Endothelial pathology in preeclampsia

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Chapter 4

Thrombomodulin expression in the kidney is regulated by anti-angiogenic factors, *in vitro* and *in vivo*

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ABSTRACT

Background

Levels of soluble thrombomodulin are increased in preeclampsia. Whether this increase reflects a change in functional thrombomodulin on the glomerular endothelium, and if these changes affect anti-coagulative and cytoprotective signaling is unknown. Hence, we investigated thrombomodulin signaling and regulation in preeclampsia patients and experimental models of endothelial dysfunction.

Methods

Kidney tissue from eleven preeclampsia patients and 22 pregnant women was collected. Kidneys from five pigs with metabolic syndrome after a high-fat diet, five pigs with diabetes after streptozotocin injection and five control pigs and from twelve mice treated with endoglin or vascular endothelial growth factor A (VEGF-A) receptor inhibiting antibodies were collected. Human umbilical vein endothelial cells were treated with VEGF-A or a soluble VEGF-receptor (sFlt-1). Gene expression of thrombomodulin, endothelial protein C receptor and tissue factor was investigated with qPCR. Thrombomodulin protein expression was investigated with immunohistochemistry.

Results

In preeclampsia, kidney thrombomodulin was increased compared to pregnant control subjects and this increase was correlated with podocyte nephrin expression ($P<0.01$). Glomerular thrombomodulin was increased in diabetic pigs and in mice treated with anti-angiogenic compounds. In diabetic pigs, mRNA expression of thrombomodulin, endothelial protein C receptor, and VEGF-A increased (all $P<0,05$). sFlt-1 decreased endothelial thrombomodulin mRNA in vitro.

Conclusions

Renal endothelial thrombomodulin expression is increased during anti-angiogenic conditions, in vitro and in vivo. Increased thrombomodulin expression is accompanied by increased podocyte nephrin expression, indicative of a protective effect on the glomerulus. These results indicate an attempt of glomerular endothelial cells to maintain cytoprotection; investigating pathways through which thrombomodulin expression is

increased in endothelial cells could reveal clues to restore or prevent endothelial kidney damage.

ABBREVIATIONS

APC	Activated protein C
VEGF-A	Vascular endothelial growth factor A
VEGFR-2	Vascular endothelial growth factor receptor 2
soluble Flt-1	sFlt-1
DN	Diabetic nephropathy
HUVEC	Human umbilical vein endothelial cell

INTRODUCTION

Preeclampsia is a syndrome of pregnancy characterized by hypertension and proteinuria, with systemic endothelial dysfunction as the final common pathway leading to symptomatic disease.[1] Thrombomodulin is a transmembrane glycoprotein found on the endothelium of arteries, venules and capillaries.[2] It regulates endothelial cell survival, coagulation and endothelial activation through signaling via activated protein C (APC) and the endothelial protein C receptor (EPCR).[2, 3] Thrombomodulin is cleaved from the endothelial surface under inflammatory conditions, releasing a truncated, supposedly nonfunctional soluble thrombomodulin protein.[3, 4] In preeclampsia placental abundance of thrombomodulin is decreased.[5] This is accompanied by increased levels of cleaved thrombomodulin in the circulation.[6]

One of the first maternal organs symptomatically affected in preeclampsia is the kidney; proteinuria is one of the first symptoms of preeclampsia and microalbuminuria during early pregnancy is a predictor of the development of preeclampsia.[1] Kidney changes in preeclampsia are distinct; endothelial cells lose their fenestrations and become swollen, this is known as endotheliosis, and podocyte foot process effacement and podocyte loss in urine is observed.[7]

In preeclampsia, the placenta produces excessive levels of the anti-angiogenic factors soluble Flt-1 (sFlt-1) and soluble endoglin.[8, 9] sFlt-1 and soluble endoglin are soluble receptors, binding to vascular endothelial growth factor A (VEGF-A) and transforming growth factor beta, respectively, giving rise to an angiogenic imbalance when in excess.[10] VEGF-A and soluble endoglin play a pivotal role in maintaining an intact glomerular filtration barrier in the

kidney. Animal models with overexpression of sFlt-1 and soluble endoglin develop renal changes somewhat similar to those in preeclampsia, for example with glomerular endothelial cell swelling and podocyte changes.[11-13] Likewise, deviations in VEGF-A availability to the glomerulus are associated with a variety of kidney diseases in humans.[14-16] Patients treated with VEGF-A inhibitors develop similar renal lesions as in preeclampsia, including endothelial cell swelling, renal thrombi and proteinuria.[17, 18]

The angiogenic imbalance of preeclampsia might (partially) contribute to the renal lesions in preeclampsia through impairing glomerular thrombomodulin signaling; thrombomodulin is a mediator of glomerular endothelial homeostasis during endothelial dysfunction. In a mouse model for diabetic nephropathy, thrombomodulin-dependent APC signaling was decreased and impairment of APC signaling resulted in glomerular endothelial apoptosis.[19] VEGF-A increases thrombomodulin expression in endothelial cells *in vitro*.[20] Also, in preeclampsia, increased sFlt-1 production in the placenta is associated with placental loss of thrombomodulin.[5]

In summary, decreased thrombomodulin signaling and expression appear to be associated with renal endothelial dysfunction and preeclampsia. However, if thrombomodulin abundance changes in the kidney in preeclampsia, and if this is associated with the glomerular lesions of preeclampsia is unknown. We set out to investigate if kidney thrombomodulin expression is changed in preeclampsia, and if this change could be caused by the angiogenic imbalance of preeclampsia. Therefore, thrombomodulin abundance was studied in kidney tissue from human patients with preeclampsia and pregnant controls. Further, thrombomodulin expression and abundance were studied in disease models for endothelial dysfunction: porcine models for diabetes and metabolic syndrome and mice treated with anti-angiogenic compounds. Lastly, thrombomodulin expression was studied in an in-vitro model for glomerular endothelial cells under anti-angiogenic conditions.

MATERIALS AND METHODS

Patient samples

To collect renal tissue from women who died from the consequences of preeclampsia and control subjects, a nationwide search of the Dutch Pathology Registry was conducted. This is a network of all pathology laboratories in the Netherlands. The pathology data were linked with the

records of the National Maternal Mortality Committee of the Dutch Society of Obstetrics. Preeclampsia was defined according to the definition of the International Society for the Study of Hypertension in Pregnancy (ISSHP).[8] Two control groups were included; the first group consisted of pregnant women without a hypertensive disorder prior to or during pregnancy, who died from a cause unrelated to hypertension, and the second group consisted of non-pregnant women with a history of chronic hypertension. Paraffin-embedded kidney samples from 11 women with preeclampsia, 22 normotensive pregnant controls and 14 non-pregnant hypertensive controls were available for this study. The patient characteristics of these groups have been described before.[21] This study was approved by the Medical Ethics Committee of the Leiden University Medical Center (P12.107).

Porcine models for metabolic syndrome and diabetes

To further study the association between endothelial dysfunction, angiogenic imbalance and thrombomodulin expression and abundance, samples from porcine models for metabolic syndrome and diabetes were collected. The control group of five pigs received a diet consisting primarily of barley, wheat and soy bean oil. The metabolic syndrome group of ten pigs received a cafeteria diet with a high content of lard, fructose, sucrose and added cholesterol. Five of the cafeteria diet fed pigs were additionally treated with low-dose streptozotocin (STZ) after five months, with the dose of STZ being adjusted individually to induce non-insulin dependent diabetes to model type two diabetes. After seven months, paraffin and snap-frozen kidney tissue was collected as described by Jonker et al.[22]

sFLt-1/ endoglin mouse model

To study the effect of anti-angiogenic conditions on endothelial thrombomodulin expression in vivo, six C57/Bl6 mice were treated two times per week with VEGF-receptor inhibiting antibodies (10 mg/kg, DC101, BioXcell) or anti-endoglin antibodies (M1043, kindly provided by Tracon Pharmaceuticals, San Diego USA) or with a combination for five weeks. Six mice were treated with appropriate IgG control antibodies for five weeks. Afterwards, mice were sacrificed and kidney tissue was collected, processed and stained for thrombomodulin expression as described below. Animal

experiments were approved by the animal ethics committee of the Leiden University Medical Center, the Netherlands.

Histology and immunohistochemistry

Human renal sections were scored by a renal pathologist blinded with respect to cases and control subjects to evaluate histological changes, e.g. endotheliosis, glomerulosclerosis, podocyte count and parietal cell activation in human renal samples, as described before.[21] For immunohistochemistry, sections were deparaffinized, antigen retrieval was performed with citrate (for the thrombomodulin antibodies) or with Tris EDTA (for the fibrin antibody), and peroxidase was blocked by incubating the sections in a hydrogen peroxide solution for 20 minutes. Kidney samples from humans and pigs were incubated with a mouse monoclonal thrombomodulin antibody (1:200, Leica Biosystems, Danvers), a nephrin antibody, or a fibrin antibody (1:200) for one hour, and kidney samples from mice were incubated with a rabbit polyclonal thrombomodulin antibody (1:200 Santa Cruz) for one hour. Binding of the primary antibody was visualized with labeled anti-mouse or labeled anti-rabbit polymer (DAKO, Belgium) and diaminobenzidine as a chromogen. As positive and negative controls, human placenta and mouse skin samples were used.

Quantification of immunohistochemistry

In human patients and in mouse specimen, thrombomodulin, nephrin and fibrin staining were scored as being either present, when more than 10% of the capillary walls in the glomeruli were stained positively, or absent, when less than 10% of the capillary walls in the glomeruli was stained positively.

PCR

Quantitative PCR was performed to quantify placental mRNA expression of thrombomodulin, VEGF-A, Flt-1, tissue factor and endothelial protein C receptor. RNA was isolated with TRIzol® (Lifetechnologies, San Francisco, CA, USA). Synthesis of cDNA was performed with AMV reverse transcriptase (Roche, Basel, Switzerland), and SYBR Green quantitative PCR was performed according to the manufacturer's protocol (Bio-Rad Laboratories Inc, Hercules, CA, USA). Primer sequences are described in Supplementary Table 1. Expression was measured by the comparative threshold cycle method

and normalized to hypoxanthine phosphoribosyl transferase and GAPDH expression. A melting curve analysis was performed to verify the specificity of amplification.

Glomerular endothelial cell and podocyte cell culture experiments

Human umbilical vein endothelial cells (HUVECs) that were confluent for two days were incubated with VEGF-A (20 ng/ml; R&D Systems) for two, four, six and eight hours. To determine the effect of sFLT-1 on VEGF-A-induced endothelial activation, HUVECs were incubated for four hours with sFLT-1 (0, 10, 100 or 1000 ng/ml; R&D Systems) in the presence of VEGF-A (20 ng/ml). These experiments were performed three times. Cell lines were negative for mycoplasma contamination. [23] RNA isolation and PCR were performed as described above.

Statistical analyses

Categorical data were analyzed using the chi-square test. Mean differences in normalized mRNA expression levels between two groups were analyzed with the unpaired t-test for normally distributed data or with the Mann–Whitney U test for skewed distributions. A p-value below 0.05 was considered significant. All analyses were performed using the SPSS statistics software (IBM, version 20).

RESULTS

Thrombomodulin immunohistochemistry in human samples

Thrombomodulin abundance in glomeruli was studied in 11 kidney specimens from women with preeclampsia, from 22 pregnant control subjects and from 14 non-pregnant hypertensive control subjects. Thrombomodulin was present in 9 out of 11 (82%) kidney samples from preeclampsia patients and in 9 out of 22 (41%) kidney samples from the pregnant control subjects. In non-pregnant hypertensive control subjects, thrombomodulin was present in 3 out of 14 (21%) kidney samples. The staining pattern was significantly more frequently positive in preeclampsia patients as compared to pregnant controls (P = 0.026) and as compared to non-pregnant hypertensive controls (P = 0.003). These results are illustrated in Figure 1.

Thrombomodulin expression and renal lesions of preeclampsia

Thrombomodulin protein abundance was compared with the histopathological changes and fibrin- and nephrin abundance in the kidney specimen, as markers of excessive coagulation and podocyte viability, respectively. Thrombomodulin expression correlated with activation of parietal epithelial cells ($P = 0.023$), but not with endotheliosis, podocyte count or endothelial fibrin deposits ($P > 0.05$). Further, glomerular thrombomodulin expression correlated with podocyte nephrin expression ($P < 0.01$). Fibrin deposits were observed in similar quantities in specimen from patients with preeclampsia and from controls.

Thrombomodulin expression in endothelial dysfunction porcine model

Thrombomodulin protein abundance and mRNA expression were studied in porcine models for diabetes and metabolic syndrome, and controls; thrombomodulin protein abundance was increased in the diabetes model; staining in glomeruli was observed in 40% of control pigs, in 40% of pigs with metabolic syndrome and in 80% of pigs with diabetes ($P<0.05$). Examples of staining in glomeruli and negative glomeruli are depicted in figure 2. Kidney thrombomodulin mRNA expression was increased approximately 2-fold in pigs with metabolic syndrome ($P < 0.05$) and in pigs with diabetes mellitus as compared to control pigs. This increase was accompanied by an increase in mRNA expression of the co-receptor EPCR in the metabolic syndrome pigs ($P < 0.05$) and diabetes mellitus pigs as compared to controls. Tissue factor expression was not different between case and control groups. sFlt-1 mRNA was increased in kidneys from pigs with metabolic syndrome and diabetes mellitus as compared to controls (both $P < 0.05$), but VEGF-A mRNA was increased as well in the case groups and there was no difference between cases and controls with respect to the VEGF-A/sFlt-1 ratio. These results are illustrated in Figure 3.

Thrombomodulin immunohistochemistry in mice treated with anti-angiogenic compounds

Thrombomodulin abundance was investigated in kidney tissue from mice treated with VEGF-receptor inhibiting antibodies and anti-endoglin antibodies and control mice (Figure 4). Thrombomodulin expression was present in a diffuse and overall staining pattern among glomeruli in 50% of

treated mice and in 20% of control mice (P > 0.05). Thrombomodulin staining in peritubular capillaries was present in all treated and control mice.

In vitro experiments

To investigate the possible effect of the angiogenic imbalance on thrombomodulin expression in vitro, HUVEC cells were incubated with VEGF-A and sFlt-1. Adding sFlt-1 to HUVEC cells cultured in VEGF-enriched medium decreased thrombomodulin mRNA expression approximately twofold (Figure 5).

DISCUSSION

Soluble thrombomodulin is cleaved from the endothelial surface, e.g. by matrix metalloproteinases, under inflammatory conditions.[3] We have now shown that this increased thrombomodulin cleaving in preeclampsia and diabetes does not lead to decreased thrombomodulin abundance on the endothelium. Instead, increased thrombomodulin abundance was observed under these conditions, implicating a 'rescue-mechanism' initiated by endothelial cells to compensate for the pro-inflammatory, procoagulant environment. However, this 'rescue-mechanism' is not sufficient to prevent kidney damage. The above described mechanism has been proposed before in glomerulonephritis.[24]

Angiogenic factors play a substantial role in maintaining the glomerular filtration barrier. This study underlines that angiogenic factors regulate renal thrombomodulin expression, *in vitro* and *in vivo*. In vitro, sFlt-1 transfection of human umbilical vein endothelial cells resulted in downregulation of thrombomodulin expression. Contrastingly, glomerular thrombomodulin abundance increased in mice treated with anti-angiogenic compounds, and in pigs with diabetes and metabolic syndrome. Moreover, kidney thrombomodulin abundance was increased significantly in preeclampsia patients compared to pregnant controls.

Our results raise the question why sFlt-1 decreases thrombomodulin *in vitro*, but why thrombomodulin expression is increased under anti-angiogenic circumstances *in vivo*. Because thrombomodulin is known to be downregulated by hyperfiltration and shear stress, transforming growth factor beta and endotoxins, which are all increased in preeclampsia and diabetes, one would expect a decrease in glomerular endothelial

thrombomodulin expression, but our study revealed the opposite.[9] A possible explanation could be that one of the major isoforms of VEGF-A, VEGF-A 165, can be stored in the glomerular basement membrane through binding to heparin sulphate proteoglycans, so local levels of VEGF-A might even be increased under the endothelium despite increased levels of circulating sFlt-1.[25]

Interestingly, VEGF-A and sFlt-1 mRNA expression increased in diabetic pigs, resulting in a net unchanged VEGF-A/sFlt-1 ratio, implicating unchanged levels of freely circulating VEGF-A. However, thrombomodulin expression was still increased in these cases. Recently, sFlt-1 was revealed to have an effect on podocytes independent of VEGF-A.[26] sFlt-1 binds to lipid rafts on the podocyte cell surface and initiates nephrin phosphorylation and cytoskeleton changes.[26] We propose that the changes observed in preeclamptic and diabetic nephropathy do not merely result from decreased VEGF-A availability, but from independent sFlt-1 signalling to pericytes and podocytes as well.

Glomerular endothelial thrombomodulin expression is associated with increased expression of nephrin in podocytes and parietal epithelial cell activation in our study. This implies cross-talk across the glomerular basement membrane, between endothelial cells and podocytes. Known players in glomerular cross-talk are VEGF-A and endothelin-1.[10] VEGF-A produced in podocytes reaches the endothelial cells either through diffusion or transport through heparin sulphate proteoglycans, where it binds to the VEGFR-2 on the endothelium and stimulates survival and cell structure maintenance.[25] Lack of podocyte VEGF-A results in endothelial cell abnormalities. Endothelial cells become activated and produce endothelin-1, which binds to receptors on the podocyte's cell surface, resulting in podocyte damage. Thrombomodulin is a strong inhibitor of endothelial activation; enhanced thrombomodulin signaling under anti-angiogenic conditions could therefore suppress endothelin-1 expression and interfere with the glomerular damage loop.

Soluble thrombomodulin has been applied as therapy in humans during sepsis and results in decreased mortality.[27] Perhaps treatment with soluble thrombomodulin could have a beneficial effect on glomerular endothelium, and subsequently on glomerular cross talk and endothelin expression, under anti-angiogenic conditions as well. Illustrative, soluble thrombomodulin administration appears to be a promising way to restoring glomerular

function in mouse models of inflammatory kidney disease.[28] However, since thrombomodulin expression is already elevated in preeclampsia and diabetic nephropathy, it might not be the rate-limiting step in controlling the glomerular damage loop; effects of a further increase of thrombomodulin signaling in these pathologies still have to be investigated.

In sum, this research provides evidence for the hypothesis that angiogenic factors regulate renal endothelial thrombomodulin expression, *in vitro* and *in vivo*. Further, we illustrated that increased soluble thrombomodulin levels under inflammatory conditions are accompanied by increased endothelial thrombomodulin expression, and not by thrombomodulin loss. Investigating pathways through which thrombomodulin expression is increased in endothelial cells could reveal clues to restore or prevent endothelial damage in the kidney.

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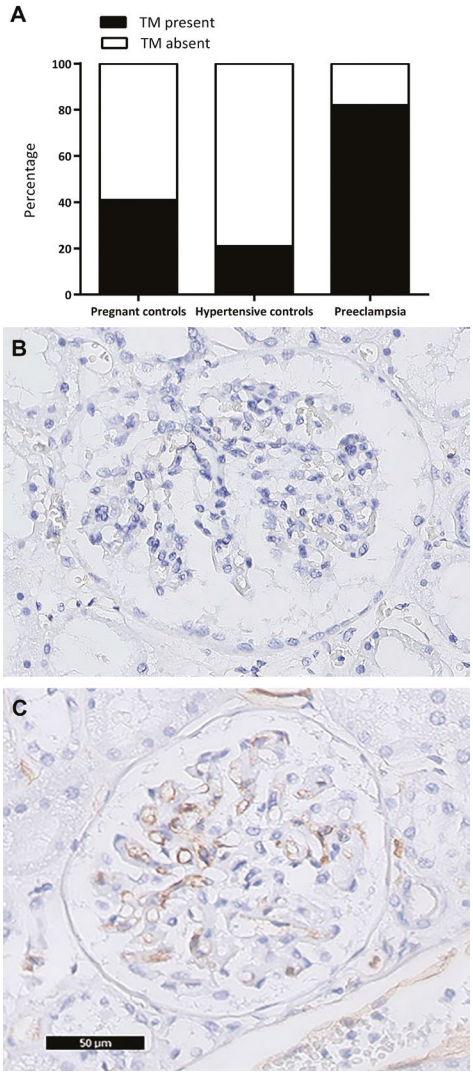
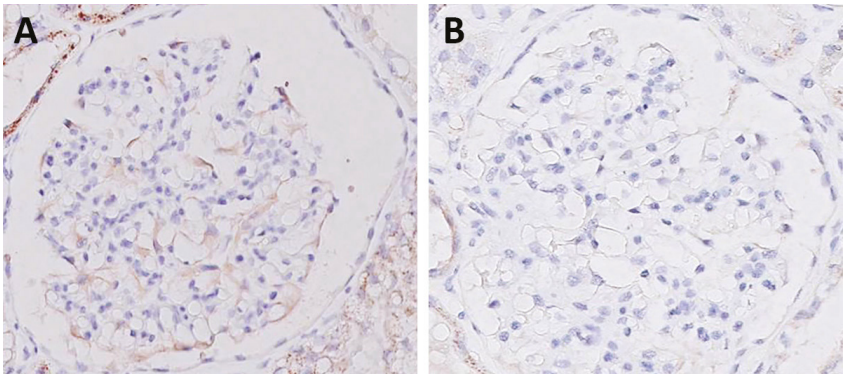
FIGURES & LEGENDS

Figure 1 thrombomodulin expression in kidney samples from human preeclampsia patients and controls



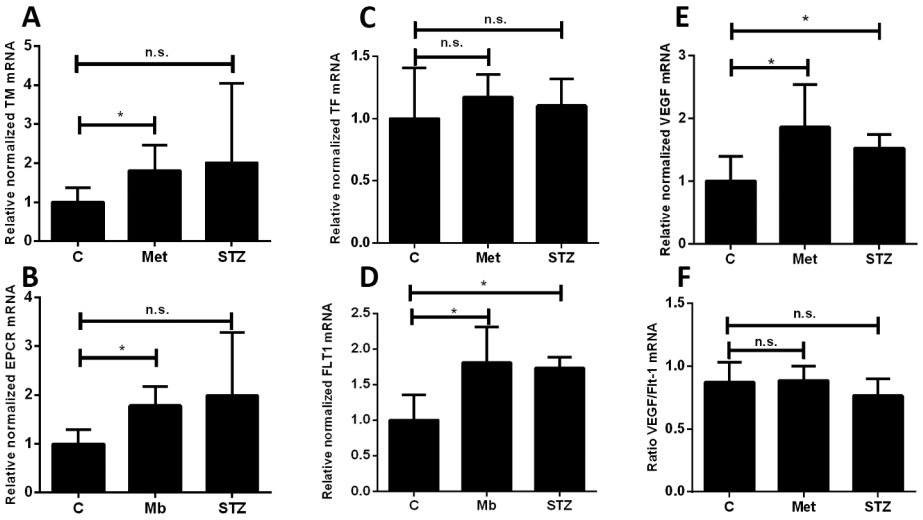
A) shows the percentage of kidney samples where thrombomodulin was absent or present in pregnant controls, hypertensive controls and preeclampsia patients.
B) shows a control kidney sample stained for thrombomodulin; there is no positive staining.
C) shows a preeclamptic kidney sample stained for thrombomodulin, there is positive staining along the capillary walls in the glomerulus and in the peritubular capillaries.

Figure 2 Thrombomodulin staining in porcine kidneys



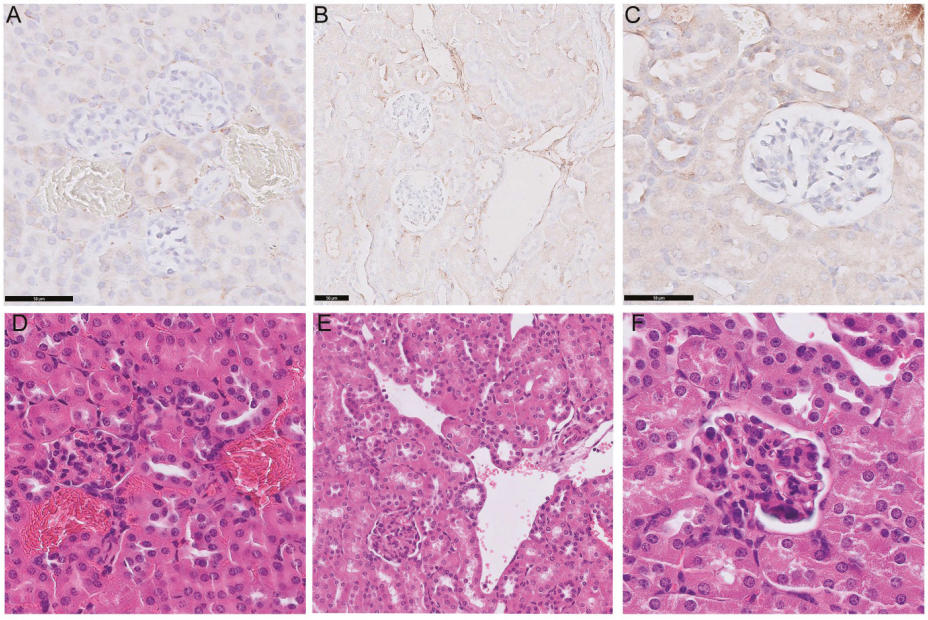
A) glomerulus from diabetic pig, immunohistochemically stained for thrombomodulin, with a positive staining pattern.
B) glomerulus from control pig, immunohistochemically stained for thrombomodulin, with a negative staining pattern.

Figure 3 mRNA expression in pig kidney tissue



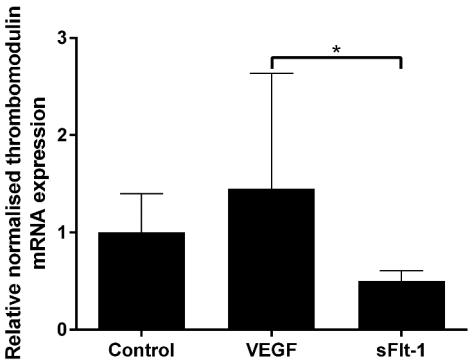
A) relative normalized thrombomodulin mRNA expression in cases and controls. B) relative normalized EPCR expression in cases and controls. C relative normalized tissue factor mRNA expression in cases and controls. D relative normalized Flt-1 mRNA expression in cases and controls. E relative normalized VEGF-A mRNA expression in cases and controls. F VEGF-A/Flt-1 mRNA ratio in cases and controls. C) control group; Mb: metabolic syndrome group; STZ: streptozotocin-treated group;*: P<0.05; n.s.: not significant; TM: thrombomodulin; TF: tissue factor.

Figure 4 Thrombomodulin expression in kidneys from mice treated with VEGF-receptor inhibiting antibodies and anti-endoglin antibodies



A) and B) thrombomodulin staining in big vessels of a mouse treated with anti-angiogenic compounds
C) thrombomodulin staining in parietal epithelial cells in a mouse treated with anti-angiogenic compounds.
D-F) HE-staining of the same regions as in A-C.

Figure 5 Thrombomodulin mRNA expression in HUVEC cells treated with VEGF and sFlt-1



Thrombomodulin relative mRNA expression in HUVEC cells incubated with VEGF, or with VEGF and sFlt-1.*: P<0,05