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Endothelial dysfunction and inflammation in diabetic nephropathy

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Chapter 7

Summary, General discussion, and Perspectives

SUMMARY

Diabetic nephropathy is one of the major causes of end-stage renal disease and is typically characterised by vascular damage. Although traditional therapies such as glycaemic control have been found to be beneficial, at least to some degree[1], the percentage of patients with diabetes who progress to end-stage renal disease has remained unchanged over the past two decades. Therefore, there is an urgent need for new preventive and therapeutic options for diabetic patients, particularly patients with diabetic nephropathy. Developing new treatment strategies requires a better understanding of the mechanisms that lead to the development of diabetic nephropathy. In the work described in this thesis, we investigated various mechanisms by which the immune system plays a role in the development of diabetic nephropathy.

A meta-analysis performed by our group revealed that a polymorphism in the apolipoprotein C1 gene (*APOC1*), which encodes apolipoprotein C-I (apoCI), is associated with an increased risk of developing diabetic nephropathy[2]. Recently, Bouillet *et al.* reported that diabetic patients have higher plasma levels of apoCI compared to non-diabetic subjects[3]. In addition, apoCI increases the inflammatory response in activated macrophages *in vitro*[4], and this increased response can exacerbate the development of diabetic nephropathy[5]. In the work described in **Chapter 2** of this thesis, we investigated whether overexpressing *APOC1* leads to the development of kidney damage using transgenic mice that overexpress the human *APOC1* gene (*APOC1*-tg mice); wild-type littermates served as the control group; in addition, we investigated the role of macrophages in this process. To complement these findings, we also examined the presence of glomerular apoCI deposits in renal autopsy material obtained from diabetic patients with and without diabetic nephropathy, as well as material from non-diabetic controls. We found that *APOC1*-tg mice – but not wild-type mice – develop albuminuria, kidney dysfunction, and nodular glomerulosclerosis; *APOC1*-tg mice also have increased numbers of glomerular inflammatory M1 macrophages. Compared to wild-type macrophages, *APOC1*-tg macrophages also have an increased inflammatory response upon activation *in vitro*. Furthermore, we found

that patients with diabetic nephropathy have a higher prevalence of glomerular apoCI deposits compared to both diabetic patients without nephropathy and non-diabetic controls. These apoCI deposits were co-localised with glomerular macrophages. Taken together, these results suggest that apoCI may exacerbate the development of diabetic nephropathy by increasing the inflammatory response in activated glomerular macrophages. Therefore, apoCI may represent a promising new therapeutic target for patients who are at risk for developing diabetic nephropathy.

Vascular endothelial growth factor A (VEGF-A) plays a role in the migration of both monocytes and macrophages and facilitates the extravasation of leukocytes by activating endothelial cells[6]. In animal models, glomerular VEGF-A levels are higher in animals with diabetes compared to non-diabetic controls[7], and glomerular VEGF-A levels are correlated with increased severity of diabetic nephropathy[8]. In the work described in **Chapter 3** of this thesis, we investigated whether treatment with soluble fms-like tyrosine kinase-1 (sFLT-1), an inhibitor of VEGF-A, can reduce diabetes-induced renal pathology in type 1 diabetic mice. The severity of diabetic nephropathy was measured in diabetic mice that were transfected with the *sFlt-1* gene 15 weeks after the induction of diabetes; these findings were compared to an age-matched, non-transfected group of diabetic mice. We found that overexpressing *sFlt-1* in diabetic mice significantly improves kidney function, resolves diabetes-related kidney damage, and reduces endothelial cell activation and inflammation; specifically, treatment reduced the number of glomerular macrophages and reduced the glomerular levels of tumour necrosis factor alpha (TNF-alpha) protein. *In vitro,* sFLT-1 decreases VEGF-A–induced endothelial cell activation. Taken together, these data suggest that inhibiting VEGF-A with sFLT-1 can reduce the severity of diabetic nephropathy, thereby reducing glomerular inflammation and supporting cellular repair mechanisms.

Activation of endothelial cells is a key factor in inflammation. Upregulation of surface activation markers on endothelial cells leads to an increase in leukocyte extravasation[9]. Both VEGF-A and endoglin are critical mediators of endothelial cell function and health, and both are associated with endothelial cell activation[10,11]. Although research has suggested that VEGF-A and endoglin interact[12], the precise

mechanism by which these two proteins interact is currently unclear. In the work described in **Chapter 4** of this thesis, we investigated: *i*) whether glomerular endoglin expression is associated with diabetic nephropathy; *ii*) whether reducing endothelial endoglin expression affects endothelial cell activation and/or monocyte adhesion, and – if so – by which mechanism; and *iii*) whether glomerular endoglin expression is correlated with endothelial cell activation in patients with diabetic nephropathy. Compared to non-diabetic control mice, we found that diabetic mice have increased glomerular levels of endoglin protein, localised primarily in glomerular capillary walls. *In vitro*, compared to endothelial cells expressing normal levels of endoglin, endothelial cells that express approximately 30% lower levels of endoglin have reduced activation upon stimulation with VEGF-A, as well as reduced monocyte adhesion. Reducing endoglin expression also increases the activation of the Akt serine/threonine kinase (Akt) upon VEGF-A stimulation, thereby reducing ATF-2 (activating transcription factor 2)–mediated expression of endothelial cell activation markers. Furthermore, we found that the expression of glomerular vascular cell adhesion molecule-1 (VCAM-1) is significantly higher in patients with diabetic nephropathy compared to non-diabetic controls. Interestingly, the glomerular level of endoglin protein was correlated with the glomerular level of VCAM-1 protein in these patients. These data suggest that targeting endoglin – thereby reducing endothelial cell activation and subsequent inflammation – may have therapeutic value in patients who are at risk for developing diabetic nephropathy.

In patients with diabetes, activation of the complement system plays a role in the development of complications in a variety of organs[13]. However, the role of the complement system in diabetic nephropathy is poorly understood. In the work described in **Chapter 5** of this thesis, we studied the prevalence of specific complement components in renal vascular compartments in a large autopsy cohort consisting of diabetic patients both with and without diabetic nephropathy. We found that complement activation is associated with several factors, including more severe classes of diabetic nephropathy, reduced kidney function, and the presence of histological lesions; these results were supported by examining a small cohort of renal biopsies obtained from patients with diabetic nephropathy. Specifically,

we found a high prevalence of glomerular IgM and complement factor C1q deposits, as well as an absence of glomerular mannose-binding lectin (MBL) deposits. These findings suggest that the classical pathway of the complement system plays a role in the development of diabetic nephropathy.

APOC1-tg mice, which express human *APOC1*, develop nodular glomerulosclerosis by 15 months of age. This relatively slow disease progression makes this model less suitable for studying therapeutic and preventive interventions. To overcome this limitation, as described in **Chapter 6** of this thesis, we attempted to accelerate the progression of glomerulosclerosis in *APOC1*-tg mice by transfecting these mice with *sFlt-1* at 8 weeks of age; we then asked whether systemically increasing sFLT-1 expression for 15 weeks accelerates the development of glomerulosclerosis. We found that transfecting *APOC1*-tg mice with *sFlt-1* does not accelerate the development of glomerulosclerosis within the time period studied. In contrast, we found that *sFlt-1*–transfected *APOC1*-tg mice have fewer glomerular macrophages compared to non-transfected *APOC1*-tg mice. These data suggest that sFLT-1 treatment has an anti-inflammatory effect.

GENERAL DISCUSSION

Diabetes mellitus develops when pancreatic β-cells are functionally impaired or lost, which leads to reduced or absent levels of insulin production, or when cells become less sensitive to insulin. Consequently, changes in insulin secretion, changes in insulin sensitivity, or both, lead to perturbations in glucose homeostasis, which – via various processes – leads to the development of clinical complications, including nephropathy.

Clinically, hyperglycaemia can be controlled using a variety of therapies, including: *i*) the administration of exogenous insulin; *ii*) the use of drugs to either increase insulin secretion or decrease the release of glucose from the liver; *iii*) the use of drugs to increase the utilisation of glucose by skeletal muscle and fat; and *iv*) delaying the absorption of dietary glucose[14]. Studies have found that early, intensive glucose control therapy in patients diagnosed with type 1 or type

2 diabetes can reduce the development of microvascular complications, lower the risk of impaired glomerular filtration rate, and slow the progression towards end-stage renal disease[15-17]. However, a recent meta-analysis by Ruospo *et al.* revealed that both type 1 and type 2 diabetic patients who follow an intensive glucose control regimen have the same risk of kidney failure, death, and cardiovascular events as patients who follow a less stringent blood glucose control regimen[18]; this finding may be attributed to the negative side effects associated with intensive glucose control (e.g. hypoglycaemia). Moreover, only marginal benefits were obtained with respect to the onset and progression of microalbuminuria and myocardial infarction. In addition, although the progression of diabetic nephropathy can be delayed by standard treatment regimens such as glucose control therapy, the percentage of patients who develop end-stage renal disease has not decreased over the past two decades[1]. Taken together, these findings suggest that other factors – acting either alone or in combination with hyperglycaemia – play an important role in the development of diabetic nephropathy. Therefore, new preventive and therapeutic strategies are urgently needed. To reach this goal, a better understanding of the mechanisms underlying the development and reversal of diabetic nephropathy is needed.

One mechanism by which hyperglycaemia may lead to diabetic nephropathy is by increasing inflammation at both the local and systemic levels. In the work described in this thesis, we investigated a variety of mechanisms by which the immune system may play a role in the development of diabetic nephropathy.

Apolipoprotein C1

ApoCI regulates lipid metabolism by slowing the catabolism of triglyceride-rich lipoproteins, primarily by inhibiting the enzyme lipoprotein lipase (LPL), thereby increasing plasma triglyceride levels[19]. ApoCI also plays a role in inflammation by increasing the binding of lipopolysaccharide (LPS) to macrophages via CD14/MD2/TLR4 (cluster of differentiation 14/myeloid differentiation protein-2/toll-like receptor 4), thereby increasing the expression of TNF-alpha by these cells[4,20]. This is relevant in the setting of diabetes, as diabetic patients have

increased plasma levels of LPS due to altered gut microbiota; specifically, these patients have increased numbers of Gram-negative bacteria and increased gut permeability, a condition known as metabolic endotoxaemia[21]. Furthermore, these patients can also have increased numbers of renal glomerular and interstitial macrophages[22]. The production of TNF-alpha by glomerular macrophages has been proposed to serve as the key mediator of diabetic nephropathy[5].

The finding that a polymorphism in the *APOC1* gene is associated with an increased risk of developing diabetic nephropathy, as well as the finding that plasma apoCI levels are elevated in patients with type 1 or type 2 diabetes compared to non-diabetic controls, supports the notion that apoCI plays a role in the development of diabetic nephropathy. Taken together, these data suggest that apoCI facilitates the development of diabetic nephropathy by promoting hyperlipidaemia, by increasing the inflammatory response in activated glomerular macrophages, or both.

As discussed in **Chapter 2** of this thesis, *APOC1* overexpression is causally associated with the development of glomerulosclerosis. *APOC1*-tg mice (which overexpress the human *APOC1* gene) develop nodular glomerulosclerosis, which is reminiscent of patients with class III diabetic nephropathy. Our data suggest that hyperlipidaemia may not be the driving force behind the development of glomerulosclerosis in these mice, as we found no difference between *APOC1*-tg mice and wild-type mice with respect to renal cortex triglycerides, total cholesterol, or phospholipid levels. In contrast, we found higher numbers of glomerular macrophages in *APOC1*-tg mice compared to wild-type mice, and peritoneal macrophages isolated from *APOC1*-tg mice develop an increased inflammatory response upon LPS stimulation *in vitro* compared to wild-type macrophages. This finding may have clinical relevance, as we also found that the prevalence of glomerular apoCI deposits is higher among patients with diabetic nephropathy compared to both diabetic patients without nephropathy and non-diabetic control subjects. Moreover, in patients with diabetic nephropathy these apoCI deposits co-localise with glomerular macrophages. Taken together, this suggests that glomerular macrophages in patients with diabetic nephropathy may also have an increased inflammatory response; this response is likely augmented by apoCI, and – consistent

with our findings with *APOC1*-tg mice – may facilitate the development of diabetic nephropathy. Therefore, reducing the expression of *APOC1* may be a viable therapeutic approach for delaying or even preventing the development of diabetic nephropathy by reducing the production of inflammatory cytokines in glomerular macrophages.

The use of statins has been shown to benefit diabetic patients by lowering low-density lipoprotein (LDL) cholesterol levels[23]. Interestingly, a genome-wide association study found that changes in LDL cholesterol levels following statin therapy are associated with a polymorphism in the *APOC1* gene[24]. Statins may be clinically beneficial to diabetic patients by reducing *APOC1* expression, particularly in macrophages (Figure 1). This notion has been confirmed in *in vitro* experiments in which treating macrophages with statins reduces the expression and secretion of apoCI by these cells[25]. Thus, the statin-induced decrease in plasma lipids (due to the effect of apoCI on lipid metabolism, as discussed above) may be an indirect – albeit positive – side effect, and statins may provide clinical benefits by reducing both local and systemic inflammation.

With the exception of an increased risk of developing new-onset diabetes mellitus, no side effects have been reported in statin-treated patients with chronic kidney disease[26] or cardiovascular disease[27]. This suggests that statins are relatively safe for use in patients. Therefore, statins should be considered for all patients with diabetes, as this treatment may prevent or delay the development of diabetic nephropathy in these patients by reducing apoCI-mediated inflammation; this approach may be most beneficial to diabetic patients who have the specific polymorphism in the *APOC1* gene associated with an increased risk of developing diabetic nephropathy.

Despite these promising findings, further study is needed in order to determine the precise role of apoCI in the pathogenesis of diabetic nephropathy, the precise mechanism(s) by which overexpressing *APOC1* facilitates the development of glomerulosclerosis, and the potential clinical value of therapeutic and preventive interventions for diabetic patients with and without diabetic nephropathy. The induction of diabetes in this model is a first step towards investigating the putative role of apoCI in the development of diabetic nephropathy. However, this model is not ideally suited for addressing these ques-

tions, as it takes up to 15 months for *APOC1*-tg mice to develop nodular glomerulosclerosis. Furthermore, using a modified model in which *APOC1*-tg mice develop glomerulosclerosis more rapidly, macrophage-depletion techniques and/or bone marrow transplantation between *APOC1*-tg and wild-type mice may provide important insight with respect to the relative contribution of apoCI in lipid metabolism and/or inflammation as the driving force in the development of glomerulosclerosis in this model.

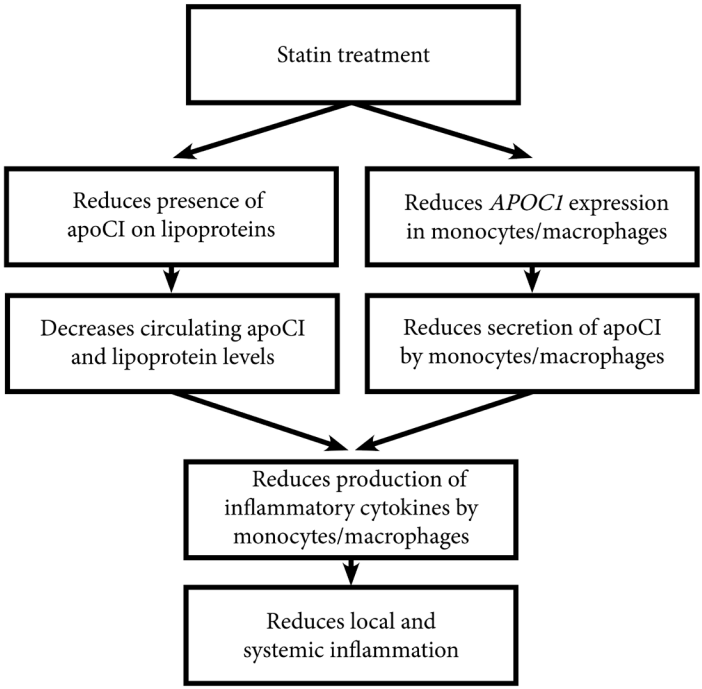

Figure 1: Hypothetical scheme by which statins may confer clinically beneficial effects. Statins reduce expression of the *APOC1* gene, reducing apoCI in lipoproteins, thereby reducing the inhibitory effect of apoCI on lipid uptake and lowering serum lipoprotein levels. Reduced *APOC1* expression also reduces the release of apoCI by monocytes/macrophages. Reduced circulating apoCI levels – by reduced apoCI on lipoproteins, by reduced secretion of apoCI by monocytes/macrophages, or both – leads to diminished activation of monocytes/macrophages, thereby reducing both local and systemic inflammation.

Vascular endothelial growth factor A

VEGF-A is a critical factor in the maintenance and survival of endothelial cells and is also involved in the migration of monocytes and macrophages[6]. Glomerular VEGF-A levels are tightly regulated in order to ensure that they remain at physiological levels; if these levels are either too high or too low, kidney disease can occur, primarily due to changes in the renal endothelium[28].

Glomerular VEGF-A levels are higher in animal models of diabetic nephropathy compared to healthy control animals. Moreover, increased glomerular VEGF-A levels are associated with more severe kidney damage in a mouse model of type 1 diabetes[8]. Treating animal models of diabetes with anti-VEGF-A prevents the development of albuminuria and glomerular hypertrophy[29-31]; however, the results are inconsistent[32], possibly due to differences in the type and/or dose of anti-VEGF-A therapy used. Nevertheless, whether anti-VEGF-A treatment can reverse existing diabetes-related kidney damage in diabetic animals has not been studied, and whether this treatment can reduce the number of glomerular macrophages remains unknown. In the work described in **Chapter 3** of this thesis, we show that treating diabetic mice with the VEGF-A inhibitor sFLT-1 restores kidney function and morphology, likely by reducing the glomerular infiltration of macrophages.

In cancer patients, treatment with anti-VEGF-A antibodies (e.g. bevacizumab) has been shown to be effective as an anti-cancer therapy[33]; however, these antibodies can increase the risk of proteinuria and hypertension in these patients. Therefore, use of these antibodies would not be suitable for patients with diabetic nephropathy, as it may actually worsen their diabetic nephropathy, rather than providing a treatment. In contrast, treating diabetic mice with sFLT-1 restored albuminuria to normal levels, suggesting superior performance compared to anti-VEGF-A antibodies. It is important to note, however, that the patient's levels of sFLT-1 should be monitored closely, as a long-term increase in systemic sFLT-1 levels can lead to proteinuria, kidney lesions, or both, primarily by affecting the function of endothelial cells[34].

In the work described in **Chapter 6**, we tested the hypothesis that transfecting 8-week-old *APOC1*-tg mice with *sFlt-1* can accelerate the

development of glomerulosclerosis, thereby providing a potentially more suitable model for studying therapeutic and preventive interventions; thus, we effectively induced a long-term systemic increase in sFLT-1 levels in these mice. However, rather than accelerating the development of glomerulosclerosis in *APOC1*-tg mice, overexpressing sFLT-1 for 15 weeks had no effect on kidney morphology or function compared to age-matched, non-transfected *APOC1*-tg mice; specifically, transfected mice did not develop glomerulosclerosis within the investigated time frame. In contrast, *APOC1*-tg mice that were transfected with *sFlt-1* had significantly fewer glomerular macrophages compared to age-matched, non-transfected *APOC1*-tg mice. Furthermore, atopic dermatitis – a skin disease characterised by scaling, lichenification, excoriation, and pruritus – develops in *APOC1*-tg mice by six weeks of age due to an infiltration of inflammatory cells, including macrophages[35], and this was completely resolved in *APOC1*-tg mice following *sFlt-1* transfection.

The aforementioned findings, which are described in **Chapters 3** and **6** of this thesis, suggest that treatment with sFLT-1 can reduce inflammation in the kidney by antagonising VEGF-A–induced activation of glomerular endothelial cells and migration of monocytes and macrophages. However, *APOC1*-tg mice that overexpress sFLT-1 still have increased activation of glomerular endothelial cells, and renal *Vegf-a* expression in these mice was similar to age-matched wild-type mice. This finding suggests that the reduced kidney inflammation following sFLT-1 treatment may be independent of inhibited VEGF-A. It is therefore interesting to speculate that sFLT-1 has functions other than sequestering VEGF-A, suggesting that an sFLT-1–based therapy may be more effective than therapies based on anti-VEGF-A antibodies, which only inhibit VEGF-A signalling.

The first evidence in support of this hypothesis was published recently by Jin *et al.*[36], who reported that sFLT-1 binds directly – and independently of VEGF-A – to lipid rafts in podocytes via glycosphingolipid monosialodihexosylganglioside, thereby modulating the morphology and function of these cells. sFLT-1 can bind to other cell types as well, including endothelial cells, thus supporting the notion that sFLT-1 has a direct effect on these cells. Interestingly, monocytes also express glycosphingolipid monosialodihexosylganglioside, and its

expression in monocytes is increased upon differentiation to macrophages[37] and upon exposure to inflammatory stimuli[38], suggesting that sFLT-1 may be able to bind to these cells via a similar process as it binds to podocytes (i.e. via glycosphingolipid monosialodihexosylganglioside). Furthermore, preincubating leukocytes with sFLT-1 prevents their subsequent VEGF-A–stimulated migration by downregulating the *FLT-1* promoter[39], thereby preventing leukocyte migration via a mechanism independent of sequestering VEGF-A. sFLT-1 has been used to treat various immune-mediated diseases, including vascular disease[40,41], arthritis[42,43], sepsis[44], and psoriasis[45], by reducing both local and systemic inflammation, by reducing the number of tissue macrophages, or both. Therefore, we hypothesise that sFLT-1 – in addition to functioning as a 'decoy' receptor for VEGF-A – modulates the inflammatory response in monocytes and macrophages. Preliminary data from our group support this hypothesis. For example, we found that differentiating monocytes into macrophages in the presence of sFLT-1 alters the morphology of the resulting macrophages compared to the morphology of monocytes differentiated in the absence of sFLT-1. In addition, when stimulated with LPS, macrophages that were differentiated in the presence of sFLT-1 have a different inflammatory response compared to macrophages that were differentiated in the absence of sFLT-1. Specifically, they have reduced expression of TNF-alpha and IL-6. These data support our hypothesis that sFLT-1 can modulate the immune response by regulating monocyte differentiation and macrophage-mediated inflammation.

Vascular endothelial growth factor A, endoglin, and endothelial cell activation

Activation of glomerular endothelial cells is a key step in the development of kidney inflammation[9]. Therefore, maintaining the health and function of glomerular endothelial cells is an important component in preventing kidney disease. VEGF-A is an important mediator of a variety of processes in endothelial cells, including their activation, proliferation, and migration, as well as angiogenesis[6]. Endoglin has been proposed to play a role in signalling via VEGF receptor 2 (VEGFR2), thereby modulating the response of endothelial cells upon sti-

mulation with VEGF-A. This notion is supported by the finding that endoglin and VEGFR2 are co-localised in endosomes, and by the finding that endoglin prevents arteriovenous malformation by changing blood flow–induced cell migration[12]. Finally, VEGF-A–induced angiogenesis is impaired in endoglin-deficient endothelial cells[11].

In the work described in **Chapter 4** of this thesis, we show that endoglin plays a role in the activation of endothelial cells and consequently affects adhesion between endothelial cells and leukocytes. Under physiological conditions, VEGF-A activates endothelial cells by inducing phosphorylation of VEGFR2 and the downstream kinase ERK1/2 (extracellular signal-regulated kinases 1 and 2), which phosphorylates ATF-2 in the nucleus, activating this transcription factor. Activated ATF-2 then drives the expression of endothelial cell activation markers[46]. Our data show that endothelial cells with reduced endoglin expression have increased levels of phosphorylated Akt and decreased levels of activated ATF-2 upon stimulation with VEGF-A (Figure 2), resulting in impaired activation of these cells compared to endothelial cells that express normal levels of endoglin.

Upon binding VEGF-A, VEGFR2 associates with the proto-oncogene tyrosine-protein kinase Src (Figure 2A)[47]. In the presence of VEGF-A, Src also induces the internalisation and degradation of endoglin via lysosomes. The association between Src, VEGFR2, and endoglin is a critical step in angiogenesis as well as other VEGF-A–induced endothelial cell functions, including proliferation, migration, and capillary tube formation[48]. Furthermore, endoglin co-localises with VEGFR2 in specific endosomes[12], promoting the activation of ERK1/2 and the subsequent activation of endothelial cells[49]. Taken together, these findings suggest that endoglin is associated with VEGFR2 at the cell surface upon stimulation with VEGF-A (possibly via Src) and that endoglin is critical for the internalisation and signalling activity of VEGFR2.

On the other hand, reduced levels of endoglin protein may prevent the internalisation of VEGFR2 (Figure 2B). This notion is supported by the increased levels of activated Akt in endothelial cells with reduced endoglin expression. At the cell surface, Akt – but not ERK1/2 – can be activated via membrane-bound VEGFR2[49]. In addition, recycling of internalised VEGFR2 to the plasma membrane increases when endoglin expression is reduced (i.e. VEGFR2 degradation is reduced)[12], possibly

via reduced endoglin-mediated lysosomal degradation of VEGFR2[48]. The resulting increased level of activated Akt inhibits the phosphorylation of ATF-2, thereby reducing endothelial cell activation. This suggests that endoglin mediates the activation – and other cellular functions – of endothelial cells following VEGF-A stimulation by driving the internalisation and downstream signalling of VEGFR2.

Interestingly, the anti-endoglin antibody TRC105 reduces endoglin levels at the cell surface via a Src-mediated process and increases the internalisation and degradation of endoglin (Figure 2C), thereby preventing VEGF-A–induced angiogenesis[48], possibly due to decreased internalisation of VEGFR2 and reduced signalling via ERK1/2.

Although TRC105 is a promising treatment option for patients with a variety of cancer types[50], a common adverse event is telangiectasias[51], which causes capillary malfunction. Interestingly, telangiectasias can also develop in patients with a mutation in either activin receptor-like kinase 1 (ALK1) or ALK5 – thus affecting endoglin-mediated signalling via TGF-beta (transforming growth factor beta) – as well as in heterozygous endoglin-deficient mice[52]. This suggests that – like VEGF-A – the levels of endoglin must be maintained at physiological levels. Interestingly, Gordon *et al.* recently reported that some cancer patients whose cancer progressed when treated with bevacizumab (an anti-VEGF-A antibody) alone experienced a reduction in tumour volume when treated with both bevacizumab and TRC105[53]. Although patients receiving this combination therapy developed hypertension and proteinuria, these and other side effects were less severe than when patients received monotherapy with either bevacizumab or TRC105 alone, even though both drugs in the combination therapy were given at their respective recommended monotherapy doses. This finding suggests that the combination therapy provided superior regulation of the endoglin and VEGFR2 signalling pathways. Our own data show that treatment with sFLT-1 is clinically beneficial in terms of resolving kidney lesions and by restoring kidney function in diabetic mice. Furthermore, we found that endoglin is associated with glomerular endothelial cell dysfunction in patients with diabetic nephropathy and plays a role in endothelial cell activation and monocyte adhesion *in vitro*. Therefore, a combination of anti-endoglin and anti-VEGF-A (i.e. sFLT-1) may have therapeutic benefits in patients with diabetic

nephropathy and warrants future study.

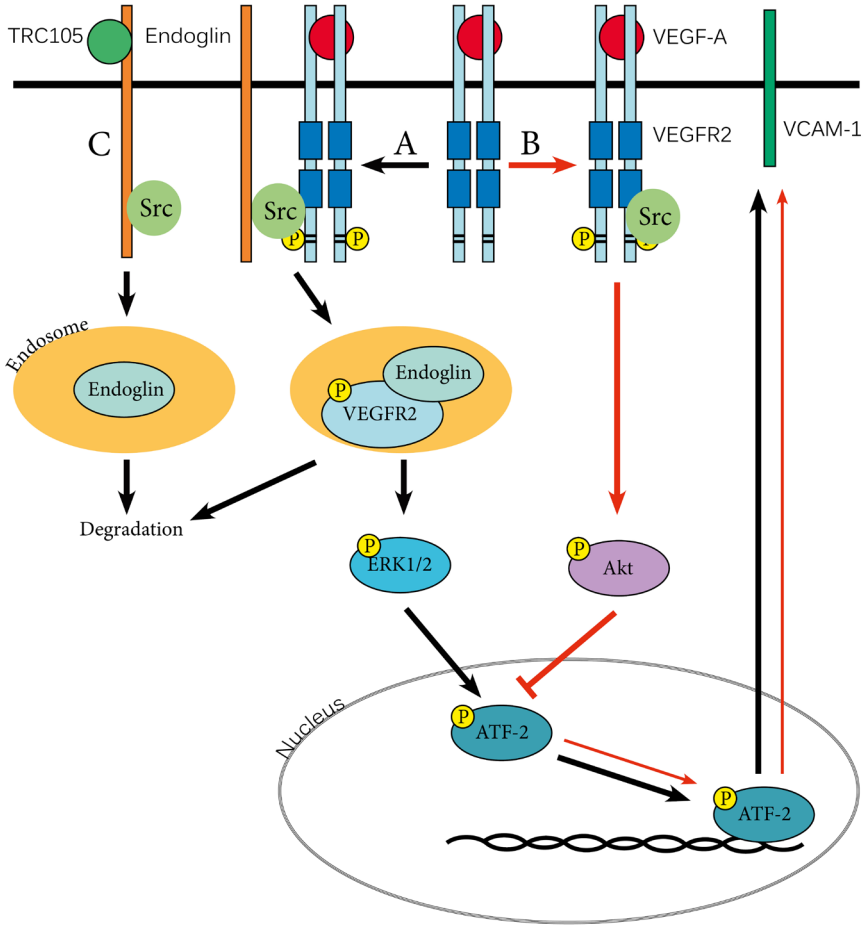

Figure 2: Schematic representation of VEGF-A–induced endothelial cell activation. A. VEGF-A binds to VEGFR2, which in turn becomes phosphorylated (P) and associates with both Src and endoglin. Phosphorylated VEGFR2 is then internalised via endosomes and subsequently activates ERK1/2. Phosphorylated ERK1/2 then translocates to the nucleus, where it activates the transcription factor ATF-2, thereby activating the endothelial cell. B. In the absence of endoglin, activated VEGFR2 remains at the cell surface and activates Akt, which then inhibits the phosphorylation of ATF-2, thereby reducing endothelial cell activation. C. Binding of the anti-endoglin antibody TRC105 to endoglin results in the Src-mediated internalisation and degradation of endoglin, thereby preventing VEGFR2 internalisation and the subsequent activation of the endothelial cell.

Complement activation

The complement system is another important mediator of endothelial cell dysfunction and vascular disease. The complement system plays a role in both innate and adaptive immunity and consists of three pathways that are activated by specific stimuli. Several components in the complement system play a role in endothelial dysfunction and vascular disease in patients with diabetes[54-56]; these components include soluble C3a and C5a (protein fragments that result from the cleavage of C3 and C5, respectively), as well as C5b-9. Given that endothelial cells express receptors for C3a and C5a, as well as complement regulators on the cell surface[57,58], these cells are likely a direct target of the complement system. Although the complement system itself can give rise to complement-mediated damage[59], complement activation can also lead to the recruitment of other immune cells such as macrophages via C3a and C5a. These macrophages may then promote the development of kidney damage via the production of macrophage-derived inflammatory cytokines, including TNF-alpha[5].

Evidence suggests that the complement system plays a role in the development of diabetes and diabetes-related complications. For example, Hillian *et al.* reported that activation of the classical complement system plays a role in the development of insulin resistance, as C1q-deficient mice are protected from hepatic insulin resistance and complement activation induced by consuming a high-fat diet[60]. In addition, intracellular signalling via CD59 (cluster of differentiation 59) – also an inhibitor of C5b-9 formation – in pancreatic islet cells is required for the efficient release of insulin, as siRNA-mediated knockdown of CD59 prevents this release[59]. CD59 expression in pancreatic islet cells is decreased in diabetic animals[61] compared to non-diabetic controls, suggesting that this downregulation of CD59 in pancreatic islet cells in diabetes contributes to altered glucose metabolism by decreasing insulin production.

In the work described in **Chapter 5** of this thesis, we show that complement activation is correlated with the severity of diabetic nephropathy; specifically, the prevalence of complement activation is higher in patients with more severe diabetic nephropathy and is associated with decreased eGFR (estimated glomerular filtration rate). In addition, we show that the prevalence of complement deposits is

higher in patients with type 1 diabetes than in patients with type 2 diabetes; this may be due to the longer average disease duration in patients with type 1 diabetes, as these patients are exposed to hyperglycaemia longer than type 2 diabetic patients, and this could lead – either directly or indirectly – to activation of the complement system. Our data suggest that the classical complement pathway is activated in patients with diabetic nephropathy, as glomerular C1q deposits are highly prevalent among these patients; in contrast, glomerular deposits of MBL were observed only rarely in these patients. Additional evidence that the classical complement pathway is activated in these patients comes from the finding that the prevalence of glomerular IgM deposits is significantly higher in patients with diabetic nephropathy than in non-diabetic subjects, and these glomerular IgM deposits both co-localise and are correlated with glomerular C1q and C4d deposits.

These data suggest that complement activation via naturally occurring antibodies (i.e. IgM) may be the result of renal vascular damage in patients with diabetic nephropathy. IgM antibodies play an important role in clearing damaged cells via intracellular antigens that become externalised during apoptosis, or under hypoxic conditions, or both. Binding of IgM antibodies to hypoxic or apoptotic cells may therefore serve to activate the complement system in patients with diabetic nephropathy[62-66]. This notion is supported by the finding that the presence of C4d and IgM is highly prevalent among patients with other renal microangiopathies[67,68].

Nevertheless, whether complement activation is the cause or the consequence of diabetic nephropathy remains unclear and should be addressed in a prospective cohort study. Regardless, complement activation clearly contributes to the progression of diabetic nephropathy, as rats with type 2 diabetes treated with complement inhibitors have reduced albuminuria, fewer histological changes, and improved kidney function compared to untreated, type 2 diabetic rats[69]. The increasing body of evidence showing the activation and/or involvement of the complement system in diabetic nephropathy suggests that inhibiting the complement system may represent a viable therapy for preventing, slowing, or even reversing diabetic nephropathy. Given that complement inhibitors such as eculizumab are already approved and have been shown to be clinically beneficial in several diseases, and given

that many new inhibitors are now being tested in clinical trials[13,70], clinical trials designed specifically to test the effect of inhibiting the complement system in patients with diabetic nephropathy may be performed in the near future.

FUTURE PERSPECTIVES

Diabetes mellitus has traditionally been considered a metabolic disease characterised by obesity, high blood glucose levels, high blood pressure, high serum triglyceride levels, and low levels of high-density lipoproteins. However, evidence that the immune system plays a role in this disease and in diabetes-related complications suggests that the picture is more complicated than previously believed. Although current therapies designed to treat patients with diabetic nephropathy – including glycaemic control, blood pressure control, and reducing serum lipids – are clinically beneficial to some degree, they remain insufficient for reducing the risk of these patients progressing to end-stage renal disease[1], giving rise to the notion that other factors must also be involved in the pathogenesis of diabetic nephropathy. The results of the work described in this thesis suggest that the focus of treatment strategies for patients with diabetic nephropathy may need to shift towards interfering with the immune system in order to reduce kidney inflammation.

Although the research described in this thesis provides several promising new targets (e.g. apoCI, the complement system, endoglin, and sFLT-1) for treating diabetic nephropathy with respect to inhibiting the immune system and reducing kidney inflammation, several questions remain. Our results demonstrate that sFLT-1 treatment reduces kidney inflammation and restores kidney morphology and function. However, future research should investigate whether the beneficial properties of sFLT-1 are mediated by sequestering VEGF-A (i.e. an indirect effect on immune cells) or by binding to leukocytes (i.e. a direct effect on immune cells). If sFLT-1 has a direct immune-modulating effect (as we hypothesise), then sFLT-1 treatment may also be beneficial for patients with other diseases in which inflammation plays a key role. Importantly, the optimal dose of sFLT-1 should

be determined, as a long-term increase in systemic sFLT-1 levels can result in kidney damage.

Interestingly, treating cancer patients with both anti-endoglin and anti-VEGF-A is more beneficial than either treatment alone, although the underlying mechanism is currently unclear. Although our results provide important mechanistic insights, future studies should be designed in order to determine the precise mechanism by which VEGF-A, endoglin, and VEGFR2 interact. Nevertheless, the addition of anti-endoglin antibodies to sFLT-1 treatment may counteract the potential side effects of sFLT-1 monotherapy. In addition, prolonged immunosuppressive therapy can result in complications that may limit the effectiveness of sFLT-1 treatment, including endothelial dysfunction and infection. Furthermore, given that complement activation contributes to the development of diabetic nephropathy, prospective studies designed to investigate complement deposits in diabetic nephropathy may reveal whether complement activation is the cause or the consequence of diabetic nephropathy. This would be an important finding, as it would offer insight into the treatment window during which complement inhibitors should be given to diabetic patients (i.e. before or after these patients develop diabetic nephropathy). In the short term, we recommend that patients with diabetic nephropathy – as well as patients who are at risk for developing diabetic nephropathy – receive statins, as this treatment is relatively save and is beneficial in terms of preventing and/or treating cardiovascular disease in general. Furthermore, statins may also be beneficial for treating diabetic patients by reducing apoCI-mediated kidney inflammation.

Our central conclusion based on the work described in this thesis is that the immune system plays a clear role in the development and progression of diabetic nephropathy. Therefore, future studies regarding the treatment and prevention of diabetic nephropathy should focus on targeting the immune system and kidney inflammation.

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