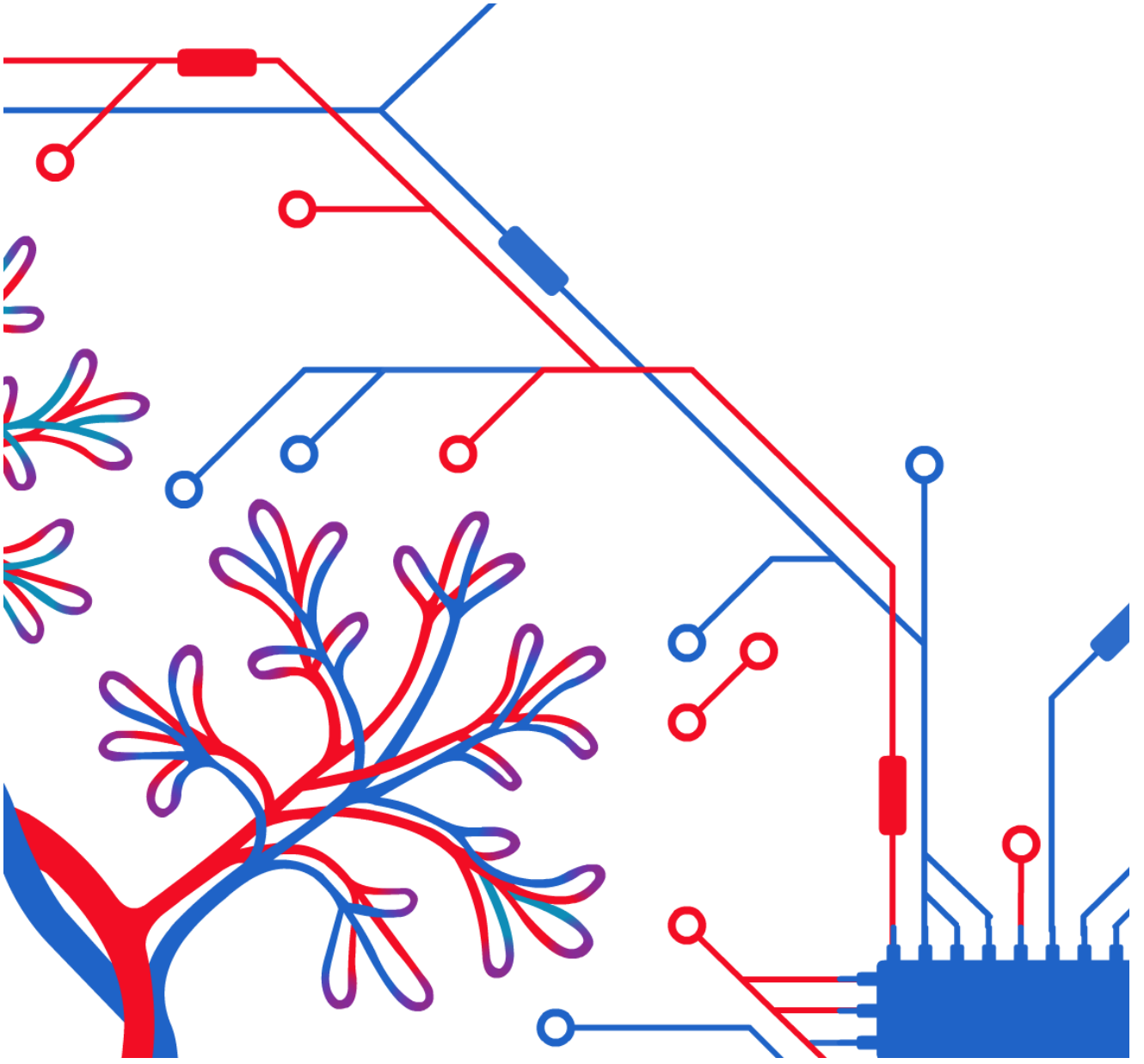
Preeclampsia, the Renin-Angiotensin-Aldosterone System and beyond

Koen Verdonk



Preeclampsia, the Renin-Angiotensin-Aldosterone System and beyond
Thesis, Erasmus University, Rotterdam. With summary in Dutch and English.
ISBN: 978-94-6203-831-8
Cover and chapter design: Simon Hattinga Verschure
Printing: CPI Koninklijke Wöhrmann (www.cpibooks.com/nl/)

Preeclampsia, the Renin-Angiotensin-Aldosterone System and beyond

Preeclampsie, het renine-angiotensine-aldosteron systeem en meer.

Proefschrift

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de rector magnificus
Prof.dr. H.A.P. Pols
en volgens besluit van het College voor Promoties

De openbare verdediging zal plaatsvinden op

woensdag 3 juni 2015 om 13.30 uur

door

Koen Verdonk

geboren te Groningen

Promotiecommissie

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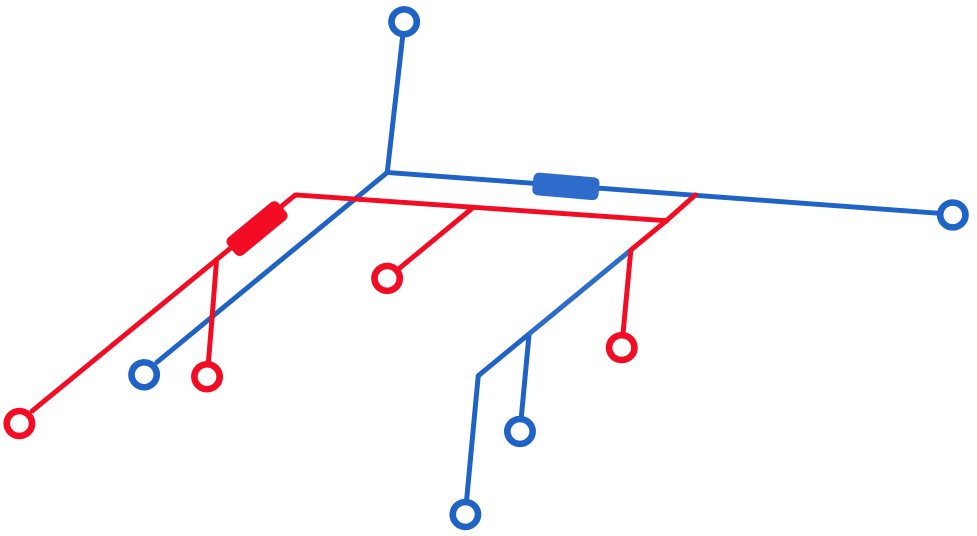

Chapter 1

General introduction

Angiotensin II, the active end-product of the renin-angiotensin-aldosterone system (RAAS), exerts its effects via angiotensin II type 1 (AT1) and type 2 (AT2) receptors. AT1-receptors mediate all well-known effects of angiotensin II, such as vasoconstriction, sodium retention, inflammation and tissue remodeling. Blockade of the RAAS, by inhibiting the formation of angiotensin II or blocking the AT1-receptor, is very effective in the treatment of cardiovascular disease. AT2-receptors are believed to counteract the effects mediated by the AT1-receptor. Therefore, stimulation of this receptor may be an interesting target for the treatment of cardiovascular disease. C21 is a selective AT2-receptor agonist, recently developed aiming to treat cardiovascular disease.
However, the general view that AT2-receptors exclusively exert beneficial effects has been challenged. For example in pathological models, their function sometimes mimics that of AT1-receptors, inducing vasoconstriction and cardiac hypertrophy.[1, 2] Yet, given its upregulation in various pathological conditions, the AT2-receptor remains a promising target for treatment, allowing effects beyond blood pressure-lowering, for example in stroke, reducing aneurysm formation,[3, 4] inflammation and myocardial fibrosis.

Recent interest has focused on the presence of renin and angiotensinogen in urine, as markers of RAAS activity within the kidney. Indeed, if the renal levels of both proteins reflect their production at renal tissue sites, their measurement would be a simple tool to determine whether the renal RAS is upregulated, thereby reinforcing the need for (more intensive) treatment with a RAS blocker. Obviously, before drawing this conclusion, we need to be certain that these urinary proteins, like albumin, do not simply reflect breakdown of the glomerular filtration barrier, i.e., that they are kidney- and not plasma-derived. If plasma-derived, their clinical value on top of a much cheaper urinary albumin measurement remains questionable.

Pregnancy demands major changes of the cardiovascular system, and this involves, among others, activation of the RAAS, allowing an aldosterone-dependent increase in the extracellular volume compartment. Remarkably, a relative resistance to the angiotensin II pressor response develops simultaneously to prevent the increase in blood pressure that normally is a result of RAAS activation when extracellular volume is increased. The increase in volume, the degree of RAAS activation and the diminished pressor response to angiotensin II are less pronounced in preeclampsia, a pregnancy-associated condition of elevated blood pressure and proteinuria. In animal models displaying excessive RAAS activation a preeclampsia-like syndrome develops during pregnancy, and in this situation the aldosterone/renin ratio is elevated compared to normal pregnancy.

Whether the RAAS plays a role in the pathophysiology of preeclampsia or whether it is just an epiphenomenon has still to be elucidated.
New insights into the pathogenesis of preeclampsia have revealed a major role for vascular endothelial growth factor (VEGF), the soluble VEGF-type 1 receptor inhibitor sFlt-1, and low levels of placental growth factor (PlGF, a member of the VEGF family). The ratio of sFlt-1 and PlGF has shown to be increased in preeclampsia. Whether this ratio can be utilized as a predictor for preeclampsia or its complications is under investigation.[5]

Aims of the thesis

In **Part 1** the function of the AT2-receptor in health and disease is discussed as well as the therapeutic potential of a novel agonist for this receptor. The aim of **Chapter 2** is to summarize the evidence that the AT2-receptor truly antagonizes AT1-receptor-mediated effects and what the role of the AT2-receptor is in diseased states. **Chapter 3** aims to clarify contradicting findings about the function of C21, a selective AT2-receptor agonist. While investigating the role of the RAAS in normal pregnancy and pregnancy complicated by preeclampsia, we also aimed to find out whether the function of the AT2-receptor changes in preeclampsia (**Chapter 11**).

In **Part 2** we investigate the use of RAAS components in urine as potential markers for intrarenal RAAS activity. In **Chapter 4** we summarize currently known evidence about the existence of a local RAAS in the kidney and we aim to find components in urine that could be a proxy for this activity. **Chapter 5** compares a novel method to measure renin with established methods of renin measurement in diverse body fluids both under physiological and pathological conditions.

Part 3 of this thesis covers recent developments about the role of angiogenic imbalance in the pathogenesis of preeclampsia, and the value of the sFlt-1/PlGF ratio as a biomarker to diagnose preeclampsia and to predict its prognosis. **Chapter 6** summarizes recent findings in the pathogenesis of preeclampsia. The aim of **chapter 7** is to provide an overview of the effects of a disturbed angiogenic balance in preeclampsia and how this relates to the well-known adaptations of the RAAS observed in this condition.
The quantification of proteinuria is still a cornerstone in the diagnosis of preeclampsia. In **chapter 8** we compare the much easier to use protein-to-creatinine ratio with the 24-hour urinary protein measurement in diagnosing clinically relevant proteinuria in patients suspected of preeclampsia.

In **chapter 9** we investigate the usefulness of the sFlt-1/PlGF ratio biomarker for distinguishing preeclampsia from other causes of hypertensive and proteinuric disease during pregnancy. The use of this ratio for the diagnosis of preeclampsia is further explored in **chapter 10**, where this test is applied in a high-risk patient cohort.
Chapter 11 aims to investigate the consequences of a disturbed angiogenic balance on the systemic RAAS. Additionally we investigated if an activated renal RAAS is present in preeclamptic women and whether this is reflected by components of the RAAS in urine.

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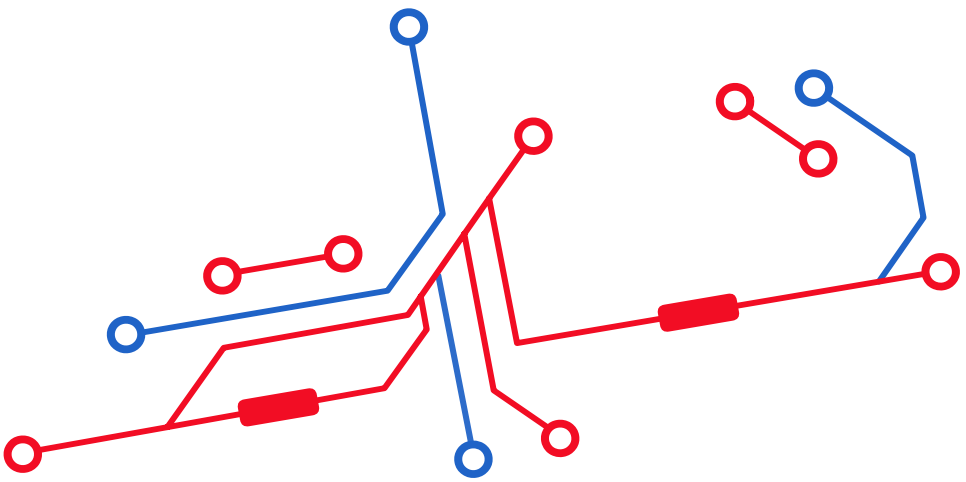
PART 1



Chapter 2
Angiotensin II type 2 receptor agonists: where should they be applied?

Expert Opin Investig Drugs. 2012 Apr;21(4):501-13.

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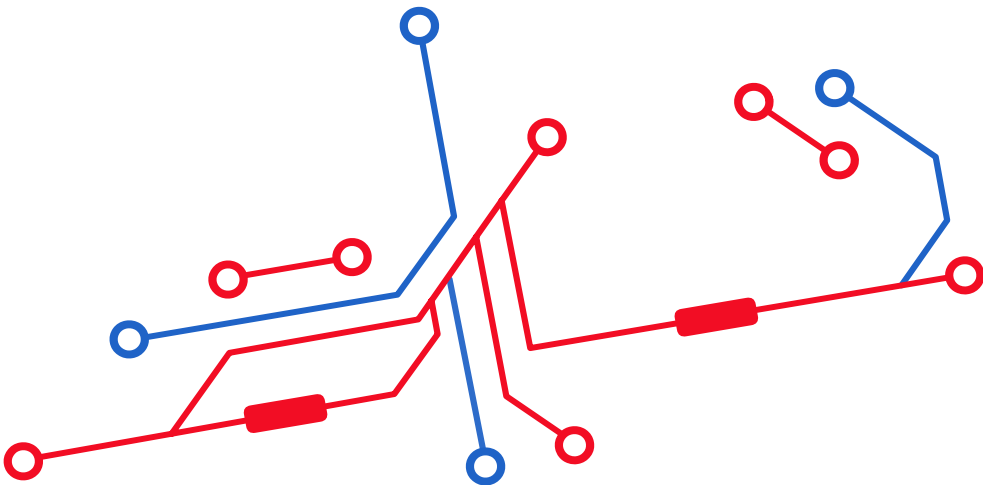

ABSTRACT

Introduction: Angiotensin II, the active endproduct of the renin–angiotensin system (RAS), exerts its effects via angiotensin II type 1 and type 2 (AT_1, AT_2) receptors. AT_1 receptors mediate all well-known effects of angiotensin II, ranging from vasoconstriction to tissue remodeling. Thus, to treat cardiovascular disease, RAS blockade aims at preventing angiotensin II–AT_1 receptor interaction. Yet RAS blockade is often accompanied by rises in angiotensin II, which may exert beneficial effects via AT_2 receptors.

Areas covered: This review summarizes our current knowledge on AT_2 receptors, describing their location, function(s), endogenous agonist(s) and intracellular signaling cascades. It discusses the beneficial effects obtained with C21, a recently developed AT_2 receptor agonist. Important questions that are addressed are do these receptors truly antagonize AT_1 receptor-mediated effects? What about their role in the diseased state and their heterodimerization with other receptors?

Expert opinion: The general view that AT_2 receptors exclusively exert beneficial effects has been challenged, and in pathological models, their function sometimes mimics that of AT_1 receptors, for example, inducing vasoconstriction and cardiac hypertrophy. Yet given its upregulation in various pathological conditions, the AT_2 receptor remains a promising target for treatment, allowing effects beyond blood pressure-lowering, for example, in stroke, aneurysm formation, inflammation and myocardial fibrosis.

Keywords*:* angiotensin, angiotensin II type 2 receptor agonist, C21, heterodimer, hypertrophy, inflammation, Marfan's syndrome, natriuresis, neuroprotection, vasorelaxation

1. INTRODUCTION

The renin–angiotensin system (RAS) not only is involved in the regulation of blood pressure and body fluid homeostasis but also plays a major role in the pathophysiology of hypertension, renal disease and heart failure. From a therapeutic point of view, the RAS pathway is of great interest as inhibition at several different levels of the pathway has been proven to be effective for the treatment of hypertension and cardiovascular disease. Angiotensin (Ang) II is considered to be the main biologically active component of the RAS and its formation is initiated by renin cleaving Ang I from angiotensinogen, which in turn is converted into Ang II by angiotensin-converting enzyme (ACE). Although Ang II can be formed in the circulation, it is now generally accepted that the generation at tissue sites is of greater importance. Such generation depends on the uptake of liver-derived angiotensinogen and kidney-derived renin from the circulation and occurs extracellular (e.g., on the cell surface) rather than intracellular.[1-10] A recent suggestion that cardiac renin is mast cell derived could not be confirmed.[11, 12] Ang II mediates its effects via Ang II type 1 (AT_1) and Ang II type 2 (AT_2) receptors. AT_1 receptors are widely expressed throughout the body and mediate all well-known effects of Ang II including vasoconstriction, sympathetic activation, sodium and water retention, production of reactive oxygen species (ROS),

tissue remodeling (cell growth, migration, proliferation, hypertrophy and survival) and inflammation. AT_1 receptors also play an important role in the negative feedback system within the RAS by inhibiting renin synthesis and release.[13,14] During AT_1 receptor blockade, Ang II levels rise and stimulation of the unblocked AT_2 receptors might subsequently contribute to the beneficial effects of this class of drugs. [15] Although their function is still not fully elucidated, AT_2 receptors in general are believed to function as an endogenous antagonist of the AT_1 receptor.[16-23] However, there also are reports of AT_2 receptors mimicking the function of the AT_1 receptor.[24-29] The development of selective AT_2 receptor agonists might lead to a better understanding of the properties of this receptor under (patho)physiological conditions and could even provide a new avenue for the treatment of cardiovascular diseases. In this paper, we review the role of the AT_2 receptor under (patho)physiological conditions and the potential therapeutic value of AT_2 receptor stimulation.

2. THE AT2 RECEPTOR: EXPRESSION AND SIGNALING

The AT_2 receptor gene was first cloned in 1993.[30] The AT_2 receptor gene shares 34% sequence homology with its AT_1 receptor counterpart and encodes for a protein of 363 amino acids with a molecular mass of 41 kDa. In contrast to the AT_1 receptor, which is mapped to autosomes, the AT_2 receptor is located on the X chromosome in both humans and rodents. Autoradiography, ligand binding and in situ hybridization experiments have shown that AT_2 receptors are the predominant subtype in the fetus. Its expression rapidly declines after birth, when the AT_1 receptor becomes the dominant subtype.[31-37] In contrast with this generally accepted concept, Yu and coworkers recently showed that AT_2 expression gradually increased from fetal life to adulthood.[38] The reason for this discrepancy is unknown, but it is clear that not all techniques are equally suitable to quantify AT_2 receptor expression. Some recognize only the mature AT_2 receptors on the membrane, whereas others may simultaneously detect immature, cytoplasmic AT_2 receptors. In addition, the specificity of the AT_2 receptor antibodies is not always clear. Thus, it is important to keep in mind that we do not know whether mRNA and/or protein expression always correlates with actual binding to the receptor and what the functional consequences of expression are. In general, AT_2 receptors can be detected in a variety of adult tissues, including uterus, ovary, adrenal medulla, heart, blood vessels and brain.[39] AT_2 receptor expression not only varies per tissue, but also depends on age, gender and species. With respect to gender, AT_1:AT_2 receptor ratios have been reported to be lower in females than males, possibly because estrogens upregulate AT_2 receptor expression and downregulate AT_1 receptor expression.[40,41] Indeed, ovariectomy induces a male-like AT_1:AT_2 receptor ratio, which could be reversed by estrogen-replacement therapy.[40] In addition, AT_2 receptor expression is subject to changes in expression during pregnancy and pathological conditions such as hypertension, heart failure and vascular injury.[13,39]

In 1995, two groups simultaneously reported that deletion of the AT_2 receptor in mice led to an increased pressor response to Ang II.[42,43] In comparison with wild-type mice, baseline blood pressure was increased in the hemizygous $AT_2^{-/y}$ receptor mice of Ichiki and coworkers, whereas no baseline blood pressure differences where found in the model of Hein and coworkers.[42,43] Mutants lacking the AT_2 receptor gene showed a lower body temperature and impaired exploratory behavior. Remarkably, despite its wide expression in the fetus, the AT_2 receptor does not seem to be required for embryonic development, as no morphological and developmental differences were found between homozygous $AT_2^{-/-}$ or hemizygous $AT_2^{-/y}$ receptor mice and their wild-type controls. Possibly, AT_2 receptor knockout mice displayed a delayed expression of calponin and h-caldesmon after birth.[44] During pregnancy, Ang II levels are elevated. Because the fetus is also exposed to these high Ang II levels, it has been postulated that vasodilatory AT_2 receptors play a role in the regulation of Ang II responsiveness in order to prevent fetal hypertension.[45]

Based on the sequence homology between AT_1 and AT_2 receptors, one would expect that receptor binding is accomplished in a similar way. Binding studies with angiotensin peptides to wild-type (closed) and constitutively active (pre-activated; N111G mutant) AT_1 receptors have demonstrated that the first two residues of Ang II (Asp^1 but in particular Arg^2) play an important role in the 'pre-activation' process of the receptor. Their binding allows the receptor to adopt a more relaxed conformation, subsequently facilitating the binding of the C-terminal five residues of Ang II that switches this 'pre-activated' receptor into the fully active conformation.[46,47] However, studies investigating the relationship of the various amino acid residues of Ang II and AT_2 receptor binding demonstrated that the affinity toward the AT_2 receptor is less sensitive to modifications of the amino acid residues that are required for binding to the AT_1 receptor (Arg^2, Tyr^4, His^6 and Phe^8).[48-50] Interestingly, the affinity profile of several Ang II analogs toward the AT_2 receptor was similar to that of the constitutively active N111G mutant of the AT_1 receptor, thereby suggesting that, unlike the AT_1 receptor, the AT_2 receptor has a 'relaxed' confirmation.[49] Whether AT_2 receptors are constitutively active like the N111G mutant of the AT_1 receptor remains to be answered, although several investigators have reported that AT_2 receptor expression exerts cellular effects without ligand binding.[51,52]

Binding studies have shown that the Ang peptides, Ang III, Ang IV and Ang-(1 – 7) all show some degree of affinity for AT_2 receptors. The rank order for binding to the AT_2 receptor that can be found in literature is Ang II ≥ Ang III >> Ang IV > Ang-(1 – 7), although some studies report Ang III to have a slightly higher affinity for the AT_2 receptor than Ang II and/or Ang IV to have a lower affinity than Ang-(1 – 7).[50,53-55] In comparison with Ang II, Ang III, Ang IV and Ang-(1 – 7), all displayed a higher preference for binding to the AT_2 receptor as compared with the AT_1 receptor,[53] thereby supporting the concept that endogenous Ang II metabolites potentially counteract the actions of Ang II, which are mediated via the AT_1 receptor.

In line with this concept, several studies have demonstrated that Ang III is the endogenous agonist of the AT_2 receptor, mediating natriuresis, vasorelaxation in the coronary circulation and aldosterone release.[18, 56-58] Since its discovery in 1989, several signaling pathways have been linked to the AT_2 receptor. [59] Despite the fact that some pathways have been well characterized, AT_2 receptor signaling remains a complex matter because several signaling cascades are not fully defined and their exact function is not always clear. This is even further hindered by the fact that the signaling is likely to vary in the different tissues and is depending on age, sex, species and (patho)physiological conditions. Up to now, the following major signaling cascades have been linked to the AT_2 receptor.

2.1 Coupling to G-proteins

Both AT_1 and AT_2 receptor are seven-transmembrane domain receptors and belong to the G-protein-coupled receptor (GPCR) superfamily. Although the coupling of G-proteins to AT_1 receptors has been extensively described, this is less well established for the AT_2 receptor. A limited number of studies has shown that AT_2 receptors couple to $G_i\alpha_2$, $G_i\alpha_3$ [55,60] and $G\alpha_{q/11,}$ [61] but whether such coupling underlies its function under all conditions is still unknown.

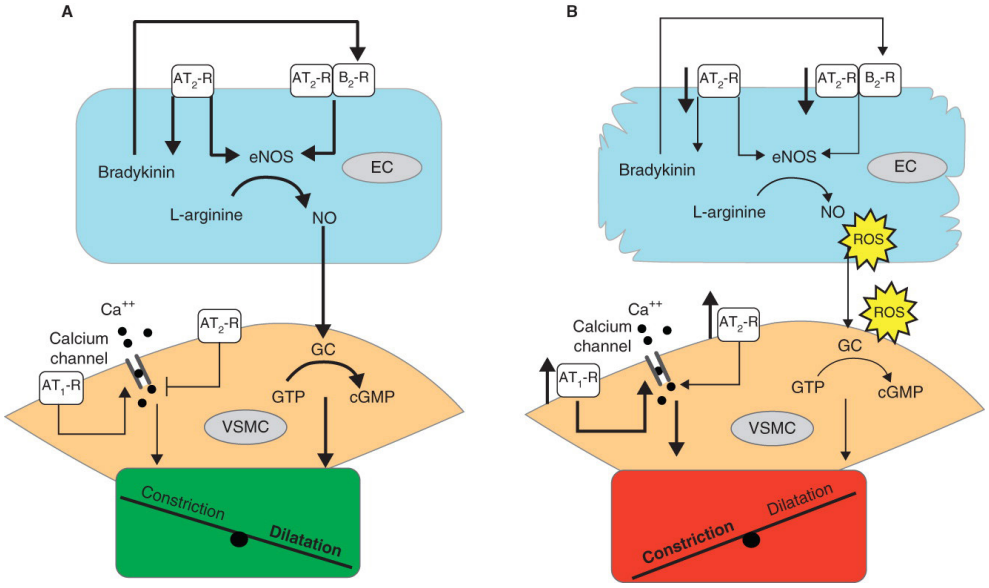

Figure 1. Vascular AT_2 receptor function under physiological (A) and pathological (B) conditions, where vasodilation and vasoconstriction predominate, respectively. Vasoconstrictor dominance might be the result of reduced endothelial AT_2 receptor expression, combined with upregulated reactive oxygen species generation and vascular smooth muscle cell AT_2 receptor expression. See text for further explanation.

2.2 Activation of the nitric oxide/guanosine cyclic 3', 5'-monophosphate pathway

Stimulation of the AT_2 receptor in endothelial cells has been demonstrated to activate the production of NO by endothelial NO synthase (NOS).[62] Activation of NOS can either occur directly via AT_2 receptor stimulation or indirectly via stimulation of the bradykinin type 2 (B_2) receptor by endogenously formed bradykinin, which is released upon AT_2 receptor stimulation.[63-66] The direct interaction between AT_2 receptors and NOS possibly involves the calcineurin/nuclear factor of activated T-cell pathway.[67] NO subsequently stimulates guanylyl cyclase to produce cGMP, which mediates effects in the vascular smooth muscle cells (VSMC) (**Figure 1**). This pathway involving AT_2 receptors, B_2 receptors, NOS, NO and cGMP contributes to vasodilation, natriuresis and the inhibition of renin production.[65, 66, 68,69]

2.3 Activation of protein phosphatases

Three specific phosphatases have been linked to AT_2 receptor activation: mitogen-activated protein kinase (MAPK) phosphatase 1, SH2-domain-containing phosphatase 1 and serine/threonine phosphatase 2A. Growth factors, including Ang II via the AT_1 receptor, mediate their growth-promoting actions through tyrosine kinase receptors and several kinase-driven phosphorylation steps. Activation of the AT_2 receptor counteracts these growth-promoting actions by dephosphorylation through subsequent activation of phosphatases. In addition to the inhibitory effect on growth, dephosphorylation (e.g., of extracellular signal-regulated kinase 1/2 (ERK1/2)) also seems to play an important role in the stimulation of apoptosis,[70] although anti-apoptotic effects also have been described.[71] Additionally, the activation of phosphatases is involved in the anti-inflammatory effect upon AT_2 receptor stimulation by decreasing activation of nuclear factor-κB (NF-κB), cyclooxygenase-2 (COX-2) and inhibiting the JAK-STAT pathway, resulting in a reduced transcription of pro-inflammatory cytokines and prostaglandins.[72-74]

2.4 Activation of phospholipase A2

AT_2 receptor stimulation by Ang II induces phospholipase A2 to release arachidonic acid (AA), which subsequently activates the Na^+/HCO_3^- symporter [75] and increases the delayed rectifier K^+ current,[76] thereby influencing intracellular pH and polarity. In addition, cytochrome P450-dependent metabolism of AA results in the formation of epoxyeicosatrienoic acids (EETs), which activate MAPKs [77] and induce ant-inflammatory effects.[78] Importantly, AA and EETs have both been linked to AT_2- and B_2 receptor-mediated vasorelaxation.[79,80]

2.5 Interaction with promyeolytic leukemia zinc finger protein

Promyeolytic leukemia zinc finger protein (PLZF) is a transcription factor, which has been associated with both transcriptional repression and activation. Target genes of PLZF include hox genes,[81] the promoter of the p85α subunit of the phosphatidylinositol-3

(PI3K-p85α) and cyclin A.[82,83]

PLZF is highly expressed in the heart and certain areas of the brain. [82,84] During development, it has been shown to act as a growth repressor and to exert pro-apoptotic functions.[81] PLZF can bind to the intracellular C-terminal part of the AT_2 receptor and (pro) renin receptor.[82, 85] Although it was reported that the AT_2 receptor is not internalized upon binding of Ang II in the vasculature.[86] Senbonmatsu and coworkers demonstrated that Ang II stimulation provoked internalization of the AT_2 receptor–PLZF complex and subsequent translocation of PLZF to the nucleus involving G_i.[82] This interaction not only has been reported to be involved in the development of cardiac hypertrophy but also is believed to play a role in neuroprotection.[82,84, 86]

2.6 Interaction with the AT2 receptor-binding protein

AT_2 receptor-binding protein (ATBP) is expressed in at least three different isoforms termed ATBP50, ATBP60 and ATBP135.[87] ATBP50, also known as AT_2 receptor-interacting protein 1 (ATIP1) [8]or mitochondrial tumor suppressor 1 (MTUS1), is the most abundant isoform and is expressed in various organs such as heart, lung, kidney, uterus and brain.[87-89] Like the G-proteins and PLZF, ATBP binds to the C-terminal tail of the AT_2 receptor and regulates the transport of the AT_2 receptor from the Golgi apparatus to the cell membrane.[87] ATBP mediates an inhibitory effect on MAPKs and anti-proliferative effects.[87,88] The inhibitory effect of ATBP requires expression of the AT_2 receptor but not ligand activation, suggesting this pathway is constitutively active, although the effect is increased after stimulation with Ang II.

In general, ligand binding in GPCRs initiates signal-transduction pathways through receptor dimerization. Although the AT_2 receptor does not behave like a typical GPCR, it is known that the AT_2 receptor can dimerize with other AT_2 receptors and/or other GPCRs. As AT_1 and B_2 receptors are co-expressed in various tissues, it is not surprising that several papers have reported AT_1/AT_2 and AT_2/B_2 receptor heterodimers, which can interact through receptor cross talk (Figure 1). Transfection studies in fetal fibroblasts have shown that the AT_2 receptor functions as endogenous antagonist of the AT_1 receptor and that heterodimerization does not require AT_2 receptor activation.[61] In line with this finding, van Esch and coworkers demonstrated that AT_2 receptor-induced vasodilation depends on simultaneous AT_1 receptor activation, as no AT_2 receptor-mediated responses were observed in AT_1 receptor-deficient animals.[17] In contrast to these findings, Miura and coworkers suggested that both the AT_1 and AT_2 receptors prefer to form homodimers and that the AT_2 receptor antagonizes the effects of the AT_1 receptor through cross talk in the cytoplasm.[90] To what degree this is a constitutive effect of the AT_2 receptor (not requiring an agonist) remains unclear.[52,90] The existence of AT_2/B_2 receptor heterodimers has been confirmed in rat pheochromocytoma cells by applying fluorescence resonance energy transfer.[91] Heterodimer formation not only appeared to be dependent on the receptor number that was expressed, but also required AT_2 receptor stimulation.

As a consequence of heterodimer formation, it is possible that AT_2 receptor activation results in B_2 receptor activation without intermediate bradykinin synthesis.[16]

3. AT_2 RECEPTOR FUNCTION UNDER (PATHO)PHYSIOLOGICAL CONDITIONS

3.1 Vascular system

The presence of the AT_2 receptor is reported in various vessel types including rat aorta, mesenteric and uterine arteries. In these arteries, the AT_2 receptor has been found on endothelial cells as well as on VSMC.[92-95] The expression varies during life and can be influenced by diverse pathologic conditions, such as pressure overload or vascular damage.[96,97] Both in vivo and in vitro studies support the concept of AT_2 receptor-mediated vasodilation. The number of studies in humans is very limited.[16,98,99] Most studies agree that the vasodilatory action of the AT_2 receptor involves endothelial NO synthesis and cGMP production, possibly through bradykinin and its B_2 receptor.[16] Stimulation of the AT_2 receptor inhibits AT_1 receptor-mediated superoxide formation. Since superoxide decreases the bioavailability of NO, such suppression would also increase NO levels.[100] An AT_2 receptor-mediated endothelium-independent relaxation has been reported via large conductance, calcium- and voltage-activated potassium (BK_{Ca}) channels.[101] Furthermore, deletion of the AT_2 receptor provided evidence that the AT_2 receptor inhibits ACE synthesis. In $AT_2^{-/y}$ receptor mice, the ACE levels are twice as high compared with wild-type mice. This rise in ACE was also seen in mice treated with the AT_2 receptor antagonist PD123319.[102] Taken together, the AT_2 receptor counteracts AT_1 receptor-mediated vasoconstriction via multiple mechanisms. However, most, if not all, of the above studies have been performed in (young) normotensive animals. Ang II induced enhanced constrictor responses in old normotensive animals as well as in spontaneously hypertensive rats (SHR).[25-27, 103] Interestingly, this enhanced constrictor response could be partially inhibited by PD123319, pointing to the involvement of AT_2 receptors as mediators of vasoconstriction. ROS scavenging of NO has been proposed as a possible mechanism for this loss in function of the AT_2 receptor, combined with decreased expression of the AT_2 receptor on endothelial cells (Figure 1).[25,103] Since endothelium removal did not change the constrictor response of the AT_2 receptor, it is even possible that the constrictor AT_2 receptors are located on the VSMC, as opposed to the endothelial location of the relaxant AT_2 receptors.[26] According to one study, constrictor and dilatory AT_2 receptors coexist in the human forearm.[98] Lowering blood pressure, even with non-RAS blockers, suppressed the constrictor effect of the AT_2 receptor and allowed the return of bradykinin/NO-mediated relaxant responses.[26, 104]

Distinct from the acute effects of AT_2 receptor described above, there is growing evidence that the AT_2 receptor has beneficial effects on vascular remodeling. In various studies, the AT_2 receptor was upregulated after vascular injury, while the expression of the AT_1 receptor remained unchanged.[105,106] In AT_2 receptor-transfected VSMC, AT_2 receptor stimulation facilitated serum-deprivation-induced apoptosis,

and in vivo transfection of balloon-injured rat carotid arteries with the AT_2 receptor gene showed a marked reduction of the neointima.[97,105] These findings were supported in an AT_2 receptor knockout mouse where the formation of the neointima and the proliferation of smooth muscle cells were greater than that in wild-type mice after injury of the femoral artery.[107]

AT_2 receptor-mediated vascular remodeling was also apparent in an animal model for Marfan syndrome.[108] Selective blockade of the AT_1 receptor inhibited aneurysm progression via decreased transforming growth factor-β (TGFβ)-mediated activation of ERK1/2, and AT_2 receptor signaling was indispensable for this inhibition. Indeed, ACE inhibition and the treatment of Marfan mice lacking the AT_2 receptor with an AT_1 receptor antagonist did not affect ERK activation. There was a small decrease in aortic root size under these conditions, most likely as a consequence of blood pressure lowering.[108]

3.2 Heart

AT_2 receptor expression in the heart varies among species, for example, in humans cardiac AT_2 receptor expression is more abundant than rodents.[109] Furthermore, AT_2 receptors expression in the heart has been reported to increase under pathological conditions as well as in response to combined ACE inhibition and AT_1 receptor blockade.[110-115] On the one hand, this may result in attenuated responses to AT_1 receptor-mediated pressor, growth and fibrotic effects, possibly in a bradykinin- and NO-dependent manner, thereby improving cardiac hemodynamics post-myocardial infarction (MI).[20, 21, 116-119] However, studies in transgenic and knockout animals have yielded conflicting results, and according to some, the absence of the AT_2 receptor in knockout animals prevents the development of cardiac hypertrophy when induced by Ang II infusion or pressure overload or has no effect at all.[4, 29, 106] Moreover, AT_2 receptor overexpression has also been shown to induce cardiac hypertrophy and fibrosis.[28, 120] Thus, like in the vessel wall, AT_2 receptors may be both protective and deleterious, possibly depending on the disease state and/or their location. This is supported by data obtained in the coronary vascular bed, showing AT_2 receptor-mediated dilatation in normotensive animals but not in SHR.[25,57] In these studies, the vasoconstrictor effects of Ang III were greatly enhanced in the presence of the AT_2 receptor antagonist PD123319 starting at Ang III concentrations in the low nanomolar range, whereas PD123319 enhanced the Ang II-induced constriction only at submicromolar Ang II concentrations. Additionally, aminopeptidase A inhibition and blockade of NOS enhanced the vasoconstrictor effects of Ang II to a similar degree as PD123319 (J.H.M. van Esch; unpublished data), suggesting that Ang III generation from Ang II by aminopeptidase A is responsible for AT_2 receptor-induced vasodilation following Ang II application and that this dilation occurs in an NO-dependent manner. This phenomenon did not occur in the coronary circulation of SHR due to the absence of counterregulatory AT_2 receptors and/or a change of the AT_2 receptor phenotype from relaxant to vasoconstrictor.[25]

3.3 Kidney

Studies using AT_2 receptor-deficient animals revealed a rightward shift in the pressure–natriuresis curves.[121] Although this suggests an important role for the AT_2 receptor in mediating natriuresis, it should be taken into account that AT_1 receptors were upregulated in this model. Subsequent studies by Padia and coworkers demonstrated that AT_1 receptor blockade induced an enhanced natriuretic response, which could be abolished by AT_2 receptor blockade.[18] Intrarenal infusion of Ang II or Ang III both induced AT_2-receptor mediated natriuresis in Sprague–Dawley (SD) rats during AT_1 receptor blockade.[18] The natriuretic response to Ang III was augmented by inhibition of aminopeptidase N, whereas inhibition of aminopeptidase A abolished AT_2-receptor mediated natriuresis.[56, 122] This suggests that Ang III is the endogenous agonist for AT_2 receptor-mediated natriuresis. By contrast, Ang III failed to induce AT_2 receptor-mediated natriuresis and AT_2 receptor translocation in the renal proximal tubule cells of SHR.[123] Aminopeptidase N inhibition restored the defective AT_2 receptor-mediated natriuresis in SHR suggesting that the development of hypertension in SHR underlies an enhanced breakdown of Ang III by aminopeptidase N.[124] The AT_2 receptor modulates sodium reuptake via Na^+, K^+-ATPase in the proximal tubules and by counteracting the AT_1 receptor-induced bicarbonate reabsorption.[125,126] Such counteracting effects may also underlie the suppression of renal fibrosis.[127] Yet recently it has been described that the AT_1 and AT_2 receptors may also act synergistically in the kidney, because similar to the AT_1 receptor, the AT_2 receptor inhibits renin release in the kidney, most likely through the NO/cGMP pathway.[69]

3.4 BRAIN

AT_2 receptors are expressed in the brain, although in most brain nuclei the AT_1 receptor is predominant.[128] Studies in AT_2 receptor knockout mice revealed that the AT_2 receptor influences the expression of more than 62 genes in the brain, including genes linked to blood pressure regulation, learning and cognition, and neuroprotection.[129] Intracerebroventricular injection of Ang II increases blood pressure via AT_1 receptor stimulation, and AT_2 receptors counteract this rise in blood pressure.[130] However, in contrast to this antagonistic interaction, both receptor subtypes act synergistically with regard to the regulation of water intake.[130] The impaired drinking response in AT_2 receptor knockout mice might also result from the impaired exploratory behavior and locomotor activity in AT_2 receptor knockout mice. In wild-type mice, the AT_2 receptor is expressed in areas related to learning and control of motor activity.[131] Notable evidence for the involvement of the AT_2 receptor in learning and cognition is a case report about a woman with no AT_2 receptor expression resulting in a mental retardation because of a balanced x;7 chromosomal translocation.[132] Furthermore, 8 of 590 male patients with mental retardation were found to have sequence changes in the AT_2 receptor gene, including one frameshift and three missense mutations.[132] Additionally, a deleterious role for the AT_2 receptor has been described in relation to Alzheimer's disease, where $G\alpha_{q/11}$ dysfunction was found to be due to amyloid β-triggered AT_2 receptor oligomerization.[133,134]

The neuroprotective properties of the AT_2 receptor in stroke have been studied extensively. Stroke is a major contributor to mortality, morbidity, and disability in industrialized countries. RAS blockade, in particular AT_1 receptor antagonism, appears to result in stroke prevention, and this effect occurs independently of the blood pressure-lowering effect of RAS blockers.[135] An attractive explanation is that the elevated Ang II concentrations during AT_1 receptor blockade stimulate AT_2 receptors in the brain. These receptors are upregulated after brain injury.[136] They promote cell differentiation and regeneration in neuronal tissue and reduce oxidative stress in the ischemic brain.[137-139] In AT_2 receptor knockout mice, the ischemic area was significantly larger than wild-type mice after transient middle cerebral artery occlusion, and this observation was paralleled by an in crease in superoxide production and NADPH oxidase activity.[139] The cerebroprotective role of AT_2 receptor stimulation may relate to its anti-apoptotic effects and/or its capacity to increase neuronal survival and neurite outgrowth, possibly in an NO-dependent manner.[71,140,141]

4. AT_2 RECEPTOR STIMULATION

As discussed earlier, angiotensin metabolites other than Ang II may act as endogenous ligands of the AT_2 receptor, and some effects previously attributed to Ang II might in fact have been mediated by Ang III, Ang IV or Ang-(1 – 7). [18,56,57, 142-146] Attempts to generate a selective agonist for the AT_2 receptor initially yielded the Ang II-derived peptide CGP42112A, a partial agonist. This drug confirmed the hypotensive and anti-proliferative effects, the stimulation of neurite outgrowth and the neuroprotection in a stroke model that have been attributed to AT_2 receptor stimulation.[86,147-153] Additionally, CGP42112A application resulted in AT_2 receptor-induced anti-inflammatory effects, as evidenced by NF-κB inhibition, suppression of COX2 synthesis, and blockade of STAT3 phosphorylation and TNF-α production.[74,154] The fact that CGP42212A is a partial agonist, combined with its short half-life, however, hampered its further use in in vivo studies.[147]

In 2004, Hallberg and Alterman reported the synthesis of compound 21 (C21), the first selective non-peptide AT_2 receptor agonist.[155,156] C21 displays a K_i of 0.4 nM for the AT_2 receptor and a K_i of > 10 μM for the AT_1 receptor.[155] C21 is derived from L162,313, a non-peptide angiotensin receptor agonist with a similar affinity for the AT1 and AT2 receptors.[156-158] Initial studies reported that C21 has an oral bioavailability of 20 – 30% and an estimated half-life of 4 h in plasma.[155] It induces the outgrowth of neurite cells, stimulates p42/p44 MAPK and enhances duodenal alkaline secretion in SD rats. C21 lowered MAP by ~ 25 mmHg in anesthetized SHR but not in SD rats. Remarkably, this response was not affected by AT2 receptor blockade, suggesting that it did not involve AT2 receptor stimulation. Bosnyak and coworkers reported a vasodepressor response after administration of C21 (300 ng/kg per minute) in conscious SHR (but not WKY rat) on top of low-dose AT1 receptor antagonism, which could be blocked by the AT2 receptor antagonist PD123319.[159] In the absence of AT1 receptor blockade,

no blood pressure-lowering effects were observed in a wide range of models, including the stroke-prone hypertensive rat (SHR-SP), the 2-kidney, 1-clip (2K1C) hypertensive SD rat and in post-MI Wistar rats and C57BL/6 mice.[160-163] When infused over a short time period at a concentration of up to 300 ng/kg per minute, C21 induced a modest rise in MAP (~ 4 mmHg) in male SD rats, which could be abolished by PD123319, whereas at a high dose (1000 ng/kg per minute) it increased MAP by 20 mmHg in an AT_1 receptor-dependent manner.[159, 164] Similarly, when given chronically to SHR-SP at a dose of 694 ng/kg per minute, C21 increased systolic blood pressure by ~ 20 mmHg in an AT_1 receptor-dependent manner.[165] Yet in the latter study, C21 did not lower blood pressure on top of AT_1 receptor antagonism – in fact, C21 exerted no effect at all on blood pressure under these conditions, possibly because the applied AT_1 receptor blocker dose had already resulted in maximum blood pressure-lowering effects. Taken together, these data demonstrate C21-induced relaxation via AT_2 receptors or unknown mechanisms, as well as constriction via both AT_2 and AT_1 receptors, the latter requiring high doses. AT_1 receptor blockade appeared a prerequisite to observe AT_2 receptor-mediated hypotensive effects in vivo, although this is not a universal finding.[165] In vitro, C21 relaxed conductance and resistance vessels of rats and mice, both with and without AT_1 receptor blockade.[159]

Yet despite the lack of a clear blood pressure-lowering effect in most studies, administration of C21 generally did result in beneficial organ-protective effects. C21 improved post-MI remodeling in the Wistar rat, evidenced by an ameliorated cardiac function and a reduction of inflammatory cytokines (IL-1β, IL-2 and IL-6) and pro-apoptotic markers (caspase-3 and Fas-ligand).[162] However, these data were recently challenged.[163] C21 reduced vascular injury and myocardial fibrosis in SHR-SP, as evidenced by an improved endothelial function, and a reduction in oxidative stress, collagen content, fibronectin and inflammatory cell infiltration.[165] C21 increased renal blood flow and natriuresis in anesthetized SD rats.[164] In SHR-SP, when fed a high-salt diet, chronic treatment with C21 reduced renal damage by preventing infiltration of inflammatory cells, collagen accumulation and neo-expression of vimentin as well as by suppressing the increase in plasma renin activity and proteinuria.[160] In 2-kidney, 1-clip (2K1C) hypertensive SD rats, C21 treatment inhibited early renal inflammation by reducing the renal levels of TNF-α, IL-6 and TGF-β_1 and by increasing the NO and cGMP levels. The anti-inflammatory effects of C21 were confirmed by Rompe et al. in fibroblasts, who demonstrated that C21 reduced the TNF-α-induced IL-6 expression.[23] This involved the activation of protein phosphatases, an increased synthesis of EETs and inhibition of NF-κB activity.[23] In the brain, C21 has been shown to mediate neuroprotective effects by inducing neurite outgrowth, delaying the onset of brain abnormalities in SHR-SPs and enhancing cognitive function in an Alzheimer's disease mouse model.[155, 160, 166]

Whether all effects of C21 described above are attributed to direct AT_2 receptor stimulation remains to be determined, since not all studies simultaneously co-administered AT_2 receptor antagonists and/or made use of AT_2 receptor-deficient animals.

5. EXPERT OPINION AND CONCLUSIONS

In general, experimental studies have shown that AT_2 receptor stimulation results in vasorelaxatio and natriuresis in healthy animals and suppresses growth,fibrosis, hypertrophy and inflammation in the diseased state, thereby counteracting the deleterious actions of the AT_1 receptor.[16-18, 20-23, 167] To date, our knowledge on AT_2 receptor function in humans is limited to a few studies showing, at most, modest vasodilatory effects.[16,98,99] Yet the rise in Ang II during AT_1 receptor blockade is real and multiple preclinical studies support the concept that this Ang II (or one or more of its metabolites), via stimulation of the non-blocked AT_2 receptor, contributes to the beneficial effect of these drugs. All these conclusions rely on the use of a single AT_2 receptor antagonist, PD123319, and/or the application of AT_2 receptor knockout animals. Simultaneously, studies are now available that support an opposite view of the AT_2 receptor,[24-29,98,133,134] suggesting that its function might become deleterious (e.g., becoming pro-hypertensive and pro-hypertrophic) in the diseased state. This may relate to its expression at new sites and/or its heterodimerization with new receptor partners.

The availability of selective AT_2 receptor agonists (CGP42112A and C21) should help to clarify these issues. Yet CGP42112A is a peptide, which acts as a partial agonist, limiting its in vivo application, and C21 exerts effects, for example, on blood pressure, that are not necessarily linked to AT_2 receptor stimulation. The latter may, at least in part, be a dose-related phenomenon, since lower doses (presumably allowing AT_2 receptor-specific effects only) did not affect blood pressure. This is somewhat surprising given the wealth of studies supporting AT_2 receptor-mediated vasorelaxation. One explanation might be that CGP42112A- or C21-induced relaxation via AT_2 receptors can be observed only during AT_1 receptor blockade, although this was not the case in all studies. [86,149,150,159,165] Alternatively, the possibility that AT_2 receptors exert both relaxant and constrictor effects should be considered. Concomitant AT_1 receptor blockade does not appear to be a prerequisite for all C21-induced effects, particularly those with regard to the prevention of end-organ damage. Clearly at this stage, when interpreting the neuroprotective and anti-inflammatory effects of C21, we need definite in vivo proof that these are truly AT_2 receptor mediated.[155,160-162,165] Given its upregulation in various pathological conditions, the AT_2 receptor remains a promising target for treatment, allowing effects beyond blood pressure-lowering, for example, in stroke, aneurysm formation, inflammation and myocardial fibrosis.[23,105,106, 108, 110-114, 160,161,165,168] Indeed, Vicore Pharma, the patent holder of the new AT_2 receptor agonist C21, will soon decide about the possible indication(s) for C21 now that a first trial has been planned for 2012.[169]

Preferably, in parallel with such a trial, studies should unravel why and how the phenotype of AT_2 receptors changes under pathological conditions, in order to obtain a better prediction of the cardiovascular side effect profile of AT_2 receptor agonists.

ARTICLE HIGHLIGHTS

AT_2 receptors are generally assumed to counteract the deleterious actions of the AT_1 receptor, by stimulating vasorelaxation and natriuresis and by suppressing growth, fibrosis, hypertrophy and inflammation.

Compared with the physiological state, AT_2 receptor expression is upregulated under pathological conditions such as hypertension, heart failure and stroke.

The view that AT_2 receptors exclusively exert beneficial effects has been challenged, and under certain (pathological) conditions AT_2 receptors have been reported to mimic AT_1 receptor function, for example, inducing vasoconstriction and hypertrophy.

Selective AT_2 receptor agonism could provide a new therapeutic avenue for the treatment of cardiovascular diseases (e.g., stroke, aneurysm formation and post-myocardial infarction), but as AT_2 receptor stimulation may not always be beneficial, a careful analysis of its side-effect profile, focusing on hypertension and cardiac hypertrophy, is warranted.

ACKNOWLEDGEMENTS

This study has been performed within the framework of the Dutch Top Institute Pharma, project T2-301 (Renin-angiotensin system blockade beyond angiotensin II).

DECLARATION OF INTEREST

The authors state no conflict of interest and have received no payment in preparation of this manuscript.

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Chapter 3
Compound 21 Induces Vasorelaxation via an Endothelium- and Angiotensin II Type 2 Receptor-Independent Mechanism

Hypertension. 2012 Sep;60(3):722-9.

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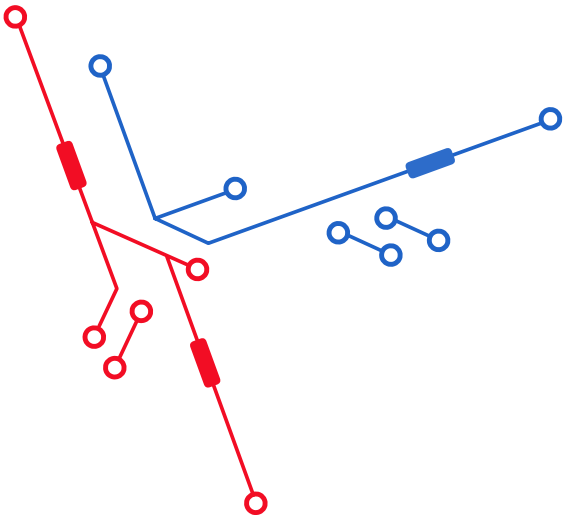

ABSTRACT

Angiotensin II type 2 (AT_2) receptor stimulation has been linked to vasodilation. Yet, AT_2 receptor-independent hypertension and hypotension (or no effect on blood pressure) have been observed in vivo after application of the AT_2 receptor agonist compound 21 (C21). We, therefore, studied its effects in vitro, using preparations known to display AT_2 receptor-mediated responses. Hearts of Wistar rats, spontaneously hypertensive rats (SHRs), C57Bl/6 mice, and AT_2 receptor knockout mice were perfused according to Langendorff. Mesenteric and iliac arteries of these animals, as well as coronary microarteries from human donor hearts, were mounted in Mulvany myographs. In the coronary vascular bed of Wistar rats, C57Bl/6 mice, and AT_2 receptor knockout mice, C21 induced constriction followed by dilation. SHR hearts displayed enhanced constriction and no dilation. Irbesartan (angiotensin II type 1 receptor blocker) abolished the constriction and enhanced or (in SHRs) reintroduced dilation, and PD123319 (AT_2 receptor blocker) did not block the latter. C21 relaxed preconstricted vessels of all species, and this did not depend on angiotensin II receptors, the endothelium, or the NO-guanylyl cyclase-cGMP pathway. C21 constricted SHR iliac arteries but none of the other vessels, and irbesartan prevented this. C21 shifted the concentration-response curves to U46619 (thromboxane A_2 analog) and phenylephrine (α-adrenoceptor agonist) but not ionomycine (calcium ionophore) to the right. In conclusion, C21 did not cause AT_2 receptor-mediated vasodilation. Yet, it did induce vasodilation by blocking calcium transport into the cell and constriction via angiotensin II type 1 receptor stimulation. The latter effect is enhanced in SHRs. These data may explain the varying effects of C21 on blood pressure in vivo.

INTRODUCTION

Stimulation of the angiotensin (Ang) II type 2 (AT_2) receptor mediates vasorelaxant,[1–6] natriuretic,[7] growth-suppressing,[8] and antifibrotic[9] effects. As such, it seems to counteract Ang II type 1 (AT_1) receptor-mediated effects.[10] However, opposite findings have been reported as well, and according to some studies, Ang II type 2 (AT_2) receptor effects mimic those of the AT_1 receptor (eg, inducing vasoconstriction[11,12] and hypertrophy[13]). Our knowledge on AT_2 receptor function is largely based on the use of the AT_2 receptor antagonist PD123319, AT_2 receptor-deficient ($AT_2R^{-/y}$) animals, and the peptidic AT_2 receptor agonist CGP42112A. The use of the latter is hampered by its partial agonistic properties. In 2004, Wan et al[14] reported the synthesis of compound 21 (C21), the first selective nonpeptide AT_2 receptor agonist. C21 has an oral bioavailability of 20% to 30% and an estimated half-life of 4 hours in plasma. Administration of C21 in various cardiovascular disease models, including the postmyocardial infarction Wistar rat[15]; the stroke-prone hypertensive rat[16,17]; and the 2-kidney, 1-clip hypertensive Sprague-Dawley (SD) rat,[18] resulted in beneficial organ-protective effects.

The vasorelaxant properties of C21 are less straightforward. C21 lowered mean arterial pressure by ≈25 mm Hg in anesthetized spontaneously hypertensive rats (SHRs) but not in SD rats.[14] Remarkably, this response was not affected by AT_2 receptor blockade, suggesting that it did not involve AT_2 receptor stimulation. Bosnyak et al[19] reported a vasodepressor response after administration of C21 (300 ng/kg per minute) in conscious SHRs (but not Wistar-Kyoto rats) on top of low-dose AT_1 receptor antagonism, which could be blocked by the AT_2 receptor antagonist PD123319. In the absence of AT_1 receptor blockade, no blood pressure–lowering effects were observed in stroke-prone hypertensive rats; 2-kidney, 1-clip hypertensive SD rats; post-myocardial infarction Wistar rats; and C57BL/6 mice.[15–18,20] When infused over a short time period, 300 ng/kg per minute of C21 induced a modest rise in mean arterial pressure (≈4 mm Hg) in male SD rats, which was not seen in combination with PD123319.[21] A 3.3-fold higher dose increased mean arterial pressure by 20 mm Hg in male SHRs in an AT_1 receptor-dependent manner.[19] Similarly, C21 transiently increased systolic blood pressure by ≈20 mm Hg in an AT_1 receptor-dependent manner when given orally to stroke-prone hypertensive rats at a dose of 1 mg/kg per day.[17] Yet, in the latter study, C21 did not lower blood pressure on top of AT_1 receptor antagonism; in fact, C21 exerted no effect at all on blood pressure, possibly because the applied AT_1 receptor blocker dose had already resulted in maximum blood pressure-lowering effects. Taken together, these in vivo data demonstrate C21-induced relaxation via AT_2 receptors or unknown mechanisms, as well as constriction via both AT_2 and AT_1 receptors, the latter requiring high doses. AT_1 receptor blockade appeared a prerequisite to observe AT_2 receptor-mediated hypotensive effects in vivo, but this is not a universal finding and may depend on the degree of AT_1 receptor blockade.[17,19,20] In vitro data on C21-induced relaxation are scarce, despite the wide range of in vitro studies supporting AT_2 receptor-induced relaxation in multiple vascular beds. No human data are available. Therefore, it was the aim of the present study to investigate C21-induced vasodilation/constriction in vitro, taking into consideration species- (including humans), pathology- (hypertension), and concentration-related effects and carefully considering its blockade by AT receptor antagonists or in AT_2 receptor-deficient ($AT_2R^{-/y}$) mice. We made use of preparations that, in previous studies, displayed clear AT_2 receptor-mediated vasodilation.[1,2,11,12,22]

METHODS

Animal Studies

Male Wistar rats (337±5 g; n=45), male SHRs (320±3 g; n=18), and male C57BL/6 mice (29±1 g; n=10) were obtained from Harlan. Male $AT_2R^{-/y}$ (28±1 g; n=10) bred on a C57BL/6 background were obtained from the animal facilities of the Charité (Campus Benjamin Franklin, Berlin, Germany). C57BL/6 and $AT_2R^{-/y}$ mice were genotyped to verify AT_2 receptor expression. All of the experiments were performed under the regulation and permission of the animal care committee of the Erasmus MC.

Animals were anesthetized with pentobarbital (60 mg/kg IP). Rat hearts were excised and placed in ice-cold Tyrode buffer,[22] whereas mouse hearts were placed in modified Krebs-Henseleit buffer,[23] both gassed with 95% O_2/5% CO_2. Subsequently, iliac and mesenteric arteries were removed and either used directly or after overnight storage in cold, oxygenated Krebs-Henseleit solution. Such storage does not affect responsiveness.[24,25]

Human Studies

Human coronary microarteries were obtained from 5 heart-beating donors (2 men and 3 women, age 41±7 years) who died of noncardiac causes (3 cerebrovascular accident, 1 head trauma, and 1 suicide) <24 hours before the heart was taken to the laboratory. Hearts were provided by the Rotterdam Heart Valve Bank after removal of the heart valves for transplantation purposes. The study was approved by the ethics committee of the Erasmus MC. Human coronary microarteries were isolated and stored in Krebs-Henseleit, as described before.[1]

Langendorff Preparation

Rat and mouse hearts were perfused according to Langendorff, as described previously.[22,23] Gassed perfusion buffer was used to superfuse the mouse hearts to prevent temperature fluctuations. Coronary flow (CF) was measured with a flow probe (Transonic systems). After a stabilization period of 30 minutes, baseline values of CF were obtained. Next, bolus injections (100 µL) of perfusion buffer were applied 3 times to determine injection-induced changes in CF. Subsequently, concentration-response curves (CRCs) to C21 (kindly provided by Vicore Pharma) were constructed via bolus injections, in the absence or presence of the AT_1 receptor antagonist irbesartan (provided by Sanofi-Synthelabo) or PD123319. Blockers were present in the perfusion buffer starting 15 minutes before the first bolus injection.

Mulvany Myograph

Human coronary microarteries (diameter, ≈750 µm), rat iliac arteries (diameter, ≈800 µm), rat mesenteric arteries (diameter, ≈150 µm), mouse iliac arteries (diameter, ≈350 µm), and mouse abdominal aortas (diameter, ≈550 µm) were cut into ring segments of ≈2-mm length. In some rat iliac artery segments, the endothelium was removed by gently rolling the vessel after insertion of the tip of small-angled forceps into the lumen. Segments were mounted in a Mulvany myograph with separated 6-mL organ baths containing gassed Krebs-Henseleit buffer at 37°C, as described previously, and tension was normalized to 90% of the estimated diameter at 100 mm Hg of effective transmural pressure. After a 30-minute stabilization period, the maximal contractile response was determined by exposing the vessels to 100 mmol/L of KCl. Thereafter, vessels were preincubated for 30 minutes in fresh buffer in the absence or presence of 1 µmol/L of irbesartan, 1 µmol/L of PD123319, 100 µmol/L of N^G-nitro-L-arginine methyl ester (L-NAME), 10 µmol/L of 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one, 200 µmol/L of hydroxocobalamin, 10 µmol/L of Y27632, or 0.1 to 100.0 µmol/L of C21, and CRCs were constructed to C21, phenylephrine, U46619, or ionomycin.

C21-induced relaxation was studied after preconstriction with U46619 (10–100 nmol/L) or 30 mmol/L of KCl.

AT2 Receptor Binding Studies

HEK 293 cells stably expressing rat AT_2 receptors under geneticin selection (kindly provided by Dr. W.G. Thomas, University of Queensland, Brisbane, Queensland, Australia) were grown in DMEM supplemented with 10% FCS, 100 U/mL of penicillin/streptomycin, and 200 µg/mL of geneticin. For binding studies, the cells were trypsinized and seeded in 48-well plates (Corning) at a density of 5×10^4 cells per well. Cells were allowed to attach for 48 hours. The plates were then placed on ice and washed once with ice-cold Hanks' balanced salt solution, followed by another wash with cold Hanks' balanced salt solution supplemented with 0.1% BSA (binding buffer). After removal of binding buffer, C21, vehicle, or PD123319 (to determine nonspecific binding) was added in 100 µL of cold binding buffer and allowed to incubate for 20 minutes. Next, 50 µL of binding buffer containing 25000 cpm of ^{125}I-Ang II was added. After 4 hours of incubation, binding buffer was removed, and wells were washed twice with Hanks' balanced salt solution. Subsequently, cells were lysed with 0.1 mol/L of NaOH, and radioactivity was counted in a gamma counter.

Spectral Analysis

To determine the molecular interaction between C21 and hydroxocobalamin, solutions containing hydroxocobalamin (200 µmol/L) and C21 (0.1–1.0 mmol/L) or NaCl (0.1–1.0 mmol/L; negative control) were prepared. Absorption spectra (300–560 nm) were determined using a UV mini-1240 spectrophotometer (Shimadzu). The pH values of individual solutions were measured afterward and were within the range of 6.7 to 7.0.

Data Analysis

Data obtained with the Langendorff preparation were recorded and digitalized using WinDaq waveform recording software (Dataq Instruments) and Labchart software (AD Instruments). After a manual selection of the desired signals preinjection and postinjection, data were analyzed using Matlab (Mathworks Inc) and Labchart. CRCs were analyzed as described before[26] using GraphPad Prism 3.01 (Graph Pad Software Inc) to determine the maximum effect (E_{max}) and pEC_{50} ($=-^{10}\log EC_{50}$) values. Statistical analysis was performed by 1- or 2-way ANOVA, followed by post hoc evaluation according to Bonferroni. $P<0.05$ was considered significant.

RESULTS

AT2 Receptor Binding Studies

C21 concentration-dependently prevented ^{125}I-Ang II binding to AT_2 receptor-transfected HEK-293 cells (inhibition constant, 1.02±0.14 nmol/L; n=3; Figure 1). In addition, 1 µmol/L of PD123319 displaced ^{125}I-Ang II binding to these cells by 96.9±1.6% (n=4).

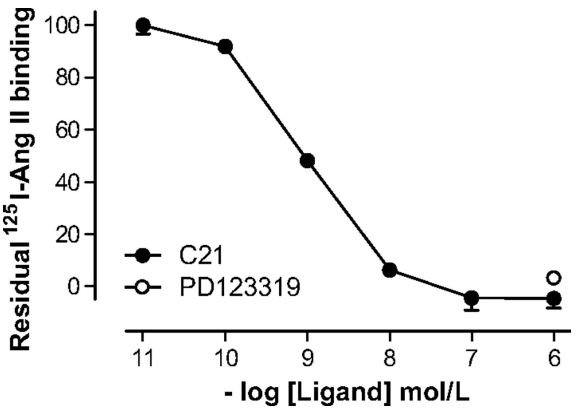

Figure 1.
Displacement of angiotensin (Ang) II type 2 (AT_2) receptor-specific ^{125}I-Ang II binding in AT_2 receptor-transfected HEK-293 cells by compound 21 (C21) or PD123319 (mean±-SEM of n=3–4).

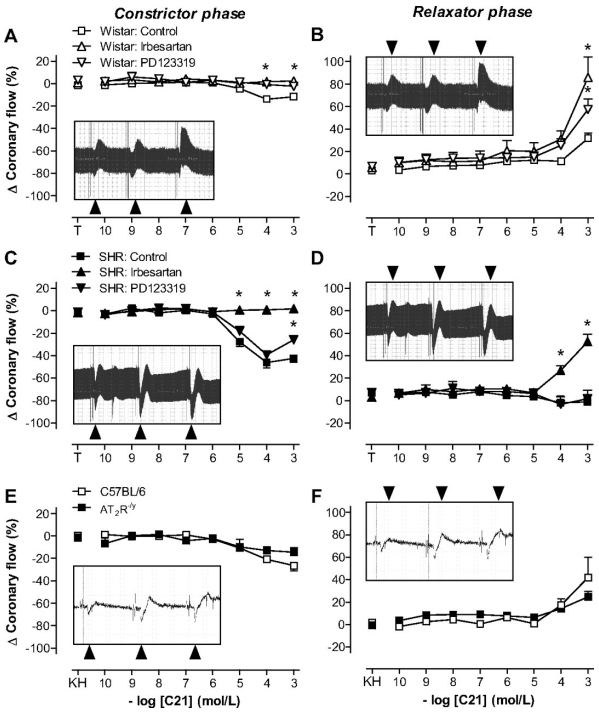

Figure 2.
Biphasic effect of compound 21 (C21) bolus injections (100 µL) on coronary flow in Wistar rats (A and B), spontaneously hypertensive rats (SHRs; C and D), and C57BL/6 versus AT2 receptor-deficient (AT2R−/y) mice (E and F) in the absence or presence of irbesartan or PD123319. Baseline flow values were 9.2±0.4, 11.3±0.7, 1.3±0.1, and 1.7±0.3 mL/min in Wistar rats, SHRs, and C57BL/6 and AT2R−/y mice. Inlay, Original tracing showing the effect of the highest C21 concentration. Arrows, The constrictor and relaxant phases. The x axis displays the concentration of the agonist in the injection fluid. Data are mean±SEM of n=4 to 7 and represent percentage change from baseline. T and KH represent a bolus injection of Tyrode's (A through D) or Krebs-Henseleit (E and F) buffer, respectively. *P<0.05 vs control.

Langendorff Preparation

At concentrations >1 µmol/L (in the injection fluid), C21 induced a biphasic response in the coronary circulation of the Wistar rat (n=4), a CF decrease (constrictor phase) of maximally 14±3% (Figure 2A) followed by a CF increase (relaxant phase) of maximally 32±10% (Figure

2B). Irbesartan and PD123319 (n=4–5) abolished (P<0.05) the CF decrease and enhanced the CF increase (P<0.05). In SHRs, the constrictor effects of C21 were greatly enhanced (E_{max}, 48±4%; P<0.05 versus Wistar rat; Figure 2C), whereas its relaxant effects were abolished (n=7; Figure 2D). Irbesartan (n=4) fully abolished the C21-induced coronary constriction (P<0.05) in SHRs and allowed the return of the relaxant response to C21 (P<0.05). PD123319 (n=4) partially reduced the constrictor response (P<0.05) but did not induce

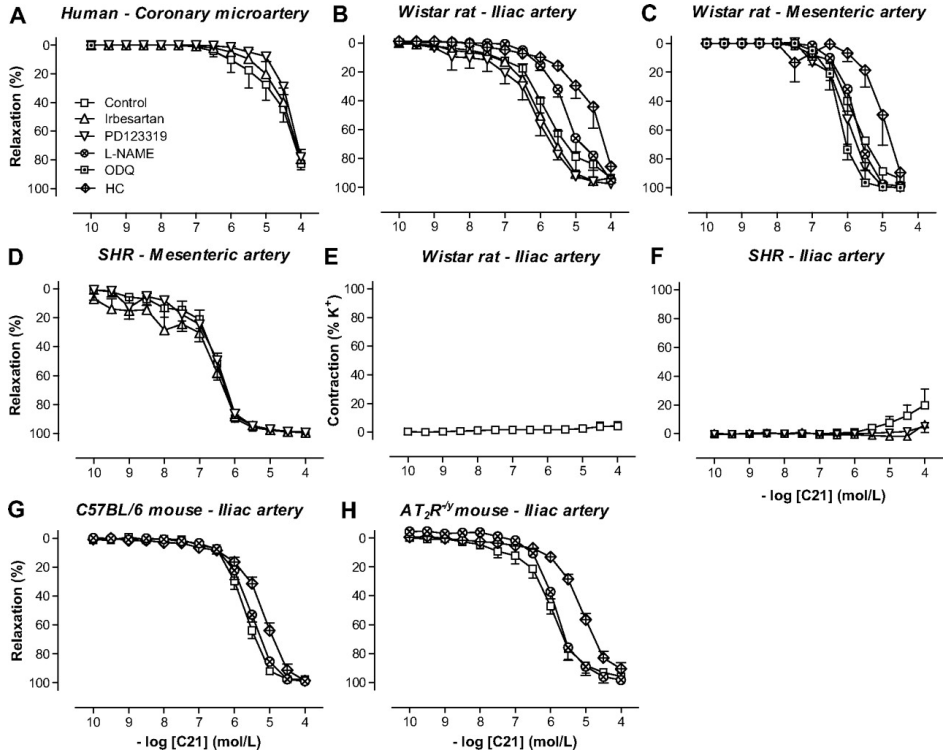

Figure 3.

Effect of compound 21 (C21) in KCl-preconstricted human coronary microarteries (A), U46619-preconstricted Wistar rat iliac arteries (B), Wistar rat mesenteric arteries (C), and spontaneously hypertensive rat (SHR) mesenteric arteries (D), as well as in U46619-preconstricted iliac arteries of C57BL/6 (G) and AT2 receptor-deficient (AT2R−/y) mice (H), in the absence or presence of irbesartan, PD123319, NG-nitro-L-arginine methyl ester (L-NAME), 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), or hydroxocobalamin (HC). E and F show the C21-induced constrictor responses (or absence thereof) in iliac arteries of Wistar rats and SHRs. Data are mean±SEM of n=6 to 10 and have been expressed as a percentage of the maximum contraction-induced KCl or U46619.

relaxation. Results in C57BL/6 and $AT_2R^{-/y}$ mice (n=4; Figure 2E and 2F) mimicked those in Wistar rats.

Mulvany Myograph

C21 concentration-dependently relaxed preconstricted human coronary microarteries

(pEC_{50}, 4.8±0.3; E_{max}, 83±4.2%; n=7; Figure 3A), Wistar rat iliac arteries (pEC_{50}, 5.6±0.2; E_{max}, 93.2±4.0%; n=7; Figure 3B),
Wistar rat mesenteric arteries (pEC_{50}, 5.8±0.3; E_{max}, 93.8±6.6%; n=3; Figure 3C), and SHR mesenteric arteries (pEC_{50}, 6.6±0.2; E_{max}, 93.2±2.6%; n=2; Figure 3D). C21 did not constrict human coronary microarteries (n=2; data not shown) or Wistar rat iliac arteries (n=4; Figure 3E). It did, however, constrict SHR iliac arteries (n=4; Figure 3F), and both irbesartan (n=4) and PD123319 (n=3) blocked this constrictor effect (Figure 3F).

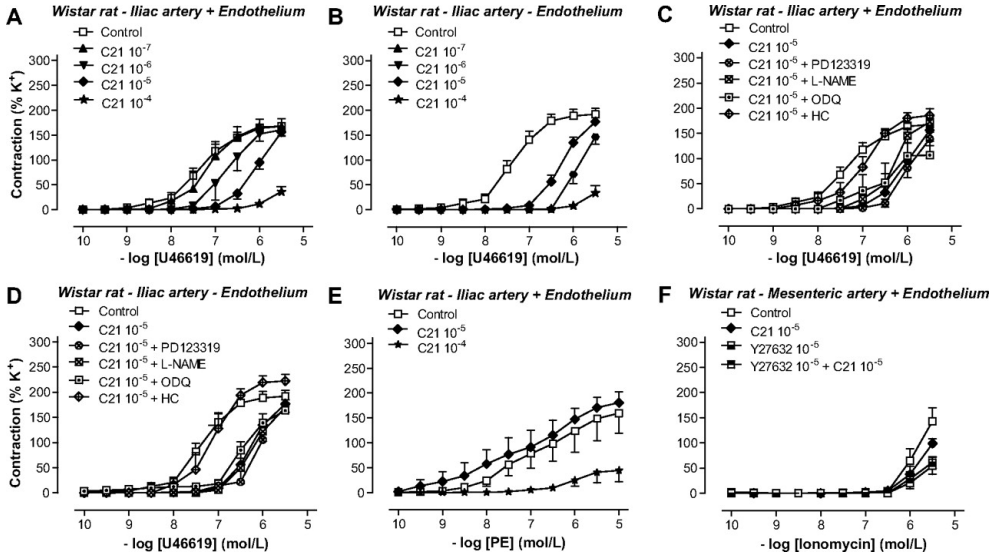

Figure 4.
Effects of U46619 (A through D), phenylephrine (E), or ionomycin (F) in the absence or presence of increasing compound 21 (C21) concentrations with or without endothelium, PD123319, NG-nitro-L-arginine methyl ester (L-NAME), 1H-[1,2,4]oxadiazolo[4,3-a] quinoxalin-1-one (ODQ), hydroxocobalamin (HC), or Y27632 in iliac (A through E) and mesenteric (F) arteries of the Wistar rat. Data are mean±SEM of n=3 to 19 and have been expressed as a percentage of the contraction induced by 100 mmol/L of KCl.42

To study the mechanism underlying the C21-induced vasorelaxation, we focused on AT receptors, the NO pathway, and calcium entry. The NO scavenger hydroxocobalamin shifted the C21 CRCs in Wistar rat iliac arteries (pEC_{50}, 4.7±0.2; E_{max}, 85.5±8.4%; n=4; Figure 3B) and rat mesenteric arteries (pEC_{50}, 5.1±0.2; E_{max}, 89.5±7.0%; n=3; Figure 3C) ≈5- to 10-fold to the right ($P<0.05$), whereas irbesartan, PD123319, the endothelial NO synthase inhibitor L-NAME, or the guanylyl cyclase inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one were without effect. Irbesartan and PD123319 also did not affect the C21 responses in SHR iliac arteries (n=4; Figure 3D).

C21 relaxed preconstricted mouse iliac arteries (pEC_{50}, 5.7±0.1; E_{max}, 97.8±1.4; n=8; Figure 3G) and mouse abdominal aortas (pEC_{50}, 5.7±0.1; E_{max}, 98.4±0.3%; n=9; data not shown). Like in the rat, hydroxocobalamin but not L-NAME shifted the C21 CRCs in these arteries 4- to 10-fold to the right (P<0.05). Results in iliac arteries (n=6–10; Figure 3H) and abdominal aortas of $AT_2R^{-/y}$ mice (n=5–10; data not shown) were identical to those in C57BL/6 mice. U46619 concentration-dependently constricted rat iliac arteries (n=21; Figure 4A). C21 (at a concentration of 1, 10, and 100 µmol/L, respectively) shifted the U46619 CRCs ≈8, ≈25, and >80-fold to the right (P<0.05 for all), and hydroxocobalamin but not endothelium removal, irbesartan, PD123319, L-NAME, or 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one prevented this shift (Figure 4B through 4D). Similar observations were made for phenylephrine (n=3–4; Figure 4E). In contrast, 10 µmol/L of C21 did not affect contractions induced by the calcium ionophore ionomycin either alone or on top the RhoA-kinase inhibitor Y27635 (n=7; Figure 4F). As expected, Y27635 did suppress the ionomycin-induced contraction (n=7; P<0.05; Figure 4F).

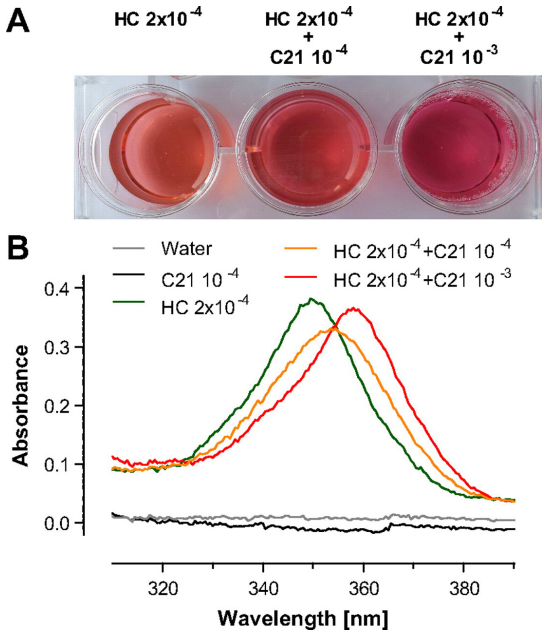

Figure 5.
Compound 21 (C21)–induced alteration of the absorption spectrum of hydroxocobalamin (HC). Please note that NaCl has no such effect.

Spectral Analysis

Spectral analysis (300–560 nm) of solutions containing 200 µmol/L of hydroxocobalamin demonstrated a peak absorption at a wavelength of 349.5 nm. In the presence of 100 µmol/L and 1 mmol/L of C21, the peak absorption was shifted to 354.5 and 358.0 nm, respectively, whereas this was unaffected in the presence of equimolar NaCl concentrations (Figure 5).

Spectral analysis of solutions containing 100 µmol/L of C21 or water did not reveal any absorption peaks within this wavelength range.

DISCUSSION

The present study does not reveal C21-induced, AT_2 receptor-mediated vasodilation in any of the models tested, despite the fact that such vasodilation has been demonstrated previously in these models.[1,2,11,12,22] Simultaneously, we were able to confirm that C21 binds with high affinity to AT_2 receptors.[14,27] Yet, C21 did induce relaxant, as well as constrictor effects, in full agreement with the diversity of C21 effects on blood pressure in a wide range of models, ranging from decreases to increases of ≤25 mm Hg.[14,19–21] Our study now shows at what concentrations these effects occur and provides the mechanisms that potentially underlie these phenomena. Remarkably, the constrictor effects appeared to be enhanced under pathological conditions, because they were best observed in the coronary vascular bed and iliac artery of the SHR. Irbesartan blocked these constrictor effects, suggesting that they were AT_1 receptor mediated. This agrees with the well-established AT_1 receptor upregulation in SHRs.[28] Clearly, therefore, C21 is capable of stimulating AT_1 receptors, as has also been suggested based on in vivo studies.[19,20]

Interestingly, in both Wistar rats and SHRs, PD123319 partially blocked the coronary constrictor effects of C21 (Figure 2), and partial blockade was also observed in C21-constricted iliac arteries of the SHR. Moreover, in male SD rats, Hilliard et al[21] reported inhibition of a C21-induced rise in mean arterial pressure by PD123319. A unifying explanation of these findings is the existence of AT_1/AT_2 receptor heterodimers, coupling to net dilatory/constrictor effects, depending on their ratio and/or location. Alternatively, it should be considered that PD123319, at the applied concentration of 1 µmol/L, exerted a modest degree of AT_1 receptor blockade in our studies.

The biphasic coronary effects of C21 in Wistar rats were mimicked in C57BL/6 mice. In SHRs, after the enhanced coronary constrictor response to C21, a dilator phase was virtually absent. Blocking the initial constrictor effect with irbesartan enhanced the subsequently occurring vasodilation in Wistar rats and reintroduced coronary vasodilation in SHRs. At first sight, this supports an unmasking of AT_2 receptor-mediated coronary vasodilation. However, PD123319 did not block the C21-induced coronary vasodilation in Wistar rats, and a similar vasodilation occurred in $AT_2R^{-/y}$ mice. Moreover, as discussed above, PD123319, if anything, blocked vasoconstriction and enhanced coronary dilation. In addition, in a previous study we were unable to detect AT_2 receptor-mediated vasodilation in the SHR coronary vascular bed.[11] Thus, a non-AT receptor-dependent mechanism must underlie the coronary relaxant effect of C21.

Indeed, all of the responses in isolated vessels, including human coronary microarteries, in our study support a C21-induced, AT_2 receptor-independent vasorelaxation. The concentrations at which this effect occurred were in the micromolar range, that is, well above the nanomolar affinity for AT_2 receptors. Complete relaxation of preconstricted vessels requi-

red a C21 concentration of ≈1 to 10 µmol/L in rodents and 10 to 100 µmol/L in humans.

Concentrations of 1 µmol/L were sufficient to shift the constrictor curves to U46619 ≈10-fold to the right. Most in vitro studies investigating the effects of C21 applied concentrations of ≥0.1 µmol/L.[14,19,29] The single (to the best of our knowledge) previous study investigating C21-induced effects in rodent vessels reported C21-induced relaxations that are comparable to those observed here. However, this study stopped its CRCs at 1 µmol/L of C21, and, thus, no E_{max} or pEC_{50} could be determined.[19] Remarkably, this study observed larger effects (≈25% to 30% relaxation at 1 µmol/L of C21) in the SHR aorta than in the mouse aorta or rat mesenteric artery (≈10% to 20% relaxation).[19] This is unexpected because of the AT_2 receptor phenotype shift reported for SHRs, allowing AT_2 receptors to induce constriction instead of relaxation.[11,12] Unfortunately, no studies with PD123319 were performed to confirm that the relaxant effects of C21 in the SHR aorta truly involved AT_2 receptor stimulation.

In vivo, C21 has been infused at doses ranging from 0.05 to 5.00 µg/kg per minute or was applied IP or orally at doses ranging from 0.3 to 10.0 mg/kg per day.[14–21,30] Given its distribution volume of 3 times total body water, its half life of ≈4 hours, and a bioavailability of ≈30%,[14] this is expected to result in C21 plasma levels ranging from 0.1 to 5.0 µmol/L, that is, well within the range applied here. Such levels (up to >10000-fold above the reported inhibition constant for the AT_2 receptor) are also in agreement with the fact that C21 induced AT_1 receptor-mediated effects in vivo, because its inhibition constant for AT_1 receptors is >10000 times above that for AT_2 receptors.

C21-induced relaxation occurred in an endothelium-independent manner and could be blocked by the NO scavenger hydroxocobalamin but not the NO synthase inhibitor L-NAME or the guanylyl cyclase inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (Figures 3 and 4). This initially suggested a role for nonendothelial NO synthase–derived NO-like factors like S-nitrosothiols.[31] However, an alternative explanation is that the cobalt group of hydroxocobalamin inactivated C21 through interaction with its imidazole ring. Indeed, spectral analysis subsequently confirmed this concept. Thus, most likely, the effect of hydroxocobalamin is attributed to its capacity to bind/inactivate C21 and does not involve NO scavenging.

C21 concentration-dependently shifted the CRCs to both the thromboxane A_2 agonist U46619 and the α-adrenoceptor agonist phenylephrine to the right and fully relaxed U46619- and KCl-preconstricted vessels, which demonstrates that its relaxant effects are not related to a specific receptor. Importantly, C21 did not alter the constrictor response to the calcium ionophore ionomycin. This raises the possibility that C21, instead of directly interfering with contractile (Ca^{2+}-dependent) responses, blocks calcium transport into the cell, thus preventing responses that depend on extracellular calcium, like vasoconstriction.

C21 did not block the RhoA-Rho kinase pathway, which has been reported previously to underlie AT_2 receptor-mediated vasodilation.[32]

PERSPECTIVES

Despite overwhelming data supporting AT_2 receptor-mediated vasodilation,[3–6,33–35] for instance, in the preparations investigated in these studies,[2,11,12] we were unable to demonstrate such vasodilation in response to the AT_2 receptor agonist C21 in rat, mouse, and human vessels. Yet, our current study does support C21-induced vasorelaxation, albeit in an AT receptor-independent manner, possibly involving blockade of Ca^{2+} transport into the cell. Simultaneously, C21 is capable of activating AT_1 receptors, thereby causing vasoconstriction. Taken together, this combination of both relaxant and constrictor effects can help explaining the hypertensive and hypotensive effects of C21 in vivo, including even the absence of such effects.[15–17,20,36] It rules out the application of C21 as an antihypertensive agent. Clearly, studies observing organ-protective effects of chronic C21 application should now carefully determine to what degree these effects truly depend on AT_2 receptor activation, by using either selective AT_2 receptor antagonists or by simultaneously studying the effects in AT_2 receptor knockout models. To guarantee AT_2 receptor selectivity, it appears that C21 infusion rates should stay well below 0.5 µg/kg per minute.

SOURCES OF FUNDING

This study has been performed within the framework of the Dutch Top Institute Pharma, project T2-301 (Renin-Angiotensin System Blockade Beyond Angiotensin II).

DISCLOSURES

None.

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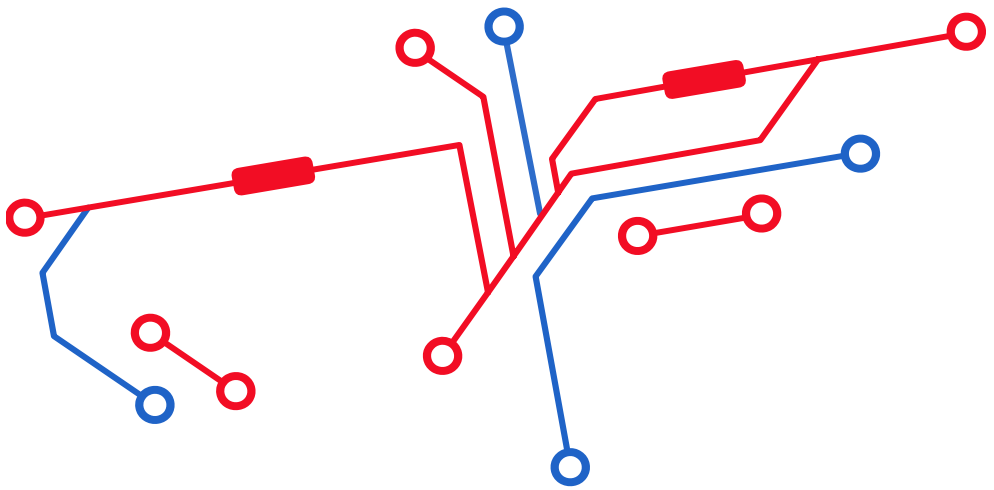
PART 2



Chapter 4
Urinary Markers of Intrarenal Renin-Angiotensin System Activity In Vivo

Curr Hypertens Rep. 2013 Apr;15(2):81-8.

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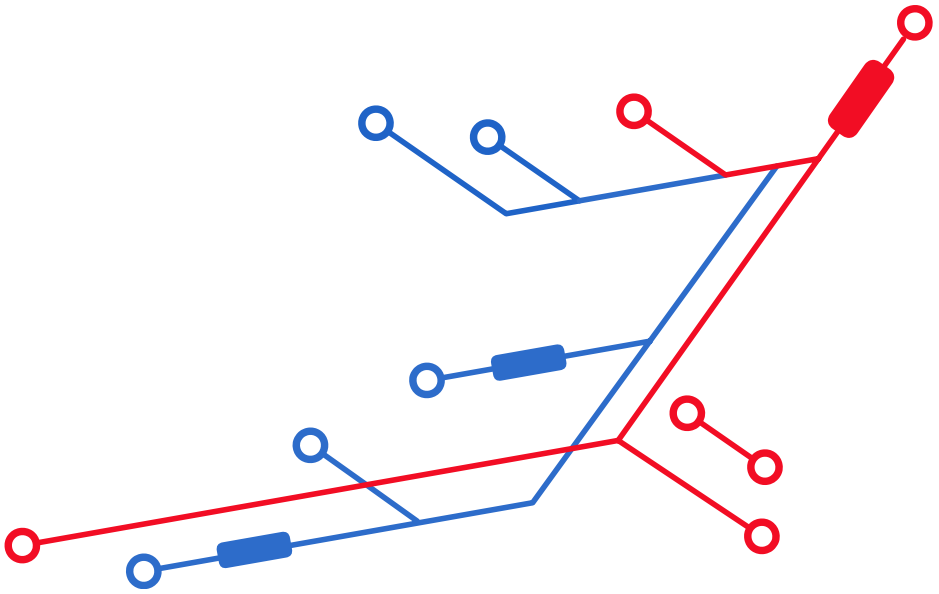

ABSTRACT

Recent interest focuses on urinary renin and angiotensinogen as markers of renal renin-angiotensin system activity. Before concluding that these components are independent markers, we need to exclude that their presence in urine, like that of albumin (a protein of comparable size), is due to (disturbed) glomerular filtration. This review critically discusses their filtration, reabsorption and local release. Given the close correlation between urinary angiotensinogen and albumin in human studies, it concludes that, in humans, urinary angiotensinogen is a filtration barrier damage marker with the same predictive power as urinary albumin. In contrast, in animals, tubular angiotensinogen release may occur, although tubulus-specific knockout studies do not support a functional role for such angiotensinogen. Urinary renin levels, relative to albumin, are >200-fold higher and unrelated to albumin. This may reflect release of renin from the urinary tract, but could also be attributed to activation of filtered, plasma-derived prorenin and/or incomplete tubular reabsorption.

INTRODUCTION

Renal angiotensin (Ang) II levels are several orders of magnitude higher than circulating Ang II levels, and it is, therefore, generally believed that renal Ang II originates at renal tissue sites. Indeed, making use of infusions of 125I-labeled Ang I and II, we were able to show that, despite significant uptake of circulating 125I-labeled angiotensin II, >90 % of renal Ang II could not be explained on the basis of such uptake, and thus truly is derived from local synthesis.[1] Where in the kidneys such generation occurs, and what the effects of locally generated Ang II are, has been discussed elsewhere in detail. [2–4] An obvious question is what determines such generation, i.e., where do the renin, angiotensinogen and angiotensin-converting enzyme (ACE) originate that together generate renal Ang II? A role for non-ACE enzymes (e.g., chymase) seems unlikely given the virtual absence of renal Ang II following ACE knockout.[5]

Renin is made in the juxtaglomerular apparatus, and released into the interstitial space, from where it may reach the circulation via diffusion across the peritubular capillaries. Proximal tubular fluid, however, also contains renin, suggesting that circulating renin is filtered in the kidney.[6] Unexpectedly, renin expression has also been observed in the principal cells of the collecting duct.[7] Kang et al. suggested that such expression is upregulated in the diabetic kidney, and contributes to the elevated levels of prorenin (the inactive precursor of renin) in patients with diabetic nephropathy.[8]

Circulating, liver-derived angiotensinogen diffuses into the interstitium, reaching interstitial fluid levels that are comparable to those in blood.[9]

In addition, angiotensinogen mRNA has been demonstrated in the proximal straight tubule, suggesting that angiotensinogen synthesis may also occur locally in the kidney, independently of its synthesis in the liver.[4]

Circulating ACE plays little, if any, role, and, thus, renal Ang II generation will depend entirely on locally expressed, membrane-bound ACE in the kidney.[10] Indeed, in the human kidney, ACE is abundant in the brush border of the proximal tubule, and, remarkably, usually absent in endothelial cells of any vessel type.[11] Endothelial neoexpression of ACE comes into play in different diseases, e.g., diabetes mellitus and chronic arterial hypertension.[11] Recent interest focuses on the occurrence of both renin and angiotensinogen in urine, as markers of renal renin-angiotensin system (RAS) activity, potentially reflecting the disease state.[2, 13] If indeed the renal levels of both proteins reflect their production at renal tissue sites, their measurement would be a simple manner to determine whether the renal RAS is upregulated, thus reinforcing the need for treatment with a RAS blocker. However, before drawing this conclusion, we need to be certain that these urinary proteins, like albumin, do not simply reflect breakdown of the glomerular filtration barrier, i.e., that they are kidney- and not plasma-derived. If plasma-derived, their clinical value on top of a much cheaper urinary albumin measurement needs to be proven.

This review summarizes all current findings on urinary angiotensinogen and renin. It will also discuss the presence of prorenin in urine. Given the less abundant literature on urinary ACE, it does not focus on this RAS component. Moreover, it will also not address urinary aldosterone and Ang II, since these peptides are much smaller and thus highly likely to easily filter from the circulation. Indeed, all urinary aldosterone is plasma-derived, and thus urinary aldosterone is a well-established parameter of changes in circulating aldosterone.[12]

URINARY ANGIOTENSINOGEN

Animal Studies. Infusing Ang II (40 ng/min) in rats on top of a high-salt diet (8 %) not only raised the renal Ang II content, but also increased the urinary angiotensinogen excretion 4-fold, from 1 to 4 nmol/day.[14] At a rat urinary volume of ≈10 mL/day, this corresponds with urinary angiotensinogen levels of 100–400 pmol/mL. Given earlier reports on angiotensinogen expression in proximal tubular cells,[15] and the fact that in this study urinary angiotensinogen excretion correlated with renal Ang II but not plasma Ang II, it was suggested that urinary angiotensinogen reflects renal Ang II production. Surprisingly, Ang II in fact stimulated renal angiotensinogen synthesis, resulting in both elevated renal angiotensinogen levels and increased urinary angiotensinogen excretion, thus, potentially creating a positive feed-forward loop.[16] According to this concept, the rise in renal Ang II content following Ang II infusion involves de novo Ang II formation in the kidney from locally generated angiotensinogen.[17] When interpreting these results, it should be kept in mind that Ang II also raised blood pressure and is known to stimulate hepatic angiotensinogen production.[18]

In combination with the deleterious effects of Ang II on glomerular barrier function, occurring, at least in part, due to the rise in blood pressure, an alternative explanation of these findings is therefore that they reflect increased filtration of circulating, liver-derived angiotensinogen, particularly when its levels are increased.[19] Moreover, infusing 125I-labeled Ang II results, via Ang II type 1 (AT1) receptor-mediated endocytosis, in renal 125I-Ang II levels that, per gram wet weight, are ≈4-5-fold higher than the plasma 125I-Ang II levels per mL at steady state.[20-22] Thus, the rise in renal Ang II following Ang II infusion in rats could be easily attributed to AT1 receptor-mediated endocytosis of infused Ang II. Interestingly, under high-salt conditions, male rats displayed a higher rise in urinary angiotensinogen excretion than female rats during Ang II infusion.[23] Although this could be attributed to the higher renal angiotensinogen mRNA expression in males exposed to Ang II and high salt, an alternative explanation is the higher degree of proteinuria in the male rats of this study, which would be accompanied by increased filtration of circulating angiotensinogen. The urinary angiotensinogen levels in this study ranged from ≤0.1 (control) to >30 ng/day (after Ang II infusion) per gram body weight. At a body weight of ≈250 g and a urinary volume of ≈10 mL/day, this would correspond with urinary angiotensinogen concentrations of 2.5–750 ng/mL (0.04–12 pmol/mL). Thus, the control angiotensinogen levels in this study are >1000-fold lower than those in the above described studies.

Rats exposed to deoxycorticosterone acetate plus high salt display hypertension but not elevated Ang II levels. In this model, urinary angiotensinogen remained in the normal range (1 nmol/day).[24] Yet, the Dahl salt-sensitive rat, when put on a high-salt diet (suppressing the circulating RAS) did display inappropriately elevated renal and urinary angiotensinogen levels in the face of hypertension.[25] Other models evaluating renal dysfunction in the presence of low-to-normal circulating RAS activity (e.g., mice with diabetic nephropathy or IgA nephritis and spontaneously hypertensive rats) also displayed elevated urinary angiotensinogen levels. This could be suggestive for an activated renal RAS, as is believed to exist in diabetes mellitus.[26-29] If, however, this involves increased renal angiotensinogen expression and the subsequent release of this angiotensinogen into urine, the question is whether the increased renal angiotensinogen expression is the cause or consequence (feed-forward concept) of the elevated renal Ang II levels. The urinary angiotensinogen excretion in diabetic mice amounted to 250 µg/day (vs. 20 µg/day in controls) at three days after the induction of diabetes. Interestingly, in that same time period, while on insulin treatment, the urinary volume also increased ≈10-fold (from 1–1.5 to 10–15 mL/day), implying that the actual urinary angiotensinogen concentration did not change. The urinary angiotensinogen concentration in IgA nephritic mice was 15 ng/mL, threefold higher than in control mice. Assuming a mouse urinary volume of ≈1 mL/day, this would imply that these mice excreted 15 ng angiotensinogen/day (vs. 5 ng/day in controls), i.e., >1000-fold less than the mice of the diabetes study.

Taken together, the current animal studies report a wide range (>1000-fold) of urinary angiotensinogen concentrations, even in normal controls, and these levels are, confusingly, presented either per animal per day, per g body weight per day or per mL, and in either grams or moles. Only rarely, plasma and urinary angiotensinogen levels have simultaneously been determined.[27] Unfortunately, the plasma levels were often reported in densitometric units on the basis of Western blot analysis,[25] thus not allowing a comparison with the actual urinary levels of angiotensinogen. This raises the need for a reference protein in urine, preferably of identical size. An obvious candidate is albumin, which has a comparable molecular weight (67 kD vs. 65 kD), and is a well-established marker of nephropathy. Since urinary albumin is generally believed to be entirely plasma-derived, a careful comparison of the urinary albumin and angiotensinogen levels would clarify to what degree urinary angiotensinogen is plasma-derived as well.

HUMAN STUDIES

Unfortunately, data on the urinary albumin levels in the above animal studies are scarce. Yet, ample studies have evaluated the two proteins in urine in humans. In fact, in these studies, without exception, a strong correlation was observed between urinary angiotensinogen and albumin.[12, 13, 30, 31] To study angiotensinogen independently of this relation, Saito et al. selected 28 type 1 diabetes mellitus patients without microalbuminuria, and still observed a 3-fold rise (from 4 to 12 µg angiotensinogen/g creatinine) in urinary angiotensinogen versus 21 controls, although this rise was borderline for significance ($P = 0.045$). In type 2 diabetes mellitus patients, urinary angiotensinogen (10–150 µg angiotensinogen/g creatinine) correlated strongly with the albumin/creatinine ratio, and predicted the annual decline in the estimated glomerular filtration rate (eGFR) over a 10-year period. Not surprisingly, the patients with both albuminuria and high urinary angiotensinogen levels showed the worst decline of eGFR, and a higher incidence of renal and cardiovascular composite endpoints.[30] Unfortunately, no rigorous statistical analysis was performed to determine the independency of the effect of angiotensinogen and albumin on eGFR decline. Such an analysis was performed in 201 patients with chronic kidney disease (eGFR <60 mL/min/1.73 m2 or presence of albuminuria at ≥30 mg/24 hr).[31] Their urinary angiotensinogen levels were 6-fold elevated vs. 201 controls (26.3 vs. 4.4 µg/g creatinine), and correlated highly significantly with urinary albumin. Moreover, both urinary angiotensinogen and urinary albumin correlated negatively with eGFR. However, when correcting for albumin, the angiotensinogen-eGFR association remained significant. Thus, elevated urinary angiotensinogen levels on top of elevated urinary albumin levels independently determined eGFR decline.

Yet, the analysis did not correct for the elevated plasma angiotensinogen levels that were observed in the patients with chronic kidney disease of this study, and thus it cannot be

excluded that the independent effect of urinary angiotensinogen in reality, via glomerular filtration, reflects the activation of the systemic RAS. Yamamoto et al. confirmed the association between urinary angiotensinogen and eGFR decline in patients with chronic kidney disease, and additionally showed that the AT1 receptor antagonist losartan reduced urinary angiotensinogen.[32] Importantly, losartan also reduced plasma angiotensinogen, albeit to a lesser degree. Unfortunately, no comparison with urinary albumin was made in this study.

In hypertensive patients with a preserved kidney function, the urinary angiotensinogen/creatinine ratio correlated with blood pressure and the urinary albumin/creatine ratio.[13] Treatment with RAS blockers decreased the urinary angiotensinogen/creatinine ratio in these patients to levels that were also observed in normotensive controls (from 25 to 14 µg/g creatinine). This decrease was comparable to that in urinary albumin (from 132 to 29 mg/g creatinine). A correlation between blood pressure and the urinary angiotensinogen/creatinine ratio was even observed after excluding patients with diabetes mellitus or patients who were receiving antihypertensive treatment, in the Bogalusa Heart Study.[33] Lantelme et al. observed a relationship between urinary angiotensinogen and blood pressure only in women with essential hypertension and a low plasma renin/aldosterone ratio, and not in men with this type of hypertension, nor in patients with other types of essential hypertension. [34]

Elevated urinary angiotensinogen/creatinine ratios have been observed in IgA nephropathy in several studies, and kidney biopsies of such patients confirmed upregulated expression of angiotensinogen and enhanced Ang II immunoreactivity.[5, 36] Treatment of patients with IgA nephropathy with an AT1 receptor antagonist (valsartan) reduced urinary angiotensinogen.[36] Furthermore, the sodium sensitivity index (i.e., the reciprocal of the slope of the pressure-natriuresis curve drawn by linking the datapoints obtained during a normal- and a low-salt diet) in IgA nephropathy patients correlated positively with the log-transformed urinary angiotensinogen/creatinine ratio (17.5 vs. 7.9 µg/g, normal vs. low-salt), but not with the urinary protein excretion.[37] However, when analyzing the data, protein excretion was neither corrected for creatinine, nor analyzed with a non-parametric approach despite its usual non-normal distribution, thus making a true comparison with angiotensinogen difficult.

Summarizing, the variation in urinary angiotensinogen levels in humans is much smaller than in rodents, and the levels in healthy individuals are in a very close range: usually <10 µg/g creatinine, or, at a creatinine level of ≈1 g/L, 10 ng angiotensinogen/mL, i.e., 0.2 pmol/mL. This is several orders of magnitude lower than the urinary levels reported in rodents. Human values were in most cases log-transformed prior to analysis. Studies that also determined urinary albumin or protein usually found a close correlation between these parameters and urinary angiotensinogen, including the changes that occurred following RAS blockade.

In the rare cases that the associations between urinary angiotensinogen and disease parameters were independent of or different from albumin (or protein), either the albumin (or protein) data were differently expressed (i.e., not corrected for creatinine and non-log-transformed) or the changes in circulating angiotensinogen were not taken into consideration.[31, 37] The latter is of the utmost importance, to rule out that urinary angiotensinogen truly behaves independently of circulating angiotensinogen. Indeed, when correcting urinary angiotensinogen for plasma angiotensinogen in 101 hypertensive patients with or without diabetes mellitus, we observed that urinary angiotensinogen in all aspects mimicked urinary albumin.[12]

FILTRATION VERSUS LOCAL SYNTHESIS.

An elegant study in mice by Nakano et al. has recently investigated to what degree infused human angiotensinogen filters into urine, simultaneously making a comparison with albumin.[38] Both proteins were fluorescently labeled and applied via bolus injection into the carotid artery. Immediately thereafter, fluorescence was measured in Bowman's space and the glomerular capillaries to determine glomerular permeability. The data revealed that almost no labeled human angiotensinogen showed up in Bowman's space (four times less than labeled albumin), and that its urinary levels were below the detection limit. Increased sieving of angiotensinogen did occur with an increasing level of glomerulosclerosis, but not to a degree that would explain the rise in urinary angiotensinogen in this condition. Here it should be acknowledged that both Richoux et al. and Pohl et al.[19, 39] have demonstrated that angiotensinogen does occur in the ultrafiltrate, but is largely removed via endocytotic uptake in the tubulus, in a megalin-dependent manner. Endocytosed angiotensinogen is subsequently degraded, and the amount of angiotensinogen in the proximal convoluted tubules correlated closely with the plasma level of angiotensinogen.[39]

Importantly, in the Nakano et al. study,[38] the glomerular permeability of infused human angiotensinogen correlated with the urinary albumin levels, but not with the urinary rodent angiotensinogen levels. The authors therefore proposed that endogenous urinary angiotensinogen originated in the kidney, most likely in the tubules. They detected urinary mouse angiotensinogen levels of ≈2 ng/90 minutes versus ≈2000 ng/mL in plasma. In rats developing glomerulosclerosis, the urinary angiotensinogen levels were ≈30 ng/90 minutes at young age, and these levels increased to ≈100 ng/90 minutes at older age. Given the urinary volume of rodents of at most a few mL per day, this implies that in these animals the endogenous angiotensinogen levels in urine, like in all previous rodent studies, were several orders of magnitude above those in humans.

When considering the absence of human angiotensinogen in rodent urine in the above study, it should be taken into account that the application of a bolus injection will not result in a stable steady-state level of angiotensinogen in blood.

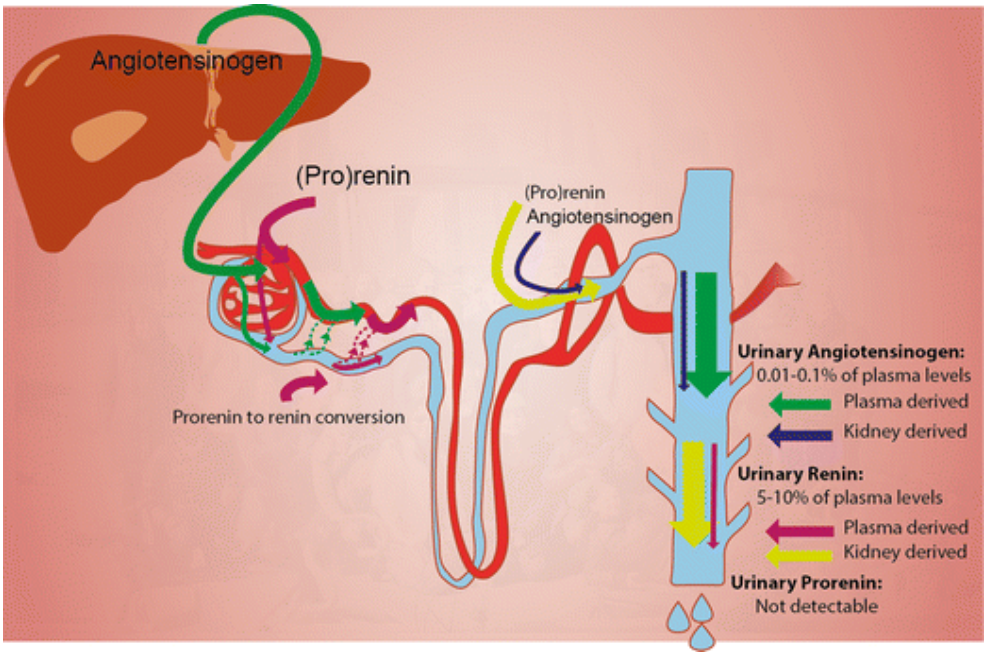

Figure 1.
Overview of the origin of urinary renin and angiotensinogen. In humans, angiotensinogen in urine correlates closely with albumin, and thus, like albumin, reaches urine via glomerular filtration from plasma. Levels range from <10–150 µg/g creatinine, or (assuming a creatinine content of ≈1 g/L), <10–150 ng/mL (i.e., <0.2–2 pmol/mL). This corresponds with <0.01–0.1 % of the plasma angiotensinogen levels in humans. In rodents, urinary angiotensinogen levels are usually much higher, and range from <0.5–4000 pmol/day, or (assuming a urinary volume of ≈1 mL/day in mice and ≈10 mL/day in rats) <0.1–400 pmol/mL. This implies that the urinary angiotensinogen levels in rodents sometimes are higher than their plasma levels. Such high levels could reflect angiotensinogen release from renal tissue sites, possibly the proximal tubule. Urinary renin levels in humans were 5–10 % of the plasma renin levels, i.e., 1–2 orders of magnitude higher than the corresponding urinary levels of albumin and angiotensinogen. Prorenin was undetectable in urine. Most likely, therefore, urinary renin has 3 sources: filtration from plasma, release from the urinary tract (possibly the collecting duct), and intrarenal conversion of (plasma-derived) prorenin to renin

Nakano et al. measured circulating angiotensinogen at one time point (90 minutes after injection), and found it to be equal to the level of endogenous mouse angiotensinogen. However, angiotensinogen, like renin, normally diffuses into the interstitium,[9, 40] reaching interstitial levels that are comparable to those in plasma.[41–43] Consequently, a substantial amount of the infused human angiotensinogen would be expected to diffuse into the interstitial space in the first few hours post-injection, thereby potentially limiting its accumulation in urine. Normal angiotensinogen levels in human urine are ≈0.2 pmol/mL (see above), versus ≈1200 pmol/mL in plasma, i.e., the urinary levels are at most 0.01 % of the plasma levels.

It is, therefore, likely that, in mice, the urinary levels of human angiotensinogen, in view of its whole-body distribution, remained <0.01 % of its post-injection plasma level, i.e., (far) below the detection limit of the applied assay. A similar line of reasoning applies to a study where the authors failed to detect human angiotensinogen in rat urine following its intravenous injection.[24]

A unifying concept, given the >100-fold higher urinary angiotensinogen levels in rodents, might be that in humans urinary angiotensinogen is exclusively plasma-derived, hence, its close correlation with albumin, whereas in rodents it is indeed largely kidney (proximal tubule)-derived (see Fig. 1). To investigate the physiological importance of such kidney-derived angiotensinogen, a kidney-specific angiotensinogen knockout mouse has been developed.[44] Unexpectedly, such mice had renal angiotensinogen and Ang II levels that were identical to those in control mice, both under normal conditions and following podocyte injury. Thus, it was concluded that, even if angiotensinogen is synthesized in the kidney, it has no functional role, i.e., it does not contribute to renal angiotensin production. This contradicts the feed-forward loop between Ang II and renal angiotensinogen,[16] but would still imply that (non-functional) angiotensinogen in the urine of rodents provides an indication of the amount of Ang II to which the kidney (proximal tubule) is exposed. Clearly, evidence is now needed to what degree this holds true in animal models with severe glomerular damage, resulting in increased filtration of plasma proteins, including albumin and angiotensinogen.

URINARY RENIN AND PRORENIN

Renin was originally described in human urine by Lumbers and Skinner.[45, 46] They found no relationship with urinary creatinine or urinary protein, nor with plasma renin. On average, the urinary renin level was ≈7 % of that in plasma. Remarkably, although women normally have lower plasma renin levels than men,[47] their urinary renin levels were higher. Natriuretic therapy (spironolactone + chlorothiazide) elevated plasma renin 5-fold, but only modestly increased urinary renin. As a consequence, renin clearance (mL plasma/24 hours) fell by >50 %, whereas protein clearance fell by ≈20 %. The authors concluded that during sodium depletion either increased tubular reabsorption of renin had occurred, or that urinary renin originated from the urinary tract (tubular sites and/or the collecting duct). In case of the latter, such local renin release then apparently remained relatively constant in the face of increased circulating renin levels.

Yukimura et al. confirmed the modest role of urinary excretion in the metabolism of renin, and were unable to demonstrate prorenin in the urine of dogs,[48] Unlike Lumbers and Skinner, these authors did see a rise in urinary renin excretion when elevating plasma renin in dogs, although in this case plasma renin was elevated by injecting semipurified kidney renin.

Mazanti et al. found that renin in mouse urine was two orders of magnitude lower than in plasma, and also observed an increase in urinary renin following injection of exogenous renin.[49] Importantly, when blocking tubular reabsorption with lysine, urinary renin rose >100-fold, without altering plasma renin.[49] This suggests that normally, ultrafiltered renin is reabsorbed almost completely. Indeed, Pohl et al. confirmed such reabsorption, and suggested that it involves megalin, since immunoreactive renin could not be demonstrated in the proximal tubulus of mice lacking tubular megalin.[19] Nielsen et al. observed very little, if any, prorenin in mouse urine.[50] However, after lysine, prorenin levels in urine became detectable, and the authors were able to estimate that the urinary clearance of prorenin in the absence of tubular reabsorption was ≈10-fold lower than that of renin.

In a recent study involving 101 diabetic and non-diabetic patients with or without hypertension, we observed urinary renin levels that were around ≈6 % of those in plasma,[12] identical to the observations by Lumbers and Skinner.[45, 46] Prorenin was undetectable in urine in our hands, and this was not due to prorenin-renin conversion in urine, since adding prorenin to urine did not result in renin generation. Renin in urine was unrelated to urinary albumin or angiotensinogen, and relative to these proteins >200-fold higher. Urinary renin did not correlate with plasma renin or prorenin. Unexpectedly, urinary renin decreased in patients treated with a RAS blocker (despite their elevated plasma renin levels), and increased in diabetics (despite their lower plasma renin levels). This would be in agreement with the concept that urinary renin is derived from the distal nephron, i.e., the collecting duct, and that Ang II stimulates the release of renin/prorenin from this site, as opposed to its inhibitory role towards renin release from the juxtaglomerular cells. Indeed, several animal studies support the concept of renin synthesis in the principal cells of the collecting duct, and its upregulation by Ang II and diabetes mellitus.[7, 8, 51]

The inability to detect prorenin in human urine may be so for several reasons. First, concentrations may be too low to detect, implying that prorenin is not filtered to the same degree as renin, reabsorbed to a greater degree, and/or not released from the collecting duct. Second, proteolytic cleavage of filtered prorenin may have occurred in the kidney, during the formation of urine. Clearly, such cleavage does not occur in urine per se (given our inability to observe prorenin-renin conversion when adding prorenin to urine ex vivo), although at the same time it is known that the kidneys are the only sites in the body where prorenin-renin conversion occurs. [52, 53]

In summary, it appears that circulating renin is filtered in the glomerulus (given its presence in proximal tubular fluid), but subsequently highly efficiently reabsorbed in the proximal tubule (see Figure 1). This resembles the renal handling of circulating angiotensinogen.[19, 54] For unknown reasons, filtration of circulating prorenin tends to occur to a lesser degree, and is likely to be followed by proteolytic cleavage to renin in the urinary system, thus explaining why urinary prorenin is undetectable in most studies. In addition, there may be

Ang II-stimulated renin release from the collecting duct.
This would explain why urinary renin levels are far above those of albumin and angiotensinogen, and do not run in parallel with changes in plasma renin.

CONCLUSION

In humans, urinary angiotensinogen correlates closely with urinary albumin, and when carefully correcting for changes in circulating angiotensinogen, there is as yet no convincing evidence that there are conditions where urinary angiotensinogen behaves independently of urinary albumin. Therefore, urinary angiotensinogen, like albumin, clearly is a marker of renal disease, and the measurement of both proteins offers the same information. Interestingly, using a proteomics approach, Alge et al. recently identified urinary angiotensinogen and albumin as the two strongest biomarkers in patients with acute kidney injury predicting the need for renal replacement therapy.[55] These investigators subsequently confirmed the prognostic predictive power of urinary angiotensinogen in 97 patients who underwent cardiac surgery for the development of acute kidney injury. Unfortunately, they did not verify the prognostic power of urinary albumin, despite the many studies that support such a role.[56–58]

In rodents, there may be a release of non-functional angiotensinogen into urine from proximal tubular synthesis sites. Ang II stimulates such release. Remarkably, this angiotensinogen neither contributes to the renal angiotensinogen content nor to the renal Ang II levels, implying that all functionally renal angiotensinogen is liver-derived. Liver-derived angiotensinogen may reach renal tissue sites via diffusion into the interstitium and/or glomerular filtration. [44]

Urinary renin potentially has three sources: filtration from plasma, release from the collecting duct, and intrarenal conversion of (plasma-derived) prorenin to renin. Normally, filtered renin is highly efficiently reabsorbed, and the same applies to filtered prorenin. Thus, most urinary renin would then be derived from the collecting duct. Only when greatly elevating circulating renin (e.g., by infusing exogenous renin or by blocking the RAS highly efficiently), tubular reabsorption falls short, allowing plasma renin to also accumulate in urine. The same will occur when plasma prorenin levels are elevated, provided that filtered prorenin is converted to renin in the urinary system. Thus, an alternative explanation for the elevated urinary renin levels in diabetics vs. non-diabetics is that it is the consequence of their elevated prorenin levels.[12, 47] Clearly, we need prospective studies evaluating the use of urinary renin as an independent marker, e.g., to establish the renal efficacy of RAS blockers and renal RAS activity in diseases such as diabetes mellitus and CKD.

ACKNOWLEDGMENT

E.J. Hoorn is supported by a grant from The Netherlands Organisation for Scientific Research (NWO, Veni grant, 91612140).

DISCLOSURE

No potential conflicts of interest relevant to this article were reported.

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Chapter 5
Methodologic Issues in the Measurement of Urinary Renin

Clin J Am Soc Nephrol. 2014 Jul;9(7):1163-7.

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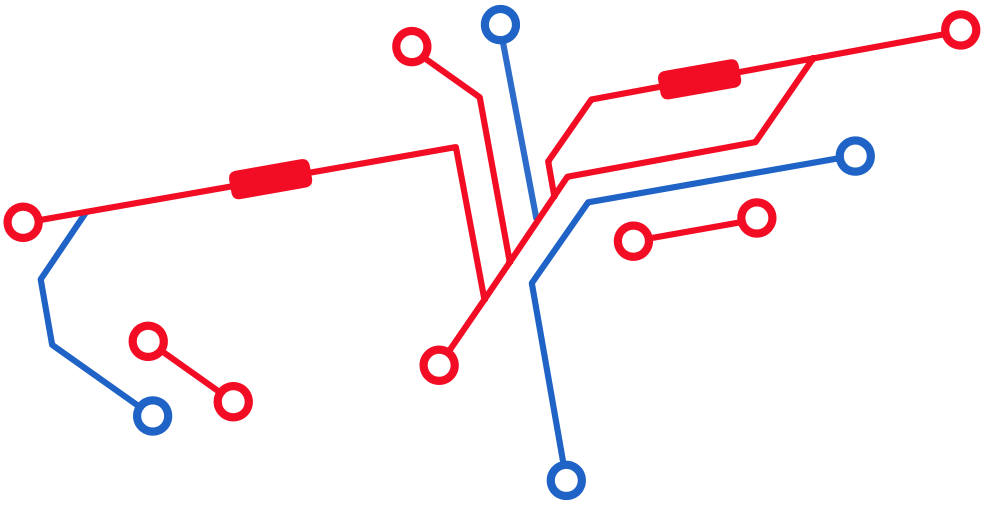

Abstract

Background and objectives

Alge et al. recently reported that urinary renin may be a prognostic biomarker for AKI after cardiac surgery. However, their urinary renin levels far exceeded published plasma renin levels, whereas normally, urinary renin is <10% of plasma renin. This result raises questions about the specificity of the new Quantikine Renin ELISA Kit used in the work by Alge et al., which is claimed to detect total renin (i.e., renin and prorenin). Therefore, this study tested this assay.

Design, setting, participants, & measurements Plasma and urine from 30 patients with hypertension, diabetes, or preeclampsia and 10 healthy pregnant women (randomly selected from sample sets obtained earlier to investigate urinary renin-angiotensin system components) were used to compare the ELISA with a validated renin immunoradiometric assay and an in-house enzyme kinetic assay. Measurements were performed before and after in vitro prorenin activation, representing renin and total renin, respectively.

Results Total renin measurements by ELISA, immunoradiometric assay, and enzyme kinetic assay were highly correlated. However, ELISA results were consistently ≥10-fold higher. The ELISA standard yielded low to undetectable levels in the immunoradiometric assay and enzyme kinetic assay, except after prorenin activation, when the results were ≥10-fold lower than the ELISA results. In plasma, prorenin activation increased ELISA results by 10%–15%. Urine contained no detectable prorenin.

Conclusions The ELISA renin kit standard is prorenin, and its immunoreactivity and enzymatic activity after conversion to renin do not match the International Reference Preparation of human renin that has been used to validate previous immunoradiometric assays and enzyme kinetic assays; in fact, they are at least 10-fold lower, and thus, any measurements obtained with this ELISA kit yield levels that are at least 10-fold too high. The ELISA antibodies detect both renin and prorenin, with a preference for the former. Given these inconsistencies, urinary renin levels should be measured by established renin assays.

INTRODUCTION

Renin-angiotensin system (RAS) components in urine are currently being evaluated as renal disease markers, the underlying concept being that they reflect the activity of the renal RAS independent from the circulating RAS and that the renal RAS contributes to renal disease [1,2]. For instance, Alge et al.[3] recently suggested that urinary angiotensinogen is a prognostic biomarker for the progression of AKI after cardiac surgery. In a subsequent study, Alge et al.[4] additionally studied urinary renin to improve prognostic power. Remarkably, the urinary renin-to-creatinine ratios in that study, involving patients between 58 and 79 years old, ranged from 114 to 2894 pg/mg.

Given the urinary creatinine concentration in such patients (approximately 1 mg/ml), these results would translate to urinary renin levels of 114–2894 pg/ml [5,6]. Such levels are several orders of magnitude higher than the urinary renin levels reported by others (approximately 1–2 pg/ml; range=0.03–157 pg/ml) [7–9] and even substantially higher than the plasma renin levels in cardiac patients.[10] This finding raises questions about the specificity of the ELISA used in the works by Alge et al. to measure renin (Quantikine ELISA; R&D Systems, Abingdon, UK).[4] One reason for this discrepancy might be that the ELISA simultaneously detects prorenin, the inactive precursor of renin (i.e., it actually measures total renin [renin and prorenin]), and not renin. However, although prorenin levels in plasma are generally 10-fold higher than plasma renin levels, urine contains no detectable prorenin, thereby ruling out this possibility.[7]

Classically, renin is measured immunoreactively, making use of antibodies that recognize the active site of renin, or enzyme kinetically (i.e., based on its capacity to generate angiotensin I [Ang I]).[11] The latter is more sensitive but can obviously not be used in samples from patients treated with a renin inhibitor. In the present study, we compared the new ELISA kit with both an established renin immunoradiometric assay (IRMA) and an in-house enzyme kinetic assay (EKA). We focused on urine samples obtained from various patient groups but also tested plasma samples.

MATERIALS AND METHODS

Samples Urine samples were obtained from 10 hypertensive patients (six men and four women; age=58±10 years), 10 patients with diabetes mellitus (eight men and two women; age=59±10 years), 10 healthy pregnant women (age=33±4 years), and 10 women with preeclampsia (age=31±5 years). From the latter two groups, plasma samples were also available. The former two groups have been described previously, and the latter two groups are part of an ongoing study investigating the RAS in pregnancy and preeclampsia.[7,8] The four patients groups were chosen on the basis of availability and their wide variety in renin and prorenin levels; diabetic patients and preeclamptic women have relatively low renin and high prorenin levels versus hypertensive patients, and pregnant women have relatively high renin and prorenin levels.[12,13] The 10 representatives from each group were chosen in a random manner. All studies were approved by the Medical Ethical Review Board and performed in accordance with the Declaration of Helsinki and Good Clinical Practice. Informed consent was obtained from each subject. Blood was collected in EDTA tubes and centrifuged, and plasma was stored at −20°C until analysis. Urine was frozen without inhibitors and also stored at −20°C until analysis. The samples from the healthy pregnant women and the women with preeclampsia had not been thawed before, whereas the samples from the diabetic and hypertensive patients had been thawed one time. This information is important, because repetitive thawing and freezing of plasma, but not urine, is known to cause prorenin activation (i.e., prorenin-to-renin conversion) without

affecting the total amount of renin and prorenin.

Biochemical Measurements

Total Renin Measurement in Plasma and Urine by Quantikine ELISA.

Total renin in urine and plasma was measured by Quantikine ELISA, which makes use of a 96-well plate precoated with a (pro)renin antibody (i.e., an antibody that recognizes both renin and prorenin). After binding renin and prorenin, a secondary (pro)renin-specific enzyme-linked monoclonal antibody is added, which after washing, is incubated with a substrate solution that allows a color to develop in proportion to the amount of renin bound in the initial step. The color is subsequently detected making use of a microplate reader (VersaMax ELISA Microplate Reader; Molecular Devices, Sunnyvale, CA). The detection limit is 4.4 pg/ml, and the standard curve ranges from 31.3 to 2000 pg/ml.

Renin and Total Renin Measurement in Plasma by Cisbio IRMA.

Renin in plasma was measured with an IRMA (Renin III; Cisbio, Gif-sur-Yvette, France), making use of an active site-directed radiolabeled antibody.[11] Total renin can also be measured with this assay either after converting prorenin to renin with trypsin (proteolytic activation; i.e., the prosegment is cleaved off) or by incubating the sample for 48 hours with 10 µmol/L aliskiren.[14] The latter procedure induces a conformational change in the prorenin molecule (nonproteolytic activation; involving the removal of the prosegment from the enzymatic cleft), allowing its recognition by the active site-directed antibody. The detection limit of the IRMA is 1 pg/ml, and the standard curve ranges from 2.5 to 320 pg/ml.

Renin Measurement in Urine by Cisbio IRMA or EKA.

Renin in urine was measured by either IRMA or EKA.[11] The latter involves the incubation with excess sheep angiotensinogen and angiotensinase inhibitors and the subsequent detection of the generated Ang I by radioimmunoassay. The detection limit of the EKA is 0.05 ng Ang I/ml per hour. Ang I–generating activities were converted to renin concentrations based on the fact that 1 ng Ang I/ml per hour corresponds with 2.6 pg human renin/ml. [15] Before their application in the IRMA, the urine samples from the patients with hypertension and diabetes were concentrated 5-fold with Amicon Ultra-10 centrifugal filter devices (EMD Millipore, Cork, Ireland). This concentration step was not applied to the samples from pregnant women because of their higher renin content. Prorenin activation did not increase the renin content of the urine samples,[7] suggesting that urine does not contain prorenin.

Statistical Analyses

Results are expressed as mean and SD. Levels that were below the detection limit were considered to be equal to the detection limit to allow for statistical evaluation. Differences between levels before and after prorenin activation were assessed by paired t tests.
Data were logarithmically transformed before analysis in case of non-normal distribution. Correlations between assay results were assessed by linear regression. $P<0.05$ was considered statistically significant. Statistical analyses were performed with SPSS (version 21;

IBM).

RESULTS

Urinary renin in 40 samples, when measured by IRMA or EKA, amounted to 1.9±2.2 pg/ml. Results were identical after prorenin activation (data not shown), suggesting that urine does not contain prorenin. When measuring these same urine samples in the ELISA, which measures renin and prorenin (i.e., total renin), total renin levels were ≥10-fold higher than levels measured by IRMA or EKA (Figure 1A). An identical 10-fold–higher value was obtained when detecting the IRMA renin standard in the ELISA (Figure 1B). Importantly, urinary total renin levels, when measured by ELISA, were below the detection limit in 26 samples compared with 1 sample when applying the IRMA or EKA. Assuming that the levels in the samples that were below the detection limit equaled the detection limit, the urinary total renin levels on the basis of the ELISA were 20±31 pg/ml.

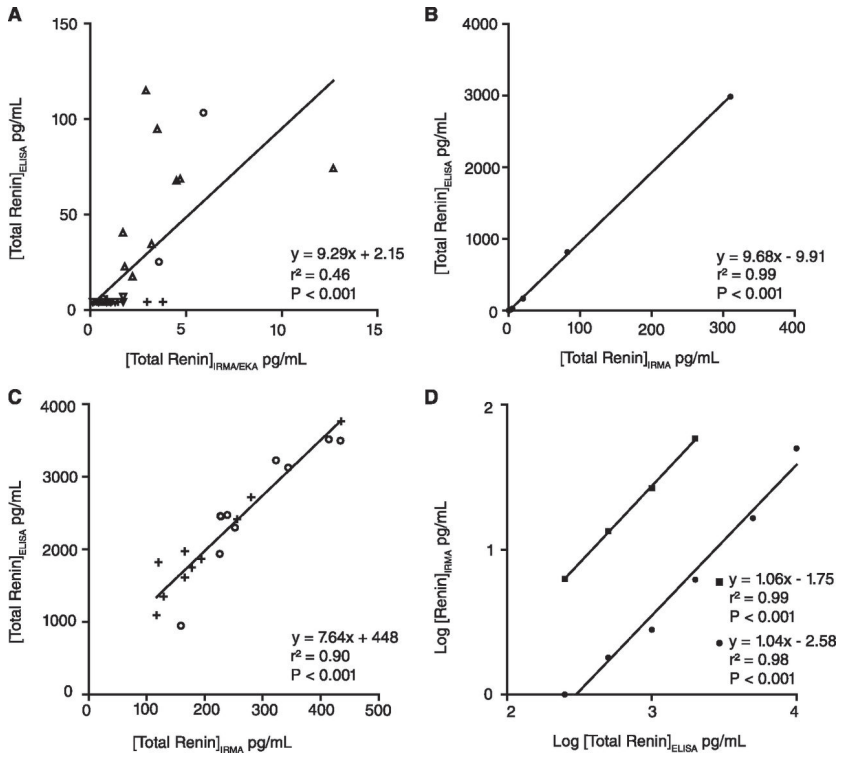

Figure 1.

Comparison of immunoradiometric assay (IRMA) and ELISA total renin measurements. (A) urine samples; (B) IRMA renin standard; and (C) plasma samples. Samples were obtained from patients with hypertension (▽), diabetes (Δ), and preeclampsia (○) and healthy pregnant women (+). D compares the ELISA standard before (●) and after (■) prorenin activation; not enough standard was available to have the same number of observations after prorenin activation as before prorenin activation. In C, the relationship between the two measurements was not different when limiting it to samples that were above the detection limit.

Plasma renin in 20 samples, when measured by IRMA, was 30±18 pg/ml and increased to 244±100 pg/ml after prorenin activation (P<0.001). The latter values represent renin and prorenin (i.e., total renin). The same samples, when measured by ELISA, yielded approximately 10-fold higher total renin levels (2315±810 pg/ml) (Figure 1C). Taken together, these data suggest that the ELISA is less sensitive than the other two approaches and yields total renin levels that are ≥10-fold higher.

The most logical explanation of the above findings is that the ELISA standard differs from the IRMA standard. We therefore measured the ELISA standard in both the EKA and IRMA before and after prorenin activation with trypsin. Figure 1D shows the comparison between IRMA and ELISA. Before prorenin activation, the ELISA standard yielded levels in the IRMA that were approximately 300-fold lower than expected, whereas after activation, the levels were approximately 30-fold lower than expected. Results obtained by EKA were identical to results by IRMA (data not shown). Given the approximately 10-fold increase in both renin immunoreactivity and enzymatic activity after prorenin activation, it is clear that the ELISA standard is actually prorenin. The standard curve of the ELISA is claimed to run from 31.3 to 2000 pg/ml. In reality, it now seems to be from 1 to 67 pg/ml.

Finally, because the ELISA antibodies detect renin and prorenin equally well, it should not matter whether samples are measured before or after nonproteolytic prorenin activation by aliskiren. However, when measuring 8 individual plasma samples (4 samples from healthy pregnant women and 4 samples from women with preeclampsia randomly chosen from 10 samples of each group that were used in this study) before and after aliskiren treatment pairwise in the ELISA, we consistently observed that the levels detected after activation (2626±756 pg/ml) were 10%–15% higher than before activation (2361±719 pg/ml; P<0.01). This finding suggests that at least one of two antibodies applied in this assay has a preference for renin.

DISCUSSION

Urinary renin is increasingly believed to serve as a prognostic biomarker.[1,4,7,8] It may be filtered from plasma or could be kidney-derived.[1] Normally, urinary renin levels are 5%–10% of plasma renin levels.[7-9] Particularly, under conditions where plasma renin levels are high, a substantial percentage of urinary renin may be plasma-derived.[8] Thus, to what degree urinary renin yields information independent from the circulating RAS remains to be determined. Alge et al.[4] recently reported that urinary renin, together with urinary angiotensinogen, associates with AKI after cardiac surgery. Their urinary renin levels were measured with the Quantikine Renin ELISA Kit and exceeded published plasma renin levels.[11,12] However, the present study reveals that this ELISA does not provide reliable renin levels. In fact, the kit simultaneously detects both renin and prorenin, and thus, given that in most body fluids prorenin greatly exceeds renin (up to 100-fold), it provides information on prorenin.[11,12,16] Remarkably for a renin kit, the ELISA uses recombinant prorenin as a standard,

and even more concerning, this prorenin has not been calibrated against the International Reference Preparation of human renin. As a consequence, its total renin (renin and prorenin) results are more than one order of magnitude from published total renin levels over the past 3 decades. Indeed, the normal range claimed by the manufacturer is 201±1851 pg/ml in plasma and nondetectable to 96.7 pg/ml in urine (i.e., >10-fold higher than the levels reported earlier by others in normal subjects).[7,8,11,12]

The Quantikine prorenin standard, when converted to renin, yielded ≥10-fold lower levels than expected in the well established Cisbio renin IRMA and our in-house renin EKA. According to the manufacturer's manual, the ELISA kit relies on antibodies that detect both renin and prorenin (i.e., antibodies that recognize epitopes on renin's main body and not its active site or the prosegment). Nevertheless, our data consistently showed that the ELISA kit results after prorenin activation were 10%–15% higher than before activation, which is suggestive of a renin preference of at least one of two antibodies. Unfortunately, the manufacturer did not provide information on the precise epitopes recognized by these antibodies, and thus, we cannot explain this renin preference.

Given the noncalibrated standard of this ELISA, it is not surprising that Alge et al.[4], when using this kit, obtained urinary renin levels that are far above the normal range in plasma[11] and, most likely, even above the plasma levels in patients who developed AKI after cardiac surgery. It should be noted that plasma renin values were not reported in their study but that their highest urinary total renin levels (2894 pg/ml) exceed the plasma levels of total renin measured in heart failure patients treated with RAS blockers.[10]

Clearly, the urinary renin levels detected with this kit do not reflect reality, and realistic values would only be obtained when taking into consideration that the ELISA standard contains ≥10 times less prorenin than claimed. Without such correction, the ELISA results do not allow comparison with published urinary renin levels. However, even when corrected for the lower prorenin content of the ELISA standard, the kit does not offer a greater sensitivity than the Cisbio IRMA or the EKA. Nevertheless, in view of the correlation between the levels measured with this kit and the levels measured with validated assays (Figure 1), it is still possible that urinary renin is a prognostic biomarker for AKI after cardiac surgery.

We suggest that, in future studies, urinary (and plasma) renin levels should be measured by established renin assays that have been rigorously validated by making use of the International Reference Preparation of human renin.[11] EKAs are most sensitive and can be applied directly to nonconcentrated urine samples. However, they depend on the availability of angiotensinogen and require the measurement of Ang I by radioimmunoassay. IRMA kits are easier to handle but display a lower sensitivity.

Ideally, urine samples obtained from healthy individuals should, therefore, be concentrated approximately 5-fold before the application of such assays. The concentration step is not required when studying samples from patients with an activated RAS.

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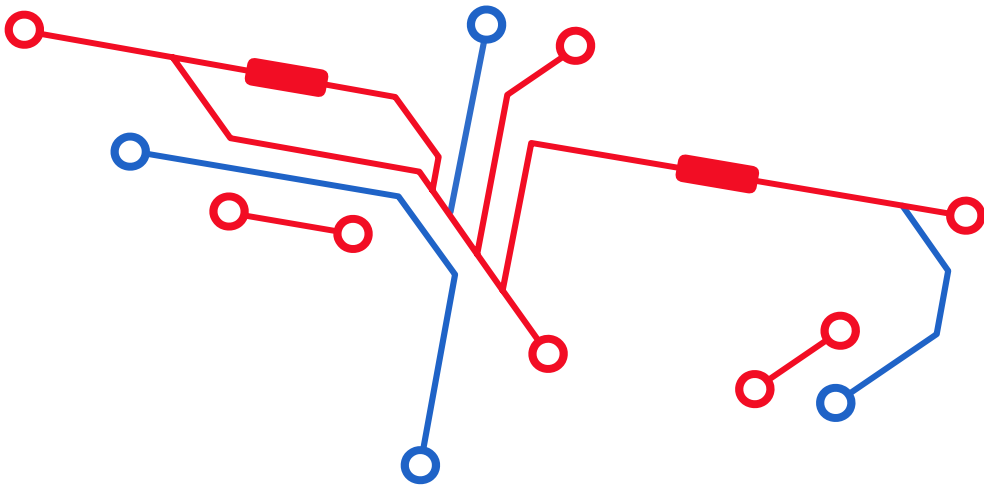
PART 3



Chapter 6
Nieuwe inzichten in pathogenese van pre-eclampsie
De rol van angiogeneseremmende factoren

Ned Tijdschr Geneeskd. 2011;155:A2946.

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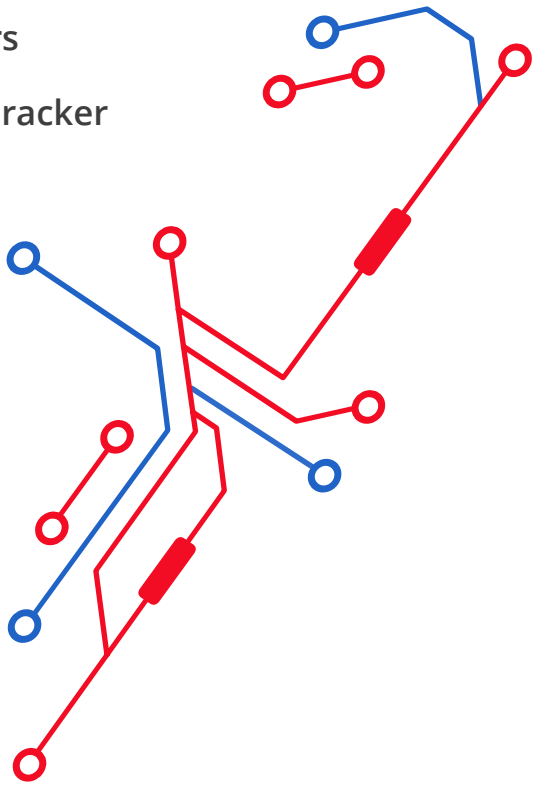


SAMENVATTING

De pathogenese van pre-eclampsie kent 2 fasen. De eerste fase wordt gekenmerkt door insufficiënte placentatie, de tweede fase door een sterk verhoogde afgifte uit de placenta van 2 angiogeneseremmende factoren, namelijk vrij circulerende vormen van fms-achtig tyrosinekinase 1 (sFlt-1) en van endogline (sEng).
In de maternale circulatie remmen sFlt-1 en sEng de effecten van de vasculair-endotheliale groeifactor (VEGF) en transformerende groeifactor ß (TGFß). Dit resulteert in endotheelcel-activatie en inflammatie en leidt uiteindelijk tot het klinisch beeld van pre-eclampsie.
De stijging van de plasmaconcentraties van sFlt-1 en sEng gaat 6-8 weken vooraf aan het optreden van pre-eclampsie.
Onderzocht wordt of de stijging in plasmaconcentraties van sFlt-1 en sEng in combinatie met een dalende concentratie placentaire groeifactor een bruikbare voorspeller voor pre-eclampsie vormen.

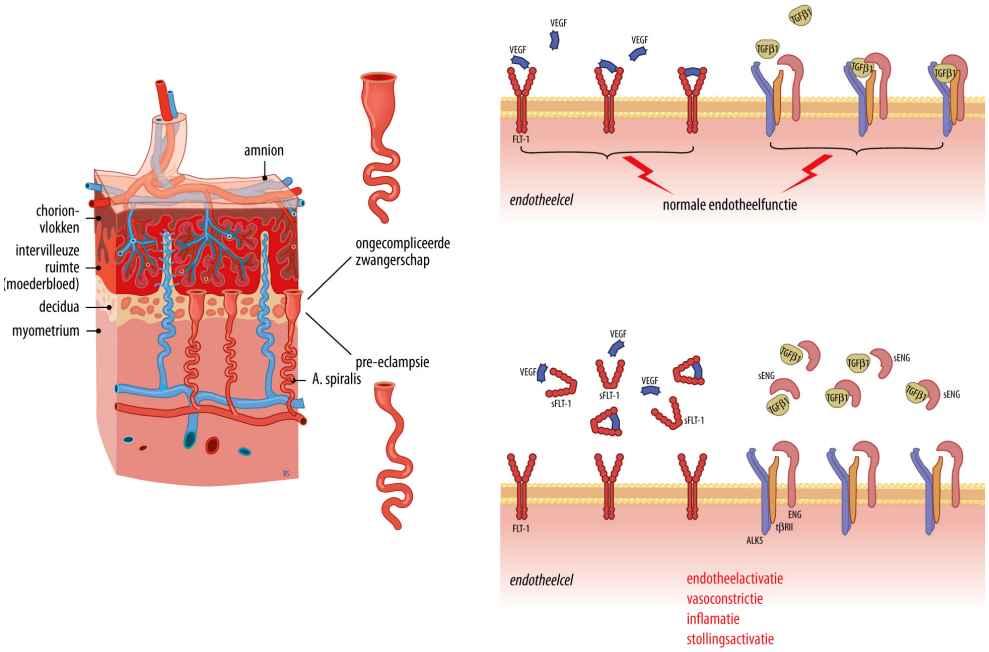
INTRODUCTIE

Pre-eclampsie, gekenmerkt door hypertensie en proteïnurie, is de belangrijkste oorzaak van maternale morbiditeit en sterfte tijdens de zwangerschap in de westerse wereld.[1] Er begint tegenwoordig meer inzicht te komen in het ontstaan van pre-eclampsie. Bij het ontstaan speelt een verhoogde placentaire productie van 2 angiogeneseremmende factoren een belangrijke rol, zo heeft recent onderzoek laten zien. Deze factoren zijn de oplosbare vormen van de receptor voor vasculair-endotheliale groeifactor (VEGF) en van endogline (‘soluble endoglin’, sEng). Algemeen wordt aangenomen dat insufficiënte placentatie leidt tot verhoogde productie van deze factoren, die de angiogenese remmen.[1-4]
In dit artikel gaan wij in op insufficiënte placentatie en de pathofysiologische rol van de angiogeneseremmende factoren. Ook bespreken wij de rol van auto-antistoffen tegen de angiotensine II-type 1-receptor bij het ontstaan van pre-eclampsie. Tevens wordt een verband gelegd met het optreden van een pre-eclampsie-achtig syndroom bij patiënten die behandeling ondergaan met angiogeneseremmers wegens kanker.

Insufficiënte placentatie

Gestoorde placentatie wordt gezien als een belangrijke factor bij het ontstaan van pre-eclampsie. Normaal wordt de placentatie gereguleerd door een samenspel van een groot aantal groeifactoren en cytokines met bijbehorende receptoren.[4] Voor een adequate bloedtoevoer naar de placenta is remodellering van de uteriene spiraalarteriën essentieel.[5]
De remodellering vindt plaats door migratie en differentiatie van foetale cytotrofoblastcellen in de spiraalarteriën, die daardoor veranderen van relatief nauwe weerstandsvaten in wijde capaciteitsvaten (figuur 1). Tijdens deze invasie verandert de cytotrofoblastcel fenotypisch van een epitheelcel in een endotheelcel.
Het mechanisme achter deze migratie en differentiatie is nog niet volledig opgehelderd.

Wel staat vast dat placentaire zuurstofspanning en deciduale 'natural killer'(NK)-cellen bij de placentatie zijn betrokken.[6] Bij pre-eclampsie zijn de invasie en differentiatie van cytotrofoblastcellen in de spiraalarteriën gestoord. Hierdoor schiet de gewenste adaptatie van de spiraalarteriën tekort.[6] Dit leidt via opeenvolgende episodes van ischemie en reperfusieschade tot oxidatieve stress en endoplasmatisch-reticulumstress in de placenta.[7] Hierdoor en door de hypoxie zelf nemen de expressie en productie van ontstekingsfactoren en angiogeneseremmende factoren in de placenta sterk toe. Het vrijkomen van deze factoren in de maternale circulatie veroorzaakt gegeneraliseerde endotheelcelactivatie, die leidt tot vasoconstrictie, inflammatie en stollingsactivatie, wat bijdraagt aan het klinisch beeld van pre-eclampsie.



Figuur 1.

De rol van vasculair-endotheliale groeifactor (VEGF) en transforme- rende groeifactor β (TGFβ) bij ongecompliceerde zwangerschap (boven) en pre- eclampsie (onder). In de ongecompliceerde zwangerschap veranderen spiraalar- teriën van relatief nauwe weerstandsvaten in wijde capaciteitsvaten. Bij dit proces zijn foetale cytotrofoblastcellen betrokken, die veranderen in endotheel- cellen. VEGF en TGFβ zijn noodzakelijk voor de endotheelfunctie. Bij pre-eclampsie is de verwijding van de spiraalarteriën gestoord. Er is een sterk ver- hoogde placentaire afgifte van 2 angiogeneseremmende factoren, namelijk vrij circulerende vormen van de VEGF-receptor ('soluble fms-like tyrosine kinase 1' (sFlt-1)) en van endogline (Eng). Deze factoren vangen VEGF en TGFβ weg, wat leidt tot endotheelcelactivatie en inflammatie (Flt-1 = fms-achtig tyrosine- kinase 1; Alk5 = 'activin receptor-like'-kinase-5; TβRII = TGFβ-receptor II).

Insufficiënte placentatie wordt veroorzaakt door genetische, immunologische, vasculaire en omgevingsfactoren.[4] Vasculaire factoren verklaren waarom pre-eclampsie vaker voorkomt bij zwangerschap op oudere leeftijd en bij zwangeren met onderliggende microvasculaire problematiek, zoals diabetes mellitus, chronische hypertensie en systemische lupus erythematodes (SLE). Hierbij moet worden aangetekend dat insufficiënte placentatie niet altijd leidt tot pre-eclampsie, maar bijvoorbeeld ook gepaard kan gaan met alleen intra-uteriene groeivertraging of zelfs een normaal verlopende zwangerschap.

Angiogeneseremmende factoren en auto-antistoffen

Zoals gezegd blijken 2 angiogeneseremmende factoren een belangrijke rol te spelen in de pathofysiologie van pre-eclampsie, namelijk sEng en een vrij circulerende vorm van een VEGF-receptor. Type 1-VEGF-receptor is een synoniem voor 'fms-like tyrosine kinase 1' (Flt-1), een membraangebonden receptor op onder andere de endotheelcel. Voor de vrij circulerende ('soluble') vorm hiervan gebruiken we de afkorting 'sFlt-1'. Flt-1 is een receptor die wordt gestimuleerd door VEGF en door de placentaire groeifactor (PLGF). Deze 2 groeifactoren worden, evenals sFlt-1, gesynthetiseerd door de placenta en zijn essentieel voor trofoblastfunctie en -overleving.

De angiogeneseremmende factoren zijn ontdekt door genexpressieonderzoek van placenta's van zwangeren met en zonder pre-eclampsie. Uit dit onderzoek bleek dat de placentaire expressie van sFlt-1 en sEng bij pre-eclampsie sterk verhoogd is.[8, 9] Alternatieve 'splicing' (zie uitlegkader) van het DNA dat codeert voor Flt-1 leidt tot de vorming van de oplosbare vorm sFlt-1. Bij de oplosbare vorm ontbreken het transmembrane en intracellulaire domein van de receptor (zie figuur 1). Eenmaal in de maternale circulatie remt sFlt-1 de angiogenese door binding aan VEGF en PLGF (zie figuur 1). Dat sFlt-1 een rol speelt bij het ontstaan van pre-eclampsie is aangetoond in gentransfectie-experimenten.[8] Introductie van het gen voor sFlt-1 in zwangere ratten veroorzaakte hypertensie, proteïnurie en glomerulaire endotheliose, een nierafwijking die kenmerkend is voor pre-eclampsie.[8,10] Toediening van VEGF kon dit beeld voorkomen, wat erop wijst dat de afwijkingen veroorzaakt werden door antagonisme van de effecten van VEGF.[11] In ongecompliceerde zwangerschappen is de plasmaconcentratie van sFlt-1 gedurende de eerste 7 maanden van de zwangerschap relatief constant, om de laatste 2 maanden van de zwangerschap te stijgen. Deze stijging is veel sterker bij zwangeren met pre-eclampsie.[12]

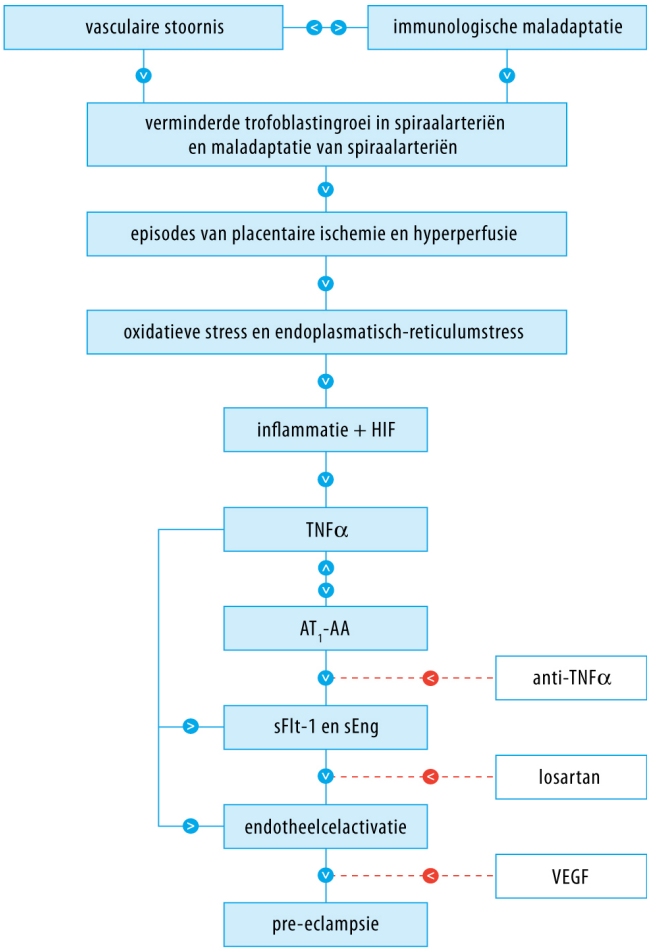
Angiogenese is onontbeerlijk voor tumorgroei. Daarom worden angiogeneseremmers, in de vorm van monoklonale antistoffen tegen VEGF of VEGF-receptorblokkers, tegenwoordig toegepast bij diverse vormen van kanker.[13] Al snel na de introductie bleek onverwachts dat deze middelen bij een deel van de patiënten hypertensie, proteïnurie en de voor pre-eclampsie kenmerkende glomerulaire endotheliose veroorzaakten.[14] Deze bevinding ondersteunt indirect de rol van angiogeneseremmende factoren bij het ontstaan van pre-eclampsie.

ENDOGLINE

Endogline is een membraangebonden coreceptor voor transformerende groeifactor β1 en β3, (TGF-β1 en -β3), met hoge expressie op syncytiotrofoblasten en endotheelcellen.[15] Endogline moduleert de functie van TGF-ß en speelt een rol bij de angiogenese en regulatie van de vaattonus door activering van het stikstofmonoxide-synthase. De oplosbare vorm, sEng, is een proteolytisch splitsingsproduct van het membraangebonden endogline.
In ongecompliceerde zwangerschappen stijgt de plasmaconcentratie van sEng in geringe mate tijdens de laatste maanden van de zwangerschap. Bij vroege pre-eclampsie is deze stijging veel sterker.[16] Analoog aan de gentransfectie-experimenten met sFlt-1, veroorzaakte verhoogde expressie van sEng bij zwangere ratten hypertensie en proteïnurie.[9]
Los van de zwangerschap kunnen mutaties in het gen dat codeert voor endogline erfelijke hemorrhagische teleangiëctasie (ziekte van Rendu-Osler-Weber) veroorzaken.[17] Dit is een autosomaal overervend ziektbeeld, gekenmerkt door arterioveneuze malformaties en focaal capillairverlies. Dit experiment van de natuur bevestigt de rol van endogline bij de angiogenese.

AUTO-ANTISTOFFEN STIMULEREN ANGIOTENSINE II-RECEPTOR

In 1999 werden voor het eerst bij pre-eclampsie IgG-auto-antistoffen beschreven die de angiotensine II-type 1-receptor (AT_1) stimuleren; bij gezonde zwangerschappen kwamen deze auto-antistoffen niet voor.[18] Deze antistoffen waren gericht tegen een epitoop van de AT_1-receptor aan de buitenzijde van de celmembraan. Dat auto-antilichamen membraanreceptoren kunnen stimuleren is een bekend fenomeen. Een klassiek voorbeeld is de ziekte van Graves, waarbij stimulerende antistoffen tegen de thyreoïdstimulerend-hormoon(TSH)-receptor op schildkliercellen hyperthyreoïdie veroorzaken.
In een recente studie waren AT_1-auto-antistoffen (AT_1-AA) aantoonbaar bij 70% van de zwangerschappen gecompliceerd door pre-eclampsie, maar ook bij 20% van de gezonde zwangerschappen.[19] AT_1-AA zijn ook aangetoond bij zwangeren die geen pre-eclampsie ontwikkelden maar wel bij echo-doppleronderzoek een verminderde uteroplacentaire doorbloeding hadden.[20] Ook bij ratten met verminderde uteroplacentaire doorbloeding, die model staan voor zwangeren met pre-eclampsie, zijn AT_1-AA aangetoond.[21] Op grond van deze bevindingen concluderen wij dat AT_1-AA niet geheel specifiek zijn voor pre-eclampsie en dat de productie wordt gestimuleerd in situaties van uteroplacentaire hypoperfusie. Het is interessant dat AT_1-AA niet alleen zijn aangetoond bij pre-eclampsie, maar ook bij maligne hypertensie, afstoting van niertransplantaten en hypertensie.[22]
IgG of gezuiverd AT_1-AA van vrouwen met pre-eclampsie en geïnjecteerd in zwangere muizen veroorzaakte pre-eclampsie, inclusief de kenmerkende glomerulaire afwijkingen.[23] De pre-eclampsie kon worden voorkomen door de angiotensine II-receptorblokker losartan.[23] Dit experimenteel onderzoek bevestigt dat AT_1-AA pre-eclampsie kunnen veroorzaken.



Figuur 2
Pathogenese van pre-eclampsie. De eerste fase wordt gekenmerkt door insufficiënte placentatie, leidend tot hypoxie en oxidatieve stress. In de tweede fase is er een sterk verhoogde afgifte uit de placenta van 2 angiogenese-remmende factoren, namelijk vrij circulerende vormen van fms- achtig tyrosinekinase 1 (sFlt-1) en van endogline (sEng). Deze verhoogde afgifte wordt gemedieerd door tumornecrosisfactor α (TNFα) en auto-antistoffen die de angiotensine II-type 1-receptor (AT1-AA) stimuleren. In experimenten bij proefdieren kon dit proces worden onderbroken door TNFα-remmers en de angiotensine II-receptor-blokker losartan. ook toediening van vEGf, dat bindt aan sFlt-1, kon pre-eclampsie bij proefdieren voorkómen (HIF = hypoxie- induceerbare factor).

WAT ZET AAN TOT AANMAAK VAN SFLT-1, SENG EN AT1-AA?

Een voor de hand liggende vraag is hoe de placentaire productie van de angiogeneseremmende factoren sFlt-1 en sEng wordt aangezet. Op grond van experimenteel onderzoek kan geconcludeerd worden dat zowel hypoxie als ontstekingsmediatoren betrokken zijn bij de verhoogde productie van deze factoren en van AT1-AA (figuur 2).

Hypoxie Insufficiënte placentatie is de initiële fase van pre-eclampsie. Men veronderstelt dat door insufficiënte placentatie de zuurstofspanning in de placenta periodiek daalt. Deze verminderde zuurstofspanning leidt middels verhoogde productie van de hypoxie-induceerbare transcriptiefactor-1 tot een toegenomen productie van angiogeneseremmende factoren.[24] In celkweken van cytotrofoblasten kon worden aangetoond dat de productie van sFlt-1, maar niet die van VEGF of PLGF, sterk toeneemt bij hypoxie, evenredig met de ernst van de hypoxie.[25]

Tumornecrosisfactor α Er zijn ook aanwijzingen dat inflammatie, al of niet secundair aan de hypoxie, een rol speelt bij de toegenomen productie van sFlt-1 en sEng. Onderzoek in zwangere ratten heeft laten zien dat verminderde uteroplacentaire perfusie, geïnduceerd door het aanbrengen van clips om de aorta en ovariële arteriën, leidde tot een verhoogde productie van sFlt-1, sEng en AT_1-AA.[21] In dit model werd ook een verhoogde productie van tumornecrosisfactor α (TNFα) aangetoond. TNFα is een cytokine dat vooral geproduceerd wordt door geactiveerde macrofagen. Deze ontstekingsmediator activeert endotheelcellen. Hierdoor komen adhesiemoleculen tot expressie die leukocyten binden. Daarnaast activeert TNFα T_1-helpercellen. In eigen onderzoek vonden we dat de plasmaconcentraties van TNFα hoger waren in zwangeren met pre-eclampsie dan in gezonde zwangeren met een vergelijkbare zwangerschapsduur.[26]
Zeer recent werd aangetoond dat AT_1-AA verkregen van vrouwen met pre-eclampsie en geïnjecteerd in zwangere muizen, leidden tot de vorming van sEng en sFlt-1.[23,27] De vorming van deze factoren door blootstelling aan AT_1-AA was ook aantoonbaar in humane placenta's.[23] De vorming van sEng en sFlt-1 kon worden geremd door antistoffen tegen TNFα of de oplosbare TNFα-receptorblokker. Op grond van deze bevindingen blijken AT_1-AA de versterkte placentaire productie van angiogeneseremmende factoren te stimuleren, met TNFα als mediator. Dit proces kan worden geblokkeerd door de angiotensine II-receptorblokker losartan en door TNFα-remmers (zie figuur 2).

HET PRAKTISCH BELANG VAN DE NIEUWE INZICHTEN

De kennis dat AT_1-AA, sFlt-1 en sEng een rol spelen bij de pathogenese van pre-eclampsie heeft nog niet geleid tot klinische interventies die op deze factoren zijn gericht. Toediening van VEGF voorkomt wel pre-eclampsie bij proefdiermodellen voor deze aandoening.[11] Wellicht dat deze behandeling in de nabije toekomst ook een optie is voor patiënten met pre-eclampsie.

Ondertussen wordt onderzocht of de angiogeneseremmende factoren bruikbare biomarkers zijn voor het voorspellen van pre-eclampsie in een relatief vroeg stadium van de zwangerschap.[16] Hoewel er nog geen specifieke behandeling voor pre-eclampsie is, kan een eenvoudige manier van voorspellen nuttig zijn voor tijdige verwijzing naar een expertisecentrum. Daarnaast zou het een hulpmiddel kunnen zijn om onderscheid te maken tussen pre-eclampsie en hypertensie die niet aan de zwangerschap gerelateerd is of andere aandoeningen zoals SLE.
Tijdens de laatste 2 maanden van normotensieve zwangerschappen stijgen de plasmaconcentraties van sFlt en sEng en daalt die van PLGF, zo werd in verschillende retrospectieve studies aangetoond.[12] De stijging van sFlt-1 en sEng en de daling van PLGF is meer uitgesproken bij vrouwen die later pre-eclampsie ontwikkelen dan bij normotensieve zwangerschappen.

Deze veranderingen treden 6-8 weken eerder op dan het klinisch beeld van pre-eclampsie. Als pre-eclampsie eenmaal aanwezig is, zijn de stijging van sFlt-1 en sEng en daling van PLGF het meest uitgesproken.
Aangezien de plasmaconcentratie van sFlt-1 sterker stijgt en die van PLGF sterker daalt bij zwangeren met pre-eclampsie dan bij normotensieve zwangerschappen, zou vooral de ratio sFlt-1/PLGF een goede screeningstest kunnen zijn om pre-eclampsie te voorspellen.[12] Een dergelijke test is recent op commerciële basis geïntroduceerd. De toegevoegde waarde van zo'n test ten opzichte van andere voorspellers van een risico op pre-eclampsie is nog onduidelijk.
Wij denken dat AT1-AA minder geschikt zijn om pre-eclampsie te voorspellen dan de concentraties sFlt-1, sEng en PLGF. Dit heeft te maken met de ingewikkelde bioassay waarmee de antistoffen bepaald worden en met het feit dat ze soms ook bij normale zwangerschappen aantoonbaar zijn.[19]

CONCLUSIE

Er is veel vooruitgang geboekt in het ophelderen van factoren die een rol spelen bij het ontstaan van pre-eclampsie, dankzij genexpressieonderzoek van placenta's van vrouwen met en zonder pre-eclampsie en de ontwikkeling van diermodellen voor pre-eclampsie.
De eerste fase van pre-eclampsie wordt gekenmerkt door een gestoorde placentatie met oxidatieve stress, endoplasmatisch-reticulumstress en inflammatie als gevolg. De tweede fase wordt gekenmerkt door overproductie van placentaire angiogeneseremmende factoren die, eenmaal in de circulatie, bijdragen aan het ontstaan van de aandoening.
De bepaling van de ratio van sFlt-1- en PLGF-concentraties kan in de nabije toekomst wellicht gebruikt worden als screeningstest om pre-eclampsie te voorspellen. Daarnaast is het denkbaar dat de nieuwe inzichten leiden tot een meer causale behandeling van zwangeren met pre-eclampsie en betere maternale en foetale prognose van deze aandoening.

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Chapter 7
The renin–angiotensin–aldosterone system in preeclampsia: the delicate balance between good and bad

Clin Sci (Lond). 2014 Apr;126(8):537-44.

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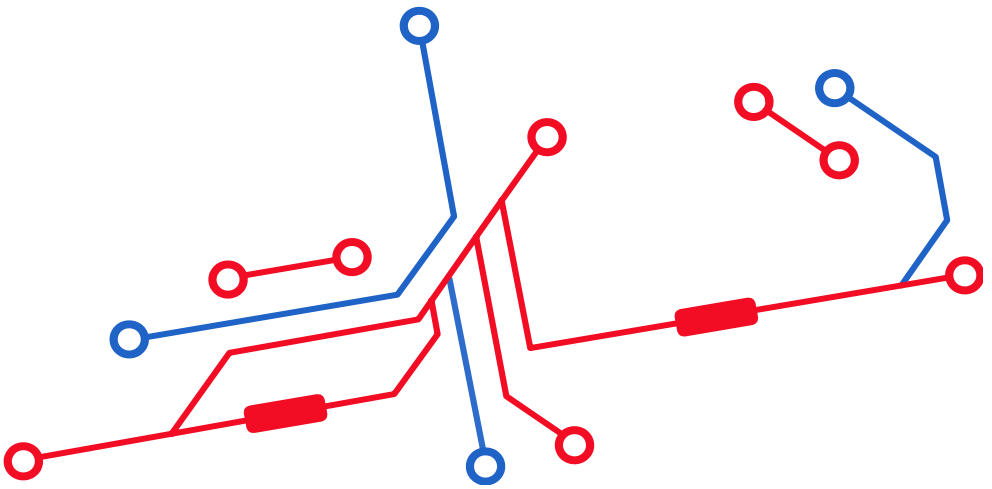

ABSTRACT

Pregnancy demands major changes of the cardiovascular system, and this involves, among others, activation of the RAAS (renin–angiotensin–aldosterone system), allowing an aldosterone-dependent increase in volume. Remarkably, a relative resistance to the pressor response of AngII (angiotensin II) develops simultaneously to prevent the increase in blood pressure that would normally accompany RAAS activation. The increase in volume, the degree of RAAS activation and the diminished pressor response to AngII are less pronounced in preeclampsia. However, animal models displaying excessive RAAS activation also result in a preeclampsia-like syndrome, and the aldosterone/renin ratio is elevated in preeclampsia compared with a normal pregnancy. New insights into the pathogenesis of preeclampsia have revealed a major role for VEGF (vascular endothelial growth factor), VEGF-inactivating sFlt-1 (soluble fms-like tyrosine kinase-1) and AT_1 (angiotensin II type 1) receptor autoantibodies. The last mentioned activate AT_1 receptors, thereby potentially suppressing circulating renin and aldosterone. VEGF, both directly and indirectly (by increasing capillary density), affects adrenal aldosterone synthesis. The present review summarizes all of the recent findings regarding RAAS regulation in preeclampsia compared with normal pregnancy, concluding that factors such as sFlt-1 and AT_1 receptor autoantibodies disturb the delicate balance that normally results in a volume increase and a diminished vasoconstrictor response to AngII in pregnant women. It is possible that there are non-parallel changes in the circulating and renal RAAS in preeclampsia, which are potentialhly reflected by the urinary levels of renin.

INTRODUCTION

Preeclampsia is a condition unique to pregnancy characterized by new-onset hypertension and proteinuria after 20 weeks of gestation. Despite many years of research, the aetiology of the disease is still unknown. Hypertensive disorders, including preeclampsia, are among the most common complications of pregnancy, and they constitute a major cause of maternal, fetal and neonatal morbidity and mortality worldwide. Management of preeclampsia consists of treating the elevated blood pressure and the prevention of seizures when preeclampsia progresses to eclampsia, but there is no definite cure other than.[1]

Currently, two stages in the development of preeclampsia are distinguished. The first stage consists of poor placentation as a result of abnormally shallow cytotrophoblast invasion in the maternal spiral arteries supplying the placenta. As a consequence the extent of the widening of the spiral arteries is insufficient, causing impaired blood flow to the placenta. This leads to the second stage of preeclampsia, which consists of repeated periods of placental hypoxia, resulting in an increased production of ROS (reactive oxygen species), HIF-1α (hypoxia-inducible factor-1α) and TGF-β1 (transforming growth factor-β1). These factors, in turn, increase the production of a splice variant of the VEGF (vascular endothelial growth factor) receptor sFlt-1 (soluble fms-like tyrosine kinase-1).

sFlt-1 can be found at high concentrations in the maternal circulation during preeclampsia and acts as an anti-angiogenic factor by binding free VEGF and PlGF (placental growth factor). A decreased availability of these factors affects the growth and development of the placenta and fetus, but it also affects the mother and is considered to be responsible for the clinical features of preeclampsia when present at increased amounts in the maternal circulation (Figure 1). In particular, the health of endothelial cells and kidney function including the maintenance of the glomerular filtration barrier strongly depend upon these growth factors.[1]

Increased levels of sFlt-1 can be detected 5 weeks prior to the onset of the clinical symptoms, and decreased levels of PlGF can be detected even earlier. Many studies have already been performed investigating sFlt-1 and PlGF for the prediction and diagnosis of preeclampsia with promising results. The highest sensitivity and specificity for predicting and diagnosing preeclampsia are obtained using the sFlt-1/PlGF ratio, where a ratio above 85 was used as the cut-off for a positive test.[2]

Since there is no cure or therapy to prevent preeclampsia, the usefulness of the test is currently limited to predict a poor pregnancy outcome in patients with preeclampsia and to distinguish between other diseases in pregnancy with preeclampsia-like symptoms.[3,4]

Animal models confirm the importance of sFlt-1 in the pathogenesis of preeclampsia. In rats where the perfusion of the placenta is surgically reduced by partially clamping the blood flow to the placenta, increased sFlt-1 levels have been reported. [5] Furthermore introducing sFlt-1 via a viral factor in pregnant rats gives a preeclampsia-like syndrome, with hypertension and proteinuria.[6]

The RAAS (renin–angiotensin–aldosterone system) (Figure 2) is involved in both stages of the disease. Importantly, it is generally believed that some RAAS components, prorenin in particular, are synthesized in the uteroplacental unit, thus allowing local AngII (angiotensin II) production independently of AngII production in the systemic circulation (for an extensive overview see Herse et al.).[7]

The present review discusses preeclampsia-related alterations in various components of the RAAS, some of which were first reported >20 years ago, focusing in particular on their recently elucidated link with placental hypoxia and anti-angiogenic factors. Given its role as a regulator of blood pressure and fluid homoeostasis, changes in the RAAS should be evaluated in view of the changes in the cardiovascular system that occur in normal pregnancy and in a pregnancy complicated by preeclampsia. The haemodynamic changes and changes in the extracellular volume occurring during pregnancy and preeclampsia will therefore be reviewed first.

HAEMODYNAMIC CHANGES IN PREGNANCY AND PREECLAMPSIA

Pregnancy demands major changes in the cardiovascular system. During pregnancy there is a 30–50% increase in the extracellular fluid and a 30–40% increase in plasma volume.

The increase in plasma volume is extremely important, as it is a major determinant of organ perfusion. The driving force for the increase in extracellular volume seems to be a decrease in the systemic vascular resistance, as reflected by a fall in the systolic and diastolic blood pressure in early gestation. It is generally believed that vasodilating factors such as nitric oxide play an important role in the decrease in vascular resistance. [8] Underfilling of the circulation owing to generalized vasodilation may result in a compensatory activation of the RAAS, leading to water and sodium retention (Figure 1). Related to the increase in plasma volume and vasodilation, cardiac output increases by 30–50% during pregnancy via increases in both stroke volume and heart rate.[9]

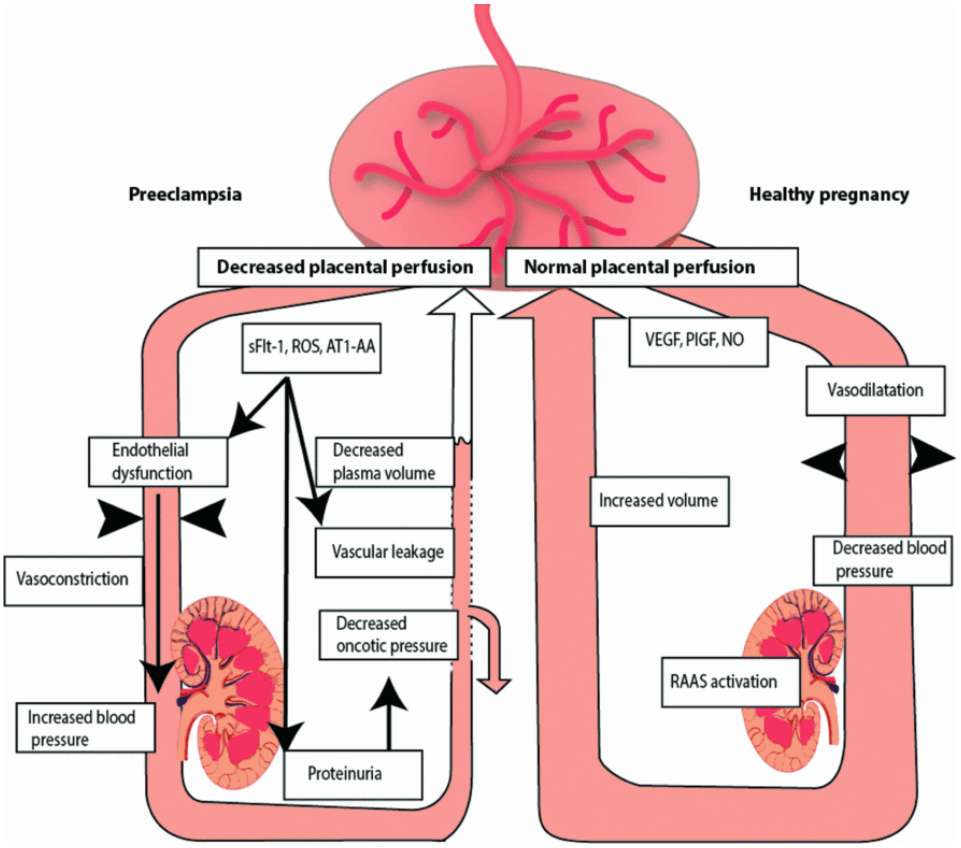

Figure 1

Vascular system and perfusion of the placenta in a pre-eclamptic and a healthy pregnancyIn pre-eclampsia endothelial dysfunction leads to increased vascular resistance, blood pressure elevation, increased vascular permeability and a decrease in oncotic pressure. This will further decrease circulating volume and diminish placental perfusion. In response, the placenta will produce more anti-angiogenic vasoactive factors such as sFlt-1, AT_1-AA and ROS, causing further vasoconstriction and deterioration of placental perfusion.

Furthermore, the renal blood flow and glomerular filtration rate markedly increase during pregnancy, and peak at approximately 50% above non-pregnant levels in the second trimester.[10] As compared with normal pregnancy, the expansion of plasma volume and the decrease in vascular resistance are less pronounced in preeclampsia. This decreased expansion in plasma volume can be detected as early as weeks 14–17 of gestation.[11] Most probably as a direct consequence of the decreased plasma volume, cardiac output is also lower in preeclampsia than in normal pregnancy.[12] Uterine and umbilical artery Doppler flow measurements have revealed that pregnancies complicated either by preeclampsia or growth restriction are characterized by a compromized uteroplacental flow,[11] suggesting that the normally occurring expansion of plasma volume and increase in cardiac output are essential for the maintenance of a sufficient uteroplacental flow.
In summary, besides hypertension and proteinuria, preeclampsia, as compared with normal pregnancy, is characterized by a relatively high vascular resistance, a reduced intravascular volume, a reduced cardiac output and a decrease in uteroplacental flow. Remarkably, despite this reduction in circulating volume, most components of the circulating RAAS are down-regulated in preeclampsia compared with normal pregnancy. Whether this is a physiological response to the increased blood pressure or an active contributor to the pathophysiology of preeclampsia is an unresolved question.

ANGIOTENSINOGEN

Oestrogens stimulate the synthesis of angiotensinogen, resulting in an increase in the levels of circulating angiotensinogen during the first 20 weeks of gestation. Angiotensinogen levels in preeclampsia are comparable with those in normal pregnancy; however, there appears to be a relative increase in the so-called high-molecular-mass form of angiotensinogen in preeclampsia. When expressed as a percentage of the total angiotensinogen, this form is present at <5% in non-pregnant women. In normal pregnancy, it increases, possibly owing to release from the placenta, to 16%,[13] whereas in women with pregnancy-induced hypertension it is 28%. [14] Recently, it was reported that high-molecular-mass angiotensinogen is a complex of proMBP (proform of eosinophil major basic protein) with normal angiotensinogen. [15] Importantly, the kinetics of the cleavage by renin of proMBP–angiotensinogen are dramatically impaired and may contribute to the reduced PRA (plasma renin activity) observed in preeclampsia. [16]
Besides formation of this complex, two isoforms of the angiotensinogen molecule were found when solving its crystal structure at a 2.1 Å (1 Å=0.1 nm) resolution. [17] These two forms represent the reduced and oxidized forms of a labile disulfide bridge in the molecule. Oxidation results in a structural change which preferentially interacts with (pro)renin receptor-bound renin. Given the increased oxidative stress in preeclampsia, the amount of oxidized angiotensinogen in this disease is increased.[17]

However, the resulting enhanced angiotensin generation by renin, when interacting with oxidized angiotensinogen, contrasts with the reduction in PRA that is normally observed in preeclampsia.[16] Combined with recent studies raising doubt on the role of the (pro)renin receptor as a renin-binding receptor *in vivo*,[18] the clinical significance of these findings therefore remains uncertain.

Polymorphisms of the angiotensinogen gene that increase plasma angiotensinogen levels have also been associated with preeclampsia.[19] The underlying mechanism remains unclear, but it might involve the reduction in renin that is the consequence of such elevated angiotensinogen levels.[20] Interestingly, in this regard, pregnant mice overexpressing murine angiotensinogen fail to maintain their volume overload, possibly because they were unable to up-regulate renin expression in the distal nephron.[21] Whether this has relevance to the clinical syndrome of preeclampsia remains to be determined. Mating of female mice overexpressing human angiotensinogen with males expressing human renin (both of which cannot react with their mouse counterparts) leads to a preeclampsia-like syndrome with hypertension, proteinuria and the well-known histological abnormalities of the kidney.[22] Importantly, this model relies on the release of placenta-derived human renin into the circulation, where it reacts with maternal angiotensinogen.

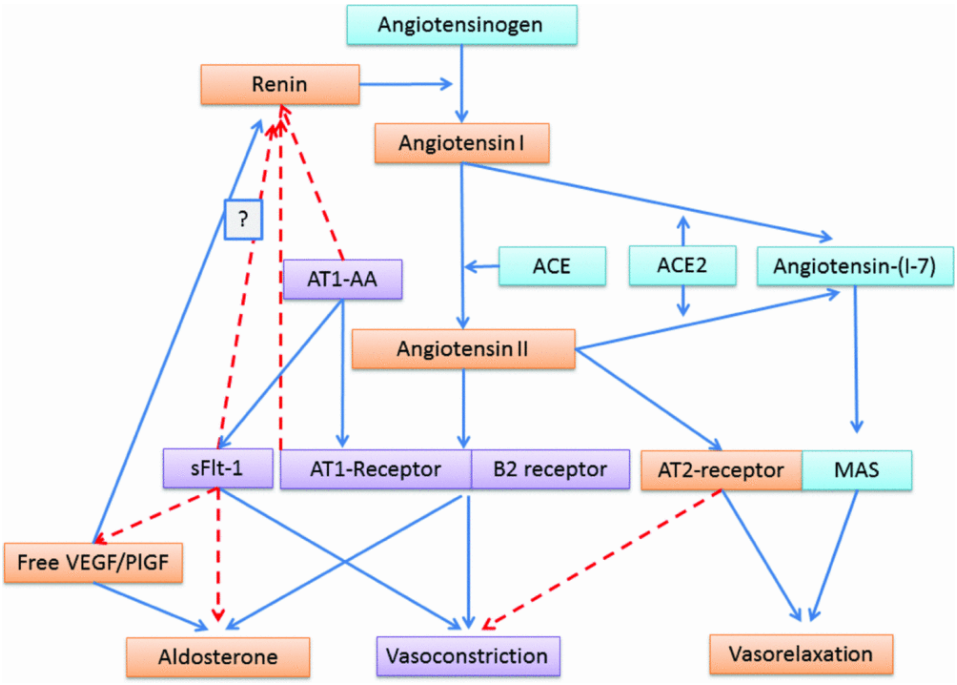

Figure 2

The RAAS in pre-eclampsia compared with a normal pregnancy

Orange squares indicate suppressed levels and purple squares increased levels. Blue squares indicate levels comparable with healthy pregnancy. Solid arrow lines indicate stimulation and broken lines inhibition.

When mating female mice overexpressing human renin with male mice overexpressing human angiotensinogen no such phenotype occurred. A similar transgenic animal model in rats also resulted in impaired spiral artery remodelling and a reduced placental perfusion.[23] However, it should be realized that in both of these models the circulating RAAS activity is excessively high,[24] as opposed to the human situation, were preeclampsia associates with a relatively low degree of RAAS activity.

PRORENIN AND RENIN

In early pregnancy there is a 4–5-fold increase in the levels of renin's inactive precursor prorenin. This prorenin is mainly derived from the ovaries and, to a lesser extent, from the uteroplacental unit and the kidneys.[25–27] Why the ovaries synthesize and release prorenin in such massive amounts is still unknown. Following the discovery of the (pro)renin receptor,[28] it was thought that this receptor might bind and activate prorenin *in vivo*, thereby for the first time providing a function for prorenin. However, the nanomolar affinity of this receptor for prorenin implies that its (picomolar) concentrations in the plasma are several orders below the levels required for binding, even in pregnancy.[29] Nevertheless, this receptor might play a role in prorenin-synthesizing organs, for instance the ovary, where such concentrations are likely to occur. Importantly, prorenin levels are elevated in preeclampsia compared with normal pregnancy, and, in women with Type 1 diabetes, high plasma prorenin associates with an increased risk for the development of preeclampsia.[30]

Renin, unlike prorenin, is exclusively derived from the kidneys. Its levels are elevated in pregnancy, most probably as a compensatory mechanism in response to the fall in vascular resistance and blood pressure at the beginning of pregnancy. Renal biopsies taken from patients with preeclampsia a few days after pregnancy contained almost no renin-positive juxtaglomerular cells.[31] This is unlikely to be the result of depletion, since, if anything, renin is lower in preeclampsia than in a normal pregnancy. Thus it seems that renin synthesis in preeclampsia is suppressed. These low(er) renin levels in preeclampsia are counterintuitive in the face of the reduced plasma volume in this condition. Potential renin suppressors are the elevated atrial natriuretic peptide levels in preeclampsia,[32] the increased sensitivity to AngII and AT_1-AAs agonistic autoantibodies against the AT1 receptor (AngII type 1 receptor). The last two interfere with the negative-feedback loop between renin and AngII, since AT_1 receptor stimulation suppresses renin release. Finally, VEGF blockade with the angiogenesis inhibitor sunitinib not only increases blood pressure and induces a preeclampsia-like syndrome involving renal dysfunction, proteinuria, glomerular endotheliosis and activation of the ET-1 (endothelin-1) system, but also suppressed renin.[33] Thus VEGF removal by the elevated sFlt-1 levels might be a further explanation for the decrease in renin in preeclampsia.

ANGII

As a result of the increased levels of angiotensinogen and renin, AngII is increased in a normal pregnancy. Not surprisingly, given the lower levels of angiotensinogen and renin, AngII is lower in preeclampsia compared with normal pregnancy. Interestingly, historical experiments by Gant et al. showed that twice as much AngII is needed to get a 20 mmHg increase in blood pressure in pregnant compared with non-pregnant women.[34] Women with preeclampsia, on the other hand, do not show this resistance to AngII, which can already be observed as early as week 10 of gestation and thus well before the onset of clinically apparent symptoms. This increased sensitivity was still present 8 months after pregnancy.[35] There are several explanations for this increase in sensitivity. First, the adipose tissue of patients with preeclampsia displays elevated AT_1 receptor expression. Increased AT_1 receptor expression may also be present in other tissues.[7] Secondly, preeclampsia is characterized by an increased heterodimerization of the AT_1 receptor with the bradykinin type 2 receptor. Since this prevents AT_1 receptor internalization, the AngII response cannot be diminished by removing the AT_1 receptors from the membrane.[36] Thirdly, the balance between constrictor AT_1 receptors and dilator AT_2 receptors (AngII type 2 receptors) receptors may be disturbed.[3] Finally, it has been reported that increased neutrophil infiltration resulting in excessive ROS production and RhoA (Ras homologue family member A) kinase activation leads to increased vascular reactivity in preeclampsia.[37] Of note, since this conclusion was reached by constructing two AngII concentration–response curves in a row in human omental arteries, without correcting for tachyphylaxis, it needs to be interpreted with caution.[38]

AT_1 RECEPTOR AUTOANTIBODIES

In 1999, Wallukat et al. reported that autoantibodies that stimulate the AT_1 receptor are present in women with preeclampsia, raising the possibility that preeclampsia is an autoimmune disease.[39] AT_1-AA levels have been shown to correspond with disease severity.[40] There are multiple ways by which AT_1-AA might contribute to the pathogenesis of preeclampsia. First, AT_1-AA will induce vasoconstriction via stimulation of AT_1 receptors on vascular smooth muscle cells. Secondly, AT_1-AA–AT_1 receptor interaction up-regulates PAI-1 (plasminogen-activator inhibitor-1), sFlt-1 and NADPH oxidase, which are all linked to the pathogeneses of the disease.[7] Indeed, NADPH up-regulation enhances ROS formation,[41] whereas PAI-1 decreases trophoblast invasion, resulting in decreased placental function. The question arises as to why these antibodies are produced in the first place. Initially it was thought that the AT_1-AA were cross-reacting against the antigenic region of the parvovirus B19; however, no evidence for this theory could be found subsequently.[42,43] An activation of the immune system seems to play an important role, since treating rats with rituximab prevented the mobilization of B-lymphocytes, thereby suppressing the production of AT_1-AA.[20]

Furthermore, infusion of inflammatory cytokines into pregnant rats results in the production of AT_1-AA.[22] Moreover, the question of why the autoimmune response is directed against the AT_1 receptor, and whether there are other vasoactive autoantibodies in preeclampsia, remains to be elucidated.

ACE2 and angiotensin-(1–7)

Ang-(1–7) angiotensin-(1–7) is an angiotensin metabolite, generated by ACE2 (angiotensin-converting enzyme 2) from AngI (angiotensin I) or AngII (Figure 2), that has received a lot of attention, in part, because binding to its receptor Mas counteracts the AT_1-receptor-mediated effects.[3] In parallel with AngII, its levels are elevated during normal pregnancy, but relatively reduced in preeclampsia.[44] The simplest explanation is that this is the consequence of a reduced degree of RAAS activity. Interestingly, pregnant ACE2-knockout mice display a higher blood pressure during pregnancy, a reduced gain of weight (indicative of a reduced volume increase) and deliver smaller pups.[45] This may be due to the expected increase in AngII (in the absence of its metabolism by ACE2), a reduction in the levels of Ang-(1–7) or both.

In apparent contrast with these observations, Sykes et al.[46] showed in early pregnancy that women who developed preeclampsia had higher Ang-(1–7) levels than women who remained healthy during pregnancy. Further analysis revealed that this difference was only present in women with female fetuses.[46] In this regard, it should be realized that Ang-(1–7) is also associated with the inhibition of angiogenesis in animal models for tumour growth.[47] Obviously, angiogenesis is an essential aspect of pregnancy, and whether Ang-(1–7) is good or bad in this situation remains to be determined.

Aldosterone

Consistent with the changes in renin and AngII, aldosterone is increased during pregnancy and is relatively low in pregnancies complicated by preeclampsia. A (too) low aldosterone level might be responsible for the decreased volume expansion in preeclampsia, and the resultant poor placental perfusion. Indeed, the proliferation of cultured human trophoblasts increased upon aldosterone stimulation, and aldosterone levels correlated with placental weight in both rats and humans.[48] Furthermore, blocking mineralocorticoid receptors with spironolactone resulted in a decreased umbilical flow in mice, either owing to interference with the local vascular effects of aldosterone or to a general reduction in volume. [48, 49]

In line with these observations, gain-of-function variants of the *CYP11B2* (cytochrome P450, family 11, subfamily B, polypeptide 2; also known as aldosterone synthase) gene reduced the risk of developing preeclampsia.[50]

Furthermore, an increase in volume transiently decreases blood pressure in preeclampsia,[51] and supplemental NaCl exerts similar hypotensive effects in women that display neither the increase in aldosterone production nor the blood pressure fall expected during pregnancy.[52]

The aldosterone/renin ratio is elevated in normal pregnancy. This suggests that additional factors on top of AngII determine aldosterone synthesis. Indeed, a recent study has demonstrated that VEGF stimulates aldosterone production by adrenocortical cells.[53] Furthermore, in rats overexpressing the VEGF-inactivating sFlt-1, capillary rarefaction of the adrenal glands was observed and plasma aldosterone and sFlt-1 levels were inversely correlated.[53] This suggests that VEGF can also stimulate aldosterone production indirectly, i.e., by enhancing adrenal capillary density.[53] These observations suggest that VEGF might be the additional aldosterone-stimulating factor in pregnancy. Simultaneously, they offer an explanation for the lower aldosterone levels in preeclampsia.

In line with the above studies, infusion of IgG from women with preeclampsia (most probably containing AT_1-AA) resulted in hypertension and proteinuria in pregnant mice.[54] Interestingly, in non-pregnant mice, such an IgG injection resulted in an increase in aldosterone (most probably via stimulation of the AT_1 receptor), whereas in pregnant mice it led to a decrease in aldosterone production, most probably because infusion of AT_1-AA stimulated the production of sFlt-1. As described above, sFlt-1 facilitates the capillary rarefaction of the adrenal glands and VEGF infusion could counteract this phenomenon.[53]

Buhl et al. reported another mechanism that might explain the decrease in aldosterone levels in preeclampsia.[55] They observed that urinary plasminogen is greatly elevated in preeclampsia, and correlated negatively with urinary aldosterone. This was due to plasminogen proteolysis and the subsequent stimulation of ENaC (epithelial sodium channel) by plasmin, resulting in sodium retention and aldosterone suppression.

Although a major role for the (relative) lack of aldosterone in the pathogenesis of preeclampsia may be present, it has also been suggested that the plasma volume changes precede the changes in aldosterone, both in preeclampsia and in patients with fetal growth restriction.[11] Moreover, although aldosterone levels in preeclampsia are lower than in normal pregnancy, relative to renin they are actually higher.[56] This implies that aldosterone stimulants other than VEGF and renin come into play, e.g. AT_1-AA, the increased sensitivity to AngII and/or ET-1.

CONCLUSIONS AND REMAINING QUESTIONS

A key event in the pathogenesis of preeclampsia is the decreased perfusion of the placenta. Most probably this is the result of impaired widening of the maternal spiral arteries.[1] In the second phase underfilling of the maternal vascular system may contribute to the pathogenesis of the disease. At this second stage there is systemic endothelial dysfunction, causing high systemic vascular resistance and an increase in blood pressure.

In combination with an increase in vascular permeability and a decrease in oncotic pressure, circulating volume is decreased further, thereby worsening the placental perfusion. In turn, the placenta will produce more vasoactive factors such as sFlt-1, AT_1-AA and ROS, causing further vasoconstriction and thereby resulting in a vicious cycle.
In line with this theory is that interrupting this vicious circle by increasing the circulating volume with intravenous fluid replenishment can temporarily lower blood pressure. However, this is unlikely to be effective at the later stages of the disease, because then vascular leakage and hypo-albuminaemia, resulting in a decreased plasma oncotic pressure, reduce the capacity to retain fluid in the intravascular compartment.
The RAAS can interfere in two ways with this vicious circle, via sodium and water retention (via aldosterone and directly via AngII) and via an increase in vascular resistance. The first part might be beneficial, since an increase in volume in view of maintaining tissue perfusion is crucial. The second part might be deleterious since the increase of vascular resistance is part of this vicious circle.
In pregnancy, the RAAS supports the increase in volume by increasing the production of aldosterone, while simultaneously developing a relative resistance to the pressor response of AngII.[34] How the latter occurs is not completely clear. Possibly, high levels of aldosterone counteract AngII-induced vasoconstriction and/or there is an up-regulation of vasodilator AT_2 receptors. [49,57] Importantly, the increase in aldosterone exceeds that in renin and AngII, and the resulting increase in the aldosterone/renin ratio suggests that other aldosterone stimulants have come into play. Indeed, one such new player is VEGF.[53]
In preeclampsia, the RAAS is suppressed and, as we described previously, this is somewhat unexpected in view of the reduced circulating volume. It is hard to say whether the suppression of the RAAS is inappropriate and deleterious or a healthy protection against a further increase in blood pressure. Indeed, transgenic animal models with an excessive RAAS activity show almost all of the features of a pregnancy complicated with preeclampsia.[22,23] We propose that in preeclampsia the RAAS has lost the delicate balance that in normal pregnancy results in a volume increase and a diminished vasoconstrictor response to AngII. RAAS suppression could be a logical consequence of both the increased pressor response to AngII and the elevated levels of AT_1-AA in preeclampsia. Clearly, a crucial question is why the pressor response to AngII remains enhanced. A possibility is that either there are no counterbalancing AT_2 receptors and/or that the AT_2 receptors display a constrictor phenotype in this disorder.[58] When suppressing the RAAS, aldosterone levels should decrease in parallel. Yet in preeclampsia the aldosterone/renin ratio is increased even further as compared with normal pregnancy. This is not due to VEGF-dependent aldosterone production, since VEGF activity, if anything, is diminished in preeclampsia.[6] It may relate to the increased pressor response to AngII and the elevated levels of AT_1-AA, which might keep aldosterone levels, at least relative to renin, elevated, even when circulating AngII levels are low.

Furthermore, there may be alternative aldosterone stimulants such as ET-1, as the ET-1 system is activated in preeclampsia.[59] Future studies should therefore carefully determine the factors that affect plasma aldosterone (renin, AngII, VEGF, AT_1-AA, ET-1 and the sFlt-1/PlGF ratio) to obtain a full understanding of its 'inappropriate' level in preeclampsia. In addition, given the observation that pregnant mice overexpressing murine angiotensinogen were unable to up-regulate renin expression in the distal nephron (as normally occurs in pregnancy) and consequently develop hypovolaemia, it might be worthwhile to study the renal RAAS in preeclampsia in more detail, e.g. by measuring renin in urine.[60] Evidence indicates that urinary renin levels reflect renal RAAS activity more accurately than plasma renin levels.[61,62] Such information will tell us to what degree changes in the circulating and renal RAAS run in parallel in normal pregnancy and preeclampsia. This is particularly relevant in view of our observation that VEGF blockade with sunitinib suppresses renin and simultaneously induces a preeclampsia-like syndrome.[33]

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Chapter 8
Variation of urinary protein to creatinine ratio during the day in women with suspected preeclampsia

BJOG. 2014 Dec;121(13):1660-5.

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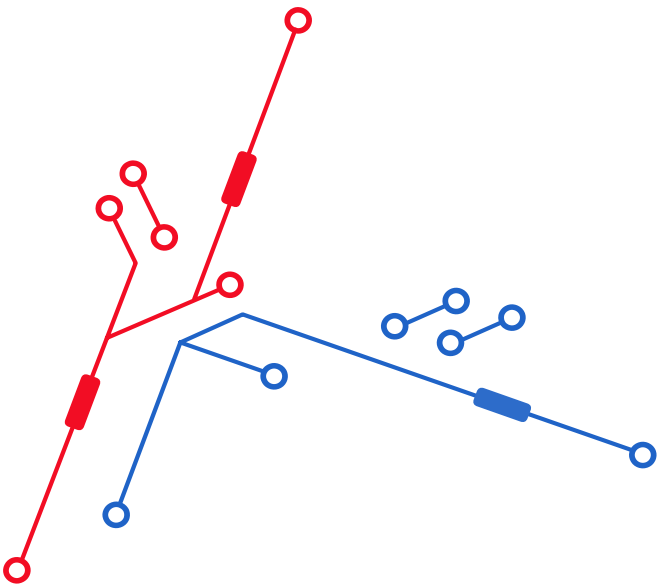

ABSTRACT

Objective To investigate the stability throughout the day of the protein to creatinine ratio (PCR) in spot urine, to demonstrate whether the PCR is a valid alternative for 24-hour protein investigation in pregnant women.

Design Prospective study.

Setting Tertiary referral university centre.

Population Women suspected of having preeclampsia, admitted to the Erasmus Medical Centre.

Methods Twenty-four-hour urine collections and simultaneously three single voided 5-ml aliquots were obtained at 8 a.m., 12 a.m. (noon) and 5 p.m. A PCR was measured in each specimen and compared with the 24-hour protein excretion.

Main outcome measures The 24-hour proteinuria and PCR measured in spontaneous voids.

Results The PCRs correlated strongly with each other and with the 24-hour protein excretion but did show variation throughout the day (mean coefficient of variation 36%; 95% confidence interval 31–40%). The coefficient of variation was unrelated to the degree of 24-hour proteinuria. Receiver operating characteristics curves to discriminate between values below and greater than or equal to the threshold of 0.3 g protein per 24-hour had an area under the curve of respectively 0.94 (8 a.m.), 0.96 (noon) and 0.97 (5 p.m.). Sensitivities at 8 a.m., noon and 5 p.m. were respectively 89%, 96% and 94%; specificities were 75%, 78% and 78% with the proposed PCR cut-off of 30 mg/mmol (0.26 g/g) (National Institute for Health and Care Excellence guidelines). There is no evidence of a difference between the three measurement times regarding the sensitivities and specificities.

Conclusion

The PCR determined in spot urine varies throughout the day but is a valid alternative for 24-hour urine collections in pregnant women. It is especially useful to rapidly identify clinically relevant proteinuria.

INTRODUCTION

Preeclampsia is diagnosed when after 20 weeks of gestation repeated blood pressure measurements equal or exceed 140/90 mm Hg in combination with the *de novo* proteinuria of ≥0.3 g/24-hour.[1,2] The reference standard for the latter measurement is the quantity of protein in a 24-hour urine collection, a very cumbersome procedure, prone to errors related to inaccurate and/or incomplete collections, problems that become exaggerated during pregnancy.[3-5] Indeed, imprecise outpatient urine collections have been reported to approach 50% in some studies; as a consequence, patients are therefore often admitted to the hospital to quantify the degree of proteinuria.[6-9]

The qualitative proteinuria dipstick test is not a reliable alternative.

Its readings are affected by whether the urine is concentrated or diluted, as well as by its pH, resulting in an unacceptable number of false-positive and false-negative test results.[10] In recent years, caregivers have turned to measuring the protein to creatinine ratio (PCR) in single voided urine and this has become the preferred and recommended approach for quantifying proteinuria in non-pregnant subjects.[3, 11, 12] In this respect, the recently published National Institute for Health and Care Excellence guidelines recommend using a spot PCR ≥ 30 mg/mmol as an alternative to ≥0.3 g/day in 24-hour collections to diagnose clinically relevant proteinuria.[13] However, use of the PCR on a single voided urine as an alternative to the 24-hour measurement assumes that the urinary excretion rates of protein and creatinine throughout the day are relatively constant. Creatinine excretion shows very little variation, but we could not find verification for protein excretion. We therefore explored the constancy of spot PCR at different time points during the day and their relationship with simultaneously obtained 24-hour urinary protein excretion. Specifically we assessed the diagnostic accuracy of the PCR in women hospitalised to determine whether or not they had preeclampsia.

METHODS

Women suspected of having developed preeclampsia, admitted to the Department of Obstetrics and Gynaecology's inpatient service at the Erasmus Medical Centre in Rotterdam were recruited for a protocol, the study was completed in 2 years. All volunteers gave consent following detailed explanation of the study goal using a protocol approved by the hospital's ethics review committee. Suspected preeclampsia was defined as *de novo* hypertension, with blood pressure ≥140/90 mmHg after 20 weeks of gestation and a urine protein dipstick reading ≥1+. Pregnant women with chronic hypertension who developed new-onset proteinuria after mid-gestation were also asked to participate. Women with urinary tract infection, pre-existing proteinuria or having a delivery before the 24-hour urinary collection was completed, were excluded.

During the urine collection period the women were on bed rest with bathroom privileges. The collection began at midnight with 5-ml separate aliquots saved for PCR testing from requested spontaneous voids at approximately 8 a.m., 12 a.m. (noon) and 5 p.m. Nurses monitored the completeness of the 24-hour collection, and when errors occurred, the procedure was stopped and restarted at midnight the next day.

Creatinine was measured by an enzymatic assay (CREA plus; Roche Diagnostics, Germany; inter-assay coefficient of variation < 3%) and protein was measured by a colorimetric assay (Roche Diagnostics; inter-assay coefficient of variation < 1.6%). Dipsticks (Albustix; Siemens Healthcare Diagnostics Inc., Tarrytown, NY, USA) were visually analysed. A single plus represented 0.3 g/l. Statistical analysis was performed with SPSS Statistics 18 (SPSS Inc., Chicago, IL, USA). Proteinuria, defined as ≥ 0.3 g in the 24-hour collection was used as the reference standard to compare with the respective PCRs.

A cut-off value of the PCR of 30 mg/mmol (0.26 g/g)[1, 13] was used to calculate the sensitivity and specificity of PCR at the three time points.
PCRs at 8 a.m., noon and 5 p.m. were compared using the Wilcoxon signed-rank test and for each subject the coefficient of variation for the three time points of the PCR was computed. For the whole group, Spearman's bivariate analysis was used to calculate correlation coefficients between the PCRs at the three time points and between the PCRs and the 24-hour urinary protein excretion. For the three PCRs, receiver operating characteristics (ROC) curves were constructed and areas under the curve (AUC) were calculated. The Cochran *Q*-test was used to test if there were differences in sensitivity and specificity at the different time points.
Optimal cut-off levels were determined with the Youden's index by finding the maximum value of the sum of sensitivity and specificity (thereby taking equal weights of the sensitivity and specificity). Intra-class correlation coefficients were calculated to evaluate the agreement of the three PCRs at the different time points. Values were log-transformed in all analyses to obtain normal distributions, except for calculating the intra-individual coefficient of variation of the PCR. A value of $P \leq 0.05$ (two-sided) was considered to indicate a significant difference.

RESULTS

One hundred and twelve women were recruited, six were excluded because of incomplete data, and one because of pre-existing proteinuria. None had evidence of a urinary tract infection. Of the 105 evaluable women, five had a creatinine excretion of < 5.5 mmol (0.6 g) per 24-hour, suggesting incomplete 24-hour urine collection.[14] In one of these patients PCR was positive. However, excluding these patients had no effect on the main outcomes of the study so these patients were not excluded and their data are shown in the results.
Clinical characteristics of the study population are presented in Table 1. The 24-hour proteinuria varied from 0.05 to 17.04 g with 73 women (70%) having a 24-hour proteinuria exceeding 0.3 g. Median (interquartile range) PCR was 60.0 mg/mmol (23.3–178.5 mg/mmol) at 8 a.m., 79.2 mg/mmol (28.2–233.9 mg/mmol) at noon and 73.3 mg/mmol (24.6–747.1 mg/mmol) at 5 p.m. The PCR of 8 a.m. was lower than the PCR at noon (60 mg/mmol versus 79.2 mg/mmol; $P = 0.002$) but did not differ from the PCR at 5 p.m. (60 mg/mmol versus 73.3 mg/mmol; $P = 0.167$), the PCR at noon was higher than the PCR at 5 p.m. (79.2 mg/mmol versus 73.3 mg/mmol; $P = 0.044$). The average coefficient of variation of the three PCRs was 36% (95% confidence interval (95% CI) 31–40%). The intra-individual coefficient of variation did not correlate with the amount of proteinuria (Spearmans rho = −0.08, $P = 0.41$). The intra-class correlation coefficients were very good with 0.91 (95% CI 0.86–0.94) for 8 a.m. versus noon, 0.90 (95% CI 0.86–0.93) for noon versus 5 p.m. and 0.90 (95% CI 0.85–0.93) for 8 a.m. versus 5.00 p.m.

Proteinuria	<0.3 g/24 hours	≥0.3 g/24 hours	Total
Number	32	73	105
Nullipara	19 (59%)	40 (55%)	59 (56%)
Gestational age (weeks)	33.1 (30–36)	31 (28–35)	31 (29–35)
Age (years)	30 (27–35)	31 (29–34)	31 (28–34)
Serum creatinine (µmol/l)	57 (48–61)	60 (52–67)	58 (50–65)
Serum uric acid (mmol/l)	0.36 (0.28–0.45)	0.36 (0.29–0.40)	0.36 (0.29–0.40)
Proteinuria (g/day)	0.18 (0.13–0.21)	1.27 (0.6–3.01)	0.61 (0.24–1.70)

Table 1.

Characteristics of the study population (total and subdivided according to values < 0.3 g/24 hours and ≥ 0.3 g/24 hours)

	PCR 8 a.m.	PCR 12 a.m.	PCR 5 p.m.
PCR, median (25th–75th centile)	60 (26–173)[c]	79 (29–229)	73 (25–193)[d]
True positives/false positives[a]	65/8	70/7	68/7
True negatives/false negatives[a]	24/8	25/3	25/4
True positives/false positives[b]	59/1	69/4	67/3
True negatives/false negatives[b]	31/14	28/4	29/5
Area under the ROC curve (95% CI)	94 (90–98)	96 (92–99)	97 (94–100)
Sensitivity,% (95% CI)[a]	89 (80–95)	96 (88–99)	94 (86–98)
Specificity,% (95% CI)[a]	75 (57–89)	78 (60–91)	78 (60–91)
Sensitivity,% (95% CI)[b]	81 (72–90)	95 (89–100)	93 (90–96)
Specificity,% (95% CI)[b]	97 (93–100)	88 (76–99)	91 (81–100)

Table 2.

Sensitivities and specificities, for different cut points of PCR to diagnose proteinuria (≥0.3 g/24 hours)

[a] PCR cut-off 30 mg/mmol.

[b] Classifying according to the optimal cut-off for the protein creatine ratio (PCR cut-off 8 a.m. 50.4 mg/mmol, PCR cut-off 12 a.m. 42.4 mg/mmol, PCR cut-off 5 p.m. 35.4 mg/mmol).

[c] PCR at 8 a.m. is lower than PCR at noon P = 0.002.

[d] PCR at 5 p.m. is lower than PCR at noon P = 0.044.

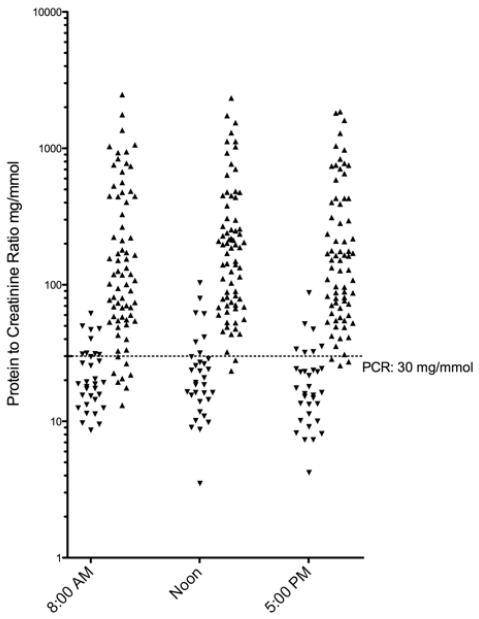

Figure 1.
Scatterplot of individual protein–creatinine ratios obtained at 8 a.m., 12 a.m. and 5 p.m. stratified for proteinuria <0.3 (inverse triangles) or ≥0.3 g per 24-hour (triangles). Note the logarithmic scale on the vertical axis.

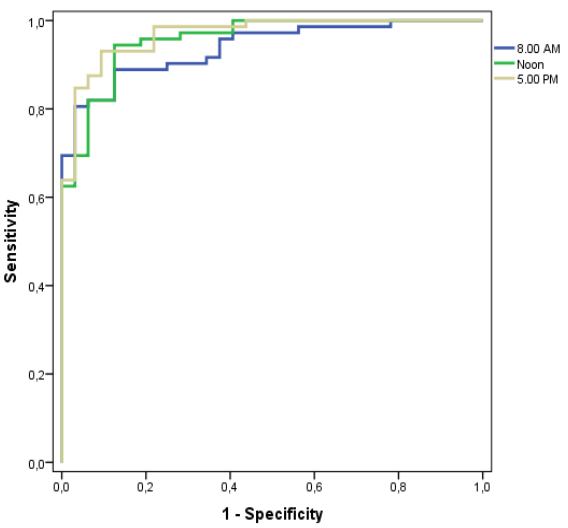

Figure 2.
Receiver operating characteristics (ROC) curve to diagnose proteinuria (≥0.3 g/24-hour) according to PCR 8 a.m., PCR 12 a.m. and PCR 5 p.m. The areas under the curve of PCR at 8 a.m., 12 a.m. and 5 p.m. are 0.94 (95% CI 0.90–0.98), 0.96 (95% CI 0.92–1.00) and 0.97 (95% CI 0.94–1.00), respectively.

The distribution of the PCR values stratified for the positive and negative outcomes of the 24-hour urinary protein collection is shown in Figure 1. The PCR in one or more of the samples was a false negative in ten women (7.3%) at a PCR cut-off of 30 mg/mmol. The 24-hour proteinuria in these ten women was relatively low, varying from 0.3 to 0.6 g with an average value of 0.4 g. The ROC curves of the three PCRs to discriminate between 24-hour proteinuria of <0.3 versus ≥0.3 g/24 hours are shown in Figure 2.

The three PCRs discriminated well, with AUCs varying from 0.94 to 0.97. Optimal cut-off values were 50.4 mg/mmol at 8 a.m., 42.4 mg/mmol at noon and 35.4 mg/mmol at 5 p.m. as determined by the Youden's index. Sensitivities and specificities for different cut-off points of PCR to diagnose proteinuria are given in Table 2. There were no significant differences between the three measurement times regarding the specificities (Cochran *Q*-test: $P = 0.89$). The same applied to the sensitivities ($P = 0.12$).

DISCUSSION

Main findings

Studying inpatients suspected of having preeclampsia we found that the PCRs during the day are well correlated but are subject to variation. Nonetheless, ROC analyses indicate that PCRs at different daytime points are quite accurate to confirm or reject the presence of the threshold for diagnosing 24-hour proteinuria, as reflected by the AUCs of >0.94 or more. In 7.3% of women with a 24-hour proteinuria of ≥0.3 g one or two of the PCRs was below the generally accepted cut-off value of 30 mg/mmol and hence was false negative. In these women proteinuria, albeit above the cut-off value of ≥0.3 g, was relatively low with an average value of 0.4 g per day.

Strengths and limitations

To our knowledge no other studies have looked at the variation of the spot PCR during the day in the manner described here. The difference from other studies is that we not only compared three spot PCRs against the 24-hour urine collection, but also looked at the variation of the three spot PCRs throughout the day and measured the within-subject variation in PCR.

Although the nurses closely monitored the urinary collection, the total creatinine excretion in the 24-hour collection was relatively low in several subjects suggesting under-collection of urine. Another limitation concerning the generalisability of this study is that subjects were hospitalised for 24 hours and had bed rest. However, even in a situation where there is little variance in the activity pattern, there was still a considerable variability in the PCR during the day. One could emphasise that the activity pattern of non-hospitalised patients is more diverse, which could also affect the variation of the PCR.[4] Moreover, 70% of our patients had proteinuria. The performance of the test will be worse in a cohort of patients with a relatively low prevalence of proteinuria. This study might be underpowered for detecting differences in the sensitivity and specificity of the PCR during the day, because the primary objective of this study was to determine the possible variation of the PCR during the day in a spot urine. Further studies are needed in women with minimal proteinuria and in ambulant patients.

Interpretation

Multiple studies have been performed to investigate the accuracy of the PCR in pregnant women. In general these studies compared the spot PCR against 24-hour urine collection. In a systematic review and meta-analysis Cote et al. concluded that the accuracy of the PCR to rule-out proteinuria is reasonable using a cut-off value of 30 mg/mmol.[8] With this cut-off point, sensitivity and specificity were respectively 84% and 76%.[8] In another systematic review it has been estimated that the optimum threshold for PCR has to be between 35 and 40 mg/mmol (0.30 and 0.35 mg/g) for diagnosing clinically relevant proteinuria, but that sensitivity (83.6%) and specificity (76.3%) of the PCR are relatively poor.[15] In the latter review the authors performed a subgroup analysis for hypertensive pregnant women with a positive dipstick test for proteinuria. In this subgroup, sensitivity and specificity improved with respective values of 86% and 95% when using a threshold of the PCR above 23 mg/mmol.[15]

In our study there was, in fact, some variation in the PCR throughout the day, which appeared unrelated to the degree of proteinuria. Apart from the underlying renal lesion, a variety of factors such as posture, exercise, emotional factors and blood pressure may influence protein excretion, which is in accordance with the finding that the 8 a.m. PCR, after an overnight rest, is lower than the PCR at noon. Furthermore, this resulted in a somewhat higher optimal cut-off value of 50.4 mg/mmol for diagnosing clinically relevant proteinuria. In our population the prevalence of proteinuria was large, with 70% of the women having a proteinuria of at least 0.3 g per 24 hours, and in all women the urinary dipstick test for proteinuria was positive. Given the variation in PCR throughout the day, it should be emphasised that in a cohort of patients with a relatively low prevalence of proteinuria the performance of a PCR test result ≥30 mg/mmol will be worse.[15]

Twenty-four hour urine collection is time-consuming and often inaccurate. Incomplete outpatient collections have been reported to approach 50% in some studies.[3] Even in this study, where 24-hour urine collections were performed while patients were hospitalised and supervised by the nurses, collections were probably incomplete in almost 5% of the participants, as reflected by the low 24-hour urinary creatinine excretion. Collection errors may be due not only to over- and under-collection, but also to gestation-related changes in the urinary tract, including dilatation or transient obstruction of the ureters when gravidas assume certain postures and the enlarged uterus presses on the point where the iliac arteries cross the ureters.[4, 5] It has been proposed therefore, that the 24-hour or any other form of timed urine collection should be replaced by the determination of the PCR in a single voided urine, as has become the standard approach in non-pregnant subjects.[12]

Two studies have investigated the variation of the PCR throughout the day in 6- to 8-hour collections.[14, 16] Gonsales Valério et al. investigated 75 hypertensive pregnant women after a gestational age of 20 weeks, recording the PCR at arrival as well as during four subsequent 6-hour periods.[14] The mean at each period was fairly constant, but, unfortunately,

no information about the within-subject variation in PCR was reported. In concordance with our study PCRs at the different time-points and 24-hour protein excretion were strongly correlated with correlation coefficients of 0.8 or higher.[14] Contrary data emerged from the study by Lindow and Davey, who measured PCR values in three 8-hour samples in 22 pre-eclamptic women with indwelling catheters, comparing these values to the protein excreted in 24 hours. The PCR appeared to vary considerably and the correlation with 24-hour collections was poor.[16] Few details were given but if these patients were ill enough to require catheterisation, than a changing glomerular filtration rate might have invalidated the results.

CONCLUSION

PCR determined in single voided urine can be used as an alternative for 24-hour urine collections in pregnant women with preeclampsia or suspected of having this condition. However, the PCR measured in spontaneous voids varies throughout the day, which appears to be independent of the amount of proteinuria. While this knowledge should be taken into account when it is used as a substitute of a 24-hour proteinuria measurement in women suspected of having preeclampsia, the ratio appears an acceptable alternative, especially when time is a factor to distinguish gestational hypertension from preeclampsia. Since all our patients were hospitalised further studies are necessary to determine whether our results also apply to the outpatient setting.

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Chapter 9
Differential Diagnosis of Preeclampsia Remember the Soluble Fms-Like Tyrosine Kinase 1/Placental Growth Factor Ratio

Hypertension. 2012 Oct;60(4):884-90.

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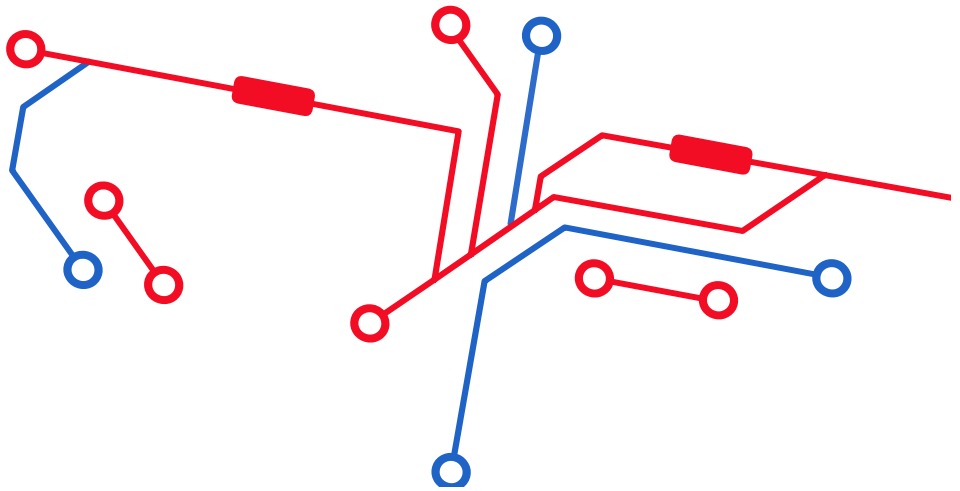

INTRODUCTION

Preeclampsia is a pregnancy-associated condition, clinically characterized by hypertension, proteinuria, and progressive edema, affecting 3% to 5% of all pregnancies.[1] Preeclampsia can occur in previously healthy women and in women with underlying conditions, such as hypertension, lupus nephritis, or the antiphospholipid syndrome (APS).[1,2] Conversely, pregnancy can be a trigger to activate underlying diseases. A well-known example is lupus nephritis, which can flare during pregnancy, especially when the disease is still active in the months preceding pregnancy or during conception.[3] In the United States there are yearly ≈4500 pregnancies with systemic lupus erythematosus (SLE), and of these pregnancies, 13% to 35% are complicated by preeclampsia and 14% to 65% by a lupus flare.[2] The clinical features of a lupus nephritis flare closely mimic those of preeclampsia. Differentiation between these 2 conditions on clinical grounds can therefore be a challenge, particularly because lupus nephritis itself predisposes to preeclampsia.[2,3] However, such differentiation is critical to optimal management: a lupus flare requires initiation or intensification of immunosuppressive therapy, whereas delivery of the child and the placenta is the only treatment currently available for severe preeclampsia.

In recent years, an imbalance between proangiogenic and antiangiogenic proteins derived from the placenta has been suggested to play a role in the pathophysiology of preeclampsia, and measurement of these proteins in the maternal circulation is coming of age as a tool to diagnose or to provide prognostic information of this condition.[4,5] Compared with normal pregnancies, the serum concentration of the antiangiogenic soluble Fms-like tyrosine kinase 1 (sFlt-1) is markedly increased, whereas that of placental growth factor (PlGF) is decreased in preeclampsia.[4,6–10] Because of the reciprocal changes of these markers, the ratio of sFlt-1/PlGF appears to be a superior marker of (early onset) preeclampsia compared with the individual values of these markers.[7,11] In the 3 complicated pregnancies of this grand round, we used the sFlt-1/PlGF ratio as a biomarker to differentiate among superimposed preeclampsia, activation of the underlying disease, or the combined occurrence of these 2 conditions. For all 3 of the patients, sFlt-1 and PlGF were measured by precise ELISA on a fully automated Elecsys system (Roche Diagnostics).[12]

CASE 1

A 41-year–old woman gravida 6, para 0, was admitted to our hospital at 21+1 gestational weeks because of progressive edema in the legs and shortness of breath. In the past 4 weeks she had gained 25 kg in weight. The medical history included an autoimmune hepatitis, diagnosed at 15 years of age. The hepatitis evolved in liver cirrhosis, complicated by portal hypertension with splenomegaly, esophageal varices, prophylactically treated with band ligation, and thrombocytopenia because of hypersplenism. This woman also has an APS with deep venous thrombosis in the right leg and spontaneous spleen infarctions with positive tests for lupus anticoagulant and antiphospholipid antibodies. For the APS, lifelong treatment with the vitamin K antagonist acenocoumarol was initiated. Antinuclear antibo-

dies (ANAs) were incidentally positive. The autoimmune hepatitis had responded well to prednisone and azathioprine, and 2 years before admission, immunosuppressive therapy was discontinued. The obstetric history was as impressively complicated as her general medical history. She had had 5 pregnancies resulting in early miscarriages. Other than the APS, no other explanations for the recurrent pregnancy loss, such as parental karyotype or uterine abnormalities, could be diagnosed. The current pregnancy had occurred spontaneously.

Blood pressure (BP) at a gestational age of 4 weeks was 110/60 mm Hg, serum creatinine concentration 64 µmol/L (reference range, 35–62 µmol/L), and platelet count 40 × 10^9/L (174–391 × 10^9/L), whereas liver enzymes were within the reference range. At admission she was treated with low molecular weight heparin (LMWH) at a therapeutic dose.

At physical examination we saw a moderately ill woman with severe edema of the lower extremities and vulva and slightly elevated central venous pressure. BP was 150/84 mm Hg, pulse rate 83 bpm, and respiration rate 12/min. Oxygen saturation at room temperature was 97%. At cardiac auscultation, a grade 2/6 early systolic ejection murmur was heard. Auscultation of the lungs was unremarkable. Fetal ultrasonography was normal. Laboratory examination revealed elevated values of creatinine (120 µmol/L [reference range 35–71 µmol/L]) and uric acid (0.59 mmol/L [reference range, (0.12–0.34) mmol/L]). Serum concentrations of total protein (52 g/L [reference range, 57–69 g/L]) and albumin (25 g/L [reference range, 26–45 g/L]) were reduced. Serum concentrations of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and lactate dehydrogenase (LD) were <1.5 times elevated. Serum concentration of total bilirubin (20 µmol/L [reference range, 0–14 mol/L]) was elevated, whereas serum haptoglobin (<0.05 g/L [reference range, 0.28–2.01 g/L]) was reduced. The hemoglobin concentration was 6.9 mmol/L (reference range, 6.8–8.7 mmol/L) and the platelet count 38 × 10^9/L. ANA titer was 1:80, antidouble-stranded DNA antibody was negative, and anti SS-A antibody was weakly positive. Analysis of urinary sediment showed 3+ for erythrocytes and 1+ for leukocytes. Urinary casts were absent. Proteinuria was 0.5 g per day. Maternal echocardiography showed a mild degree of mitral valve insufficiency and evidence of elevated right ventricular pressure, suggesting pulmonary hypertension. Abdominal ultrasonography excluded the possibility of an inferior caval or renal vein thrombosis, but a portal vein thrombosis was diagnosed. Pulmonary embolism was excluded on a contrast-computed tomography of the lungs, but bilateral pleural effusion was visible. Within the days after admission, edema further increased and BP rose. Because of the portal vein thrombosis, the dose LMWH was increased, guided by the measurement of antifactor Xa levels. During the hospital stay, renal function deteriorated with a rise in serum creatinine to 200 µmol/L and a further increase in proteinuria to 1.2 g/d. The platelet count decreased to 18 × 10^9/L and hemoglobin to 6.0 mmol/L (reference range, 6.8–8.7 mmol/L). Liver enzymes further increased (AST, 156 U/L [reference range, 0–33 U/L] and ALT, 59 U/L [reference range, 0–33 U/L]).

LD increased to 331 U/L (reference range, 0–447 U/L) and total bilirubin to 26 µmol/L. Regarding the differential diagnosis, lupus nephritis; superimposed preeclampsia; hemolysis, elevated liver enzymes, low platelets syndrome; catastrophic APS; and hepatorenal syndrome were considered. Because of the possibility of lupus nephritis, intravenous methylprednisolone was initiated. The hypertension was treated with α-methyldopa and nicardipine. Despite this treatment, her clinical condition deteriorated. Five days after admission, the measurements of sFlt-1 of 84 339 pg/mL and of PlGF of 55.3 pg/mL, resulting in a sFlt-1/PlGF ratio of 1525, strongly suggested the diagnosis of superimposed preeclampsia (Table). Based on this high ratio, the deterioration of the maternal clinical condition and the very early gestational age, implying a poor pregnancy outcome, pregnancy was terminated. At gestational age of 22+0 weeks, labor was induced. Eight hours later an infant boy (body weight, 325 g; P<10) with an APGAR score of 0 was vaginally delivered. The placental weight was 130 g (P10–25), with a maturation exceeding the pregnancy duration. A few regions with infarctions (<5%) were present.

Postpartum furosemide, enalapril and later also spironolactone were given to treat the edematous state and elevated BP. Nine days after delivery the patient could be discharged. Twelve weeks after discharge liver enzymes were in the reference range. Serum protein had increased to 68 g/L and albumin to 33 g/L and platelet count was 50×10^9/L.

CASE 2

A 28-year–old woman, gravida 1, para 0, was referred from another hospital to our department at 27+0 gestational weeks because of proteinuria. Eleven years before admission she was diagnosed with SLE with positive ANA and antidouble-stranded DNA antibodies and manifestations of painful joints, Raynaud phenomenon, and proteinuria attributed to a grade IV lupus nephritis. In the past she had been treated with cyclophosphamide, prednisone, and azathioprine. Prednisone was discontinued 2 years before admission, but azathioprine was continued. The SLE was stable for several years with a proteinuria of ≈3 g per day and a normal serum creatinine concentration. At the first antenatal appointment at 7 weeks of gestation, no signs of active disease were present, and azathioprine treatment was discontinued.

At admission a not acutely ill patient was seen with pronounced edema of the lower extremities and labia. The body weight was 84 kg (65 kg before pregnancy), BP 140/90 mm Hg, and heart rate 84 bpm. Physical examination of heart and lungs was normal, and no signs of active SLE were present. The cardiotocogram and fetal ultrasonography were normal. Biochemical analysis showed a uric acid of 0.27 mmol/L and a serum creatinine of 96 µmol/L. Serum values of AST, ALT, LD, and haptoglobin were normal. C-reactive protein was <1 mg/L, the ANA titer was 1:160, and antidouble-stranded DNA antibody and anti-SS-A antibodies were negative. The hemoglobin concentration was reduced (5.6 mmol/L), but platelet and leukocyte counts were normal. Examination of the urine showed a proteinuria of 14.5 g per day and a 1+ dipstick for hemoglobin.

Parameter	Patient 1	Patient 2	Patient 3
Maternal age, y	41	28	27
Gravida, Para	6, 0	1, 0	1, 0
GA at admission	21 wk+1 d	27 wk	27 wk+6 d
Medical history	Autoimmune hepatitis, liver cirrhosis, thrombocytopenia, antiphospholipid syndrome	Lupus nephritis, hypothyroidism	Lupus nephritis, pulmonary embolism
Differential diagnosis	Lupus flare, preeclampsia/HELLP Catastrophic antiphospholipid syndrome, hepatorenal syndrome	Lupus flare and/or preeclampsia	Lupus flare and/or preeclampsia
sFlt-1, pg/mL	84 339	1070	14 082
PLGF, pg/mL	55.3	379.0	58.7
sFlt-1/PlGF ratio	1525	2.8	240
ANA titer	0,0972	0,153	0,097
Anti-dsDNA (0–10)*, IU/mL	Negative	Negative	10
C3 (0.84–1.68), g/L	0.3	0.8	1.02
C4 (0.16–0.42), g/L	0.02	0.12	0.14
Uric acid (0.12–0.34), mmol/L	0.59	0.27	0.39
LD (0–447), U/L	331	389	218
Creatinin (35–62), µmol/L	158	103	104
ALT (0–33), U/L	59	47	6
AST (0–33), U/L	156	48	14
Hemoglobin (6.8–8.7), mmol/L	6.9	5.6	7.6
Platelets (150–370), ×109/L	18	121	218
GA at delivery	22 wk	33 wk+1 d	31 wk+1 d
Birth weight (percentile), g	325 (<10)†	1680 (10–25)	1350 (20–50)

Table 1.

Characteristics of the 3 Patients

HELLP indicates hemolysis, elevated liver enzymes, low platelets syndrome; GA, gestational age; LD, lactate dehydrogenase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ANA, antinuclear antibodies; dsDNA, double-stranded DNA; PlGF, placental growth factor; sFlt-1, soluble Fms-like tyrosine kinase 1.

*Values between brackets in the parameter column indicate reference range. †This was a still birth.

At admission she was treated with a prophylactic dose of LMWH and furosemide 40 mg, twice daily. Because a lupus flare was suspected, 60 mg daily of prednisone, 50 mg twice daily of azathioprine, and 200 mg daily of hydroxychloroquine were prescribed. Despite extensive immunosuppressive therapy, proteinuria increased to 20 g per day and edema progressed. In the urinary sediment 1+ erythrocytes, as well as hyaline and granular casts, were detectable. Also, BP increased to 150/100 mm Hg and the patient developed headache and nausea. The cardiotocogram remained normal. In the differential diagnosis we considered a lupus flare most likely, but superimposed preeclampsia could not be excluded. To assist in the diagnosis we measured sFlt-1 and PlGF serum concentrations. sFlt-1 was 1070 pg/mL and PlGF 379 pg/mL, resulting in a ratio of 2.8 (Table). Based on these findings we considered the diagnosis of superimposed preeclampsia highly unlikely. For the lupus flare intravenous pulse therapy with methylprednisone was given, after which prednisone (60 mg daily) was restarted. The pulse therapy had a favorable effect on her general well being, but proteinuria did not decline and serum albumin concentration decreased to 20 g/L. Furosemide was increased to treat the edema but without detectable effect. α-Methyldopa and labetalol were given to treat the hypertension. At 33+1 weeks, labor was induced because of severe, treatment-resistant edema. A healthy baby boy of 1680 g (P10–25) was delivered. The weight of the placenta (180 g) was below the fifth percentile for the pregnancy duration. Infarctions <5% of the placenta volume were present. After delivery, BP remained high and was treated with enalapril, hydrochlorothiazide, nifedipine, labetalol, and spironolactone. The lupus nephritis was treated with hydrochloroquine and mycofenolate-mofetil, whereas prednisone was tapered off. Four weeks after delivery the proteinuria had decreased to 2.0 g per day and serum albumin concentration increased to 30 g/L. Serum creatinine at that time was 77 µmol/L.

CASE 3

A 27-year–old woman, gravida 1, para 0, was referred because of edema and proteinuria at 27+6 gestational weeks. Six years before admission she was diagnosed with class IV lupus nephritis complicated by a severe nephrotic syndrome, treated with prednisone and mycofenolate-mofetil. Four years before admission the lupus nephritis was in complete remission. Serum creatinine concentration by that time was 96 µmol/L and proteinuria 1.5 g/d. She continued to use 3 doses daily of 500 mg of mycofenolate-mofetil and 10 mg/d of prednisone. Because of her wish to become pregnant, mycofenolate-mofetil was replaced by 3 doses daily of 50 mg of azathioprine. BP at 7+5 gestational weeks was 120/75 mm Hg and urinary dipstick 2+ for protein. At 27+4 weeks of gestation she developed edema. BP at this time was 136/87 mm Hg, and urinary dipstick revealed 4+ protein. Serum creatinine was 60 µmol/L, uric acid 0.31 mmol/L, serum albumin 30 g/L, hemoglobin 7.6 mmol/L, and platelet count 141 × 10^9/L. Serum concentrations of AST, ALT, and LD were within the reference range. The ANA titer was 1:80 and the antidouble-stranded DNA antibody concentration 10 IU/mL (reference range, 0–10 IU).

Medication, apart from azathrioprine, consisted of calcium carbasalate, 100 mg daily, and LMWH at a therapeutic dose.

At admission to our hospital, her symptoms were slight headache and edema. At physical examination she appeared in good health. The BP was 135/90 mm Hg, and 2+ ankle edema was present. The cardiotocogram was unremarkable. Ultrasound examination of the uterus revealed a structurally normal fetus with an estimated weight of 1300 g (P84–90). At laboratory evaluation, serum uric acid was 0.39 mmol/L, serum creatinine 75 µmol/L, total protein 53 g/L, and albumin 30 g/L, whereas complement factor C4 concentration was slightly decreased (Table). The hemoglobin concentration was 7.3 mmol/L and platelet count 144 × 10^9/L. Urinary analysis showed hyaline, leukocyte, and granular casts; proteinuria was 4.5 g per day. AST, ALT, and LH were normal. In the differential diagnosis, lupus nephritis flare, superimposed preeclampsia, or a combination of both conditions was considered. Because of the stable clinical condition and early gestational age, the decision was to try to prolong the pregnancy duration. At 30+0 weeks of gestation she developed more headache. Body weight progressively increased (5 kg in 4 days) because of fluid retention, and BP further increased (160/105 mm Hg), as did the proteinuria. Serum creatinine increased to 103 µmol/L, and platelet count decreased to 121 × 10^9/L. On advice of the consulted nephrologist, prednisone was started. To treat the high BP, methyldopa and nifedipine were prescribed, but BP remained elevated and proteinuria increased to 21 g per day. To assist in the diagnosis of superimposed preeclampsia, serum angiogenic factors were measured. The sFlt-1 concentration was 14 082 pg/mL and PlGF 58.7 pg/mL, resulting in a ratio of 240 (Table). Because of this high ratio, a further rise in BP, progressive edema, and development of ascites, it was decided to terminate the pregnancy. At 31+1 week she delivered by cesarean section. An infant boy of 1350 g, APGAR score 9 after 1 minute and 10 after 5 minutes, was born. The placental weight was 200 g (reference 340 g) with a maturation exceeding the pregnancy duration. The histopathology of the placenta was compatible with preeclampsia as well as SLE. After delivery, BP increased further, for which magnesium and nicardipine were given intravenously. Six days after delivery she was discharged. BP at that time was 135/80 mm Hg while using enalapril, nifedipine, and methyldopa. Immunosuppressive therapy consisted of azathioprine and prednisone. Postpartum azathioprine was replaced by mycofenolate-mofetil because of further deterioration of the lupus nephritis. Twelve weeks postpartum she was doing well. Serum creatinine by that time was 90 µmol/L and proteinuria 3.3 g/d.

DISCUSSION

We first briefly elaborate on the various problems that complicated the pregnancies of our patients before further discussing the diagnostic usefulness of the sFlt-1/PlGF ratio in superimposed preeclampsia. Patient 1 had liver cirrhosis because of autoimmune hepatitis and an APS. Pregnancy in liver cirrhosis is rare, and, when it occurs, the risk of hepatic

decompensation is high.[13,14] In a recent study the overall maternal complication rate in patients with autoimmune hepatitis was ≈40%.[15] Women with cirrhosis had the highest risk of complications, including hepatitis flare and liver decompensation, but preeclampsia was not observed.[15] We wondered whether the preeclampsia in this patient was complicated by the hemolysis, elevated liver enzymes, low platelets syndrome. Because of the hypersplenism, platelet count was already low before pregnancy and further declined during the course of pregnancy, whereas LD as a sign of hemolysis modestly increased, yet remaining below the proposed cutoff value of 600 U/L.[16] Interpretation of the significance of the reduced haptoglobin concentration as a hemolysis parameter in this patient was difficult, because cirrhosis may reduce its synthesis. Serum concentrations of AST and ALT were within the reference range before and after pregnancy and moderately increased (AST 5-fold and ALT 2-fold, upper limit of the reference range) at the time that preeclampsia was diagnosed.

Next to liver cirrhosis, patient 1 had an APS with positive lupus anticoagulant and antiphospholipid antibodies. The presence of either of these factors increases the risk of developing preterm preeclampsia by a factor 10 and is associated with other obstetric complications, including recurrent pregnancy loss.[13,14,17,18] This was exemplified by our patient, because her previous 5 pregnancies ended in early miscarriages. In this patient several diagnoses were considered, including lupus nephritis because of the deterioration of renal function for which intravenous methyprednisolone was given. The outcome of the subsequently determined sFlt-1/PlGF ratio of 1525 was consistent with the diagnosis of superimposed preeclampsia, aiding in the decision to terminate the pregnancy.

Patient 2 was known to have lupus nephritis that had been in remission for 4 years while she was still on azathioprine treatment. At 7 weeks of gestation, azathioprine was discontinued by her referring obstetrician. This was an unfortunate decision, because flares of lupus are reported to occur in 30% to 60% of pregnant SLE patients.[19–22] At admission to our department triple immunosuppressive therapy was initiated, and immunosuppressive therapy was later intensified, but proteinuria and BP increased, raising the question of the presence of superimposed preeclampsia. The low sFlt-1/PlGF ratio of 2.8 argued against this diagnosis. Despite this low ratio, the progressive edema, attributed to the severe proteinuria and hypopalbuminemia, required induction of labor.

Patient 3 was treated with azathioprine during her pregnancy because of a lupus nephritis. Despite this treatment, proteinuria progressively increased to 21 g per day. Furthermore, BP rose and kidney function deteriorated. In this patient, a lupus flare or a lupus flare plus superimposed preeclampsia was considered. The result of sFlt-1/PlGF ratio of 240 favored the latter possibility.

sFlt-1/PlGF Ratio as a Diagnostic Test

The sFlt-1/PlGF ratio in our patients was used to assist in the distinction between superimposed preeclampsia from activation of underlying diseases. sFlt-1 is a splice variant of the fms-like tyrosine kinase or vascular endothelial growth factor receptor 1, lacking the transmembrane and cytoplastic domain of the receptor. sFlt-1 is detectable in serum at low concentrations outside of pregnancy.[23] During normal pregnancy, the placental production of sFlt-1 steadily increases until labor.[5,7,24] Circulating free PlGF concentrations increase with advancing normal gestation, peaking at ≈30 weeks of gestation and subsequently declining slightly near term.[7,9,25] Compared with normal pregnancy, the serum concentration of sFlt-1 is considerably higher and that of PlGF lower in preeclampsia, especially in early onset preeclampsia.[7] Because of the reciprocal changes of circulating values of sFlt-1 and PlGF in preeclampsia compared with values in normal pregnancy, the ratio of these 2 markers as a diagnostic test for preeclampsia has been advocated.[7] Meanwhile fast and accurate assays working on existing diagnostic platforms, including a point-of-care assay, have been developed.[7,11,12,26,27] The diagnostic performance of the sFlt-1/PlGF ratio is better than that of the individual values of sFlt-1 and PlGF.[7,11] With a cutoff value of 85, the reported sensitivity and specificity of the sFlt-1/PlGF ratio are 89% and 97% (area under the receiver operating characteristic curve, 0.97) for preterm preeclampsia and 74% and 89% (area under the receiver operating characteristic curve, 0.87) for late-onset preeclampsia.[7] In the 2 patients who we considered to have preeclampsia, the sFlt-1/PlGF ratios clearly exceeded the cutoff value of 85.[7] The low sFlt-1/PlGF ratio of 2.5 in the second patient was below the 25th percentile of the ratio observed in healthy pregnancies, thereby making the diagnosis of preeclampsia highly unlikely.[7]

Apart from being a test to diagnose preeclampsia, recent research has shown that the sFlt-1/PlGF ratio is also strongly associated with subsequent maternal and perinatal adverse outcomes.[8] In women presenting at <34 weeks of gestation, the sFlt-1/PlGF ratio performs better than other currently available clinical signs and laboratory tests, like BP, and serum concentrations of creatinine, uric acid, and ALT in the prediction of adverse outcomes.[8] Furthermore, the sFlt-1/PlGF has been found to discriminate well between patients with preexisting hypertension or gestational hypertension and patients with superimposed preeclampsia.[28–30]

Factors Influencing the sFlt-1/PlGF Ratio

To be a useful diagnostic test to differentiate activation or new onset of an underlying condition mimicking preeclampsia from (preterm) preeclampsia, the sFlt-1/PlGF ratio should not or only to a minimal extent be affected by the underlying condition. Patients 2 and 3 both had a lupus nephritis flare, but notwithstanding that the pregnancy duration was comparable, their sFlt-1/PlGF ratios differed considerably. In patient 2, the sFlt-1/PlGF ratio was between the reported 2.5 to 25th percentile of normal pregnancy, and in patient 3 it was considerably higher than the proposed cutoff value of 85.[7]

These findings are reassuring, but obviously more data are required to ascertain that a lupus flare itself has no substantial influence on the sFlt-1/PlGF ratio.
Data about the relationship between SLE and proangiogenic and antiangiogenic factors during pregnancy is limited and not uniform.[31–33] In a retrospective case-control study concerning 52 SLE pregnancies (blood sampled between 22 and 32 weeks of gestation), the serum concentration of sFlt-1 was significantly higher in the SLE pregnancies with (superimposed) preeclampsia (n=18) than in those without preeclampsia
(1768 versus 1177 pg/mL), whereas the serum PlGF concentration between the 2 groups did not differ.[33] Further prospective studies with repeated determination of the sFlt-1/PlGF ratio are required to clarify whether SLE during pregnancy, either active or not, has influence on the sFlt-1/PlGF ratio and whether the rise in this ratio, characteristic for early onset preeclampsia, is still fully present. Of potential concern is a recent finding by Rosenberg et al showing that heparin treatment during pregnancy is associated with increased sFlt-1 levels.[34] This increase was detectable after the 28th week of pregnancy in women who, for various reasons, were treated with prophylactic or therapeutic doses of mostly LMWH but in whom pregnancy course and outcome were uneventful. In women on heparin, the serum sFlt-1 concentration in the third trimester was ≈2 times higher than in women off heparin (4596 versus 2612 pg/mL). The heparin-induced increase in sFlt-1 concentration is attributed to shedding of the extracellular domain of the Flt-1 receptor from the placental tissue, caused by heparin displacing the sFlt1 heparin-binding site from the extracellular matrix.[34,35] Heparin treatment during pregnancy was without effect on the serum concentrations of vascular endothelial growth factor, PlGF, or soluble endoglin.[34] Our first described patient was treated with a therapeutic LMWH dose from the early beginning of gestation because of her APS. Our second and third described patients started with a prophylactic LMWH dose at 7 weeks of gestation. Because no repeated measurements were performed, we cannot exclude the possibility that the elevated sFlt-1 concentrations in patients 1 and 3 were attributable, at least in part, to heparin treatment. Contrary to the study of Rosenberg et al,[34] the elevated sFlt-1 concentration in patient 1 was already present in the second trimester of pregnancy. Moreover, in both patients, the serum sFlt-1 concentrations were considerably higher (84 339 in patient 1 and 14 082 in patient 3) than those reported by Rosenberg et al.[34] The effect of intrauterine growth restriction on circulating sFlt-1 or PlGF levels or their ratio has been reported in several studies.[10,36–38] The results of these studies are conflicting, which in part might be related to the used definition of intrauterine growth restriction, the time of blood sampling in relation to gestational age, and whether preeclampsia had been excluded. In a relatively large study, Chaiworapongsa et al reported higher sFlt-1 levels in small-for-gestational-age pregnancies compared with control pregnant women, but these levels were still considerably lower than in preeclamptic women.[36] Moreover, in line with other reports, sFlt-1 levels were elevated only in small-for-gestational-age patients with abnormal uterine artery Doppler

velocimetry examination.[36,39]

New Developments

The angiogenetic markers here discussed have an excellent diagnostic accuracy to distinguish (superimposed) preeclampsia from other pregnancy-related conditions associated with hypertension and proteinuria but are not sensitive enough to serve as an early screening test.[4,5] Meanwhile, proteomic, metabolomic, and gene expression profiling of maternal plasma, urine, or peripheral blood mononuclear cells have been applied to search for new biomarkers and potential pathogenetic pathways.[40] It should be acknowledged that these techniques are not suited for simple, low-cost routine clinical prediction of preeclampsia but for the identification of new biomarkers.[41]

Since early 2000, an imbalance between antiangiogenic and proangiogenic factors has been recognized as an important pathogenetic mechanism in the development of preeclampsia.[23] At this moment the only remedy for preeclampsia is delivery of the child and placenta, and albeit beneficial for the health of the mother, it is associated with neonatal morbidity and mortality and high healthcare expenditure. A promise for the future is that normalization of the angiogenic imbalance can be achieved either by administration of proangiogenic factors or removal of antiangiogenic factors.[42,43] In a clinical pilot, the possibility of extracorporeal removal of sFlt-1 using a negatively charged dextran cellulose column to absorb sFlt-1 in 3 patients with very preterm preeclampsia has been reported.[42] This pilot showed that, with this approach, elevated circulating sFlt-1 levels can be reduced, leading to a reduction in proteinuria and stabilization of BP without negative maternal or fetal effects.[42] Thus, the sFlt-1/PlGF ratio can be applied as a diagnostic tool in the differential diagnosis of preeclampsia and has potential as a biomarker to initiate and monitor new treatment options in preterm preeclampsia with the objective to prolong pregnancy and to improve its outcome.

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Chapter 10
sFlt-1 and PlGF measurements and their ratio for the diagnosis and prognosis of preeclampsia in a high-risk cohort

Submitted

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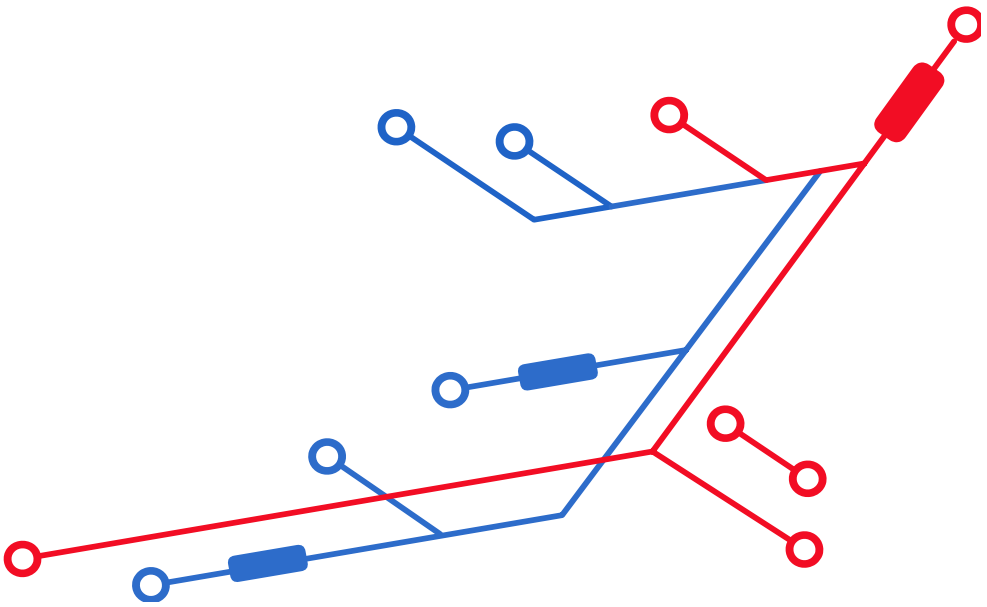

INTRODUCTION

Hypertensive disorders in pregnancy are the major cause of maternal and neonatal morbidity and mortality worldwide.[1] It is clinically important to distinguish preeclampsia (PE) from other forms of hypertension and proteinuria in pregnancy since patients with PE are at high risk for adverse pregnancy outcome.[2] The clinical diagnosis of PE is defined as de novo hypertension and proteinuria at or after 20 weeks of pregnancy. Although PE seemed to be a clearly defined disease, recently the American College of Obstetricians and Gynecologists (ACOG) removed (massive) proteinuria as an imperative criterion for the diagnosis of PE because proteinuria is poorly predictive for the course of the disease and the adverse pregnancy outcome.[3]

PE can severely affect both mother and child. Serious events in the mother are the HELLP syndrome, acute kidney failure, pulmonary edema, subcapsular liver hematoma, eclampsia, and cerebral hemorrhage. The most serious threats for the fetus are intrauterine growth restriction (IUGR), premature delivery, placental abruption and fetal/neonatal death.[4] Choosing between temporizing management and delivery is repeatedly a weighing of maternal versus fetal risks. The soluble Fms-like tyrosine kinase 1 (sFlt-1)/placental growth factor (PlGF) ratio has been introduced in the past decade as an aid to diagnose PE and as a valuable instrument to provide prognostic information.[5-7] This ratio of two biomarkers, produced by the placenta, has excellent test properties for diagnosing PE.[7] Before 34 weeks of gestation sensitivities and specificities of over 90% are reported.[8] However, when used after 34 weeks in pregnancy, the test performs less well due to a physiological rise in sFlt-1 and a decreased level of PlGF at the end of normal pregnancies.[9] After 34 weeks of gestation, sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were 58.3%, 93.3%, 82.4%, and 80.8%.[10]

Most studies validating this biomarker for diagnostic or prognostic purposes have been performed in patients suspected of PE because of an increased blood pressure, proteinuria, or other symptoms associated with PE, such as headache, visual disturbances, right upper quadrant pain or edema.[11-12] In these populations, the sFlt-1/PlGF test has been compared with parameters known to have limited value for prognostic purposes such as blood pressure, proteinuria and elevated liver enzymes for the prediction of adverse pregnancy outcome.[2] It is thus unclear whether this test has any additional value in patients with an established diagnosis of PE.

In a cohort of pregnant women at high risk of PE and adverse pregnancy outcome we scrutinized the utility of the sFlt-1/PlGF ratio in the decision-making of patients with hypertensive-proteinuric disorders and we analyzed the frequency of disagreement between the clinical diagnosis of PE and the sFlt-1/PlGF test. In addition we investigated whether sFlt-1/PlGF test predicts the risk of adverse maternal and/or fetal outcome more accurately than the diagnosis of PE based on the traditional criteria. Moreover, we explored which biomarker correlated best with specific parameters of disease severity.

METHODS

Patients

From September 2011 until August 2013 patients with suspected or with confirmed PE were recruited into this prospective study. Patients were suspected of PE if they presented with (preexisting) hypertension, proteinuria or any symptoms associated with PE such as right upper quadrant abdominal pain or headache with visual disturbances. The Erasmus MC Medical Ethics Committee approved the study protocol and written informed consent was obtained from all participants. After information about the aim of the study, participants were asked to donate a blood sample for measurement of sFlt-1 and PlGF at the time of admission either in the outpatient clinic or clinic because of suspicion for PE. Clinical characteristics of the patients were acquired on the same day as blood collection at inclusion. The diagnosis of PE was based on clinical judgment and routine laboratory findings at inclusion, whereas values of sFlt-1 and PlGF were determined after delivery to prevent any influence of this information on decision making of the clinicians. After inclusion the pregnancy course of each patient was followed and clinical characteristics and pregnancy outcome were obtained from the medical records after delivery. PE was defined according to the International Society for the Study of Hypertension in Pregnancy (ISSHP).[13] According to this, PE refers to de novo hypertension (systolic blood pressure (SBP) of ≥140 and/or a diastolic blood pressure (DBP) of ≥90 mmHg or taking antihypertensive medication for GH) and proteinuria (PCR ≥30 mg/mmol or ≥300mg/24 h) at or after 20 weeks of pregnancy. The PE group was further divided into superimposed PE (supPE), and HELLP syndrome (hemolysis, elevated liver enzymes and low platelets). SupPE was diagnosed in women with chronic hypertension with new onset of proteinuria or a sudden increase of blood pressure or appearance of thrombocytopenia and increased liver enzymes, or a sudden increase of proteinuria in patients with a preexisting proteinuria.

In consonance with Chappell et al.[12] we have used the fullPIERS minus transfusion of blood product definition for the adverse outcome, since a standard definition for adverse pregnancy outcome is missing. Adverse outcome was defined as the occurrence of one or more complication(s) of PE within two weeks after blood sampling. Maternal adverse outcomes were: development of the HELLP syndrome, pulmonary edema, subcapsular liver hematoma, cerebral hemorrhage, seizure, maternal death and acute renal failure (absolute increase in the serum creatinine concentration of ≥0.3 mg/dL (26.4 micromol/L) from baseline; ≥50% increase in serum creatinine; or oliguria with <0.5 mL/kg per hour over a period of 6 hours). Adverse fetal outcomes were: premature delivery (delivery <37 weeks), extreme premature delivery (delivery <32 weeks), intra uterine growth restriction (IUGR; weight below the 10th percentile) and fetal/neonatal death.

sFlt-1 and PlGF assays

SFlt-1 and PlGF were measured using commercially available assays on the Elecsys platform (Roche Diagnostics), as previously described.[9] After centrifuging the samples were stored at -80°C until analysis. For each sample the sFlt-1/PlGF ratio was calculated.[7-10,12]

Statistical Analysis

Comparisons were performed using the Mann-Whitney U-test for non-parametrically distributed data, or the t-test for normally distributed data. Values are reported as mean ± standard deviation (SD) for continuous data, as median and the interquartile range for marker levels, and as a percentage for categorical data. For the diagnosis of PE at inclusion, the diagnosis of PE as a final diagnosis and the prediction of adverse outcome, sensitivity, specificity, PPV and NPV were calculated for the sFlt-1/PlGF ratio using the previously reported cut-off point of ≥85.[14] A cut-off value of ≥85 for the sFlt-1/PlGF ratio was used because others have previously shown that a ratio ≥85 has a high sensitivity and specificity of respectively 82% and 95%.[15] Furthermore, this ratio seems to predict the occurrence of adverse maternal and fetal outcomes related to PE in patients without a clinical diagnosis of PE.[16] For the correlation of sFlt-1, PlGF and their ratio with the severity of the disease, Spearman's Rho correlation coefficient was used. A binary logistic regression analysis was performed to obtain the odds ratios for comparison of the different models to predict adverse pregnancy outcome. Statistical Package for Social Sciences (SPSS) software, version 21.0, was used for analysis. For all tests a value of $P<0.05$ was considered to be significant.

RESULTS

General findings

A total of 96 patients were enrolled in the current study, of whom two had a dizygotic twin pregnancy. The demographic and clinical characteristics at the time of blood sampling for sFlt-1/PlGF measurement are listed in Table 1. Fifty-three (55%) patients met the clinical criteria for PE at the time of blood sampling, 32 women (33%) had preexisting hypertension, and 19 (20%) had preexisting proteinuria. The prevalence of ≥1 antihypertensive medication use was 43 (81%) in the PE and 19 (44%) in the non PE group. In addition, 12 (13%) women developed HELLP syndrome, 4 (4%) women had pulmonary edema, and 5 (5%) had acute renal failure and three (3%) of them developed this within 14 days after blood sampling for sFlt-1 and PlGF determination (Table 2). In total 64 out of 96 women delivered preterm (<37 weeks), and 37 (58%) of them delivered prior to 32 weeks. Nineteen infants had IUGR (Table 2). There were no significant differences in age or gestational age at study entry between patients with or without PE. Systolic and diastolic blood pressure, protein-to-creatinine ratio (PCR), the sFlt-1 and the sFlt-1/PlGF ratio among patients with PE were higher than in the non-preeclamptic group (Table 1).

The outcome of pregnancy stratified according to the clinical diagnosis of PE and a positive or negative ratio is given in Table 2. Out of 53 patients clinically diagnosed with PE, 47

(89%) had a ratio ≥85 and 37 out of the 43 patients (86%) without clinical diagnosis of PE had a ratio <85. Maternal complications (38%) were highest in patients with a clinical diagnosis of PE and a positive ratio (Table 2). In the 6 patients with a positive test but no clinical diagnosis of PE, 1 maternal complication occurred. No maternal complications were recorded in the 6 patients with a clinically diagnosed PE with a negative test. In the 37 patients without PE and a negative test also no adverse outcomes were reported. Irrespective of the clinical diagnosis of PE fetal/neonatal complications were considerably lower (14% versus 72%, p < 0.001) in patients with a negative ratio compared to those with a positive ratio.

	No Preeclampsia	Preeclampsia	P
N	43	53	
Age, years	32 ± 6	32 ± 5	0.921
GA at inclusion, weeks	30 ± 6	30 ± 4	0.804
GA at delivery, weeks	36 ± 4	31 ± 4	<0.001
SBP, mm Hg	130 (120-140)	143 (132-150)	0.001
DBP, mm Hg	80 (76 -90)	90 (85-90)	0.012
PCR, mg/mmol	27 (15 – 297.7)	107 (63 – 341)	<0.001
Preexisting hypertension, n	19 (44.2%)	13 (24.5%)	0.066
≥ 1 Anti-hypertensives, n	19 (44%))	43 (81%)	0.001
Preexisting proteinuria, n	14 (32.6%)	5 (9.4%)	0.004
sFlt-1, pg/ml	2766 (1712 – 7823)	15069 (9968 - 22356)	<0.001
PlGF, pg/ml	232 (121 – 434)	52 (27 - 93)	<0.001
sFlt-1/PlGF Ratio	12 (4 – 66)	344 (128 – 644)	<0.001

Table 1. Characteristics of patients at time of inclusion. The diagnosis of preeclampsia is based on clinical criteria. GA: Gestational Age; SBP: systolic blood pressure; DBP: diastolic blood pressure; PCR: protein-to-creatinine ratio.

Patients with clinically diagnosed PE and a ratio <85 (false negative test). Six of the 53 patients (11%) with a clinically diagnosis of PE had a ratio <85 (Table 3). Gestational age at time of blood sampling varied from 20 to 37 weeks. Two of the patients (nrs. 2 and 3 in Table 3) had an adverse pregnancy outcome. One patient (nr. 2) was diagnosed with symptomatic preeclampsia and she had a twin pregnancy. The other patient had a ratio of almost 83. This high ratio was largely due to the low value of PlGF of 55 pg/ml. Her pregnancy was complicated by IUGR. In the other 4 patients pregnancy outcome, notwithstanding the clinical diagnosis of PE, was uncomplicated.

The duration between admission and blood sampling for determination of the ratio and delivery varied from 0 to 113 days.

	Positive test with PE at inclusion	Positive test without PE at inclusion	Negative test with PE at inclusion	Negative test without PE at inclusion
N	47	6	6	37
Interval between test and delivery, days	7 ± 9	9 ± 11	26 ± 44	44 ± 36
GA at delivery, weeks	31 ± 3	30 ± 3	36 ± 1	37 ± 4
Birth weight, g	1356 (914-1781)	1300 (734-1373)	2620 (1760 - 2736)	2837 (2235 - 3522)
Fetal/neonatal complication				
- IUGR, n	11 (23%)	2 (33%)	1 (17%)	5 (14%)
- Extreme prematurity, n	28 (60%)	6 (100%)	0 (0%)	3 (8%)
- NICU stay, days	11 ± 13	6 ± 4	2 ± 2	2 ± 7
- Fetal/neonatal death, n	4 (9%)	1 (17%)	1 (17%)	1 (3%)
Maternal complication				
- HELLP syndrome, n	11 (23%)	1 (17%)	-	-
- Pulmonary edema, n	4 (9%)	-	-	-
- Acute renal failure, n	3 (6%)	-	-	-
sFlt-1, pg/ml	16041 (113146-23455)	12016 (9468-14284)	4762 (2971-8256)	2606 (1593-5751)
PLGF, pg/ml	44 (27-80)	50 (29-69)	194 (91-437)	259 (156-478)
sFlt-1/PlGF ratio	369 (198-697)	293 (173-389)	43 (4-70)	8 (3-34)

Table 2. Pregnancy outcome stratified by clinical diagnosis and sFlt-1/PlGF ratio.

Positive test, sFlt-1/PlGF ratio ≥85; Negative test, sFlt-1/PlGF ratio <85; GA, gestational age; IUGR, Intrauterine growth retardation; NICU, neonatal intensive care unit; Extreme prematurity, gestational age at delivery <32 weeks.

Case	1#	2*,[1]	3	4	5	6
Maternal age, years	28	41	40	36	30	34
GA at inclusion, weeks, days	20.6	29.6	35.0	36.0	36.2	37.0
SBP, mm Hg	120	159	130	160	159	141
DBP, mm Hg	80	99	90	100	87	86
PCR, mg/mmol	34.8	18.7	54.9	39.5	45.5	66.7
Preexisting hypertension	No	No	No	Yes	No	No
Antihypertensive drug use	Yes	No	No	Yes	No	No
Preexisting proteinuria	No	No	No	No	No	No
sFlt-1, pg/ml	1144	12632	4505	5018	3580	6797
PlGF, pg/ml	298	235	55	152	853	104
Ratio	4	54	83	33	4	66
Days from inclusion to delivery	113	31	8	2	1	0
GA at delivery, weeks, days	37.0	34.2	36.1	36.2	36.3	37.0
Adverse outcome	-	Neonatal death[2]	IUGR	-	-	-

Table 3.

Characteristics of the patients with a ratio <85 and clinically diagnosed with preeclampsia at time of inclusion (false negative ratio).

* Dizygotic twin pregnancy.

[1] Symptomatic PE with headache, vomiting, tachycardia, epigastric pain, blurred vision and seeing stars.

[2] Diamniotic, dichorionic twin pregnancy of which one fetus had multiple severe abnormalities (microcephaly, tetralogy of Fallot and severe intrauterine growth restriction, the other fetus was healthy).

GA, gestational age; SBP, systolic blood pressure at diagnosis, DBP: diastolic blood pressure at diagnosis; PCR, protein-to-creatinine ratio at diagnosis.

Initially this patient presented in another hospital with a BP of 156/95 mmHg and a PCR of 92 mg/mmol. PE was diagnosed and methyldopa was started. Thereafter she was transferred to our hospital, because of PE at a very early GA. At study entrance she had a normal BP with the use of methyldopa.

Case	1	2	3	4	5*	6
Maternal age, years	27	20	35	34	39	30
GA at inclusion, weeks, days	27.6	28.3	28.6	30.5	33.3	21.3
SBP, mm Hg	140	140	120	140	150	160
DBP, mm Hg	90	90	80	80	90	105
PCR, mg/mmol	1548	13.1	15.7	12.9	24.4	22.7
Preexisting hypertension	Yes	No	No	No	No	Yes
Antihypertensive drug use	Yes	No	No	No	Yes	No
Preexisting proteinuria	Yes	No	No	No	No	No
Other preexisting diseases	SLE, LN IV	-	SLE	-	-	-
sFlt-1, pg/ml	14082	10495	11647	12385	14893	6388
PlGF, pg/ml	59	76	34	67	42	13
Ratio	239	138	343	185	355	491
Days from inclusion to delivery	15	4	5	4	11	28
GA at delivery, weeks, days	31.0	29.0	29.4	31.2	35.0	25.3
Days from inclusion to diagnosis	12	4	2	3	9	10
Final diagnosis	supPE	GH	GH	PE	supPE	supPE
Adverse outcome	-	IUGR	-	HELLP	IUGR	Still birth[1]

Table 4.

Characteristics of the patients with a ratio ≥ 85 without a clinically diagnosed preeclampsia at time of inclusion (false positive ratio).

* Dizygotic twin pregnancy.

[1]Stillbirth, severe IUGR early in pregnancy.

GA, gestational age; SBP, systolic blood pressure at diagnosis, DBP: diastolic blood pressure at diagnosis; PCR, protein-to-creatinine ratio at diagnosis; SLE, systemic lupus erythematosus; LN, lupus nephritis class IV; supPE, superimposed preeclampsia; GH, gestational hypertension; HELLP, hemolysis elevated liver enzymes and low platelet count; IUGR, intrauterine growth restriction.

Patients with no clinical diagnosis of PE and a ratio of >85 (false positive test)

In 6 of the 43 patients (14%) without clinical diagnosis of PE at inclusion, the ratio was ≥85 (Table 4). Gestational age at time of blood sampling varied from 21 to 37 weeks. Of these 6 patients 3 developed superimposed PE, 1 PE with HELLP and 2 gestational hypertension. Adverse pregnancy outcome (still birth and IUGR) occurred in 3 patients. The duration between inclusion and blood sampling for determination of the ratio and delivery varied from 0 to 28 days (Table 4).

	sFlt-1/PlGF ratio of ≥85			Clinically diagnosed PE
	PE at inclusion	Final diagnosis PE	Adverse outcomes	Adverse outcomes
Sensitivity (%)	86	74	86	75
Specificity (%)	88	90	77	71
PPV (%)	89	93	73	67
NPV (%)	86	67	88	79

Table 5.

Sensitivity, specificity, positive and negative predictive values for the ratio cutoff point of ≥85 for diagnosing preeclampsia with clinical preeclampsia as reference and for adverse outcomes. Sensitivity, specificity, positive and negative predictive values for adverse outcomes based on clinically diagnosed PE are also provided.

PE, preeclampsia; PPV, positive predictive value; NPV, negative predictive value.

Sensitivity and specificity

With a cutoff value of the ratio ≥85 the sensitivity for the clinical diagnosis of PE at admission was 86%. This value modestly decreased for the final clinical diagnosis of PE (Table 5). Compared to the clinical diagnosis of PE, sensitivity and specificity for prediction of adverse outcomes were better for the ratio using a cutoff value of ≥85 (Table 5).

Correlations of sFlt-1, PlGF or their ratio with disease severity and outcome

PlGF concentration was considerably lower in the preterm than in the term infants, respectively 53 pg/mL (28-115) versus 279 pg/mL (158-533) (P<0.001). PlGF was also lower in pregnancies complicated by IUGR (44 pg/mL (26-66). The sFlt-1/PlGF ratio correlated positively with blood pressure, PCR, the serum concentrations of creatinine and uric acid, adverse outcome and stay at the NICU, but inversely with gestational age at delivery and birth weight percentile corrected for gestational age (Table 6).

When comparing sFlt-1 and PlGF concentrations separately with variables of disease severity and outcome it became clear that sFlt-1 correlated positively with maternal variables of disease severity and PlFG concentration with fetal/neonatal variables of disease severity (Table 6). In a binary logistic regression model, patients with an established clinical diagnosis of PE had an 8 (3-20) times increased risk for having an adverse pregnancy outcome (Table 7A; Model 1). After adjustment for gestational age at time of measurement (Model 2), this risk was 9 (4 - 23). Patients with a positive test had a 19 (6 - 58) times increased risk for adverse pregnancy outcome, and 29 (9-86) when correcting for gestational age at testing. When both risk factors (clinical diagnosis and ratio) were combined and corrected for gestational age at testing (Table 7B; Model 3), only a positive sFlt-1/PlGF ratio remained significant with an odds ratio of 22 (5 – 91).

	Ratio	sFlt-1	PlGF
SBP, mm Hg	.409**	.324**	-.341**
DBP, mm Hg	.252*	.229*	-.241*
PCR, mg/mmol	.279*	.314**	-.141
Serum creatinine, µmol/l	.229	.416**	.042
Serum uric acid, mmol/l	.372**	.500**	-.144
Period from inclusion to delivery, days	-.656**	-.696**	.499**
GA at delivery, weeks	-.699**	-.439**	.736**
Birth weight, percentile	-.304**	-.154	.318**
Apgar score 5 min	-.392**	-.271**	.393**
Stay at NICU, days	.565**	.401**	-.629**
Any adverse outcome 1	.642**	.460**	-.587**

Table 6. Correlations of sFlt-1, PlGF and their ratio with variables of severity of the disease and outcome.

[1]Any form of adverse pregnancy outcome as mentioned in the Methods.

*P<0.05; **P<0.01.

SBP, systolic blood pressure; DBP, diastolic blood pressure; PCR, protein-to-creatine ratio;

GA, gestational age; NICU, neonatal intensive care unit.

R^2		Odds ratio	P value
0.25	Clinical diagnosis	7.0 (2.8 – 17.4)	<0.001
0.44	Positive test	19 (6.4 – 58.2)	<0.001
0.31	Model 1: Clinical diagnosis#	9 (3.5 – 23.1)	<0.001
0.53	Model 2: Positive test#	29 (9.4 – 86.3)	<0.001

Table 7A.

Odds for adverse outcome of pregnancy.

Corrected for GA at time of measurement <34 weeks.

R^2			Odds ratio	P value
0.53	Model 3#:	- Clinical diagnosis	1 (0.4 – 6.1)	0.59
		- Positive test	22 (5.4 – 90.9)	<0.001

Table 7B.

Odds for adverse outcome of pregnancy.

Corrected for GA at time of measurement <34 weeks.

DISCUSSION

To our best knowledge this is the first study comparing the sFlt-1/PlGF ratio with the clinical diagnosis of PE for the prediction of adverse outcome of pregnancy. We separated our cohort in positive test results (sFlt-1/PlGF ratio ≥85) and negative test results (sFlt-1/PlGF ratio <85) and we then compared the ratio with the clinical diagnosis of PE and for the prediction of adverse outcome of pregnancy. We found a considerably increased risk for an adverse pregnancy outcome when the ratio was above the suggested cut-off value of 85 in patients clinically diagnosed with PE. Thus, this study confirms the accuracy of the sFlt-1/PlGF test for the diagnosis of PE and the prediction of PE-related adverse outcomes.

In our study, binary logistic regression analysis revealed that a positive test is a better predicting factor for poor pregnancy outcome than the clinical diagnosis of PE. This finding is important because in some of our patients clear discrepancies were present between the clinical diagnosis and the test findings. In accordance with previous studies we found that even patients who did not meet the classical diagnostic criteria for PE, but who did have a positive test, had a comparable incidence of adverse outcomes as patients with a clinical diagnosis of PE and a positive test (Table 2).[15-17]

This suggests that either there is a shortcoming of the current definition of PE, that the

diagnosis has been missed, and/or that the clinical syndrome of PE was still in its developing phase. Although experienced clinicians made the diagnosis, the high prevalence of preexisting hypertension and proteinuria in our population of respectively 32% and 20% might have hampered the diagnostic process in some of the patients. Particularly for these patients, the sFlt-1/PlGF test may be a helpful tool for diagnostic and as a consequence decision-making purposes.[18]

A point of concern is the observation that 6 patients with clinically diagnosed PE, of whom 1 with hypertension and symptomatic PE, had a negative sFlt-1/PlGF ratio. It should be remarked that 4 of these patients were tested after 35 weeks of pregnancy, at which timepoint the ratio has shown to lose its accuracy.[9] Indeed the two patients with a negative test who were tested at 20.6 weeks and 29.6 weeks of pregnancy remained pregnant for 113 and 31 days respectively, indicating that a negative test before 35 weeks of pregnancy may predict a prolonged duration of the pregnancy even when PE is diagnosed on the basis of classical criteria. Of note, two patients with a false-negative sFlt-1/PlGF test experienced adverse events. One adverse event was caused by severe, non-viable, congenital malformations. The other was caused by IUGR. The relatively high ratio in this case was mainly due to the very low PlGF of 55 pg/mL eight days prior to delivery. On the other hand, we had 6 patients with a positive test, but no clinical diagnosis of PE at inclusion. As displayed in Table 4, these women were diagnosed with superimposed PE, HELLP syndrome or gestational hypertension and they all delivered preterm within 28 days after inclusion. Our findings support previous findings that an elevated ratio can detect disease and adverse outcomes in some individual cases earlier than achievable by routine clinical and laboratory assessment and that the ratio is helpful to distinct between preexisting conditions and superimposed PE.[19] For example, patient 1 in Table 4 was a very complicated case. She was known with class IV lupus nephritis, and she therefor was suspected of both exacerbation of SLE and superimposed preeclampsia. At time of inclusion, we considered a lupus flare most likely, because of a slight increase in anti-double-stranded DNA antibody whereas complement factor C4 concentration was slightly decreased. The clinical diagnosis of superimposed preeclampsia was eventually made 12 days after inclusion, because of further increase in blood pressure and proteinuria and a decrease in platelet count. Knowing this ratio at inclusion would have allowed making the diagnosis of superimposed PE at an earlier time point.

Interestingly, we found strong correlations of sFlt-1, PlGF and the ratio with well-known parameters for the severity of the disease and outcome. sFlt-1 was positively correlated with relevant maternal parameters such as blood pressure, proteinuria and uric acid, whereas PlGF was positively correlated with fetal adverse outcomes like gestational age at delivery, IUGR and prolonged stay at the NICU.

As reported recently a low PlGF is strongly linked with low birth weight and therefore can

add valuable information to identify pregnancies with a potential adverse fetal outcome, necessitating urgent delivery. [19]

Strengths and limitations

Some limitations of our study should be mentioned. First, the study was performed in a single center and the sample size of this study is limited. Furthermore, we only measured the sFlt-1/PlGF ratio once, while as shown in Table 5, the PPV of the ratio slightly improves whereas the NPV moderately decreases when comparing the initial and final clinical diagnosis of PE. Follow-up of the patients with repetitive measurements could thus result in a reduction of the number of false positive ratios, related to the evolution of the clinical course of their pregnancy. Schools et al. showed the importance of repeated measurements for prediction of preeclampsia and pregnancy outcome.[20] In a cohort of patients with a lower incidence of PE than in our cohort, the PPV and NPV values might be lower. Studies indicate that a negative test has a high value to predict an uncomplicated pregnancy course with a favorable outcome.[11] In addition, when comparing with other studies there is no standard definition for adverse pregnancy outcome. We have used the FULLPIERS minus transfusion of blood product definition, as also used by Chappell et al.[12] For the generalizability and comparability of studies, a general definition for adverse outcome of a preeclamptic pregnancy is warranted. Although this study has a relatively small population, only a few studies have included as many severe preeclamptic patients presenting before 34 weeks of gestation as our study. Moreover, most studies only compared a high versus a low ratio for the prediction of adverse outcome. Since it is well known that the clinical diagnosis of PE greatly increases the chance for getting an adverse outcome, strength of this study is that we also compared positive sFlt-1/PlGF tests with the confirmed clinical diagnosis of PE for the chance of adverse outcome.

CONCLUSION

In a cohort of pregnant women with clinical PE or at high risk of PE we have shown that a sFlt-1/PlGF ratio ≥85 is associated with a higher odds for adverse pregnancy outcome than the clinical diagnosis of PE. Its additional value for diagnostic purposes remains limited since most patients with a positive test do have the clinical diagnosis of PE. Yet, particularly in pregnant women with preexisting hypertension and/or proteinuria, measurement of the ratio may be valuable for making the correct diagnosis. Finally, due to its strong correlation with clinical parameters, the sFlt-1/PlGF ratio may be useful for research purposes as the clinical definition of PE is not always straightforward.[3]

ACKNOWLEDGEMENTS

We wish to thank Roche Diagnostics for funding the sFlt-1 and PlGF tests.
We wish to thank Mrs. Annie Vereecken (pharmacist-biologist, CEO AML laboratory Antwerp) for measuring sFlt-1 and PlGF.

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Chapter 11
Association Studies Suggest a Key Role for Endothelin-1 in the Pathogenesis of Preeclampsia and the Accompanying Renin–Angiotensin–Aldosterone System Suppression

Hypertension. 2015, in press

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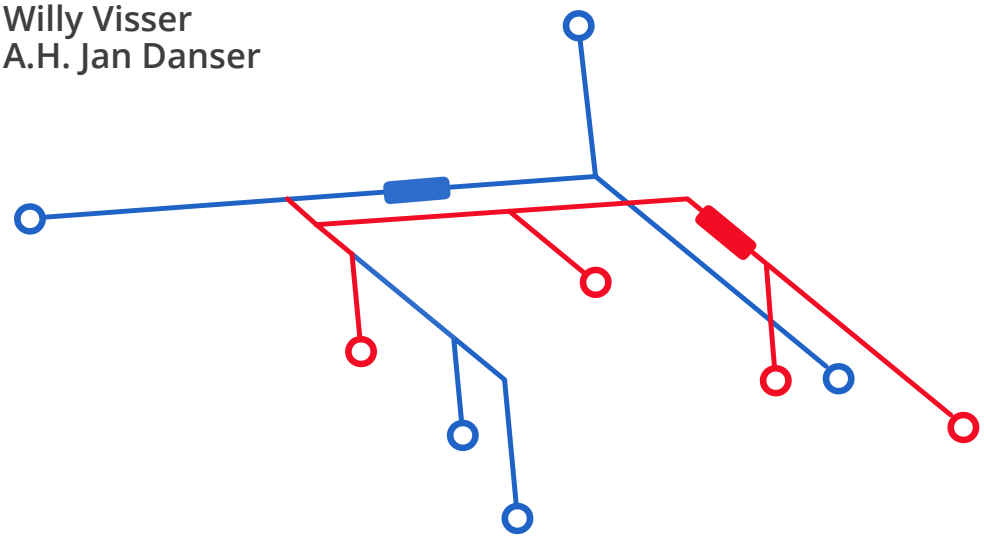

ABSTRACT

Women with preeclampsia display low renin-angiotensin-aldosterone system activity and a high anti-angiogenic state, the latter characterized by high levels of soluble Fms-like tyrosine kinase-1 (sFlt-1) and reduced placental growth factor (PlGF) levels. To investigate whether renin-angiotensin-aldosterone system suppression in preeclampsia is due to this disturbed angiogenic balance, we measured mean arterial pressure (MAP), creatinine, endothelin-1, and renin-angiotensin-aldosterone system components in pregnant women with a high (≥85; n=38) or low (<85; n=65) sFlt-1/PlGF ratio. Plasma endothelin-1 levels were increased in women with a high ratio, whereas their plasma renin activity and plasma concentrations of renin (PRC), angiotensinogen and aldosterone were decreased. Plasma renin activity-aldosterone relationships were identical in both groups. Multiple regression analysis revealed that PRC correlated independently with MAP and plasma endothelin-1. Plasma endothelin-1 correlated positively with sFlt-1 and negatively with PRC, and urinary protein correlated with plasma endothelin-1 and MAP. Despite the lower plasma levels of renin and angiotensinogen in the high-ratio group, their urinary levels of these components were elevated. Correction for albumin revealed that this was due to increased glomerular filtration. Subcutaneous arteries obtained from preeclampsia patients displayed an enhanced, AT_2 receptor-mediated response to angiotensin II. In conclusion, a high anti-angiogenic state associates with endothelin-1 activation, which together with the increased MAP may underlie the parallel reductions in renin and aldosterone in preeclampsia. Since endothelin-1 also was a major determinant of urinary protein, our data reveal a key role for endothelin-1 in the pathogenesis of preeclampsia. Finally, the enhanced angiotensin responsiveness in preeclampsia involves constrictor AT_2 receptors.

INTRODUCTION

Preeclampsia (PE) is a pregnancy-related disorder, clinically characterized by the new onset of proteinuria and hypertension in the second half of pregnancy, with a great impact on maternal and fetal morbidity and mortality worldwide.[1] A better understanding of the pathogenic mechanisms underlying PE might help identifying biomarkers that allow early diagnosis and treatment of PE. Recently, disturbances in angiogenic balance (favoring anti- over pro-angiogenic factors), elevated endothelin-1 (ET-1) levels, and a suppressed renin-angiotensin-aldosterone system (RAAS) have been reported.[2-4] As a consequence, the ratio of the anti-angiogenic soluble Fms-like tyrosine kinase-1 (sFlt-1) and the pro-angiogenic placental growth factor (PlGF) is now believed to be a reliable biomarker for the diagnosis of PE.[5] In fact, patients with a ratio ≥85 have a poor pregnancy outcome independent of their clinical diagnosis compared to patients with a ratio <85.[5]

Of interest, treatment of cancer patients with anti-angiogenic drugs (which, like sFlt-1, prevent the actions of vascular endothelial growth factor (VEGF)) resulted in hypertension, proteinuria, renin suppression and elevated ET-1 levels.[6] Animal studies with anti-angio-

genic drugs additionally revealed that the renal histological changes observed during such treatment, in particular glomerular endotheliosis, resembled the renal alterations observed in PE.[7] From the observation that the dual $ET_{A/B}$ receptor antagonist macitentan prevented both the rise in blood pressure and proteinuria during anti-angiogenic treatment it appeared that ET-1 is causally involved in these PE-like side effects.[8]

A suppressed RAAS in PE is counterintuitive given the reduced circulating volume in this disorder.[2, 9] Gennari-Moser et al. have proposed that VEGF stimulates aldosterone production, both directly and indirectly, the latter by enhancing adrenal capillary density.[10] On this basis, a rise in sFlt-1, via VEGF inactivation, should suppress aldosterone levels in PE. Buhl et al. observed that urinary plasmin levels are elevated in PE. The resulting ENaC activation might also suppress aldosterone.[11] Finally, the occurrence of angiotensin (Ang) II type 1 (AT_1) receptor autoantibodies in PE, which stimulate the AT_1 receptor, should suppress renin (negative feedback loop) but increase aldosterone. Their presence may contribute to the high Ang II sensitivity in PE. Careful analysis of the plasma aldosterone/plasma renin concentration (PRC) ratio in PE might shed light on these possibilities. In the past, this ratio has been shown to be elevated in pregnant women, the highest ratio in fact occurring in PE.[4] However, given the substantial angiotensinogen rises in pregnancy (and to a lesser degree in PE), Ang I generation for a given level of renin may differ greatly between pregnant and non-pregnant women. Therefore, the plasma aldosterone/plasma renin activity (PRA) ratio might be more appropriate to investigate this relationship. Given the renal damage in PE, urinary RAAS component measurement could provide additional diagnostic information, provided that such components are truly kidney- (and not plasma-) derived.[12]

In the present study, we hypothesized that RAAS suppression in PE is the consequence of the disturbed angiogenic balance and/or the resulting rise in ET-1, and that urinary RAAS components provide additional diagnostic information, e.g., on the degree of renal dysfunction. To address these hypotheses, we measured sFlt-1, PlGF, ET-1 and RAAS components in plasma and urine of pregnant women with sFlt-1/PlGF ratios ≥85 or <85. We additionally investigated what Ang II receptor (type 1 or type 2) contributes to the enhanced Ang II responsiveness in PE, making use of subcutaneous arteries obtained after caesarean delivery.

METHODS

Human studies

Patients were recruited between March 2012 and February 2013 at the Erasmus MC and midwifery Rotterdam west. The Erasmus MC Medical Ethics Committee approved the study protocol, and written informed consent was obtained from all subjects before participation. Blood pressure was measured 3 times with an automated blood pressure monitor (Omron 705 CP-2 Healthcare) in sitting position. The first reading was excluded and the subsequent two were averaged.

Mean arterial pressure was calculated with the formula: MAP = 2/3(diastolic blood pressure)+1/3(systolic blood pressure). On the same day, blood and urine were collected, processed and stored at -20°C until analysis. Two groups of women were included. The first group consisted of patients with (suspected) preeclampsia, characterized by hypertension and/or proteinuria, or having preeclampsia-like complaints like headache with visual disturbances, and abdominal pain in the right upper quadrant. Exclusion criteria were coexisting diabetes (gravidarum) and inability to obtain informed consent. The second group consisted of healthy pregnant women, matched for gestational age. Exclusion criteria were a history of preeclampsia, hypertension, proteinuria, diabetes (gravidarum), or inability to obtain informed consent.

Additionally, a second cohort of patients included consisting of women with preeclampsia, defined as de novo hypertension and proteinuria in the second half of pregnancy (blood pressure ≥ 140/90 mm Hg, and proteinuria > 300 mg/24 hours after the 20th week of gestation), and healthy pregnant women, with no history of preeclampsia, hypertension, proteinuria, or diabetes. These women all delivered via caesarean section, which was performed because of fetal or maternal well-being in the case of preeclampsia, or a child presenting in breech position, or a previous caesarean section in the case of healthy pregnant women.

From these women abdominal subcutaneous tissue was collected during a caesarean section for the evaluation of their microvascular function.

Rat studies

Male Wistar Kyoto rats (280-300 gram) obtained from Charles River, were housed in individual cages and maintained on a 12-h light/dark cycle, having access to standard laboratory rat chow and water ad libitum. The VEGF inhibitor sunitinib (Pfizer) was administered for 8 days by oral gavage at 3 different doses (7, 14 or 26.7 mg/kg.day; n=6-14) as described previously.[6] At the end of each experiment, rats were euthanized with 60 mg/kg pentobarbital i.p. and blood was sampled for measurement of circulating endothelin-1. All experiments were performed under the regulation and permission of the Animal Care Committee of the Erasmus MC.

Biochemical measurements

Endothelin-1 was measured by chemiluminescent ELISA (QuantiGlo, R&D systems; detection limit 0.34 pg/mL). Plasma renin concentration and plasma prorenin (the latter after its conversion to renin by trypsin), as well as plasma renin activity (PRA), were measured by enzyme-kinetic assay as described before (detection limit 0.05 ng angiotensin I per ml.hr).[13] Plasma and urinary angiotensinogen were measured as the maximum quantity of angiotensin I that was generated during incubation with excess recombinant renin (detection limit 0.5 pmol/mL),[13] while urinary angiotensinogen was additionally measured by commercial ELISA (IBL International; detection limit of 0.01 pmol/mL).

Aldosterone was measured by solid-phase radioimmunoassay (Diagnostic Products Corporation; detection limit 11 pg/mL). Urinary aldosterone, but not plasma aldosterone, was extracted according to kit instructions (recovery >86%). Soluble Fms-like tyrosine kinase-1 (sFlt-1) and placental growth factor (PlGF) measurements were performed on the fully automated Roche Elecsys system (Elecsys PlGF, human PlGF, and Elecsys sFlt-1, sFlt-1) as described previously,[14] and the sFlt-1/PlGF ratio was calculated for each sample. Sodium, potassium, creatinine, albumin and total protein were measured with routine laboratory methods.

Microvascular function

Arteries (diameter 1-2 mm) were isolated from abdominal subcutaneous tissue and processed either directly or after overnight storage. They were cut into segments of ≈2 mm length and mounted in a Mulvany myograph (Danish Myo Technology) with separated 6-mL organ baths containing Krebs bicarbonate solution, aerated with 95% O2 and 5% CO2, and maintained at 37°C. Following a 30-min stabilization period, the optimal internal diameter was set to a tension equivalent to 0.9 times the estimated diameter at 100 mm Hg effective transmural pressure, as described before.[15] Endothelial integrity was verified by observing relaxation to 10 nmol/L substance P after preconstriction with 10 nmol/L of the thromboxane A2 analogue U46619. Subsequently, to determine the maximum contractile response, the tissue was exposed to 100 mmol/L KCl. Next, after a 30-min equilibration in fresh organ bath fluid, segments were pre-incubated for 30 min with the AT1 receptor antagonist irbesartan, the AT2 receptor antagonist PD123319 (both 1 µmol/L) or vehicle. Thereafter, concentration-response curves were constructed to angiotensin II.

Statistical analysis

The sFlt-1/PlGF ratio (≥85 or <85) was used to subdivide the patients in 2 groups. Based on a pilot study in preeclampsia patients and healthy pregnant controls, displaying urinary renin levels, respectively, of 2.1 (0.05-158) (median and range) and 0.25 (0.05-5.0) ng Ang I/ml.hr, a minimum of n=36/group was calculated to be sufficient to detect a 50% difference in urinary renin between the 2 groups with 80% power and 5% significance. Urinary renin was chosen for this power analysis given the limited knowledge about this parameter, as opposed to the substantial differences in most plasma parameters measured in this study that have already been reported by others.[2] Continuous variables are given as mean and standard deviation (SD) for normally distributed data and as median and interquartile range for non-parametrically divided data. Differences between groups were tested with the Student's t-test in case of the former and with the Mann-Whitney U-test in case of the latter. For the entire group of subjects Pearson's correlation coefficient was calculated to assess the correlation between two continuous variables. Multiple linear regression analysis was applied to determine the variables affecting circulating renin, aldosterone and endothelin-1. Non-parametrically distributed data were log-transformed before correlation or regression analysis was performed.

All statistical analyses were calculated with IBM SPSS Statistics 21 (IBM Corporation). Concentration-response curves were analyzed with Prism 5.0 for Windows (GraphPad software inc.) to obtain the maximum effect (Emax) and pEC50 (-10logEC50). Two way ANOVA was used for comparison, followed by a Bonferroni post-hoc evaluation to correct for multiple testing.

RESULTS

Rat studies

An 8-day treatment with the VEGF inhibitor sunitinib dose-dependently increased plasma ET-1 levels in Wistar Kyoto rats (Figure 1A).

Human studies

Population characteristics

Of the 103 pregnant women included, 65 had a sFlt-1/PlGF ratio <85 (negative test) and 38 had a ratio ≥85 (positive test). There were no differences in gestational age at inclusion. Patients with a positive test had a higher blood pressure and delivered on average 8 weeks earlier than patients with a negative test. As expected, treatment with methyldopa, calcium antagonists and magnesium sulfate predominated in the group with the positive test (Table 1). Plasma ET-1 and creatinine levels were increased in patients with a positive test, whereas PRA, PRC, and plasma angiotensinogen, aldosterone and albumin were decreased in these patients (Table 1). Plasma ET-1 correlated positively with plasma sFlt-1 (Figure 1B). Plasma prorenin, the aldosterone/PRA ratio, and the PRA-aldosterone relationship were identical in both groups (Table 1 and Figure 2A).

Urinary protein, albumin, angiotensinogen, renin and prorenin were increased in patients with a positive test, whereas aldosterone was decreased in the urine of such patients (Table 2). Urinary Na^+ levels were identical in both groups, also after correction for K^+. For the analysis of prorenin differences, samples that yielded levels below the detection limit (11 [31.4%] in the women with a positive test, 33 [54.1 %] in women with a negative test) were excluded. There was no difference in urinary creatinine levels between the two groups, and outcomes were identical with and without correction for creatinine. Angiotensinogen measurements by EKA and ELISA were significantly correlated (Figure 2B; $r=0.33$, $P<0.001$), although levels determined with the ELISA were consistently 2-3-fold lower. This was most likely due to the fact that the ELISA standard, when determined twice in our EKA (applying Ang I as standard), yielded ≈2-fold higher levels than predicted (0.37 vs. 0.15 pmol/mL and 1.03 vs. 0.62 pmol/mL).

Determinants of plasma aldosterone, PRC, plasma ET-1, mean arterial pressure (MAP) and urinary protein/creatinine ratio (uPCR)

Aldosterone. Plasma sFlt-1, MAP, uPCR, and plasma ET-1 correlated negatively with plasma aldosterone, while a positive correlation was observed with plasma PlGF, gestational age at measurement, plasma angiotensinogen, PRC and PRA (Table S1). Next, parameters

Characteristic/Biochemical parameters	sFlt-1/PlGF ratio <85	sFlt-1/PlGF ratio ≥85	P-value
Number	65	38	
SBP, mm Hg	119 ± 14	144 ± 13	<0.001
DBP, mm Hg	72 ± 12	88 ± 9	<0.001
MAP, mm Hg	87 ± 12	106 ± 9	<0.001
G.A. inclusion, weeks + days	29+2 (26+4 – 34+5)	29+0 (25+3 – 31+4)	0.221
G.A. birth, weeks + days	38+2 (37+1 – 39+1)	30+6 (27+2 – 32+0)	<0.001
sFlt-1, pg/mL	1912 (1275 – 3143)	15044 (10151 – 22749)	<0.001
PlGF, pg/mL	369 (247 – 613)	31.6 (14.9 – 55.5)	<0.001
sFlt-1/PlGF ratio	4.75 (2.2 – 11.5)	565 (203 – 919)	<0.001
Creatinine, µmol/L	51 (44 – 58)	56 (51 – 66)	0.01
Albumin, g/L	36 (35 – 38)	31 (28 – 34)	<0.001
Renin, ng Ang I/mL.hr	17.9 (13.0 – 24.7)	8.3 (5.8 – 11.2)	<0.001
Prorenin, ng Ang I/mL.hr	172 (141 – 228)	198 (151 – 249)	0.224
Angiotensinogen, pmol/mL	6163 (5364 – 7008)	4433 (3719 – 5204)	<0.001
PRA, pmol Ang I /mL.hr	4.9 (3.7 – 7.2)	1.6 (1.4 – 2.3)	<0.001
Aldosterone, pg/mL	407 (290 – 822)	185 (121 – 378)	<0.001
Aldosterone/PRA ratio	90.3 (59.2 – 136.7)	103.1 (56.7 – 175.8)	0.414
Endothelin-1, pg/mL	0.69 (0.61 – 0.85)	1.88 (1.19 – 2.49)	<0.001
Methyldopa	9 (13.8%)	29 (76.3%)	<0.001
Calcium channel antagonists	1 (1.5%)	16 (42.1%)	<0.001
Magnesium sulfate	0	7 (18.4%)	<0.001

Table 1.

Characteristics and biochmical parameters in plasma of women with a sFlt-1/PlGF ratio <85 and patients with a ratio ≥85.

SBP, DBP, systolic, diastolic blood pressure; G.A., gestational age; sFlt-1, soluble Fms-like tyrosine kinase-1; PlGF, placental growth factor; PRA, plasma renin activity.

displaying a significant correlation with plasma aldosterone were added into a multiple linear regression model (using PRA as a representative of both PRC and angiotensinogen, and deleting uPCR in view of its strong relationship with MAP). Under such conditions, only gestational age at measurement and PRA remained significant determinants (Table S2). Replacing PRA in the model by PRC and plasma angiotensinogen revealed that the association with PRA was due to PRC (P=0.006) and not plasma angiotensinogen (P=0.649) (data not shown).

Biochemical parameter	sFlt-1/PlGF <85	sFlt-1/PlGF ≥85	P-value
Creatinine, µmol/L	7.7 (5.2-10.9)	6.9 (4.2 – 11.9)	0.615
PCR, mg/mmol	10.0 (8.0-13.6)	110 (35.6 – 247)	<0.001
ACR, mg/mmol	0.58 (0.28 – 1.11)	78.6 (10.3 – 198)	<0.001
Angiotensinogen (EKA) / creatinine, pmol/mmol	0.45 (0.30 - 0.83)	0.93 (0.40 – 1.64)	0.003
Angiotensinogen (ELISA) / creatinine, pmol/mmol	0.08 (0.04 - 0.14)	0.25 (0.14 – 0.53)	<0.001
Renin/creatinine, ng Ang I/ mL.hr/mmol	0.10 (0.04 – 0.17)	0.20 (0.08 - 0.5)	0.002
Prorenin/creatinine, ng Ang I/mL.hr/mmol	0.01 (0.01 – 0.04)	0.05 (0.01 – 0.23)	<0.001
Aldosterone/ creatinine, pg/mmol	8522 (4163 – 13151)	3012 (1605 – 4596)	0.001
Sodium/creatinine, mmol/mmol	11.4 (8.5 – 15.6)	9.6 (3.6 – 15.4)	0.054
Potassium/creatinine, mmol/mmol	7.7 (5.3 – 10.9)	4.4 (3.6 – 6.4)	<0.001
Sodium/Potassium ratio, mmol/mmol	1.4 (1.0 - 2.5)	1.7 (0.9 - 2.6)	0.76

Table 2.

Biochemical measurements in urine of women with a sFlt-1/PlGF ratio <85 and patients with a ratio ≥85. PCR, protein/creatinine ratio; ACR, albumin/creatinine ratio; EKA, enzyme-kinetic assay.

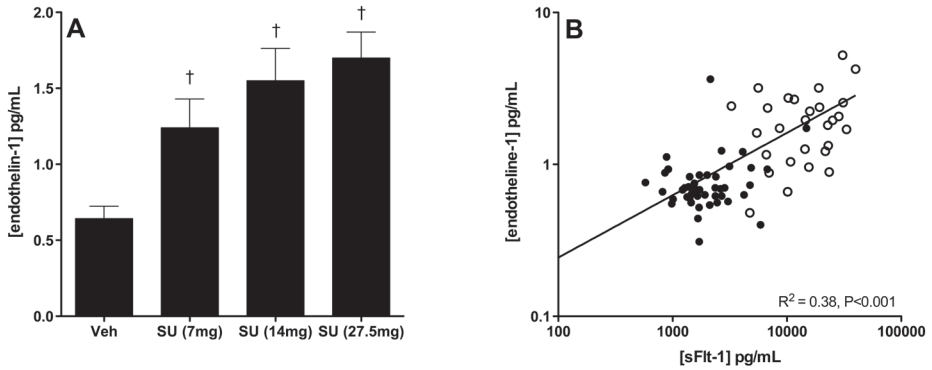

Figure 1.

Plasma endothelin-1 levels in rats treated with different doses of the VEGF inhibitor sunitinib (SU: 7, 14 and 26.7 mg/kg/day) or vehicle (Veh) (panel A), and versus sFlt-1 in pregnant women (panel B), subdivided according to sFlt-1/PlGF ratio (≥ 85, open circles; <85, closed circles).

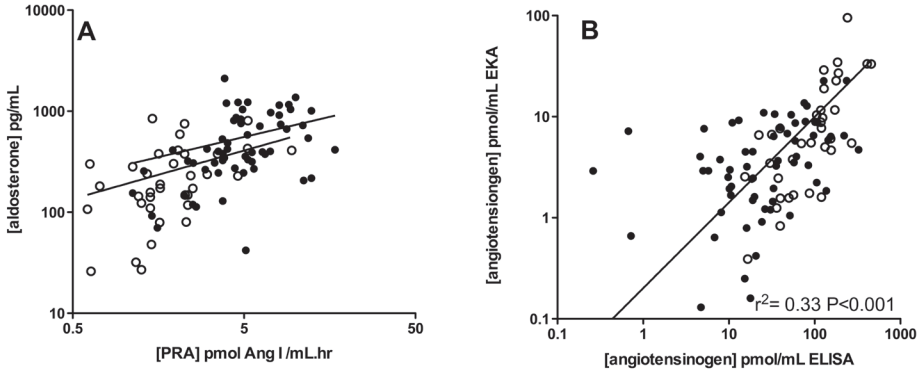

Figure 2.

A, PRA versus aldosterone in pregnant women, subdivided according to sFlt-1/PlGF ratio (≥ 85, open circles; <85, closed circles). The relationship was identical in both groups. B, angiotensinogen measurement by ELISA versus that by enzyme-kinetic assay (EKA) in urine.

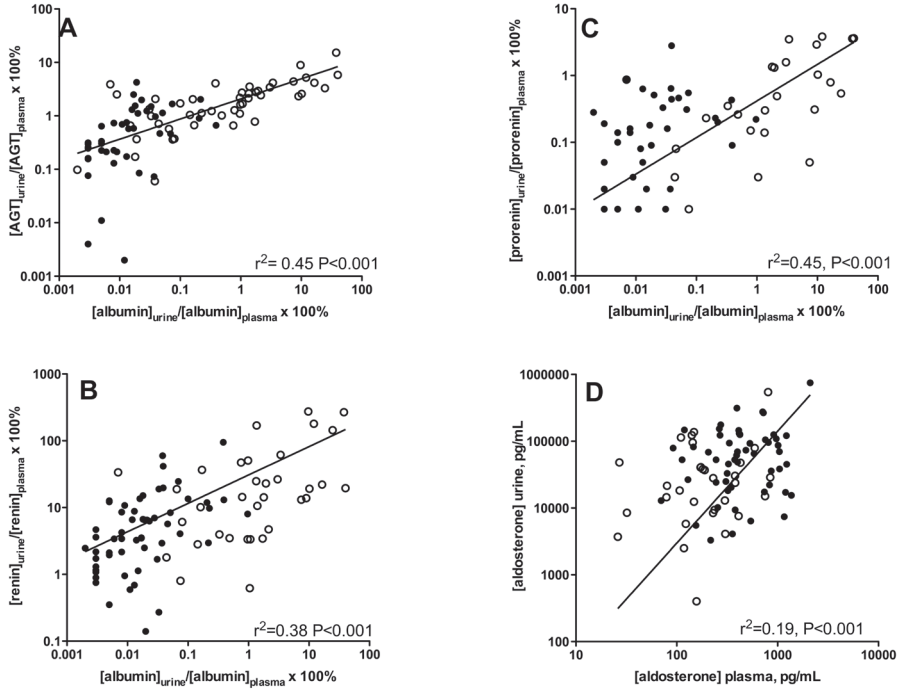

Figure 3.

Correlations between the urine/plasma concentration ratio of albumin and that of angiotensinogen (Aogen, panel A), PRC (renin, panel B), and prorenin (panel C) in pregnant women, subdivided according to sFlt-1/PlGF ratio (≥ 85, open circles; <85, closed circles). Panel D compares plasma and urinary aldosterone levels.

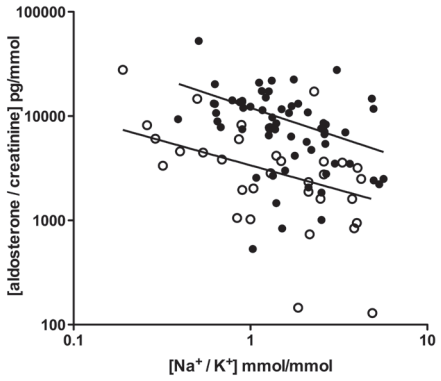

Figure 4.
Urinary Na^+ ratio versus urinary aldosterone in pregnant women, subdivided according to sFlt-1/PlGF ratio (≥ 85, open circles; <85, closed circles).

PRC. PRC correlated positively with PRA, plasma PlGF, plasma angiotensinogen and plasma aldosterone, and negatively with plasma sFlt-1, MAP, uPCR, and plasma ET-1 (Table S1). There was no relationship with gestational age at measurement. When incorporating all significant parameters into a multiple regression model (excluding uPCR like above), only **MAP** and plasma ET-1 remained significantly correlated with PRC (Table S3). Aldosterone was kept out of this analysis because of its well-known strong positive correlation with renin, mediated by renin-induced angiotensin generation.

ET-1. Plasma ET-1 correlated positively with plasma sFlt-1, creatinine, MAP and uPCR, and negatively with plasma PlGF, PRC, PRA, plasma angiotensinogen and plasma aldosterone (Table S1). There was no relationship with gestational age at measurement. Adding all independent significant parameters in a multiple linear regression model, revealed that only the relationships with sFlt-1 and PRC remained significant (Table S4).

MAP and uPCR. MAP and uPCR correlated highly significantly with each other, and displayed identical positive correlations with plasma sFlt-1 and plasma ET-1, and negative correlations with PRA, plasma angiotensinogen and plasma PlGF. Additionally, uPCR correlated positively with plasma creatinine, and negatively with PRC and plasma aldosterone. Multiple linear regression analysis revealed that only plasma PlGF and uPCR correlated independently with MAP (Table S5), while plasma PlGF, plasma ET-1 and MAP determined 69% of uPCR variation (Table S5).

Origin of urinary RAAS components

At first sight, the opposite changes in urinary renin and angiotensinogen in urine versus plasma in the 2 groups (see Tables 1 and 2) appear to indicate a differential regulation of the circulating and renal RAAS. However, these data should be interpreted in view of the increased albumin/protein levels in the urine of patients with a high ratio.

Figures 3A-3C therefore compare the plasma/urine ratio of albumin with the ratios of renin, prorenin and angiotensinogen. The highly significant positive relationships with the albumin ratio for the 3 proteins, which all have a molecular mass that is comparable to that of albumin, strongly suggest that their elevated urinary levels simply reflect the same phenomenon that underlies the elevated urinary albumin/protein levels: increased filtration. To establish the relationship for prorenin, we excluded urinary samples that yielded levels below the detection limit (n=44).

Urinary aldosterone changes paralleled plasma aldosterone changes, and both parameters were highly correlated (Figure 3D). Here we did not correct for albumin, given the much lower molecular weight of aldosterone. Urinary aldosterone levels for a given level of urinary sodium (corrected for potassium, a measure for the aldosterone effect on the collecting duct) were lower for women with a positive test (Figure 4).

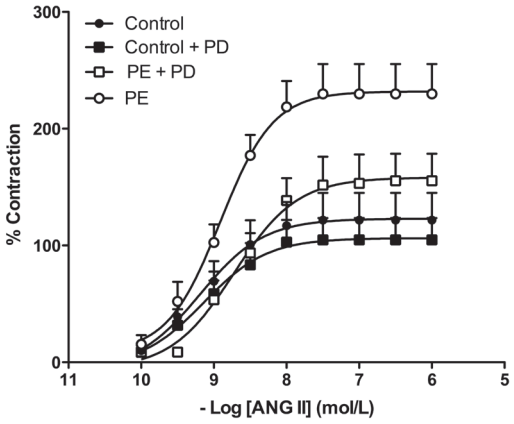

Figure 5.
Angiotensin (ANG) II concentration-response curves in patients with preeclampsia (PE) and healthy pregnant controls with and without the AT_2 receptor antagonist PD123319.

Microvascular function

Ang II CRCs were obtained in subcutaneous arteries from 9 patients with PE (age 31±4 years, gestational age 29± 3.5 weeks, MAP 111 ± 7 mm Hg) and 8 healthy controls (age 35±5 years, gestational age 39± 0.5weeks, MAP 81 ± 8 mm Hg; $P<0.05$ vs. PE for all). Maximum constrictor responses to KCl and relaxant effects to substance P were identical in both groups (data not shown). As expected, the Ang II E_{max} was twice as large in PE vessels as compared to healthy vessels (Figure S1), while Ang II potencies in both vessel types were identical (pEC_{50} 8.9 ± 0.15 vs. 9.0 ± 0.13). Irbesartan completely abolished all Ang II responses (data not shown), whereas PD123319 normalized the enhanced response in PE ($P<0.05$), without having an effect in healthy vessels (Figure 5).

Variable	Log [Aldosterone, pg/mL]	Log [Renin, ng Ang I / mL.hr]	Log [Endothelin-1, pg/mL]	Log [uPCR, mg/mmol]	MAP,mm Hg
Log [Aldosterone, pg/mL]	-	0.51*	-0.33*	-0.36*	-0.15
Log [Renin, ng Ang I / mL.hr]	0.51*	-	-0.49*	-0.43*	-0.08
Log [Endothelin-1, pg/mL]	-0.33*	-0.49*	-	0.70*	0.24*
MAP, mm Hg	-0.31*	-0.48*	0.57*	0.62*	-
Log [uPCR, mg/mmol]	-0.36*	-0.43*	0.70*	-	0.62*
Log [Plasma creatinine, µmol/L]	-0.15	-0.08	0.24*	0.28*	0.17
Log [sFlt-1, pg/mL]	-0.27*	-0.35*	0.62*	0.65*	0.60*
Log [PlGF, pg/mL]	0.52*	0.47*	-0.60*	-0.69*	-0.69*
G.A. measurement, weeks	0.36*	0.00	0.07	-0.01	0.16
Log [Angiotensinogen, pmol/mL]	0.38*	0.27*	-0.38*	-0.42*	-0.27*
Log [PRA, pmol Ang I / mL.hr]	0.60*	0.88*	-0.54*	-0.51*	-0.55*

Table S1.

Univariable regression to assess variables affecting the plasma levels of aldosterone, renin and endothelin-1 levels, and the urinary protein/creatinine (uPCR) ratio.

MAP, mean arterial blood pressure; G.A., gestational age; sFlt-1, soluble Fms-like tyrosine kinase-1; PlGF, placental growth factor; PRA, plasma renin activity.

Variable	Coefficient	P-value
Log [sFlt-1, pg/mL]	0.050	0.706
Log [PlGF, pg/mL]	0.288	0.085
G.A. measurement, weeks	0.272	0.009
MAP, mm Hg	0.099	0.479
Log [PRA, pmol Ang I /mL.hr]	0.519	<0.001
Log [Endothelin-1, pg/mL]	-0.030	0.809

R Square 0.558

Table S2.

Multiple linear regression to assess variables affecting plasma aldosterone levels.

MAP, mean arterial blood pressure; G.A., gestational age; sFlt-1, soluble Fms-like tyrosine kinase-1; PlGF, placental growth factor; PRA, plasma renin activity.

Variable	Coefficient	P
Log [sFlt-1, pg/mL]	0.117	0.480
Log [PlGF, pg/mL]	0.039	0.844
G.A. measurement, weeks	0.283	0.498
MAP, mm Hg	-0.335	0.050
Log [Endothelin-1, pg/mL]	-0.306	0.039
Log [Angiotensinogen, pmol/mL]	0.052	0.695

R Square 0.307

Table S3.

Multiple linear regression to assess variables affecting plasma renin levels. MAP, mean arterial blood pressure; G.A., gestational age; sFlt-1, soluble Fms-like tyrosine kinase-1; PlGF, placental growth factor.

Variable	Coefficient	P-value
Log [sFlt-1, pg/mL]	.319	.021
Log [PlGF, pg/mL]	-.061	.731
G.A. measurement, weeks	.109	.307
MAP, mm Hg	.134	.366
Log [Renin, ng Ang I /mL.hr]	-.262	.022
Log [Aldosterone, pg/mL]	-.069	.579
Log [Plasma creatinine, µmol/L]	.211	.056

R Square 0.515

Table S4.

Multiple linear regression to assess variables affecting plasma endothelin-1 levels. MAP, mean arterial blood pressure; G.A., gestational age; sFlt-1, soluble Fms-like tyrosine kinase-1; PlGF, placental growth factor.

	MAP		uPCR	
Variable	**Coefficient**	**P-value**	**Coefficient**	**P-value**
Log [sFlt-1, pg/mL]	.058	.621	.144	.201
Log [PlGF, pg/mL]	-.481	.001	-.301	.032
G.A. measurement, weeks	.107	.207	.077	.350
Log [Endothelin-1, pg/mL]	.014	.904	.350	.001
Log [PRA, pmol Ang I / mL.hr]	-.137	.167	.084	.384
Log [Plasma creatinine, µmol/L]	-.134	.136	-.071	.417
Log [uPCR, mg/mmol]	.294	.024	-	-
MAP	-	-	.273	.024
R Square	0.66		0.69	

Table S5.

Multiple linear regression to asses variables affecting mean arterial blood pressure (MAP) and the urinary protein/creatinine ratio (uPCR).

G.A., gestational age; sFlt-1, soluble Fms-like tyrosine kinase-1; PlGF, placental growth factor.

DISCUSSION

In this study, we have used a cut-off of 85 of the sFlt-1/PlGF ratio to distinguish patients with a high and low anti-angiogenic state, rather than making a subdivision based on the clinical diagnosis of PE. This was done mainly in view of our purpose to investigate the relation between the anti-angiogenic state, the endothelin system and the RAAS. It also minimizes the risk of including patients with pre-existing disease like systemic lupus erythematosus.[16] However, it should be noted here that a re-analysis on the basis of a subdivision according clinical diagnosis yielded identical results (data not shown).

A high anti-angiogenic state, as reflected by elevated sFlt-1 levels, is associated with increased ET-1 levels. Given the observation that ET-1 also rises in cancer patients and rats treated with the VEGF inhibitor sunitinib (Figure 1A),[6] the most logical explanation of this ET-1 rise is that it is the direct consequence of VEGF inhibition, either through VEGF inactivation by sFlt-1 (in PE) or through interference with VEGF signaling (with sunitinib). Elevated ET-1 levels have been reported earlier in PE.[17, 18] Our study now suggests that this ET-1 elevation is a major determinant of both the blood pressure rise and proteinuria in this disorder, as well as the RAAS suppression (Figure 5). Again a parallel may be drawn with the ET-1-mediated side effects in sunitinib-treated patients, i.e., hypertension and proteinuria, which were accompanied by renin suppression.[6] ET receptor blockade prevented these side effects in sunitinib-treated rats,[8] while also in two rat models for PE (reduced uterine perfusion pressure and sFlt-1 injection) ET receptor antagonism completely blocked the hypertensive response.[19, 20]

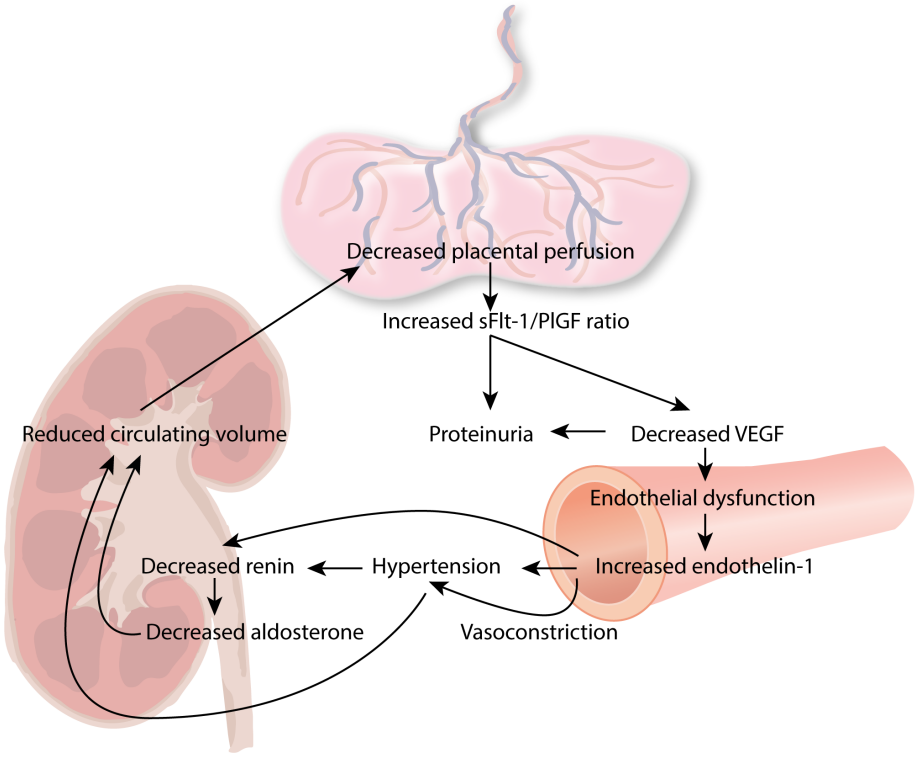

Figure 6.

Unifying model summarizing all findings from this study. Decreased perfusion of the placenta (most probably resulting from impaired widening of the maternal spiral arteries[2]), results in placental hypoxia and soluble Fms-like tyrosine kinase-1 (sFlt-1) release. SFlt-1 binds free placental growth factor (PlGF) and vascular endothelial growth factor (VEGF), thereby inactivating these factors, increasing the sFlt-1/PlGF ratio, and inducing endothelial dysfunction. As a consequence, endothelin-1 production is turned on, which not only induces hypertension and proteinuria but also suppresses renin release. Such suppression will also occur due to the rise in blood pressure. The renin suppression is accompanied by a parallel aldosterone suppression, illustrating that the latter is entirely due to diminished angiotensin generation. Diminished renin-angiotensin-aldosterone system activity combined with high blood pressure results in a reduced circulating volume, thereby further decreasing placental perfusion.

Moreover, ET-1 has been reported to suppress renin release in animal models.[21, 22]
The cause of the RAAS suppression in PE has always been elusive. Given the reduced circulating volume in PE the opposite should have occurred.[2] Apparently, both ET-1 and the rise in blood pressure overrule this response.

Importantly, our data show a similar PRA-aldosterone relationship and aldosterone/PRA ratio in patients with a high and low anti-angiogenic state. This implies that the decrease in aldosterone levels in PE is the simple consequence of reduced Ang I-generating activity. Gennari-Moser et al. have recently reported that VEGF stimulates aldosterone production by enhancing adrenal capillary density.[10] Additionally, they observed that sFlt-1 overexpression in rats reduced aldosterone levels, resulting in an inverse correlation between sFlt-1 and aldosterone. Our data fully confirm this inverse relation in humans, but multiple regression analysis subsequently revealed that it was actually due to the sFlt-1-induced rise in ET-1 which suppressed renin. Moreover, had aldosterone been selectively reduced in PE, this should have resulted in a reduced aldosterone/PRA ratio. No such reduction was observed. In fact, if anything, previous studies reported an elevated aldosterone/PRC ratio in PE versus healthy pregnant women.[4] Here it should be considered that pregnant women display elevated prorenin and angiotensinogen levels.[2] Prorenin may interfere in the immunoreactive renin assay by crossreacting with the antibody that recognizes renin's active site,[23] and the high (up to 4-5-fold) angiotensinogen levels will lead to a higher PRA (and consequently aldosterone) level for a given PRC as compared to nonpregnant women. From this point of view, it is better to compare aldosterone with PRA, since this parameter takes into account the changes in angiotensinogen and is not affected by prorenin crossreactivity. Summarizing, our data do not support a selective, renin-independent downregulation of aldosterone in PE (related to a reduced adrenal capillary density), but rather an ET-1-mediated overall RAAS suppression (Figure 6).

Buhl et al. found that urine of patients with PE contains high levels of plasmin, which will activate collecting duct ENaC current,[11] and might thus further suppress the RAAS. Since plasma sodium is kept within narrow ranges and urinary sodium is largely diet dependent, it is difficult to obtain direct evidence for this theory in an observational study. However, in line with the theory that sodium could be retained by activation of ENaC, we observed that urinary sodium levels for a given level of aldosterone were lower in patients with a high anti-angiogenic state as compared to healthy pregnant women.

Since RAAS components in urine are believed to reflect renal RAAS activation,[24, 25] and given the severe renal pathology in PE, we quantified urinary renin, prorenin, angiotensinogen and aldosterone to investigate their biomarker value. In line with previous studies, urinary prorenin levels were often below detection limit.[26, 27] When considering only the urinary prorenin levels that were detectable, it could be calculated that urinary prorenin levels were 0.21 (0.12 – 0.45) and 0.51 (0.19 – 1.47) % (P = 0.007 for difference) of plasma prorenin in women with a low and high anti-angiogenic state, respectively (Figure 3). In reality, when also including the samples with undetectable prorenin levels, these percentages would have been even lower, and possible as low as those observed for angiotensinogen (0.07 (0.03 – 0.11) and 0.16 (0.05 – 0.34) % of plasma angiotensinogen; P = 0.001).

In contrast, urinary renin levels, relative to PRC, were up to 90-fold higher (3.47 (1.15 – 8.79) and 14.4 (3.95 -36.5) % of PRC; P<0.001). Because of the comparable molecular weights of all 3 proteins, at first sight these data seem to indicate selective renal renin release into urine, particularly in women with a high anti-angiogenic state. However, when comparing the urine/plasma ratios of all 3 proteins with the ratio of a protein of comparable weight, albumin (displaying urinary levels that are 0.014 (0.005-0.033) and 1.35 (0.25 - 5.40) % of plasma albumin in women with a low and high anti-angiogenic state, respectively;
P< 0.007), strong correlations were found in all cases. In other words, renin, prorenin and angiotensinogen enter urine, like albumin, via glomerular filtration, and their elevated levels in women with a high anti-angiogenic state are the simple consequence of increased filtration. This leaves the question why urinary renin (relative to its plasma levels) is so much higher than urinary angiotensinogen or prorenin. Urinary renin levels are too low to significantly affect urinary angiotensinogen, and thus ex-vivo Ang I generation cannot explain the low urinary angiotensinogen levels. Clearly therefore, either glomerular filtration of prorenin and angiotensinogen is greatly reduced as compared to renin, and/or renin reabsorption in the proximal tubulus is much less efficient. Since Nielsen et al.found that, after blocking tubular reabsorption with lysine, the urinary clearance of prorenin was still ≈10-fold lower than that of renin, the former explanation seems the most likely. [28] Finally, urinary aldosterone fully paralleled plasma aldosterone, in line with the concept that urinary aldosterone, like plasma aldosterone, is adrenal-derived. Thus, except for the fact that urinary aldosterone is several orders of magnitude higher than plasma aldosterone (making it easier to detect), urinary aldosterone offers no additive biomarker value for PE, nor do urinary angiotensinogen, renin and prorenin.
Women with PE are known to display greater Ang II responses than normal pregnant women. Theoretically, such enhanced responses should result in higher aldosterone levels and a reduced renin release, via AT_1 receptors in the adrenal and kidney, respectively. Collectively, this would lead to an higher aldosterone/PRA ratio. Yet, we did not observe an altered ratio, arguing against the concept of enhanced AT_1 receptor stimulation in PE. Our data in arteries from patients with PE confirm the enhanced response, but show that it is due to constrictor AT_2 receptors. Normally, such receptors induce vasodilation, but their phenotype often changes under pathological conditions, like hypertension and aging.[29, 30] The mechanism behind this change from dilator to constrictor is unknown, but may involve a disturbed endothelial function and reduced NO availability, heterodimerisation with other receptors (including the AT_1 receptor) and/or a different location of the AT_2 receptor (vascular smooth muscle cells versus endothelial cells).[31] If the enhanced response is indeed due to AT_2 receptors, this explains why there is no change in the aldosterone/PRA ratio.

PERSPECTIVE

Our data shed new light on the counterintuitive RAAS suppression in PE, and suggest that ET-1 has a key role not only in the pathogenesis of this disorder (contributing both to the high blood pressure and proteinuria), but also as a renin suppressor. The reduced urinary sodium/aldosterone relationship in women with a high anti-angiogenic state support a second reason for RAAS suppression: the occurrence of plasmin in urine, which selectively activates ENaC.[11] We did not find evidence for specifically reduced aldosterone levels in patients with an anti-angiogenic state. Urinary RAAS components, when corrected for albumin, fully reflected the alterations in the circulating RAAS in PE, and thus offered no additive value as biomarkers. Unfortunately, despite the central role for ET-1 in PE, ET receptor antagonism currently is no treatment option in human pregnancy because of its teratogenic effects.[32] In order to develop treatment modalities for PE beyond ET receptor blockade, future studies should unravel why elevated sFlt-1 levels in PE, like VEGF inhibition in cancer patients, increase ET-1 levels. Such studies might involve animal models of PE utilizing VEGF antagonism, and should take into consideration that ET-1 has been reported to reciprocally affect the expression of VEGF and its receptors.[33, 34]

DISCLOSURES

None.

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Chapter 12
Summary, discussion and future perspectives
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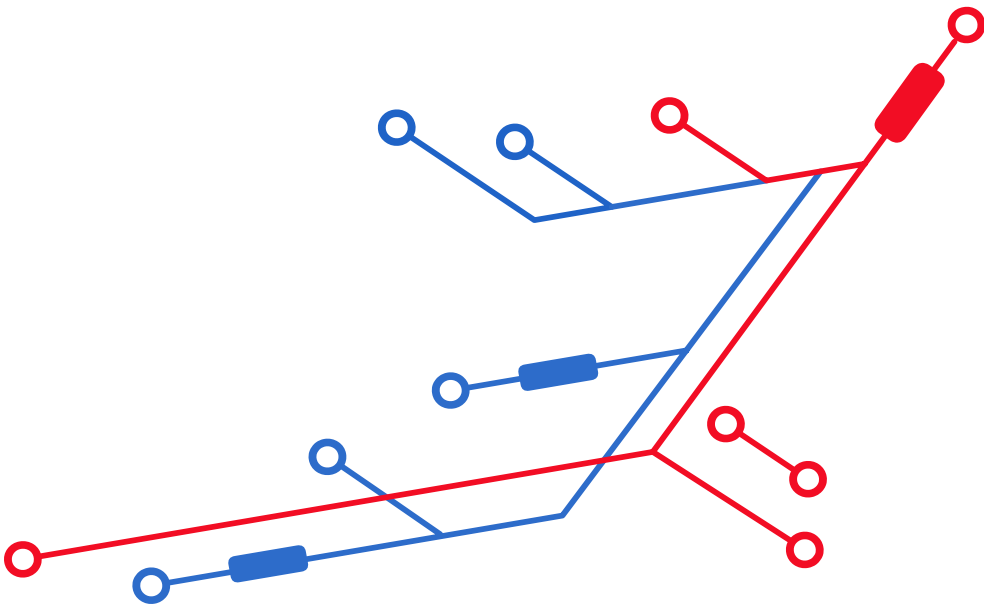

Chapter 12

Summary, discussion and future perspectives

THE AT2-RECEPTOR (CHAPTER 2,3)

The renin-angiotensin-aldosterone system (RAAS) plays an essential role in the regulation of blood pressure and body fluid homeostasis, but also contributes importantly to the pathophysiology of hypertension, renal disease and heart failure. Clinically, the RAAS is of great interest as inhibition at different levels has been proven to be an effective therapy for hypertension, cardiovascular and renal disease.

Angiotensin II mediates its effects via angiotensin II type 1 (AT1) and angiotensin II type 2 (AT2) receptors. AT1-receptors are widely expressed throughout the body and mediate the well-known effects of angiotensin II, including vasoconstriction, sympathetic nervous system activation and sodium and water retention. While experimental studies show that stimulation of the AT2-receptor counteracts these effects by inducing vasorelaxation and natriuresis in healthy animals, our knowledge about AT2-receptor function in humans is limited to a few studies, showing, at most, modest vasodilatory effects upon AT2-receptor stimulation.[1] In contrast to the beneficial effects, recent studies suggest that the AT2-receptor function might become deleterious (e.g., becoming pro-hypertensive and pro-hypertrophic) in the diseased state.[2, 3] In our study in women with preeclampsia we found a greater angiotensin II vasoconstrictor response in isolated small arteries than in the arteries of normal pregnant women, which was due to constrictor AT2-receptors. The mechanism behind this change from dilator to constrictor function is unknown, but may involve a reduced NO availability due to endothelium dysfunction, heterodimerisation with other vasoconstrictor receptors (including the AT1-receptor) and/or a different location of the AT2-receptor (vascular smooth muscle cells versus endothelial cells).[2, 4]

In the past decade C21, a selective AT2-receptor agonist, has been developed that could help to clarify these issues. However, despite overwhelming data supporting AT2-receptor-mediated vasodilation, for instance in the preparations investigated in Chapter 3, we were unable to demonstrate AT2-receptor agonistic vasodilation in response to the C21 in rat, mouse, and human vessels. However, we found support for C21-induced vasorelaxation, but in an AT receptor-independent manner, possibly by preventing calcium influx into the cell. Furthermore, we found that C21 is capable of activating AT1-receptors, resulting in vasoconstriction. This combination of vasorelaxant and vasoconstrictor effects of C21 can provide an explanation for both the hypertensive and hypotensive effects of C21 demonstrated in *in vivo* models.[5, 6]

URINARY RAAS (CHAPTER 4, 5, 11)

In chapter 4 we summarize the currently known literature about RAAS components measured in urine. Indeed, recent interest has focused on urinary renin and especially angiotensinogen as markers of renin-angiotensin system activity within the kidney.
In humans, urinary angiotensinogen correlates closely with urinary albumin, and when correcting for circulating angiotensinogen, evidence is lacking that there are conditions where urinary angiotensinogen behaves differently from urinary albumin. Therefore, urinary angiotensinogen, like albumin, should be considered as a marker of renal disease, and the measurement of urinary angiotensinogen does not offer additional information about the renal RAAS.
Urinary renin potentially has three sources: filtration from plasma, release from the collecting duct, and intrarenal conversion of (plasma-derived) prorenin to renin. Normally, filtered renin is highly efficiently reabsorbed, and the same applies to filtered prorenin. Thus, most urinary renin would then be derived from the collecting duct. Only when greatly elevating circulating renin (e.g., by infusing exogenous renin or by blocking the RAS highly efficiently), tubular reabsorption falls short, allowing plasma renin to accumulate in urine.[7] The same will occur when plasma prorenin levels are elevated, provided that filtered prorenin is converted to renin in the urinary system.[7]
Pregnancy and in particular preeclampsia are well-suited conditions to study whether the renal RAAS can be reflected by its components in urine, given the severe renal pathology in preeclampsia, and the upregulation of the RAAS in pregnancy. To investigate the value of urinary RAAS components as biomarkers, we quantified urinary renin, prorenin, angiotensinogen and aldosterone and compared them to albumin. We found prorenin, renin and angiotensinogen to be increased in urine of preeclamptic women. However, when comparing the urine/plasma ratios of these proteins with the urine/plasma ratio of albumin, we found strong correlations for all proteins. In other words, renin, prorenin and angiotensinogen enter urine, like albumin, via glomerular filtration, and their elevated levels in women with preeclampsia are the simple consequence of increased filtration. In contrast to the other RAAS components, we found urinary aldosterone to be perfectly correlated to its plasma levels, most likely related to its much smaller size. Moreover, we found a reduced urinary sodium/aldosterone relationship in women with a high anti-angiogenic state. We hypothesized in chapter 11 that this might be caused by the occurrence of plasmin in urine of preeclamptic patients, which selectively activates sodium channels, resulting in sodium and water retention and in turn suppression of the RAAS.

DIAGNOSIS OF PREECLAMPSIA (CHAPTER 7, 8, 9, 10)

The clinical diagnosis of PE is defined as de novo hypertension and proteinuria at or after 20 weeks of pregnancy.

Although preeclampsia is a clearly defined disease, it should be taken into account that both measurements are subject to great variation during the day and therefore are not ideal as a diagnostic tool. For the measurement of proteinuria, 24-hour urine collection is still the gold standard, but this method is time-consuming and prone to errors. The protein- to-creatinine ratio (PCR) is determined in a single voided urine and can be used as an alternative for 24-hour urine collections. We found that the PCR measured in spontaneous voids varies throughout the day (individual variation coefficient of +/- 36%), which appears to be independent of the amount of proteinuria. Nevertheless, the PCR appears to be an acceptable alternative, especially when time is a factor to distinguish gestational hypertension from preeclampsia. An imbalance between antiangiogenic and proangiogenic factors has been recognized as an important pathogenetic mechanism in the development of preeclampsia. Measurement of antiangiogenic (soluble Fms-like tyrosine kinase 1, sFlt-1) and proangiogenic (placenta growth factor, PlGF) proteins in the maternal circulation has come of age as a tool to diagnose preeclampsia or to provide prognostic information of this condition.[8] Compared with normal pregnancies, the serum concentration of sFlt- is markedly increased, whereas that of PlGF is decreased in preeclampsia. The ratio of sFlt-1/PlGF appears to be a superior marker of (early onset) preeclampsia compared with the individual values of these markers.[9] In a cohort of pregnant women with clinical preeclampsia or at high risk of preeclampsia we found that a sFlt-1/PlGF ratio ≥85 is associated with a higher odds for adverse pregnancy outcome than the clinical diagnosis of preeclampsia. However, we found limited additional value for diagnosing purposes, since most patients with a positive test do have the clinical diagnosis of preeclampsia. Yet, particularly in pregnant women with preexisting hypertension and/or proteinuria, measurement of the ratio may be a valuable aid for making the correct diagnosis. Finally, due to its strong correlation with clinical parameters, we found the sFlt-1/PlGF ratio to be useful for research purposes as we have used this ratio to discriminate between preeclamptic pregnancies and other hypertensive and proteinuric diseases in pregnancy in chapter 11. A promise for the future is that normalization of the angiogenic imbalance can be achieved either by administration of proangiogenic factors or removal of antiangiogenic factors.

ANGIOGENIC FACTORS, RAAS AND PREECLAMPSIA (CHAPTER 5, 6, 11)

A reduced placental perfusion plays a key role in the initial phase of preeclampsia. Once the disease becomes apparent the maternal vascular system is relatively underfilled, and there is systemic endothelial dysfunction, causing high vascular resistance and a rise in blood pressure. In combination with an increase in vascular permeability and a decrease in oncotic pressure (because of reduced plasma levels of albumin), circulating volume is reduced, thereby impairing placental perfusion. In turn, the placenta will produce more vasoactive and antiangiogenic factors such as sFlt-1, AT1-receptor auto-antibodies and reactive oxygen species, causing further vasoconstriction, resulting in a vicious cycle.

The RAAS can interfere in two ways with this vicious circle, by sodium and water retention and an increase in vascular resistance. The first part might be beneficial, since an increase in volume in view of maintaining tissue perfusion is crucial. The second part might be deleterious since the increase of vascular resistance is part of this vicious circle.
In normal pregnancy, the RAAS supports the increase in volume by increasing the production of aldosterone, while simultaneously developing a relative resistance to the pressor response to angiotensin II. In preeclampsia, renin activity and aldosterone are suppressed and the vasodilator function mediated by AT2-receptors is altered in a constrictor function as we showed in subcutaneous arteries obtained from preeclamptic women. It appears therefore that in preeclampsia the RAAS has lost its delicate balance that in normal pregnancy contributes to volume increase and is associated with a diminished angiotensin II-mediated vasoconstrictor response.
As opposed to the reduced plasma renin activity and aldosterone levels, endothelin- 1 (ET-1) levels are increased in preeclamptic women.[10] This rise in ET-1 levels is strongly correlated with the sFlt-1/PlGF ratio.

PERSPECTIVE

Stimulation of the AT2-receptor remains a promising treatment target, given its upregulation under pathological conditions, allowing effects beyond blood pressure-lowering, for example, in stroke and aneurysm formation. Based on our observations in preeclampsia, one has to keep in mind that activation of this receptor can also cause vasoconstriction instead of vasodilatation. Although an important role for endothelial dysfunction seems to be a logical explanation for the observed negative effects of this receptor, fully satisfactory proof for this theory is still lacking. Others have suggested that heterodimerization with the AT1-receptor could cause the negative effects upon AT2-receptor stimulation.
At this stage, when interpreting effects of the AT2-receptor agonist C21, proof is needed that these effects are truly AT2-receptor mediated by using either selective AT2-receptor antagonists or by studying the effects of C21 in AT2-receptor knockout mice.
The sFlt-1/PlGF ratio has been introduced in the past decade as an aid to diagnose preeclampsia and as an instrument to provide prognostic information with regard to pregnancy continuation.[8] We have shown additional benefit of this test in a selected cohort of pregnant women with preexisting hypertension and proteinuria, but further prospective studies are needed to establish its clinical value in a less complicated patient population.
Angiogenesis inhibition either via high levels of sFlt-1, as occurs in preeclampsia, or induced by antiangiogenic drugs in patients with cancer, causes hypertension, proteinuria and renin suppression.[11] We found that ET-1 is likely a mediator of these effects, but the reason for the increase in ET-1 levels under these conditions is still unclear and needs clarification. Recently a direct inhibitory effect of endothelin on renin production was shown, via stimulation of endothelin receptor on the juxtaglomerular cells.[12]

It would be interesting to see whether intervention of the endothelin system in patients with early severe preeclampsia with the ultimate aim to avoid an imminent pregnancy termination would be a therapeutic option. A caveat is that direct blockade of the ET-1 receptor might be teratogenic.[13]

Besides effects on the ET-1 system, it has been reported that a disturbed angiogenic balance also influences different aspects of sodium handling. VEGF determines capillary density in the adrenal glands, thereby potentially affecting aldosterone production, and has shown to contribute to the development of skin lymphatic capillaries.[14] Normally, the skin buffers Na^+, and lymphatic capillaries are responsible for the subsequent removal of Na^+. Recent studies suggest that without a fully developed lymphcapillary network a high-salt diet contributes to the development of hypertension.[14]

Furthermore, disturbed VEGF signaling affects the glomerular microvasculature leading to proteinuria, but also to the appearance of plasmin in (pre-)urine. Plasmin in turn activates the renal tubular epithelial sodium transporter, causing sodium retention and thereby an increase in blood pressure.[15] Because of the reduced circulating volume and the suppressed RAAS, some researchers suggest that restoring circulating volume by sodium suppletion could be protective against the development of preeclampsia.[16] Our finding that suppression of the RAAS seems to be related to the increased ET-1 levels (and maybe also because of activated renal tubular epithelial sodium transporters), suppletion of sodium seems to intervene in an illogical manner in the pathophysiology of the disease, and might even have adverse effects. More logical treatment options might be restoring angiogenic balance by removing sFlt-1 or the addition of pro-angiogenic factors, blockade of the ET-1 receptor (preferably by drugs not crossing the placenta) or the blockade of the renal tubular epithelial sodium transporter.[17] Since preeclampsia is a disease only present in humans, animal models have several limitations and do not well represent the human situation. The clinical similarities found in preeclamptic women and in patients treated with VEGF inhibitors point to a common pathophysiological mechanism and may enable to develop relevant experimental models that will help to explore new treatment options.

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Nederlandse samenvatting

DE ANGIOTENSINE TYPE 2 RECEPTOR (HOOFDSTUKKEN 2 EN 3)

Het renine-angiotensine-aldosteron systeem (RAAS) speelt een belangrijke rol bij de regulatie van de bloeddruk en het ontstaan en onderhouden van orgaanschade aan hart en nieren ten gevolge van een hoge bloeddruk. Medicamenteuze blokkade van het RAAS is daarom zeer succesvol gebleken in de behandeling van hypertensie, nierziekten en hart- en vaatziekten.

Een groot deel van de RAAS activiteit gaat via angiotensine II stimulatie van de angiotensine type 1 (AT1) en de type 2 (AT2)-receptor. De AT1-receptor komt overal in het lichaam voor en zorgt onder andere voor vasoconstrictie, activatie van het sympathisch zenuwstelsel, retentie van water en zout, hypertrofie en inflammatie. De AT2-receptor kan gedeeltelijk de effecten van de AT1-receptor antagoneren, hetgeen voordelen kan hebben onder pathofysiologische omstandigheden. Echter bij mensen is enkel een antagonerend effect op de vasoconstrictie waargenomen. Bovendien zijn er ook studies die rapporteren dat stimulatie van de AT2-receptor onder bepaalde omstandigheden leidt tot toegenomen constrictie van de vaten en hypertrofie van het hart, bij hypertensieve en ook bij oudere ratten.

Opvallend is dat wij in arteriën geïsoleerd uit buikvet van vrouwen met pre-eclampsie een toegenomen constrictie van de arteriën zien bij het toedienen van angiotensine II (hoofdstuk 10). Door gebruik te maken van een selectieve blokker voor de AT2-receptor (PD12339) blijkt deze toegenomen constrictie voor een groot deel toe te schrijven te zijn aan de AT2-receptor. Echter de manier waarop de AT2-receptor van een vasodilaterende naar een vasoconstrictieve functie wisselt is niet geheel duidelijk geworden. Mogelijke verklaringen kunnen zijn dat, de voor pre-eclampsie kenmerkende endotheel dysfunctie, essentieel is voor een goede AT2-receptor functie, of dat heterodimerisatie van de AT2-receptor met andere receptoren zorgt voor een veranderde functie.

In de afgelopen jaren is compound 21 (C21) ontwikkeld als een specifieke agonist voor de AT2-receptor. We beschrijven in hoofdstuk 2 de tot nu toe bekende data over de effecten van C21, waaronder anti-inflammatoire en vasodilaterende eigenschappen. Vanzelfsprekend werden deze toegeschreven aan stimulatie van de AT2-receptor. Wij beschrijven echter in hoofdstuk 3 AT2-receptor-onafhankelijke, C21 gemedieerde, vasorelaxatie in de aanwezigheid van een blokker van de AT2-receptor en zelfs in transgene muizen zonder deze receptor. Tevens laten wij zien dat C21 in staat is om de AT1 receptor te stimuleren, wat leidt tot vasoconstrictie. Deze resultaten bieden hiermee een verklaring voor zowel de bloeddrukverhogende als -verlagende effecten die zijn toegedicht aan C21. Studies naar de effecten van C21 op de AT2-receptor dienen daarom altijd een controle groep te hebben waarbij de AT2-recptor geblokkeerd dan wel genetisch verwijderd is.

RAAS COMPONENTEN IN URINE (HOOFDSTUKKEN 4 EN 5)

In hoofdstuk 4 bespreken wij de huidige kennis met betrekking tot RAAS componenten in de urine. De afgelopen jaren heeft dit onderwerp veel aandacht gekregen, met name angiotensinogeen en renine in urine als maat voor de RAAS-activiteit en als voorspeller van nierschade ten gevolge van dit geactiveerde RAAS.
Echter het blijkt dat de uitscheiding van angiotensinogeen, wat een soortgelijke molecuulgrootte heeft als albumine, zeer sterk correleert met de uitscheiding van albumine. Omdat er geen enkele situatie/ziekte bekend is waarbij de mate van uitscheiding van deze twee eiwitten niet van elkaar verschilt is het zeer waarschijnlijk dat angiotensinogeen, net als albumine, een marker is voor nierschade zonder verdere toegevoegde waarde.

Renine gemeten in de urine heeft drie mogelijke bronnen: Filtratie uit plasma, lokale productie in de nier en prorenine uit het plasma dat in de nier/urine wordt omgezet naar renine. Normaal gesproken wordt al het door de nier gefiltreerde renine terug gewonnen, net als prorenine. Echter bij een sterk toegenomen aanbod van renine of prorenine lukt het de nier niet alle renine terug te winnen en wordt het meetbaar in de urine.
In de zwangerschap en met name bij patiënten met pre-eclampsie is er een toegenomen eiwituitscheiding in de urine en tevens een (sterk) verhoogde activiteit van het RAAS. Dit bleken ideale omstandigheden om de uitscheiding van RAAS componenten in de urine te bestuderen. Hiertoe hebben we renine, prorenine, angiotensinogeen en aldosteron gemeten in de urine en vergeleken met albumine. Hierbij bleek dat prorenine, renine en angiotensiongeen verhoogd waren in de urine van vrouwen met pre-eclampsie. Echter wanneer wij naar de urine/plasma ratio's van deze waarden keken en deze vergeleken met die van albumine werd duidelijk dat deze zeer sterk correleerden met elkaar. Dit betekent dat, net als bij albumine, deze onderdelen van het RAAS, passief verloren gaan in de urine door een verminderde barrière functie van de nier bij patiënten met pre-eclampsie. Daarentegen bleek aldosteron in de urine, zoals al eerder beschreven, goed te correleren met zijn eigen plasma waardes. Interessant was dat de verhouding natrium en aldosteron in de urine lager was bij patiënten met pre-eclampsie. Plasmine, ook een eiwit uitgescheiden in de urine, zou hierbij een rol kunnen spelen. Plasmine is namelijk in staat om selectief natrium kanalen in de nier te stimuleren, wat ervoor zorgt dat meer water en zout wordt geresorbeerd. Dit kan op zijn beurt weer resulteren in een verdere onderdrukking van het RAAS zoals wij die zien bij patiënten met pre-eclampsie.

DE DIAGNOSE VAN PRE-ECLAMPSIE (HOOFDSTUKKEN 8,9 EN 10)

De klinische diagnose van pre-eclampsie is gedefinieerd als een hypertensie en proteïnurie in de tweede helft van de zwangerschap. Ondanks deze duidelijke definitie kan het stellen van de diagnose lastig zijn, omdat beide metingen onderhevig zijn aan een forse variatie gedurende de dag. Om proteïnurie vast te stellen, dient de eiwit uitscheiding in de 24-uurs urine gemeten te worden. Deze methode, welke op dit moment de gouden standaard is, kost veel tijd en is foutgevoelig. Als alternatief voor het 24 uur verzamelen van urine kan de eiwit/creatinine ratio bepaald worden in een portie urine. Wij beschrijven in hoofdstuk 7 dat de eiwit/creatinine ratio ook onderhevig is aan een forse variatie gedurende de dag (variatiecoëfficiënt +/- 36%). Desondanks lijkt deze test in veel gevallen toch een geschikte methode om zonder vertraging de diagnose pre-eclampsie te kunnen stellen.
Sinds een jaar of 10 is duidelijk geworden dat een onbalans tussen factoren die vaatnieuwvorming remmen en factoren die dit stimuleren een belangrijke rol spelen in de pathogenese van pre-eclampsie.
Ten opzichte van normale zwangerschappen is de anti-angiogene groeifactor, soluble Fms-like tyrosine kinase 1 (sFtl-1), sterk verhoogd en de pro-angiogene factor, placenta growth factor (PlGF), sterk verlaagd bij patiënten met pre-eclampsie. De hoogste sensitiviteit en specificiteit voor het stellen van de diagnose pre-eclampsie kan worden behaald door de ratio van deze twee factoren te nemen, sFlt-1/PlGF. Het meten van deze ratio kan bovendien mogelijke complicaties van pre-eclampsie voorspellen (hoofdstuk 10).
Bij patiënten met al langer bestaande proteïnurie en hypertensie kan het stellen van de diagnose een uitdaging zijn. We beschrijven in hoofdstuk 9 hoe het gebruik van de sFlt-1/PlGF ratio bij patiënten met systemische lupus erythematosus (een ernstige auto-immuun ziekte, tevens gekenmerkt door hypertensie en proteïnurie) helpt om deze aandoening te onderscheiden van pre-eclampsie. Dit is van belang, omdat, behalve symptoombestrijding, het beëindigen van de zwangerschap de enige behandeling is voor patiënten met pre-eclampsie, terwijl systemisch lupus erythematosus goed behandeld kan worden met hoge doses corticosteroïden.
Of in een normale populatie zwangere vrouwen het kostenefficiënt en veilig is om enkel de ratio te gebruiken voor de diagnose en prognose van pre-eclampsie moet nog blijken uit vervolg studies.

PRO-ANGIOGENE FACTOREN, HET RAAS EN PRE-ECLAMPSIE (HOOFDSTUKKEN 6, 7 EN 11)

Verminderde bloedtoevoer naar de placenta speelt een hoofdrol in de ontstaansfase van pre-eclampsie. De placenta gaat door deze verminderde perfusie tal van factoren produceren, waaronder anti-angiogene factoren, en deze zorgen voor endotheelschade bij de moeder, met als gevolg hypertensie en proteïnurie. Door de toegenomen vasoconstrictie, en de afgenomen oncotische druk door het verlies van eiwit, neemt de perfusie van de

placenta verder af.
De placenta gaat vervolgens weer meer anti-angiogene factoren produceren, hetgeen leidt tot een vicieuze cirkel. Het RAAS kan op twee manieren ingrijpen op deze vicieuze cirkel. Een voordeel kan zijn dat activatie van het RAAS zorgt voor een toename van het circulerend volume door water en zout vast te houden. Een groot nadeel is dat het ook zorgt voor een toename van de vaatweerstand doordat angiotensine II via de AT1-receptor zorgt voor vasoconstrictie. In een normale zwangerschap is het RAAS geactiveerd en zorgt een toegenomen productie van aldosteron voor water en zout retentie, terwijl de vaten relatief ongevoelig worden voor de constrictoire effecten van angiotensine II. Vrouwen met pre-eclampsie hebben echter een verlaagd aldosteron en een toegenomen gevoeligheid voor angiotensine II (hoofdstuk 11). Zoals eerder beschreven, zou het verdwijnen van de vasodilaterende functie van de AT2-receptor hierbij een rol kunnen spelen.
In tegenstelling tot de verminderde plasma spiegels van het RAAS, is endotheline-1 verhoogd bij patiënten met pre-eclampsie. Deze stijging is daarbij sterk gerelateerd aan de hoogte van de sFlt-1/PlGF ratio.

Curriculum Vitae

Koen Verdonk werd geboren op 21 maart 1984 te Groningen. Na het voltooien van het V.W.O. aan het Vincent van Gogh college te Assen in 2003 startte hij met de opleiding Geneeskunde aan de Erasmus Universiteit te Rotterdam. Tijdens de eerste twee jaar van zijn opleiding Geneeskunde was hij wedstrijd roeier bij a.r.s.r Skadi, waar hij zowel in een 4+ als een 8+ geroeid heeft. Hij was verder actief lid bij de Medische Faculteits Vereniging te Rotterdam, waar hij een bestuursfunctie vervulde als Vice-Praeses en Commissaris Onderwijs Interne van 2005 tot 2006. Daarnaast was hij actief betrokken bij de studenten inspraak en was hij verkozen tot lid van de Universiteitsraad Rotterdam waar hij plaats nam in het praesidium. Tijdens zijn wetenschapsstage deed hij onderzoek aan de lange termijn effecten van mineralocorticoïdreceptor blokkade. Na het behalen van zijn doctoraal examen begon hij in 2010 onder leiding van dr. Anton H. van den Meiracker, dr. Willy Visser, prof. dr. Eric A.P. Steegers en prof. dr. A.H. Jan Danser aan zijn wetenschappelijk onderzoek naar het renine-angiotensine-aldosteron systeem bij zwangere vrouwen met pre-eclampsie. Tijdens zijn onderzoek heeft hij een poos plaats genomen in het bestuur van de promovendi vereniging Promeras. Momenteel voltooid hij zijn opleiding Geneeskunde en hoopt hij begin 2016 af te studeren en vervolgens te beginnen aan de opleiding Interne Geneeskunde.

Dankwoord

Met het schrijven van dit dankwoord sluit ik een mooie periode in mijn leven af. Met veel plezier kan ik terugkijken op een zeer fijne en leuke tijd. Het is een voorrecht dat ik dit boekje heb mogen schrijven en ik ben hiervoor veel mensen dankbaar. Om te beginnen met Ton van den Meiracker. Dankzij Ton maakte ik kennis met het doen van onderzoek. Ondanks een donkere eenzame start in de archieven van het Erasmus MC werd ik gegrepen door het wetenschappelijk onderzoek. Je leerde mij met veel geduld dat precisie en consistentie onontbeerlijk zijn voor een mooie publicatie. Daarnaast wist je met een bijzondere taalgevoel elke kromme zin weer recht te maken. We hebben veel leuke en gezellige congressen met elkaar gehad en hopelijk gaan er nog veel volgen.
Met Willy Visser maakte ik pas kennis toen ik was aangenomen voor het promotie traject. Dankzij jouw enorme enthousiasme en onuitputtelijke energie en betrokkenheid werd ik telkens weer gemotiveerd als het even tegenzat. Door jouw enthousiasme was er ook altijd wel wat te onderzoeken. Van bloed samples begraven achterin een vriezer uit de vorige eeuw, tot een compleet manuscript, wat de basis was voor een hoofdstuk uit dit boekje.
Jan Danser wil ik bedanken voor zijn geduld en hulp bij het voltooien van het proefschrift. Hoe laat het ook was en hoeveel dagen na de gestelde deadline ik ook iets opstuurde, je keek het zeer kritisch na, corrigeerde het en stuurde het vaak nog hetzelfde uur weer terug. Deze snelheid en efficiëntie en het feit dat het wel lijkt of je van elk artikel over het RAAS weet waar en wanneer het gepubliceerd is, was onmisbaar voor het schrijven van dit proefschrift.
Eric Steegers wil ik ook graag bedanken. Onze bijeenkomsten waren voor mij een goede stimulans om de voortgang en de resultaten op een rij te zetten en het overzicht te behouden. Daarnaast was je uitgebreide basale en klinische kennis over pre-eclampsie belangrijk voor het ontstaan van dit proefschrift.

Prof.dr. J.A.M. van der Post, prof.dr. S. Sleijfer en prof.dr. R. Zietse wil ik bedanken voor het plaatsnemen in mijn commissie.

Joep, van jou heb ik geleerd wat onderzoeken in de praktijk inhoudt. Het bedenken van een protocol, het doen van vaatstudies en het omgaan met tegenvallende resultaten. Ik vond het super gezellig op alle congressen, en het was erg fijn dat ik af en toe bij je in het wiel mocht rijden door de polders van Rotterdam en omstreken.
Joke en Titia hartelijk dank voor de hulp bij de inclusie van alle patiënten, ondanks dat er naast de PEDRAS en de URAAS nog veel meer studies waren. En niet te vergeten dank voor de dropjes. Edith en Evelien en iedereen van de (voormalig) BD wil ik bedanken voor de samenwerking bij de PEDRAS en URAAS-studie.

Frank, Ingrid, Usha, Richard, Janette, bedankt voor jullie hulp met het vergaren van alle resultaten. Zonder jullie hulp was de standaard deviatie twee maal zo hoog en mijn promotie traject twee keer zo lang geweest.
Nils, Marcel en Frank, jullie hebben me begeleid bij mijn eerste stapjes op het lab. Ondanks al jullie hulp had ik al snel een mooie “wall of shame” bij elkaar gepipetteerd. Het was duidelijk, de westernblot was mij niet op het lijf geschreven. Op het lab van de 14e was er gelukkig labwerk dat geschikter was voor mij, namelijk vaatjes. Dankzij Joep, Els, Richard en Wendy kreeg ik dit snel onder de knie.Het was leuk en gezellig om met jullie dagen op het lab door te brengen.
BD-zuipkeet / Lunch en borrel app: Anton, Arthur, David, Frank, Luit, Luuk, Lodi, Mahdi, Manne, Marcel, Matej, Nils, Stijn, Thijs, dankzij jullie heeft het boekje ruim een jaar (extra) vertraging opgelopen, maar ik had geen bijeenkomst willen missen!
Jorie, Pieter, het was super leuk om met jullie rondjes, voor, tijdens of na het werk te rennen!
Mijn opvolgster Langeza Saleh wil ik alvast bedanken voor de samenwerking en het enthousiasme waarmee je het onderzoek hebt voortgezet.
Verder wil ik mijn kamergenoten, Ilse, Manne, Usha, Rene, Bruno, Sieneke en Kathera bedanken voor hun gezelligheid en hun tolerantie naar mijn onrust. Ook de andere collega’s Birgitte, Hisko, Katie, Kayi, Stephanie en Paula bedankt voor jullie gezelligheid en de frequent gevulde snoepjes pot.
Wesley en Manne bedankt dat jullie paranimfen willen zijn. Manne, speciaal omdat hij helemaal uit Berlijn komt en Wesley voor het extra werk wat hij daardoor heeft moeten doen.
Verder wil ik mijn vrienden bedanken voor het geduld dat ze op wisten te brengen om naar de onderzoekersverhalen te luisteren en met name Thijs, waarmee ik elk nieuw wetenschappelijk idee dan wel dwaling uitgebreid door kon nemen in de koffiebar.
Bob bedankt voor je scherpe blik en correcties in wazige tijden. Simon bedankt voor je creatieve ontwerp van dit boekje.

Tot slot wil ik mijn familie bedanken.
Lieve pap en mam, ik ben een enorme geluksvogel dat jullie mijn ouders zijn. De vanzelfsprekendheid waarmee jullie er altijd voor ons zijn geeft zoveel steun dat ik elke uitdaging, waaronder een promotie, met vertrouwen durf aan te gaan. Erik en Bianca bedankt voor alle gezelligheid tijdens de vakanties en de gezellige weekendjes in ons kuuroord Assen.

Lieve Mouna, bedankt dat je er altijd voor me bent, ook tijdens het schrijven van dit boekje. Je bent met je liefde, stoerheid en doorzettingsvermogen voor mij de belangrijkste bron van energie en motivatie. Ik hou van je en ik heb heel veel zin in onze toekomst samen.

PhD portfolio

Name PhD student	Koen Verdonk
PhD period:	2010-2015
Erasmus MC Department:	Internal Medicine, Division of pharmacology and Vascular Medicine
Promotors:	Prof. dr. A.H.J. Danser, Prof. dr. E. A.P. Steegers
Supervisor:	Dr. A.H. van den Meiracker, Dr. Willy Visser
Research School:	Cardiovascular Research School Erasmus University Rotterdam (COEUR)

PhD training

General academic skills

Biomedical English Writing and Communication	2012	4
Research Integrity	2012	1

Indepth courses (e.g. Research school, Medical Training)

Coeur Cardiovascular Medicine	2011	1.5
Coeur Pathophysiology of ischemic heart disease	2012	1.5
Coeur cardiovascular Pharmacology	2013	1.5
Coeur Heart failure research	2012	1.5
Coeur Molecular biology in atherosclerosis and cardiovascular research	2010	1.5
Winterschool Dutch Kidney Foundation	2011	2
NSH course vascular biology, Papendal	2012	2

Presentations

Presentations at the internal medicine and division of Pharmacology	2010-2013	3

International conferences

High blood pressure Research - Scientific session 2010, Washington DC (USA)	2012	1,2
Wetenschapsdagen Interne Geneeskunde - Antwerpen	2011	0,6
High blood pressure Research - Scientific session 2012, Orlando, (USA)	2012	1,2
XVIII ISSHP world congress, Geneva	2012	1,2
High blood pressure Research - Scientific session 2013, Whashington DC, USA	2013	1,2
High blood pressure Research - Scientific session 2014, New Orleans (USA)	2014	1,2

Didactic skills

Supervising practicum:		
V.O. Farmacologische beinvloeding van het autonome zenuwstelsel	2010-2014	4,8
COEUR course 3, pharmacology of isolated blood vessels	2012	0,4
Supervising Master's theses of 4 medical students	2010-2014	3

Other

Board member Promeras	2012-2014	3

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