# PRORENIN AND DIABETES MELLITUS

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# PRORENIN AND DIABETES MELLITUS

(prorenine en diabetes mellitus)

**PROEFSCHRIFT**

Ter verkrijging van de graad van doctor
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door

Antonius Adrianus Maria Franken

geboren te Bergen op Zoom

**PROMOTIECOMMISSIE:**

| | |
|---|---|
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| Overige leden: | Prof. Dr. P.T.V.M. de Jong |
| | Prof. Dr. J.C. Birkenhager |
| | Prof. Dr. A.J.M. Donker |

Men weet eigenlijk slechts, zolang men weinig weet;

hoe meer men weten gaat, hoe meer men gaat twijfelen.

Goethe

CONTENTS page

**Part of this thesis is based on the following articles:**

1. Franken AAM, Derkx FHM, Schalekamp MADH, Man in 't Veld AJ, Hop WCJ, van Rens GH, de Jong PTVM. Association of high plasma prorenin with diabetic retinopathy. J of Hypertension 1988;6 (suppl 4):S461-463.

2. Schalekamp MADH, Franken AAM, de Jong PTVM. Plasma prorenin and diabetic retinopathy. In: MacGregor GA, Sever PS (eds) Current Advances in ACE Inhibition. Churchill Livingstone 1989:139-143.

3. Franken AAM, Derkx FHM, Man in 't Veld AJ, Hop WCJ, van Rens GH, Peperkamp E, de Jong PTVM, Schalekamp MADH. High plasma prorenin in diabetes mellitus and its correlation with some complications. J Clin Endocrinol Metab 1990;71:1008-1015.

4. Danser AHJ, van den Dorpel MA, Deinum J, Derkx FHM, Franken AAM, Peperkamp E, de Jong PTVM, Schalekamp MADH. Renin, prorenin and immunoreactive renin in vitreous fluid from eyes with and without diabetic retinopathy. J Clin Endocrinol Metab 1989;68:160-167.

5. Franken AAM, Derkx FHM, Blankestijn PJ, Janssen JAMJL, Mannesse CK, Hop W, Boomsma F, Weber R, Peperkamp E, de Jong PTVM, Schalekamp MADH. Plasma prorenin as an early marker of microvascular disease in patients with diabetes mellitus. Diabete & Metabolisme 1992;18:137-143.

6. Franken AAM, Dullaart RPF, Derkx FHM, Schalekamp MADH. Blood pressure and prorenin in relation to the progression of nephropathy in IDDM patients with slightly elevated urinary albumin excretion. A 2 year follow-up. Submitted

# Chapter 1

# GENERAL INTRODUCTION

## 1.1 Circulating and local renin-angiotensin systems

*Circulating renin-angiotensin system; the Classic concept.*

Until a decade ago the renin angiotensin system (RAS) was considered to be a classic endocrine negative feedback system regulating blood pressure and sodium-potassium homeostasis (1). The aspartic protease, renin, the initiating enzyme of the RAS-cascade, is secreted by the juxta-glomerular cells of the kidney, in response to a variety of stimuli, such as a drop of the transmural pressure gradient across the juxtaglomerular cells, reduced delivery of sodium to the distal tubular macula densa sites, and activation of beta - adrenoreceptors located in the juxtaglomerular cells.

Renin released by the kidney circulates in plasma and cleaves angiotensinogen, produced by the liver, thereby generating the biologically inactive decapeptide angiotensin I (Ang I). Angiotensin I in turn is converted in plasma into the octapeptide angiotensin II (Ang II) that is biologically highly active, by angiotensin converting enzyme, a dipeptidyl aminopeptidase whose concentration is especially high in the pulmonary vascular endothelium. Ang II is conveyed by arterial blood to the peripheral tissues, where it exerts its effects by interaction with specific Ang II receptors.
Ang II increases peripheral vascular resitance through powerful constrictor effects on vascular smooth muscle. Ang II reduces renal sodium excretion by acting directly on renal blood vessels and on renal tubular cells and by acting indirectly via increased aldosterone secretion from the adrenals (Figure 1).

*Circulating versus local renin-angiotensin systems; the new concept*

The classic concept of the circulating RAS, the activity of which depends on the regulated secretion of renin from the kidney, has been extended in the last years. An increasing number of studies has now demonstrated the existence of local tissue angiotensin generating systems which may operate, at least in part, independently of the circulating RAS (2,3).

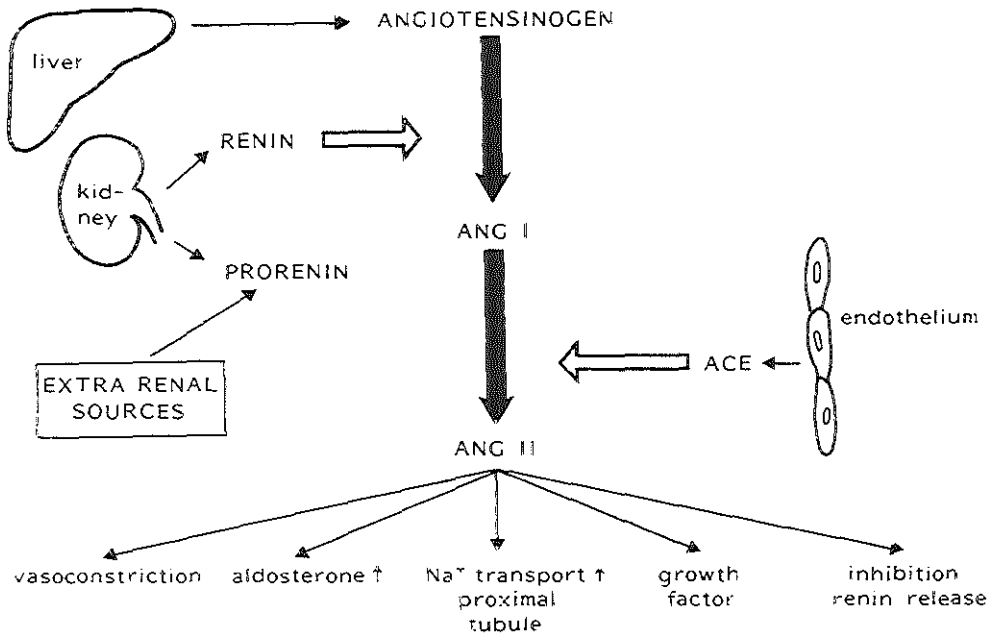


Figure 1: The classic concept of the renin-angiotensin system

According to one hypothesis, the primary function of the circulating RAS is not the systemic delivery of Ang II to tissues, but rather the delivery of renin and possible also angiotensinogen to tissues; A main fraction of Ang I and AII is generated locally at tissue sites by the action of plasma-derived renin (or perhaps prorenin) originating from the kidney on locally synthesized or plasma-derived angiotensinogen. The locally formed Ang I may then be converted in situ to Ang II or may enter the circulation and converted at the luminal site of the endothelial cells lining the blood vessels (4-6, Figure 2).

The detection of mRNA for renin and angiotensinogen in some tissues is strong evidence for local synthesis of these components in these tissues. The existence of a complete RAS system in the kidney has been well documented( 7,8). Ang II formation in extrarenal RAS tissues has been demonstrated for instance in the adrenals (9,10), the pituitary (9,10), the brain (11), testis (10), ovary (11,12) and in vascular tissue(13-17).

Most, if not all, renin in circulating plasma originates from the kidney, but a major fraction of prorenin in plasma is of extrarenal origin. It has been shown that testis and ovary can indeed secrete prorenin into the cicrculation (18-20).

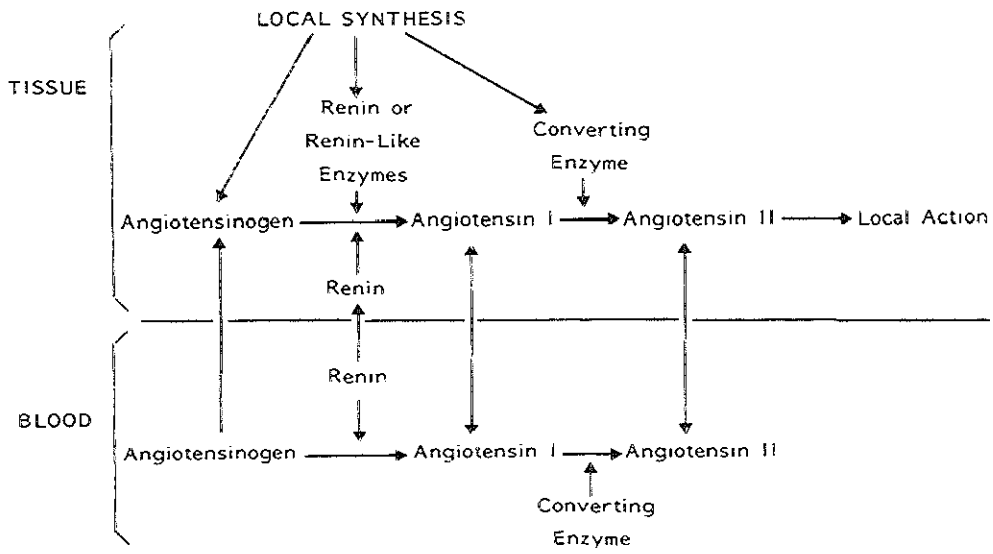


Figure 2: Circulating renin-angiotensin system versus local renin-angiotensin system: interactions and possible angiotensin generation sites.

*Prorenin:*

In the kidney, prorenin is synthesized in the juxtaglomerular cells, where it is converted to renin. The kidney secretes both renin and prorenin into the circulation.

Since the initial identification of inactive renin in amniotic fluid by Lumbers (21) in 1971, much progress has been made in understanding the relationship between prorenin and renin. This progress was possible partly as a result of the purification of native human renin with the generation of antibodies against renin (22-24) and cloning of the human renin gene sequences. These events made it possible to study renin gene expression and to produce recombinant prorenin and renin and to provide knowledge of their complete amino acid sequences (25-28).

Cellular processing and secretion of prorenin and renin in the juxtaglomerular cells can follow two pathways: the regulated pathway or the constitutive pathway.

Preprorenin is the primary translation product of renin messenger RNA (25-28).

The 23-aminoacid signal, or "pre" sequence, directs insertion of the protein into the lumen of the rough endoplasmatic reticulum and this is associated with rapid cleavage to form prorenin (29,30). In this inactive state, prorenin traverses the rough endoplasmatic reticulum and Golgi apparatus where it is glycosylated. The extent of glycosylation is variable, both in the renin produced by the kidney and that produced by extrarenal sources (28,31,32).

From the Golgi apparatus, prorenin can be sorted to one of three compartments, which include two secretory pathways (regulated and constitutive) and the lysosomes.

Prorenin has been demonstated to bind to mannose 6-phosphate receptors, which appear to facilitate its entry into the lysosomal compartment, where it is degraded to renin (33). The percent of cellular renin that enters this compartment is unknowm, but probably small (approximately 5%) (33).

Prorenin can also be sorted to the regulated pathway, in which prorenin is stored in secretory granules. Active renin is the primary product of the regulated secretory pathway (34,35). Prorenin-to-renin conversion appears to occur in the secretory granule and release of renin from the granules occurs after stimulation by a secretagogue. Several different proteases have been implicated as potential processing enzymes, for instance in the kidney a thiol protease resembling cathepsin B (37). This proccessing enzyme cleaves the amino terminal 43-amino acid prosegment, which allows exposure of the active site of renin.

Prorenin can also enter the constitutive pathway, in which prorenin is the secretory product (34,35). This pathway is not regulated acutely and delivers prohormone to the cell surface at a constant rate (37).

Two human tissues have been identified that primarily secrete prorenin; an ovarian leiomyosarcoma and the decidua of pregnant women (18,38).

*Regulation of prorenin ; observations in humans*

It is unknown which factors determine whether the Golgi apparatus sorts prorenin to either the regulated or the constitutive pathway (39).

The kidney releases both renin and prorenin. Acute stimulation of the juxtaglomerular cells, for instance exercise, isoproterenol infusion or a first dose frusemide leads to a prompt rise of plasma renin with no change in plasma prorenin (40,41). Chronic stimulation, for instance sodium restriction, captopril treatment leads to a rise of both plasma renin and prorenin.

The stronger the stimulus the higher the percentage contribution of renin to the level of total renin (prorenin plus renin) in plasma. The proportion of the total amount of circulating prorenin that is secreted by the kidneys is probably also increased under these circumstances (40-44). In other words, acute modulation of the juxtaglomerular cells leads to alterations in the renin release from the regulated secretory pathway with little effect on the constitutive pathway, although marked stimulation of the regulated pathway appears to result in decreased constitutive release of prohormone and chronic modulations leads to both renal renin release and prorenin release from the constitutive secretory pathway. The nature of the mechanisms regulating these relationships between prorenin and renin secretion is unknown.

Normal human plasma contains prorenin in a concentration about ten times higher than that of active renin. Plasma of nephrectomized patients contains little or no renin but the concentration of prorenin is sometimes as high as in normal individuals (45,46). In average the prorenin concentration in nephrectomized patients is about one third of that in controls. Thus, about one third of the plasma prorenin in normal subjects appears to be of extrarenal origin.

*Extrarenal sources of plasma prorenin; observations in humans.*

In pregnant women the plasma concentrations of both renin and prorenin are increased but, in contrast with what one sees during chronic stimulation of the release of renin by the kidneys, the percent increase in plasma prorenin is much greater. This is already the case in the first trimester of pregnancy (47,48). There is now good evidence that the ovary is the main source of this increase in plasma prorenin. Plasma prorenin is about two times higher in the luteal phase of the menstrual cycle than in the follicular phase. An increase in prorenin at the time of ovulation or shortly thereafter comparable to the increase in pregnancy has been reported in women who were treated with gonadotropins because of an in vitro fertilization program (49,50). The prorenin concentration in the ovarian follicular fluid of these women is about 40 times higher than the concentration in plasma (51,52).

In a woman without functioning ovaries, who became pregnant after transfer of a donated fertilized oocyte, pre-pregnancy plasma levels of prorenin and renin were normal and renin rose two-fold during pregnancy in this patient as it does in normal pregnant women. Prorenin, however, did not show the normal rise in the first eight weeks. In addition plasma prorenin remained low throughout pregnancy( 53). Because of these findings it seems likely that the ovary is an important source of the elevated plasma prorenin in pregnancy, not only in the first trimester but also in the last.
Renin mRNA expression suggests that the decidua is the major source of renin in the uterus and placenta. Renin gene expression was not detected in amnion, basal plate chorion, myometrium or the placental villi (18).
The lack of renin gene expression, but positive immunostaining in chorion, suggests that this menbrane does not produce prorenin but is capable of taking prorenin that is synthesized by the decidua and transporting it into the amniotic fluid, which is known to contain high quantities of prorenin.
It is not known whether prorenin is involved in the reproductive physiology, either locally or systemically.

Another well known source of plasma prorenin are the renin-secreting tumors. Most tumors arise from the kidney (nephroblastoma, juxtaglomerular cell tumor or reninoma), are malignant, and are associated with hypertension and hypokalemia. They secrete both renin and prorenin, but mainly prorenin into the circulation (54-56). A series of fifteen extrarenal renin-secreting tumors has been reported; 14 of these were in women, and seven were located in the reproductive tract (38). All tumors were malignant. Some tumors were highly vascularized, for instance in the case of an angiolymphoid hyperplasia with eosinophilia, glioblastoma multiforme, haemangiopericytoma and alveolar soft-part sarcoma and a close correlation and the degree of neovascularization has been repoted (57,58).

In conclusion, it seems that extrarenal RAS systems such as in the ovary and renin-secreting tumors have in common that they are located in highly vascularized tissues and that they secrete mainly prorenin, via the constitutive pathway, into the extra-cellular space. Whether the prorenin secreted by this pathway is subsequently activated in situ or is taken up by other tissues and then activated, remains to be determined.

*Functions of angiotensin II*

Ang II is, at least in humans, the most important biologically active endproduct of the RAS. Apart from its well-known role in blood pressure regulation Ang II has mitogenic and angiogenic properties. Ang II has beyond its direct vasoconstrictive and aldosterone synthesis-promoting effects to control adequate capillary perfusion, some growth modulating effects. Ang II appears to be a growth regulator in the kidney. It binds to specific cell surface receptors present on a number of different renal cell types including mesangial, vascular smooth muscle, tubular and interstitial cells, and activates many

of the intracellular signalling pathways associated with cell growth in the kidney (59,60). Ang II has been shown to stimulate DNA and protein synthesis in cardiovascular tissues (61) and induces hyperthrophy without cellular proliferation in cultured aortic smooth muscle cells (62). Whether AII stimulates vascular smooth muscle cell proliferation, directly or via potentiating other growth factors (EGF, PDGF) is still under discussion (59,63).

Anyway, after renal ischaemia Ang II restores blood flow to tissues below an aortic ligature by stimulating the development of collateral circulation (64). Furthermore, implantation of Ang II in the avascular rabbit cornea facilitated not only the activation of preexisting collateral pathways but also new vessel formation (65).

In the ovary luteinization of the follicle takes place following ovulation. This coincides with rapid and extensive neovascularization of the developing corpus luteum.

It is possible that local generation of prorenin and formation of Ang II are involved in neovascularization of the developing corpus luteum.

Captopril, an angiotensin converting enzyme inhibitor, not only lowered blood pressure, but was also more effective than hydralazine and propanolol in preventing increaments in aortic smooth muscle content and medial smooth musle content weight during development of hypertension in the spontaneously hypertensive rat (66).

Recently Wang et al (67) have shown that captopril treatment in one-kidney, one-clip hypertensive and normotensive rats resulted in reduction in cross-sectional wall area of aortas and arterioles in both normotensive and hypertensive rats, as well as a reduction in the number of small arterioles.

*Uptake of renin or prorenin by vascular tissue*

Labeled Ang I infusion studies showed that the major fraction of Ang I and Ang II in circulating plasma is produced locally at the tissue sites rather than in circulating plasma (68,69). The Ang I production rates in the various tissues correlated with PRA, suggesting that most of the renin responsible for local ANG I production is kidney-derived rather than dependent on in situ synthesized renin.

Other indirect evidence of vascular uptake of renin is based on experiments in rats after bilateral nephrectomy (70-72). Direct evidence of vascular renin uptake was shown by demonstrating renin uptake in aortic tissue after renin injection in bilaterally nephrectomized rats (73,74). Infusion of recombinant human prorenin in rhesus monkeys shows direct evidence for uptake of prorenin (75) and demonstrated that the infused prorenin had none of the effects of renin and that circulating prorenin is not the precursor for circulating renin (76).

*Possible physiological role of prorenin*

The only well-established role for prorenin is that it is a mandatory intracellular precursor for the synthesis of active renin.

In vivo there is no evidence for activation of prorenin in plasma, this is illustrated by the complete absence of renin activity in nephrectomized human subjects (77). However, prorenin can be activated in vitro by limited proteolysis with trypsin or other proteases, this results in cleavage of the prosequence and the irreversible formation of active renin (78). Interestingly, prorenin can also be activated by acid or cold temperature which results in a reversible activation (79,80).

Prorenin can be reversibly activated in vitro when acidified to pH 3.3 and only to be deactivated when incubated at neutral pH and 37°C and when prorenin is chilled to 0°C it develops reninlike activity that disappears when the sample is warmed to 37°C.

It has been suggested that acidification or freezing causes the prorenin molecule to physcally unfold its prosegment (open position) and thus to expose its active site, while refolding and shielding of the active site takes place upon neutralization or warming (81, Figure 3 ).

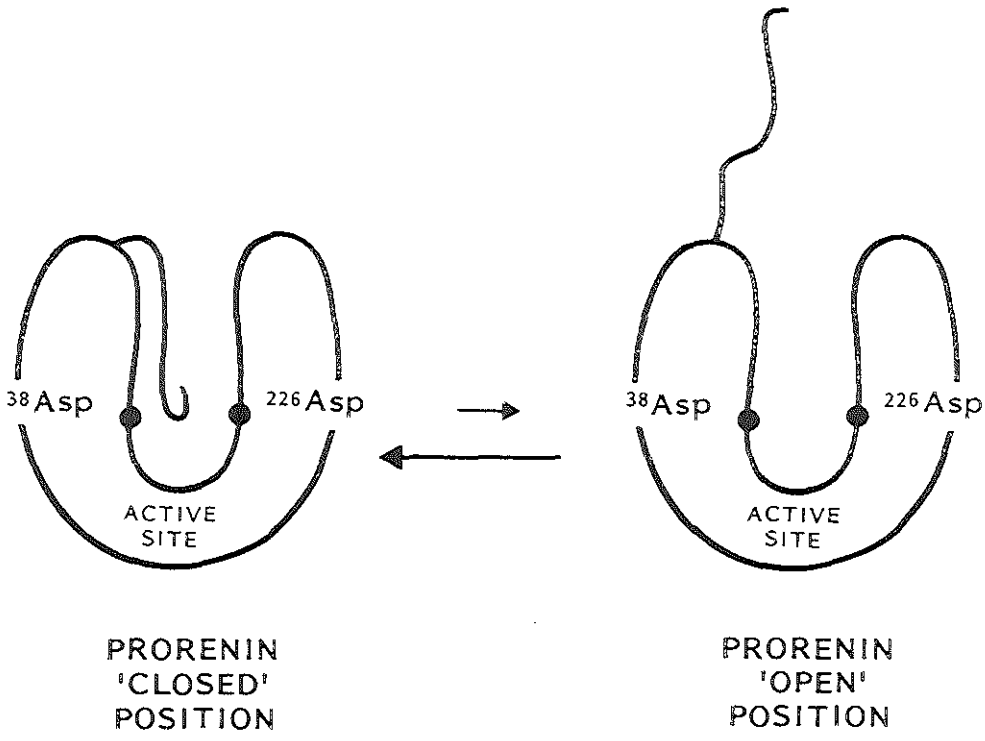


Figure 3: Hypothetical activation of prorenin by unfolding the prosegment.

This observation made Sealy et al to speculate that conditions may exist in target organs in vivo that favor the "open" form of prorenin (figure 3). They hypothesised that prorenin can bind to specific receptors which activate prorenin without cleavage of the prosegment. If the prorenin receptor were located near converting enzyme and angiotensin II receptor, in the presence of angiotensinogen, the activated prorenin could cleave angiotensin I from angiotensinogen which in turn could be activated by converting enzyme to angiotensin II (81).
After infusion of human prorenin into monkeys high concentrations of prorenin or renin protein (which could not be distinguished) were localized immunohistochemically in the glomerular region of the afferent arteriole where very high Ang II concentrations were detected with immunohistochemistry (82). In their model prorenin is to generate localized high concentrations of Ang II causing regional vasodilation by rendering tissues insensitive (tachyphylactic) to the vasoconstrictor effect of circulating Ang II or by releasing vasodilator substances (82,83).
In animals and humans, organs with high rates of blood flow- kidney, eye, ovary, pregnant uterus and placenta- all produce high concentrations of prorenin. An abnormal plasma prorenin could be the cause of the renal and retinal hyperperfusion that occurs in diabetes mellitus and is thought to predispose people to the development of microvascular disease. (84).

## 1.2 The renin-angiotensin system and diabetes mellitus

In 1972 Christlieb et al were the first who reported upon the RAAS and diabetes mellitus. They were interested in factors involved in initiating and sustaining the hypertension which frequently accompanies both type I and type II diabetes mellitus (85,86).

*The renin-angiotensin system in ketoacidosis*
Christlieb et al began their humun studies in patients with uncontrolled diabetes.In patients with ketoacidosis rather high plasma levels of renin activity (PRA) and aldosterone were found, suggesting a state of secondary hyperaldosteronism. Shortly after treatment (4-48 hours) with insulin, PRA levels decreased to normal (85). Several other groups confirmed these observations (87-89). The plasma levels of angiotensin II (Ang II) are also high in most ketotic patients (90). The high renin levels have been attributed mainly to fluid volume depletion and possibly also to a direct effect of metabolic acidosis on renin release (88).
Circulating catecholamines, especially norepinephrine, are elevated in established ketoacidosis, and return to normal after metabolic control (90).

*The renin-angiotensin system in uncontrolled nonketotic diabetes*

In non-ketotic diabetes PRA has been variously reported to be high, normal or low (91-96). This variability may perhaps be explained by differences in the severity of metabolic disease and by differences in the type of diabetes ( type I and type II), and by the presence or absence of complications ( nephropathy, autonomic neuropathy). With improved control the levels of PRA, Ang II returned to normal (96).

Basal levels of catecholamines are unchanged by poorly controlled diabetes; when metabolic control was improved supine and upright plasma norepinephrine concentrations did not change (97,98).

Christensen et al (99) measured PRA, Ang II, glomerular filtration rate (GFR) and renal plasma flow (ERPF) in newly diagnosed type I diabetic (IDDM) patients before and during initial insulin treatment.

Both GFR and ERPF were elevated at the start and fell significantly after 8 days of insulin treatment. PRA an renin substrate (angiotensinogen) were normal at the start and remained unchanged during treatment. Plasma Ang II was low initially and also remained unchanged.

*The renin-angiotensin system in diabetes with and without microvascular complications*

Christlieb et al (92) extended their research to diabetics with or without diabetic complications. In patients without clinical evidence of microvascular complications the RAS appeared to function normally, as jugded from the fact that PRA were normal and PRA responded normally to stimuli of sodium depletion and upright posture .

These results were confirmed by other authors (100-104). On the other hand, an increased vasopressor responsiveness to exogenous Ang II was found in normotensive, normoalbuminuric patients without retinopathy (105).

Again, Christlieb et al (92,105) were the first to report about low PRA levels and a blunted response to stimuli in diabetic patients with nephropathy and/or severe orthostatic hypotension. Suppression of the RAS when diabetes is complicated with clinical nephropathy and autonomic neuropathy has been reported by several other authors (106-109).

In patients with retinopathy as the only obvious diabetic complication normal basal PRA and normal responsiveness of PRA were described by Christlieb et al (110). In such patients the vascular reactivity to both Ang II and norepinephrine was found to be normal and diastolic blood pressure was higher in the group with retinopathy.

Drury et al (102-104) also reported on higher PRA ( supine and erect) in type I diabetics with proliferative retinopathy as compared to diabetics without complications and to normal persons, matched for age, sex and bodyweight. Patients with proliferative diabetic retinopathy (PDR) had higher blood pressures ( both systolic and diastolic) and worse metabolic control, and the inverse relationship between PRA and blood pressure and sodium excretion which was seen in normal controls and diabetics without

complications, was not apparent in the group with proliferative retinopathy (102-104).

It is difficult to compare the results published by different groups, because of heterogenity of the study populations, the different definitions of nephropathy.In addition, earlier studies could not differentiate between incipient or overt proteinuria. Finally, control groups were sometimes used withthout adequate matching and last but not least different methods were used for measuring the components of the RAS.

The plasma level of renin in most studies was measured as PRA with an enzyme kinetic assay. Results of this assay are affected by assay conditions, such as pH, the use of angiotensinase inhibitors, and the concentration of renin substrate in the plasma.

Diabetic patients without clinical evidence of microvascular disease have a normally acting RAS; their plasma levels of PRA, renin and aldosterone seem to be normal under basal and stimulated conditions.Patients with clinical evidence of complications seem to have a depressed RAS; the plasma levels of PRA, Ang II and aldosterone are all low (111-113).

*Mechanisms of hyporeninemia in diabetes mellitus*

Possible mechanism for this hyporenemia in complicated diabetes are (92):
1) expanded blood volume due to sodium retention or plasma hyperosmolarity resulting from hyperglycaemia, 2) destruction of juxtaglomerular cells due to nephropathy, 3) decreased activity of the the sympathetic nervous system, 4) defective renin synthesis due to insulin deficiency and 5) decreased renin substrate.

*Expanded blood volume.*

There is evidence for sodium retention in diabetes mellitus.
It has been observed both in type I or type II diabetes regardless of patients age, sex, presence or absence of proteinuria, retinopathy and peripheral neuropathy (116).
Moreover sodium retention was evident in both normo- or hypertensive subjects. Blood and plasma volumes were normal in normotensive diabetics, and reduced in hypertensive diabetics (97,114-116).
Feldt-Rasmussen et al (113) measured the plasma renin concentration and the concentration of Ang II and aldosterone in patients with normoalbuminuria, microalbuminuria and overt nephropathy in relation to exchangeable sodium and blood volumes. They found normal renin levels and suppressed angiotensin II and aldosterone levels regardless of the presence or absence of albuminuria. The plasma levels of norepinephrine and epinephrine were also reduced in patients with albuminuria. Exchangeable sodium was elevated in most diabetics, especially in patients with overt nephropathy. Blood and plasma volumes were normal.
The increase in exchangeable sodium was accompanied with an increased extracellular volume. Blood pressure was significantly correlated with exchangeable sodium in the group with microalbuminuria.

There is substantial evidence that sodium retention in diabetes is mainly due to enhanced tubular reabsorption of sodium (117). As mechanisms underlying the sodium retention have been proposed an increased glucose- coupled tubular reabsorption of sodium (118) or an insulin induced increased tubular reabsorption (119,120). The observed increased vascular reactivity to Ang II and norepinephrine might be a physiological response to sodium retention. Sodium retention is likely to play a role in the blood pressure rise observed at the very early stages of diabetic nephropathy. Hyperfiltration as observed in the early stages is probably a compensating mechanism for sodium retention , so that blood pressure is kept within normal limits.

*Destruction of juxtaglomerular cells.*
Hyalinization of the afferent glomerular arteriole offers another explanation for renin suppression in longstanding diabetes mellitus. The process is usually segmental and is unlikely to explain renin supression in patients with short duration of their diabetes.

*Decreased activity ofthe sympathetic nervous system.*
Autonomic diabetic neuropathy with secondary diminshed activity of the sympathetic nervous system may also affect renin release (106,107). Low circulating levels of catecholamines and an increased pressor response to norepinephrine have been observed (121-124). It is well established that renin secretion is under control of beta adrenoceptor stimulation. However, patients with othostatic hypotension due to autonomic neuropathy had normal PRA both supine and upright (105).

*Defective renin synthesis.*
Originally Christlieb et al (92) considered insulin-deficiency as a cause of a defect in renin synthesis. However, rather high PRA levels were found in diabetic ketoacidosis, a clear state of insulin-dependency. Defective renin synthesis as a explanation for low PRA was suggested in 1974 by Day et al (125) after the discovery of large amounts of big renin, an inactive form of renin, in plasma of patients with diabetes mellitus and proteinuria. Deleiva et al (126) describes in 1976 two diabetics with nephropathy and hyporenemic hypoaldosteronism and high levels of inactive renin. It was suggested that high levels of inactive renin represented a defect in the conversion of inactive renin to active renin, a defect caused by renal diabetic damage. A near complete block in renin production could result in very low plasma renin accompanied by high prorenin and low aldosterone.
The inactive renin of kidneys and plasma, later on, turned out to be prorenin, the precursor of mature renin, derived from preprorenin (127).
From now on attention was directed from renin to prorenin and the prorenin-renin conversion.

*Decreased renin substrate.*

There is no evidence for a renin substrate deficiency according to Feldt-Rasmussen in type I diabetes with albuminuria or overt nephropathy (113).

## Prorenin and diabetes mellitus

In their initial survey, Day et al discovered that the plasma of patients with diabetes and proteinuria contained large amounts of inactive renin. Bryer-Ash (128) et al showed that prorenin levels rose to unusualy high levels in diabetic patients with albuminuria after intravenous administration of the diuretic furosemide.

The largest increments occurred in patients whose active renin did not rise to the usual level after furosemide treatment. In normal subjects the increase in prorenin was very small after furosemide regardless of the PRA response.

Similar responses of inactive renin in diabetics after furosemide were reported by Manchandia et al (129).

The kidneys are the most likely source of the increased furosemide-stimulated rise in plasma prorenin in the diabetic patients (130).

Elevated prorenin in diabetic nephropathy may reflect a diminished conversion of prorenin to renin. In patients with retinopathy but without overt proteinuria , however , Bryer-Ash et al (131) reported on significantly raised plasma prorenin levels, suggesting a relation between elevated plasma prorenin and retinopathy, a relation that is partly independent of nephropathy.

Luetscher et al (132) examined the relation between prorenin and the presence of diabetic microvascular complications. Plasma renin and inactive renin (prorenin) was measured in 235 diabetic patients (type I and type II) and in 90 controls. In the controls there was a significant relation between plasma prorenin and age ($r=0.30$, $p<0.001$).

Among type I diabetics plasma prorenin was significantly correlated with age ($r=0.26$, $p<0.01$), duration of diabetes ($r=0.36$, $p<0.01$), and the presence of microvascular complications (retinopathy and/or albuminuria of more than 50 mg/l).

In type II diabetes the correlation between prorenin and age did not reach statistical significance, as it was for type I, however plasma prorenin was correlated with duration of diabetes ( r=0.36, 0.01) and also with the presence of diabetic complications.

Figure 4 shows the relation between plasma prorenin and the presence of microvascular complications. The highest values of plasma prorenin were found in the patients with albuminuria. Plasma renin levels did not differ in controls and patients with or without diabetic complications.

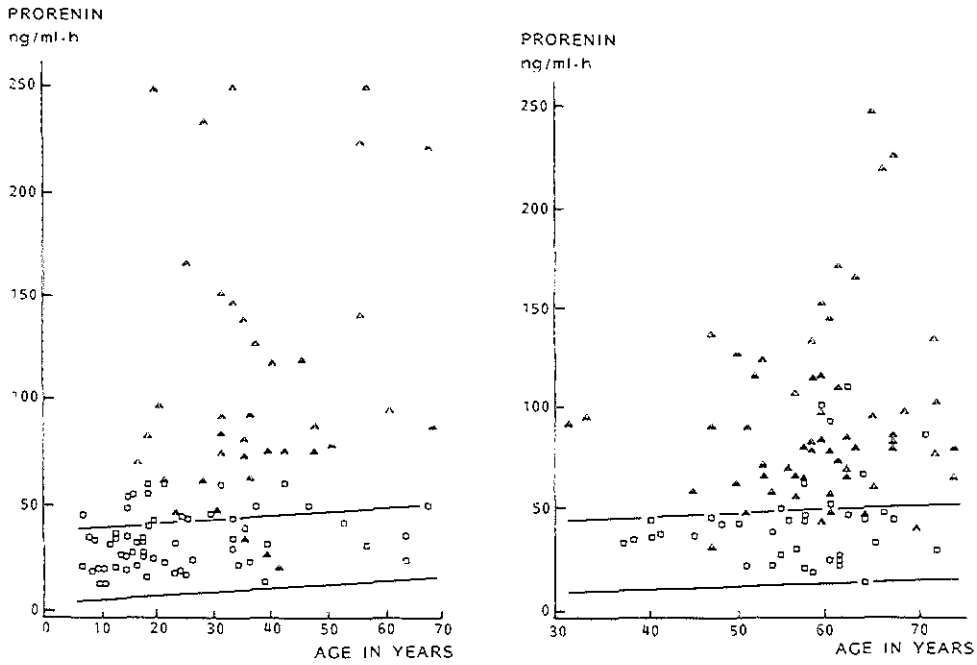


Figure 4: Plasma prorenin in relation to age and the presence of microvascular complications in type I (left) and type II (right) diabetes. Patients without complications are represented by squares and those with complications (retinopathy, neuropathy or albuminuria) by triangles. Dashed lines delimit 95 per cent of values in controls. Prorenin is expressed as nanograms angiotensin I per milliliter of plasma per hour. From Luetscher et al. (132).

It was proposed that the plasma level of prorenin may be used as a marker of microvascular complpications. Luetscher suggested that the excess of plasma prorenin is secreted by kidneys.

The kidneys might have a limited ability to process prorenin to active renin. However, a extra-renal source of the elevated plasma prorenin was not excluded by Luetscher. The reason for this inability, according to Luetscher et al, might be an alteration in the intracellular renin biosynthesis, for instance non-enzymatic glycosylation of one of the prorenin- processing enzymes. Cathepsin B, a kidney protease that can convert in active renin to active renin, becomes inactive after incubation with high concentrations of glucose (133-135).

Loss of adrenergic stimulation, as is seen with diabetic autonomic neuropathy, may lead to decreased secretion of renin by the juxtaglomerular cells and it has been postulated by two groups of investigators (136,137) that a compensatory increase in the synthesis and secretion of prorenin by these cells is the cause of the increase of porrenin in diabetes mellitus.

Whether increased plasma prorenin is related to kidney involvement or visa versa is an important question. The two processes, one leading to albuminuria, and the other to increased plasma prorenin, may be independent, although they seem to converge as the renal laesion progresses. Prospective studies were needed to solve this problem.

Luetscher et al (138) investigated the relation between plasma prorenin and micro-albuminuria in IDDM and NIDDM patients. Both in IDDM and NIDDM normotensive patients plasma prorenin was correlated with increasing levels of albuminuria. This relation was not consistently present in the hypertensive subjects, in three of nine hypertensive patients with gross albuminuria the plasma prorenin value was in the normal range.

Other data reported by Luetscher (139) show that in type II diabetics with hypertension the association between microvascular complications and prorenin is less reliable, even when they excluded patients with antihypertensive drugs which could effect plasma prorenin level.

Amemiya et al (140) reported higher levels of prorenin along with the progress of the degree of albuminuria in IDDM patients. The ratio of active renin and total renin was even lower in the normoalbuminuric patients as compaired with non-diabetic controls, suggesting an altered synthesis of renin with lower conversion rate of prorenin to active one as early as in the normoalbuminuric stage.

Prospective data reported by Wilson et al (141) suggest that in adolescent diabetics a rise of prorenin even precedes the development of microvascular complications. Among the patients who had had diabetes for more than four years, the mean plasma prorenin activity in those (n=7), in whom retinopathy or overt albuminuria developed was significantly higher 18 months before the complication appeared than in those (n=20) in whom no complication appeared during a similar period of observation.

They used, however, the presence of overt albuminuria in stead of microalbuminuria as a sign of microvascular involvement. Patients with either intermittent or continious microalbuminuria had higher levels of plasma prorenin than patients without microalbuminuria, but the difference was not statistically significant, only overt microalbuminuria was significantly related to plasma prorenin. Their data also suggest retinopathy being a more important factor related to plasma prorenin than intermittent and continious microalbuminuria.

In Figure 5 the incidence of microangiopathy related to plasma prorenin in 34 patients with previously uncomplicated diabetes during 12-54 months of follow-up is shown.

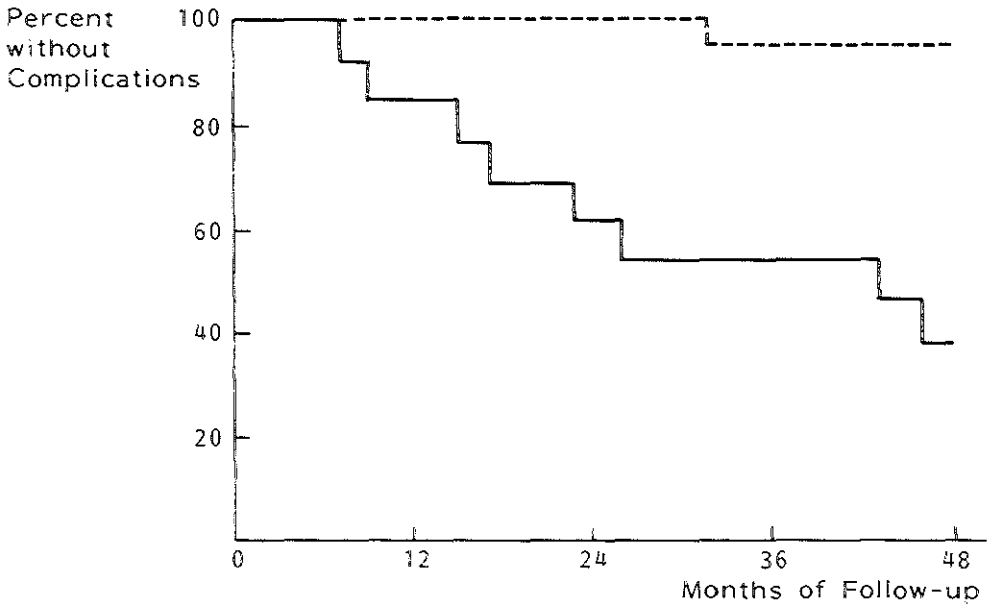


Figure 5: Incidence of microangiopathy (retinopathy or overt albuminuria) in 34 patients with previously uncomplicated type I diabetes mellitus during 12-54 month of follow-up. The dashed line denotes patients whose plasma prorenin activity was consistently within the normal range; a complication developed in only one of the 21 patients. The solid line denotes patients whose plasma prorenin activity exceeded the upper limit of normal (11.5 ng angiotensin I /l.s); a new complication developed in 8 of the 14 patients. From Wilson and Luetscher (141).

In conclusion, in longstanding diabetes mellitus complicated by microvascular disease, the plasma level of prorenin is often 2-3 times normal, whereas renin is below the normal range. The cause of the elevated plasma prorenin is unknown. There are two main possibilities, first an increase in the renal or extrarenal synthesis of prorenin and secondly a reduced renal or extrarenal clearance of prorenin.

## 1.3 Aim of the thesis

The questions we hoped to answer by the studies in this thesis are:

1. Is an increased circulating level of prorenin indeed related to the presence of microvascular disease in diabetes mellitus and, if so, with which type of complication (retinopathy, nephropathy, or neuropathy) is it associated ?.
2. What is the source of the increased circulating prorenin in longstanding diabetes mellitus?.
3. Is the RAS, local and/or systemic, involved in the pathogenesis of diabetic microvascular complications?.
4. Is it possible to use plasma prorenin measurement as an early marker of microvascular disease, especially in respect to microalbuminuria?.

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## Chapter 2

# HIGH PLASMA PRORENIN IN DIABETES MELLITUS AND ITS CORRELATION WITH SOME COMPLICATIONS


## SUMMARY

Plasma prorenin is abnormally high, whereas renin is normal or even low, in many patients with longstanding diabetes mellitus complicated by microvascular disease. Nephropathy or autonomic neuropathy have been put forward as a cause. We found that in 223 consecutive diabetics prorenin correlated positively with serum creatinine, the presence of macroalbuminuria(>250 mg/L) and the presence of diabetic retinopathy, particularly the proliferative type. This correlation did not depend on the presence of neuropathy or on whether the patient was on insulin or not. It was also independent of sex, age, duration of diabetes, blood pressure and the blood levels of glucose and HbA1c. The association between elevated prorenin and retinopathy remained significant after adjustment for creatinine and the presence of macroalbuminuria. Of the whole group of diabetics 94 consecutive patients were assessed for the presence of microalbunimuria (30-300 mg/24h). Independently of the presence of micro- or macroalbuminuria, the mean level of prorenin was not above normal in the patients without retinopathy and was 2-3 times normal in those with proliferative retinopathy. Thus, retinopathy appears to be a more important determinant of abnormally high prorenin than nephropathy. In addition, the renal vein-to-artery ratio of prorenin in 7 diabetics with both advanced nephropathy and proliferative retinopathy was not elevated, despite the high peripheral vein prorenin level and the impaired renal perfusion. Thus the abnormally high prorenin in these patients could not be explained by abnormal secretion by the kidneys. Finally, prorenin was not high in 16 non-diabetics with loss of sympathetic activity due to chronic autonomic neuropathy, which indicates that, in the absence of diabetes, this type of autonomic failure is not sufficient to cause the high prorenin levels seen in diabetics. Our findings are evidence that abnormally high plasma prorenin in diabetes is not an immediate consequence of altered glucose metabolism. This abnormality is related to the development of microvascular disease in the eye and the kidney, and is, at least in part, due to either decreased clearance of prorenin from the circulation or increased production from extrarenal sources or both.


## INTRODUCTION

Prorenin is an inactive precursor of renin, which in plasma is the rate limiting enzyme responsible for the formation of angiotensin II, a peptide with important vascular actions. In longstanding diabetes mellitus complicated by microvascular disease the plasma

level of prorenin is often 2-3 times normal (1,2), whereas renin is within or below the normal range (3,4). The cause of the elevated plasma prorenin in diabetes is unknown. Prorenin is synthesized in the renal juxtaglomerular cells, where it is converted to renin. The kidney releases both renin and prorenin into the circulation (5,6), and it is possible that in diabetic nephropathy the quantity of prorenin released by the kidneys is disproportionally high because of abnormal enzyme processing or secretion (7).

Diabetic autonomic neuropathy has also been put forward as a cause. Loss of normal adrenergic stimulation of the renal juxtaglomerular cells might lead to a decrease in the secretion of renin stored in these cells and to a compensatory increase in the synthesis and secretion of prorenin. High plasma levels of prorenin have indeed been documented in some diabetics with autonomic neuropathy (8,9). However, autonomic neuropathy was not the only diabetic complication in these patients.

In the present study we examined in a group of diabetics the statistical correlations between the plasma level of prorenin and the presence of nephropathy, neuropathy and retinopathy. We also studied a group of non-diabetic patients with loss of sympathetic function due to autonomic neuropathy in order to examine whether loss of adrenergic stimulation, in the absence of diabetes and its complications, is associated with an elevated plasma prorenin. We finally measured plasma prorenin in the renal veins of patients with diabetic nephropathy in an attempt to demonstrate increased secretion of prorenin by the kidney.

## SUBJECTS AND METHODS

*Outpatients with diabetes mellitus.*

This group (n=223) consisted of all diabetics attending our outpatient facilities in three consecutive months, with the exception of patients with overt cardiac failure, hepatic disease or renal failure (serum creatinine above 200 µmol/L). Patients on drugs likely to affect the plasma level of renin were also not included. Some clinical characteristics of these patients are given in Table 1. In the study group 136 were on insulin, 82 on oral antidiabetic drugs and 5 on diet alone. The patients were seen by an internist and an ophthalmologist, who were unaware of the results of the renin measurements. Non-timed overnight urine collections obtained at two different days were tested for the presence of protein. Macroalbuminuria was defined as albumin above 250 mg/L in each of the two samples. An assessment of microalbuminuria was made in the patients visiting the outpatient clinic in the last two months excluding those with macroalbuminuria and those with a history of kidney or urinary tract disease. Clinical characteristics of these patients are given in Table 2. Microalbuminuria was defined as albumin between 30-300 mg/24h in at least two out of three consecutive 24 hour urine collections (10).

Diabetic retinopathy was assessed by direct and indirect ophthalmoscopy after pupillary dilatation and in most cases also by fluorescein angiography. Grading of the

severity of diabetic retinopathy was based on the findings at ophthalmoscopy and was done by the ophthalmologist.

Table 1. Clinical characteristics of diabetic patients with and without macroalbuminuria

| | | | Macroalbuminuria | | p value |
|---|---|---|---|---|---|
| | | | absent | present | |
| Sex | female/male | n | 89/72 | 26/36 | ns |
| Age | median | yr | 56 | 54 | ns |
| | range | | 21-87 | 20-92 | |
| Duration | median | yr | 9 | 15 | p< 0.05 |
| of diabetes | range | | 1-55 | 1-47 | |
| Insulin treatment | on insulin/not on insulin | n | 95/66 | 41/21 | ns |
| Neuropathy | absent/present | n | 127/34 | 44/18 | ns |
| Diabetic | absent/present | n | 84/77 | 15/47 | p < 0.001 |
| retinopathy | background/proliferative | n | 49/28 | 11/36 | p < 0.001 |
| HbA1c | mean (sd) | % | 9.8 (1.2) | 10.1 (0.9 ) | ns |
| Serum creatinine | mean (sd) | µmol/L | 82 (18) | 101 (20) | p < 0.001 |
| | above 110 µmol/L | n | 10 | 19 | p < 0.001 |
| Blood pressure | | | | | |
| systolic | mean (sd) | mmHg | 149 (24) | 148(21) | ns |
| diastolic | mean (sd) | mmHg | 81 (12) | 86(12) | ns |
| Renin | geometric mean | mU/L | 12.5 | 15.7 | ns |
| | 95% conf.interval | | 2.4-65.0 | 3.3-76.4 | |
| Prorenin | geometric mean | mU/L | 196 | 373 | p < 0.001 |
| | 95% conf.interval | | 44.1-873 | 88.0-1579 | |

ns = not significant (p > 0.05).

Table 2. Clinical characteristics of diabetic patients with and without microalbuminuria

| | | | Microalbuminuria | | p value |
|---|---|---|---|---|---|
| | | | absent | present | |
| Sex | female/male | n | 36/38 | 10/10 | ns |
| Age | median | yr | 49 | 55 | $p < 0.05$ |
| | range | | 21-76 | 38-86 | |
| Duration of diabetes | median | yr | 11 | 13 | ns |
| | range | | 1-39 | 1-45 | |
| Insulin treatment | on insulin/not on insulin | n | 43/31 | 12/8 | ns |
| Neuropathy | absent/present | n | 62/7 | 17/3 | ns |
| Diabetic retinopathy | absent/present | n | 39/35 | 6/14 | ns |
| | background/proliferative | n | 17/18 | 9/5 | ns |
| HbA1c | mean (sd ) | % | 9.7(1.2) | 10.3 (0.9) | ns |
| Serum creatinine | mean (sd ) | µmol/L | 79 (19 ) | 80 (20 ) | ns |
| | above 110 µmol/L | n | 4 | 2 | ns |
| Blood pressure | | | | | |
| systolic | mean (sd ) | mmHg | 144 (24) | 160 (21) | $p < 0.05$ |
| diastolic | mean (sd ) | mmHg | 79 (19 ) | 86 (12) | ns |
| Renin | geometric mean | mU/L | 11.4 | 7.6 | ns |
| | 95% conf. interval | | 2.1-61.8 | 1.6-35.1 | |
| Prorenin | geometric mean | mU/L | 166 | 163 | ns |
| | 95% conf. interval | | 43.0-632 | 40.1-653 | |

ns = not significant ($p > 0.05$).

For the purpose of the present analysis, diabetic retinopathy was categorized as either background retinopathy or proliferative retinopathy. Background retinopathy was characterized as the presence of microaneurysms, whether or not combined with hemorrhages and hard exudates (11). Eyes with preproliferative retinopathy, characterized by multiple cotton wool spots, venous abnormalities, beading and duplications, intraretinal microvascular abnormalities and diffuse large blot hemorrhages, were also categorized as background diabetic retinopathy. Patients with new vessels anywhere in the retina in either eye were categorized as having proliferative retinopathy. Of the 65 patients with proliferative diabetic retinopathy 55 had been treated by laser photocoagulation of one or both eyes.

Sensory or motor fibre neuropathy was defined as diminished sense of vibration at the lower or upper extremities and loss of Achilles or patellar tendon reflexes (12), which generally is associated with other signs of neuropathy or with symptoms, such as sensory loss, distal paresthesia or pain. No attempt was made to exclude or diagnose autonomic neuropathy but none of the patients had symptomatic orthostatic hypotension.

Table 3. Plasma levels of prorenin in various categories of diabetics

| Factor | Category | n | Prorenin mU/L | Significance |
|---|---|---|---|---|
| Sex | Female | 117 | 194 (43.0-844) | $p < 0.001$ |
| | Male | 106 | 291 (63.6-1330) | |
| Insulin | On insulin | 87 | 203 (49.5-827) | $p < 0.01$ |
| | Not on insulin | 136 | 262 (51.5-1330) | |
| Proteinuria | Absent | 161 | 193 (43.2-848) | $p<0.0001$ |
| (>300 mg/L) | Present | 62 | 368 (85.7-1580) | |
| Neuropathy | Absent | 172 | 219 (46.0-1040) | ns |
| | Present | 51 | 276 (54.6-1400) | |
| Retinopathy | Absent | 99 | 165 (46.5-589) | |
| | Background | 60 | 228 (51.6-1010) | $p < 0.01$ |
| | Proliferative | 64 | 416 (101-1710) | $p < 0.001$ |

Prorenin is given as geometric mean and 95% confidence interval. ns = not significant ($p > 0.05$). The difference between patients with and without neuropathy was of borderline significance ($p = 0.06$).

*Patients with diabetic nephropathy.*

Serum creatinine in these patients (n=7) was above 300 µmol/L and total effective renal plasma flow measured by 131I-Hippuran clearance (13) was less than 130 mL/min. They also had proliferative diabetic retinopathy. There were no symptoms or signs of autonomic neuropathy. Orthostatic hypotension was absent and the Valsalva response was normal. Peripheral venous plasma prorenin was elevated (above 360 mU/L) in each patient. The patients were studied at the time they were undergoing aortic angiography in preparation of receiving a kidney transplant. Blood was sampled from the aorta and renal veins before the contrast injection was given.

*Non-diabetic patients with chronic autonomic neuropathy.*

These patients (n=16, age 34-80 yr) have been described in detail (14) and some relevant clinical data are given in Table 3. All had severe incapacitating orthostatic hypotension. Disruption of the integrity of the baroreflex arc with loss of normal sympathetic function was confirmed by the absence of a systolic blood pressure overshoot in phase 4 of the Valsalva response. The resting plasma level of norepinephrine was low in the patients with pre- or postganglionic lesions. In all patients plasma norepinephrine did not respond to headup tilt on a tilting table for 30 min causing a blood pressure drop of 20-25 mmHg systolic and 10-15 mm Hg diastolic.

*Blood sampling.*

In the group of outpatients with diabetes blood samples for measuring renin and prorenin were drawn from an antecubital vein after the patients had rested for 30 min in the sitting position. Blood samples for measuring creatine, glucose and glycosylated hemoglobin (HbA1c, normal range 5.5-7.3 %) were also taken. In the non-diabetics with autonomic neuropathy peripheral venous blood was collected in the lying position. During the renal vein catheterisation studies blood was sampled from the renal vein on each side and at the same time from the abdominal aorta.

*Measurements of renin, prorenin and norepinephrine.*

The plasma levels of renin and prorenin were measured with the enzyme kinetic assay, in which the angiotensin I generated by plasma in vitro was quantitated by radioimmunoassay (15). For measuring prorenin, the proenzyme was quantitatively converted to renin at 4 °C by immobilized trypsin (5,16). That we were measuring 'true' prorenin, and not a renin-like enzyme, was supported by comparing the results of the enzyme kinetic assay with those of a direct radioimmunoassay (16,17). The latter was a solid phase sandwich assay in which two monoclonal human renin antibodies, which reacted equally well with kidney renin and chorionic cell culture prorenin, were used. Measurements in plasma samples from diabetics with prorenin levels ranging from 100-1000 mU/L, as determined by the indirect enzyme kinetic assay, showed excellent agreement between the direct and indirect assays. The specific enzymatic activity of

in vitro activated prorenin in plasma of diabetics was 0.8 (0.1) mU/ng (mean,sd, n=8), which is not different from the specific activity of purified human kidney renin (17).

Normal levels of renin and prorenin in males (n=50, age 21-54 yr) were 18.2 ( 7.5-52) and 166 mU/L (65-300) mU/L respectivily (geometric mean, range). In normal women in the first 2-12 days of the menstrual cycle (n=54, age 20-37 yr) renin was 15.4 (4.7-59) mU/L and prorenin was 151(62-357) mU/L (16).

Plasma norepinephrine was measured by high performance liquid chromatography (14). In normal subjects (n=57, age 28-78 yr) plasma norepinephrine was 1.5 (0.6-3.6 nmol/L (geometric mean,range).

Table 4. Correlations of (log) prorenin with various factors in diabetes

| | r | Significance |
|---|---|---|
| Age | 0.07 | ns |
| Duration of diabetes | 0.24 | $p < 0.001$ |
| Systolic blood pressure | - 0.07 | ns |
| Diastolic blood pressure | 0.02 | ns |
| Serum creatinine | 0.37 | $p < 0.001$ |
| Blood glucose | 0.11 | ns |
| HbA1c | 0.09 | ns |

r = Pearson correlation coefficient. ns = not significant ($p > 0.05$).

*Assessment of macro- and microalbuminuria.*

Macroalbuminuria was assessed in non-timed overnight urine samples with dipstick (Albustix, Miles, Naperville, ILL, USA), which has a detection limit of 250 mg/L. In case of a trace reaction, the urine sample was tested by the quantitative biuret reaction. Macroalbuminuria was defined as dip-stick positive or biuret reaction of protein above 250 mg/L in two out of two overnight urine samples. Microalbuminuria was assessed in three consecutive 24 hour urine collections with radial immunodiffusion in agarose gel (18). The gel contained 30 µL undiluted antiserum against human serum albumin (Dakopatts, Glostrup, Denmark) per 10 mL gel. The lower limit of detection was 2 mg/L and the interassay variability was 8 %. Microalbuminuria was defined as albuminuria of 30-300 mg/24h in at least two of the 24 hour urine collections, while all three 24 hour urine collections were dipstick-negative.

Table 5. Effects of diabetic retinopathy on mean (log) prorenin and standard errors (SE) estimated by multiple regression analysis

| Factor | Diabetic retinopathy grouping not included | | | Diabetic retinopathy grouping included | | |
|---|---|---|---|---|---|---|
| | Effect | SE | P value | Effect | SE | P Value |
| Constant (intercept) | 0.94 | 0.26 | | 1.09 | 0.24 | |
| Creatinine | 0.024 | 0.005 | <0.0001 | 0.023 | 0.05 | <0.001 |
| Creatinine square | -0.000097 | 0.000024 | <0.0001 | -0.000092 | 0.000022 | 0.0001 |
| Insulin therapy | 0.08 | 0.04 | 0.06 | 0.02 | 0.04 | 0.7 |
| Proteinuria (>250 mg/L) | 0.19 | 0.05 | <0.001 | 0.10 | 0.05 | <0.05 |
| No retinopathy | Not tested | | | -0.10 | 0.05 | <0.05 |
| Proliferative diabetic retinopathy | Not tested | | | 0.19 | 0.06 | 0.001 |

Effects denote mean increase of (log) prorenin by: 1 U creatinine or creatinine square, insulin therapy *vs.* no insulin therapy (1 *vs.*0), proteinuria present vs. absent (1 *vs.*0), no diabetic retinopathy *vs.* background diabetic retinopathy (1 *vs.*0), and proliferative diabetic retinopathy *vs.* background diabetic retinopathy (1 *vs.*0).

Table 6. Plasma levels of prorenin, renin and norepinephrine in non-diabetics with chronic autonomic neuropathy

| Patient no. | Site of lesion | Etiology | Prorenin mU/L | Renin mU/L | Norepinephrine nmol/L |
|---|---|---|---|---|---|
| 1-6 | Preganglionic | Parkinson's disease | 141 | 5.1 | 0.62 |
| | | | 59-380 | 1.9-15.3 | 0.19-2.6 |
| 7-11 | Postganglionic | Amyloidosis | 189 | 10.2 | 0.33 |
| | | | 121-327 | 5.0-30.4 | 0.09-1.2 |
| 12 | Postganglionic | Progressive AF | 103 | 8.1 | 0.26 |
| 13 | Postganglionic | Progressive AF | 271 | 0.8 | 0.75 |
| 14 | Postganglionic | Progressive AF | 176 | 0.9 | 0.14 |
| 15 | Afferent | Iatrogenic | 176 | 4.2 | 2.0 |
| 16 | Afferent | Iatrogenic | 236 | 14.6 | 3.2 |

Data in Parkinson's disease and amyloidosis are given as geometric means and range. AF = autonomic failure. Iatrogenic = after radical neck dissection with preoperative irradiation.

Table 7.Renin and prorenin across the kidneys of patients with proliferative diabetic retinopathy and endstage nephropathy

| | | | | | |
|---|---|---|---|---|---|
| Sex | | | female/male | n | 2/5 |
| Age | | | median (range) | yr | 51 (41-60) |
| Duration of diabetes | | | median (range) | yr | 21 (7-38) |
| Insulin treatment | | | on insulin/not on insulin | n | 5/2 |
| Albuminuria | | | mean (sd) | g/L | 3.8 (0.8) |
| Serum creatinine | | | median (range) | µmol/L | 650 (311-730) |
| Blood pressure | | systolic | mean (sd) | mm Hg | 179 (34) |
| | | diastolic | mean (sd) | mm Hg | 94 (10) |
| $^{131}$I-hippurate clearance | | | mean (sd) | mL/min | 61 (38) |
| $^{131}$I-hippurate extraction | | right kidney | mean (sd) | | 0.36 (0.09) |
| | | left kidney | mean (sd) | | 0.30 (0.07) * |
| Renin | right kidney | artery | geometric mean (range) | mU/L | 9.4 (4.7-22.8) |
| | | vein | geometric mean (range) | mU/L | 14.7 (8.2-27.7) |
| | | vein/artery ratio | mean (sd) | | 1.58 (0.28) ** |
| Renin | left kidney | artery | geometric mean (range) | mU/L | 9.4 (4.8-21.7) |
| | | vein | geometric mean (range) | mU/L | 15.5(10.0-29.7) |
| | | vein/artery ratio | mean (sd) | | 1.69 (0.35) ** |
| Prorenin | right kidney | artery | geometric mean (range) | mU/L | 464 (316-914) |
| | | vein | geometric mean (range) | mU/L | 471 (330-982) |
| | | vein/artery ratio | mean (sd) | | 1.02 (0.06) *** |
| Prorenin | left kidney | artery | geometric mean (range) | mU/L | 505 (308-896) |
| | | vein | geometric mean (range) | mU/L | 526 (321-963) |
| | | vein/artery ratio | mean (sd) | | 1.04 (0.03) *** |

* = no significant difference between right and left kidney. ** = significantly different from 1.00, $p < 0.001$
*** = not significantly different from 1.00 (no net secretion of prorenin).

*Statistical analysis.*

Differences between groups for discrete data were analyzed by CHI square test or by the Fisher exact test when appropriate. Differences for continuous data were analyzed with the unpaired t-test or with the Student-Newman-Keuls test for multiple comparisons, with the exception of the age of the patient and the duration of diabetes, which were analyzed with the median test (19). Renin and prorenin were not normally distributed. Logarithmic transformation yielded apparently normal distributions with similar standard deviations in all groups. These transformed values were used for statistical analysis. Multiple regression analysis was performed with the use of the SPSS/PC+ program 1986 (SPSS Inc., Chicago, ILL, USA).

## RESULTS

*Correlations of prorenin with diabetic complications.*

In Table 1 the patients are grouped according to the presence or absence of persistent proteinuria. The groups did not significantly differ in female/male ratio, age, HbA1c and systolic and diastolic pressure or in the prevalence of insulin treatment and neuropathy. The prevalence of diabetic retinopathy was higher in the group with persistent proteinuria, and retinopathy in this group was more often of the proliferative type. Diabetes was of longer duration and serum creatinine was also higher in these patients. The plasma level of prorenin was nearly two times higher but renin was not significantly different.

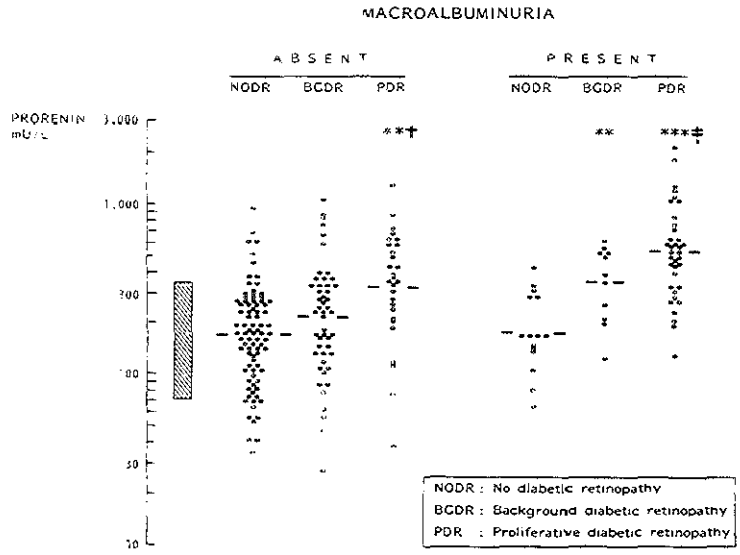


Figure 1. Plasma prorenin in patients without diabetic retinopathy (NODR), with background diabetic retinopathy (BGDR) or with proliferative diabetic retinopathy (PDR). The patients are subdivided in those with and without macroalbuminuria. Horizontal lines indicate geomatric means. Note the logarithmic scale.
*=p<0.05, **=p<0.01, ***=p<0.001, for difference from the group without diabetic retinopathy.
†=p<0.05,‡=p<0.01, for difference from the group with background diabetic retinopathy.Hatched bar, normal range.

In Table 2 the patients are grouped according to the presence or absence of microalbuminuria. Again the groups did not significantly differ in female/male ratio and HbA1c or in the prevalence of insulin treatment and neuropathy. There was also no difference in serum creatinine. Systolic blood pressure was higher in the patients with microalbuminuria than in those without albuminuria. In contrast with the data obtained

when the diabetics were grouped according to whether they had macroalbuminuria or not, no differences in the plasma levels of prorenin or renin were found between the patients with and without microalbuminuria.

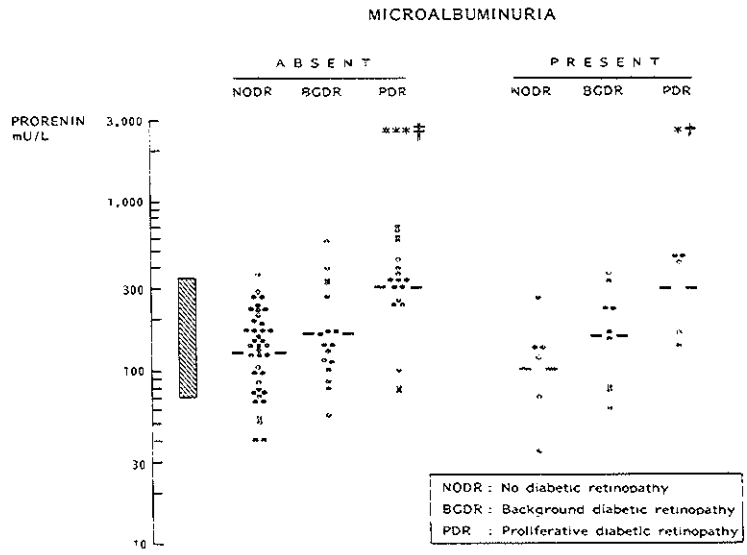


Figure 1. Plasma prorenin in patients without diabetic retinopathy (NODR), with background diabetic retinopathy (BGDR) or with proliferative diabetic retinopathy (PDR). The patients are subdivided in those with and without microalbuminuria. Horizontal lines indicate geomatric means. Note the logarithmic scale.
*=p <0.05, **=p <0.01, ***=p<0.001, for difference from the group without diabetic retinopathy.
†=p<0.05,‡=p<0.01, for difference from the group with background diabetic retinopathy.Hatched bar, normal range.

Prorenin was higher in the patients with diabetic retinopathy than in those without retinopathy, and the levels were highest in those with proliferative diabetic retinopathy, no matter macro- or microalbuminuria was present or not (Fig.1 and 2). The mean level of prorenin was 2-3 times normal in the patients with proliferative retinopathy but was not above normal in the patients without retinopathy, and this was the case both in the presence and absence of macro- or microalbuminuria.

Reninin the patients with proliferative retinopathy was also higher than in those without retinopathy but the difference was much smaller than for prorenin and the difference was only seen in the group with macroalbuminuria. There was no difference in renin between the patients with background retinopathy and those without retinopathy, and this was true for the groups with macroalbuminuria or microalbuminuria as well as for the group without albuminuria.

The influence of various factors on the plasma level of prorenin was examined by multiple regression analysis (Tables 3-5). The factors studied were: sex, age, duration of diabetes, insulin treatment, blood glucose, HbA1c, systolic and diastolic blood pressure, neuropathy, serum creatinine, macroalbuminuria and, finally, retinopathy. Statistically significant correlations were found with sex, duration of diabetes, insulin treatment, serum creatinine, proteinuria and retinopathy. In case of neuropathy, prorenin was also generally increased but this finding was not statistically significant.

The factors most strongly correlating with prorenin were insulin treatment, serum creatinine, macroalbuminuria and retinopathy. The correlation with creatinine was not linear and the term creatinine square was introduced in the regression equation (Table 6). Once allowance was made for the presence of retinopathy, categorized as background diabetic retinopathy or proliferative diabetic retinopathy, the relations of prorenin with sex, duration of diabetes and insulin treatment were no longer significant. This can be explained by interrelations between the various factors. In 34 percent of males proteinuria was present as compared to 22 percent of females. Also, males had higher ($p < 0.001$) creatinine levels than females. Finally with regard to the duration of diabetes, it appeared that longer duration generally implied insulin treatment and macroalbuminuria.

The multiple regression analysis therefore shows that high prorenin was associated with nephropathy and retinopathy and that other factors did not significantly contribute to this association. The analysis further indicated that the association with retinopathy was, at least in part, independent of the association with nephropathy. In contrast with the findings on prorenin, no significant correlations were found between the plasma level of renin and the various factors mentioned above.

*Plasma prorenin in non-diabetics with autonomic neuropathy.*

Supine plasma renin was below the normal range in two patients with a preganglionic lesion and in two with a postganglionic lesion. In none of the patients with autonomic neuropathy did plasma renin rise after headup tilt for 30 min, despite a blood pressure drop of 20-25 mm Hg systolic and 10-15 mmHg diastolic. Plasma prorenin was within the normal range in all patients (Table 6).

*Renal vein plasma prorenin in diabetics with advanced nephropathy.*

Arterial plasma prorenin was markedly elevated in these patients (Table 7). The renal vein-to-artery prorenin ratio was 1.02 (0.03) on the right side and 1.04 (0.03) on the left (mean,sd). In subjects with untreated essential hypertension (n=18) the ratio is 1.03 (0.05) on the right side and 1.04 (0.06) on the left (20) . Thus the ratio in the diabetes was not higher than in subjects with essential hypertension, despite the markedly reduced renal plasma flow in the former.

Peripheral vein renin in the diabetics was at the lower end of the normal range.

The renal vein-to-artery ratio of renin was 1.58 (0,28) on the right side and 1.69 (0,35) on the left. In subjects with untreated essential untreated essential hypertension the ratio is 1.28 ( 0.13) on the right side and 1,30 (0,14) on the left (20). The higher ratio in the diabetics is a consequence of the lower renal perfusion.

## DISCUSSION

Luetcher et al. (2) were the first to report that the plasma level of prorenin is increased in patients with longstanding diabetes and they suggest that prorenin might serve as a marker of microvascular disease. Our study offers further evidence in support of this. We found that in our patient population the elevated prorenin was associated with the presence of diabetic retinopathy, particularly the proliferative type, and that a number of confounding factors, i.e. sex, duration of diabetes, blood pressure, the blood levels of glucose and HbA1c or the prevalence of neuropathy or insulin treatment, did not significantly contribute to this association.

In contrast with prorenin, plasma renin is normal or low in most diabetics (3,4). In the patients in whom proliferative diabetic retinopathy was associated with macroalbuminuria we found plasma renin to be somewhat higher than in the groups without these complications. Higher renin levels in diabetics with albuminuria have also been reported by others (21). This finding might be an artifarct caused by inadvertent activation of prorenin during blood sampling and processing. Samples from such patients contain relativily large quantities of prorenin and inadvertent cryoactivation of prorenin as little as one percent is already sufficient to raise the measured level of renin by 50 % (16).

Autonomic neuropathy is not uncommon in diabetes. Loss of adrenergic stimulation may lead to decreased secretion of renin by the juxtaglomerular cells and it has been postulated that a compensatory increase in the synthesis and secretion of prorenin by these cells is the cause of the increase in plasma prorenin in diabetes (8,9). However, our observations in non-diabetics with autonomic neuropathy do not support this explanation. Our data demonstrate that autonomic neuropathy in the absence of diabetes and its complications does not cause plasma prorenin to rise to the levels that are found in diabetics, even when there is serious loss of adrenergic function.

It is possible that, independently of the presence of autonomic neuropathy, the processing of prorenin to renin in the juxtaglomerular cells is somehow impaired and that the secretion of prorenin from these cells is increased when the kidney is affected by diabetic microvascular disease (7).

The mean level of prorenin was not above normal in the patients without retinopathy both in the presence and absence of macro- or microalbuminuria, whereas the mean level of prorenin was markedly elevated in the patients with proliferative retinopathy, not only when retinopathy was associated with macro- or microalbuminuria but also when albuminuria was absent. These findings suggest that retinopathy is a more important

determinant of abnormally high prorenin than nephropathy.

We measured the renal vein-to-artery ratio of prorenin in diabetics who had endstage nephropathy and in whom peripheral vein plasma prorenin was elevated. The ratio in these patients was not significantly different from unity (no net secretion) and was not higher than in essential hypertension, despite the fact that renal plasma flow was much lower. Thus, the high plasma level of prorenin in the diabetics could not be fully explained by abnormal secretion by the kidneys.

This leaves us with two possibilities. One is reduced extrarenal clearance of prorenin and the other is increased extrarenal production. Non-enzymatic glycosylation of prorenin might lead to reduced clearance from the circulation. We found, however, no significant correlation between the levels of prorenin and HbA1c. It has been postulated that a cellular receptor exists, which binds the prosegment of prorenin (22). Such binding might initiate internalization of prorenin, for instance in the vascular endothelium. A number of endothelial functions is known to be impaired in diabetes (23-25) and this may also be true for prorenin internalization, with reduced clearance of prorenin from the circulation as a result.

With regard to the possibility of increased production of prorenin at extrarenal sites, it is of interest that large amounts of (pro)renin have been found in highly vascularized extrarenal tumors (26,27). Neovascularization is an important feature of diabetic microvascular disease. In this context it might be relevant that the vitreous of the eye contains prorenin in concentrations that are, relative to albumin and other plasma proteins, much higher than in plasma (17). Prorenin is also present in extracts of the retina in concentrations too high to be explained by trapped plasma (28). It is also interesting that, for a given level of albumin, the level of prorenin in the vitreous from eyes with retinal detachment due to proliferative diabetic retinopathy was found to be higher than in eyes with spontaneous retinal detachment (17). It seems unlikely that the eye is an important source of circulating prorenin because of the small contribution of the eye to total body blood flow but extensive areas of microvascular disease, possibly associated with new vessel formation, elswhere in the body might produce enough prorenin to increase its level in plasma.

At any rate, the present study appears to indicate that the high plasma levels of prorenin found in diabetics are not an immediate consequence of altered glucose metabolism. These high levels are related to the development of microvascular disease in the eye and the kidney, and they are, at least in part, due to either decreased clearance of prorenin from the circulation or increased production at extrarenal sites or both.

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## Chapter 3


### Summary

Renin, prorenin and immunoreactive renin were present in vitreous and subretinal fluid of eyes from subjects with and without diabetic retinopathy. Renin substrate, albumin, transferrin and immunoglobulin G were also found in these ocular fluids. In many samples renin levels were close to the detection limit of the assay. The levels of renin substrate, albumin, transferrin and immunoglobulin G varied widely among ocular fluid samples, but in each individual sample the levels were, relative to each other, similar to those in plasma. In contrast, the prorenin level in ocular fluid was up to 100 times higher than expected on the basis of the plasma protein content of ocular fluid. Moreover, there was little difference in prorenin concentrations between samples with a low and a high plasma protein content. Prorenin, relative to albumin and other plasma proteins, was higher in vitreous fluid from eyes with proliferative diabetic retinopathy complicated by traction retinal detachment than in eyes of non-diabetic subjects with spontaneous retinal detachment. It appears that prorenin (and possibly renin) in ocular fluid is controlled by an active and specific process, possibly local synthesis within the eye. In view of the vascular actions of angiotensin II, an intraocular renin-angiotensin system may play a role in diabetic retinopathy.


### Introduction

The kidney secretes both renin and prorenin, an inactive precursor of renin, into the circulation. Plasma of nephrectomized patients contains little or no renin but it does contain prorenin (1,2), in concentrations sometimes as high as those in normal individuals. It thus appears that extrarenal production can make a major contribution to the level of prorenin in plasma, whereas most, if not all, renin in plasma is secreted by the kidneys. Synthesis of renin or prorenin and other components of the renin-angiotensin system is known to occur at various extrarenal sites, for instance adrenal (3,4) pituitary (3,4), testis (3,4), brain (5), and ovary (6-8). Cultured human chorionic cells (9) and ovarian thecal cells (8) release prorenin into the medium and there is good evidence that in women with hyperstimulated cycles and during pregnancy, the ovary, probably the corpus luteum, releases prorenin into plasma (7,10).

A common feature of the organs in which synthesis of renin or prorenin occurs is their extensive vascularization (11). The eye, particularly the retina and uveal tract, is such a highly vascularized organ. Angiotensin II-binding sites have been found in retinal blood vessels (12), and transvitreal infusion of angiotensin I and II produces constriction of the retinal arteries (13). The retina contains angiotensin converting enzyme activity (14) and this enzyme is also found in aqueous fluid (15). Here we report measurements of

renin, prorenin, immunoreactive renin, renin substrate and various plasma proteins in aqueous, vitreous and subretinal fluid. The ocular fluid samples were obtained at the time of cataract extraction or vitrectomy and the protein concentrations in these samples were compared with those in simultaneously obtained plasma. Our study included eyes affected by proliferative diabetic retinopathy because the renin-angiotensin system has been implicated in neovascularization (16).

## Subjects and methods

*Non-diabetic subjects*

Aqueous fluid was collected at the time of cataract extraction from 21 subjects (15 women and 6 men; mean age, 68 yr; range, 26-86 yr). Four subjects were receiving a diuretic and six a α-adrenergic antagonist.

Vitreous fluid aspirates were obtained from 16 subjects (8 women and 8 men; mean age, 52 yr; range, 20-82 yr). The samples were collected at the time of pars plana vitrectomy, which was performed because of recurrent retinal detachment due to proliferative vitreoretinopathy. Four subjects were receiving a diuretic, in 3 of them combined with a α-adrenergic antagonist.

Subretinal fluid was obtained from 18 subjects (8 women and 10 men; (mean age) 59 yr; range, 8-76 yr), with rhegmatogenous retinal detachment, which is a type of retinal separation precipitated by a hole or a tear in the retina. In this type of detachment fluid accumulates between the retinal pigment epithelial layer and the neural retina. The retinal detachments had occurred between 1 day and 3 months (median, one week) before subretinal fluid collection. Three subjects were receiving a diuretic, in 2 of them combined with a α-adrenergic antagonist.

*Diabetic subjects*

Vitreous fluid was obtained from 15 diabetic subjects with proliferative diabetic retinopathy (8 women and 7 men; mean age, 51 yr; range, 28-71 yr). Vitrectomy was performed because of traction retinal detachment. The duration of diabetes ranged from 6-32 yr. Twelve subjects were receiving insulin, 5 were receiving a diuretic and 1 was receiving a α-adrenergic antagonist.

Aqueous fluid can only be collected at the time of cataract extraction. In diabetic subjects, however, this procedure may stimulate proliferative retinopathy. Cataract extraction is therefore not performed in eyes affected by proliferative diabetic retinopathy. Consequently, aqueous fluid could not be collected from such eyes. We also were unable to collect subretinal fluid from diabetic subjects with traction retinal detachment because drainage of subretinal fluid is rarely performed in these subjects and, if it is performed, the approach is via the transvitreal route, so that the sample is heavily contaminated with material from the vitreous.

n subjects with a rhegmatogenous retinal detachment, subretinal fluid is removed via the transscleral route, where no such contamination occurs.

*Collection of ocular fluid samples*

Approximately 0.1 mL aqueous fluid was collected with a tuberculin syringe and a 25-gauge needle. The needle was introduced at the limbus of the cornea through the groove of the cataract incision. A 0.3-1.0 mL sample of vitreous fluid was aspirated before substitution fluid was infused into the vitreous. Subretinal fluid was aspirated transsclerally, after local diathermic coagulation of the choroid.

The ocular fluid samples were free of macroscopically visible blood and were frozen at -70 °C immediately after collection. A peripheral venous blood sample was drawn simultaneously with the collection of ocular fluid. Blood for determination of renin, prorenin, immunoreactive renin, renin substrate, albumin, transferrin and immunoglobulin G (IgG) was collected in tubes containing 0.1 volume of 0.13 mol/L trisodium citrate. The blood was immediately centrifuged at 3000 x g for 10 minutes at room temperature, and 1-mL aliquots of plasma were stored at -70 °C. Blood for determination of angiotensin II was collected in prechilled tubes containing 0.1 volume of 0.06 mmol/L pepstatin-A, 0.125 mol/L disodium EDTA and 0.025 mol/L phenantroline in order to block renin, angiotensin converting enzyme and angiotensinases, respectively. The blood samples were immediately centrifuged at 3000 g for 10 minutes at 4 °C, and 2-mL aliquots of plasma were stored at -70 °C.

*Analytical methods*

Renin was measured in duplicate by enzyme-kinetic assay, in which the samples were incubated at 37 °C and pH 7.5 with saturating amounts of sheep renin substrate in the presence of inhibitors of angiotensinases and angiotensin converting enzyme. The generated angiotensin I was quantitated by RIA (17). For measuring prorenin in plasma, prorenin was converted into renin by incubation with Sepharose-bound trypsin (0.25 mg/mL) for 48 hours at 4 °C. Previous studies, including measurements of total immunoreactive renin (renin plus prorenin), indicated that the prorenin to renin conversion in plasma is complete after incubation with the immobilized trypsin under these circumstances and that destruction of renin or prorenin does not occur (18). Experiments in which known quantities of purified human kidney renin were added to ocular fluid demonstrated that in some samples destruction of renin did occur with this method. This destruction might be due to the low content of serine protease inhibitors in ocular fluids as compared to plasma. Therefore, in ocular fluid we chose to use plasmin to convert prorenin into renin (17,19). For this purpose the sample was incubated with plasmin at a final concentration of 0.5 µmol/L for 48 hours at 4 °C before the assay.

Comparison of the results of the enzyme-kinetic assay in plasmin-activated ocular fluid samples with the results of the assay of total immunoreactive renin in non-activated

samples demonstrated that the conversion by plasmin was complete without any loss of prorenin or renin; the specific enzymatic activity of plasmin-activated prorenin in ocular fluid samples was not different from the specific activity of purified kidney renin and plasma renin (see Results). Plasmin at the concentration mentioned above cannot be used to activate prorenin in native whole plasma because of its high content of plasmin inhibitors.

The concentrations of renin and prorenin measured by the enzyme-kinetic assay were expressed as milliunits per L using the WHO human kidney renin standard 68/356 (WHO International Laboratory for Biological Standards, London, United Kingdom) as reference standard. The lower limit of detection was 0.5 mU/L and the interassay variability at low concentrations of renin or prorenin (2-5 mU/L) was 11 % for both renin and prorenin. Immunoreactive renin was measured in duplicate with a sandwich assay (18,20) using the monoclonal antibodies R 3-27-6 and R 3-36-16 (Ciba-Geigy, Basel, Switzerland). The two monoclonal antibodies recognize different epitopes of the renin molecule and react equally well with human kidney renin and chorionic cell culture prorenin. The assay was carried out in polystyrene tubes (Star Tubes, code 4-70319; Nunc, Roskilde, Denmark). The inner surface of these tubes was coated with antibody R 3-27-6 (21). Immunoreactive renin in the assay sample is quantitatively bound to this antibody. The amount of solid phase-bound immunoreactive renin was measured with antibody R 3-36-16, which had been radiolabeled with 125I. The results of this assay were expressed as nanograms per L using highly purified human kidney renin (Ciba-Geigy) as a standard. One milliunit of the WHO human kidney renin standard corresponded to 1.41 ng of the Ciba-Geigy standard. The lower limit of detection was 5 ng/L, and the interassay variability was 8%.

The concentration of renin substrate was determined as the maximum quantity of angiotensin I that was generated during incubation at 37 iC and pH 7.5 with an excess of purified active human kidney renin in the presence of inhibitors of angiotensinases and angiotensin converting enzyme (18). The lower limit of detection was 1 nmol/L and the interassay variability was 10 %.

Immunoreactive angiotensin II was measured by radioimmunoassay after SepPak (Waters, Milford, MA, USA) extraction of the sample (22). The lower limit of detection was 2 pmol/L and the interassay variability was 15%.

Albumin, transferrin and IgG were measured by single radial immunodiffusion (LC and NOR-Partigen plates, Behringwerke, Marburg, Germany) according to the method of Mancini et al (23).

*Data analysis*

Plasma proteins enter the vitreous mainly by diffusion. One of the reasons why the concentrations of these proteins are low in vitreous fluid is that they have to cross a relatively impermeable barrier. Breakdown of this so-called blood-retinal barrier leads to increased diffusion of plasma proteins into the eye. The rate of diffusion of a given protein

is related to its molecular size and plasma concentration. In accordance with this is the fact that the concentrations of the different proteins relative to each other are similar in plasma and vitreous fluid (24,25).

Thus, unless certain specific uptake processes exist, for which in the eye no evidence is available with regard to any of the proteins mentioned in this paper, one would expect a relatively high intraocular albumin concentration (due to partial breakdown of the blood-retinal barrier) to be accompanied by a proportionally high concentration of plasma proteins of comparable size. Therefore, we chose to take the vitreous fluid/plasma concentration ratio of albumin as an index of the integrity of the blood-retinal barrier, an abnormally high ratio being an indication of breakdown of this barrier. By multiplying this ratio with the level of a given protein in plasma, the level of this protein in ocular fluid can be estimated, assuming that, as mentioned above, this protein is transferred from the blood into the vitreous and vice versa by mechanisms that are qualitatively and quantitatively the same as those for the transfer of albumin. For example, for renin substrate the calculation would be as follows:

$$[RS_{oc}] = [RS_{pl}] \times [ALB_{oc}]/[ALB_{pl}],$$

in which RS is renin substrate, ALB is albumin, oc is ocular fluid, pl is plasma, and brackets denote the concentration.

If our assumptions are correct, the calculated concentrations should be equal or at least closely correlated to the actually measured concentrations. Therefore, the two sets of data were analyzed by linear regression.

For analyzing differences between diabetic and nondiabetic subjects unpaired t-tests were performed after logarithmic transformation of the data.

Values were considered significant if $p < 0.05$.

## Results

*Non-diabetic subjects*

The levels of renin in many vitreous and aqueous fluid samples were at or below the detection limit of the assay (0.5 mU/L), which is less than 5 % of the level in plasma. In subretinal fluid the renin level was about 20 % of that in plasma (Table 1). Prorenin was detectable in all samples of vitreous and aqueous fluid; its level in vitreous fluid was about 20 % and in aqueous fluid about 5 % of that in plasma. In subretinal fluid the prorenin level was as high as in plasma. Renin and prorenin concentrations in the fluid compartments of the eye were in the order: subretinal fluid > vitreous fluid > aqueous fluid. The levels of renin substrate in subretinal, vitreous and aqueous fluid were 10, 5 and 0.5 % of those in plasma, respectively. Thus, they too were in the order: subretinal fluid > vitreous fluid > aqueous fluid. This was also true for the levels of albumin, transferrin and IgG (Table 2). There was no correlation between the levels in ocular fluid

and those in plasma for any of the proteins.

As described under Data analysis above, a theoretical concentration in ocular fluid for each protein was predicted based on the albumin content of the sample. For renin substrate, transferrin and IgG the calculated and measured values were linearly correlated in both vitreous and subretinal fluid, and the slopes of these correlation lines were not significantly different from 1.0 (Tables 3 and 4). Renin substrate and transferrin concentrations in ocular fluid could, in fact, be accurately predicted by these calculations. The IgG level measured was systematically about 2 times lower than that calculated, which may be due, at least in part, to its larger molecular size as compared to that of albumin and the other proteins.

For prorenin the findings were different. The prorenin levels in both vitreous and subretinal fluid varied much less than the levels of the other proteins (Tables 1 and 2). Furthermore, calculating prorenin concentrations on the basis of the albumin content of the sample yielded much lower (down to 1/100th) values than those actually measured, particularly in samples with a low plasma protein content (intact blood-retinal barrier). The prorenin level in subretinal fluid was higher than that in vitreous fluid, even when corrections were made for the higher plasma protein content in subretinal fluid samples (Fig. 1). In both vitreous and subretinal fluid the slopes of the regression lines describing the correlation between the measured and calculated prorenin concentrations were significantly different from 1.0, thereby indicating the different behavior of prorenin as compared to albumin and other plasma proteins.

Table 1. Levels of prorenin, renin, and renin substrate in ocular fluids.

| | n | Prorenin (mU/L) | | Renin (mU/L) | | Renin substrate (nmol/L) | |
|---|---|---|---|---|---|---|---|
| | | Eye | Plasma | Eye | Plasma | Eye | Plasma |
| Non-diabetic subjects | | | | | | | |
| Aqueous vs. plasma | 21 | 4.4 | 163 | < 0.5 | 9.8 | 5.1 | 1080 |
| | | 2.0-8.7 | 36.7-453 | ND-0.5 | 1.5-62.9 | 1.6-15.4 | 898-1430 |
| Vitreous vs. plasma | 16 | 34.5 | 174 | < 1.0 | 17.3 | 54.5 | 1120 |
| | | 17.4-61.9 | 67.0-396 | ND-2.8 | 3.7-51.0 | 3.0-630 | 791-3030 |
| Subretinal vs. plasma | 18 | 132 | 128 | 2.4 | 14.3 | 107 | 1230 |
| | | 36.8-305 | 65.1-251 | 0.9-6.4 | 4.3-105 | 10-1430 | 841-2250 |
| Diabetic subjects | | | | | | | |
| Vitreous vs. plasma | 15 | 61.0 | 357 | < 2.0 | 17.7 | 61.0 | 1030 |
| | | 19.0-172 | 121-679 | ND-3.5 | 4.4-127 | 7.0-1000 | 628-2040 |

Shown are the geometric mean and range. ND, Not detectable. In vitreous fluid and plasma the levels of prorenin, but not those of renin substrate, were higher in diabetic than in non-diabetic subjects ($p < 0.01$).

Table 2. Levels of albumin, IgG, and transferrin in ocular fluids.

| | n | Albumin (g/L) | | IgG (g/L) | | Transferrin (g/L) | |
|---|---|---|---|---|---|---|---|
| | | Eye | Plasma | Eye | Plasma | Eye | Plasma |
| Non-diabetic subjects | | | | | | | |
| Aqueous vs. plasma | 21 | 0.19 | 33.1 | ND | ND | ND | ND |
| | | 0.06-0.45 | 28.3-39.4 | | | | |
| Vitreous vs. plasma | 16 | 1.55 | 33.0 | 0.19 | 11.0 | 0.18 | 2.44 |
| | | 0.11-20.2 | 22.9-42.5 | 0.03-0.70 | 6.61-14.4 | 0.02-1.74 | 1.60-2.99 |
| Subretinal vs. plasma | 18 | 3.03 | 35.4 | 0.48 | 11.2 | 0.45 | 2.57 |
| | | 0.39-28.5 | 29.2-41.9 | 0.07-5.10 | 7.0-18.2 | 0.06-3.97 | 2.08-3.44 |
| Diabetic subjects | | | | | | | |
| Vitreous vs. plasma | 15 | 1.51 | 29.2 | 0.19 | 9.2 | 0.14 | 2.06 |
| | | 0.32-17.1 | 21.7-36.8 | 0.04-0.96 | 3.88-16.7 | 0.06-0.37 | 1.50-2.70 |

Shown are the geometric mean and range. The levels of albumin, IgG, and transferrin in both vitreous and plasma did not differ between diabetic and non-diabetic subjects. ND, Not done.

The data on prorenin shown in Tables 1, 3 and 4 and Figs. 1-3 were obtained by the enzyme-kinetic assay. That prorenin measured by this assay is, in fact, prorenin is supported by the excellent agreement with the measurements of immunoreactive renin (Fig. 4). The mean specific enzymatic activity of in vitro activated prorenin was 0.7 ± 0.2 (± SD) mU/ng (n=9) in vitreous fluid and 0.6 ± 0.2 mU/ng (n=10) in subretinal fluid. These values are not different from the specific activity of renin from plasma and kidney (18).

Immunoreactive angiotensin II was 11.1 ± 1.8 pmol/L in vitreous fluid (n=12) compared to 17.5 ± 1.3 pmol/L in plasma. In subretinal fluid (n=15) it was 14.8 ± 1.6 pmol/L compared to 23.9 ± 2.0 pmol/L in plasma.

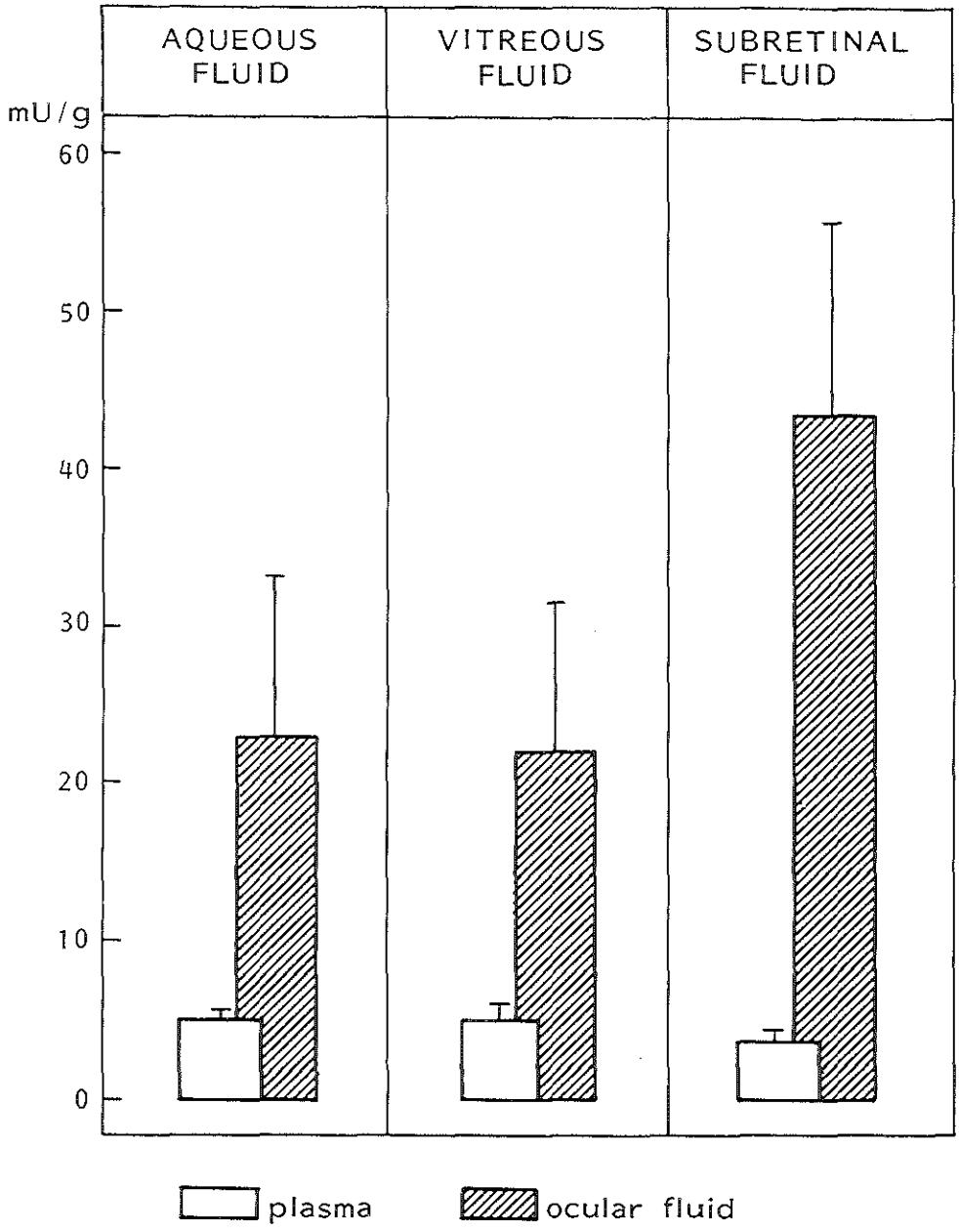


Figure 1. Mean (SE) prorenin/albumin concentration ratio in ocular fluids and plasma of nondiabetic patients.

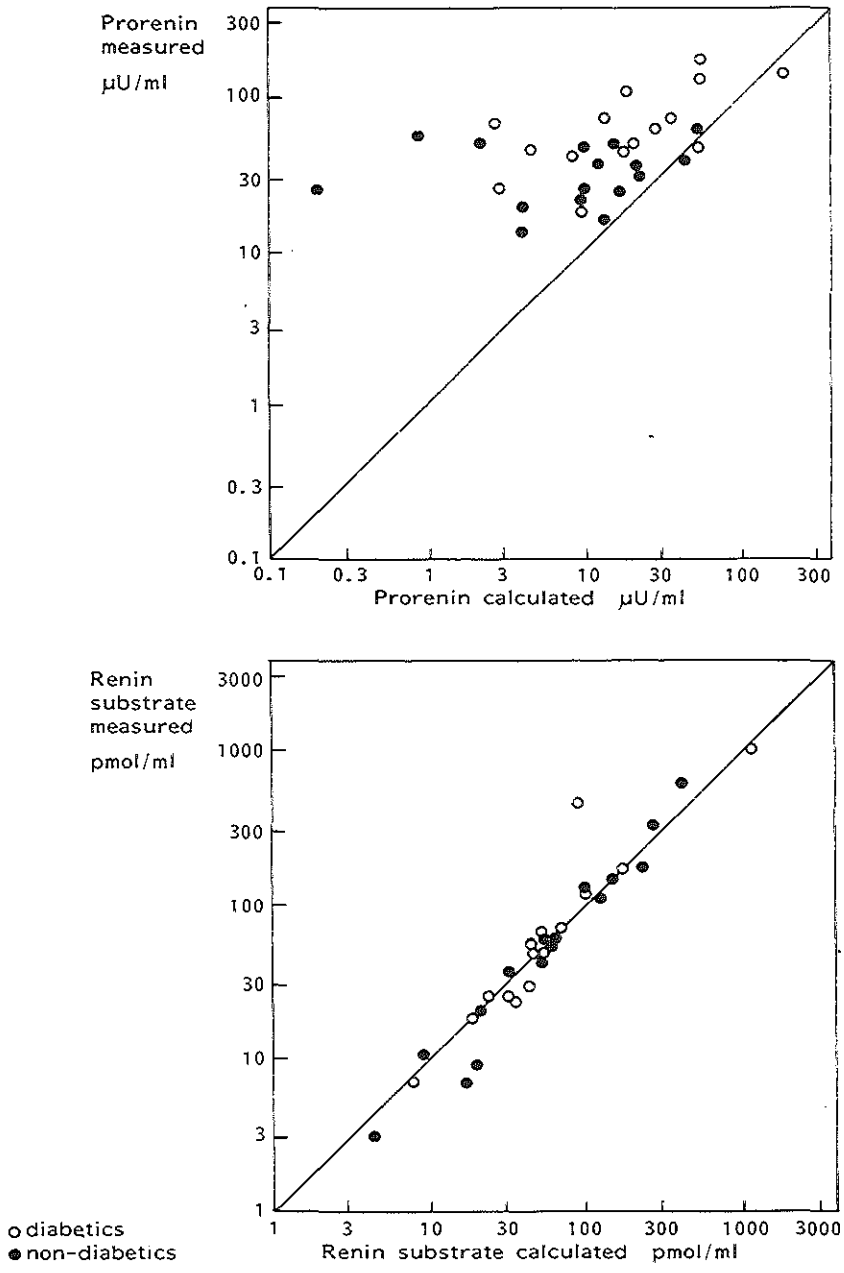


Figure 2. Measured versus calculated concentrations of prorenin (top panel) and renin substrate (bottom panel) in vitreous fluid. For an explanation of the calculation see text (Data analysis). The slopes and significance levels of the correlations are given in Table 3. o diabetic, ● non-diabetic.

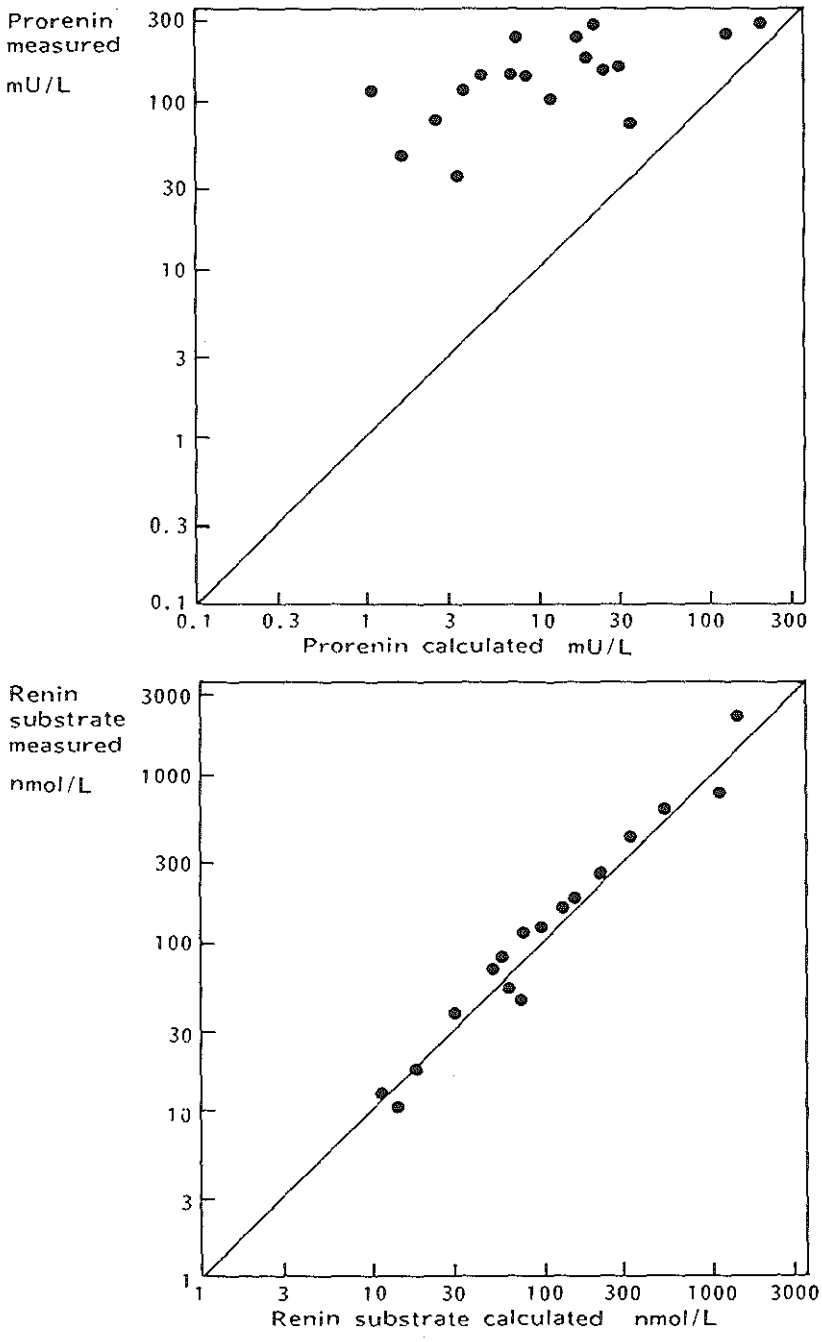


Figure 3. Measured versus calculated concentrations of prorenin (top panel) and renin substrate (bottom panel) in subretinal fluid. For an explanation of the calculation see text (Data analysis). The slopes and significance levels of the correlations are given in Table 4.

Table 3. Correlations between the measured and calculated concentrations of proteins in vitreous fluid.

| Protein | Regression Line | r | p |
|---|---|---|---|
| Prorenin | | | |
| Non-diabetic subjects | $y = 0.015^{a}x + 1.523$ | 0.05 | NS |
| Diabetic subjects | $y = 0.328^{a}x + 1.370$ | 0.64 | < 0.05 |
| Renin substrate | $y = 1.024x - 0.028$ | 0.97 | < 0.0001 |
| IgG | $y = 1.063x - 0.344$ | 0.91 | < 0.0001 |
| Transferrin | $y = 0.899x + 0.066$ | 0.89 | < 0.0001 |

y is the log (measured concentration); x is the log (calculated concentration). For renin substrate, IgG, and transferrin, data from diabetic and nondiabetic subjects were combined because no differences were found for these proteins between the two groups (Tables 1 and 2).

[a]Slope different from 1.0, $p < 0.0001$.

Table 4. Correlations between the measured and calculated concentrations of proteins in subretinal fluid.

| Protein | Regression Line | r | p |
|---|---|---|---|
| Prorenin | $y = 0.271^{a}x + 1.839$ | 0.61 | < 0.01 |
| Renin substrate | $y = 0.975x + 0.064$ | 0.98 | < 0.0001 |
| IgG | $y = 0.941x - 0.302$ | 0.99 | < 0.0001 |
| Transferrin | $y = 0.934x + 0.262$ | 0.94 | < 0.0001 |

y is the log (measured concentration); x is the log (calculated concentration).

[a]Slope different from 1.0, $p < 0.0001$.

*Diabetic subjects*

The results of the renin substrate, albumin, transferrin, and IgG measurements in vitreous fluid and plasma of the diabetic subjects were similar to those in the non-diabetic subjects (Tables 1 and 2). The levels of renin and prorenin in vitreous fluid were higher in the diabetic than in the non-diabetic subjects. Prorenin was also higher when allowance was made for differences in plasma protein content of the samples. In the diabetic subjects the prorenin concentration of vitreous fluid correlated with the plasma prorenin concentration ($r=0.78$, $n=15$, $p < 0.001$). In the non-diabetic subjects there was no significant correlation between the levels of prorenin in vitreous fluid and plasma.

As in the non-diabetic subjects, prorenin in vitreous samples with low plasma protein content was much higher (up to 25 times) than expected on the basis of the albumin content of the samples.

Immunoreactive angiotensin II was 9.0 ± 2.5 pmol/L in vitreous fluid (n=15) compared to 9.9 ± 2.9 pmol/L in plasma.

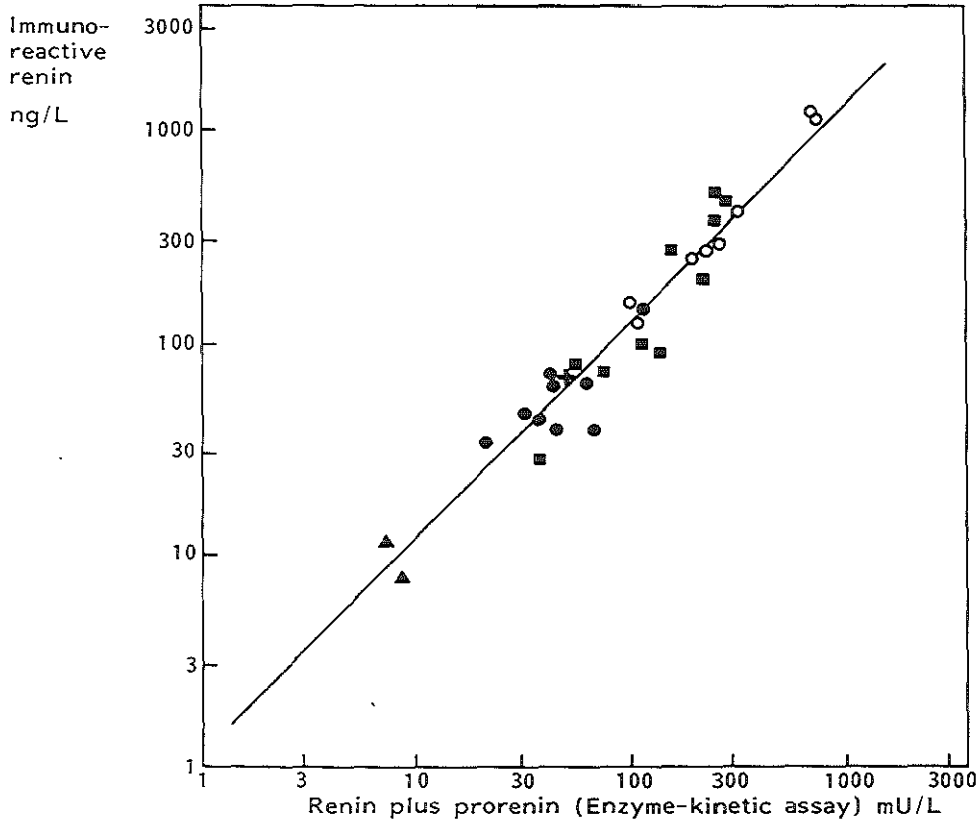


Figure 4. Total renin (prorenin plus renin) measured by enzyme-kinetic assay versus immunoreactive renin ($r=0.97$, $p < 0.0001$). o plasma, ■ subretinal fluid, ● vitreous fluid, ▲ aqueous fluid, + WHO human kidney renin standard.

## Discussion

The levels of albumin (mol wt, 69 K), transferrin (mol wt, 90 K), IgG (mol wt, 150 K) and renin substrate (mol wt, 65 K) in vitreous fluid differed widely from sample to sample, but in each individual sample the levels were, relative to each other, comparable to those in plasma. The IgG level in vitreous fluid, relative to that of albumin, was systematically somewhat lower than in plasma, probably due to its larger molecular size (25). These results are in agreement with earlier findings that most soluble protein in the vitreous is derived from plasma (24-26).

The plasma protein content of normal vitreous fluid has been estimated to be 0.5-2 % of that in plasma (25,26). In our study vitreous fluid from eyes with recurrent retinal detachment due to proliferative vitreoretinopathy contained higher levels of plasma proteins. These higher values probably reflect partial breakdown of the blood-retinal barrier in such eyes (27).

The blood-retinal barrier is formed by the tight junctions between the endothelial cells of the retinal capillaries and the tight junctions between the retinal pigment epithelial cells, the latter restricting the transfer of plasma proteins escaping from the capillaries of the choroid (28). Proteins from plasma may enter the vitreous as a result of focal cellular necrosis, opening of the intercellular junctions, or vesicular transport and formation of transcellular channels. The rate of diffusion of proteins through such discontinuities in the blood-retinal barrier depends upon the concentration gradient across this barrier, the molecular size of the proteins and the number and area of discontinuities. Our results are in accordance with the contention that diffusion through these pores is the main mechanism of transfer of plasma proteins to the vitreous.

This process, however, does not appear to hold true for prorenin (mol wt, 54 K). The concentration of prorenin, relative to that of albumin, was much higher in vitreous fluid than in plasma, and the prorenin level in vitreous fluid also was little influenced by its plasma protein content. Thus, prorenin may enter the vitreous by a mechanism that is different from that of albumin and other plasma proteins. This mechanism is selective for prorenin and may involve an active process. Receptor-mediated transcellular transport is such a selective mechanism, but as yet there is no evidence for the existence of cell-membrane receptors of prorenin. Our findings raise the possibility that not all the prorenin in the vitreous is derived from plasma but that some of it is produced in the eye.

As described under Data analysis above, the vitreous level of prorenin that has crossed the blood-retinal barrier by passive diffusion in the same way as albumin can be estimated by multiplying the vitreous/plasma concentration ratio of albumin by the plasma prorenin level. By subtracting this calculated level of plasma-derived prorenin from the level actually measured, we estimated the level of prorenin that entered the vitreous by some process that is different from diffusion out of the circulation. In most

samples of vitreous fluid the estimated level of prorenin that had entered the vitreous by such a diffusion-independent process was more than 4 times higher than the estimated level of prorenin that had entered the vitreous by passive diffusion from blood. Thus, relative to the total amount of prorenin in vitreous fluid, the contribution of plasma-derived prorenin crossing the blood-retinal barrier merely by diffusion appears to be small. The implicit assumption underlying these calculations is that albumin and prorenin leave the vitreous in the same way, that is by free diffusion into the aqueous fluid (29), where the concentrations of these proteins were much lower than in the vitreous.

The concentrations of albumin, transferrin, IgG and renin substrate were 2-3 times higher in subretinal fluid than in vitreous fluid. This was to be expected, since subretinal fluid from eyes with retinal detachment is more or less a concentrate of vitreous fluid (27). Vitreous fluid enters the subretinal space through the hole(s) of the retina, and water is actively absorbed from the subretinal space by the retinal pigment epithelium. Again, the findings for prorenin were different. The concentration of prorenin, relative to albumin, was much higher in subretinal fluid than in plasma, particularly in subretinal samples with low plasma protein concentrations. Moreover, relative to albumin and other plasma proteins, prorenin was 2 times higher in subretinal than in vitreous fluid. If it is assumed, on the basis of the evidence discussed above, that most of the prorenin in the vitreous is not derived from plasma but is produced in the eye, the difference in prorenin content between subretinal and vitreous fluid may suggest that the subretinal compartment is closer to the site of prorenin production.

Not only were the prorenin concentrations of vitreous and subretinal fluid higher than expected, so too were the renin (mol wt, 48 K) concentrations. The data on renin, however, are more difficult to interpret than those on prorenin because in many samples renin was at or below the detection limit of the assay and because some prorenin to renin conversion may have occurred during storage and handling of the samples. Even as little as 1 % conversion will result in a large percentage increase of renin in these samples. Immunoreactive angiotensin II also was found in samples of vitreous fluid, in concentrations comparable to those in plasma. Further work is needed to answer the question of its origin.

That the levels of albumin and IgG in vitreous fluid from eyes affected by proliferative diabetic retinopathy were higher than the levels in normal eyes can be explained by the increased permeability of the blood-retinal barrier in this condition (30).

The higher vitreous level of prorenin, relative to those of albumin and other plasma proteins, in the diabetic subjects as compared to non-diabetic subjects is more difficult to explain. The same arguments in favor of the hypothesis that, generally, diffusion from the blood contributes little to the total amount of prorenin in the vitreous, apply to both diabetic and non-diabetic subjects. It seems, therefore, unlikely that the higher level of prorenin in vitreous fluid of the diabetic subjects (2 times that in non-diabetic subjects) was caused by the higher level in plasma (also 2 times that in non-diabetic subjects).

It might be the other way around; increased release or leakage of prorenin from the eye affected by proliferative diabetic retinopathy may contribute to the increased prorenin level in plasma.
This possibility is further supported by the finding that, in contrast with other proteins, the plasma concentration of prorenin in diabetic subjects correlated significantly with the concomitant vitreous prorenin concentration. Considering the fact that in some diabetic subjects the blood-retinal barrier for plasma proteins was still relatively intact (low vitreous/plasma albumin concentration ratio), whereas in others it was extremely leaky, no such correlation was to be expected, if diffusion from plasma into the vitreous was the main mechanism of transfer of prorenin.
An elevated plasma prorenin level in diabetic subjects has been found to be associated with microvascular complications, including retinopathy (31). Evidence is accumulating that neovascularization is initiated by diffusible chemical factors arising from ischemic areas of the retina (32). Renin and angiotensin have been found in cultured neuronal and glial cells from rat brain (33,34); both cell types are abundantly present in the retina. Angiotensin II acts on vascular tone and has mitogenic and trophic actions on vascular smooth muscle and other cells (35). In fact, it has been reported to promote neovascularization (16). An intraocular renin-angiotensin system may, therefore, play a role in proliferative diabetic retinopathy.

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## Chapter 4

# THE EFFECT OF PANRETINAL PHOTOCOAGULATION ON PLASMA PRORENIN IN DIABETIC PATIENTS WITH PROLIFERATIVE RETINOPATHY.


### Summary

There is strong evidence for the presence of a local renin-angiotensin system in the eye. Plasma prorenin is abnormally elevated in diabetic patients, particularly when diabetes is complicated by proliferative retinopathy. In view of the angiogenic properties of Ang II a local RAS may be involved in the development of diabetic retinopathy and part of the elevated plasma prorenin might be derived from the eye. Panretinal photocoagulation leads to regression of ocular neovascularization.

We studied the effect of photocoagulation on plasma prorenin levels in 12 diabetic patients with untreated proliferative retinopathy. Before treatment plasma prorenin ranged from 236 mU/l to 901 mU/l with a geometric mean of 442 mU/l, which is more than two times higher than in normals.

Although, the mean plasma prorenin declined significantly four weeks after the last lasertreatment, no uniform effect on plasma prorenin was shown.

In some patients plasma prorenin remained unchanged, in others it declined, however in most patients prorenin did not become normal.

Therefore no definite conclusion can be made regarding the effect of panretinal photocoagulation on plasma prorenin levels.

It is unlikely that leakage of prorenin into the circulation from eyes affected with proliferative retinopathy has a major contribution to the high plasma prorenin level. In view of the low ocular plasma flow and the relative long half life of prorenin, high levels of prorenin are to be expected in the ocular compartments. However, such high levels have not been measured in vitreous fluid from eyes with proliferative retinopathy. The high plasma prorenin is probably to be explained by a decreased clearance from the circlution or by increased production from renal or other than ocular extrarenal sites or by both.


### Introduction

Diabetic retinopathy is a major cause of blindness and visual morbidity in the Western world. Two of the severe blinding complications in diabetes are macular oedema and the development of neovascularization.

The mechanism by which neovascularization develops is not known. However, there is a strong indication that retinal hypoxia may lead to proliferative retinopathy (1-3). Evidence is accumulating that diffusible chemical factors from ischaemic areas are

involved in this process (4).

Diabetes mellitus is frequently accompanied by specific abnormalities of the renin-angiotensin system (RAS). Prorenin, the inactive precursor of renin, is elevated in plasma of patients with longstanding diabetes with microvascular complications, such as nephropathy and retinopathy (5). Recent data showed an association between high plasma prorenin and the presence of proliferative retinopathy, independent of age, sex, insulin-dependency, metabolic control, neuropathy and, at least in part, independent of the presence of nephropathy as manifested by albuminuria (6).

There is substantial evidence for the presence of a local renin-angiotensin system in the eye. This evidence is based on measurements of renin and prorenin in vitreous fluid and other ocular fluid compartments in diabetics and non-diabetics (7) and on immunohistochemical studies(8). Recent data from Wagner et al (9) demonstrated expression of renin, angiotensinogen and angiotensin-converting-enzyme mRNAs in human eyes.

Theoretically, one could speculate that part of the elevated prorenin in plasma in patients with proliferative retinopathy might be derived from the eye.

Angiotensin II has mitogenic and angiogenic properties (10-13) and because of these vascular actions, a local renin-angiotensin system in the eye may be involved in the pathogenesis of diabetic retinopathy.

Panretinal photocoagulation has been demonstrated to markedly reduce the risk of severe visual loss by regression of neovascularization (14-16).

The mechanism by which panretinal photocoagulation has this effect is not known. Regression of the new vessel formation is not only achieved by destruction of the area of neovascularization itself, but often also by destruction of non-related retinal areas with capillary non-perfusion. Some studies have shown that panretinal photocoagulation improves the oxygen supply to the inner retina by destroying the outer retina so that choroidal oxygen flux can reverse inner retinal hypoxia and the production of the putative vasoproliferative factor is stopped (17-20). In other words panretinal photocoagulation may work by eliminating the source of the angiogenic factor.

If renin or other components of the RAS are involved in this proces, and if part of the elevated prorenin in plasma of patients with proliferative retinopathy originates from the eye, what is the effect of panretinal photocoagulation on the plasma level of prorenin in such patients.

## Subjects and methods

We examined 265 diabetic (type I and 2) patients attending our outpatient facilities in eight consecutive months. Patients with overt cardiac failure, hepatic disease or renal failure (serum creatinin 200 umol/l or above) were not included. Diabetic retinopathy was assessed by direct and indirect ophthalmoscopy after pupillary dilatation.

Retinopathy was categorized as background retinopathy (BGDR) or proliferative retinopathy (PDR). Previous photocoagulation (date, argon versus xenon, focal versus panretinal) and previous vitrectomy were noted.

Blood was taken to measure serum creatinine, blood glucose, glycosylated hemoglobin and plasma renin and prorenin. Blood samples for renin and prorenin were drawn from an anticubal vein after the patients had been rested for 30 minutes in the sitting position. In patients, undergoing photocoagulation treatment, samples of renin and prorenin were taken one day before, one day after, one week after and 4 weeks after each photocoagulation session.

Non-timed overnight urine collections were obtained at two different days and were tested for the presence of albuminuria by dipstick method. Macroalbuminuria was defined as albumin above 250 mg/l in each of the two samples, which is the detection limit of the dipstick method. In patients in which the dipstick test was negative, three 24 hour urine collections were assessed for microalbuminuria.

From the group of patients with proliferative retinopathy (n=84) 22 patients had not been treated before and needed extensive panretinal photocoagulation. In these patients we studied the effect of laser treatment on the levels of plasma prorenin and renin. Fluorescein angiography was performed to assess the presence of capillary non-perfusion. These patients had intraretinal or preretinal new vessels with fibrosis on or within 1 disc diameter of the optic disc in both eyes. The patients were graded as having high-risk characteristics according to The Diabetic Retinopathy Study Research Group classification (15), and required extensive panretinal photocoagulation treatment.
There were no substantial lens opacities or vitreous hemorrhages sufficient to interfere with laser treatment. Since treatment of patients with such high-risks cannot be postponed, it was not possible to study a matched control group or to randomize between direct or postponed treatment. Panretinal blue argon laser photocoagulation was performed with the Goldman three mirror lens or with the Rodenstock panfunduscope in 1-3 sessions per eye within a period of 3-12 weeks. All four quadrants of each eye were treated. The area between the temporal arcades was spared.
Laser parameters were: coagulation time between 0.1 and 0.3 sec., energy 0.2 to 0.8 Watt, spotsize 500 um for the Goldmann three mirror lens and 200 um for the Rodenstock panfundoscope. The intensity was such that a slight blanching was visible. Two eyes had also a transconjuctival equatorial cryocoagulation treatment of the retina.

### Analytical procedures

The plasma levels of renin and prorenin were measured with the enzyme-kinetic assay, in which the angiotensin I generated by plasma in vitro was quantified by radioimmunoassay. Prior to assay prorenin was converted to renin at 4°C by immobilized trypsin in the presence of human serum albumin (21,22). Normal levels (79 non-diabetic

males, 81 non-diabetic females, age 48 +15 yr, mean =SD) of renin and prorenin were 14.5 (3.6-61.4) and 154 (4.1-358) mU/l respectively (geometric mean and 95% confidence interval).

Macroalbuminuria was assessed in non-timed overnight urine samples with dipsticx (Albustix, Miles, Naperville, ILL, USA), which has a detection limit of 250 mg/l. Macroalbuminuria was defined as dip-stick positive or biuret reaction of protein above 250 mg/l in two out of two overnight urine samples. Microalbuminuria was assessed in three consecutive 24 hour urine collections with radial immunodiffusion (23). The lower limit of detection was 2mg/l and the interassay variability was 8 %. Microalbuminuria was defined as albuminuria of of 30-300 mg/24h in at least two of 24 hour urine collections.

## Statistics

Data on plasma prorenin and renin from the group of 223 consecutive patients were compared by the Mann-Whitney U test.
Data on plasma prorenin and renin from the group of untreated patients with PDR before and after panretinal photocoagulation were compared with the One-way analysis of variance for repeated measurements.

## Results

From the group of 265 consecutive diabetics 84 patients had proliferative retinopathy. In this group 62 patients had been treated with photocoagulation in the past and 9 patients had undergone a vitrectomy. In this group of 62 patients there were 44 males, mean age was 53 years and mean duration of diabetes was 17 years. In 15 patients the diabetes was treated with oral medication only.
Plasma prorenin in patients with proliferative retinopathy ranged from 75-2133 mU/l with a geometric mean of 400mU/l , which is more than two times higher than in normal persons. Plasma renin ranged from 4.2-53.3 with a geometric mean of 15.8 mU/l, which is not different from the normal range.
Out of the 84 patients with proliferative retinopathy 22 patients had not been treated before and they, according to the stage of their proliferative eye disease, required panretinal photocoagulation in both eyes. In these patients, after informed consent, the efect of panretinal photocoagulation on plasma prorenin were studied. Three patients became blind in one eye and were excluded because of the fact that they could not be treated in both eyes. Seven patients did not complete the study or were exluded for several reasons. One patient moved to another country, two patients developed detoriation of the renal function (serum creatinine above 200 umol/l), three patients were put on ACE-inhibitor therapy by their own physician and one patient did not complete the photocoagulation therapy.

Twelve patients completed the study and their clinical data are shown in Table 1. The female-male ratio was 9 to 3. The age ranged between 23-76 years with a mean of 52 years. All but three patients were treated with insulin. The duration of the diabetes ranged from 3-40 years with a mean of 20 years. Four patients were treated with diuretics and 1 patient with a beta blocker and 1 patient with a alpha and beta blocker because of coexisting hypertension. The hypertension was well controlled and antihypertensive therapy was not changed during the study period.
Six patients showed albuminuria (5 macroalbuminuria and 1 microalbuminuria) as a sign of diabetic nephropathy.

Table 1. Clinical characteristics of diabetic patients with untreated proliferative retinopathy

| patient | sex | age | duration of diabetes | treatment of | serum creatinine | albuminuria | treatment of |
|---|---|---|---|---|---|---|---|
| | yr | yr | yr | diabetes | µmol/L | mg/24h | hypertension |
| 1 | m | 63 | 3 | glibenclamide | 102 | 12 | furosemide |
| 2 | m | 49 | 21 | insulin | 200 | >300 | furosemide |
| 3 | m | 61 | 40 | glibenclamide | 139 | >300 | metoprolol |
| 4 | m | 64 | 16 | insulin | 115 | 17 | furosemide |
| 5 | m | 37 | 23 | insulin | 95 | >300 | |
| 6 | m | 45 | 25 | glibenclamide | 148 | >300 | labetolol |
| 7 | f | 75 | 17 | insulin | 80 | 5 | |
| 8 | f | 23 | 18 | insulin | 73 | 25 | |
| 9 | f | 28 | 11 | insulin | 63 | 5 | |
| 10 | m | 44 | 16 | insulin | 184 | >300 | |
| 11 | f | 55 | 25 | insulin | 82 | 30 | |
| 12 | m | 76 | 22 | insulin | 166 | 25 | |

Table 2 and Figure 1 show the plasma levels of prorenin and renin before treatment, one week after the first coagulation session and four weeks after the last session. Plasma prorenin before treatment ranged from 236 mU/l to 901 mU/l with a geometric mean of 442 mU/l, ,plasma renin ranged from 3.7- 40.6 mU/l with a geometric mean of 16.3 mU/ Plasma prorenin and renin was not changed the day after the coagulation session. Mean plasma prorenin decreased significantly from 442 U/ml to 302 uU/l four weeks after the last lasertreatment. Mean plasma renin did not change significantly during folluw-up. It should be noted that in 6 patients no decrease in plasma prorenin could be demonstrated. In 5 out of 6 patients in whom plasma prorenin decreased, plasma prorenin remained above the normal range.

Table 2. Plasma prorenin and renin before and after panretinal photocoagulation

| | Prorenin (mU/L) | | | Renin (mU/L) | | |
|---|---|---|---|---|---|---|
| patient | before treatment | one week after first laser treatment | four weeks after last laser treatment | before treatment | one week after first laser treatment | four weeks after last laser treatment |
| 1 | 717 | 598 | 283 | 27.6 | 23.6 | 15.3 |
| 2 | 901 | 1120 | 427 | 29.2 | 20.1 | 16.1 |
| 3 | 457 | 370 | 231 | 39.7 | 12.8 | 16.2 |
| 4 | 360 | 346 | 352 | 40.6 | 24.4 | 38.5 |
| 5 | 447 | 506 | 415 | 21.0 | 27.8 | 20.4 |
| 6 | 451 | 428 | 480 | 3.7 | 6.5 | 9.4 |
| 7 | 300 | 291 | 230 | 10.9 | 16.6 | 10.3 |
| 8 | 582 | 402 | 505 | 43.3 | 19.6 | 23.8 |
| 9 | 236 | 162 | 203 | 5.1 | 6.7 | 5.5 |
| 10 | 410 | 272 | 179 | 7.2 | 4.8 | 4.0 |
| 11 | 458 | 320 | 211 | 17.6 | 10.2 | 5.6 |
| 12 | 334 | 430 | 463 | 8.5 | 14.8 | 22.1 |

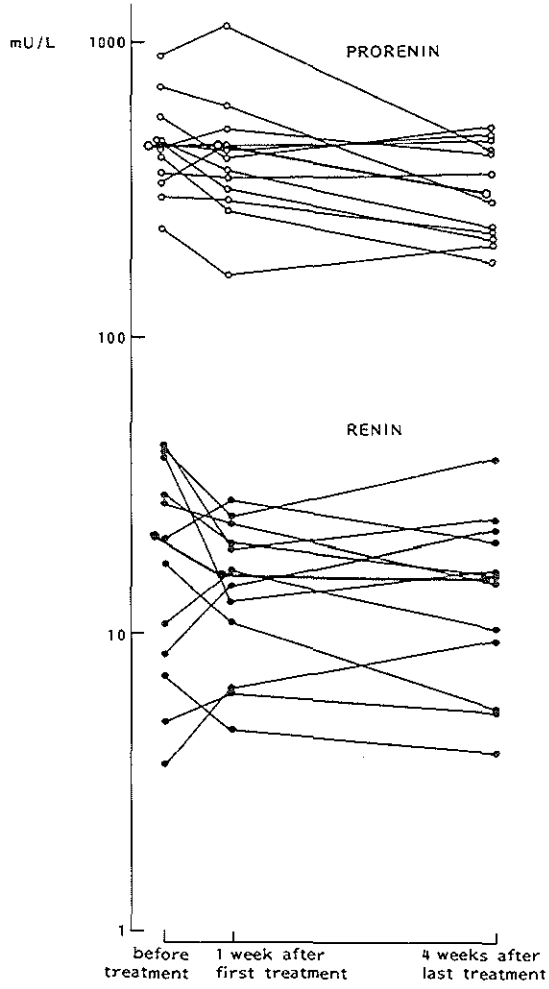


Figure 1. The effect of panretinal photocoagulation on plasma prorenin and renin levels in 12 patients with proliferative diabetic retinopathy.

## Discussion

There is strong evidence for the presence of a local renin-angiotensin system in the eye. In view of the angiogenic and mitogenic vascular actions of angiotensin II, the most important biologically active end product of the renin-angiotensin sytem, it is conceivable that a local renin-angiotensin system in the eye is involved in the development of diabetic proliferative retinopathy. The vitreous fluid from eyes affected by proliferative diabetic retinopathy contains an abnormally high concentration of prorenin and one wonders whether some of it may leak into the circulation and whether this may contribute to the abnormally high levels of prorenin in plasma.

Our data show no uniform effect of panretinal photocoagulation on the plasma levels of prorenin. At any rule, there was no acute effect (one day). In some patients prorenin remained unchanged also after a longer follow-up period, others showed a decrease in prorenin but it should be noted that in most patients prorenin did not become normal. Although it is true that prorenin in vitreous fluid from eyes with proliferative diabetic retinopathy with retinal detachment is higher than in eyes with spontaneous retinal detachment, it is perhaps a priori unlikely that leakage of prorenin into the circulation leads to high plasma levels that are observed in diabetics.

The ocular plasma flow is extremely low (24-26) as compared with total body plasma flow and the plasma half life of prorenin has been estimated to be in the order of 1-2 hours (27,28). This would mean that the veno-arterial prorenin gradient would be very high, more than 1000 mU/l, if release of prorenin from the eye would be the cause of the abnormally high level in circulating plasma.

If this were the case, one would expect to find very high levels of prorenin in some compartments of the eye. In fact, such high levels have not been measured. The highest levels of prorenin we measured in vitreous fluid in eyes with proliferative diabetic retinopathy were 172 mU/L (7). The highest levels of total renin (prorenin + renin) measured in tissues from bovine eyes were 15.3 ng Ang I per g tissue per hour in pigment epithelium-choroid as compared with 6.8 ng Ang I per g tissue per hour in plasma (29).

In other words the high circulating plasma prorenin in patients with PDR cannot be fully explained by production from the affected eyes.

However, this does not rule out any role for a local ocular RAS system in the development of diabetic retinopathy. Most of the elevated plasma prorenin is probably derived from production by renal or other extrarenal sources or may be explained by a decreased renal or extrarenal clearance of prorenin or both.

In conclusion, an elevated plasma prorenin is related to the presence of proliferative retinopathy partly independent of the presence of albuminuria and panretinal photocoagulation treatment may lead to decline of the elevated plasma prorenin level suggesting a relation between them.

Based upon this data long-term clinical studies should be performed to examine whether

ACE inhibitor treatment has a favourable effect on the development and progression of retinal neovascularization in diabetic subjects.

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## Chapter 5

# PLASMA PRORENIN AS AN EARLY MARKER OF MICROVASCULAR DISEASE IN PATIENTS WITH DIABETES MELLITUS


### Summary

To test the hypothesis that a high plasma prorenin can be used as an early marker of microvascular complications in patients with diabetes mellitus plasma prorenin was measured in 44 patients with urinary albumin excretion between 30 and 300 mg/24h (microalbuminuria) and 120 patients with urinary albumin excretion below 30 mg/24 h (normoalbuminuria). A high plasma prorenin was associated with diabetic retinopathy, particularly the proliferative type, serum creatinine and the 24h urinary albumin excretion rate. Plasma prorenin was not correlated with age, duration of diabetes, glyco-sylated hemoglobin, blood glucose, and blood pressure. The association between elevated plasma prorenin and retinopathy remained significant after adjustment for serum creatinine and albumin excretion. Independent of the presence or absence of micro-albuminuria, the mean plasma level of prorenin was not above normal in patients without retinopathy and was 2 to 3 times normal in patients with proliferative retinopathy. Thus retinopathy appears to be an important determinant of abnormally high plasma prorenin. Angiotensin converting enzyme (ACE) was elevated in the patients with diabetes mellitus as compared to control subjects but the plasma levels of ACE in diabetics with normoalbuminuria was not significantly different from the group with microalburinuria. Plasma prorenin was not associated with ACE. A plasma level of prorenin of 225 mU/L had a sensitivity of 0.71 and a specificty of 0.71 for detecting.the presence of microalbuminuria.


### Introduction

Human plasma contains two forms of renin : a) prorenin the enzymatically inactive precursor of renin and, b) renin the active enzyme (1,2). In plasma of normal subjects only 10 percent of the total renin concentration circulates in the form of renin and this percentage increases if the total renin concentration increases (2). The kidney is the major source of renin in plasma. The kidney is also an important source of plasma prorenin (3,4) but part of the circulating prorenin originates from extrarenal sites such as the ovaries and testis (5.6)

In patients with diabetes mellitus plasma prorenin is often high (7-10), whereas renin is normal or low (11,12). There is a close association between a high level of plasma prorenin and diabetic complications (10). In diabetics without overt nephropathy plasma prorenin correlated positively with serum creatinine, the presence of macroalbuminuria

(Albustix positive urine) and the presence of retinopathy.

The association between elevated plasma prorenin levels and retinopathy remained significant after adjustment for nephropathy.

Diabetic nephropathy will develop in about 40 percent of the patients with insulin dependent diabetes mellitus and is characterized by persistent macro-albuminuria, a decrease in glomerular filtration rate and an increase in blood pressure. This stage is preceeded by a stage of incipient nephropathy characterized by urinary albumin excretion below the detection limit of the Albustix but above the normal excretion of 30 mg/day (microalbuminuria 30-300 mg/24h), and a normal or even high glomerular filtration rate. Microalbuminuria is regarded as a predictor of overt diabetic nephropathy (13).

In the present study we examined whether plasma prorenin could be used as a marker of microvascular disease in a group of 120 diabetic patients with a normal urinary albumin excretion and 44 patients with incipient diabetic nephropathy.

## Subjects and methods

*Patients.*

Patients (n=164) attending our outpatient clinic were included in the study. Patients with a history of kidney, urinary tract, liver or heart disease were not included and also patients on drugs likely to affect the plasma level of renin were not included. All patients had serum creatinine of 120 µmol/L or below. Some clinical characteristics of the patients are given in Table 1. In the study group 127 patients were on insulin, and 37 patients were on oral antidiabetic drug treatment. An assessment of the 24 h urinary albumin excretion was made on three occasions in a period of one month. Microalbuminuria was defined as albumin excretion between 30-300 mg/24h, mean of three determinations (14).

*Ophthalmoscopy.*

Ophthalmoscopy after pupillary dilatation was done by the ophthalmologist who was unaware of the results of the renin measurements. Diabetic retinopathy was categorized as either background retinopathy or proliferative retinopathy. Background retinopathy was characterized by the presence of microaneurysms, whether or not combined with hemorrhages and hard exudates (15). Eyes with preproliferative retinopathy, as characterized by multiple cotton wool spots, venous abnormalities, beading and duplications, intraretinal microvascular abnormalities and diffuse large blot hemorrhages, were also categorized as background diabetic retinopathy. New vessels anywhere in the retina in either eye were categorized as proliferative retinopathy. Of the 45 patients with proliferative diabetic retinopathy 34 had been treated by laser photocoagulation of

one or both eyes 3-24 months before the study.

*Neurological assessment.*
Sensory or motor fibre neuropathy was defined as diminished sense of vibration at the lower or upper extremities and loss of Achilles and/or patellar tendon reflexes (16), which generally is associated with other signs of neuropathy or with symptoms, such as sensory loss, distal paresthesia or pain. None of the patients had symptomatic orthostatic hypotension.

*Blood sampling.*
Blood samples for renin and prorenin were drawn from an antecubital vein after the patients had rested for 45 min in the supine position. Blood was also taken for serum creatinine, blood glucose, glycosylated hemoglobin (HbAc1) , angiotensin-converting enzyme (ACE) and noradrenaline.

*Measurements of plasma renin and prorenin.*
Measurements were made with an enzyme kinetic assay, in which the in vitro generated angiotensin I was quantitated by radioimmunoassay (17). For measuring prorenin, the proenzyme was converted to renin by trypsin (3,18). Normal levels of renin and prorenin in 255 normal subjects aged 18-62 yr were 14.3 (4.4-47.4) mU/L (geometric mean and 95 % confidence interval) for renin and 140 (64-305) mU/L for prorenin.

*Measurement of plasma noradrenalin and angiotensin converting enzyme .*
Plasma noradrenalin was measured by high performance liquid chromatography (19) and angiotensin-converting enzyme (ACE) by an enzyme kinetic assay (20) . In normal subjects (n=57) aged 24-57 yr plasma noradrenalin was 1.5 (0.4) nmol/L mean (SD) and ACE 14.9 ( 2.1 ) U/L.

*Urinary albumin.*
Albumin was measured by radioimmunoassay. The lower limit of detection was 1 mg/L and the interassay variability was 9 %.

*Statistical analysis.*
Differences between groups for discrete data were analyzed by CHI square test. Differences for continuous data were analyzed with the unpaired t-test or with the Student-Newman-Keuls test for multiple comparisons. Renin and prorenin were not normally distributed. Logarithmic transformation yielded apparently normal distributions with similar standard deviations in all groups. These transformed values were used for statistical analysis. Multiple regression analysis was performed with the use of the SPSS/PC+ program 1986 (SPSS Inc., Chicago, ILL, USA).

TABLE I. – *Clinical characteristics of diabetic patients with and without microalbuminuria*

| | *Microalbuminuria* | | *P value* |
|---|---|---|---|
| | *Absent* $n = 120$ | *Present* $n = 44$ | |
| Sex (No. of female/male) | 56/64 | 16/28 | ns * |
| Age (yr) | | | |
| Median | 42 | 46 | ns |
| Range | 17-76 | 20-76 | |
| Duration of diabetes (yr) | | | |
| Median | 13 | 16 | ns |
| Range | 1-44 | 20-76 | |
| Insulin treatment (no. on insulin/ not on insulin) | 89/31 | 31/13 | < 0.01 |
| Neuropathy (no of subjects; absent/present) | 88/32 | 20/24 | < 0.01 |
| HbA1c [%; mean (SD)] | 8.6 (1.1) | 8.8 (1.5) | ns |
| Serum creatinine (μmol/l) [mean; (SD)] | 80 (16) | 85 (21) | ns |
| Blood pressure (mmHg) | | | |
| Systolic [mean; (SD)] | 135 (23) | 141 (27) | ns |
| Diastolic [mean; (SD)] | 77 (11) | 80 (13) | ns |
| Diabetic retinopathy (no.) | | | |
| Absent | 63 | 12 | |
| Background | 31 | 13 | < 0.01 |
| Proliferative | 26 | 19 | |

* $p > 0.05$

## Results

### *Correlation of prorenin with diabetic complications*

In Tables 1 and 2 the patients are grouped according to the absence or presence of microalbuminuria. The groups did not differ significantly in sex, age, duration of diabetes, the use of insulin and blood pressure. Blood glucose, glycosylated hemoglobin, plasma angiotensin converting enzyme and noradrenalin were also not significantly different between the two groups. Patients with microalbuminuria had an increased prevalence of retinopathy and neuropathy in comparison to diabetics with normoalbuminuria.

The plasma levels of prorenin in the patients without and with micro-albuminuria and with various degree of diabetic retinopathy are depicted in Figure 1 and Table2 . The plasma level of prorenin in diabetics with normoalbuminuria was significantly higher ($p<0.01$) than plasma prorenin in age- and sex-matched healthy subjects. The highest plasma levels of prorenin were found in the the patients with proliferative retinopathy both in the normoalbuminuric group as well as in the group with microalbuminuria. Plasma renin was lower in diabetics with or without microalbuminura than in control subjects ($P<0.05$). In 72 patients plasma prorenin and 24 h urinary albumin excretion was measured at three occasions. The coefficient of variation was 9 %(1-56), median and (range) for prorenin and 30%(1-135) for albuminuria. Thus there was much less variabilty ($p<0.001$) in plasma prorenin than in 24 h urinary albumin excretion.

TABLE II. – *Plasma renin, prorenin, noradrenalin and ACE in patients with and without microalbuminuria*

| | *Microalbuminuria* | | *P value* |
|---|---|---|---|
| | *Absent* | *Present* | |
| Noradrenalin [pmol/l,mean ;(SD)] | 1.41 (0.58) | 1.37 (0.53) | ns * |
| ACE [U/l,mean ;(SD)] | 15.6 (4.4) | 17.6 (4.3) | ns |
| Renin [mU/l, geometric mean]<br>range | 11.0<br>2.5-48.4 | 10.5<br>2.2-39.0 | ns |
| Prorenin [mU/l, geometric mean]<br>range | 169<br>52-548 | 264<br>71-990 | < 0.01 |

* $p > 0.05$

The influence of the various factors on the plasma level of prorenin was analyzed by multiple logistic regression analysis. The factors studied were: sex, age, duration of diabetes, insulin treatment, blood glucose, glycosylated hemoglobin, systolic and diastolic blood pressure, neuropathy, serum creatinine, angiotensin-converting enzyme, noradrenalin, 24h excretion of albumin and retinopathy. (Tabs 3,4). Statistical significant correlations were found with sex, creatinine, albumin excretion and retinopathy. Plasma prorenin was not correlated with angiotensin converting enzyme or plasma noradrenaline.

The multiple logistic regression analysis showed that high prorenin was positively associated with the presence of sex (male higher than in female, $p<0.001$), proliferative retinopathy ($p<0.0001$), albumin excretion rate ($p<0.001$) and negatively with diastolic blood pressure ($p=0.002$), and that the other factors including neuropathy and serum creatinine did not significantly contribute to the high plasma level of prorenin . It also appeared that the association with retinopathy was at least in part independent of the association with nephropathy. No significant correlations were found between the plasma level renin and the various factors mentioned above.

A plasma level of prorenin of 225 mU/L was derived from a Receiver Operating Curve as the best cut-off to detect the presence of microalbuminuria. The sensitivety and specificity of the plasma prorenine level for detecting microvascular disease in diabetics was 0.60 and 0.89 respectively.

TABLE III. — *Correlation of (log) prorenin with various factors in patients with diabetes mellitus*

| *Factor* | *r* | *P value* |
|---|---|---|
| Age | 0.113 | ns * |
| Duration of diabetes | 0.237 | < 0.001 |
| Systolic blood pressure | − 0.099 | ns |
| Diastolic blood pressure | − 0.129 | ns |
| Serum creatinine | 0.323 | < 0.001 |
| Blood glucose | 0.090 | ns |
| HbA1c | 0.024 | ns |
| ACE | 0.085 | ns |
| Noradrenaline | 0.102 | ns |
| 24 h albumin excretion | 0.316 | < 0.001 |

* p > 0.05

TABLE IV. — *Plasma levels of prorenin and renin in various categories of diabetics*

| *Factor* | *Category* | *n* | *Renin* | | *Prorenin* | |
|---|---|---|---|---|---|---|
| | | | *mU/l* | *P value* | *mU/l* | *P value* |
| Sex | Female | 72 | 9.7<br>2.2-42.7 | ns | 152<br>44-530 | |
| | Male | 92 | 11.7<br>3.3-42.7 | ns | 226<br>69-748 | < 0.01 |
| Insulin | On insulin | 127 | 10.6<br>2.9-39.0 | ns | 200<br>57-703 | |
| | Not on insulin | 37 | 12.0<br>2.2-64.4 | ns | 162<br>44-587 | < 0.01 |
| Neuropathy | Absent | 108 | 11.0<br>2.6-46.0 | ns | 160<br>53-482 | |
| | Present | 56 | 10.1<br>2.5-40.0 | ns | 224<br>65-774 | < 0.01 |
| Retinopathy | Absent | 75 | 11.1<br>2.3-53.7 | | 145<br>53-393 | |
| | Background | 44 | 10.2<br>2.7-38.2 | ns | 192<br>51-723 | < 0.01 |
| | Proliferative | 45 | 12.2<br>3.4-43.7 | | 297<br>93-946 | < 0.001 |

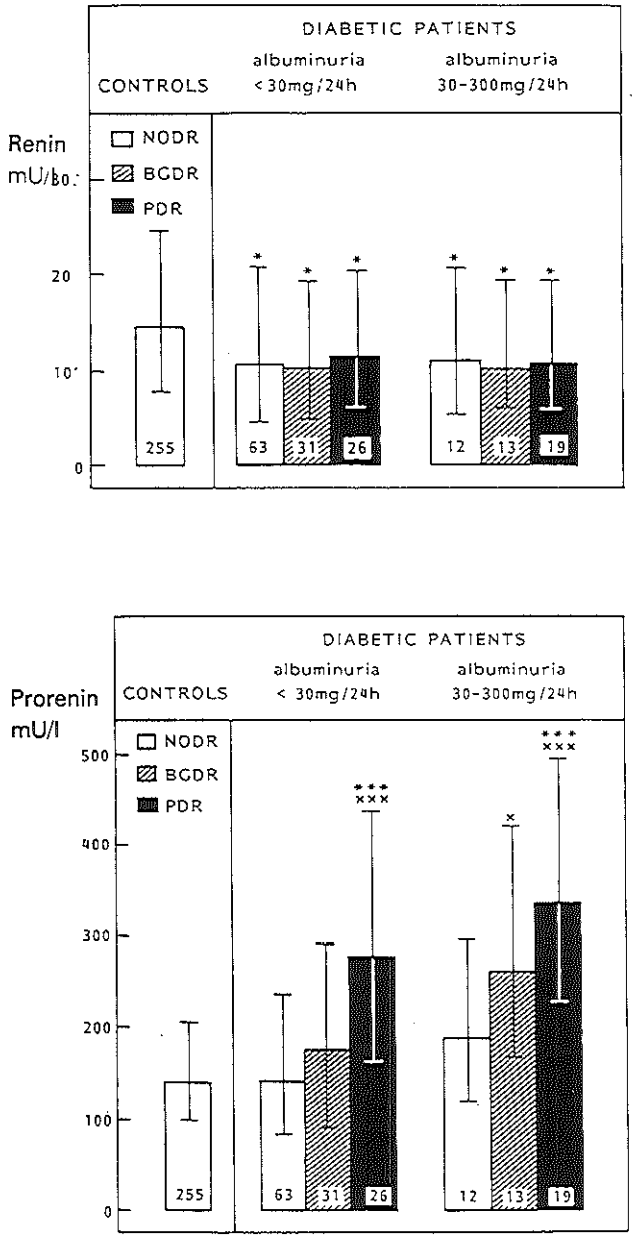


Fig.1. Plasma renin (upper panel) and prorenin (lower panel) in diabetic patients with normoalbuminuria or microalbuminuria. Patients were subdivided in those wihout retinopathy (NODR), with background retinopathy (BGDR) and those with proliferative retinopathy (PDR). The numbers in the bar are the numbers of patients in each group. * $p < 0.05$ from healthy control subjects Results are expressed as the geometric mean x / : SD

## Discussion

Luetcher et al were the first to report a close association between a high level of plasma prorenin and the presence of microvascular complications in patients with longstanding diabetes mellitus (8). Other investigators, including our group, could confirm these findings (9,10). We found that the elevated plasma level of prorenin was associated with serum creatinine, the presence of macroalbuminuria and the presence of diabetic retinopathy, particularly the proliferative type. A number of confounding factors, i.e. sex, duration of diabetes, blood pressure, blood levels of glucose and HbAc1, the prevalence of neuropathy or insuline treatment did not significantly contribute to this association. The association between plasma prorenin and retinopathy remained significant after adjustment for serum creatinine and the urinary albumin excretion rate .The present study, in a well-defined group of diabetics with incipient nephropathy confirms and extends the earlier observations made in patients with more advanced diabetic complications.

Our results indicate that plasma prorenin level might be used as a marker for the presence of microvascular disease in patients with diabetes mellitus. Plasma prorenin level was already elevated in some patients with background retinopathy and a normal urinary excretion rate of albumin. Determination of plasma prorenin offers some advantages over measurements of urinary albumin excretion, because urine collection is not required and because of the lower day-to-day variability of plasma prorenin level. Prospective studies are necessary to see whether a high plasma prorenin level precede the development of microalbuminuria and/or retinopathy. In a retrospective study by Wilson et al it appeared that an increased plasma prorenin identified a group of young patients with diabetes who were at high risk for developing retinopathy and nephropathy (21).

Serum levels of angiotensin-converting enzyme (ACE), a marker of endothelial cell function, were reported to be elevated in about 25 % of patients with diabetes mellitus (22,23). We also found that ACE was elevated in our subjects with diabetes in comparison with normal nondiabetic subjects. There was however no significant difference in the plasma levels of ACE in the patients with microalbuminuria in comparision with those with normal albumin excretion.

The pathophysiology underlying the elevated plasma level of prorenin levels in patients with diabetes mellitus is not understood. There are two possibilities; a) an increase in the renal or extrarenal synthesis of prorenin or b) a reduced clearance. Rats with chronic streptozocin-induced diabetes mellitus show a significant fall in plasma renin accompanied by an increase in plasma prorenin (24). After bilateral nephrectomy plasma renin and prorenin decrease in control rats as well as in diabetic rats suggesting a renal origin for the high plasma prorenin levels. However, we did not found an elevated renal vein-to-artery ratio for plasma prorenin in diabetic patients with

end-stage renal disease and proliferative retinopathy, despite an elevated plasma level of prorenin and an impaired renal plasma flow (9).

An increase in the extrarenal production of prorenin may also explain high plasma prorenin levels. In human vitreous or subretinal fluid the levels of prorenin are, expressed per mg albumin, in contrast to other plasma proteins, up to 100 times higher in the eye fluids than in plasma (25). Prorenin is higher in vitrous fluid from eyes with proliferative diabetic retinopathy complicated by traction retinal detachment than in eyes of nondiabetics with spontaneous retinal detachment. In the bovine ocular tissue the highest concentration of prorenin was found in the pigment epithelium-choroid layer and in the retina (26).

Misbin et al suggested that the high plasma level of prorenin is caused by autonomic dysfunction (27). Loss of adrenergic stimulation of the juxtaglomerular cells may lead to a decreased secretion of renin by the juxtaglomerular cells and then leads to a compensatory increase in the synthesis and release from prorenin by these cells in diabetic patients with autonomic insuffiency. None of our patients had overt autonomic neuropathy and we found no association between plasma noradrenaline and prorenin. Moreover, patients with autonomic neuropathy in the absence of diabetes mellitus and its complications do not have high plasma prorenin levels (9).

In summary, the high plasma prorenin in patients with diabetes mellitus is related to the development of microvascular disease and may be at least in part due to an increase production from extrarenal sources such as the eye, decreased clearance of prorenin from the circulation, or both. Plasma prorenin may be used as an early marker of diabetic microvascular disease but prospective studies are needed to prove that plasma prorenin measurements are useful to identify patients at risk for developing diabetic microvascular disease.

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## Chapter 6

# BLOOD PRESSURE AND PRORENIN IN RELATION TO THE PROGRESSION OF ALBUMINURIA IN TYPE 1 DIABETIC PATIENTS WITH SLIGHTLY ELEVATED URINARY ALBUMIN EXCRETION. A 2 YEAR FOLLOW-UP.


## Summary

Microalbuminuria is a strong predictor of clinical nephropathy in Type 1 (insulin dependent) diabetes mellitus. Patients with microalbuminuria are also prone to the development of generalized microangiopathy .

Plasma prorenin is often elevated in Type 1 diabetic patients, particularly when diabetes is complicated by retinopathy and albuminuria. An elevated plasma prorenin may therefore be a marker of microvascular complications. However, little is known about the elevation of prorenin over time, in relation to development of microalbuminuria, during the course of diabetes.

We conducted a two year prospective study in a selected group of normotensive Type 1 diabetic patients with slightly elevated levels of albuminuria. Progression of albuminuria was independently related to baseline blood pressure ($p<0.001$) and retinopathy ($p<0.01$) but not to baseline glomerular filtration rate (GFR) and metabolic control. Baseline prorenin was elevated in only 6 of the 25 patients and was not different in patients with and without progression of albuminuria.

Plasma prorenin was significantly higher in patients with exsudative or proliferative retinopathy. Thus, a relation between plasma prorenin and progression of albuminuria was not apparent in this group of patients. This may be explained by the low degree of albuminuria; prorenin may rise at later stages of renal diabetic disease. Another possiblity is that an elevated plasma prorenin is associated with a certain other aspect of microangiopathy of which albuminuria is not a representative sign.


## Introduction

Microalbuminuria is considered to be a sign of diabetic microangiopathy and is used to distinguish Type 1 (insulin-dependent) diabetic patients who are at risk for future development of diabetic nephropathy from those who are not (1-4). Microalbuminuria shows a high degree of intrapatient variability so that repeated measurements are necessary to establish the presence and worsening of microalbuminuria (5-7).

The plasma level of prorenin is often raised in Type 1 (insulin-dependent) diabetic patients and high levels are related to the presence of proteinuria and severe retinopathy (8,9). It has therefore been suggested that prorenin can be used as an other marker of diabetic microangiopathy.

The much lower day to day variability of plasma prorenin as compared to urinary albumin excretion (10) may offer advantages in identifying patients at risk of progressive microangiopathy.

It is important to determine at which time during the development of diabetic nephropathy prorenin begins to rise and to detect factors that may contribute to the progression of albuminuria early in the course of diabetic renal involvement. Mosetti et al (11) have observed increased plasma prorenin when the rate of albumin excretion exceeded 40 mg per day. Data reported by Wilson et al (12) suggest that in adolescent diabetics a rise in plasma prorenin may precede the development of renal and retinal disease. These authors, however, used the presence of macroalbuminuria as a sign of renal involvement.

In the present study we examined some clinical factors contributing to progression of albuminuria during two years follow-up in normotensive
Type 1 (insulin-dependent) diabetic patients selected to have minor elevations in urinary albumin excretion. Plasma prorenin was measured serially to investigate whether its level can be used as a marker of progressive albuminuria.

## Patients and methods

*Patients and follow up.*

The study was approved by the local medical ethics committee and all participating patients gave informed consent. Patients with Type I (insulin-dependent) diabetes mellitus and elevated urinary albumin excretion rate were selected from an out-patient population. Glucagon-stimulated C-peptide levels were less than 0.2 nmol/l.
Diabetes had to be present for at least five years and all patients self-monitored their blood glucose levels. Patients with a mean overnight urinary albumin excretion rate between 10 and 200 ug/min determined in three consecutive collections were included in this study. In overnight urine collections a cut-off level of 10 g/min represents the upper normal limit (above the 97.5 th percentile for non-diabetic patients) in our clinic. It should be noted that this inclusion criterium is lower than the internationally proposed level of 20 ug/min to define microalbuminuria.
The exclusion criteria were: urinary tract infection or renal tract abnormalities, hypertension ( >160/95 mmHg) and the use of antihypertensive medication. A total of 25 consecutive patients were selected for this study. Their sodium intake was unrestricted an none used a low protein diet. The prospective follow-up was started within three months after selection of the patients. All participants completed the study. The patients were studied at 4 month intervals. On each occasion three overnight urine specimens were obtained for determination of albumin excretion and one 24-h urine collection was obtained for the determination of sodium and urea excretion.

Blood pressure was measured according to the method of Riva Rocci Phase V of Korotkoff after the patients had been sitting for five minutes, and the results of three

measurements were averaged. Glomerular filtration rate (GFR) and effective renal plasma flow (ERPF) were determined at the beginning of the study, after one and two years.

Blood was drawn, from an anticubal vein after the patients had rested for 10 minutes in the sitting position, to measure renin, total renin (renin plus prorenin) and HbA1. Blood samples were centrifuged at room temperature for 10 minutes and the plasma was stored at -20 °C.

### Analytical procedures

The plasma levels of renin and total renin (renin and prorenin) were measured according to previously described methods (13,14). Prorenin was calculated as the value for total renin minus the value for renin. We here report the values for total renin as well for prorenin and renin seperately because the samples were analysed two years after blood collection. During storage at -20 °C some prorenin to renin conversion takes place, resulting in renin levels that are higher and prorenin levels that are lower than in vivo. The sum of the two, total renin, does not change.

Normal levels (geometric mean and 95% confidence interval) of renin, prorenin and total renin in 255 normal subjects aged 18-62 were 14.3 (4.4-47.4) mU/l, 140 (964-305) mU/L and 160 (65-300) mU/L respectively.

Plasma prorenin levels above 225 mU/l were considered to be abnormal (10).

Urinary albumin was measured by a commercially available double-antibody radio-immunoassay (Diagnostic Products Corporation, Apeldoorn, The Netherlands). Hemoglobin A1 was measured by colorimetry (15), reference values: 4.5 to 5.8%.

GFR and ERPF were measured simultaneously as the clearance of $^{125}$I- thalamate and $^{131}$I- hippuran, respectively (16,17). To prevent ketosis and hyperglycaemia during the renal haemodynamic studies the patients received an intravenous glucose solution and regular acting insulin. Blood glucose was monitored every half hour and kept between 4.4 and 8.4 mmol/l by adjusting glucose infusion. After 4 hours to normalize and stabilize blood glucoses, GFR and ERPF were determined over a 2-hour period.

Retinopathy was assesed by fundoscopy through dilated pupils and classified by an opthalmologist for the worse eye as : absent, microaneurysms, exsudative retinopathy, and proliferative retinopathy.

### Statistical analysis

Data are expressed as mean ± SD or as mean ± SEM (figures) except urinary albumin excretion values and renin data which are given as the geometric mean and 95% confidence intervals. Changes in variables were analyzed by two-way analysis of variance according to Friedman. In addition, paired and unpaired Wilcoxon tests and

Kruskhal Wallis analysis of variance were used when appropriate. Adjustment for multiple comparisons was carried out using Duncan's method. Differences in proportions of variables among subgroups were assessed by chi-quare statistics or Fisher exact test. The independent contribution of parameters to progression of albuminuria and to plasma renin and prorenin was evaluated by multiple regression analysis. A two-sided p-value $< 0.05$ was considered to be significant.

## Results

Progression of albuminuria was assessed by comparing the pooled urinary albumin measurements obtained during the second year of follow-up with the baseline measurements. An increase of 25% or more from baseline was defined as progression. According to this criterion, 13 patients showed progression of albuminuria.

The clinical characteristics of patients with progression (n=13) and those without progression (n=12) of albuminuria are presented in Table 1.

There were no differences with respect to age, sex distribution, diabetes duration, insulin-dose, metabolic control between the two groups.

Body mass index was higher ($p<0.02$) and retinopathy was more severe ($p<0.05$) in the progressors as compared to the non-progressors.

At the start of the study urinary albumin excretion was not different between the progressors and non-progressors ($p>0.60$). A significant difference in urine albumin excretion was seen from 16 months on ward (Fig. 1).

Systolic blood pressure was significantly higher in the progressors at the start and during the course of the follow-up period (Fig. 2). Diastolic blood pressure was not significantly different ($p<0.07$) at baseline but was higher during the follow-up period of the study in the progressors.

At the start both GFR and ERPF were not different between the groups. (Table 2). GFR was significantly decreased after two years in both groups. The decrease in ERPF after two year follow-up was not significant in either group.

Table 1. Baseline characteristics of patients according to progression of albuminuria.

| | | No progression of albuminuria | Progression of albuminuria |
|---|---|---|---|
| Age | yr | 40 (21-59) | 48 (23-58) |
| Sex | male/female | 10/2 | 11/2 |
| Duration of diabetes | yr | 20 (5-45) | 21 (10-44) |
| Body mass index | $kg/m^2$ | 23.0±1.4 | 24.8±2.5 |
| Insuline dose | U/kg/day | 0.70±0.18 | 0.79±0.29 |
| $HbA1_c$ | % | 7.3±1.5 | 7.8±1.4 |
| Systolic blood pressure | mmHg | 120±2 | 135±4* |
| Diastolic blood pressure | mmHg | 72±2 | 77±2* |
| Retinopathy | a/m/e+p[1] | 4/2/6 | 0/7/6* |
| Albuminuria | µg/min | 32 (14-72) | 25 (14-25) |

Results are expressed as mean ± SD, when data are normally distributed and as geometric mean and 95% confidence interval when data are not normally distributed.

* $p < 0.05$ from patients without progression of albuminuria. (unpaired t-test for blood pressure, and Fisher exact test for presence or absence of retinopathy.

[1]) a= retinopathy absent, m= microaneurysm, e= exsudative retinopathy, p= proliferative retinopathy.

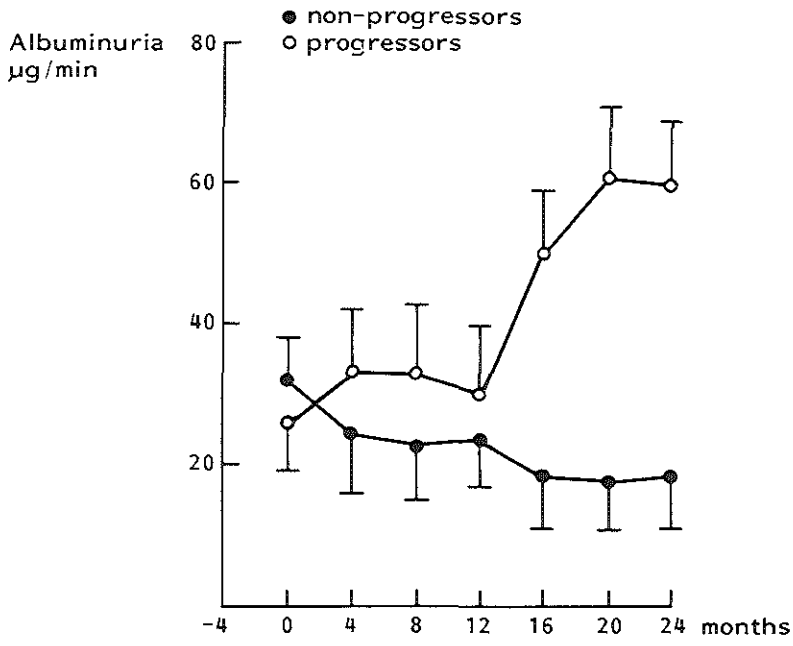


Fig.1 Albuminuria during two years follow up. Results are presented as geometric mean ± sem (antilog of mean after logarithmic transfomation). Progessors, open circles (n=13) and nonprogressors closed, circles (n=12).

Table 2. Renal hemodynamics. Comparison between patients with and without progression of albuminuria.

| Renal hemodynamic parameter | progression of albuminuria | start of study | 1 year follow-up | 2 years follow-up |
|---|---|---|---|---|
| Glomerular filtration rate (ml/min/1.73 m²) | no | 127±7 | 122±6 | 115±7** |
| | yes | 123±8 | 120±5 | 115±5** |
| Effective renal plasma flow (ml/min/1.73 m²) | no | 535±29 | 520±28 | 501±29 |
| | yes | 510±44 | 502±38 | 482±37 |

Data are mean ± SEM. ** $p < 0.01$ paired t-test with adjustment for multiple comparison for difference from value at the start of the study.

At the start both GFR and ERPF were not different between the groups. (Table 2). GFR was significantly decreased after two years in both groups. The decrease in ERPF after two year follow-up was not significant in either group.

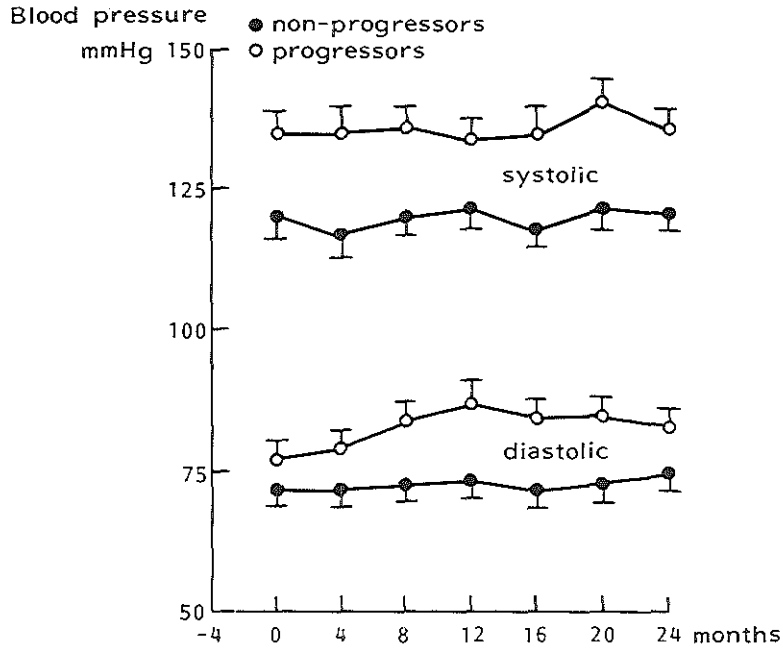


Fig.2 Systolic and diastolic blood pressure during two years follow up. Results are presented as mean ± sem. Progessors, open circles (n=13) and non-progressors, closed circles (n=12).

Metabolic control, as judged by HbA1 measurement, was comparable in the two groups during the study: HbA1, at baseline, during the first year of follow-up and during the second year of follow-up, was 7.9 ± 1.4%, 8.2 ± 1.1% and 8.0 ± 0.4% respectively in the progressors and 7.3 ± 1.5%, 8.5 ± 0.4% and 8.1 ± 0.4%, respectively, in the non-progressors.

The 24 hour urinary excretion rates of sodium and urea were not different in the two groups at the start and did not change significantly during the study. The mean urinary sodium excretion was 154 ± 48 and 170 ± 44 mmol/24h in the progressors and the non-progressors and the mean urea excretion was 371 ± 77 and 390 ± 82 mmol/24h, respectively.

Total renin (renin plus prorenin) and prorenin were not significantly different between the two groups at the start of the study ($p>0.40$).

Baseline plasma prorenin was abnormally elevated (above 225 mU/l) in only 6 out of the 25 patients. Total renin did not significantly change during the follow-up period (Table 3, Figure 3).

Table 3. Plasma levels of total renin in patients with and without progression of albuminuria.

| Progression of albuminuria | total renin (mU/L) | |
|---|---|---|
| | start of study | 2 year follow-up |
| no | 251 (184-343) | 240(187-341) |
| yes | 205 (159-265) | 185 (140-227) |

Data are expressed as geometric mean and 95% confidence interval.

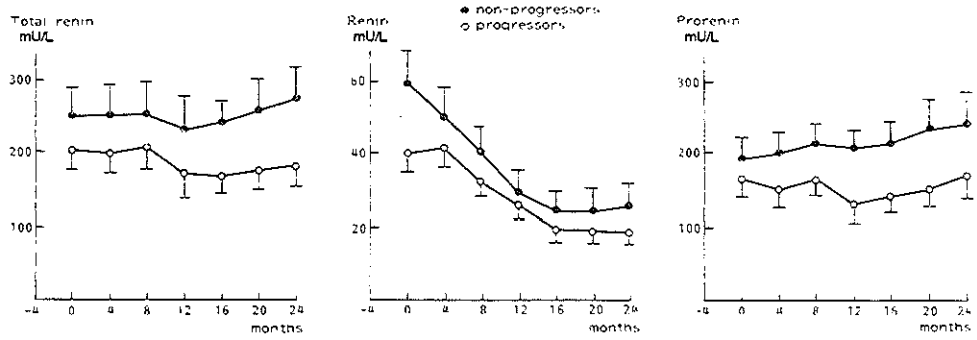


Fig.3 Plasma level of total renin, prorenin and renin during two years follow up. Results are presented as geometric mean ± sem (antilog of mean after logarithmic transfomation). Progessors, open circles (n=13) and non-progressors, closed circles (n=12).

Renin decreased significantly during follow-up (Fig. 3).
This finding can be explained by the time-related cryoactivation by which prorenin is converted to renin during handling of plasma at low temparature and storage at -20'C (see Methods). As is shown in Table 4, total renin were higher in patients with exsudative retinopahty or proliferative retinopathy.

Table 4. Plasma levels of total renin in patients with and without retinopathy at start of study after two year follow-up.

| Type of retinopathy | Total renin (mU/l) | |
|---|---|---|
| | start of study | 2 years follow-up |
| no | 150 (81-232) | 145 (97-232) |
| microaneurysm | 225 (156-316)* | 215 (147-316)* |
| exsudates and/or proliferation | 249 (177-355)* | 230 (166-130)* |

Data are expressed geometric mean and 95% confidence interval. * $p < 0.05$ with adjustment for multiple comparison after logarithmic transformation of the data, for difference with patients without retinopathy.

Progression of albuminuria (as the two years relative change in albuminuria after logaritmic transformation) was independently associated with baseline mean arterial blood pressure (p=0.001) and with the presence of retinopathy (p=0.001) but not with baseline GFR (p=0.17), baseline plasma prorenin (p=0.21) and baseline HbA1 (p=0.32).

The levels of urinary albumin excretion during the second year of follow-up was independently related to the baseline albuminuria (p<0.001), baseline mean arterial pressure (p= 0.001), but again not to baseline GFR (p= 0.17) and baseline plasma prorenin (p= 0.19). Urinary albumin excretion during follow-up was also correlated with the degree of retinoapthy at the start of the study (p= 0.01). The prorenin levels at the start of the study and during folow-up were independently related to the presence of retinopathy (p= 0.01 and 0.007), to the duration of diabetes (p= 0.03 and 0.03) and to the body mass index (p= 0.05 and 0.02). Prorenin was not related to GFR, mean arterial pressure and albuminuria either at the start of this study or during follow-up (p>0.5 for all parameters).

## Discussion

An important finding of this study was the lack of an association between the levels of prorenin and the progression of albuminuria. Progression of albuminuria was not related to baseline total renin and prorenin levels. Furthermore, plasma total renin and prorenin did not significantly change during the two years of follow-up. The present findings therefore do not support the hypothesis that patients with an early rise in albuminuria can be identified by plasma prorenin measurement.

It is possible that an elevated plasma prorenin is associated only with higher levels of microalbuminuria or even with macroalbuminuria.

Indeed, although it has been reported that a rise in plasma prorenin can precede the development of microvascular complications (12), the presence of macroalbuminuria was used as a sign of the presence of diabetic renal disease. Patients with either intermittent or continious microalbuminuria tended to have higher levels of plasma prorenin than patients without microalbuminuria, but the difference was not statistically significant. Only macroalbuminuria was significantly correlated with plasma prorenin (12). The lack of a correlation between elevated plasma prorenin and early abnormalities in albuminuria in our study is in agreement with recent prospective data in non-hypertensive Type 2 (non-insulin dependent) diabetic patients (18).

In our study the level of plasma prorenin was significantly related to the presence of rather advanced retinopathy which is in accordance with previous cross-sectional (8,10) and prospective studies (11), suggesting retinopathy to be more important factor related to plasma prorenin than microalbuminuria.

Another explanation of our results might be that elevated plasma prorenin is associated with a certain aspect of diabetc microangiopathy of which albuminuria is not a representative sign.

The mechanisms responsible for the association between high prorenin levels and diabetic complications are not known. Reduced renal clearance of prorenin due to alterations in the processing of prorenin to renin in the juxtaglomerular cells could be a mechanism. Reduced extra-renal clearance of prorenin due to impaired endothelial function or glycosylation of prorenin with impaired internalisation of plasma-derived prorenin as a result is another possibility (8,9,19,20). Finally, increased extra-renal production of prorenin through activation of local renin-angiotensin systems, for instance a local renin-angiotensin system in the eye, might also contribute to the high level of plasma prorenin in patients with diabetic retinopathy (21-23).

Another important finding of this study was the presence of a correlation between blood pressure and progression of albuminuria. Both systolic and diastolic blood pressure were significantly higher, albeit still in the normal range, in the second year in subjects with progression of albuminuria (Figure 1). These findings support the concept that a rise of blood pressure is associated with progression of renal involvement even

at a very early phase (24). This would strenghten the argument to lower blood pressure in microalbuminuric patients without hypertension (25-27). Progresssion of albuminuria was not significantly related to baseline GFR. In both groups (progressors and non-progressors) 4 patients had glomerular hyperfiltration at the start of the study. During follow-up GFR decreased independently of progression of albuminuria. Other studies have shown that glomerular hyperfiltration as such can contribute to the development of diabetic nephropathy (28,29) but this has not been consistently reported (30,31).

Improved metabolic control has been shown to delay the progression of albuminuria (32). In our study glycosylated haemoglobin was not different at the start and did not change during follow-up in both between progressors and non-progressors of albuminuria. This can probably explained by the fact that all patients were accustomed to self monitoring their blood glucose levels and thereby narrowing the range of metabolic control. Progression of albuminuria was also independently associated with the presence and severity of retinopathy suggesting a relation between the micro-angiopathy at the renal and retinal level.

In conclusion, prorenin measurement does not appear to adequately predict progression of albuminuria in Type 1 diabetic patients with slightly elevated urinary albumin excretion. Progression of albuminuria is correlated with blood pressure, even when blood pressure is in the normal range and renal involvement is at an very early stage.

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## Chapter 7

## SUMMARY

*7.1 Background and aim of research*

The renin-angiotensin system (RAS) plays an important role in the regulation of arterial blood pressure and water and salt regulation.

Until recently this system was considered to be exclusively a circulating endocrine system. Circulating renin, released by the renal juxtaglomerular cells, reacts with hepatically derived angiotensinogen (renin substrate) to form the inactive decapeptide angiotensin I (Ang I). Ang I, in turn, is converted to the biologically active octapeptide angiotensin II (Ang II) by angiotensin converting enzyme (ACE) which is bound to the endothelial cell membrane. Ang II formed in the circulation diffuses to the tissues where it stimulates specific receptors on the surface of vascular smooth muscle cells as well as on the surface of the aldosterone-producing cells in the adrenal glands.

More recent evidence, however, suggests that, in addition to the circulating endocrine RAS, there exist local or tissue renin-angiotensin systems in which Ang I and Ang II are formed in the tissues rather than in the circulation. Tissue production of these peptides may be catalyzed either by locally synthesized renin and renin substrate or by renin and renin substrate that are taken up from the plasma. Local renin-angiotensin systems have both autocrine and paracrine functions. Messenger RNA (mRNA) for renin has been demonstrated not only in the kidney but also in such organs as the adrenals, ovaries, testes, and brain, and mRNA for angiotensinogen has been found in all of these tissues as well. On the other hand, renin in the heart and blood vessel wall probably originates mainly in the kidneys and is then taken up from the circulation.

Human plasma contains not only renin but also its enzymatically inactive precursor prorenin. Virtually all plasma renin is derived from the kidney, as evidenced by the observation that circulating renin levels are extremely low or not demonstrable in anephric patients. The kidney is also an important source of circulating prorenin. However the finding that anephric patients have plasma prorenin levels that are 30-40% of normal indicates that a proportion of circulating prorenin is clearly of extrarenal origin. It is likely that such organs as the adrenals, testes, and ovaries release not renin but prorenin into the circulation. For example, the increased plasma prorenin levels seen in pregnant women are largely derived from the ovaries.

Unlike plama renin, plasma prorenin is increased in patients with diabetes mellitus who have microvascular complications. The mechanisms that account for the elevations in plasma prorenin levels seen in these patients are yet unknown. In patients receiving a beta-adrenoreceptor antagonist (beta-blocker), plasma renin levels fall and plasma prorenin levels rise.

Thus, it has been proposed that the development of autonomic neuropathy in patients

with diabetes mellitus may be responsible for the increase in plasma prorenin. Loss of sympathetic stimulation of the beta-adrenoreceptors on the juxtaglomerular cells might result in diminished secretion of renin and, as a compensatory mechanism, the synthesis and secretion of prorenin in the juxtaglomerular apparatus might be augmented

According to an alternative hypothesis, when the juxtaglomerular cells are affected by diabetic micrangiopathy, conversion of prorenin to renin in these cells decreases and secretion of prorenin consequently increases.

A third explanation for the elevated plasma prorenin levels may be increased production and secretion from an extrarenal source such as, for example, blood vessel walls damaged by diabetic micrangiopathy. It is also possible that the rise in plasma prorenin may be attributable not so much to increased production as to abnormal elimination of this prohormone from the circulation.

The first aim of our research was to address the question of whether an elevation in plasma prorenin is, in fact, an indicator of diabetic microangiopathy and, if so, with which type of complication (retinopathy, nephropathy, or neuropathy) is it associated ?.

The possibility that increased plasma prorenin may be linked to changes in carbohydrate metabolism was investigated by attempting to correlate hemoglobin A1c and blood glucose levels with prorenin levels in insulin-dependent as well as non-insulin-dependent patients.

The selective hyperproreninemia in diabetic patients may stem from increased renal or extrarenal production of prorenin or from decreased clearance, or a combination of the two. We studied the first possibility by comparing the renal arteriovenous gradient of prorenin in diabetic patients who had high prorenin levels with that in non-diabetic subjects.

Our group's previous research has shown the presence of a local RAS in the human eye. In addition, mRNAs for prorenin, angiotensinogen, and ACE have been demonstrated in the choroidal tissue. The clear correlation between elevated plasma prorenin levels and the presence of diabetic retinopathy pointed to the eye as a probable source of the increased prorenin. Accordingly, we studied the influence of panretinal laser coagulation on plasma prorenin levels in patients with proliferative diabetic retinopathy.

*7.2 Plasma prorenin in patients with or without diabetic microvascular complications.*

In a group of 223 patients with diabetes mellitus (Chapter 2) plasma prorenin levels correlated with the presence of nephropathy as well as with the presence of retinopathy, particularly the proliferative form. Retinopathy appeared to be a stronger determinant of prorenin concentration than did nephropathy, however. Plasma prorenin was already elevated in patients with proliferative retinopathy who still had urinary albumin concentrations in the normal range below 30 mg/24h. In contrast, plasma prorenin levels remained normal in patients who had proteinuria greater than 300 mg/24h but did not have retinopathy. The highest prorenin concentrations were observed in patients with both

nephropathy and retinopathy. Multiple regression analysis showed no significant influence of age, duration of diabetes, gender, blood glucose concentration, HbA1c concentration, insulin use, or the presence of neuropathy on the correlation between prorenin and the presence of retinopathy or nephropathy.

In seven patients with both serious nephropathy and retinopathy who underwent renal arteriography in preparation for eventual renal transplant, we found no significant difference between renal vein prorenin and renal artery prorenin levels, despite a marked elevation in peripheral plasma prorenin and a diminution in renal blood flow. It is thus unlikely that the increased plasma prorenin in patients with diabetes mellitus can be attributed solely to increased renal secretion.

In contrast, we found no elevations in plasma prorenin levels in 16 patients with serious non-diabetic autonomic neuropathy. This appears to rule out autonomic diabetic neuropathy as an explanation for the elevated plasma prorenin levels found in patients with microvascular complications.

*7.3 Prorenin in the ocular fluid of patients with diabetic retinopathy*

Extrarenal renin-angiotensin systems are found predominantly in highly vascular organs. These extrarenal systems appear to release prorenin but not renin into the extracellular fluid. It is possible that the intracellular conversion of prorenin to renin may lead to the formation of Ang I and Ang II. The eye is a highly vascular organ and retinal neovascularization is an important aspect of diabetic retinopathy. Recent research has shown that Ang II can stimulate vessel wall growth. Application of Ang II to the cornea of the rabbit eye stimulates corneal neovascularization. Moreover, Ang II receptors have been demonstrated in the retinal vessels.

We investigated the presence of diverse components of the RAS in ocular fluid obtained from human eyes during surgery in diabetic as well as nondiabetic patients (Chapter 3). Prorenin was present in aqueous humor, vitreous fluid, and subretinal fluid and its concentration in vitreous and subretinal fluid was many times higher than would be expected on the basis of the serum protein content of these fluids. In particular, the vitreous fluid from patients with diabetic retinopathy contained twice as much prorenin (relative to serum albumin) as did that from nondiabetic patients, whereas vitreous angiotensinogen content (relative to serum albumin) did not differ between the two groups. It is noteworthy that the vitreous fluid contained virtually no renin. These observations are consistent with local production of prorenin in the eye. Taken together with the recent discovery of mRNA expression of renin, angiotensinogen, and ACE in human eye tissue, our findings argue for the existence of an intraocular renin-angiotensin system and suggests a role for this system in the development of retinal neovascularization in diabetes mellitus.

*7.4 Effect of panretinal photocoagulation on plasma prorenin levels in patients with diabetic retinopathy*

Proliferative retinopathy is a major cause of blindness in patients with diabetes mellitus. There is evidence that retinal hypoxia sets the stage for neovascularization and that the production of growth factors by the ischemic retina plays an important role in this process. The treatment of proliferative diabetic retinopathy consits of panretinal photocoagulation, which leads to the regression of neovascularization. Although the mechanism of action of laser treatment remains to be clarified, one hypothesis is that this theapy reduces the production of growth factors.

We studied the effect of laser treatment on plasma prorenin levels in 12 patients with previously untreated proliferative retinopathy (Chapter 4).

The rationale for these studies was that the finding of a decrease in elevated plasma prorenin levels following laser treatment would provide evidence that prorenin is produced by the diabetic eye from which it then enters the circulation. Although the mean prorenin value did indeed decrease four weeks after the last laser treatment the results were not unequivocal. Plasma prorenin levels fell in only half of the patients and, despite this decrease, remained higher than normal. Thus, definitive conclusions cannot be drawn regarding the effect of laser treatment on plasma prorenin levels. Possible causes of the ambiguity in our results include the small number of patients studied, the lack of an control group, and the use of concomitant medications that can influence plasma prorenin levels.

It is not likely that leakage of prorenin from the eye contributes significantly to the elevated plasma prorenin levels found in patients with proliferative retinopathy. Low plasma flow in the eye (0.3 ml/min) and the relative long plasma half-life of prorenin (1-2h) would lead one to expect extremely high prorenin levels in one or more ocular fluid compartments. Such levels have not been found in the extracellular vitreous fluid of patients with diabetes.

*7.5 Plasma prorenin in diabetic patients with microalbuminuria*

There is currently no biochemical marker for the development and progression of diabetic microangiopathy. The identification of such a marker would be an important contribution to the diagnosis and management of patients with diabetes. In chapter 5 we describe the relationship between prorenin and microvascular complications in 164 diabetic patients with or without microalbuminuria. These patients did not have macroalbuminuria and were thus either free of nephropathy.

Plasma prorenin levels were correlated with the presence of retinopathy as well as microalbuminuria. The magnitude of the elevation in plasma prorenin level proved to be related to the seriousness of retinopathy or nephropathy, with the highest values found in patients who had both proliferative retinopathy and albuminuria. This cross-sectional study supports the possibility that an elevated plasma prorenin level may be an early

marker of progressive microangiopathy. Although microalbuminuria has been viewed by many investigators as an indicator of progressive microvascular nephropathy, it is a highly variable parameter, influenced by many factors including posture, exercise, diet, metabolic regulation, and arterial blood pressure.

We measured plasma prorenin levels and albumin excretion in a group of 72 patients with insulin-dependent diabetes on three different occasions over a four-week period. The variation coefficient of prorenin was only 9 %, as compared with 35 % for albumin excretion. Thus, prorenin measurements yielded far less variable results than did albuminuria determinations. Plasma prorenin measurements proved to have 72% specificity and 72% sensitivity as a diagnostic test for the presence of albuminuria in a group of 140 patients with insulin-dependent diabetes mellitus. The sensitivity and specificity of the test for the presence of retinopathy and/or albuminuria were 60% and 89%, respectively. We therefore concluded that an elevated plasma prorenin level is not a sufficiently sensitive marker for the presence of microangiopathy. Nevertheless, it is possible that plasma prorenin measurements can be used to follow the course of microvascular complications in patients with diabetes mellitus. Longitudinal studies will be necessary to supplement the data from our cross-sectional studies.

It is not yet known whether the elevation in plasma prorenin represents an early or late stage of microvascular complications. There is evidence that an increased plasma prorenin level may appear prior the first clinical manifestations of microvascular complications. We undertook a two-year prospective study of plasma prorenin concentrations and the progression of albuminuria in a group of 25 normotensive patients with insulin-dependent diabetes mellitus who had slightly elevated albumin excretion (Chapter 6). We observed no relationship between the progression of albuminuria and plasma prorenin values and found no changes in plasma prorenin during the two-year follow-up period. Progression of albuminuria was related to blood pressure (although blood pressure was not elevated according to WHO criteria) and to the presence of retinopathy. Retinopathy appeared to be a stronger determinant than microalbuminuria of elevated plasma prorenin.

These findings do not support the hypothesis that plasma prorenin measurements can be used as an early indicator of progression of albuminuria. It is possible that increase in plasma prorenin appear with higher albumin excretion or that elevated plasma prorenin levels are associated with certain aspects of microangiopathy that are unrelated to the development of microalbuminuria.

*7.6 Therapeutic implications*

Drugs that influence the RAS system, such as ACE inhibitors, have been shown to reduce albuminuria in patients with type 1 diabetes mellitus and nephropathy, even in those who are normotensive (1-4). There is evidence that treatment with ACE inhibitors slows the progression of diabetic nephropathy, through an action that is partially independent of the blood pressure lowering effects of these agents (5-7).

Our research provides clear evidence for the existence of an intraocular renin-angiotensin system and suggests a role for this system in the development of diabetic retinopathy. It is possible that elevated plasma prorenin levels may reflect stimulation of a local renin-angiotensin system in tissues with microvascular abnormalities.

Drugs that affect the RAS, such as ACE inhibitors, specific renin inhibitors, and Ang II antagonists may have therapeutic potential in patients with diabetic retinopathy. Parving et al (8) recently demonstrated that an ACE inhibitor decreased protein leakage from the retinal vessels in eyes with background retinopathy. Studies of the effect of ACE inhibitors on the development and progression of retinal neovascularization in patients with diabetes mellitus are certainly warranted.

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## Chapter 8

## SAMENVATTING

*8.1 Achtergrond en doel van het onderzoek*

Het renine-angiotensine systeem (RAS) speelt een belangrijke rol bij de regulatie van de arteriële bloeddruk en de water- en zouthuishouding.

Tot voor kort werd dit systeem uitsluitend als een in het plasma circulerend endocrien systeem beschouwd. Circulerend renine, afgescheiden door de juxtaglomerulaire cellen in de nieren, reageert met angiotensinogeen afkomstig uit de lever, waarbij het inactieve prohormoon angiotensine I (Ang I) wordt gevormd. Ang I wordt, door het aan de membraan van endotheelcellen gebonden angiotensine converterend enzym (ACE), omgezet in het biologisch actieve angiotensine II (Ang II). Het in de circulatie gevormde Ang II diffundeert naar de weefsels en stimuleert specifieke receptoren op het celoppervlak van gladde spiercellen in de vaatwand en op het oppervlak van de aldosteron producerende cellen in de bijnierschors.

Behalve dit circulerende endocriene renine-angiotensine systeem zijn er inmiddels aanwijzingen voor het bestaan van lokale of weefsel renine-angiotensine systemen. Lokaal wil zeggen dat Ang I en II niet in de circulatie worden gevormd maar in de weefsels. Dit kan door lokaal gesynthetiseerd renine en renine substraat (angiotensinogeen) of door renine en renine substraat afkomstig uit het plasma. Lokale renine-angiotensine systemen hebben autocriene and paracriene functies.

mRNA's van renine en angiotensinogeen zijn o.a. aangetoond in de nier, bijnier, ovarium, testis en hersenen. Renine in het hart en de vaatwand is waarschijnlijk grotendeels afkomstig uit de circulatie en is geproduceerd door de nier.

Menselijk plasma bevat naast renine ook prorenine, de enzymatisch inactieve voorloper van renine. Vrijwel al het renine in het plasma is afkomstig van de nier; bij nierloze patiënten is het circulerende renine zeer laag of niet aantoonbaar. De nier is ook een belangrijke bron van het circulerende prorenine, echter een deel van het circulerende prorenine heeft een extra-renale oorsprong; bij nierloze patiënten is het prorenine in plasma 30-40 % van normaal. Organen, zoals bijnier, testis en ovarium, geven waarschijnlijk prorenine aan het circulerende bloed af en geen renine. Het verhoogde plasma prorenine bij zwangere vrouwen bijvoorbeeld is voor een groot gedeelte afkomstig uit het ovarium.

Plasma prorenine is, in tegenstelling tot plasma renine, verhoogd bij patiënten met diabetes mellitus met microvasculaire complicaties.

Welk mechanisme aan dit verhoogde plasma prorenine ten grondslag ligt, is nog onbekend. Bij patiënten die met een beta-adrenoceptor antagonist (beta-blokker) worden behandeld daalt het plasma renine en stijgt het plasma prorenine. Er is daarom geopperd dat het optreden van autonome neuropathie bij diabetes mellitus verantwoordelijk

zou zijn voor het verhoogde circulerende plasma prorenine. Verlies van sympathische stimulatie van de beta-adrenoceptoren op de juxtaglomerulaire cellen in de nier zou een verminderde secretie van renine geven. Hierdoor zou compensatoir een toename van de synthese en secretie van prorenine in het juxtaglomerulaire apparaat optreden.

Een andere hypothese gaat uit van een verminderde omzetting van prorenine tot renine in de juxtaglomerulaire cellen en dientengevolge een verhoogde secretie van prorenine, wanneer deze cellen door diabetische microangiopathie zijn aangetast. Een derde verklaring voor het verhoogde prorenine in plasma zou kunnen zijn dat er toegenomen productie en secretie is vanuit een extra-renale bron, bijvoorbeeld vanuit de door microangiopathie aangetaste vaatwand. Tenslotte is het mogelijk dat niet zozeer de productie van prorenine maar de eliminatie van prorenine uit de circulatie abnormaal is.

Het eerste doel van ons onderzoek had betrekking op de vraag of een verhoogd plasma prorenine inderdaad een indicator is van diabetische microangiopathie en met welke type van complicaties (retinopathie, nephropathie of neuropathie) het verband houdt. De mogelijkheid dat verhoogd prorenine verband houdt met veranderingen in het koolhydraatmetabolisme bij diabetes werd onderzocht door het hemoglobine A1c en bloed glucose te correleren met de prorenine spiegel en door zowel insuline-afhankelijke als niet insuline-afhankelijke patiënten te bestuderen.

De selectieve hyperproreninemie bij diabetes kan samenhangen met een verhoogde renale of extra-renale productie van prorenine of met een verminderde klaring of met een combinatie van beiden. De mogelijkheid van verhoogde renale productie werd onderzocht door de arterioveneuze gradient van prorenine over de nier bij patienten met diabetes met een hoog prorenine te vergelijken met de gradient bij patienten zonder diabetes mellitus.

Uit eerder onderzoek van onze groep was duidelijk geworden dat er in het menselijk oog een lokaal renine-angiotensine systeem aanwezig is. In het choroïdeale weefsel konden mRNA's prorenine, angiotensinogeen en ACE worden aangetoond. Omdat een verhoogd plasma prorenine duidelijk gecorreleerd bleek te zijn met de aanwezigheid van diabetische retinopathie zou het oog een bron van het verhoogde prorenine kunnen zijn. Daarom werd bij patienten met proliferatieve retinopathie de invloed van panretinale lasercoagulatie op de plasmaspiegel van prorenine bestudeerd.

*8.2 Prorenine in plasma van patienten met en zonder diabetische microvasculaire complicaties*

Bij een groep van 223 patienten met diabetes mellitus (hoofdstuk 2) bleek dat de plasmaspiegel van prorenine was gecorreleerd zowel met de aanwezigheid van nephropathie als met retinopathie, vooral de proliferatieve vorm.

Retinopathie leek een sterkere determinant te zijn dan nephropathie. Immers plasma prorenine was al verhoogd bij patienten met proliferatieve retinopathie maar met albuminurie die nog in het normale gebied tot 30 mg/24h lag. Verder bleek dat in de groep met een proteinurie van meer dan 300 mg/24h, maar zonder retinopathie, de plasmaspiegel

van prorenine normaal te zijn. De hoogste proreninespiegels werden gevonden bij de combinatie van nephropathie en retinopathie. Multiple regressie analyse toonde dat leeftijd, duur van de diabetes, geslacht, bloedglucose, HbA1c, insulinegebruik, en de aanwezigheid van neuropathie geen significante bijdrage leverden tot de correlatie tussen prorenine en de aanwezigheid van retinopathie of nephropathie.

Bij 7 patienten met ernstige gecombineerde nephro- en retinopathie, die een nierarteriografie moesten ondergaan in het kader van een toekomstige niertransplantatie, vonden wij geen significant verschil tussen prorenine in de niervene en de nierarterie, ondanks een sterk verhoogd prorenine gehalte in het perifere plasma en een verminderde nierdoorstroming. Dit maakt het niet waarschijnlijk dat het verhoogde prorenine bij diabetes mellitus alleen verklaard zou kunnen worden door toegenomen renale secretie.

Bij 16 patienten met een ernstige autonome neuropathie door een andere oorzaak dan diabetes mellitus, werden geen verhoogde plasma proreninespiegels aangetoond, zodat ook autonome diabetische neuropathie niet de verklaring lijkt te zijn voor het verhoogde plasma prorenine bij patienten met microvasculaire complicaties.

*8.3 Prorenine in oogvocht van patienten met diabetische retinopathie*

Extra-renale renine-angiotensine systemen worden vooral aangetroffen in vaatrijke organen. Anders dan de nier blijken de extra-renale systemen geen renine maar wel prorenine af te kunnen geven aan de extracellulaire vloeistof. Prorenine kan mogelijk, via intracellulaire conversie tot renine, leiden tot de vorming van angiotensines. Het oog is een sterk gevasculariseerd orgaan en retinale vaatnieuwvorming is een belangrijk aspect van diabetische retinopathie. Recent onderzoek heeft aangetoond dat angiotensine II vaatwandgroei kan bevorderen. Angiotensine II applicatie op de cornea van het konijne-oog stimuleert vaatnieuwvorming in de cornea. Tevens zijn er angiotensine II receptoren aangetoond in de retinale vaten.

Wij onderzochten de aanwezigheid van de diverse componenten van het renine-angiotensine systeem in oogvloeistoffen verkregen uit menselijke ogen tijdens oogoperaties bij patienten met en zonder diabetes mellitus (hoofdstuk 3). Prorenine was aanwezig in kamerwater, glasvocht en subretinaalvocht.

Het prorenine in glasvocht of subretinaal vocht was vele malen hoger dan op grond van het gehalte aan serumeiwitten van deze vochten verwacht kon worden. Bovendien bevatte het glasvocht van patienten met diabetische retinopathie tweemaal zoveel prorenine (t.o.v. serumalbumine) als dat van patiënten zonder diabetes, terwijl het gehalte aan angiotensinogeen (t.o.v. serumalbumine) niet verschilde. Opvallend was dat glasvocht vrijwel geen renine bevatte. Deze bevindingen passen bij locale productie van prorenine in het oog. Dit, tezamen met de recente ontdekking van mRNA-expressie van renine, angiotensinogeen, en ACE in humaan oogweefsel, duidt op het bestaan van een intra-oculair renine-angiotensine systeem en suggereert een rol van dit intra-oculaire systeem bij de ontwikkeling van retinale vaatnieuwvorming bij diabetes mellitus.

*8.4 Effect van panretinale photocoagulatie op de plasma prorenine spiegel bij patienten met diabetische retinopathie.*

Proliferatieve retinopathie is een belangrijke oorzaak van blindheid bij patienten met diabetes mellitus. Er zijn aanwijzingen dat retinale hypoxie ten grondslag ligt aan deze vaatnieuwvorming en dat de productie van groeifactoren door de ischaemische retina hierbij een belanrijke rol speelt. De behandeling van proliferatieve diabetische retinopathie bestaat uit panretinale photocoagulatie, welke leidt tot regressie van de vaatnieuwvorming. Het werkingsmechanisme van laserbehandeling is nog niet opgehelderd. Een van de veronderstellingen is dat lasertherapie de productie van groeifactoren vermindert.

We onderzochten het effect van laserbehandeling op de plasma prorenine spiegel bij 12 patienten met onbehandelde proliferatieve retinopathie (hoofdstuk 4). Het idee hierachter was dat, als na lasertherapie een daling wordt gevonden van het verhoogde plasma prorenine, dit zou pleiten voor productie en afgifte van prorenine naar het bloed door het diabetische oog. Hoewel de gemiddelde waarde van prorenine 4 weken na de laatste laser behandeling gedaald was, waren de resultaten toch niet éénduidig. Slechts bij de helft van de patienten daalde het plasma prorenine, en het bleef ondanks de daling nog steeds verhoogd. Definitieve conclusies omtrent het effect van laserbehandeling op de plasma proreninespiegel kunnen dan ook niet getrokken worden. Het kleine aantal onderzochte patienten, het ontbreken van een controlegroep en het gebruik van co-medicatie, die het plasma prorenine kan beinvloeden, kunnen oorzaken zijn van onze niet éénduidige resultaten.

Het is trouwens à priori niet erg waarschijnlijk dat lekkage van prorenine uit het oog in belangrijke mate bijdraagt tot het verhoogde plasma prorenine bij patienten met proliferatieve retinopathie.

Op grond van een lage plasma doorstroming in het oog (0.3ml/min) en een relatief lange plasma halfwaardetijd van prorenine (1 tot 2 uur), verwacht men extreem hoge proreninespiegels in één of meer oogcompartimenten van het oog. Echter, zulke extreem hoge spiegels zijn niet gemeten in glasvocht van ogen aangedaan door proliferatieve retinopathie.

*8.5 Prorenine in plasma van diabetes patienten met microalbuminurie*

Tot op heden is er geen geschikte (biochemische) plasmamarker voor het ontstaan en de ontwikkeling van diabetische microangiopathie bij de diabetes patient. Het bestaan van een dergelijke marker zou een belangrijke bijdrage kunnen leveren tot het diagnostisch en therapeutisch beleid van diabetes patienten. In hoofdstuk 5 beschrijven we bij 164 diabetes patienten met en zonder microalbuminurie de relatie tussen prorenine en microvasculaire complicaties. Deze patienten hadden dus geen macro-albuminurie en waren dus voor wat betreft de nephropathie in een vroeg stadium. Plasma prorenine was gerelateerd aan de aanwezigheid van zowel retinopathie als microalbuminurie. De mate van plasma prorenine verhoging was gerelateerd aan de ernst van zowel retinopathie als

nephropathie; de hoogste waarden werden gevonden bij patienten met gecombineerde proliferatieve retinopathie en albuminurie.
Deze cross-sectionele studie wijst op de mogelijkheid dat een verhoogd plasma prorenine een vroege marker zou kunnen zijn voor progressieve microangiopathie. Microalbuminurie wordt door vele onderzoekers gezien als een indicator voor progressieve microvasculaire nephropathie. Microalbuminurie is echter een zeer variabele parameter. De albumine excretie via de nier wordt beinvloed door vele factoren, zoals houding, inspanning, dieet, metabole regulatie en arteriele bloeddruk.

Bij een groep van 72 patienten met insuline-afhankelijke diabetes mellitus werden prorenine en de albumine uitscheiding op drie verschillende tijdstippen binnen een periode van 4 weken bepaald. De variatiecoefficient van de prorenine uitslagen bedroeg 9% tegenover 35% voor de albuminurie uitkomsten. Bepalingen van prorenine geven dus veel minder variabele resultaten dan bepalingen van de microalbuminurie.
De sensitiviteit en specificiteit van een plasma prorenine bepaling, in een groep van 140 patienten met insuline afhankelijke diabetes mellitus, als diagnostische test voor de aanwezigheid van albuminurie waren resp. 0.72 en 0.72. De sensitiviteit en specificiteit voor het aantonen van retinopathie en/of albuminurie was 0.60 en 0.89.
Een verhoogd plasma prorenine is dus zeker geen gevoelige indicator voor de aanwezigheid van microangiopathie. Misschien dat plasma prorenine wel gebruikt kan worden voor de follow-up van microvasculaire complicaties. Longitudinaal onderzoek dient daarom onze cross-sectionele gegevens aan te vullen.

Het is onbekend of de verhoging van het plasma prorenine in een vroeg of laat stadium van de microvasculaire complicaties optreedt Er zijn aanwijzingen dat een verhoging van het plasma prorenine reeds kan optreden voordat de eerste tekenen van microvasculaire complicaties klinisch manifest worden.

Bij een groep van 25 normotensieve patienten met insuline- afhankelijke diabetes mellitus en een slechts licht verhoogde albumine uitscheiding via de nier onderzochten wij prospectief gedurende 2 jaar de plasma prorenine concentraties en de progressie van albuminurie (hoofdstuk 6). Progressie van albuminurie was niet gerelateerd aan de plasma prorenine waarde en plasma prorenine veranderde niet tijdens de follow-up periode.
Progressie van albuminurie was gerelateerd aan de bloeddruk (ook al was deze niet verhoogd volgens de WHO criteria) en aan de aanwezigheid van retinopathie. Retinopathie leek een sterkere determinant voor verhoogd plasma prorenine te zijn dan microalbuminurie.

Deze gegevens ondersteunen niet de hypothese dat een vroege toename van microalbuminurie door plasma prorenine bepalingen opgespoord zou kunnen worden. Het is mogelijk dat pas bij een hogere albumine excretie een stijging van plasma prorenine optreedt of dat een verhoogd plasma prorenine geassocieerd is met bepaalde aspecten van microangiopathie die geen verband kenden met het ontstaan van microalbuminurie.

*8.6 Therapeutische implicaties*

Studies met geneesmiddelen die ingrijpen in het RAS syteem zoals de ACE-remmers tonen bij patienten met type I diabetes mellitus en nephropathie een reductie van de albuminurie, zelfs bij normotensieve patienten (1-4).

Er zijn aanwijzingen dat behandeling met ACE-remmers de progressie van diabetische nephropathie, deels onafhankelijk van het effect op de bloeddruk, vertraagt (5-7).

Ons onderzoek toont duidelijke aanwijzingen voor het bestaan van een intra-oculair renine-angiotensine systeem en suggereert een rol voor dit systeem bij het ontstaan van diabetische retinopathie. Het zou kunnen zijn dat een verhoogd plasma prorenine een uiting is van een gestimuleerd locaal renine-angiotensine systeem in weefsels waarin de microvasculaire afwijkingen zich ontwikkelen.

Geneesmiddelen die ingrijpen in dit systeem, zoals ACE-remmers, specifieke renine-remmers en Angiotensine II antagonisten, hebben misschien een therapeutisch effect bij diabetische retinopathie. Parving et al (8) toonden recentelijk aan dat een ACE-remmer de eiwitlekkage vanuit de retinale vaten in ogen met achtergrondsretinopathie verminderde. Studies gericht op het effect van ACE-remmers op het ontstaan en de progressie van retinale neovascularisatie bij patienten met diabetes mellitus, zijn dan ook zeker aangewezen.

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## NAWOORD

Dit proefschrift is bewerkt op de afdeling Inwendige Geneeskunde I van het Academisch Ziekenhuis Dijkzigt. Zoals vaak is ook dit werk tot stand gekomen met de hulp van velen. Zonder de ongenoemden tekort te willen doen wil ik een enkeling met name bedanken.

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## Curriculum Vitae

De schrijver van dit proefschrift werd geboren op 9 november 1956 te Bergen op Zoom. Na het behalen van het diploma Atheneum-B aan de Rijksscholengemeenschap te Bergen op Zoom in 1975, begon hij in datzelfde jaar de studie geneeskunde aan de Medische Faculteit van de Erasmus Universiteit te Rotterdam. Na het artsexamen in 1981 vervulde hij aansluitend de militaire dienstplicht als officier-arts bij de Koninklijke Landmacht. In juni 1983 starte hij met de huisartsenopleiding aan het Huisartseninstituut van de Erasmus universiteit, welke 1 juni 1984 werd afgerond.

In de periode 1 januari 1985 tot 1 januari 1990 volgde hij de opleiding interne geneeskunde op de afdeling Inwendige Geneeskunde 1 van het Academisch Ziekenhuis Rotterdam-Dijkzigt (hoofd: Prof. Dr. M.A.D.H. Schalekamp). Op 1 januari 1990 werd hij geregistreerd als internist.
Vanaf 1 april 1990 is hij werkzaam als algemeen internist in het Wilhelmina ziekenhuis te Assen.

## List of abbreviations

| | |
|---|---|
| ACE | Angiotensin converting enzyme |
| Ang I | Angiotensin I |
| Ang II | Angiotensin II |
| BGDR | Background diabetic retinopathy |
| ERPF | Effective renal plasma flow |
| GFR | Glomerular filtration rate |
| IDDM | Insulin-dependent diabetes mellitus |
| NIDDM | Non-insulin-dependent diabetes mellitus |
| NODR | No (without) diabetic retinopathy |
| PDR | Proliferative diabetic retinopathy |
| PRA | Plasma renin activity |
| RAS | Renin-angiotensin system |
| RS | Renin substrate (angiotensinogen) |