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DISSERTATION

A new model to study human microvascular reactivity in vivo

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Zusammenfassung

Einleitung: Essentielle Hypertonie ist eine der weltweit häufigsten Erkrankungen und damit eine der größten medizinischen Herausforderungen der heutigen Zeit. Erhöhter Blutdruck ist einer der wichtigsten Risikofaktoren für die Entstehung von Herz- und Kreislauferkrankungen, welche in den Industrieländern zu den häufigsten Todesursachen zählen. Als Hauptort der Regulation des Blutdrucks, spielt die Mikrozirkulation eine Schlüsselrolle in der Ätiologie und während des Fortschreitens dieser Erkrankungen. Einer der wichtigsten Faktoren der zu den pathologischen Veränderungen bei Bluthochdruck führt ist das zentrale Hormon des Renin-Angiotensin-Systems (RAS), Angiotensin II (Ang II). Nichtsdestotrotz sind bisher nur indirekte oder invasive Methoden bekannt um die Reaktivität der kleinsten Blutgefässe auf lokal applizierte Substanzen im Menschen zu untersuchen (z.B. Laser-Doppler nach Iontophorese). Aus diesem Grund war das Ziel der vorliegenden Arbeit ein neues Modell für die *in vivo* Untersuchung der humanen Mikrozirkulation zu entwickeln und den Effekt von Ang II zu ermitteln.
Methoden: Zu diesem Zweck wurden normotensive Probanden mit einem zuvor validierten biomikroskopischen Setup untersucht. Die nüchternen Teilnehmer wurden in randomisierter verblindeter Reihenfolge an je einem Auge mit Placebo und am Anderen mit Ang II in zwei verschiedenen Konzentrationen (0.001%, 0.01%) bzw. mit Phenylephrine (0.25 %) als pharmakologische Kontrolle behandelt. Es wurden je drei Arteriolen und drei Venolen pro Auge vor und nach der Gabe des jeweiligen Augentropfens auf eine DVD aufgezeichnet Anschließend wurden die Gefäßdurchmesser mittels spezieller Software gemessen und die relative Veränderung ermittelt.
Ergebnisse: Die Validierung des Models ergab einen geringen Variationskoeffizienten von 4 % für die Gefäßmessungen. Die biologische Variabilität, ermittelt durch wiederholte Messungen vor und nach Placebo, lag bei 3 %. Ang II führte in der niedrigen Dosierung (0.001 %) nicht zu einer Vasokonstriktion. Die zehnfach höhere Dosis Ang II hatte genauso wie Phenylephrine einen statistisch signifikanten Effekt auf den Gefäßdurchmesser. Die Vasokonstriktion dauerte über 10 Minuten an und ließ im Verlauf langsam nach.
Diskussion: Das vorgestellte Modell eignet sich zur *non-invasiven* Untersuchung der Mikrozirkulation des Menschen. Die Ergebnisse zeigen dass der Effekt von lokal appliziertem Phenylephrin und Ang II im Auge mit dem vorgeschlagenen Modell messbar ist. Somit eröffnet sich die Möglichkeit den Einfluss von Schlüsselhormonen aus dem RAS auf die Mikrozirkulation möglich macht.

Schlüsselwörter: Hypertonie, Mikrozirkulation, Konjunktiva, Biomikroskopie, Reaktivität, Angiotensin II

Abstract

Introduction: Primary hypertension is one of today's most relevant and challenging medical conditions. It is the leading risk factor for cardiovascular disease (CVD) representing the most common cause of death in economically developed countries. As the major site of pressure reduction, the microcirculation plays a key role in the development and progression of arterial hypertension. One of the most prominent factors contributing to microcirculatory impairment as seen in hypertension is the key peptide of the Renin-Angiotensin-System (RAS), Angiotensin II (Ang II). However, at present only indirect or invasive measurement of microvascular reactivity to locally applied drugs (e.g. laser-Doppler technique following drug delivery by iontophoresis) is available. Therefore, the aim of the present study was to develop a new model to directly study human microcirculatory reactivity in vivo, in particular, to the effects of locally applied Ang II.

Design and methods: Normotensive subjects were studied with a previously validated intravital microscopy system. Following an overnight fast, conjunctival arterioles and venules were recorded before and after application of placebo eye-drops in one eye, and Ang II (0.001%, 0.01%), or phenylephrine (0.25%) drops in the other eye (in random order). Microvascular diameter changes were measured using dedicated software.

Results: Repeated analyses of the same microvessels in five subjects revealed a variability of measurement of 4 %. The repeated measurements of conjunctival microvascular diameters following placebo eye drops resulted in a variability of diameters of 3 % for arterioles and venules. A low concentration of Ang II (0.001%) had no effect on microvascular diameters. However, the higher concentration of Ang II (0.01%) and phenylephrine induced a statistically significant vascular constriction. This constriction lasted for at least 10 minutes.

Conclusion: A new model for in vivo testing of microvascular reactivity in man with low variability to locally applied drugs is presented. Ang II acutely induced a constriction of conjunctival arterioles and venules, which lasted for at least 10 minutes. These data suggest that the proposed in vivo model is suitable for studying the RAS in the human microcirculation.

Keywords: hypertension, microcirculation, conjunctiva, intravital microscopy, reactivity, angiotensin II

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1 Introduction

1.1 General introduction

Primary hypertension is one of today's most relevant and challenging medical conditions. It is the leading risk factor for cardiovascular disease (CVD) representing the most common cause of death in economically developed countries. Despite the multifaceted available treatment options, recent epidemiological studies predicted a 60% increase in the total number of people affected by high blood pressure (BP) within the next 20 years (Kearney, et al., 2005). In recent decades immense research efforts have been undertaken in order to improve the understanding of the underlying pathophysiological mechanisms and to establish appropriate prevention and treatment strategies. Today there is general agreement that established hypertension is characterized by an approximately normal cardiac output and a proportionally increased peripheral vascular resistance (Folkow, 1982; Pries, et al., 1999). Although the exact location of resistance arteries remains unclear, there is a general consensus that the microcirculation (vessel diameter < 150µm) is responsible for the regulation of the peripheral resistance and hence arterial pressure (Christensen and Mulvany, 2001; Struijker Boudier, et al., 1992). As the major site of pressure reduction, these minute vessels play a key role in the development and progression of arterial hypertension. Several alterations of microvascular structure and function have been described in beginning and established hypertension, representing cause and consequence of high blood pressure (Antonios, et al., 1999; Noon, et al., 1997; Shore and Tooke, 1994; Sullivan, et al., 1983). One of the most prominent factors contributing to microcirculatory impairment is the key peptide of the Renin-Angiotensin-System (RAS), angiotensin II (Ang II). This octapeptide hormone is known to variably affect vascular structure and function, leading to vasoconstriction and stimulating inflammation and remodeling processes (Dzau, 2001). In line with this, recent findings suggest a link between genes of the RAS and the risk for hypertension. It is discussed whether this is partially due to an increased sensitivity or reactivity to Ang II (de Leeuw, 1992; Spiering, et al., 2000; Zhu, et al., 2003). So far, countless models designed to study the different aspects of the microcirculation have been presented. They range from necropsy- and biopsy-based examination of vessel wall composition or receptor distribution via different animal models to angiographic washout techniques in the human renal vascular system.

Structural alterations like capillary rarefaction, as consistently described in hypertensive conditions, have been reliably identified by intravital biomicroscopy in different human and animal tissues (Antonios, et al., 1999; Chen, et al., 1987). The approaches to study microvascular function *in vivo* are however limited to either invasive or indirect procedures, e.g. microscopy of dissected hamster cremaster or laser-Doppler flowmetry in various tissues. Non-invasive procedures seem to be highly advisable for several reasons. An example is the influence of tissue exposure or anesthesia on the delicate functional anatomy of the microcirculation (de Leeuw, 1992; Harper, et al., 1978). Nevertheless, to date no decent models for a non-invasive and safe examination of microvascular function are available.

In the present study, a novel approach to the investigation of microvascular reactivity is introduced. The following paragraphs discuss the various elements that contributed to the formation of the present work. At first hypertension is described in more detail, as it is one of the most prevalent medical conditions with a crucial role in the development of innumerable serious diseases. Secondly, microcirculation as the origin of vascular resistance and the center of blood pressure regulation is considered. This section includes a summary of the basic physiology and the current research regarding the alterations observed in hypertension with a focus on the role of the RAS and Ang II. Finally, the different existing models designed to study the microcirculation will be taken into account, leading to the hypotheses and the aim of the present study.

1.2 Hypertension

Primary hypertension is known to be a major public health challenge in virtually all regions of the world. It is one of the most important and independent modifiable risk factors for cardiovascular and related diseases like cerebrovascular insult and renal failure. Consequently, it is known to be the leading cause of mortality and is ranked third as a cause of disability adjusted life years (DALYs) in economically developed countries (Ezzati, et al., 2002; He and Whelton, 1997; Whelton, 1994).

The concept of elevated blood pressure as the origin of serious organ damage emerged in the early 20th century, when the prognosis of malignant hypertension for instance was worse than that of many types of cancer. In untreated cases, patients died within six to 12 month following the diagnosis (Keith, et al., 1939). Considering that there was little other treatment than a rice or low sodium diet in addition to physical rest, hypertension presented itself as a remarkably dangerous threat. Although proven to be effective in some individuals, the acceptance of the rigid anti hypertensive diet as promoted by Kempner and his colleagues was not easily achieved

(Grollmann, 1945; Kempner, 1974). The British physician-philosopher Sir George Pickering described or rather condemned it as “insipid, unappetizing, monotonous, unacceptable and intolerable” (Moser, 1997). It consisted essentially of fruit in any form and rice with a little sugar and milk. The daily amount of sodium intake was as low as 150 mg and chloride intake was reduced to 200 mg. In addition to the necessary asceticism of a patient to stick to the diet no plausible explanation for its success was at hand. At that time it was still unclear whether the effect on blood pressure was related to the low sodium intake or the consequent weight loss of the patients (Kaunitz, 1979). Possibly because of the missing rationale for its effectiveness and the enormous hassle associated with this treatment, at that time many physicians did not recommend treatment of hypertension at all. The consequences of hypertension were thought to be inevitable (Moser, 1997; Ventura, et al., 2001).

The promising results of the earliest, sometimes fairly heroic, surgical and pharmaceutical approaches to antihypertensive treatment by means of sympathectomy, injection of pyrogens or anti-malarial drugs, signalized the beginning of a new era (Freis, et al., 1958). For the first time it had been proven that blood pressure could permanently be lowered and organ damage delayed. Sensing the significance of the issue, an ever increasing amount of research on the treatment of hypertension was initiated. Subsequently developed, more specific anti-hypertensive drugs such as reserpine and hydralazine gave rise to new problems. On the one hand the new drugs provided highly effective blood pressure lowering. On the other hand they led to unexpected side effects, had a narrow therapeutic range and development of a tolerance was frequently observed. Extensive side effects beginning with fatigue, insomnia or headaches and ending with a lupus-like syndrome discouraged many patients and physicians equally to give drug treatment a chance (Sjoerdsma, 1963). The stepwise extension of medical treatment by orally effective diuretics, modulators of the autonomic nervous system through α- and β-blockers, calcium-channel blockers and inhibitors of the RAS made management of hypertensive patients considerably easier (Cohuet and Struijker-Boudier, 2005; Moser, 1997). The new drugs showed fewer side effects and were easier to control. Fixed dose combinations finally prove to substantially reduce hypertension-associated cardiovascular events (Scholze, et al., 2006). With these new treatment options at hand and growing comprehension of involved pathophysiology the threatening appearance of hypertension has ever since been reduced.

Nevertheless, observing the trends in prevalence for example in the United States one can easily see that after two decades of continuous decline, the number of patients diagnosed with hypertension is growing again since the nineties of the last century (Whelton, 2004). An analysis by Thamm et al. in the late 1990s indicated a comparable progression in the German population.

They estimated that the total prevalence of hypertension in the adult German population (17-79 years) was as high as 55.3 % (60.2 % in Men and 50.3 % in women) (Thamm, 1999). Revealing an increase of 20.9 % in males and 25.5% in females when compared to the data of the MONICA (monitoring trends and determinants in cardiovascular disease) Augsburg trial collected between 1984-95 (Gasse, et al., 2001). The recent work by Kearney et al. on the burden of hypertension pointed out that approximately 26.4 % (26.6 % of men and 26.1 % of women) corresponding with an estimated total number of 972 million adults (333 million in the economically developed countries and 639 million adults in economically developing countries) were suffering from high blood pressure (BP) in 2000. In addition a 60 % increase by 2025 was predicted promising a still greater challenge for health care systems all over the world (Kearney, et al., 2005). In 2002 the expenses caused by hypertension in Germany reached a total of € 8.13 billion in all age groups, the highest being in the population aged between 65 and 85 years. Considering the average 17 years lost by death under the age of 65 per 100000 inhabitants (Germany 1995-1997), the impact on society and its health care system becomes apparent (Noethen, 2006).

In contrast to the growing awareness of the importance of BP lowering to levels below 140/90 mmHg (systolic/diastolic), very little success in achieving this goal can be noted (Scholze, et al., 2006). A recent report compared the prevalence and control of hypertension in six European countries (UK, Finland, Germany, Italy, Spain and Sweden) to that in Canada and the USA. It indicated that in these European countries, an average of only 8 % of the treated hypertensive individuals had BP levels below 140/90 mmHg, compared with 23 % in Canada and the US (Wolf-Maier, et al., 2003). One explanation is that hypertension remains asymptomatic until late in its course. This leads on the one hand to a reduced compliance of the patient to stay on medication and on the other hand to belated initiation of treatment when most of the pathologic alterations are already established and hence less modifiable.

Several hypotheses concerning the pathogenesis of hypertension have been generated over the years. One of the most important considers alterations in the microvascular structure and function as the main cause for hypertension and CVD (Struijker Boudier, et al., 1992). As the major site of vascular resistance and therefore BP regulation, the microcirculation has been a central research interest. This may be demonstrated by the fact that between 1998 and 2001, 800 articles per year have been published in MEDLINE-listed journals on this topic (Levy, et al., 2001; Tritto and Ambrosio, 1999). The following chapter will give more detailed information about the microcirculation and its role in the development and progression of hypertension.

1.3 The microcirculation

In the early 17th century William Harvey postulated the idea of a circulation of blood through the body with the heart as a pump. Thereby challenging the, at that time, broadly accepted theories of the ancient Greek physician Galen (Wikipedia, 2006). During his life he was not able to prove his concept of a network of very minute vessels connecting arterioles and venules, basically because of lacking visual evidence. The invention of a microscope by the Dutch merchant Antonie van Leeuwenhoek finally enabled Marcello Malpighi 1661 to recognize and describe the capillary network of the lung. Thereby he provided a scientific proof of Harvey's concept generated half a century earlier. Their discovery of the terminal vascular bed consisting of a huge number of only microscopically visible vessels connecting arterial and venous vascular trees was repeatedly proven and refined during the following centuries. One of the most popular methods in this era focused on red blood cell flow in transparent fishtails and many microscopes were equipped with so called "fish-plates" (Axel R. Preis and Werner, 1996).

Experiments by Haller, Spallanzani and Poiseuille (1828) contributed almost a hundred years later to a more detailed comprehension of microvascular morphology and flow patterns. Jean Léonard Marie Poiseuille's dissertation published in 1828 led to the conclusion that the major pressure decrease in the circulation takes place in the small blood vessels of the microcirculation (Poiseuille, 1828). Today it is generally accepted that especially the small arteries and precapillary arterioles are responsible for about 40-90% of the peripheral resistance (Schmid Schönbein GW, 1986). In addition, Poiseuille and at the same time Gotthilf Hagen revealed the relation between flow resistance (R), tube length (l), viscosity (η) and the tube radius (r) and expressed it in a single formula. Today well known as the Hagen-Poiseuille law:

$$\mathbf{R = 8 \cdot l \cdot \eta / (\pi \cdot r4)}$$

Although later revised and partially corrected to fit the more dynamic behavior of blood flow in "*in vivo"* blood vessels, the equation first developed in rigid tubes is still taught to medical students around the globe to approach basic vascular and blood flow physiology (Martini, et al., 1930; Silbernagel and Despopulos, 2001).

One of the major functions of the arteriolar part of the microvascular network is to regulate blood flow and hydrostatic pressure at the capillary level to avoid disturbances in transcapillary exchange and to provide an adequate nutrient and oxygen supply. As the flow rate is proportional to the 4th power of the vessel radius (see Hagen-Poiseuille's law) a precise and careful adjustment of vascular diameter is required. Therefore the fine-tuning is realized via a large number of interrelated mechanisms. The neural system is represented by the sympathetic

nervous system and its adrenergic postganglionic nerves. It is acting via noradrenalin on the different adrenoceptors (α_1, α_2, β_1, β_2,…) in constant interaction with endothelin, nitric oxide (NO) and myogenic mechanisms (Silbernagel and Despopulos, 2001). These mechanisms reflect the autoregulatory response to intravascular pressure changes of the endothelium and the smooth-muscle cells. This response keeps capillary blood flow on a constant level even if perfusion pressure changes. Reduction in blood flow causes the production and release of vasoactive metabolites like NO, CO_2, K^+, adenosine, H^+-ions, AMP and ADP that dilate the vessel characterizing the metabolic system. Endothelial cells interact with most of the systems related to blood flow regulation. They carry receptors for mediators of vascular tone and they react to mechanical forces as for example shear stress and circumferential wall stress (Pries and Werner, 1996). Subsequently, they are involved in short-term tonus regulation of the underlying smooth muscle cells and in long-term functional and structural modulation of the microcirculation. A large number of humoral mediators are synthesized by the endothelium and exert specific actions on the endothelium. For example endothelin, the eicosanoids like prostaglandin or thromboxane A_2 and B_2, bradykinin, kallidin and most importantly again NO. The latter is synthesized via endothelial nitric oxide synthase (eNOS) and has vasoprotective effects like vasodilation, anticoagulation, antioxidative capacity, inhibition of leukocyte adhesion and smooth muscle proliferation (Schulman, et al., 2005). In addition several vasoactive hormones like catecholamines (dopamine and (nor-) adrenalin), acetylcholine, ADH (vasopressin), histamine and most importantly Ang II influence long- and short-term function of the microcirculation. Analogous to the other agents, Ang II is integrated in a complex regulation and feedback loop, the Renin-Angiotensin-System (RAS).

1.4 The Renin-Angiotensin-System

The RAS with its main effector peptide Ang II is a hormonal system that is highly relevant in cardiovascular regulation processes and in maintaining the electrolyte and water homeostasis (Oparil and Haber, 1974a; 1974b). The classical pathway leading to the formation of Ang II can be described as followed. As a reaction to various stimuli, for example in conditions of acute pressure decrease or low sodium levels, renal pressure- and osmolarity-receptors are stimulated and release the proteolytic enzyme renin from the granulated cells of the juxtaglomerular apparatus. This enzyme cleaves the liver-derived angiotensinogen to angiotensin I (Ang I) which is subsequently hydrolyzed to Ang II by the angiotensin converting enzyme (ACE) (Silbernagel and Despopulos, 2001).

This historically established idea of a single systemic RAS (as described above) has been revolutionized by a number of recent findings. Firstly the discovery of the tissue RAS demonstrated that Ang II is also synthesized by the endothelium where it exerts direct influence on endothelial key functions (Jin, et al., 1988). The synthesis occurs virtually on all tissue levels (e.g. blood vessels, heart, and brain) and is not reduced to certain organ-systems such as liver or lung. This implicates a complex interaction with the local peripheral pressure- and flow-regulation (Dzau, 2001) and underlines that Ang II has a much greater influence than systemic hemodynamics and renal function. Secondly, alternative enzymatic pathways that lead to the formation of Ang I and Ang II have been characterized. Ang I can be formed by cathepsin or tonin and then converted to either Ang II by chymase, trypsin and cathepsin or to angiotensin 1-7 (Ang 1-7) by tissue endopeptidases (Johnston and Risvanis, 1997; Kokkonen, et al., 1997). Nevertheless, valuable studies showed that local inhibition of ACE in the forearm leads to vasodilatation due to the reduced conversion of Ang I to Ang II suggesting that ACE is responsible for most of the peripheral Ang II production (Houben, et al., 2000). Only relatively recently human visceral adipose tissue has been found to synthesize virtually all components of the RAS including angiotensinogen, Ang I and Ang II with their receptors and many proteases capable of producing Ang II as described above. Via a direct autocrine feedback loop Ang II promotes adipocyte growth differentiation. These results led to the conclusion that adipocyte-derive components of the RAS play an important role in the pathogenesis of hypertension (Kershaw and Flier, 2004; Scholze, et al., 2005).

Virtually all known effects of Ang II are mediated through two main receptor subtypes which are interrelated and antagonistic to each other (Nakajima, et al., 1995). The Ang II type 1 receptor (AT_1) and the Ang II type 2 receptor (AT_2). They are heterogeneously distributed throughout peripheral and neural tissues (de Gasparo, et al., 2000). Both receptors have been cloned and belong to the same receptor family but differ markedly in their signaling cascades and biological activities (Timmermans, et al., 1993). The classically physiological but to some extend also deleterious effects of Ang II are mediated via the AT_1 receptor. Specific stimulation of this receptor through Ang II has been found to play an important role in the pathogenesis of several cardiovascular, renal and cerebral pathologies (Kim and Iwao, 2000; Lucius, et al., 1999; Unger, et al., 1996). Some of the underlying mechanisms are enhanced vasoconstriction, proliferation of vascular smooth muscle cells and inflammation of endothelial cells, leading to e.g. cardiac hypertrophy, vascular remodeling and renal fibrosis (Geisterfer, et al., 1988; Paquet, et al., 1990; Stoll, et al., 1995; Watanabe, et al., 2005). In particular, the microvascular alterations observed in hypertension such as enhanced vasoconstriction and anatomic alterations

have been related to the superoxide-mediated impairment of endothelial function via the AT_1 receptor and the stimulation of monocytes (Didion and Faraci, 2003; Dorffel, et al., 2003). The increased production of oxidative stress and the pro-inflammatory effect of Ang II have been found to be the origin of many pathologic processes seen in primary hypertension as demonstrated by the work of Nakamura and colleagues (Nakamura, et al., 1998). The consistently documented salutary effects of ACE-Inhibitors and AT_1 receptor blockers (ARBs) in treatment of hypertension and CVD firmly support these findings (Lindpaintner and Ganten, 1991; Scholze and Stapff, 1998; Unger, et al., 1998; Yusuf, et al., 2000).

The AT_2 receptor is known to be present at high density in all tissues during fetal development and only in certain tissues of the grown up (Lucius, et al., 1999). These are the adrenal medulla, uterus, ovary, vascular endothelium and specific areas of the brain. Up-regulated expression in adult tissue can occur under conditions of heart-failure, post-infarct tissue repair and skin and nervous system lesions (Kimura, et al., 1992; Stoll, et al., 1995; Unger, et al., 1996). AT_2 receptor-mediated effects thus seem to be involved in controlling and regeneration processes. Henrion et al. suggested a NO-mediated regulation of the vascular tone by stimulation of the AT_2 receptor acting as natural counterbalance to the effects of the AT_1 receptor (Henrion, et al., 2001). Recent evidence indicates a relationship between the renal and systemic sensitivity to Ang II and the genetic diversity for Ang II receptors. In addition, it has been shown that the RAS is altered in conditions of elevated BP.

1.5 Microcirculation in hypertension

In patients with beginning essential hypertension, cardiac output is elevated whereas peripheral resistance seems to be at normal levels. In later stages of the disease the major hemodynamic abnormality is a rise in vascular resistance (Sullivan, et al., 1983) which is etiologically related to the following changes on capillary level: 1) Increase in vascular tone 2) Functional and structural rarefaction of capillaries and small arterioles. 3) Vascular remodeling e.g. increase in wall: lumen ratio of the small arterioles through hypertrophy and hyperplasia of the vascular wall (Miller, et al., 1987; Prewitt, et al., 1984). All these changes can at some point be related to the effects of Ang II. Rarefaction has been associated with genetic factors leading to elevated sensitivity to vasoconstriction stimuli and structural changes (Antonios, et al., 1999; Houben, et al., 2005; le Noble, et al., 1990; Spiering, et al., 2000; Struijker Boudier, et al., 1992). Accordingly, it is considered to play a central role in connection with microvascular alterations in hypertensive patients. Deranged microcirculatory conditions in hypertension not only result in

alterations of peripheral resistance but also in severe damage of organs like the heart, kidney, brain and retina. This emphasizes the importance of early detection and appropriate treatment of such conditions. It seems advisable to start the investigation at the area of interest i.e. in the microcirculation.

1.6 Models to study the microcirculation

The investigation of the microcirculation has a long history. Discovered in the 17th century by Harvey and Malpighi, this minute network of only microscopically visible vessels slowly became objective of scientific work. First studied in the transparent tails of small fish, the microvascular network was only morphologically described. At the beginning of the 19th century studies by Poiseuille, Haller and Spallanzani among others were the first that included a more detailed description of microcirculatory morphology, functions and flow patterns. Though corrected and modified during the years their work still is the basis for recent studies of the microcirculation. In the early 20th century Krogh and co-workers developed a tissue cylinder model that enabled them to thoroughly study microvascular anatomy and physiology (Krogh, 1919; Krogh, 1930). Ever since this fundament was established, progressively more research focused on the microvascular network and its role within the entire circulation, gathering an enormous amount of knowledge and simultaneously producing ever new queries.

The assessment of the microvascular status has been carried out via different methods. Necropsy- and biopsy-based examinations of the microcirculation have led to essential findings concerning for example vessel wall composition and its changes in CVD and other diseases (Savaskan, et al., 2004; Short and Thomson, 1959). Intravital microscopy has been shown to be an accurate method to study microvascular bed *in vivo* (Harper, et al., 1978). Numerous approaches have been described in the literature. In animals the cremaster muscle and the mesentery have frequently been studied as they provide excellent microscope access to all parts of the microvasculature. This led to important insights in e.g. pressure response and hemodynamic resistance (Davis, et al., 1966; Pries, et al., 2001; Tyml, et al., 2003).

The quest for non-invasive models to visually study the smallest vessels in human beings logically led to those parts of the body where the tissue is transparent enough to gain sufficient access via an external microscope setup. These natural access points are the mouth (i.e. sublingual, lip and cheek), nailfold of hand and feet the skin in the newborn and the bulbar conjunctiva. The latter has been studied by first by Zeller in 1921 who used a 64-fold magnification and primarily described capillary abnormalities in arteriosclerosis, syphilis and

diabetes. Lack (1949) later refined the technique and added the possibility to acquire picture material from the observed area which allowed him to describe distinctive capillary patterns in hypertension (Zeller, 1921). He proposed a “hypertension pattern” of capillary pathology which was present in 98% of the studied hypertensive subjects. Although he only made structural observations, the technique was the basis for many other setups later on. In contrast to all other techniques in which solely the capillaries can be observed, conjunctival biomicroscopy offers the opportunity to study all segments of the microcirculation including arterioles, capillaries and venules. In addition the studied variables are not biased by tissue exposure, anesthesia or suction as in other models (Lindert, et al., 2002). In contrast to the importance of the microcirculation in countless medical conditions, the existing techniques of direct investigation of microvascular function are still limited. Laser-Doppler red blood cell fluximetry before and after locally applied substances via iontophoresis is to date the only available technique to non-invasively study the direct effects of drugs on the microvessels.

1.7 Aim of the study

The main task of the present study was to develop a non-invasive model to study microvascular reactivity in the human bulbar conjunctiva. It was hypothesized that microvascular diameter changes after the local application of phenylephrine could reliably be detected with the new setup. The applicability of the model for pre- and post-treatment measurements in hypertensive subjects was tested and it was hypothesized that the same blood vessels could be detected on different days and that the effect on day one was comparable to that on day two (effect reproduction). After the completed validation of the setup the focus was put on the reactivity to Ang II. It was hypothesized that the local application of Angiotensin II leads to vasoconstriction in the conjunctiva and that this effect vanishes over time. The effect was expected to be reproducible after complete remission. The long-term objective of the present study was to establish a model to assess the reactivity to vasoactive substances involved in the development and progression of hypertension.

2 Methods

2.1 Participants

The tested subjects were healthy individuals (n=48; ♀= 25; ♂= 23) with a mean age of 22 years (SD = 3) recruited via the blackboards of the Maastricht University. They had to refrain from smoking and drinking caffeine or alcohol containing beverages for at least 12 hours prior to the measurements. Experiments were carried out in the morning after an overnight fast. At the beginning of the test day, length and weight of the patients with indoor clothing but without shoes was measured. The patients were asked to lie in supine position for a 30 minute acclimatization period and BP was taken at the end of the period for a total of three times with the DANICA blood pressure automat Accutorr Plus®. The room temperature was constantly held at 23-24°C and precautions were taken to minimize external disturbances.

2.1.1 In- and exclusion criteria

The volunteers had to be healthy according to a conducted anamnesis. Intake of circulatory active medication led to exclusion from the study. During an introductory briefing medical history was taken and detailed information was obtained concerning some relevant conditions as shown in Table 2.

The present study was approved by the medical ethics committee of the university hospital Maastricht and all participants gave their written informed consent.

Table 1: Exclusion criteria

Circulatory diseases	Renal disorders	Ophthalmic pathologies	Other conditions
• Hypertension • CVD • Arteriosclerosis • Raynaud's disease	• Acute renal failure • Chronic renal failure • Renal arterial stenosis	• Recent inflammations • Frequent irritations due to contact lenses • Allergic conjunctivitis • Recent eye operation or medication • Glaucoma or reported raise in ocular pressure	• Diabetes type 1 or 2 • Medication influencing the circulation, • Drug abuse • Connective tissue diseases

2.2 Intravital microscopy unit

The microscope was equipped with a fixed 6.3-fold objective lens (numerical aperture (N.A.): 0.18) leading to a final 280 X magnification and was connected to a monochrome charge coupling device camera (CCD, Teli® Eindhoven, the Netherlands) which was positioned in the intermediate image of the objective lens. Scanning and focusing was done via a control stick attached to the mobile base of the microscope. The base platform could be two dimensionally steered in order to focus and horizontally move the rack. Alterations along the vertical axis were carried out by revolving the control stick. For incident oblique illumination purposes two tungsten 10 volt lamps were assembled and a contrast enhancing Leitz BG38 filter was placed in the illumination pathway. The green colored anti-red filter-lenses enhanced the displayed red blood cell columns to crisp black lines. The CCD-camera was connected to an analogue brightness and contrast enhancer and captured images were displayed on a monochrome monitor (Sony®) opposite the examiner. This system allowed instant control and adjustment of several essential visual parameters like brightness, contrast and focus of the recorded material. Recordings were stored via a DVD-recorder (Pioneer®) on 4.7 gigabyte DV discs for subsequent off-line analyses.

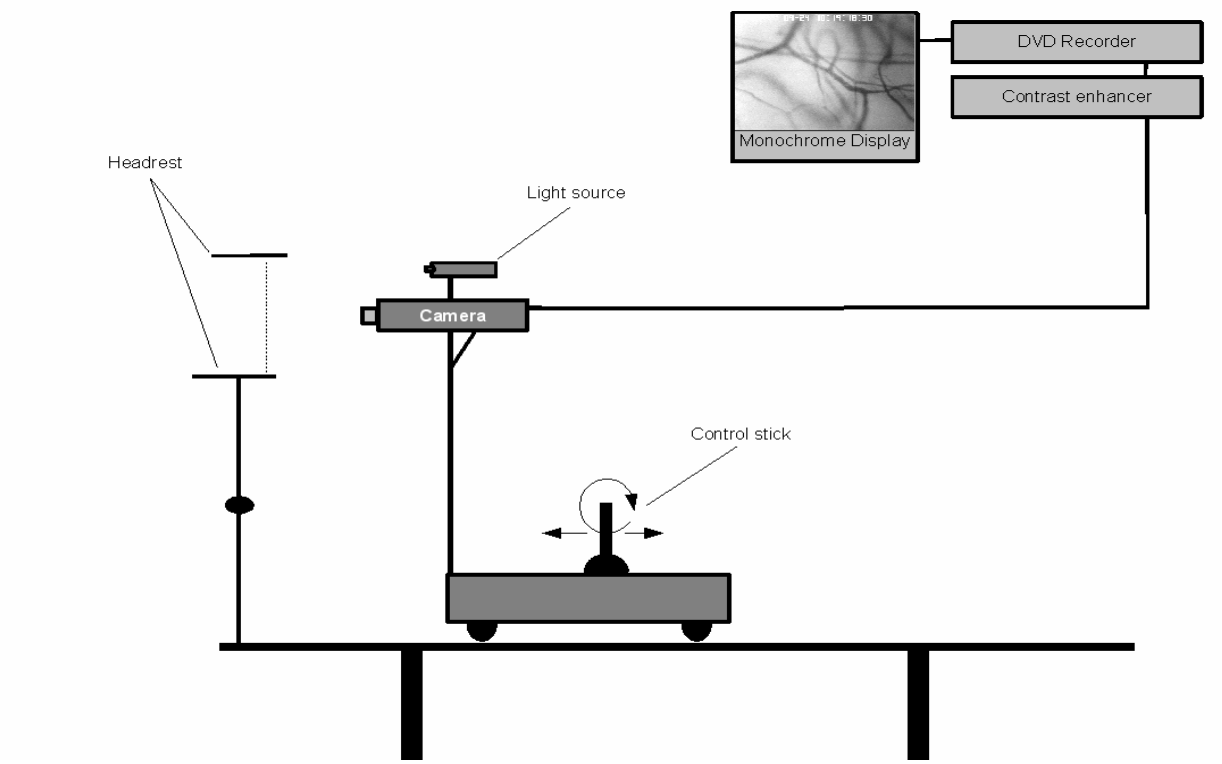

Fig. 1: Intravital microscope setup

2.3 Intravital microscopy procedure

In the following section the basic procedure for the *in vivo* investigation of the microcirculation will be described. The specific procedures concerning validation and application of the model will be explained at the end of this chapter.

All investigations were carried out in the lateral part of the bulbar conjunctiva. Both eyes of each patient were studied with the custom-build horizontal microscopy-system described above (see Figure 1). Throughout the whole recording session the subject's head was comfortably fixed by the adjustable restrainer of a slit-lamp commonly used in the ophthalmologic department. The patients were asked to sit in front of the headrest and the apparatus was adjusted to their head. To prevent larger body movements, the test persons were advised to lean against the backrest of their chair and lay their forearms on the table. In order to make a large part of the temporal conjunctiva microscopically accessible, the tested subjects were asked to look on a strategically placed red circle on the opposite wall.

A systematic scan of the conjunctiva was conducted and remarkable pairs of arteriole and venule were focused on and recorded. The scan followed a standardized pattern beginning close to the iris at the top of the eye and scanning vertically down one visual field at a time ending in the

lateral angulus ophthalmicus (see Figure 2). Conjunctival microcirculation could easily be distinguished from deeper episcleral vessels by carefully focusing on the outermost layer of the bulbus ophthalmicus. Blood vessels were identified on the basis of their branching pattern, their position in the vascular tree, and the direction of red blood cell flow at branching points i.e. convergent for venules and divergent for arterioles. The video recordings facilitated the classification of the blood vessels during the subsequent off-line analysis as the surrounding and the course of the microvessels provided useful information when some characteristic was not completely clear.

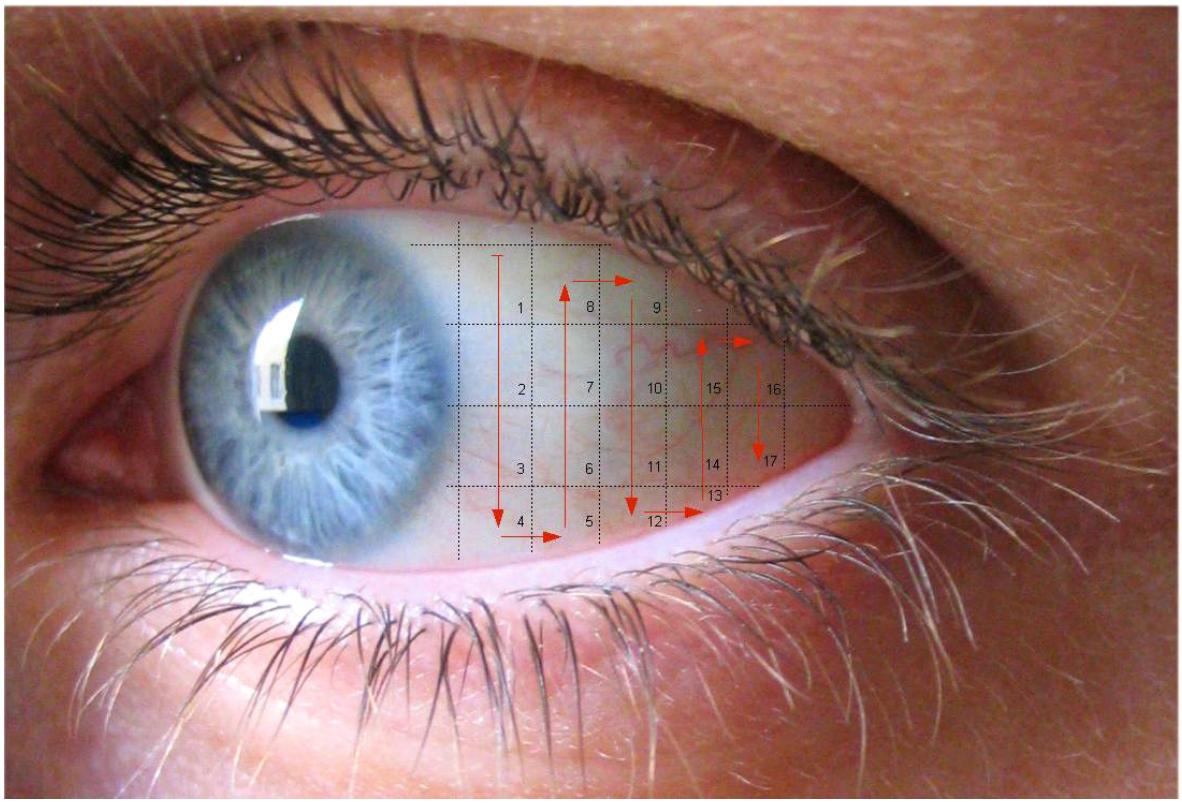

Fig. 2: Scan pattern in the bulbar conjunctiva (number of scanned fields varied between the individuals).

Microvascular diameters as represented by the red blood cell column were measured in three to four pairs of arteriole and venule per eye. These pairs had to (1) run through the conjunctiva, (2) consist of at least one arteriole and one venule per visual field, (3) be of approximately the same class, (4) be easily recognizable for later evaluation and finally had to (5) be well perfused (no sludge, stasis, or inverse flow).

Once a pair of appropriate vessels was found, the recording was initiated and the image was focused and recorded for approximately 15 seconds. This procedure was repeated until three to four pairs of conjunctival vessels were filmed. After the baseline scan was completed the eye-

drops were administered in a random order (double blind) and the same vessel pairs were recorded again for approximately five to ten seconds each. For the unlikely event of an anaphylactoid reaction or acute glaucoma, eye-wash equipment and anti-glaucoma eye drops (carbonic anhydrase inhibitor) were provided.

2.4 Local application technique

Eye drops were applied by reclining the patients head and giving one drop of the solution in the lachrymal sack while gently lifting the lower eye lid. The eye was closed at once and the canaliculi lacrimalis inferior and superior were manually obturated for 60 seconds. Primarily, this procedure helped to avoid a prompt draining of the agent through the tear channels (upper and lower) and to standardize the residence time. Furthermore the effects on circulation caused by nasal absorption of Ang II or phenylephrine were minimized.

2.5 Preparation of the eye-drop and blinding procedure

The eye drops were prepared and labeled with a code by the laboratory staff and blinding was not revealed until the image analysis was completed. For the different trials, the eye drops were individually prepared on each test day. Ang II was obtained via Clinalfa AG Switzerland and was delivered in 50µg units. The necessary amounts were diluted in 0.9% NaCl solution and aspired in a 1ml syringe for insulin injection purposes. The average volume per eye drop was 30.7 µl. It was assessed by weighing ten subsequent drops from the utilized syringe with a fine scaled laboratory balance. The low Ang II drop contained a total amount of 5µg per 0.5ml solution and the high dose Ang II drop 50 µg per 0.5ml, respectively. Thus, the average Ang II concentration per eye drop was 0.37 µg for low Ang II and 3.7µg for high Ang II. Concentration in mmol/L for low Ang II was 0.00956 and for high Ang II 0.0956. Phenylephrine eye drops with a concentration of 0.25% were obtained via the central in-house pharmacy of the university hospital Maastricht as they are commonly used in patients with conjunctivitis. One applicator contained 0.5ml NaCl O.9 % and the other contained an active agent i.e. Ang II 0.001 %, Ang II 0.01 % or phenylephrine 0.25 %, respectively.

2.6 Image analysis

After each recording session the tracks were tagged with an individual code stating the patient ID and other fundamental information like right or left eye, pre/post drop or the vessel

number within on eye. The coded tracks were systematically screened for appropriate pictures in the slow motion mode of the DVD player. Pictures were moved frame by frame to acquire focused and precise images of both arteriole and venule. These standstill pictures were acquired by a video card, transmitted to a MS Windows platform and saved as bitmap files. After organizing the files in distinct folders they were transferred to a Macintosh Performa 6320 and edited with selected Adobe Photo Shop 5® tools: 1) Auto levels for contrast and brightness 2) Video-De-Interlace filter for even fields. Afterwards the files were saved in Macintosh® image format and analyzed with a computer program designed to measure diameters of coronary blood vessels called CVA / CAAS II (Pie medical). The input pixel size and the $^{y}/_{x}$ ratio of the pixels were supplied and measurements were conducted. One segment of each pair of vessels consisting of an arteriole and a venule was analyzed before and after the administration of an eye drop. Once the file was loaded in CAAS II the examiner manually assigned the segment of interest which was subsequently analyzed by the computer (see Figure 3).

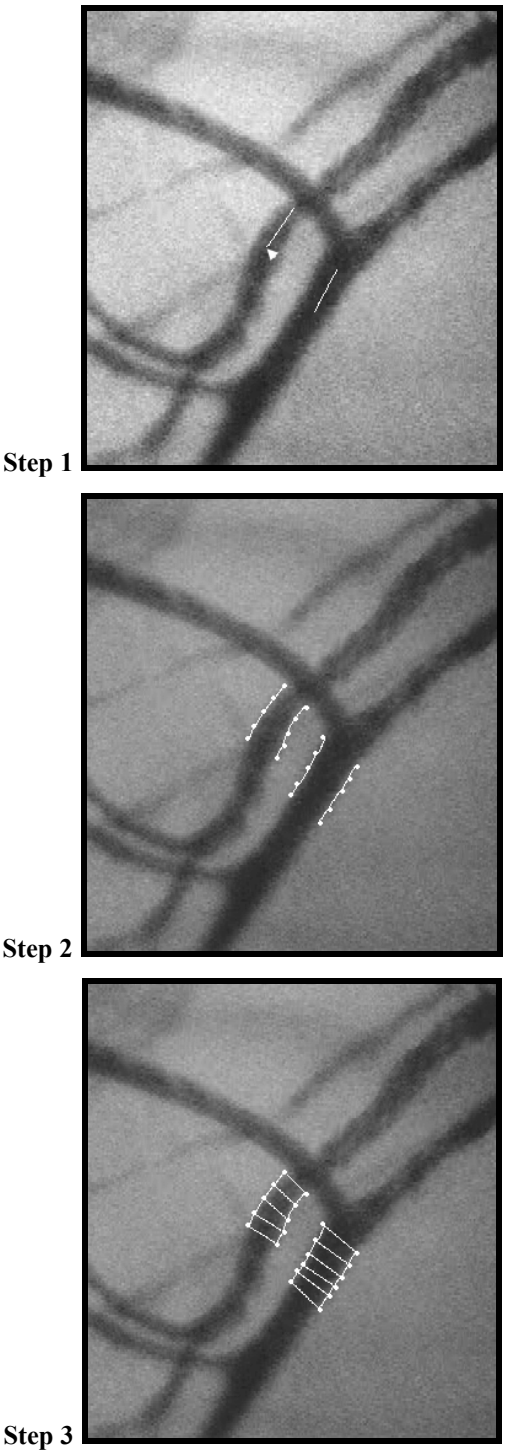

Fig. 3: Image analysis. Step 1: Segment of interest is assigned. Step 2: Vessel wall is located automatically following grey scale differences. Step 3: Vascular diameter is measured.

2.7 Validation

In order to assess the reliability and accuracy of the diameter measurements the same segment of five different vessels was repeatedly measured. The results of ten subsequent measurements were analyzed concerning their arithmetic mean, their standard deviation and their range.

The biological variability as represented by the average intraindividual variation of diameter in the conjunctival microcirculation was assessed during short- middle- and long-term intervals. The short-term variability within seconds, following e.g. a pulse wave was assessed by analyzing vascular diameters in ten consecutive screenshots (one every one to two seconds) of one vessel pair. This procedure was carried out in conjunctival recordings acquired from five patients. In order to assess the middle-term biological variability within minutes, the alterations that occurred in the eye that received the placebo eye drop were analyzed. Long-term variability was assessed by comparing the baseline vascular diameters on day one and two in the two-day trial. The measured values were analyzed according to their absolute and relative variation in diameter, range, mean and standard deviation.

The reactivity of the conjunctival microcirculation was studied double blind in healthy volunteers who received phenylephrine 0.25 % in one eye and placebo in the other in a randomized order. After each baseline scan the eye drop was administered as described above and exactly the same blood vessels were recorded again approximately 60-90 seconds following the application.

In order to test the reproducibility of the measured effects and the practical applicability of the model, six subjects were invited to a second test day. On both test days identical vessels were recorded before and after administration of the same eye drop. As the same vessels were to be found after a minimum of 24 h, not only the reproducibility of the measured effect but also the applicability of the model could be assessed. Furthermore, this two day trial was carried out to find out whether a pre- and post-treatment recording in hypertensive and other subjects would be realizable.

2.8 Application of the model

Once the validation trials were completed, the established model was applied for the first time in order to assess the reactivity to other vasoactive agents. Because of its importance in the development and progression of hypertension and CVD, Ang II was chosen. The reactivity to Ang II was measured in different setups as described in the following paragraphs.

The first group received Angiotensin II eye drops containing 0.001% ANG II (0.0956 mmol/L) corresponding to a total of 0.37 µg and placebo, respectively. The diameters of three vessel pairs was measured before and 60 seconds after the administration.

The second group received Ang II in a 10 fold higher concentration (0.01 %; 0.956 mmol/L). In addition to the basic protocol, conjunctival vessels were recorded at one and ten minutes after the administration to gather information about the duration of the measured effect. The Ang II concentration was chosen on the basis of the results after intravascular application by Jilma and colleagues in 1997 (Jilma, et al., 1997). They used a mean Ang II pressor dose of 8.44 $ng \cdot kg^{-1} \cdot min^{-1}$ equaling a total of 591 $ng \cdot min^{-1}$ for a 70 kg person and as sub-pressor dose an average of 0.62 $ng \cdot kg^{-1} \cdot min^{-1}$ equaling a total of 43 $ng \cdot min^{-1}$. Given the fact that for ocular application higher doses are necessary because of an immediate draining via the lachrymal canaliculi and the need to penetrate the vascular wall, the doses were elevated. The first group received a ten times higher than sub-pressor dose and a ten times higher than pressor dose was applied in the second group. In addition the whole amount was given in one drop instead of being fractionated over one minute. When compared to the high doses of phenylephrine (0.25 %) in usual eye drops this still was a comparatively low-dose approach.

As a variation of the second group, the microcirculation of the subjects in the time response experiments was constantly investigated over a period of 40 minutes and analysis was carried out at specific points of time. The diameter changes in conjunctival microvessels during this period were logged and a time response curve was established.

In order to assess the reproducibility of the effects seen one minute after the Ang II eye drop and to assess whether there might be an accumulative effect, a second run was added. At first a baseline scan was performed 30 minutes after the last eye drop was given in order to verify that no residual effect of Ang II was present. Afterwards a second eye drop of Ang II and placebo was given and the *de novo* constriction was measured again. The results of the two measurements were compared and the significance of the difference between the first and the second round was computed.

2.8.1 Statistical analyses

Results of the diameter measurements recorded on Microsoft Excel® spreadsheets and sorted by trial type. Absolute and relative diameter changes in µm and percent, respectively, were computed. The variability of the measurement was assessed by running descriptive statistics including mean (M), standard deviation (SD), median (Mdn), inter quartile range (IQR), variance and range. In addition the relative variability and the coefficient of variation (VC, in %) was computed. The same procedure was done for the biological variability. Additionally, a Wilcoxon signed rank test was performed comparing the baseline diameters from day one to day two and the diameters before and after administration of placebo eye drops. No significant difference between day one and day two or between pre- and post-placebo measurements was expected. Relative diameter changes after phenylephrine or Ang II were compared to placebo at one and ten minutes after the eye drops using the Wilcoxon signed rank test. It was expected that phenylephrine and Ang II would lead to a significant reduction of vascular diameter when compared to placebo. The reproducibility of the effect seen one minute after the application of Ang II was also tested by a Wilcoxon signed rank test comparing the effects of the first and the second eye drop given at a thirty minute interval. No difference in the effect was expected. Group differences concerning the participants' clinical status were tested with the Kruskal-Wallis test. *P*-values below .05 were considered statistically significant and *p*-values between .06 and .1 were considered as a trend. All analyses were performed using the statistical software package SPSS V 12.0.

3 Results

3.1 Patient characteristics

Clinical characteristics of the participants are summarized in Table 2. The Kruskal-Wallis test revealed no differences between the groups concerning age, BMI and MAP as all *p*-values varied between .9 and .2.

None of the participants reported any discomfort, itching, pain, or other ocular reaction to the applied eye drops.

3.2 Validation

3.2.1 Measurement variability

The repeated measurement (10 times) of arteriolar vessel segments revealed a low variance of .064 µm and a range from min (14.0 µm) to max (14.8 µm) of .8 µm. The venular variance was similarly low at .053 µm with a range from min (19.2 µm) to max (19.9 µm) of .7 µm. The mean diameter of the measured vessel segments was 14.4 µm (SD = .25) for arterioles and 19.5 µm (SD = .23) for venules. The relative variability of the measurement itself was .44 % for arterioles and .27 % for venules indicating a good reliability of the image analysis and diameter measurement process.

Table 2: Patient characteristics sorted by trial

	Validation		**Application**			
	Phenylephrine Reactivity	Reproducibility	Low Ang II Reactivity	High Ang II Reactivity	Repeated Challenge	Time Response
N (♂/♀)	8 (2 / 6)	5 (1 / 4)	9 (2 / 7)	13 (9 / 4)	4 (2 / 2)	6 (2 / 4)
Age, years*	21 (±2)	22 (±2)	23 (±3)	21 (±2)	23 (±2)	21 (±3)
BMI, kg/m²*	23 (±3)	25 (±4)	23 (±3)	22 (±1)	22 (±1)	21 (±1)
MAP, mmHg*	88 (±6)	85 (±5)	84 (±5)	87 (±8)	86 (±4)	85 (±7)

*Values are Means (± Standard Deviation)

3.2.2 Biological variability

Short-term variability was assessed by measuring ten consecutive screenshots (one to two second intervals) from five patients. An overall low variability of 4 % of arteriolar and venular diameters equally was found. The average diameter of the vessels studied was 15.88 µm (SD = 1.81) for arterioles and 21.28 µm (SD = .82) for venules. Recordings before and after placebo eye drops in 35 participants (i.e. 105 pairs of vessels) showed that the middle-term variability in the present population over a period of ten minutes was 3 % (SD = 10) in arterioles and 3 % (SD = 12) in venules. The average diameter was 14.3 µm (SD = 2.09) for arterioles and 19.35 µm (SD = 3.18) for venules. Long-term variability, assessed from the baseline diameters of five participants (i.e. ten eyes and 30 vessel pairs) in the two day trial, was 3 % (SD = 9) for arterioles and 2 % (SD = 6) for venules. There was no statistically significant difference between the baseline diameters on day one and two. The vessels studied belonged to the same class as in the trials before. The mean diameter was 14.21 µm (SD = 1.51) for arterioles and 19.22 µm (SD = 1.52) for venules.

3.2.3 Reactivity to phenylephrine

Application of the epinephrine derivate phenylephrine led to a statistically significant reduction of vascular diameter in arterioles (Mdn = 15 %; IQR: 9 % - 20 %), T = 1, $p < .02$, r = -.54 and in venules (Mdn = 17 %; IQR 10 % - 28 %) T = 0, $p < .02$, r = -.49 when compared to placebo. Mean baseline diameter of the blood vessels tested was 13.63 µm (SD = 2.26) for arterioles and 17.96 µm (SD = 3.24) for venules.

3.2.4 Reproducibility

The repeated investigation of the same blood vessels on two days was carried out in five subjects. The diameter reduction on day one was similar to that seen in the reactivity trial. In addition the effect could be reproduced on day two. On day one arterioles were reduced by 12 % (IQR 10 % - 21 %) T = 0, $p < .04$, $r = -.45$ and venules by 23 % (IQR 12 % - 29 %) T = 0, $p < .04$, $r = -.45$. On day two arteriolar diameter changed by -17 % (IQR -26 % - -13 %) %) T = 0, $p < .04$, $r = -.45$ and venular diameter by -21 % (IQR -36 % - -5 %) %) T = 0, $p < .04$, $r = -.39$ under identical testing conditions. There was no statistically significant difference between day one and day two; *p*-values were $p < .5$ for arterioles and $p < .67$ for venules.

3.3 Application of the model

The application trials included the reactivity to different doses of Ang II, the time response observations after the administration of Ang II and the repeated challenge of microvessels with Ang II eye drops.

3.3.1 Reactivity to low dose Ang II

In the first part of the application trial the difference in vascular diameter after one minute following the application of an eye drop containing 0.001 % Ang II or placebo was assessed in nine participants (i.e. 18 eyes and 54 vessel pairs). The captured conjunctival vessels treated with Ang II, showed no statistically significant change in diameter when compared to placebo. All *p*-values were between $p < .25$ and $p < .4$ for arterioles and between $p < .063$ and $p < .6$ for venules. The median diameter change after Ang II eye drops was 0 % (IQR -4 % - 8 %) in arterioles and 0 % (IQR -8 % - 2 %) in venules. Placebo led to an average constriction of 6 % (IQR 4 % - 12 %) in arterioles and 5 % (IQR 0 % - 14 %) in venules (see figures 4 and 5).

3.3.2 Reactivity to high dose Ang II

Arteriolar and venular diameters were significantly reduced after administration of Ang II 0.01 % solution. One minute following the application, median vascular diameter was reduced by 20% in arterioles (IQR 13 % - 23 %; T = 0, $p < .01$, $r = -.66$) and by 10 % in venules (IQR 5 % - 14 %; T = 0, $p < .03$, $r = -.48$). The second measurement ten minutes after the last application revealed a smaller but still significant 10 % reduction of diameter in arterioles (IQR 0 % - 15 %; T = 0, $p < .01$, $r = .7$), while no significant change could be established in the venules investigated (see figures 4 and 5).

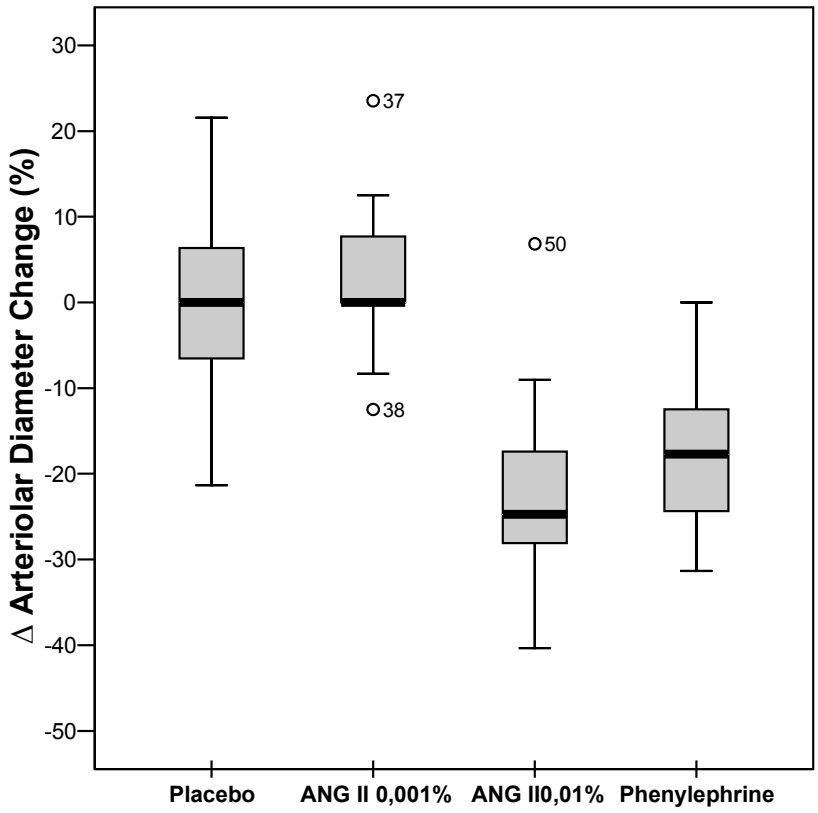

Fig. 4: Median arteriolar diameter change at one minute following the application

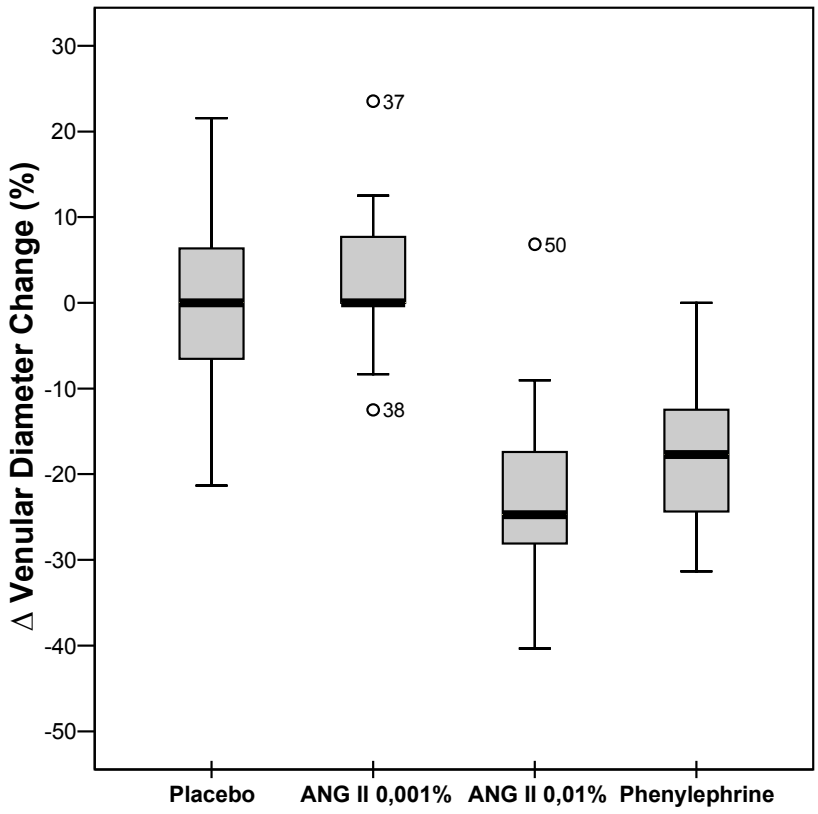

Fig. 5: Median venular diameter change at one minute following the application

3.4 Time response trial

For more information about the duration of the vasoconstriction, its course and its maximum, a time response experiment was set up (n = 5). The assessed values are displayed in Figure 6. Diameters changed in both arterioles and venules in a comparable manner. Immediately after the recording started, and thus 60 seconds after the eye drop was applied, the diameter was significantly reduced only in arterioles by 16 % (IQR: 9 % - 24 %; T = 0, $p < .04$, r = -.45). However there was no statistically significant constriction in venules after 60 seconds (Mdn -16 %; IQR: 5 % - 28 %; $p < .5$, T = 0, $r = -.15$). The effect varied slightly during the subsequent period and maximal constriction occurred three minutes after the application (art.: 17 %; IQR: 11 % - 22 %; T = 0, r = -.45, p < .04; ven.: 20 %; IQR 10 % - 27 %; T = 0, r = -.45, p < .04). The constriction disappeared gradually over the recorded period and finally after 30 minutes of observation returned to zero (see figures 6 and 7).

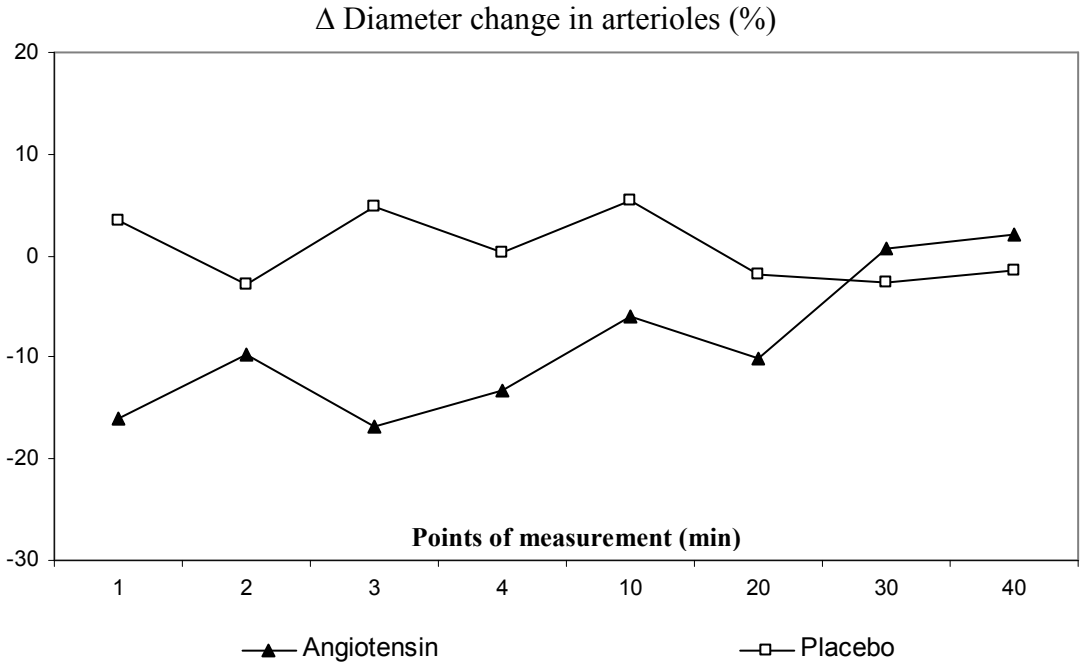

Fig. 6: Median arteriolar diameter change after the administration of Ang II 0.01% and placebo over a period of 40 minutes. X-axis shows the time points and Y-axis shows the mean relative diameter change.

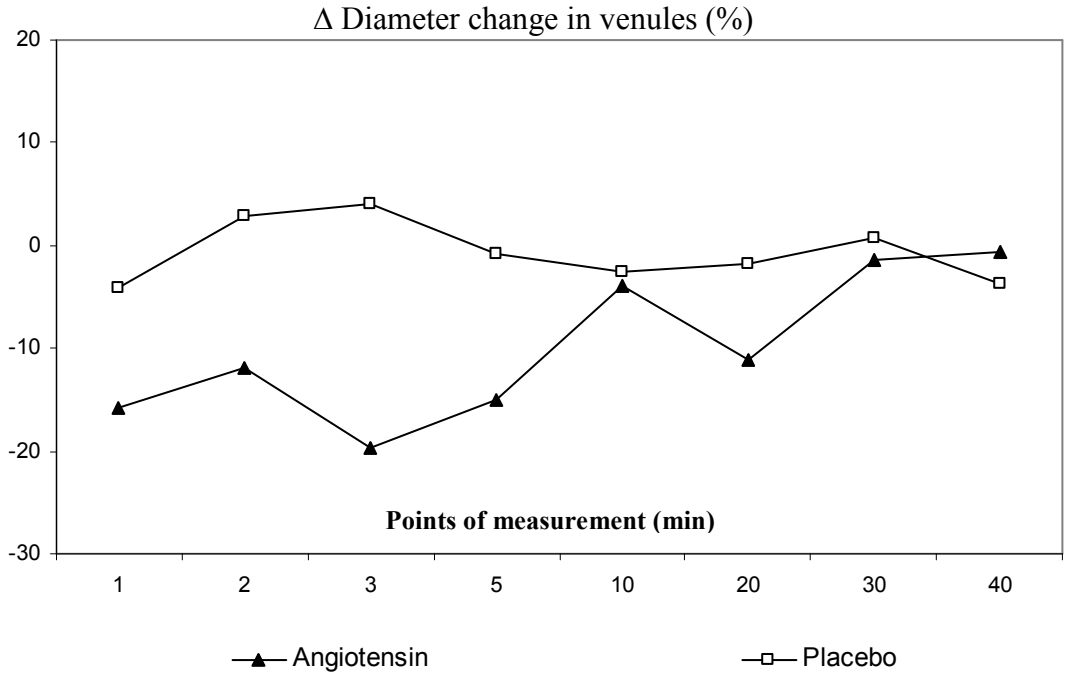

Fig. 7: Mean venular diameter change after the administration of Ang II 0.01% and placebo over a period of 40 minutes. X-axis shows the time points and Y-axis shows the mean relative diameter change.

3.5 Repeated challenge

The vascular reaction to two applications of high a dose of Ang II in quick succession was assessed in the last trial of this series. The second eye drop was applied when the effect of the first drop had vanished. The results of the antecedent experiments indicated a return to the baseline status after approximately 30 minutes and thus this period was set between the two runs. The effect on vascular diameter was not statistically significant, neither in the first nor in the second run. Nevertheless a trend could be observed when the effect of Ang II was compared to placebo (For details see Table 3). The effect did not differ significantly between the first and the second runs.

Table 3: Diameter change of arterioles and venules in round one and two.

	Arteriolar constriction*	***p*-value****	**Venular constriction***	***p*-value****
First Run	12 % (6 % - 22 %)	$p < .068$	8 % (8 % - 11 %)	$p < .066$
Second Run	17 % (10 % - 23 %)	$p < .068$	10 % (5 % - 15 %)	$p < .066$
Difference between run 1 & 2**	$p < .14$	-	p = 1	-

*Values are Median and Inter Quartile Ranges (IQR) **p-values as assessed by Wilcoxon signed rank test

4 Discussion

4.1 Validation

The aim of the present study was to establish a new model for the *in vivo* investigation of microvascular reactivity. The suggested approach provides a unique opportunity to non-invasively study microvascular function in the human bulbar conjunctiva. The validation of the setup was carried out first by assessing the variability of the measurement technique. The results of these experiments suggested a high reliability of the image analysis and diameter measurement procedure. Analyses of the different measurements revealed that although the utilized software was programmed to analyze contrast medium filled coronary arteries, the readings varied only marginally from each other. This is in line with previous findings by Houben et al. (1998) who demonstrated that monochromatic green illumination represents an adequate contrast enhancement in the conjunctiva. No participant expressed complaints concerning the comfort during the experiments. The upright sitting position was experienced as comfortable and irritations of the eye were rare as there was always enough time to relax the ocular muscles and the eye drops did not yield any side effects.

The results of the first validation experiments formed a solid basis for the successive experiments as a reliable diameter measurement and a comfortable setup were the first important obstacles to overcome. In the second part the short-term biological variability was assessed. Only 4 % variation of diameter over a period of one minute in both arterioles and venules indicated that variations caused by the technique itself (e.g. illumination or constant focusing), short-term blood pressure regulation or pulse waves were moderate. The estimated middle-term variability (~10 min) was only marginally different from that observed during short-term measurements. The 3 % variability assessed for arterioles and venules showed that during the time of the experiment diameters varied within narrow limits. The long-term variability of the baseline diameter as assessed on two different days in the same segments of identical blood vessels was analogous to the short and middle-term variability at a low level of 3 % for arterioles and 2 % for venules. The standard deviation of both middle- and long-term variation of about 10 % was surprisingly high, indicating a large range of results. Thus, the variability itself might not have been reliably assessed. Several baseline scans computed to a mean value would have been the

most suitable procedure as applied by Nagel et al. (2003). In their experiments the average of three readings of one conjunctival vessel each lasting 30 seconds formed the baseline diameter. Such a longitudinal setup might represent a more comprehensive approach for establishing a baseline value. Nevertheless, compared to the work of Nagel et al. (2003) the present model assessed the baseline diameters of three vessel pairs in each eye in order to avoid the risk of analyzing injured or atypically perfused and thus dysfunctional vessels. In addition Nagel and colleagues used a 23 % smaller magnification which complicated a classification or evaluation of the analyzed vessels. In comparison the examiner could easily assess the recorded vessels regarding their obligatory qualities in the current model. Another possibility for improving the estimation of the measurement variability was proposed by Laemmel et al. (2004). They utilized a vessel-phantom made of fluorescent microspheres to validate the accuracy of their technique. The phantom had a pre-defined diameter that could thus be compared to the results of the measurement process. This validation is very elegant but the experiences with the proposed setup from antecedent trials (Houben, et al., 1998) together with the low variation coefficient in the validation trials allowed a postponement of such efforts.

Once the variability was assessed attention was focused on the function and reactivity of the microcirculation. First the effect of an established vasoactive agent on vascular diameters was assessed and the capability of the setup to detect vascular diameter changes from pre- to post-drop readings was tested. Phenylephrine, as applied in the present study, is a commonly used substance in conditions of mild allergic or exogenous conjunctivitis leading to vasoconstriction in the inflamed tissue. Vascular diameters of three vessel pairs (arteriole and venule) were assessed before and 60 to 90 seconds after the application of phenylephrine eye drops. Comparable to the results of Nagel et al. (2003), vascular constriction reached a high level when the post drop measurement was carried out. The constriction was reliably detected and was found to be significantly stronger when compared to the effect of placebo eye drops. This finding was not surprising with respect to the effect of phenylephrine as a potent vasoconstrictor but indicated that the present model is suitable for the pre- and post-eye drop measures.

Considering the possibilities of such a direct approach to the reactivity of locally applied drugs the next step had to be the reproduction of the results and an expansion of the range of application. The reproduction of the results was carried out by testing participants in two separate testing sessions. Under identical conditions, the same blood vessels were investigated and results were compared. The videotaping function of the model enabled the examiner to assure that the same vessels were recorded and compared in both testing sessions. The effect of phenylephrine did not differ significantly between the two test days and was comparable to the

effect seen in the antecedent trial. In addition, the model met the demands concerning the reliable detection of identical conjunctival vessels on different days. Nevertheless the results should be interpreted carefully as the number of participants included in this trial was relatively small due to practical obstacles. In this third step it has been shown that the same vessels could be observed under different conditions such as pre- and post-treatment with an anti-hypertensive agent or under special conditions such as low and high sodium intake.

4.2 Application

The implementation of the new model was carried out by applying Ang II eye drops in different concentrations and measuring the effect of this peptide over a period of 40 minutes. The highly important actor of the RAS is known to play an important role in the development and progression of hypertension and CVD. One of the core theories is an increased reactivity to vasoactive agents like Ang II via different mechanisms (see Introduction 1.3.1.). The reactivity to locally applied Ang II was measured in young healthy subjects in order to validate the setup for later measurements in patients with e.g. hypertension or CVD. Ang II in a low concentration of 0.001 % did not lead to any reduction in vascular diameter. The concentration might have been too low as it is unknown how much of the applied substance finally reaches the receptors which in addition are distributed quite irregularly among ocular tissues (Savaskan, et al., 2004).

The high concentration eye drops contained 0.01% Ang II and led to a statistically significant diameter reduction after one and ten minutes in arterioles and after one minute in venules. A ten-fold concentration thus leads to an effect comparable to that of phenylephrine used in the preceding experiments. Nevertheless, AT_1 receptors are not regularly distributed in the conjunctiva and thus the constriction might have been caused by an Ang II induced impairment of vasodilatory metabolites such as NO (Rajagopalan, et al., 1996). The venular unresponsiveness to such a strong stimulus can only be explained by the general difficulty in interpreting the results measured in venules. Due to the arterio-venous feedback it is expected that if flow reduces in arterioles because of a 20 % reduction in diameter the venules will no longer be filled to an extent that their diameter remains unchanged (Silbernagel and Despopulos, 2001). The effect seen after 30 to 90 seconds slowly vanished over time and after 30 min vascular diameter returned to baseline. Not all results in this last trial were statistically significant but as the number of participants was low at least a tendency can be acknowledged. In a second trial with a comparably low number of cases the effect of Ang II was reproduced in a second run 30 minutes following the first. These results suggest that the model is suitable for the

investigation of the effect of locally applied actors of the RAS in the conjunctiva. This in turn offers a great opportunity for clinical practice. First, the setup could be used for an early detection of vascular hyper-reactivity to vasoconstrictor stimuli or for the detection of reduced vasodilator capacity in offspring of hypertensive parents or borderline hypertensives. Second, the model could be employed to control the success of treatment aimed at microvascular function and the RAS in conditions of hypertension and CVD.

4.3 Conclusions and prospective research

The proposed model represents a new approach to the non-invasive study of human conjunctival microcirculation. It is very comfortable, easy to use and has a broad area of application. The validation of the setup should nevertheless be expanded to e.g. several baseline scans, continuous readings over five to ten minutes and eye drops without preservatives. Although the influence of the examiner has been diminished by automating parts of the procedure, the inter-rater reliability will have to be assessed in the future. The influence of the experience of each examiner cannot be excluded as the vessels are still manually chosen, re-discovered and focused. A separate image capture screen where screenshots are held while the other vessels are filmed would be another helpful modification of the setup. Future investigation has to show whether other drugs such as ARBs can be applied locally and hence reveal for example an Ang II related pre-constriction leading to altered reactivity and increased BP. A next step should be the application of the model in patients with circulatory diseases such as hypertension. A specifically interesting group will consist of recently diagnosed hypertensives that have not started treatment. These patients would certainly bring up interesting information about microvascular behavior before and after treatment. In addition the treatment strategy could be adapted to the type of hypertension i.e. high output or elevated total peripheral resistance. Other fields for the application of the technique are e.g. connective tissue diseases, diabetes mellitus and in the field of critical care medicine, sepsis and eclampsia.

An interesting work by Wolf-Mayer (2003) pointed out the age distribution of hypertension. It demonstrated that especially in Germany, England and Finland an increasing number of younger individuals tend to have high BP. This underlines the importance of early detection and correct assessment of elevated BP which is frequently complicated when interpreting ambulatory BP readings (Verberk, et al., 2006). The assessment of the microvascular reactivity could reveal hemodynamic changes in the direction of hypertension earlier than ophtalmoscopy of the retina or other techniques aimed at structural rather than functional

alterations. Houben et al. (2005) recently showed that salt intake plays an important role in the functional adaptation of the microcirculation but it remains unclear whether this is just a volume effect or if possibly changes in the RAS or other regulatory systems caused the decrease in vascular density in the human conjunctiva. The proposed model might facilitate the study of causes in this complex play of interactions and finally improve the early detection and treatment monitoring of hypertension.

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Erklärung

„Ich, Philip Burgwinkel, erkläre, dass ich die vorgelegte Dissertationsschrift mit dem Thema: „A new model to study human microvascular reactivity in vivo“ selbst und ohne die (unzulässige) Hilfe Dritter verfasst und auch in Teilen keine Kopien anderer Arbeiten dargestellt sowie keine anderen als die angegebenen Quellen und Hilfsmittel benutzt habe.“

Datum Unterschrift

7 Lebenslauf

Mein Lebenslauf wird aus Datenschutzgründen in der elektronischen Version meiner Arbeit nicht mit veröffentlicht.