,240], other studies have not been able to demonstrate any such effects [241]. Very recently, the results of a large, randomized, controlled clinical trial, evaluating the effect of treatment with the ARB, candesartan, versus placebo on the incidence, regression, and progression of retinopathy in type 1 [242] and type 2 [243] diabetic patients have been published. 3326 normotensive type 1 diabetic patients without previous AHT and 1905 normotensive or treated hypertensive type 2 diabetic patients were randomized to treatment with 32 mg candesartan or placebo for three years [244,245]; however, the study was extended to four years due to a lower rate of events than expected [242,243].

In type 1 diabetic patients, candesartan had a borderline significant effect (hazard ratio (HR) for candesartan vs. placebo 0.82, *P*=0.0508) on the prespecified end-point of reducing the incidence of retinopathy by two or more steps in severity on the Early Treatment Diabetic Retinopathy Study (ETDRS) scale [120,246] (DIRECT-Prevent 1) [242], whereas this treatment had no effect on progression of pre-existing retinopathy (DIRECT-Protect-1, HR 1.02, *P*=0.85) [242]. However, post-hoc analyses showed a highly significant reduction in the incidence of a three-step or greater level of retinopathy (HR 0.65, *P*=0.0034); moreover, the final retinopathy level was more likely to improve with candesartan than with placebo in both DIRECT-Prevent 1 (odds ratio (OR) 1.16, *P*=0.0048) and DIRECT-Protect-1 (OR 1.12, *P*=0.0264) [242]. In type 2 diabetic patients (DIRECT-Protect 2), candesartan non-significantly reduced the progression of mild to moderately severe retinopathy (HR 0.87, *P*=0.20) [243], whereas there was a significant effect of candesartan on the secondary end-point of retinopathy regression (OR 1.34, *P*=0.009) [243]. In a subgroup analysis, this effect was confined to patients with mild retinopathy, whereas patients with moderate to moderately severe retinopathy did not benefit from candesartan treatment in this study [243].

Thus, despite an overall impression of a beneficial effect of candesartan on the course of diabetic retinopathy in both type 1 and type 2 diabetic patients, none of the pre-specified end-points were significantly affected by candesartan treatment in the three substudies [242,243]. Positive effects of candesartan were observed patients with no or mild retinopathy, whereas more severe stages of retinopathy did not seem to respond to candesartan treatment [242,243]. The authors speculate that more advanced stages of retinopathy, at which ischaemic changes predominate, have reached a "point of no return", where inhibition of the RAS is no longer effective [243]. If this theory is correct, the lack of a positive effect of losartan treatment in our small, short-term intervention study [3] may not be ascribed to a lack of power, too short a treatment period, or an inadequate RAS blockade; in contrast, the advanced retinopathy of our patients at inclusion may have passed a "point of no return", where pharmacological inhibition of the RAS was no longer effective.

In conclusion, even after the publication of the DIRECT study [242,243], inhibition of the renin-angiotensin system in normotensive diabetic subjects remains a promising treatment modality for reducing the incidence and progression of diabetic retinopathy, but more solid evidence for such beneficial effects is still needed.

## 6. SUMMARY AND FUTURE PERSPECTIVES

Due to the rapidly increasing global incidence and prevalence of type 2 diabetes, diabetic micro- and macrovascular complications constitute leading causes of blindness, renal failure, and cardiovascular morbidity and mortality world-wide. The aims of our investigations in subjects with type 2 diabetes were to study haemodynamic and structural abnormalities potentially associated with the presence and development of microvascular complications and to evaluate the effect of intervention with antihypertensive agents on these risk factors and complications. An overview of the major new findings from the present series of experiments in type 2 diabetic subjects is presented in **Table 3**.

We found strong associations between retinal thickness, urinary albumin excretion rate, and transcapillary escape rate of albumin in type 2 diabetic patients with maculopathy, suggesting that the pathologically increased permeability of the retinal vessels, as reflected by increased retinal thickness, corresponds to equivalent permeability changes in kidney capillaries and in small vessels throughout the circulation of the diabetic patient with microvascular complications. Hence, macular oedema seems to be a marker of widespread endothelial damage, as indicated by vascular and glomerular hyperpermeability. Addition of plasma from type 2 diabetic patients with maculopathy stimulated the expression of E-selectin in cultured human umbilical vein

**Table 3.** Major new results from the present studies in type 2 diabetic subjects.

| |
|---|
| Central retinal thickness, urinary albumin excretion rate, and transcapillary escape rate of albumin are associated in type 2 diabetic subjects with diabetic maculopathy [2] |
| Reduced nocturnal blood pressure decline ("non-dipping") is associated with micro- and macrovascular complications and predicts the progression of nephropathy [1,5,8] |
| Reduced nocturnal blood pressure decline ("non-dipping") is associated with endothelial perturbation [5] |
| Increased ambulatory pulse pressure is associated with micro- and macrovascular complications and predicts the progression of nephropathy [1,6,8] |
| Increased ambulatory pulse pressure is associated with endothelial perturbation [6] |
| Addition of plasma from type 2 diabetic subjects with diabetic maculopathy facilitates the expression of E-selectin on the surface of cultured endothelial cells [4] |
| 4 months angiotensin 2 receptor blockade with losartan 50 mg o.d. does not ameliorate diabetic maculopathy and may even aggravate macular oedema [3] |
| 12 months´ dual blockade treatment with candesartan and lisinopril reduces ambulatory pulse pressure values compared with high-dose lisinopril monotherapy [7] |

endothelial cells to a significantly higher degree than addition of plasma from non-diabetic subjects, suggesting that an exaggerated propensity to induce E-selectin expression on the surface of vascular endothelial cells may contribute to the development of maculopathy in type 2 diabetes.

Employing 24-hour ambulatory blood pressure measurements, we demonstrated that reduced nocturnal blood pressure decline ("non-dipping") and elevated pulse pressure were associated with micro- and macrovascular complications and predicted progression of nephropathy in type 2 diabetic subjects. Moreover, non-dipping and elevated pulse pressure were associated with elevated plasma levels of markers of endothelial activation in these subjects, suggesting that endothelial perturbation could represent a pathophysiological link between these haemodynamic risk factors and the development of micro- and macrovascular complications in type 2 diabetic subjects.

In a post-hoc analysis on data from the CALM II study, we found that 12 months dual blockade of the renin-angiotensin system with candesartan and lisinopril caused a significant reduction in ambulatory pulse pressure levels compared with high-dose lisinopril monotherapy, suggesting an effect of dual blockade treatment on an enhanced arterial stiffness in type 2 diabetic subjects. Considering the associations between an elevated pulse pressure and vascular complications in the previous studies, the pulse pressure lowering effect of the 12 months dual blockade treatment seems promising and advantageous for the prevention of such complications in type 2 diabetic subjects.

In a randomized, double-masked, parallel-group group study design, we found no beneficial effect of 4-months blockade of the renin-angiotensin system with losartan 50 mg o.d. compared with placebo on macular oedema and hard exudates in type 2 diabetic subjects with maculopathy. In the losartan group retinal thickness actually increased, suggesting an aggravation of macular oedema. The significance of this finding is uncertain, but the increase in retinal thickness may represent a rebound phenomenon due to a pharmacological induction of a non-dipping blood pressure pattern by the losartan treatment. Overall, the lack of effect of losartan could also be ascribed to a too short treatment period, or to a "point of no return effect", as new, larger, long-term studies point to an effect of angiotensin 2 receptor treatment in early, but not in advanced stages, of diabetic retinopathy.

**Table 4.** Suggested future studies in type 2 diabetic subjects.

| |
|---|
| Predictive effects of endothelial perturbation for the development of micro- and macrovascular complications |
| Associations between arterial stiffness (evaluated by ambulatory arterial stiffness index (AASI) pulse wave velocity (PVW), central blood pressure, and central pulse pressure), endothelial perturbation, and micro- and macrovascular complications |
| Effect of antihypertensive treatment on AASI, PVW, central blood pressure, and central pulse pressure |
| Effect of antihypertensive treatment (particularly with agents blocking the renin-angiotensin system) on earlier stages of diabetic retinopathy in larger studies with a longer treatment period |
| Effect of pharmacological restoration or enhancement of a nocturnal "dipping" blood pressure pattern with antihypertensive agents on micro- and macrovascular complications |

*Future studies* **Table 4** should focus on the predictive effects of endothelial perturbation for the development of diabetic micro- and macrovascular complications. Furthermore, the association between arterial stiffness, endothelial perturbation, and micro- and macrovascular complications in diabetes could be examined with novel indirect measures of arterial stiffness, e.g. ambulatory arterial stiffness index (AASI), as well as with more direct measures of arterial stiffness, e.g. pulse wave velocity (PVW) and central pulse pressure. The effect of antihypertensive treatment on arterial stiffness could be evaluated with the same methods. Moreover, the effect of antihypertensive treatment (particularly with agents blocking the renin-angiotensin system) on earlier stages of diabetic retinopathy could be evaluated in larger studies with a longer treatment period. Finally, the effect on diabetic micro- and macrovascular complications of restoring or enhancing a nocturnal "dipping" blood pressure pattern with antihypertensive agents could be examined.

## 7. SUMMARY

Diabetic vascular complications constitute leading causes of blindness, renal failure, and cardiovascular morbidity and mortality world-wide. We studied haemodynamic and structural abnormalities associated with the development of microvascular complications and evaluated the effect of intervention with antihypertensive agents on these risk factors and complications in type 2 diabetic patients (T2DM). Retinal thickness, urinary albumin excretion rate, and transcapillary escape rate of albumin were strongly associated in T2DM patients with maculopathy, suggesting that macular oedema is a marker of generalised vascular hyperpermeability in T2DM. Plasma from T2DM patients with maculopathy stimulated the expression of E-selectin in cultured endothelial cells. Reduced nocturnal blood pressure decline ("non-dipping") and elevated pulse pressure (PP) were associated with micro- and macrovascular complications and predicted progression of nephropathy in T2DM subjects. Non-dipping and elevated PP were associated with increased plasma levels of markers of endothelial activation in T2DM patients, suggesting that endothelial perturbation could represent a pathophysiological link between these haemodynamic risk factors and the development of vascular complications in T2DM. 4 months treatment with losartan 50 mg o.d. did not ameliorate macular oedema in T2DM patients with maculopathy. 12 months dual blockade of the renin-angiotensin system with candesartan and lisinopril reduced ambulatory PP levels compared with high-dose lisinopril monotherapy in hypertensive T2DM subjects.

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