Mechanisms of renoprotection: on the anti-inflammatory roles of thrombomodulin and soluble FLT1 in renal disease

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CHAPTER 7

Summary and General Discussion

The overall aim of this PhD project was to identify renoprotective mechanisms that maintain endothelial quiescence and inflammation, with the ultimate aim to harness these mechanisms therapeutically to halt CKD progression. Many research efforts to stop CKD progression have focused on the identification and inhibition of damaging, profibrotic and proinflammatory factors (1). In contrast, this PhD project evaluated the therapeutic potential of enhancing renoprotective mechanisms.

Considering earlier findings from our group, two known protective proteins, thrombomodulin (TM) and soluble FLT1 (sFLT1), were chosen as the experimental focus of the project. In the first part of this thesis (chapter 2 and 3), we studied the protein levels of the endothelial protein TM in glomerular diseases. In the second part (chapter 4 and 5), the anti-inflammatory effects of sFLT1 treatment were investigated in experimental models of chronic inflammation. In the final part (chapter 6), we assessed the potential interaction of sFLT1 with macrophages.

The experimental procedures and limitations specific to each chapter are detailed within the relevant chapters. This final chapter contains a summary of the main findings, followed by a general discussion of their implications.

1. Summary of Findings

1.1. *Thrombomodulin in Glomerular Endothelial Disease*

Thrombomodulin (TM) is a well-known gatekeeper of endothelial quiescence. In experimental diabetic nephropathy, TM protected against glomerulosclerosis, glomerular apoptosis, complement and inflammasome activation (2–4). These studies indicate that a dysfunction of endothelial TM contribute to the development of diabetic glomerular lesions. Despite compelling animal data, limited evidence exists for dysregulated TM expression in human kidney diseases. Therefore, in chapter 2 and 3, we investigated the differential expression of TM in two diseases of the glomerular endothelium, diabetic nephropathy (DN) and preeclampsia (PE), using autopsy kidney tissues.

During our investigations in chapter 2, we found a contrast between mouse and human TM expression in the renal endothelium. Unlike the abundant expression of glomerular TM in mice, in normal human kidneys, expression of TM antigen was restricted to the glomerular vascular pole. Diabetes was associated with reduced glomerular TM expression, irrespective of the presence of diabetic nephropathy. There was no relation between glomerular TM levels and the severity of kidney disease in terms of structure (DN class, lesions) and function (proteinuria, eGFR). However, we did observe a strong relation between lower TM expression and more macrophages within the same glomerulus. In a separate cohort, diabetes did not reduce glomerular TM mRNA expression, indicating that the loss of glomerular TM occurs through increased protein shedding. Thus, in diabetic kidneys, reduced glomerular TM is associated with glomerular inflammation, supporting the contribution of endothelial cell dysfunction to the pathogenesis of DN.

In chapter 3, TM was studied in the context of PE, a complication of pregnancy which is characterized by glomerular endothelial injury. Like in DN, women with PE have increased serum TM levels, indicative of systemic endothelial injury. Placental TM production is lowered in PE (5), raising the question where increased circulating TM originates. In this chapter, we found that in the kidneys of women with PE, glomerular TM antigen was increased compared to levels observed in normal pregnancy and hypertension, and distributed along the entire glomerular endothelium. The elevated glomerular TM levels were correlated with less glomerular endotheliosis and a smaller tuft size, suggesting an endothelial protection mechanism.

Interestingly, therapeutic endothelin-A-receptor (ETAR) blockade achieved a normalization of both loss and excess of glomerular TM in experimental animal models of DN and PE (chapter 2 and 3). In a mouse model of DN, treatment with the ETAR blocker atrasentan counteracted the loss of glomerular TM and reduced albuminuria. In contrast, in a rat VEGF-inhibition model of PE, upregulated glomerular TM levels were reduced by treatment with an endothelin-A-receptor blocker. This suggests that ETAR antagonism is capable of returning the glomerular endothelium to quiescence, regardless of the underlying condition.

Synthesizing our findings, the highly specialized glomerular endothelium expresses low levels of TM in a quiescent state, while in a diseased state, glomerular TM expression is tightly regulated according to the local demands caused by the underlying disease. Glomerular TM demonstrates a dual protective role, associated with a reduction in glomerular macrophage accumulation in diabetes, and reduced glomerular endotheliosis in PE.

1.2. *Therapeutic Potential of sFLT1 in Chronic Inflammation*

Another approach to prevent progression of kidney disease is to reduce chronic kidney inflammation. While the VEGF inhibitor sFLT1 is best known as an antiangiogenic protein that damages kidneys, sFLT1 also has potent anti-inflammatory properties in models of chronic inflammation. We previously found that low-dose sFLT1 treatment reverses pre-existent renal dysfunction and reduces macrophage infiltration in experimental DN (*6*). In chapter 4 and 5, we aimed to replicate these effects of sFLT1 in non-diabetic models of chronic inflammation.

Our data demonstrate that sFLT1 treatment effectively reduces chronic inflammation and promotes tissue repair without harming the kidneys. In chapter 4, the effects of sFLT1 on chronic inflammation were investigated in APOC1 mice, a model characterized by the spontaneous development of chronic inflammatory disease in the skin and kidneys (*7*), (8). After 2 months of treatment, sFLT1 had completely reversed the chronic skin disease and inflammation seen in APOC1 mice. Furthermore, sFLT1 treatment reduced serum cytokine levels and renal inflammation to levels observed in wild-type mice.

Next, we also found that sFLT1 treatment correlated with reduced chronic kidney inflammation and fibrosis in an experimental CKD model (chapter 5). Although the anti-inflammatory effects were less dramatic as compared to those observed in the APOC1 model, higher sFLT1 levels were associated with a reduced expression of proinflammatory and profibrotic markers in the kidney. Notably, in neither of the studies, we found any evidence of sFLT1-mediated kidney toxicity. This indicates that the low dose of sFLT1 treatment needed for anti-inflammatory effects, does not harm kidney function.

Based on the anti-inflammatory effects of sFLT1 observed in chapters 4 and 5, the main objective of chapter 6 was to study the possibility that these observations rely on a direct interaction of sFLT1 with the immune system. Together with previous reports describing that sFLT1 binds podocytes, pericytes and endothelial cells (*9*), (10), we hypothesized here that sFLT1 directly interacts with macrophages.

We found that sFLT1 binds to the cell surface of macrophages, both in vitro and in human kidney tissues. Anti-inflammatory polarization of macrophages increased their capacity to bind sFLT1, while proinflammatory activation reduced sFLT1 binding. Pretreatment of cells with heparin and heparinase III abolished binding of sFLT1, indicating an interaction between cell surface heparan sulphates and sFLT1. Double-label immunostaining suggested that sFLT1 interacts with the membrane VEGF co-receptor neuropilin-1. Finally, sFLT1 reduced the expression of chemokine recep-

tors, suggesting a regulatory function in macrophage migration. Together, these results show a new mechanism whereby sFLT1 directly modulates macrophage function.

2. General Discussion

2.1. *Thrombomodulin and Renoprotection in CKD*

Thrombomodulin was initially discovered as an endothelial regulator of coagulation, inhibiting thrombin and activating protein C (11). The subsequent discovery that *Thbd*$^{-/-}$ mice die in utero, due to a poorly understood necessity of trophoblast TM (12), led to the generation of various TM-mutant mouse lines. These models have yielded insights into the diverse anti-thrombotic, anti-apoptotic, anti-complement and anti-inflammatory mechanisms by which the multi-domain TM maintains kidney homeostasis (2), (4). Furthermore, these findings have contributed to the therapeutic use of the soluble TM ectodomain for vascular disorders, and more recently to its use as one of the human transgenes expressed in the first xeno-transplanted pig kidney (13).

An important limitation of rodent studies, is that in the case of TM, these do not parallel the human situation. Unlike the abundant TM expression in murine and rat glomeruli, in chapter 2 and 3 we found that TM is minimally present in the healthy human glomerulus. In healthy glomeruli, TM antigen is restricted to the glomerular vascular pole, and absent in peripheral glomerular capillaries. This staining pattern was recently replicated in DN and healthy kidney biopsies (14). While the human TM staining pattern underscores an interesting, understudied phenotypic diversity among human glomerular endothelial cells, the different TM expression pattern also makes mice less suitable as a model for studies on human glomerular TM function. Furthermore, the described TM kidney models are cell-unspecific, germline-penetrating models (2), (4). Since these mutant models showed TM dysfunctions in all cells during lifetime, it is difficult to conclude whether a kidney phenotype is due to ablation of glomerular endothelial TM during the disease pathogenesis, or is secondary to glomerular endothelial TM dysfunction during development, or to TM dysfunction in other cell types. Finally, the STZ model of DN used in these studies generally fails to mirror the extent of chronic kidney destruction seen in human CKD (15). Thus, animal literature on the role of glomerular TM should be interpreted with some nuance.

2.1.1. TM Regulates Glomerular Endothelial Quiescence in DN

Our findings in chapter 2 suggest that a loss of glomerular endothelial TM provides a causative link between diabetes and diabetic end-organ damage. In diabetic glomeruli, we found marked spatial correlations between a loss of TM, an increased macrophage number and a loss of endothelial glycocalyx, while finding no correlations with glomerular complement deposition and apoptosis. These findings suggest that diabetic factors cause a loss of glomerular endothelial TM, thereby activating a proinflammatory glomerular endothelial phenotype, and causing local macrophage infiltration. Therefore, the therapeutic restoration of glomerular endothelial TM, and its anti-inflammatory effects on the kidney endothelium, may be beneficial in DN. To this end, we observed that inhibition of the endothelin-A-receptor (ETAR) restores glomerular endothelial TM and reduces albuminuria in diabetic mice. It is intriguing to speculate that the ETAR blocker atrasentan delays CKD progression in patients with DN by saving glomerular thrombomodulin (16).

A causative link between the loss of glomerular endothelial TM and DN also relates to ongoing discussions about the pathogenesis of the classic Kimmelstiel-Wilson lesion of DN. This lesion is associated with a poor renal prognosis in DN patients (17). One of the theories is that this lesion is caused by local microvascular injury, based on the histological similarities between Kimmelstiel-Wilson lesions and thrombotic microangiopathy (TMA), a heterogeneous group of disorders caused by microvascular injury. Similar to glomerular TMA, Kimmelstiel-Wilson nodules show red blood cell fragments, increased plasminogen-activator inhibitor-1, mesangiolysis, microaneurysms, complement activation (18). Furthermore, a recent case report found a TM gene mutation within the ST domain (Asp486Tyr) in a patient with idiopathic nodular glomerulosclerosis and chronic TMA on renal biopsy (19). This variant and other mutations within the TM lectin domain were also associated with complement-mediated TMA before (atypical HUS) (20). Together, these lines of evidence suggest that diabetes-associated loss of TM links microvascular injury with glomerulosclerosis. Diverse molecular properties of TM may mediate this effect, including its pro-quiescent, anti-complement and anti-coagulant properties. Ultimately, molecular and cell culture studies are needed to understand how this TM mutation may mediate the development of glomerulosclerosis.

Our finding that diabetics lose glomerular endothelial TM, regardless of the presence of histological DN, warrants some discussion. Obviously, one may ask why there is no difference in glomerular TM levels between diabetics without and with nephropathy. TM may simply have no function in the diabetes-inflicted glomerular

damage. Other possible explanations are more likely. First, due to the cross-sectional nature of our autopsy study, it is possible that the DM group may have been contaminated with patients that would have developed DN at a later stage. In support of this, the duration of diabetes was significantly shorter in diabetics without DN than in diabetics with DN (9 vs. 16 years average). Since a follow-up of at least 15 years of diabetes is required before it can be established that a patient will not develop renal disease, samples taken at an earlier timepoint (in our study) may be false-negative with respect to the risk of developing renal disease. Furthermore, it is possible that the diabetes-inflicted loss of TM is important, but not sufficient, for the development of DN, and that other factors are needed to contribute to lesion development.

2.1.2. TM and Glomerular Endothelial Specialization in PE

In chapter 3, we went on to study glomerular TM in preeclampsia (PE), a complication of pregnancy that primarily affects the glomerular endothelial cells of the kidney. Renal changes in PE can also be appreciated as a type of TMA, characterized by bloodless glomeruli in which endotheliosis causes capillary occlusion, and a loss of endothelial fenestrae (21).

In women with PE, endothelial TM was upregulated compared to normal pregnancy and present along the entire glomerular tuft. High TM expression was associated with less glomerular endotheliosis, suggestive of a protective mechanism. Parallel to this, in an experimental rat model of PE kidney injury, we found that glomerular TM levels were upregulated following treatment with an intermediate dose of VEGF-inhibition (characterized by focal endotheliosis); in contrast, high-dose VEGF-inhibition (diffuse endotheliosis and fibrin deposition) did not cause an upregulation of glomerular TM. These observations suggest that TM is upregulated in PE to prevent or reverse acute glomerular endothelial cell injury, and that overt PE injury is characterized by a failure of this endothelial protective mechanism.

It is perhaps possible that TM restores glomerular endothelial cell specialization in PE. The loss of fenestrae and endotheliosis are caused by a loss of VEGF-ERK1/2 activation in PE, leading to TGFβ expression and de-specialization into a mesenchymal cell type (22). In sprouting angiogenesis, VEGF/F-actin signaling requires the TM-induced organization of podosomes (membrane microdomains), to create membrane protrusions and elicit cell migration (23). Furthermore, the maintenance and restoration of endothelial fenestrae is also maintained by VEGF signaling (24). There-

fore, glomerular endothelial TM may contribute to VEGF-dependent restoration of glomerular endothelial identity in PE. Functional studies will be needed to confirm this hypothesis.

2.1.3. Future Directions

Our results with human kidney samples in chapter 2 and 3 raise several important questions. An overarching limitation of our studies is the descriptive, retrospective design of the work. The differential regulation of glomerular TM in glomerular disease may represent many things, including inappropriate mechanisms that mediate, or compensatory mechanisms that offset pathogenic influences. Future studies on the molecular functions of TM in endothelial cells, as well as the potential use of TM as a therapeutic target are exciting to pursue next.

An alternative animal-free route for functional studies is to study the actions of distinct TM domains and of human TM mutants that have been associated with disease. In ongoing work, we have generated several TM mutant cell lines, in order to elucidate the independent functions of TM's domains and better understand the consequences of TM dysfunction. Furthermore, in order to characterize the TM membrane interactome, we have fused TM with the biotin ligase turboID. Using this strategy, it is possible to study the proteins that TM interacts with during both cell quiescence and during cell activation with proinflammatory, diabetic and antiangiogenic factors. These studies could help elucidate the specific molecular interactions that are responsible for TM's renoprotective effects.

The therapeutic potential of TM has been addressed in various mouse models of kidney disease (chapter 1). Several formulations of TM and aPC, and PAR-modulators are being investigated for the treatment of vascular disease. A recombinant soluble TM (sTM) formulation, consistent of the TM ectodomain (ART-123), is clinically used in Japan for disseminated intravascular coagulation (DIC) (25). Interestingly, one Japanese observational study reported that in patients with septic DIC and AKI, sTM treatment was associated with a nephroprotective effect (26). Those treated with sTM had a shorter dialysis-dependence and a lower 28-day mortality as compared to patients treated with anticoagulants (heparin, antithrombin). While this was a small, retrospective, observational study, the findings suggest that sTM promotes kidney repair following AKI. It will be interesting to see a replication of these findings in a randomized setting, and to follow the long-term renal outcomes following sTM therapy. As an aside, the beneficial effects of treatment with sTM in animal studies and in the observational AKI study, raise the question why intrinsic sTM—shed from

activated endothelia—lacks these nephroprotective activities. Further studies into the functional relevance of TM shedding (in diabetic glomeruli) and the action of circulating sTM, may help us inch closer to understanding the role of TM in the kidney.

3. sFLT1 and Renoprotection

3.1. *Discovery of sFLT1's Anti-inflammatory Function*

In the 2000s, a number of landmark studies described a key role of soluble FLT1 (sFLT1) in the pathogenesis of preeclampsia (27), (28). Together the studies showed that in preeclampsia, an increased placental production of sFLT1 produces a systemic VEGF deficiency, leading to glomerular injury and proteinuria. The mirroring of this kidney phenotype in patients treated with anti-cancer VEGF inhibitors (29), further confirmed the hypothesis that a systemic VEGF deficiency is toxic to the glomerular filtration barrier.

At the same time, our research group studied the development of glomerular lesions in diabetic nephropathy. Mooyaart et al. (30) found that a polymorphism in the *APOC1* gene (which encodes apolipoprotein C1) was associated with the development of CKD in diabetics. Subsequently, Bus et al. (7) found that mice carrying the human *APOC1* gene (*APOC1* mice) spontaneously develop KW lesions, which occur in advanced DN. Since KW lesions are generally a rare finding in experimental diabetic animal models (15), these observations in *APOC1* mice were highly stimulating. However, a major setback to the use of this model was the median 1.5-year duration for development of kidney lesions. Therefore, we next evaluated strategies to accelerate the kidney disease progression in *APOC1* mice. Based on the loss of glomerular VEGF in patients with DN (31), we next attempted to accelerate the kidney phenotype of *APOC1* mice by using sFLT1 (chapter 4).

To our surprise, sFLT1 did not aggravate kidney disease in *APOC1* mice at all. Instead, sFLT1 completely reversed the systemic inflammation and skin disease in *APOC1* mice (chapter 4). Given these serendipitous findings, we next aimed to replicate our findings in other kidney disease models. In streptozotocin-induced diabetic nephropathy, sFLT1 treatment also reduced chronic kidney damage and inflammation (6). In chapter 5 of this thesis, in kidney ischemia-reperfusion injury, we found that higher sFLT1 levels were correlated with less fibrosis and inflammation, suggesting a similar renoprotective effect. However, these effects were not as profound as in the previous two models.

There are a number of possible explanations for the lack of a greater anti-inflammatory effect in the IRI model (chapter 5), as compared to the APOC1 and STZ models (chapter 4, (6)). First, the duration of sFLT1 treatment may be relevant. In the APOC1 and STZ models, mice were treated with sFLT1 for prolonged times (7 and 10 weeks). In contrast, in the IRI model (chapter 4), mice were treated with sFLT1 for 4 weeks. Hence it is possible that the anti-inflammatory effects in IRI model would be more profound if sFLT1 would have been administered for a longer period.

Alternatively, the timing of sFLT1 may be important. It is possible that sFLT1 actually aggravates acute kidney injury—whilst having beneficial effects on chronic phase. In chapter 4 and in STZ mice, mice were treated with sFLT1 when damage was already induced. In chapter 4, mice instead were pretreated with sFLT1 before inducing IRI damage. It is therefore possible that sFLT1 in IRI mice increased acute damage, whilst also reducing chronic damage. In line with this, preliminary results indicate that sFLT1-ko mice that undergo bilateral IRI, have less kidney fibrosis 2 weeks after IRI, suggesting that sFLT1 aggravates the acute damage following IRI (via personal communication). In line with this, a recent study also found that in the acute phase following IRI, VEGF is protective, whilst in the chronic phase VEGF aggravates inflammation and fibrosis (32). Thus, administered at a timepoint after IRI, sFLT1 treatment may be beneficial in the IRI model.

In support of this hypothesis, we found that sFLT1 may promote the function of reparative macrophages (chapter 6). Here, we observed that macrophages bind sFLT1, and that anti-inflammatory activation of macrophages using IL-4 markedly increases their binding of sFLT1. A single-cell RNA-seq study identified five distinct macrophage subsets with alternating abundances during kidney injury and repair (murine UUO) (33). An anti-inflammatory Mrc1+ macrophage subset which shares phenotypic markers with our IL-4-activated macrophages, was associated with the repair phase following kidney injury (33). Therefore, it is possible that sFLT1 association to this macrophage subtype contributes to their beneficial functions in the repair phase after kidney injury. This suggests that appropriate timing of sFLT1 treatment, e.g. during the repair phase following kidney injury (IRI), is critical for it to mediate beneficial effects.

How would sFLT1 promote the effects of these anti-inflammatory macrophages? When we evaluated potential signaling effects of sFLT1, we found a downregulation of chemokine receptor expression, but found no effect on macrophage activation state (chapter 6). Given that sFLT1 binding may mediate cellular VEGF uptake as described in podocytes (9), a limitation is that we did not assess the effects of sFLT1/VEGF co-incubation in macrophages. Alternatively, tying in with sFLT1's tissue localization at

the cell surface glycocalyx and extracellular matrix ((34) and chapter 6), it is also possible that sFLT1 promotes the function of anti-inflammatory macrophages without altering cellular signaling, for example by acting as a guidance protein. In line with this, neuropilin-1—which we identified as a potential sFLT1 interactor in macrophages (chapter 6)—has been described to regulate the tissue positioning of macrophages in tumors (35). The role of sFLT1 in macrophage positioning would need to be validated in future in vivo studies.

3.2. *Further Investigation into sFLT1's Anti-inflammatory Mechanisms*

Even though macrophages and endothelial cells are obvious cellular candidates, it is not clear whether these are the cellular targets responsible for sFLT1's anti-inflammatory effects in vivo. Ideally, this would be tested using a sFLT1 knock-out model. By inducing a sFLT1-deletion (tamoxifen-inducible ERcre-sFLT1$^{fl/fl}$ mouse model) at different stages of chronic inflammation (e.g. R-UUO, UIRI, APOC1), the lesions and immune cell infiltrates during the acute, chronic and resolution phases could be compared to those observed in WT animals. This would highly inform on the timing and cellular mechanisms of sFLT1 function in inflammation and tissue repair. However, the generation of a sFLT1-ko model is challenging. Matsui et al. (36), (37) attempted this by deleting the 13th intron of sFLT1, theoretically abolishing production of the only murine sFLT1 isoform through alternative splicing. Their sFLT1-ko mice had more chronic inflammation and tissue injury in experimental atherosclerosis and heart failure. However, the authors also found that sFLT1-/- mice still produced generous amounts of sFLT1, indicating compensatory production of sFLT1, perhaps through uncharacterized alternative splicing mechanisms or cleavage of membrane-bound VEGFR1 (38). A potential way to overcome compensatory sFLT1 production through alternative splicing could be a mouse model in which the FLT1 gene is deleted and an intron-less FLT1 isoform is re-expressed. Alternatively, it may be worthwhile to use modulators of alternative splicing, ectodomain shedding or cellular sFLT1 secretion for generating an inducible sFLT1-deficient model (39–41).

Furthermore, it will be critical to validate the role of sFLT1 in human chronic inflammation. Prior work has suggested that tissue sFLT1 levels are reduced in patients with CKD (36), based on reduced serum levels which were measured following heparin injection (releases vessel-bound sFLT1). Still, it would be important to validate this finding within human kidney tissues, and to correlate the localization of sFLT1 to specific cell types or lesions. Currently this is impossible, since there are no anti-

bodies available that specifically target the unique C-terminal tails of the different sFLT1 variants. Even though Invitrogen has aimed to produce an anti-sFLT1 clone (#36–1100), this antibody targets both soluble and membrane-bound FLT1 isoforms (42). Future work should prioritize the development of specific anti-sFLT1 antibodies.

3.3. *Separating sFLT1's Roles in Renotoxicity and Renoprotection*

In order for sFLT1 to be considered appropriate for clinical development for the treatment of chronic inflammatory conditions, it will be important to separate its therapeutic and nephrotoxic effects. Studies in this thesis show that an anti-inflammatory dose of sFLT1 does not harm renal function. A limitation of our work has been that we used gene transfection as the method of sFLT1 delivery, making it difficult to compare the administered sFLT1 dose to other studies. Other animal studies have also investigated the effects of sFLT1 administration on kidney function and/or kidney structure. In order to identify potential causes of the discordant effects of sFLT1 on kidney function, I have summarized the methods of sFLT1 administration and the renal outcomes of these studies in Table 1.

As indicated (Table 1), there are large differences between studies with respect to the type of sFLT1 used (e.g. full-length sFLT1 plasmid, injection of the FLT1 ectodomain protein or specific protein domains), the time of follow-up, the dosage of sFLT1 used and the achieved levels of sFLT1 following treatment. Few studies used similar administration methods, limiting the comparison of renal outcomes. Furthermore, a wide range of serum FLT1 concentrations was reported, likely reflecting differences both in treatment efficacy and serum sFLT1 measurement methods. The reported kidney phenotypes caused by sFLT1 treatment can be classified in three categories: no or a protective effect on renal function; glomerular endothelial PE-like damage; and tubulointerstitial damage (Table 1).

The glomerular endothelial damage phenotype seen following experimental sFLT1 treatment is characterized by severe proteinuria, glomerular endothelial swelling and a loss of glomerular endothelial fenestrations (Table 1), mimicking the clinical presentation of PE. In animal studies, this phenotype occurs within hours after sFLT1 administration, and is rapidly reversible following treatment cessation (43), (44). sFLT1 levels need to markedly exceed VEGF: high-dose sFLT1 treatment induced severe proteinuria 3h following injection, but low-dose sFLT1 or sFLT1/VEGF co-administration had no effects (43). Similarly, administration of low doses of sFLT1 in other studies also led to a less severe glomerular phenotype (28), (45). Notably, in patients with PE,

serum sFLT1 levels are comparable to those achieved by low-dose sFLT1 regimens in animal studies, and sFLT1 concentration is poorly correlated with the extent of renal lesions (27). This suggests that in humans, VEGF-inhibition-induced glomerular endothelial injury is also defined by other predisposing factors, including diabetes (46). Taken together, VEGF inhibition by sFLT1 dose-dependently injures the glomerular filtration barrier, underscoring the potential of sFLT1-lowering strategies for PE that are currently under clinical development (41). Future studies should identify predisposing factors that contribute to toxic effects of low-dose VEGF-inhibition on the glomerular filtration barrier.

For the tubulointerstitial damage phenotype, it is not fully clear whether these changes are specific or secondary to glomerular disease. Tubular involvement is rare in patients with PE (47). In healthy animals, continuous sFLT1 administration of 2 weeks caused tubulointerstitial damage (fibrosis, reduction peritubular capillaries, increased inflammation), as well as albuminuria (48), (49). The sFLT1 titers needed for inducing tubulointerstitial toxicity were higher (3 pmol/hour (48)) compared to those needed for an anti-inflammatory effect (9 pmol per day (37)). As posited by the authors, sFLT1 may induce a VEGF-deficiency in the tubulointerstitial compartment, thereby causing a loss of peritubular capillaries, tubular hypoxia and fibrosis. However, since the glomerular phenotype was not studied and these animals also had albuminuria, it is possible that the tubulointerstitial lesions are secondary to glomerular endothelial lesions. The same holds true for a study in glomerulonephritis, where prolonged treatment with sFLT1 caused both an early elevation in proteinuria and an aggravated progression to CKD (50). In contrast, in a DN model, high-dose treatment with the VEGF-binding FLT1 D2 domain increased tubulointerstitial fibrosis and renal dysfunction, while reducing albuminuria and glomerular damage (51). Hence, it is currently unclear whether sFLT1 directly damages peritubular capillaries, in line with discordant effects of other types of VEGF-inhibition on the peritubular capillary beds (52), (53). Further investigation will be needed to assess under which conditions VEGF-inhibition may induce tubulointerstitial toxicity.

Importantly, we found no evidence of glomerular or tubulointerstitial toxicity by sFLT1 in any of our studies. This was replicated in other studies where low-dose sFLT1 also led to a suppression in tissue inflammation, but did not induce renotoxicity (37), (54). Future experiments should study the therapeutic and toxic effects of sFLT1 in parallel. Ideally, an experimental chronic inflammation model would be treated with increasing conentrations of recombinant sFLT1 protein, along with clinically licensed VEGF inhibitors (e.g. nintedanib, aflibercept, sunitinib). Analysis of a fluorescently labelled sFLT1 compound may also allow the identification of cellular interactions of

sFLT1, possibly validating the interaction between sFLT1 and the tubulointerstitial compartment. These studies could hopefully answer outstanding questions concerning the therapeutic and toxic windows, and the optimal timing and duration of sFLT1 treatment.

TABLE 1 sFLT1 treatment and renal outcomes in vivo.

Kidney phenotype	Disease model	sFLT1 formulation	Administration	Treatment follow-up	Serum sFLT1	Renal outcome	Reference
No or reduced kidney damage	Chronic inflammation (APOC1 mice)	m-sFLT1-VSV plasmid	Transfection via electroporation	8 weeks	ND	No albuminuria Reduction glomerular macrophages	Chapter 4
	Mouse AKI-to-CKD	m-sFLT1-VSV plasmid	Transfection via electroporation	4 weeks	ND	No albuminuria; No IFTA, no PTC loss; Modest reduction renal inflammation	Chapter 5
	STZ-diabetic nephropathy (WT mice)	m-sFLT1-VSV plasmid	Transfection via electroporation	10 weeks	ND	Reduction albuminuria, glomerulosclerosis, podocyte protection, reduced glomerular macrophages	Bus (6)
		h-sFLT1 gene	Dox-inducible podocyte overexpression	10 weeks	ND (Increased urine sFLT1)	No albuminuria No effect on endothelial fenestrations Reduced glomerulosclerosis	Ku (55)
	Mouse CKD-associated atherosclerosis	h-FLT1(D1–3)-His protein	Intraperitoneal protein injection 15ng/g 3x/week	10 weeks	Serum 4 ng/ml vs. undetectable in control	No albuminuria No effect on blood pressure	Onoue (54)
	Mouse cardiac fibrosis	h-FLT1(D1–3)-His protein	Intraperitoneal protein injection 15ng/g daily	2 weeks	ND	No effect on blood pressure No albuminuria	Seno (37)

TABLE 1 sFLT1 treatment and renal outcomes in vivo. (*cont.*)

Kidney phenotype	Disease model	sFLT1 formulation	Administration	Treatment follow-up	Serum sFLT1	Renal outcome	Reference
	Ovarian cancer (nude mice)	h-FLT1-Fc fusion protein	Intraperitoneal protein injection 2000 ng 3x/week	4 weeks	ND	No albuminuria No effect on blood pressure	Miyake (56)
Glomerular PE-like damage	Healthy mice	m-FLT1-Fc fusion protein	Single injection of "low" or "high" dose, ND	3–24 hours	sFLT1:VEGF molar ratio: 1:1 (low-dose) or 10:1 (high-dose)	Low-dose: no effect High-dose: severe albuminuria, glomerular endothelial swelling and detachment, slit-diaphragm dysfunction	Sugimoto (43)
		Adenoviral m-sFLT1-Fc	Intravenous adenovirus injection	10 days	Plasma sFLT1 30–80 ng/ml vs. undetectable in control	Dose-dependent albuminuria and endotheliosis, increased blood pressure (in mice with serum sFLT1 > 50 ng/ml)	Bergmann (45)
		Adenoviral m-FLT1(D1–3)-His	Intravenous adenovirus injection	2 weeks	ND	Mild increase proteinuria Decreased GEC fenestrations, no effect on capillary number No hypertension Phenotype rapidly reversible following treatment cessation	Kamba (44)
	Pregnant rats	Adenoviral m-sFLT1-Fc	Intravenous adenovirus injection, low- and high-dose	2 weeks	Serum sFlt1 7.3 ng/mL (low), 216 ng/ml (high), control ND	Dose-dependent hypertension, albuminuria, glomerular endotheliosis	Maynard (28)

TABLE 1 sFLT1 treatment and renal outcomes in vivo. (*cont.*)

Kidney phenotype	Disease model	sFLT1 formulation	Administration	Treatment follow-up	Serum sFLT1	Renal outcome	Reference
Tubulointerstitial damage	Healthy mice	m-sFLT1-Fc fusion protein	300ng/h (osmotic minipump)	2 weeks	Plasma sFLT1 400 vs. 200 pg/mL; serum VEGF 14 vs. 24 pg/mL	Increased albuminuria, BUN, hypertension; Reduction PTC, increased IFTA, increased renal macrophages; reduced endothelial glycocalyx	Wewers (48), (49), (57)
	Anti-GBM nephritis	m-sFLT1 plasmid	Intramuscular transfection with electroporation	7 weeks	Serum ND, increase in urine	Mild increase in proteinuria, glomerulosclerosis, IFTA, and reduction capillaries; no effect on renal inflammation	Hara (50)
	Db/db-diabetic nephropathy	h-FLT1(D2)-IgG1-Fc adenovirus	Intramuscular transfer	8 weeks	Serum sFLT1 400ng/ml (5–10 fold vs. control)	Increased BUN, IFTA, reduction PTC and tubular VEGF; Reduction proteinuria (increase in control mice), podocyte protection; no effect blood pressure	Kosugi (51)

m-FLT1: murine FLT1, h-FLT1: human FLT1. D: domain. ND not determined.

4. Concluding Remarks

CKD has a major impact on the mortality and quality of life of patients. A large body of evidence indicates a major role of chronic inflammation in the progressive fibrosis underlying CKD. In response, many studies have aimed to identify and target proinflammatory factors in kidney disease. This thesis evaluated the feasibility of stimulating the function of two known anti-inflammatory factors, TM and sFLT1. A dysregulation of TM was shown to elicit chronic inflammation and endothelial dysfunction in the kidneys of patients with diabetic and preeclamptic glomerulopathies. Furthermore, using mouse models of chronic inflammation, treatment with sFLT1 effectively protected against chronic inflammation and tissue damage. Our findings, combined

with the large amount of recent knowledge on pathophysiological mechanisms underlying kidney inflammation and fibrosis, are reasons for optimism. Together this work provides a basis for further development of TM and sFLT1, among other anti-inflammatory strategies, to prevent disease progression of patients with CKD.

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