Endothelial dysfunction and inflammation in diabetic nephropathy

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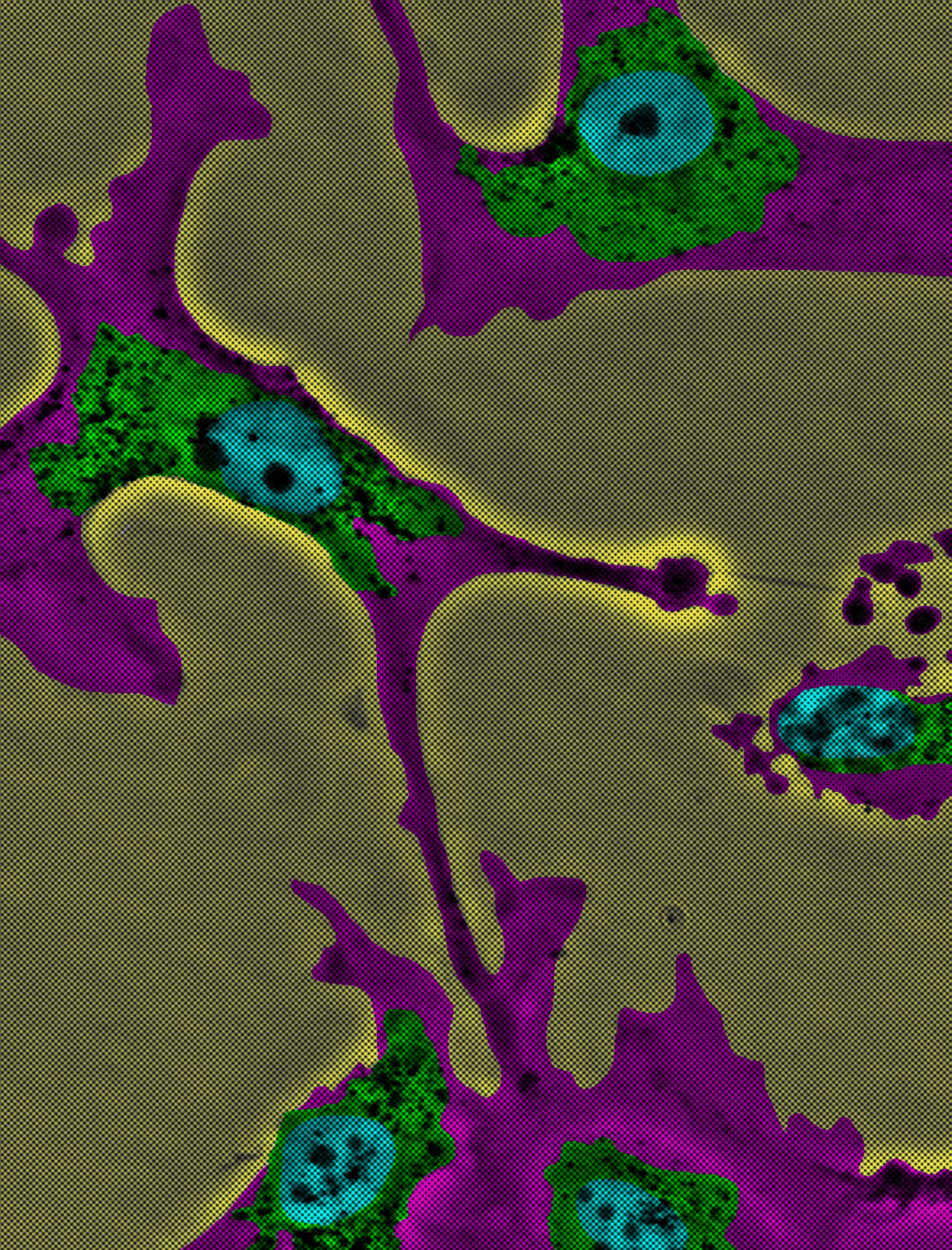
"The future belongs to those who believe in the beauty of their dreams"

*Eleanor Roosevelt**

Aan mijn ouders en broer

*It Seems to Me: Selected Letters of Eleanor Roosevelt (2001) by Leonard C. Schlup and Donald W. Whisenhunt, p. 2



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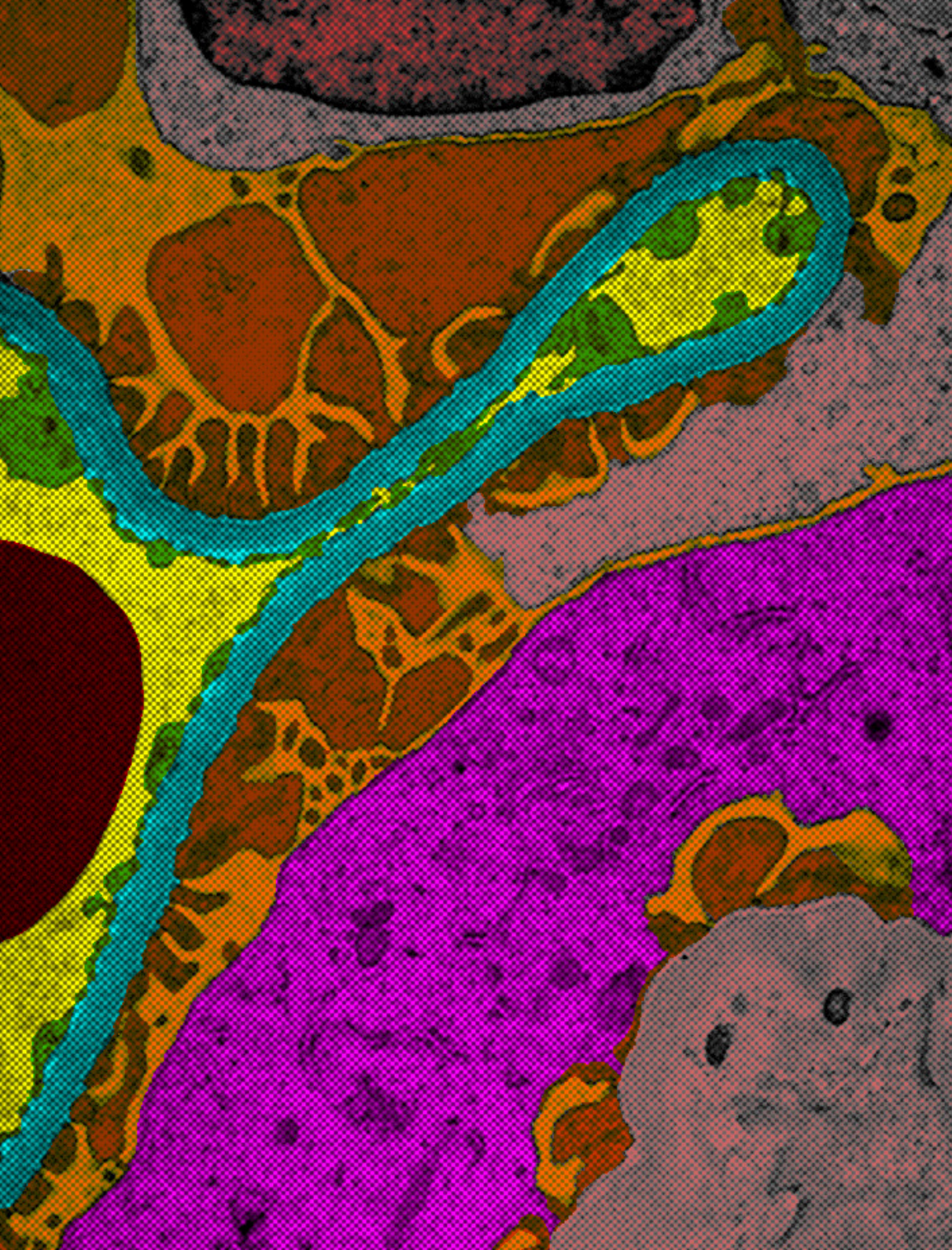

Chapter 1

Preface and Introduction

PREFACE

The first description of diabetes symptoms dates back to 1500 B.C. and was found in an ancient Egyptian medicine book called the Papyrus Ebers, containing various prescriptions for the treatment of polyuria[1]. A more precise description of diabetes was found in the works of Indian physicians around 300 B.C., who called the disease '*Madhumeh*' – a disease of sweet urine – and described signs, symptoms, acute complications, and prognoses. These physicians also differentiated between types of diabetes[2].

The term *'diabetes'* was conceived by the Greek physician Demetrius of Apamea (270 BC), and literally means 'flow through', as these patients seem to lose more fluid than they can drink. Later this was expanded to *'diabetes mellitus'*: 'honeysweet flow through'[2].

In 1869, Paul Langerhans discovered islands of cells in the pancreas – later referred to as the Islets of Langerhans – which differ from the exocrine glandular cells. Laguesse later showed that these islets have a sugar-regulating function, suggested to be the result of an internal secretion formed by those islets[1,2]. Twenty years later it was proven that the pancreas can play a role in the causation of diabetes, when Von Mering and Minkowski removed the pancreas from dogs which consequently developed diabetes[1].

In 1921, Banting and Best found the secretion substance from the pancreas that had sugar regulation functions as suggested by Laguesse, which they named *'Isletin'* (later renamed *'Insulin'*), and demonstrated that injection with this substance decreased blood sugar levels and alleviated symptoms in patients. The next important milestone in diabetes research was the discovery of the structure of insulin by Sanger, which opened the door for the synthetic development of insulin and improved treatment options for diabetics[1,2].

Type 1 and type 2 diabetes

The prevalence of diabetes is increasing worldwide, reaching epidemic proportions. In the Netherlands, the number of people with diabetes has increased rapidly from 2,8% of the population in 2001 to 4,5% in 2013, affecting around 750 thousand people[3]. There are two main types of diabetes, type 1 and type 2.

Type 1 diabetes is characterized as an autoimmune disease in which autoantibodies lead to the progressive destruction of beta (β)-cells – insulin-producing cells located in the pancreas[4]. Because insulin stimulates the transport and uptake of glucose[5], the progressive loss of β-cells eventually leads to a loss of insulin production, and consequently to hyperglycaemia.

The etiology of type 2 diabetes is multifactorial and involves complex interactions between genetic and environmental factors, but is generally accepted as a disease resulting from an unhealthy lifestyle – including a sedentary lifestyle and over-nutrition[6]. In these patients, hyperglycaemia is the result of insulin resistance and β-cell dysfunction, which lead to changes in insulin secretion[7]. Various factors can initiate insulin resistance, including defects in insulin signalling[8] and glucose uptake[5], whereas β-cell dysfunction could result from oxidative stress[9] or excess of fatty acids[10].

INTRODUCTION

The diabetes epidemic is more an epidemic of its complications. Diabetes complications are characterized by (micro)vascular disease. Although the pathophysiology linking diabetes and vascular disease is complex and multifactorial, the role of hyperglycaemia and altered growth factor expression in the development of diabetes complications is eminent. Various mechanisms have been postulated by which hyperglycaemia could lead to vascular complications.

Increased levels of glucose result in the activation of four different mechanisms: *i*) increased flux through the polyol pathway, *ii*) intracellular production of advanced glycation end products, *iii*) protein kinase C activation, and *iv*) increased hexosamine pathway activity. These four mechanisms are driven by the overproduction of superoxide by the mitochondrial electron transport chain[11]. Via these mechanisms, hyperglycaemia leads to the activation of nuclear factor-kappa B (Nf-kB), the generation of advanced glycation end products (AGEs), and oxidative stress, but also results in changes in the expression of various growth factors. Nf-kB is a key transcriptional factor involved in the regulation of pro-inflammatory[12] and pro-atherosclerotic[13] target genes in endothelial cells[14], vascular smooth muscle cells[15], and macrophages[16]. Elevated glucose levels also promote the formation of AGEs, resulting in oxidative stress[17] and changes in endothelial function (i.e. increased vasoconstriction)[18]. Additionally, hyperglycaemia can directly result in oxidative stress[19], which has been implicated in the pathogenesis of atherosclerosis and other vascular diseases[20]. Similarly, altered expression of growth factors (including vascular endothelial growth factor-A; VEGF-A) results in vascular changes such as pathological angiogenesis[21], endothelial activation and dysfunction[22], and capillary rarefaction[23]. VEGF-A is also reported to be involved in the development of atherosclerosis[24].

Endothelial cell activation is a key mediator of vascular damage and subsequent tissue damage. Endothelial cells can be activated by various stimuli resulting from the four above mentioned mechanisms[22,25,26]. Endothelial cell activation is characterized by the augmented cell-surface expression of adhesion molecules, such as vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion

molecule-1 (ICAM-1), and E-selectin[27], an enhanced permeability, and a pro-inflammatory, pro-thrombotic phenotype[28]. The increased expression of adhesion molecules on endothelial cells aids in leukocyte extravasation and subsequent tissue inflammation[29].

Target organs of diabetes complications

Microvascular disease in diabetes patients results in diabetic retinopathy, the leading cause of blindness in working-age adults; diabetic neuropathy, the leading cause of non-traumatic lower extremity amputations; and diabetic nephropathy, the leading cause of end-stage renal disease. As these complications are all characterized by vascular damage, it is no surprise that these complications are significantly associated with each other; specifically, the presence of retinopathy significantly predicts the presence of neuropathy and of nephropathy (confidence interval: 1.56–3.18 and 3.06–10.62, respectively), and accounts both for patients with type 1 diabetes and for those with type 2 diabetes[30].

Diabetic nephropathy

Diabetic nephropathy develops in around twenty to forty percent of patients with diabetes, and is the main cause of end-stage renal disease[31]. Early signs of diabetic nephropathy are albuminuria, hyperfiltration, and glomerular hypertrophy, whereas advanced stages of diabetic nephropathy are characterized by loss of podocytes and endothelial cells, and progression towards glomerulosclerosis.

Diabetic nephropathy can be diagnosed clinically or histologically. Clinical diabetic nephropathy is diagnosed *i*) when micro- or macroalbuminuria is present – which is defined as a spot urine albumin measurement of 30-299 mg/g creatinine and ≥300 mg/g creatinine, respectively – or *ii*) when the estimated glomerular filtration rate is less than or equal to 60 ml/min/1.73m2[32,33]. The diagnosis of diabetic nephropathy can also be based on histology with the use of a renal biopsy. As proposed by our group, histologically diagnosed diabetic nephropathy encompasses four classes (Figure 1)[34]: class I: mild or nonspecific light-microscopic changes and electron microscopy proven

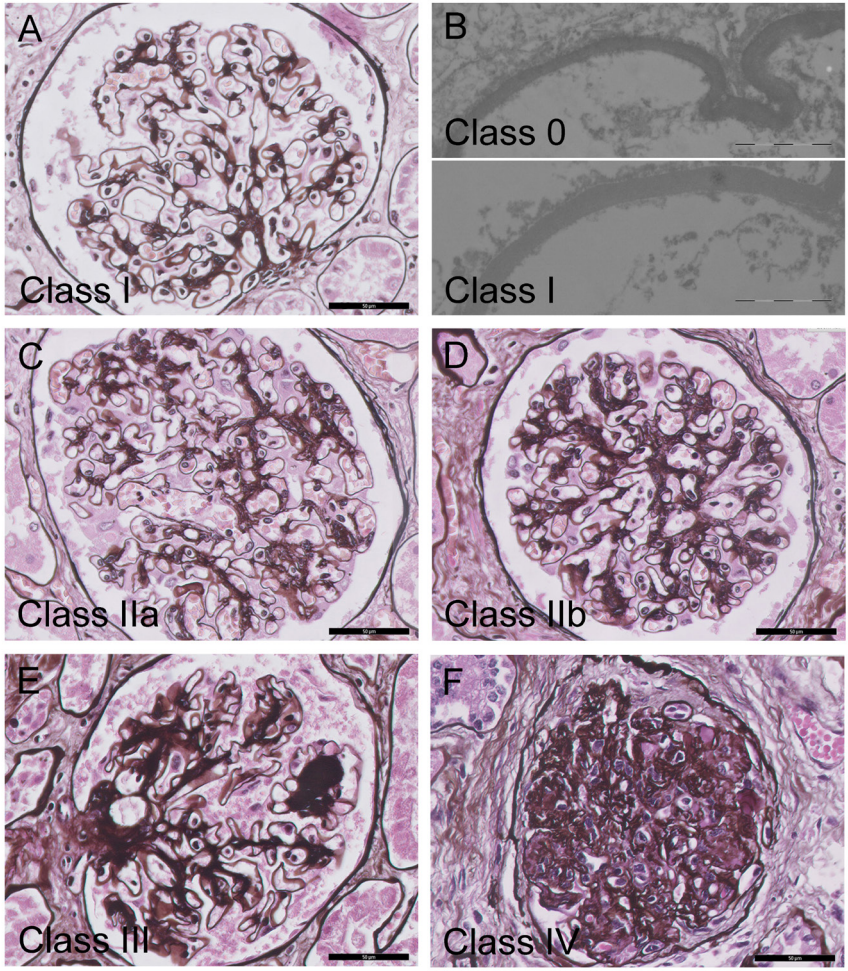

Figure 1: Histological representations of the different classes of diabetic nephropathy. Class 1 is defined as mild or non-specific histological lesions (A), together with a thickened glomerular basement membrane (B). Class 0 represents a diabetic patient without nephropathy due to normal thickness of the glomerular basement membrane (B). Class II is defined as mild (IIa; C) or severe (IIb; D) mesangial expansion in more than 25% of the observed mesangium. Class III is defined by the presence of nodular glomerulosclerosis in at least one glomerulus (E). Class IV is defined as global glomerulosclerosis in more than 50% of the glomeruli (F). Scale bars, 50 µm (A,C-F) and 2 µm (B), respectively. A and C-F: silver staining on formalin-fixed, paraffin-embedded tissue; B: electron microscopy on glutaraldehyde-fixed, epon-embedded tissue; section was contrasted with uranyl acetate and lead citrate.

glomerular basement membrane thickening; class II: mild (IIa) or severe (IIb) mesangial expansion in more than 25% of the observed mesangium; class III: Kimmelstiel-Wilson lesion in at least one glomerulus; class IV: global glomerular sclerosis in more than 50% of glomeruli[34].

As mentioned, diabetes is characterized by hyperglycaemia which plays an important role in the development of diabetes complications. However, despite glucose control regimens, the percentage of diabetes patients progressing towards end-stage renal disease has remained similar for the last two decades[35], indicating that other factors besides hyperglycaemia are involved in the pathogenesis of this disease. Various factors have been demonstrated to increase the risk of developing diabetic nephropathy, including genetic susceptibility, hypertension, and hyperlipidaemia.

Genetic susceptibility

The risk of developing diabetic nephropathy is partly attributable to genetic susceptibility. This is supported by findings that diabetic nephropathy occurs in familial clusters[36,37], and by differences in the prevalence of diabetic nephropathy between ethnicities[38]. Various gene variants are found to be associated with diabetic nephropathy, including variants of genes encoding for *i*) inflammatory cytokines (interleukins, tumor necrosis factor-alpha (TNF-alpha)), *ii*) extracellular matrix components (collagen type 4 alpha 1, laminins, matrix metalloproteinase 9), *iii*) blood pressure regulators (angiotensin I converting enzyme, angiotensin II receptor type 2), *iv*) proteins involved in endothelial function and oxidative stress (VEGF-A, nitric oxide synthase 3, catalase, superoxide dismutase 2), and *v*) proteins involved in the glucose and lipid metabolism (apolipoprotein C-I (apoCI), adiponectin, apolipoprotein E, aldose reductase, glucose transporter 1)[39,40].

Hypertension

Hypertension is defined as a systolic blood pressure over 140 mmHg, or a diastolic blood pressure over 90 mmHg, or both[41]. The prevalence of hypertension in patients with diabetes is up to three times higher than that in patients without diabetes[42]. A higher blood pressure is associated with a higher risk of adverse renal outcomes,

including micro- and macroalbuminuria, a decline in the estimated glomerular filtration rate, and the development of end-stage renal disease[43]. Blood pressure lowering therapy is effective in reducing the incidence of both micro- and macrovascular diabetic complications. Interestingly, intensive blood pressure control therapy (i.e. targeting systolic blood pressure below 120 mmHg), compared to standard, moderate regimens (i.e. targeting systolic blood pressure below 140 mmHg), does not further reduce the risk of developing cardiovascular events and even leads to more serious adverse events attributed to side effects of the antihypertensive treatment[44]. Accordingly, the American Diabetes Association recommends that patients with diabetes and hypertension should be treated to maintain a systolic blood pressure below 140 mmHg[45].

Hyperlipidaemia

The increase in lipids in the blood, such as cholesterol and triglycerides, is referred to as hyperlipidaemia. Diabetic nephropathy is associated with a lipid profile characterized by high triglyceride levels – predominantly smaller very-low-density lipoprotein (VLDL) classes[46] – and lower high-density lipoprotein (HDL) cholesterol levels[47,48]. It has been demonstrated that the content of apoCI per VLDL particle is important for the metabolism of triglycerides during the fasting and postprandial state (i.e. the increased content of apoCI per VLDL leads to less uptake of triglycerides and thus higher serum triglyceride levels), and is associated with atherosclerosis[49]. Treatment with statins in diabetic patients reduces low-density lipoprotein (LDL) cholesterol and triglyceride plasma levels, and increases levels of HDL cholesterol[50], thereby reducing the risk of vascular and renal disease. A genome-wide association study demonstrated that statin-induced changes in LDL cholesterol levels are associated with a polymorphism in the *APOC1* gene[51], potentially due to its effect on lipid metabolism[52].

Innate immune system

Diabetes has traditionally been considered a metabolic disease. But from the late 1990's, the concept of diabetes and its complications as an innate immunity-related disease started to emerge[53,54] and has been elucidated since.

The innate immune system is the first defence barrier against environmental threats, and consists of various components including monocytes and macrophages, and complement proteins. It has been demonstrated that serum levels of inflammatory markers, such as C-reactive protein, TNF-alpha, and interleukin-6, are increased in patients with diabetes compared to those in non-diabetic subjects. This increase in inflammatory markers in patients with diabetes correlates with insulin resistance and hyperglycaemia, and predicts the progression to micro- and macroalbuminuria[55-58]. Insulin resistance may be caused by the effect of TNF-alpha on endothelial cells, as TNF-alpha suppresses both the expression and the phosphorylation of the insulin receptor by these cells[59,60].

The increase in serum levels of inflammatory markers in patients with diabetic nephropathy may be the result of insulin resistance, as it has been demonstrated that insulin resistance promotes inflammation. More specifically, insulin has anti-inflammatory properties both at cellular and molecular levels. Insulin reduces the production of reactive oxygen species, inhibits various pro-inflammatory transcription factors including Nf-kB, and stimulates the expression of inhibitor of kB (IkB; an inhibitor of Nf-kB) in mononuclear cells[61,62]. Insulin also inhibits Nf-kB and monocyte chemoattractant protein-1 in endothelial cells[63]. Therefore, the loss of insulin (type 1 diabetes) and the loss of insulin signalling (type 2 diabetes) prevent the anti-inflammatory effect of insulin from being exerted.

Glomerular inflammation

Monocytes and macrophages have diverse roles in protective immunity and homeostasis, however, they also contribute to many pathological processes. The number of macrophages is increased in patients with diabetic nephropathy compared to that in non-diabetic subjects, both in glomeruli and in the interstitium[64,65]. Similar observations have been

reported in animal models of diabetic nephropathy[66-69]. The infiltration of glomerular and interstitial macrophages correlates with albuminuria and histopathological changes such as mesangial matrix expansion, fibrosis, and interstitial fibrosis and tubular atrophy (IFTA), which is suggested to develop as a consequence of macrophage-derived TNF-alpha[69]. By reducing or inhibiting renal macrophage infiltration, the development of diabetic nephropathy is attenuated[68,70], further demonstrating the involvement of macrophages in this disease.

Vascular endothelial growth factor-A

VEGF-A contributes to the renal infiltration of macrophages in at least two ways. First, it has been demonstrated that VEGF-A binds to the fms-like tyrosine kinase-1 (FLT-1) on monocytes and macrophages, thereby promoting the migration of these cells[71-73]. Second, various adhesion molecules (such as VCAM–1, ICAM–1, and E-selectin) are involved in monocyte transmigration through the vascular endothelium into the tissue[29]. These adhesion molecules are upregulated by various stimuli, including VEGF-A[22]. Therefore, VEGF–A aids in the infiltration of macrophages by stimulating monocyte and macrophage migration, and by inducing endothelial cell activation.

VEGF-A, previously known as vascular permeability factor, was first discovered in a tumor cell line[74]. There are multiple isoforms of VEGF-A which are derived from alternative splicing of exons 6 and 7. This alternative splicing gives rise to VEGF-A_{121}, VEGF-A_{165}, VEGF-A_{189}, and VEGF-A_{206}, with VEGF-A_{165} being the most frequently expressed isoform. Henceforth, VEGF-A indicates isoform VEGF-A_{165}. In the kidney, VEGF-A is mainly expressed by podocytes and tubular epithelial cells.

Vascular endothelial growth factor-A functioning

VEGF-A is involved in vasculogenesis and angiogenesis, and regulates the proliferation, migration, specialization, and survival of endothelial cells[75]. Physiological levels of VEGF-A are crucial for the development of the vasculature in glomeruli. A tight regulation of VEGF-A is important, as both downregulation and upregulation of VEGF-A result in glomerulopathies. For instance, mice with a homozygous deletion of VEGF-A are not viable and have many endothelial cell defects; podo-

cyte-specific heterozygosity for VEGF-A in mice results in proteinuria, endotheliosis, and loss of endothelial cells; and podocyte-specific overexpression of VEGF-A in mice results in a striking collapsing glomerulopathy[76]. Besides differences in VEGF-A expression, changes in serum levels of soluble FLT-1 (sFLT-1), an inhibitor of VEGF-A (as described below), also result in renal disease. Specifically, administration of sFLT-1 to rats results in hypertension, proteinuria, and glomerular endotheliosis[77].

Vascular endothelial growth factor receptors

The two main receptors for VEGF-A are FLT-1 and VEGF receptor 2 (VEGFR2) – both primarily expressed on vascular endothelial cells (Table 1). Of these receptors, FLT-1 has the highest affinity for VEGF-A. The affinity of FLT-1 for VEGF-A is about ten times as strong as the affinity of VEGFR2 for VEGF-A[78,79]. VEGF-A functioning is mainly mediated via the VEGFR2, whereas FLT-1 is considered a decoy receptor to sequester VEGF-A, thereby regulating VEGFR2 activity. sFLT-1 binds VEGF-A with the same affinity as the membrane-bound form, and is able to inhibit VEGF-A-induced mitogenesis – similar to FLT-1 – suggesting that it functions as a negative regulator of VEGF-A signalling (Table 1)[80]. However, it has recently been demonstrated that sFLT-1 has functions other than sequestering VEGF-A, as binding of sFLT-1 to podocytes (independent of VEGF-A) – via the glycosphingolipid monosialodihexosylganglioside in lipid rafts on the surface of podocytes – is crucial for podocyte functioning and morphology[81]. Interestingly, monocytes also express glycosphingolipid monosialodihexosylganglioside on their surface, and this expression is increased upon differentiation towards macrophages[82], and upon exposure to inflammatory stimuli[83]. Conceivably, sFLT-1 may be able to bind to monocytes and macrophages via glycosphingolipid monosialodihexosylganglioside in a similar fashion as the binding of sFLT-1 to podocytes, and consequently regulate monocyte/macrophage morphology and/or functioning, i.e., modulating monocyte-macrophage differentiation and immune responses.

Table 1: Main receptors of VEGF-A, their cellular expression, and their cellular effect.

	sFLT-1	FLT-1	VEGFR2
Cellular expression		- Vascular endothelium - Monocytes - Macrophages	- Vascular endothelium
Cellular effect	- Neutralizing VEGF-A - Regulating podocyte morphology and functioning	- Neutralizing VEGF-A - Migration of monocytes and macrophages	- Proliferation - Migration - Permeability - Survival

Vascular endothelial growth factor-A in diabetic nephropathy

In animal models for diabetic nephropathy, glomerular levels of VEGF-A are increased[84,85] compared to those in non-diabetic animals. The increase in glomerular VEGF-A levels contributes to the severity of diabetic nephropathy. This is supported by the finding that type 1 diabetic mice with a podocyte-specific overexpression of VEGF-A develop nodular glomerulosclerosis[86], whereas wild-type, type 1 diabetic mice only develop mesangial matrix expansion. In patients with diabetic nephropathy, glomerular VEGF-A levels are decreased compared to those in non-diabetic subjects. In these patients, the glomerular expression of VEGF-A correlates negatively with the severity of disease and positively with the number of podocytes[87,88]. In the kidney, VEGF-A is mainly expressed by podocytes, and the number of podocytes decreases during disease progression in patients with diabetic nephropathy. This is in concert with the finding that VEGF-A levels decrease during the progression of diabetic nephropathy. Therefore, we hypothesize that during early diabetes, as mimicked in animal models, glomerular VEGF-A levels are increased – potentially by the effect of hyperglycaemia on podocytes[89] – and contribute to early diabetic renal changes, whereas during the progression of diabetic nephropathy, as demonstrated in patients, glomerular levels of VEGF-A are decreased due to the loss of podocytes[88], consequently leading

to more chronic lesions of diabetic nephropathy.

Various studies report that reducing glomerular VEGF-A levels during early diabetes in animal models prevents the development of albuminuria and renal damage; however, reports are inconsistent[90-94]. This may be due to differences in the type or dose of anti-VEGF-A therapy used. Furthermore, it is unclear from these studies whether treatment to reduce glomerular VEGF-A levels improves renal histology and renal function if started when albuminuria and renal histological lesions are already present. Also, the mechanism by which anti-VEGF-A treatment is beneficial is currently unknown. For instance, it has not been studied whether anti-VEGF-A treatment in diabetic animals reduces the number of glomerular macrophages compared to that in untreated diabetic animals. This might be accomplished by inhibiting the migration of macrophages, or by reducing glomerular endothelial cell activation, or both.

Apolipoprotein C-I

ApoCI is expressed on HDL and on triglyceride-rich lipoproteins, and has several functions in both lipid metabolism and lipid transport[95]. ApoCI inhibits the uptake of lipoproteins via suppression of the lipoprotein lipase-dependent triglyceride-hydrolysis pathway[52,96], resulting in hyperlipidaemia (predominantly hypertriglyceridemia).

In addition to its role in lipid metabolism, apoCI is also involved in inflammation. ApoCI, either derived from the circulation (mainly bound to chylomicrons, VLDL, and HDL) or from macrophages[97], strongly binds to the lipid A moiety of lipopolysaccharide (LPS) – a highly inflammatory constituent of the outer membrane of Gram-negative bacteria – thereby augmenting the inflammatory response to LPS by macrophages via the cluster of differentiation 14/myeloid differentiation protein-2/toll-like receptor 4 (CD14/MD2/TLR4) pathway, including the production of TNF-alpha (Figure 2)[98,99]. By augmenting the production of TNF-alpha by macrophages, apoCI accelerates LPS-induced atherosclerosis in mice[100]. Similarly, in humans, the apoCI content of triglyceride-rich lipoproteins – including VLDL – predicts early atherosclerosis, and is associated with plaque size[49,101,102].

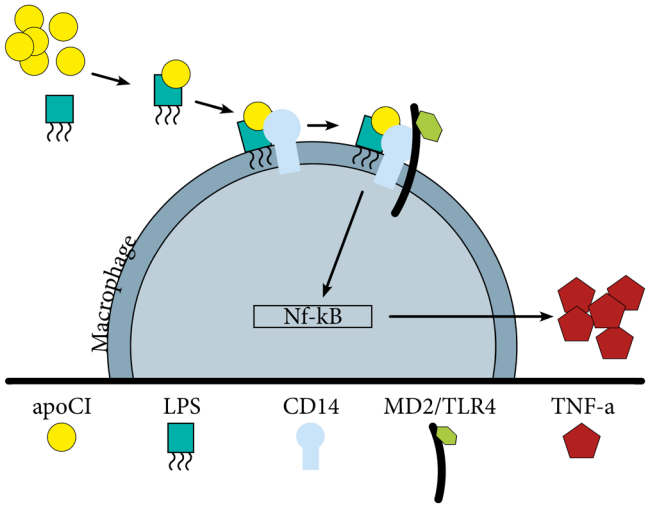

Figure 2: Schematic representation of the mechanism by which apoCI augments LPS-induced inflammatory responses in macrophages. ApoCI binds to the lipid A moiety of LPS, subsequently presenting LPS to CD14, which transfers LPS to the MD2/TLR4 complex leading to increased production of TNF-alpha via transcription of Nf-kB.

Because apoCI is associated with the progression of vascular damage, it may be involved in the progression of diabetic nephropathy as well. This notion is supported by the finding that, as mentioned earlier, a gene polymorphism in *APOC1* was found to be associated with an increased risk of developing diabetic nephropathy[40]. Interestingly, Bouillet *et al.* recently demonstrated that, compared to those in healthy control subjects, plasma levels of apoCI are increased in patients with diabetes – both type 1 diabetic patients and type 2 diabetic patients[103] – and correlate with triglyceride levels in type 2 diabetes patients (this correlation has not been investigated for type 1 diabetes patients)[104]. Furthermore, under physiological circumstances, apoCI inhibits cholesteryl ester transfer protein (CETP) activity. CETP promotes the exchange of neutral lipid species between plasma lipoproteins (i.e. cholesteryl esters and triglycerides). However, in patients with diabetes, the glycation of apoCI (as a result of hyperglycaemia) changes its electrostatic properties, thereby impairing its ability to inhibit CETP activity. This results in an increase in CETP activity in these patients compared to that in non-diabetic subjects[103], which is

associated with the development of atherosclerosis[105,106].

Although this data supports the hypothesis that apoCI may be involved in the development of diabetic nephropathy, it has to be further elucidated whether an increase in plasma apoCI levels leads to the development of diabetic nephropathy, and, if so, whether this entails the effect of apoCI on inflammatory responses by monocytes and macrophages, or that on lipid metabolism, or both.

Endoglin

As mentioned earlier, diabetic nephropathy is characterized by vascular changes, including endothelial cell activation, which aid in leukocyte extravasation and inflammation. An important molecule involved in vascular health and functioning is endoglin. Endoglin is a co-receptor for the transforming growth factor beta (TGF-β) receptor family, and modulates the signalling of TGF-β receptor type 2, activin receptor-like kinase (ALK)-1, and ALK-5[107]. Endoglin is mainly expressed on endothelial cells[108], and it is crucial for vascular development and angiogenesis as demonstrated in endoglin deficient mice[109]. This is supported by the finding that endoglin is required for VEGF-A-induced angiogenesis[110]. In various animal models of renal disease, the glomerular expression of endoglin is increased compared to that in healthy control animals. This increase in glomerular endoglin expression contributes to the severity of disease[111-113] – potentially by promoting endothelial cell activation, vascular damage[113], and macrophage infiltration[114]. Also, compared to those in non-diabetic subjects, soluble endoglin serum levels are increased in diabetes patients and correlate with endothelial dysfunction and cardiovascular damage[115]. However, whether a causal relationship exists between the altered endoglin expression levels in these patients and the observed glomerular endothelial cell activation and macrophage infiltration in their kidneys is uncertain.

VEGF-A leads to endothelial cell activation and subsequent leukocyte adhesion via binding to the VEGFR2, upon which the VEGFR2 is phosphorylated and becomes associated with proto-oncogene tyrosine-protein kinase Src (Src)[116]. Src is also associated with endoglin

upon VEGF-A stimulation, and is critical for both the internalization and degradation of endoglin via endosomes, as well as for VEGF-A/VEGFR2-induced endothelial cell functioning[116,117]. Therefore, endoglin may be a crucial co-receptor for VEGFR2 internalization and degradation, as VEGFR2 co-localizes with endoglin in endosomes (early endosome antigen-1 (EEA-1)-, ras-related protein rab-5 (Rab5)-, and ras-related protein rab-7 (Rab7)-positive endosomes)[117]. Upon internalization of the VEGFR2, extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) are activated, which in turn translocate to the nucleus, where they lead to activation of activating transcription factor 2 (ATF-2). Activation of ATF-2 subsequently leads to the transcription and translation of endothelial cell activation markers (including VCAM-1; Figure 3, black arrows)[118].

When the expression of endoglin is reduced, stimulation with VEGF-A leads to increased recycling of the VEGFR2 to the plasma membrane (i.e. less degradation of the VEGFR2), and to increased Akt serine/threonine kinase (Akt) phosphorylation, whereas ERK1/2 activation remains unchanged[119]. However, a reduced endoglin expression may also lead to decreased internalization of the VEGFR2 in endosomes. A decrease in VEGFR2 internalization may therefore result in an increased Akt activation, as it has been postulated that the VEGFR2 is able to activate Akt – but not ERK1/2 – while present on the plasma membrane[120]. Nevertheless, Akt activation is increased in endothelial cells with a reduced endoglin expression. Akt has been shown to inhibit ATF-2 phosphorylation[121], and may therefore result in less endothelial cell activation (Figure 3, red arrows). Taken together, it is possible that endoglin affects endothelial cell activation and leukocyte adhesion by altering or inhibiting *i)* the internalization process of the VEGFR2, *ii)* intracellular signalling, and *iii)* subsequent transcription of endothelial cell activation markers.

Complement activation

The complement system is an effector mechanism of the innate immune system. It has three main physiological functions: defending the host against infection, bridging the innate and adaptive immune

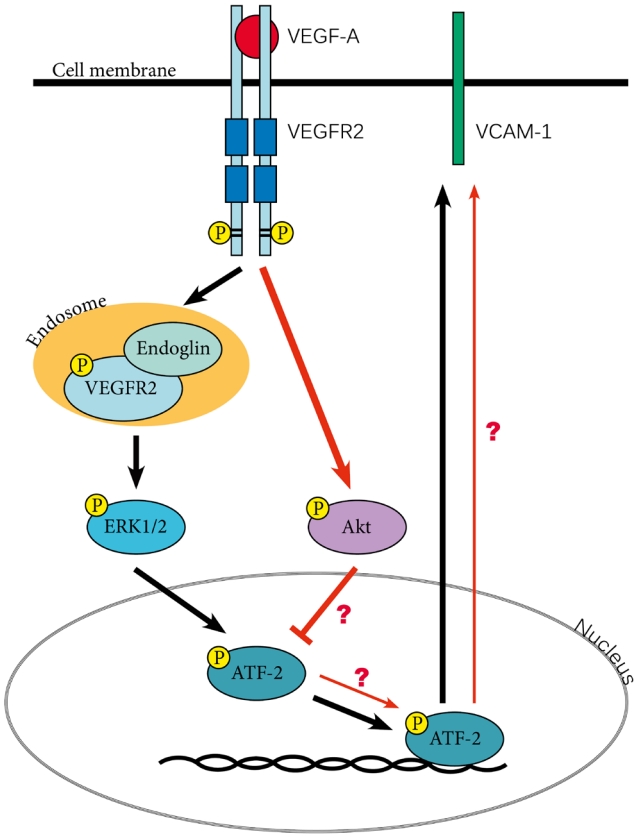

Figure 3: **Schematic representation of the mechanism of endothelial cell activation by stimulation with VEGF-A**. Binding of VEGF-A to the VEGFR2 leads to phosphorylation (P) and internalization of this receptor, which leads to increased VCAM-1 expression via the ERK1/2/ATF-2 pathway (black arrows). Endoglin co-localizes with the VEGFR2 in endosomes, and may regulate VEGFR2 internalization and degradation. When endoglin expression is absent or reduced, the activation of Akt is increased, which may inhibit ATF-2 phosphorylation and consequently the activation of endothelial cells (red arrows). ?: uncertain.

systems, and clearance of immune complexes in tissues and waste products of inflammatory injury[122]. The complement system consists of more than 35 proteins that form three pathways: the classical pathway, the lectin pathway, and the alternative pathway. Different processes initiate each pathway, but all three pathways lead to the cleavage of

complement factor (C)3 by C3 convertases, thereby initiating a final common pathway resulting in the formation of the membrane attack complex (Figure 4) – also known as C5b-9, which stimulates complement-mediated damage. In addition, protein fragments C3a and C5a, which are derived from C3 and C5, respectively, induce anaphylatoxic reactions leading to recruitment of inflammatory cells[123].

Both clinical and experimental evidence support a role for the complement system in the pathogenesis of diabetes complications[124,125]. For most diabetes complications the evidence for a role for complement activation in the progression of damage is quite strong, including diabetic retinopathy[126], diabetic cardiovascular disease[127], and diabetic neuropathy[128]. For diabetic nephropathy, however, data on this subject is scarce. Still, a growing body of evidence suggests that complement activation is also involved in the pathogenesis of diabetic nephropathy. In urine samples of patients with diabetic nephropathy, levels of C5b-9 are found to be increased[129]. Strong associations have been found between serum levels of mannose-binding lectin (MBL) and the presence of type 1 and type 2 diabetes. Serum levels of MBL correlate with renal function[130-132], suggesting the involvement of the lectin pathway in the progression of diabetic nephropathy. In addition, in renal biopsies of patients with diabetic nephropathy, the expression of C3, C4, and C9 at the protein level, and of C1q, C1r, and C1s at the mRNA level is increased compared to those in non-diabetic subjects, suggesting a role for the classical complement pathway in patients with diabetic nephropathy[133]. Furthermore, hyperglycaemia results in glycation-induced inactivation of complement regulatory proteins, including cluster of differentiation 59 (CD59) – an inhibitor of the formation of C5b-9 under physiological circumstances – leading to increased formation of C5b-9[134]. Evidence for the involvement of complement activation in the progression of diabetic nephropathy also comes from diabetic animal models. When treated with a receptor antagonist against C3a, type 2 diabetic rats have less inflammation, demonstrate an improved renal function, have less albuminuria, and a reduced deposition of extracellular matrix proteins in glomeruli compared to that in untreated diabetic rats[135], supporting the hypothesis that complement activation contributes to the progression of diabetic nephropathy.

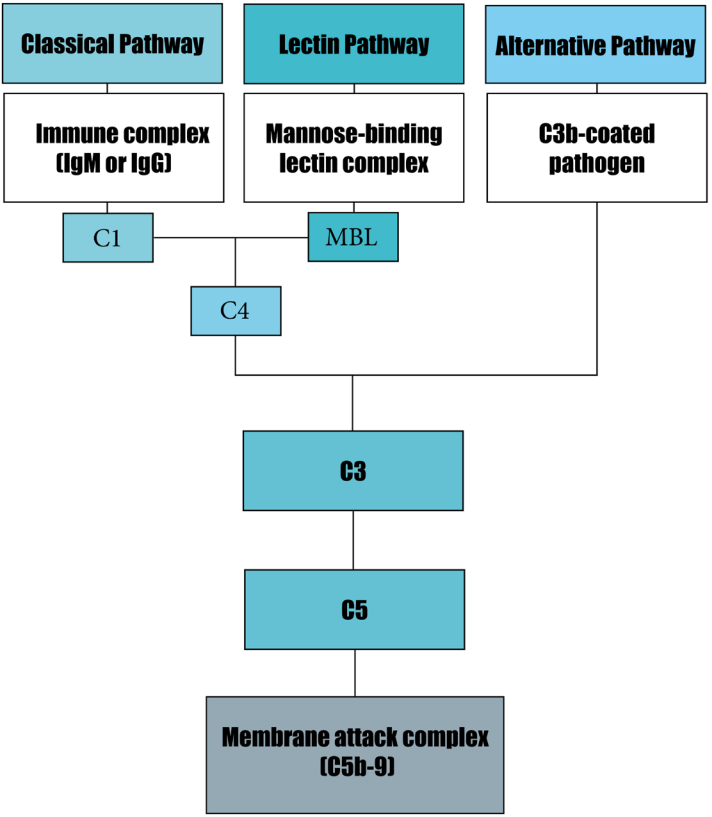

Figure 4: **Schematic overview of the complement system**. The complement system consists of three pathways: the classical pathway, the lectin pathway, and the alternative pathway – each activated by different stimuli (immune complexes, mannose-binding lectin complexes, or C3b-coated pathogens, respectively). The classical and the lectin pathway both lead to the cleaving of C4. All three pathways converge at C3, which leads to the formation of the membrane attack complex (also known as C5b-9).

Although data on complement activation in renal tissues of patients with diabetic nephropathy potentially opens a new spectrum of treatment options for these patients, these studies were thus far performed on small patient numbers, making it difficult to generalize this data to the general population.

Aims of the work described in this thesis

Current treatment to prevent or delay the progression of diabetic nephropathy involves the control of both metabolic and hemodynamic changes. Specifically, patients receive medication to lower blood glucose levels, and to reduce serum lipids and blood pressure[136]. The prevalence of some diabetes complications has been reduced with these standard treatments over the last two decades – including death from hyperglycaemic crisis, stroke, and acute myocardial infarction. However, the prevalence of end-stage renal disease in diabetes patients has not yet been reduced[35]. Therefore, new preventive and therapeutic strategies are urgently needed. In order to achieve this, a better understanding of the mechanisms behind the progression towards diabetic nephropathy is paramount. The central aim of the studies described in this thesis was to investigate the involvement of the immune system and vascular changes in the development of diabetic nephropathy.

In the work described in **chapter 2**, we investigated whether overexpressing apoCI – a molecule involved in lipid metabolism and inflammatory responses by macrophages, and associated with an increased risk of developing diabetic nephropathy – leads to the development of glomerulosclerosis in mice transgenic for human *APOC1* (*APOC1*-tg mice), and if so, by which mechanism. Additionally, we investigated the prevalence of glomerular apoCI deposits in patients with diabetic nephropathy.

In the work described in **chapter 3**, we investigated whether treatment with sFLT-1, an inhibitor of VEGF-A, reduces albuminuria and renal histopathological changes in type 1 diabetic mice. We also studied whether treatment of type 1 diabetic mice with sFLT-1 has an effect on glomerular endothelial cell activation and on the influx of glomerular macrophages.

In the work described in **chapter 4**, we investigated whether glomerular endoglin expression is associated with diabetic nephropathy in both mice and patients. Also, we studied the involvement of endoglin in VEGF-A–stimulated endothelial cell activation, and in the adhesion of monocytes to activated endothelial cells *in vitro*. Furthermore, we studied whether glomerular endoglin expression is correlated with

endothelial cell activation in patients with diabetic nephropathy.

In the work described in **chapter 5**, the aim was to elucidate whether complement activation has taken place in kidneys of patients with diabetic nephropathy, and, if so, *i*) which complement pathway is activated in these patients, *ii*) whether complement deposits are correlated with the severity of diabetic nephropathy, and *iii*) whether there are differences in complement deposition between patients with type 1 diabetes and those with type 2 diabetes.

In the work described in chapter 2, we demonstrated that glomerulosclerosis develops in *APOC1*-tg mice at 15 months of age. The relatively slow progression of the disease in this mouse model makes it less suitable to study therapeutic and preventive interventions. In the work described in **chapter 6**, we therefore aimed to accelerate the progression of glomerulosclerosis in *APOC1*-tg mice. We introduced a second hit in eight-week-old *APOC1*-tg mice by transfecting these mice with *sFlt-1*, and monitored the development of glomerulosclerosis.

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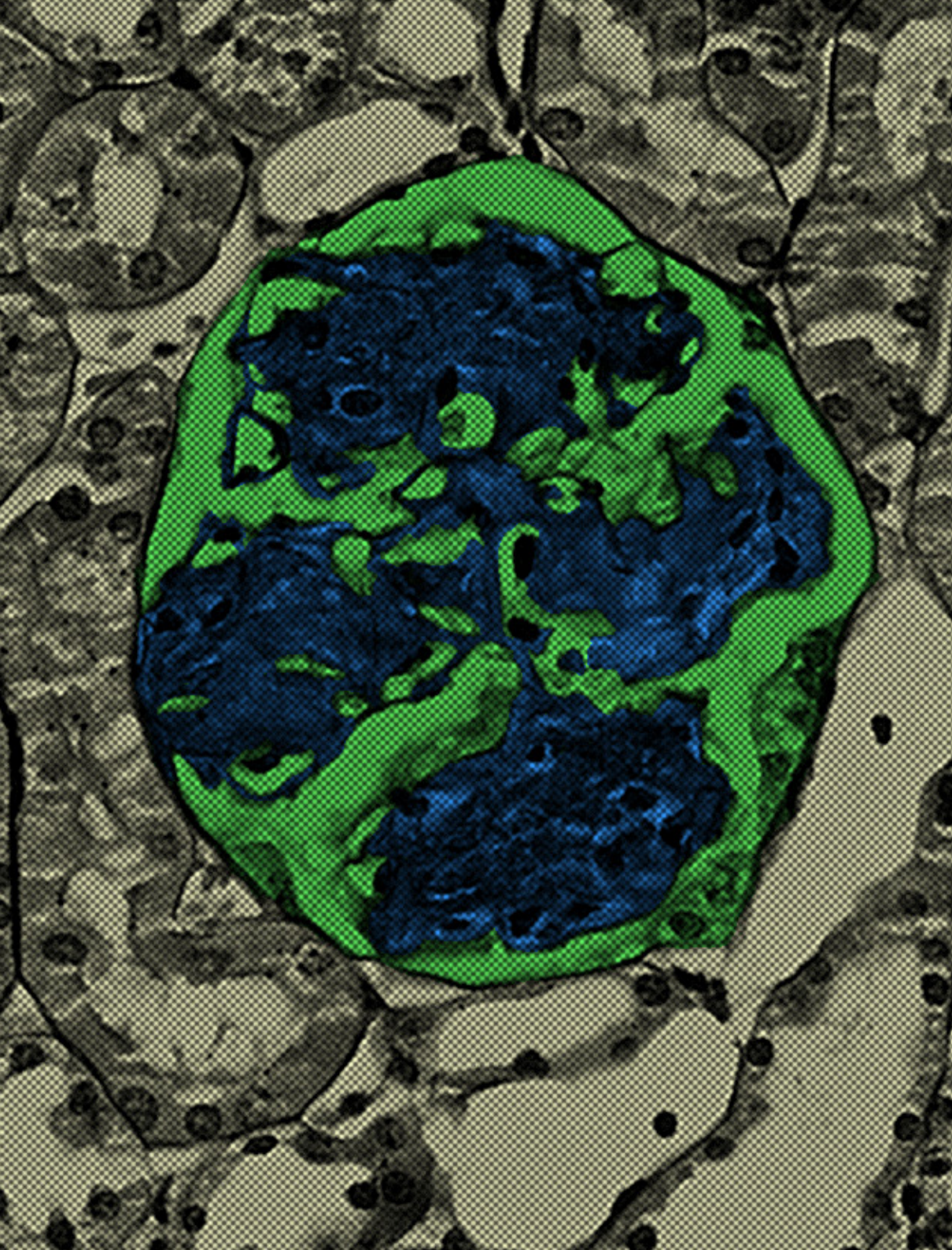

Chapter 2

Apolipoprotein C-I plays a role in the pathogenesis of glomerulosclerosis

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Abstract

Diabetic nephropathy is the leading cause of end-stage renal disease. Diabetic patients have increased plasma concentrations of apolipoprotein C-I (apoCI), and meta-analyses found that a polymorphism in *APOC1* is associated with an increased risk of developing nephropathy. To investigate whether overexpressing apoCI contributes to the development of kidney damage, we studied renal tissue and peritoneal macrophages from *APOC1* transgenic (*APOC1*-tg) mice and wild-type littermates. In addition, we examined renal material from autopsied diabetic patients with and without diabetic nephropathy and from autopsied control subjects. We found that *APOC1*-tg mice, but not wild-type mice, develop albuminuria, renal dysfunction, and glomerulosclerosis with increased numbers of glomerular M1 macrophages. Moreover, compared to wild-type macrophages, stimulated macrophages isolated from *APOC1*-tg mice have increased cytokine expression, including TNF-alpha and TGF-beta, both of which are known to increase the production of extracellular matrix proteins in mesangial cells. These results suggest that *APOC1* expression induces glomerulosclerosis, potentially by increasing the cytokine response in macrophages. Furthermore, we detected apoCI in the kidneys of diabetic patients, but not in control kidneys. Moreover, patients with diabetic nephropathy have significantly more apoCI present in glomeruli compared to diabetic patients without nephropathy, suggesting that apoCI could be involved in the development of diabetic nephropathy. ApoCI co-localized with macrophages. Therefore, apoCI is a promising new therapeutic target for patients at risk of developing nephropathy.

Introduction

Diabetic nephropathy (DN) is the leading cause of end-stage renal disease in the Western world[1]. Several factors contribute to the development of DN, including hyperglycaemia[2], hypertension[3], hyperlipidaemia[4], and genetic background[5]. Meta-analyses revealed that a polymorphism in the *APOC1* gene, which encodes apolipoprotein C-I, is associated with an increased risk of developing DN[6,7]. Moreover, a recent study reported that patients with either type 1 or type 2 diabetes have higher plasma levels of apoCI compared to non-diabetic controls[8].

In the circulation, apolipoproteins are found primarily on the surface of lipid complexes called lipoproteins (e.g., chylomicrons, very-low-density lipoproteins, and high-density lipoproteins). Apolipoproteins regulate the metabolism of lipoproteins, thereby determining the transport and redistribution of lipids among various tissues. Lipoprotein catabolism is dependent on both the apolipoproteins present on their surface and the proteins—including enzymes and receptors—with which they interact. ApoCI slows the catabolism of triglyceride-rich lipoproteins primarily by inhibiting the enzyme lipoprotein lipase (LPL), thereby increasing plasma triglycerides[9]. In addition, apoCI has inflammatory properties, as it can stimulate the production of TNF-alpha by macrophages following an inflammatory stimulus[10].

Studies performed in both human subjects and animal models revealed that macrophages play an important role in the pathogenesis of DN[11-17], and macrophage-derived TNF-alpha is believed to be a key mediator of this process[17]. Several studies regarding the role of macrophages in DN found increased number of macrophages both in glomeruli and in the interstitium. This influx of macrophages is correlated with glomerular and tubulointerstitial damage and with a decline in renal function[11-16,18,19]. Reciprocally, suppressing macrophage influx markedly reduces urinary albumin secretion, glomerular hypertrophy, and mesangial matrix expansion[14,20,21].

We hypothesise that increased expression of apoCI plays an important role in the development of kidney damage, based on three key findings: *i)* the results of the meta-analysis, which showed an association between an *APOC1* polymorphism and the development

of DN; *ii*) the finding that apoCI stimulates the inflammatory response in macrophages; and *iii*) that macrophages are involved in the development of DN. To test this hypothesis, we examined whether human *APOC1* transgenic mice develop kidney damage. Next, we investigated the role of macrophages in this process. Finally, to test whether apoCI is clinically relevant in the development of DN, we investigated the expression of apoCI in the kidneys of patients with and without DN as well as in kidneys from non-diabetic controls.

Materials and methods

Animals

In this study, we used both male and female heterozygous transgenic mice expressing human apolipoprotein C-I (*APOC1*-tg) on a C57Bl/6J background. The construct was generated by the excision of an 18-kb EcoRI fragment from a cosmid carrying the *APOE/APOC1/APOC1P1* gene region. The fragment contained a 5-kb region upstream from *APOC1* and an 8-kb region downstream from *APOC1*, including the hepatic control region element and part of *APOC1P1* pseudogene[22]. Transgenic offspring were identified by polymerase chain reaction (PCR) analysis on genomic tail-derived DNA. Male and female wild-type (WT) littermates were used as controls. Equal numbers of male and female mice were used. All mice used in this study were housed under standard laboratory conditions with free access to water and standard rodent chow (Hope Farms, Woerden, the Netherlands).

Experimental design

All animal experiments were approved by the Institutional Ethics Committee for Animal Care and Experimentation. To measure the animals' characteristics, WT and *APOC1*-tg mice (10 mice per group) were sacrificed at six months of age. The kidneys, heart, liver, brain, lungs, spleen, and pancreas were collected, and both absolute and relative weights were measured. From each organ, one piece was fixed in 4% buffered formalin and embedded in paraffin, and one piece was snap-frozen for mRNA isolation. Blood was collected in heparin tubes and used to measure white blood cells (WBCs) and haemoglobin

concentration. Blood plasma was used to measure triglycerides (TGs), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), non-HDL-C, glutamate oxaloacetate transaminase (GOT), creatinine kinase (CK), and glucose levels. Serum was used to measure blood urea nitrogen (BUN) and serum creatinine. WBCs were counted using a Muse Cell Counter (Merck, Darmstadt, Germany). A BUN colorimetric detection kit (Arbor Assays, Ann Arbor, MI, USA) was used in accordance with the manufacturer's instructions. Serum creatinine was measured using an enzymatic method on a Roche Modular P800 device (Roche, Woerden, the Netherlands) in accordance with the manufacturer's instructions. Haemoglobin, GOT, and CK were measured using Reflotron Sprint (Roche) test strips in accordance with the manufacturer's instructions. Serum creatinine was measured using an enzymatic/fluorometric creatinine assay kit (Abcam, Cambridge, UK). Plasma TGs and TC were assayed using commercially available enzymatic kits (Roche Diagnostics, Germany), and plasma glucose was measured using the glucose start reagent method (Instruchemie, Delfzijl, the Netherlands) in accordance with the manufacturer's instructions. HDL-C was measured by precipitating apoB-containing particles with 20% polyethylene glycol in 200 mM glycine buffer (pH 10), and TC was measured in the supernatant. Non-HDL-C was calculated by subtracting HDL-C from TC.

APOC1-tg mice (n=59) and age-matched WT littermates (n=19) were sacrificed at various ages ranging from 2 to 23 months. Subsequently, the kidneys were removed and subjected to histological, immunohistochemical, and mRNA analyses. From each kidney, one piece of cortical tissue was snap-frozen in pre-cooled methylbutane; a second piece was fixed in 4% buffered formalin and embedded in paraffin for histological analysis and immunohistochemistry. Frozen renal cortex samples (30 mg) from three WT and three *APOC1*-tg mice at 16 months of age were used to measure cholesterol, triglyceride, and phospholipid content as described previously for liver tissue[23].

Albuminuria

Urine albumin levels were measured using rocket immunoelectrophoresis against rabbit anti-mouse albumin, with purified mouse serum albumin (Sigma-Aldrich, Saint Louis, MO) as a standard. Urine

creatinine was measured using a creatinine assay, with picric acid, sodium hydroxide, and creatinine standards (Sigma-Aldrich). The rabbit anti-mouse albumin antibody was produced in our laboratory by immunising rabbits with an emulsion of mouse albumin and Freund's adjuvant; specificity was confirmed using western blot analysis. Serum creatinine and BUN were measured in accordance with the manufacturers' instructions (Roche and Arbor Assays, respectively) in 16-month-old mice as a measure of renal function.

Immunohistochemistry

Frozen kidney sections (3-µm thickness) were cut on a Reichert cryostat microtome. Goat anti-human apoCI (Academy Biomedical Company, Houston, TX, USA), rabbit anti-human fibronectin (Sigma-Aldrich), rabbit anti-human collagen VI (Telios Pharmaceuticals, San Diego, CA, USA), goat anti-human collagen types I, III, and IV (all from Sera Laboratories, West Sussex, UK), rat anti-VCAM-1 (Abcam), rat anti-MCP1 (Abcam), rat anti-ICAM (Abcam), rat anti-FA-11 (Abcam), rabbit anti-TNF-alpha (Abcam), rabbit anti-CD11b (Abcam), or rabbit anti-CD163 (Santa Cruz Biotechnology, Dallas, TX, USA) primary antibodies were used for immunostaining, followed by the appropriate HRP-labelled secondary antibody with diaminobenzidine as the chromogen. Double-label immunofluorescence was performed with rat anti-VCAM-1 and goat anti-von Willebrand factor (Affinity Biologicals Inc., Ancaster, Canada), followed by the appropriate fluorescent secondary antibodies, after which the slides were mounted using Vectashield plus DAPI (Vector Laboratories, Burlingame, CA, USA). Double-label immunostaining was performed with goat anti-human apoCI and mouse anti-CD68 (Dako, Glostrup, Denmark), followed by the appropriate secondary antibodies with diaminobenzidine and Vector Blue (Vector Laboratories) as the chromogens for detecting apoCI and CD68, respectively. Frozen kidney sections (10 µm thickness) of 16-month-old WT and *APOC1*-tg mice were stained with oil red O. Paraffin-embedded kidney sections (4 µm thickness) were stained with methenamine silver, periodic acid-Schiff (PAS), or Sirius red (supplementary material, Supplementary material and methods). Sections were immunostained with rabbit anti-WT1 (Abcam) or rat anti-F4/80 (Abcam) primary antibodies, followed by the appropriate HRP-labelled secondary antibody

with diaminobenzidine as the chromogen. The anti-human antibodies used for staining mouse tissue are cross-reactive with mouse tissue (data not shown).

Staining quantification

The number of podocytes in each sample was determined by counting the number of WT1-positive nuclei in 25 glomeruli. Glomerular damage was scored morphologically on PAS-stained tissues in 25 glomeruli, using a scale of 0–4 as follows: 0, normal glomeruli; 1, <10% mesangial expansion; 2, 10-25% glomerulosclerosis; 3, 25-50% glomerulosclerosis; 4, >50% glomerulosclerosis[24]. Sections were scanned, and the images were digitised using a Philips Ultra-Fast Scanner 1.6 RA (Philips Digital Pathology, Best, The Netherlands). The levels of VCAM-1, ICAM-1, and TNF-alpha expression per glomerulus were determined by measuring the surface stained by the respective antibody, relative to the total surface area of the glomerulus (ten glomeruli per section were measured) at 40X magnification (ImageJ). FA-11–positive cells were counted in 10 glomeruli per sample. PAS-stained slides were used to measure the surface area of the glomerular tuft (i.e. glomerular surface area, in μm^2) in 25 glomeruli per sample using Philips Ultra-Fast Scanner 1.6 RA software. Interstitial fibrosis was determined by measuring the surface area stained by Sirius red in five cortical fields (using a 40X objective with a fluorescence microscope) relative to the total surface area of the cortical fields (ImageJ software). Glomeruli and vessels were excluded from the analysis.

Human APOC1 and mouse apoCI expression in *APOC1*-tg mice

RNA was extracted using RNA extraction buffer (TRIzol, Ambion) from kidney, liver, brain, heart, lung, spleen, and pancreas tissues of *APOC1*-tg and WT mice, and from the liver tissue of streptozotocin (STZ)-induced diabetic WT mice 4 months after STZ injection. *APOC1* and *ApoC1* mRNA levels were measured using quantitative real-time PCR.

Macrophage isolation

WT and *APOC1*-tg mice (n=3 each) were given an intraperitoneal injec-

tion of 1 ml thioglycolate (30 g/l) on day 1 in order to increase the macrophage yield. On day 5, after the mice were sacrificed, 6 ml of sterile PBS was used for vigorous peritoneal lavage. The resulting cell suspension was centrifuged at 500 rpm for 10 min and then washed twice with PBS. The cell pellets were resuspended in 2 ml RPMI 1640 medium containing 10% FCS and 1% penicillin/streptomycin (referred to hereafter as 'medium'). The macrophages were counted in a counting chamber, and the cells were then incubated overnight in a six-well plate (with 500.000 macrophages per well in 1.2 ml of medium). After the cells were washed with medium, they were incubated for 6 h at 37˚C in the following conditions: no stimulation; 10 ng/ml LPS[25]; or 10 ng/ml LPS and 10 ng/ml anti-CD14 antibody (Zimmer Biomet, Warsaw, IN, USA). After 6 h, the medium was collected and the cells were washed three times with PBS and then dissolved in 1 ml TRIzol.

Reverse transcription and CDNA amplification

Quantitative real-time PCR (qRT-PCR) reactions were prepared using SYBR Green I master mix (Bio-Rad, Hercules, CA, USA) as recommended by the manufacturer and run on a Bio-Rad CFX real-time system. Ct (cycle threshold) values were normalised to the housekeeping gene *Hprt1*. The primer sequences used in this study are provided in the supplementary material, Supplementary materials and methods.

Human kidney samples

Renal tissue specimens were obtained from autopsy samples collected from 17 control subjects without diabetes or renal disease, from 8 patients with diabetes without nephropathy, and from 12 patients with nodular glomerulosclerosis (class III DN, based on the established histopathological classification for DN)[26]. Autopsy samples were retrieved from the pathology archives at Leiden University Medical Center. The non-diabetic control subjects, diabetic patients without nephropathy, and diabetic patients with nephropathy were 65±12, 76±12, and 72±9 years of age, respectively (p=0.086). The duration of diabetes did not differ significantly between the two diabetic groups (p=0.13).

Statistics

Because animals were randomly sacrificed between 2 and 23 months

of age, and because the relationship between glomerular damage and age produced a sigmoid-like curve for the *APOC1*-tg mice (compared to a linear relationship for the WT mice), we analysed our data by dividing mice into three age categories: ≤8 months, 8–16 months, and >16 months. We studied the various outcomes using two-way ANOVAs with an interaction term between age and genotype in the model. Model fit was assessed by visual inspection of residuals of the ANOVA models. Patient data and *in vitro* experiments were analysed using the two-tailed Student's *t* test. Differences were considered significant at $p<0.05$.

Results

Human *APOC1* transgenic mice develop glomerulosclerosis

The animals' baseline characteristics were measured at 6 months of age and are summarised in the supplementary material, Tables S1 and S2. The absolute organ weights did not differ significantly between WT and *APOC1*-tg mice; however, the relative organ weights of the kidneys and heart were significantly higher in the *APOC1*-tg mice. The *APOC1*-tg mice had hyperlipidaemia, with increased serum levels of triglycerides, total cholesterol, and non-HDL-C ($p<0.001$). In the *APOC1*-tg mice, the human *APOC1* transgene is primarily expressed in the liver and, to a lesser extent, peritoneal macrophages (Figure 1A). Accordingly, human apoCI protein was present primarily in the liver; in other organs, human apoCI protein was restricted to the blood vessels (Figure 1B-H). The liver mRNA levels of mouse *ApoC1* were 2.8-fold higher in STZ-induced diabetic mice than in WT mice ($p<0.05$; Figure 1I).

To examine whether *APOC1*-tg mice develop albuminuria, we next measured the urinary albumin:creatinine ratio at various ages (Figure 2). Compared with WT mice, the albumin:creatinine ratio was significantly higher in *APOC1*-tg mice from 14 months of age ($p<0.05$). In addition, at 16 months of age serum creatinine levels were 2.7-fold-higher in the *APOC1*-tg mice than in WT mice ($p<0.05$; data not shown). Blood urea nitrogen (BUN) levels did not differ significantly between *APOC1*-tg mice and WT mice at 16 months of age (data not shown).

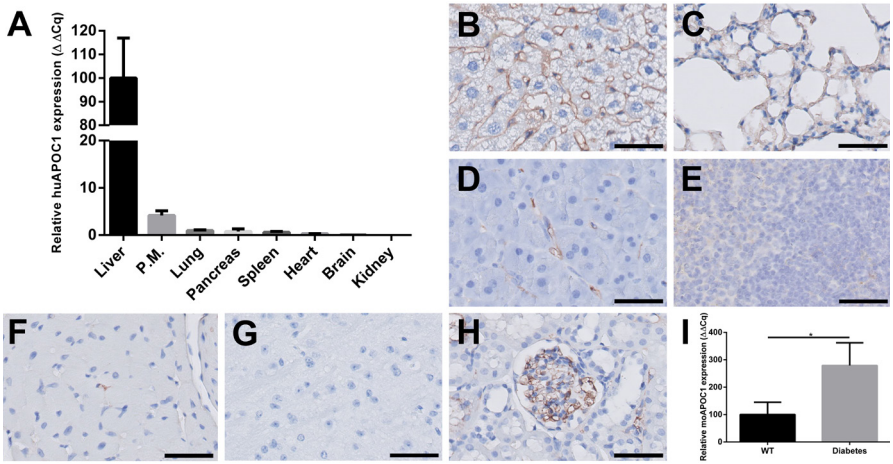

Figure 1: Transgenic mRNA and protein levels in *APOC1*-tg mice. A. Relative mRNA levels (ΔΔCq) of human *APOC1* in the organs and peritoneal macrophages (P.M.) of 6-month-old *APOC1*-tg mice. Human *APOC1* mRNA level in the liver was set to 100%. B-H. Representative images of human apoCI-immunostained liver (B), lung (C), pancreas (D), spleen (E), heart (F), brain (G), and kidney (H) samples from a 6-month-old *APOC1*-tg mouse. I. Relative mRNA levels (ΔΔCq) of mouse *ApoC1* in the liver of 6-month-old WT and STZ-induced diabetic mice. WT liver mouse *ApoC1* mRNA level was set to 100%. Scale bars indicate 50 μm.

Next, we examined the morphology of the glomeruli in *APOC1*-tg and WT mice in three age categories. Interaction effects for age and genotype were found for the glomerular damage score, glomerular hypertrophy, and the number of podocytes ($p<0.001$). At 9 months of age, the *APOC1*-tg mice began to develop mesangial matrix expansion (Figure 3B); by 15 months, this had progressed to glomerulosclerosis and was accompanied by glomerular hypertrophy (Figure 3I, J). These glomerulosclerotic lesions have a nodular-like pattern (Figure 3D) and were positive for several extracellular matrix (ECM) proteins, including collagen type I, collagen type IV, and fibronectin (supplementary material, Figure S1A-C). Lesions were also positive for collagen type VI; however, this protein was observed primarily at the periphery of the sclerotic lesions (supplementary material, Figure S1D). Decreased number of podocytes is a common feature in several kidney diseases, including DN[27]. Consistent with this finding, the number of podocytes

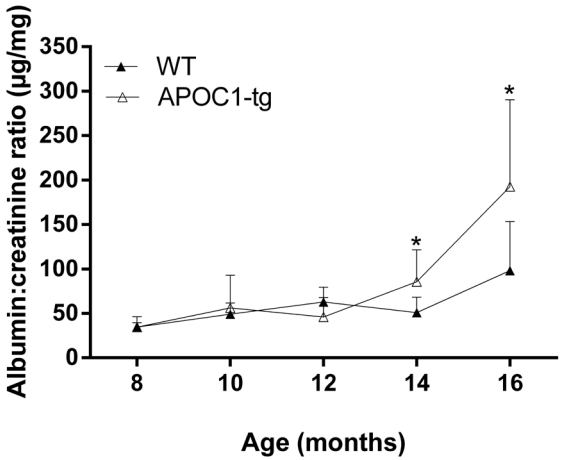

Figure 2: Time course of urinary albumin:creatinine ratio in WT and *APOC1*-tg mice. **p*<0.05, Student's *t* test.

generally decreased with age in *APOC1*-tg mice (Figure 3E, F, K). WT mice displayed no sign of either mesangial matrix expansion (Figure 3A, C, I) or podocyte loss at any time point studied (Figure 3K). Sirius red staining combined with fluorescence microscopy revealed that interstitial fibrosis increased significantly during ageing in both WT and *APOC1*-tg mice ($p<0.01$). Compared to WT mice, *APOC1*-tg mice developed significantly more interstitial fibrosis ($p<0.05$; Figure 3G, H, L). In the *APOC1*-tg mice, interstitial fibrosis was correlated with the number of podocytes ($r=-0.630$; $p<0.001$), glomerular damage ($r=0.485$; $p<0.001$), glomerular hypertrophy ($r=0.358$; $p<0.001$), and the number of glomerular macrophages ($r=0.492$; $p<0.001$). In contrast, the animals' sex was not correlated with glomerular damage ($p=0.939$), the number of podocytes ($p=0.486$), glomerular hypertrophy ($p=0.973$), or interstitial fibrosis ($p=0.542$). We also found no significant difference between *APOC1*-tg and WT mice at 16 months of age with respect to renal cortex triglyceride (3.9±0.4 and 4.6±1.7 nmol/mg tissue, respectively), total cholesterol (10.7±3.1 and 9.8±0.3 nmol/mg tissue, respectively), or phospholipid (24.3±7.4 and 22.9±0.7 nmol/mg tissue, respectively) levels. In addition, oil red O staining revealed no significant difference in renal lipid deposition between *APOC1*-tg and WT mice at 16 months of age (data not shown).

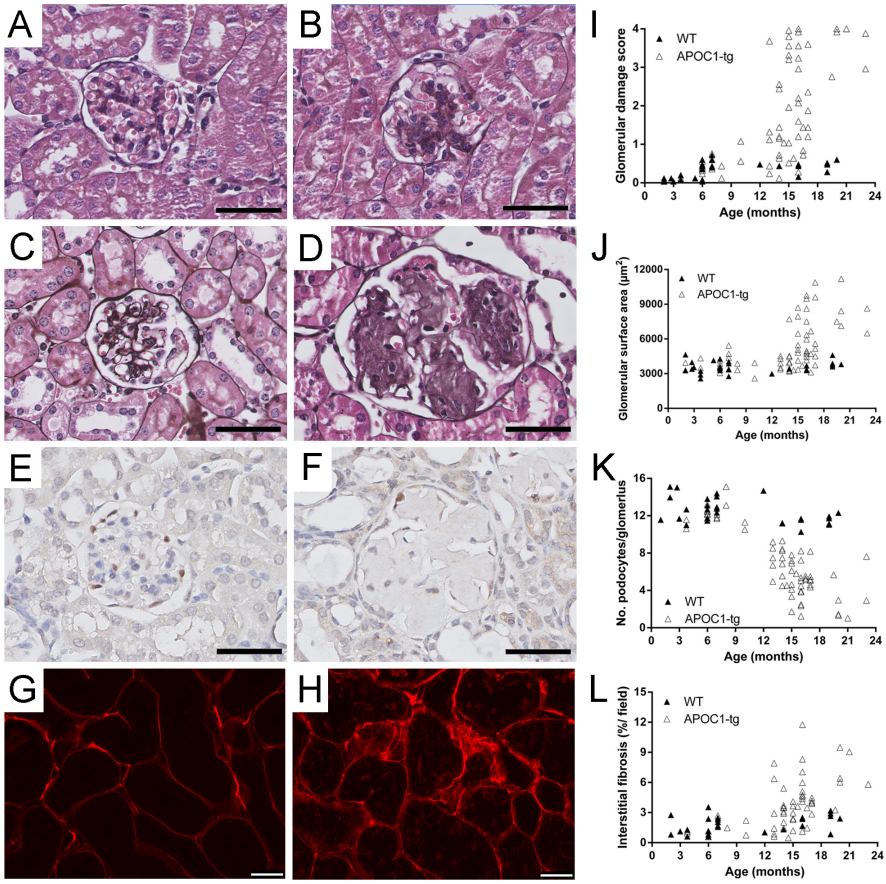

Figure 3: ***APOC1*-tg mice develop glomerulosclerosis and interstitial fibrosis.** A and C. WT mice have normal glomerular morphology at 4 months (A) and 16 months (C) of age. B and D. *APOC1*-tg mice develop kidney damage with ageing, evident as mesangial matrix expansion beginning at 9 months of age (B); this expansion progressed to severe glomerulosclerosis by 15 months of age (D). E-F. The number of podocytes decreases with age in *APOC1*-tg mice, beginning at 13 months of age (E); by 20 months, extremely few podocytes remain (F). G-H. At 20 months of age, *APOC1*-tg mice (H) develop significantly more interstitial fibrosis than age-matched WT mice (G). I. Glomerular damage score plotted against age. J. Glomerular surface area plotted against age. K. The number of podocytes per glomerulus plotted against age. L. Interstitial fibrosis per field plotted against age. The sections shown in A-D were stained with methenamine silver. The sections shown in E-F were immunostained for WT1, a marker for podocytes, and the sections shown in G-H were stained with Sirius red. Scale bars indicate 50 µm (A-F) and 25 µm (G-H).

Influx of inflammatory M1 macrophages in *APOC1*-tg mice

Next we examined whether macrophages might be responsible for the development of glomerulosclerosis in *APOC1*-tg mice. Again, we separated mice into three age categories for analysis. Even in the youngest (≤8 months) *APOC1*-tg mice had more glomerular macrophages than their WT littermates (Figure 4A, B) ($p<0.01$). This difference in glomerular macrophages in favour of *APOC1*-tg mice over WT mice remained significant across the age categories ($p<0.01$) (Figure 4C, D, K). Male and female mice did not differ with respect to the number of glomerular macrophages ($p=0.651$). Positive staining for CD11b and FA-11 on adjacent slides[28], combined with negative staining for CD163, indicates that the glomerular macrophages in *APOC1*-tg mice are M1 inflammatory macrophages. In addition, the glomeruli in *APOC1*-tg mice had greater levels of TNF-alpha than WT glomeruli ($p<0.001$) (Figure 4E, F, L), supporting the presence of M1 macrophages. Both VCAM-1 (Figure 4G, H, M) and ICAM-1 (Figure 4I, J, N) were up-regulated in *APOC1*-tg mice as early as 4 months of age, suggesting that both adhesion molecules play a role in the influx of glomerular macrophages. Double-staining for VCAM-1 and von Willebrand factor (vWF) revealed that VCAM-1 expression increased exclusively in endothelial cells (supplementary material, Figure S2). A histological examination of ICAM-1 revealed that its expression was also increased in endothelial cells.

ApoCI enhances the inflammatory response

We previously reported that apoCI increases the production of TNF-alpha in LPS-stimulated macrophages[10] by enhancing the binding of LPS to Toll-like receptor 4 (TLR4) via CD14[29]. Based on these results and findings from the present study, we hypothesised that the glomerulosclerosis seen in *APOC1*-tg mice is caused by macrophages expressing human *APOC1*, which leads to an increased cytokine response in these macrophages upon activation, ultimately stimulating mesangial cells to up-regulate production of the ECM. To test this hypothesis, we isolated peritoneal macrophages from both WT and *APOC1*-tg mice, stimulated these cells with LPS, and measured the mRNA expression levels of inflammatory and fibrogenic cytokines. Compared with WT cells, the expression levels of *Tnfa* and *Il1b* (pro-inflammatory

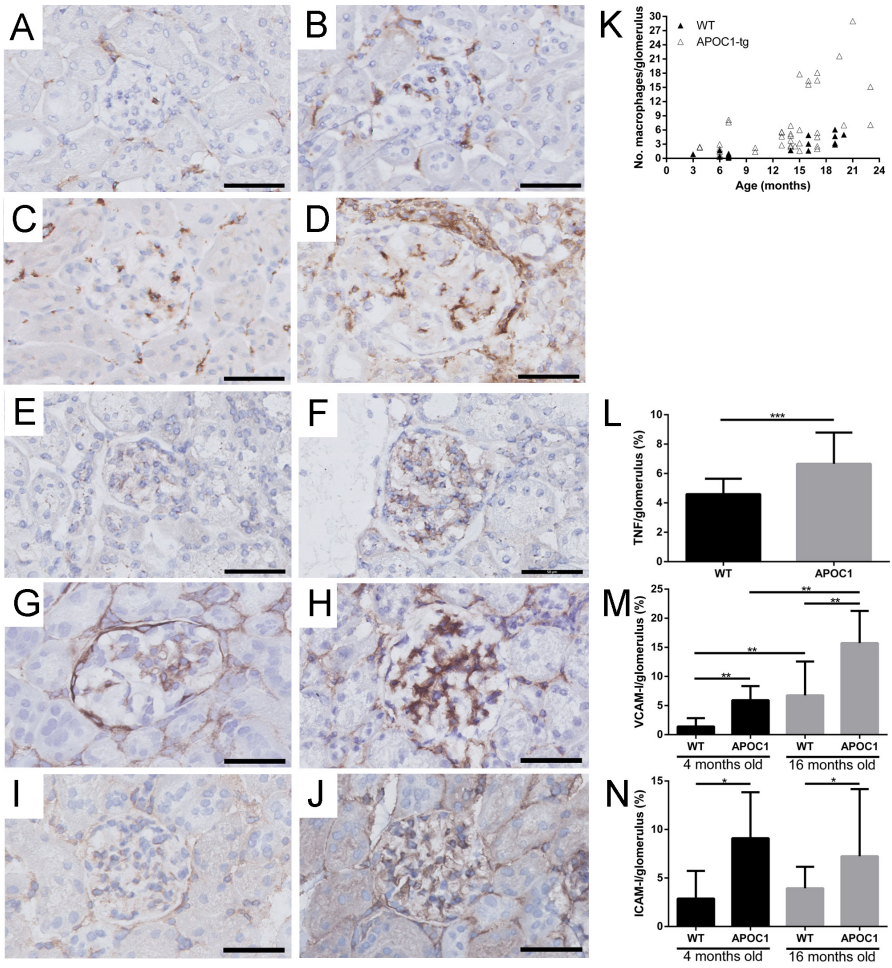

Figure 4: ***APOC1*-tg mice have increased numbers of glomerular macrophages and increased endothelial activation**. A-D. At 4 months (A, B) and 16 months (C, D) of age, *APOC1*-tg mice (B, D) have more glomerular macrophages than WT mice (A, C). E-F. At 16 months of age, TNF-alpha protein levels are higher in *APOC1*-tg mice (F) than in WT mice (E). G-J. At 16 months of age, *APOC1*-tg mice have increased expression of VCAM-1 (H) and ICAM-1 (J) compared with WT mice (G and I, respectively). K. The number of macrophages per glomerulus plotted against age. L. TNF-alpha expression per glomerulus. M. VCAM-1 expression per glomerulus. N. ICAM-1 expression per glomerulus. *p<0.05, **p<0.001, and ***p<0.001, one-way ANOVA. Scale bars indicate 50 µm.

cytokines), and the expression levels of *Tgfb1* and *Pdgfb* (both pro-fibrotic cytokines) were significantly increased in LPS-stimulated *APOC1*-tg macrophages, as well as the expression level of *Tlr4* (Figure 5). Incubating macrophages with LPS and anti-CD14 antibodies prevented the increased expression of cytokines in *APOC1*-tg macrophages (Figure 5), consistent with previous data that apoCI enhances the biological response to LPS via the CD14/TLR4 pathway[29].

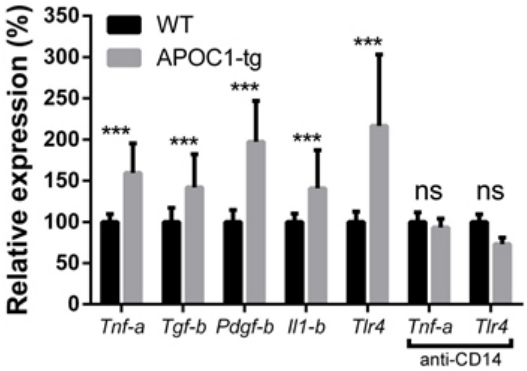

Figure 5: ***APOC1*-tg macrophages have an increased cytokine response**. Macrophages were isolated from wild-type (WT) or *APOC1*-tg mice and stimulated with LPS (10 ng/ml) or LPS and anti-CD14 (10 ng/ml) for 6 h at 37°C. The mRNA levels of *Tnf-alpha*, *Tgf-beta*, *Pdgf-beta*, *Il1-beta*, and *Tlr4* were measured. The expression levels were normalised to the respective LPS-stimulated WT control and are expressed as the mean ± sd. ***p<0.001, Student's t test.

Patients with DN have increased expression of glomerular apoCI

As a first step towards testing the hypothesis that apoCI plays a role in human DN, we studied the presence of apoCI in glomeruli of non-diabetic subjects and diabetic patients with and without DN. The patient characteristics are summarised in the supplementary material, Table S3. The glomeruli in non-diabetic controls were generally negative for apoCI (Figure 6A). In contrast, the glomeruli of kidneys obtained from diabetic patients were positive for apoCI (Figure 6B, C). The percentage of apoCI-positive glomeruli was greater in diabetic patients with nephropathy than in those without nephropathy (Figure 6D). Double-label immunostaining revealed that apoCI co-localizes with macrophages. In addition, apoCI was present in resident renal cells (Figure 6E-G).

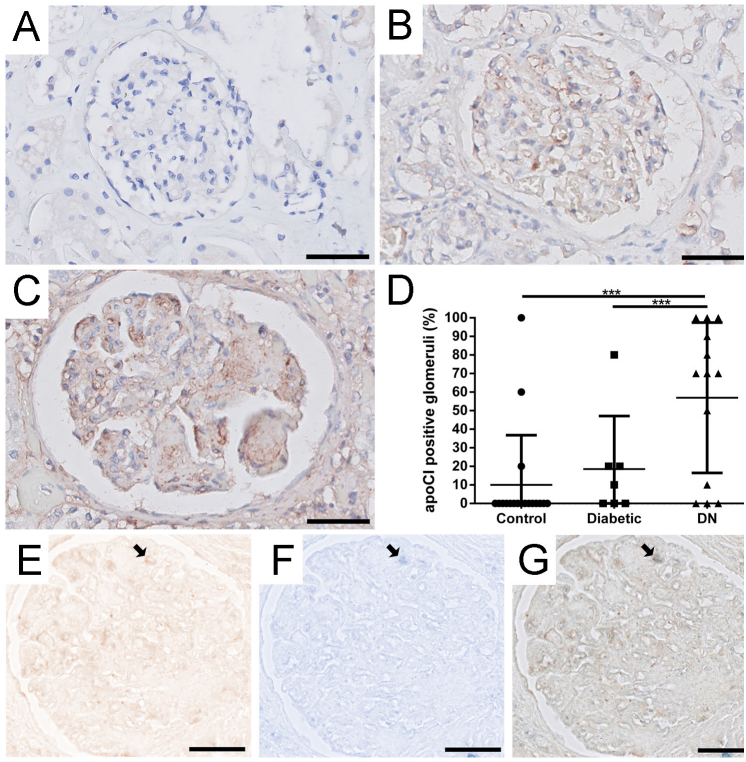

Figure 6: **Patients with DN have increased expression of glomerular apoCI**. A. Representative kidney section from a control subject showing no glomerular apoCI staining. B. Representative kidney section from a patient with diabetes without nephropathy, positive for apoCI. C. Representative kidney section from a patient with diabetes and nephropathy. D. The percentage of apoCI-positive glomeruli in control subjects and diabetic patients with and without DN. E-G. Double-label immunostaining for apoCI (E) and CD68 (F) shows co-localization of apoCI with CD68-positive macrophages (arrows); Panel G shows a merged image. ApoCI is also present in resident renal cells. ***p<0.001, one-way ANOVA. Scale bars indicate 50 µm.

Discussion

Here, we report that transgenic mice expressing the human *APOC1* gene develop glomerulosclerosis and renal dysfunction with increased albuminuria beginning at 14 months of age. The degree of kidney damage was correlated strongly with age, glomerular hypertrophy,

reduced podocyte numbers, and the number of glomerular macrophages. Moreover, apoCI increased the expression of inflammatory and fibrogenic cytokines in macrophages upon stimulation *in vitro*. Our finding that renal cortex lipid content – specifically, triglycerides, total cholesterol, and phospholipids – does not differ between *APOC1*-tg mice and WT mice suggests that the development of renal damage in *APOC1*-tg mice is not due to changes in lipid metabolism, but is likely to be due to a different function of apoCI, possibly through the activation of macrophages. This notion is supported by the finding that the increased apoCI concentration in patients with type 2 diabetes (T2D) is only partially explained by increased TG levels in these patients[30]. To further investigate the involvement of *APOC1* in the development of renal damage, we examined whether *APOC1* expression was increased in STZ-induced diabetic WT mice, an animal model for diabetic nephropathy. The mRNA levels of mouse *ApoC1* in the livers of STZ-induced diabetic WT mice were 2.8-fold higher than those in control WT mice, supporting the role of apoCI in DN. These findings are clinically relevant, as they show that apoCI plays a role in the pathogenesis of glomerulosclerosis. Interestingly, we found that, compared with controls, patients with diabetes and DN showed a significant increase in glomerular apoCI expression, supporting our hypothesis that apoCI plays a role in the pathogenesis of glomerulosclerosis in DN. Our double-label immunostaining experiments revealed that apoCI co-localized with macrophages, and apoCI was also present in resident renal cells. Given that the renal cells do not express human *APOC1* mRNA, this indicates that the apoCI present in resident renal cells was taken up either from macrophages or from the circulation.

Bouillet *et al.* recently reported that patients with type 1 diabetes (T1D) develop less nephropathy compared with T2D patients, even though T1D and T2D patients have similar increases in plasma apoCI levels compared with control subjects[8]. It is important to note that Bouillet *et al.* used proteinuria levels as a measure of nephropathy; therefore, it is possible that nephropathy was underdiagnosed. This notion is supported by our recent finding that DN lesions can develop before the onset of clinical findings; specifically, 20 out of 106 patients with histologically confirmed DN did not present with DN-associated clinical manifestations[31].

We propose that apoCI mediates the development of glomerulosclerosis in *APOC1*-tg mice via at least two distinct mechanisms. First, *APOC1* expression results in dyslipidaemia[22,32], which is reported to increase the expression of VCAM-1 and ICAM-1 in glomeruli[33,34]. These proteins mediate monocyte/macrophage infiltration and thereby contribute to the severity of DN. This is supported by the finding that diabetic ICAM-1-deficient mice have reduced glomerular infiltration of macrophages and are protected from developing renal injury[20]. Second, apoCI – either derived from the circulation[10] or expressed by macrophages – increases the inflammatory and fibrogenic response of stimulated macrophages, including increased expression of TNF-alpha and TGF-beta. These cytokines have been reported to increase the expression of ECM proteins in mesangial cells[17,35-38]. In addition to increasing matrix production, both TNF-alpha and TGF-beta downregulate expression of the protein nephrin in podocytes, thereby altering glomerular permeability and leading to albuminuria[39]. TGF-beta also induces apoptosis in podocytes[40].

Glomerular nodules, also known as Kimmelstiel-Wilson nodules, are characteristic of class III DN[26] and may be a form of microangiopathy[41]. *APOC1*-tg mice develop a renal phenotype with nodular glomerulosclerosis, with increased collagen types I, IV, and VI, increased renal dysfunction, increased glomerular hypertrophy, reduced podocyte numbers, and interstitial fibrosis – all of which are reminiscent of DN, even though these mice are not diabetic. These results support our previous finding that DN can develop without clinical manifestations[31]. With the exception of BTBR Ob/Ob mutant mice[16], nodular glomerulosclerosis is generally not observed in mice. In addition to its role in the development of glomerulosclerosis, apoCI also plays a role in the development of atherosclerosis. The apoCI content of triglyceride-rich lipoproteins (TRLs) is an independent predictor of early atherosclerosis[42] and is correlated with plaque size in carotid atherosclerosis[43]. Likewise, we previously reported that endogenous apoCI expression in hyperlipidaemia mice increased the size of atherosclerotic lesions by 60%, and this was related to an increased inflammatory response in macrophages[44]. Moreover, increased apoCI levels in TRL particles are also present in patients with DN[45], and diabetes is known to increase the risk of cardiovascular disease, including atherosclerotic disease

and congestive heart failure[46]. The finding that apoCI is involved in both nodular glomerulosclerosis and atherosclerosis supports the notion that glomerular nodules may be the result of vascular damage.

Taken together, our results obtained from *APOC1*-tg mice and patients with DN provide new insights into the role that apoCI plays in the development of glomerulosclerosis and suggest apoCI as a therapeutic target for slowing the progression of DN in high-risk patients. These data also shed new light on the mechanism by which the inflammatory and fibrogenic response is increased in macrophages, potentially leading to the development of nodular glomerulosclerosis present in *APOC1*-tg mice. Future studies should help determine whether macrophages are indeed the key contributor to the observed renal phenotype and whether *APOC1* affects the development of DN.

Acknowledgements

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Supplementary Materials

Table S1: Absolute and relative organ weights in WT and *APOC1*-tg mice at 6 months of age.

	WT	*APOC1*-tg	WT	*APOC1*-tg
Organ	**Weight, grams (mean±SD)**	**Weight grams (mean±SD)**	**Relative weight, % (mean±SD)**	**Relative weight % (mean±SD)**
Kidney (left)	0.16±0.03	0.18±0.04	0.56±0.04	0.64±0.09**
Kidney (right)	0.17±0.03	0.18±0.03	0.57±0.04	0.66±0.09***
Heart	0.15±0.03	0.20±0.03	0.52±0.04	0.58±0.07*
Liver	1.36±0.25	1.27±0.25	4.62±0.19	4.55±0.58
Brain	0.33±0.02	0.34±0.04	1.16±0.21	1.24±0.20
Lungs	0.18±0.01	0.19±0.03	0.64±0.12	0.68±0.13
Spleen	0.09±0.01	0.10±0.02	0.32±0.07	0.38±0.10
Pancreas	0.28±0.07	0.26±0.05	0.96±0.18	0.95±0.17
Total Body Weight	29.4±5.0	27.9±4.3	NA	NA

*$p<0.05$, **$p<0.01$, and ***$p<0.001$ versus WT; Student's *t* test. NA, not applicable.

Table S2: Laboratory values of WT and *APOC1*-tg mice at 6 months of age.

	WT	*APOC1*-tg
Triglycerides (mmol/L)	0.58±0.06	2.83±0.97***
Total cholesterol (mmol/L)	2.33±0.28	3.88±0.91***
HDL-C (mmol/L)	2.14±0.33	2.33±0.64
Non-HDL-C (mmol/L)	0.19±0.13	1.56±0.49***
GOT (U/L)	$0.2\pm0.1x10^{-3}$	$0.1\pm0.06x10^{-3}$
CK (U/L)	$1.2\pm0.1x10^{-3}$	$1.4\pm0.8x10^{-3}$
Haemoglobin (mmol/L)	11.03±0.71	10.91±0.72
White blood cells ($x10^9$)	2.607±0.59	2.391±0.30
BUN (mg/dl)	31.25±6.76	27.19±5.18
Serum creatinine (µmol/L)	11.0±2.7	13.0±2.8
Blood glucose (mmol/L)	11.83±1.60	11.42±2.54

Data are presented as the mean ± sd. HDL-C, high-density lipoprotein cholesterol; BUN, blood urea nitrogen; GOT, glutamate oxaloacetate transaminase; CK, creatinine kinase. *$p<0.05$, **$p<0.01$, and ***$p<0.001$ versus WT, Student's *t* test.

Table S3: Summary of patient and control subjects used for human apoCI staining.

	Autopsy control (n=17)	DM, no DN (n=8)	DM, with DN (n=12)
Age (years)	65±12	76±12	72±9
DM duration (years)	NA	12±10	23±13
HbA1c (%)	NA	7.4±0.26	7.9±0.84
Serum creatinine (µmol/ml)	NA	310±282	310±141
Blood pressure (systolic/diastolic)	NA	137/73	134/79

Data are presented as the mean ± sd; blood pressure is presented as the mean value. DM, diabetes mellitus; DN, diabetic nephropathy; NA, not applicable.

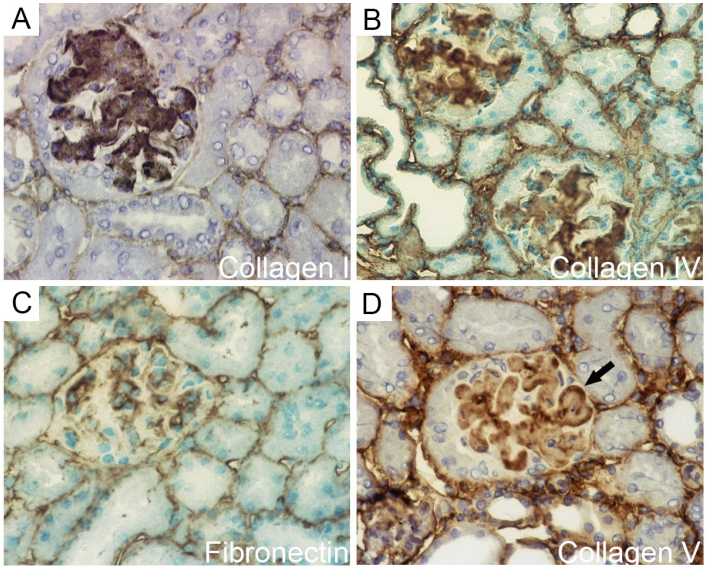


Figure S1: Sclerotic lesions contain deposits of collagen type I, collagen type IV, collagen type VI, and fibronectin. Glomerulosclerotic lesions in a 16-month-old *APOC1*-tg mice have an intense staining pattern for the extracellular matrix (ECM) proteins collagen type I (A), collagen type IV (B), and fibronectin (C). Collagen type VI is also present in the sclerotic lesions (D), but only in the peripheral areas (arrow).

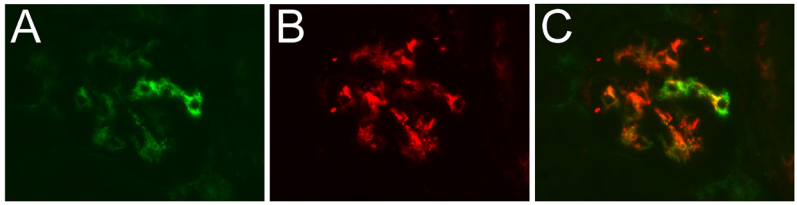


Figure S2: VCAM-1 expression in endothelial cells. A-C. Double-label immunostaining for VCAM-1 (A) and von Willebrand factor (B) in the kidneys of a 16-month-old *APOC1*-tg mouse, showing co-localisation exclusively in endothelial cells (C).

Supplemental Methods

PAS, Silver, and Sirius Red staining

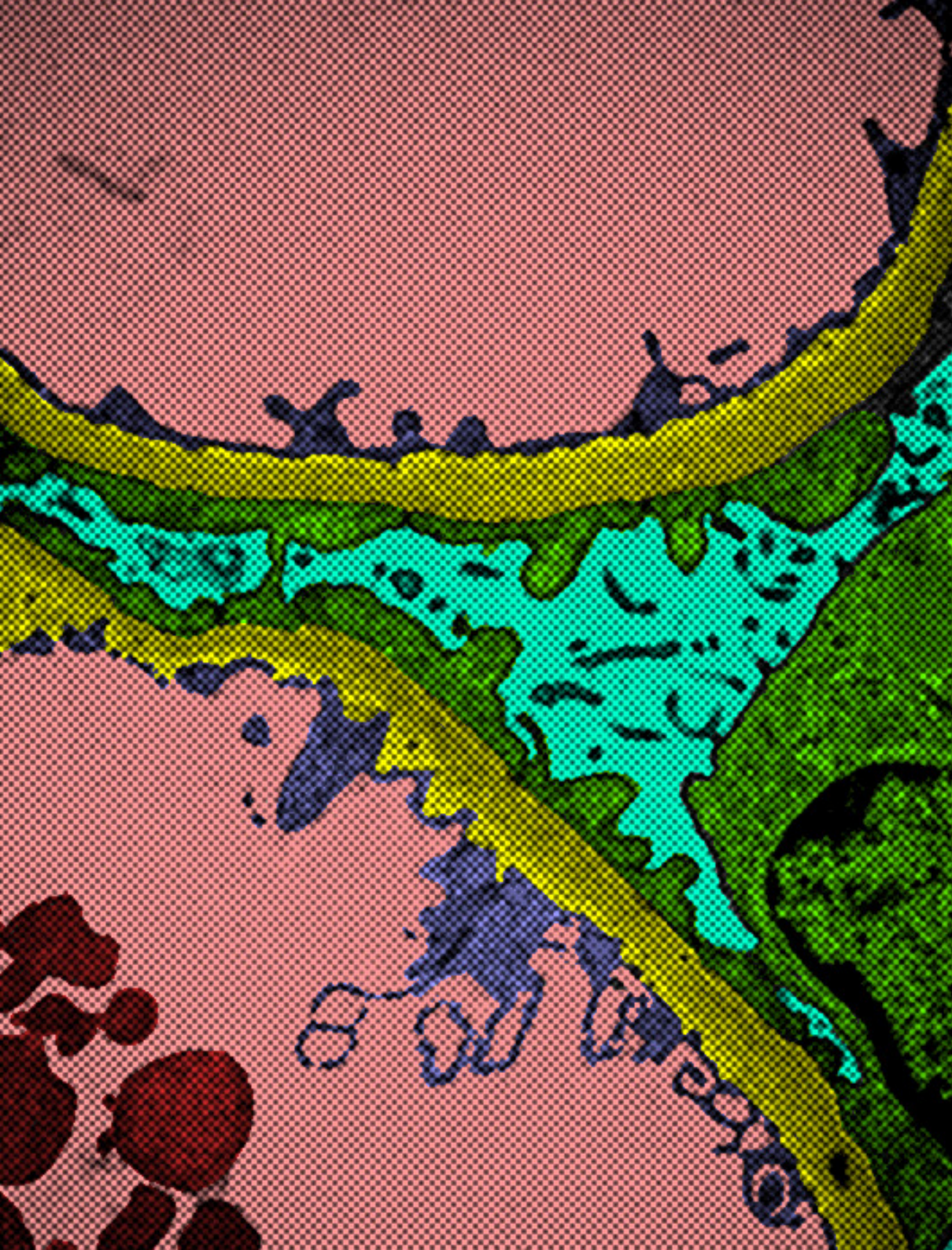
Paraffin-embedded sections (4 µm thickness) were oxidised in periodic acid at 48°C for 30 minutes, then washed with demi-water. The sections were then incubated in 5% Schiff's reagent for 20 minutes, incubated in tap water for 5 minutes, then rinsed briefly in demi-water. Finally, the sections were incubated in haematoxylin for 5 minutes, followed by incubation in tap water for 5 minutes.

For silver staining, paraffin-embedded sections (1-µm thickness) were cut and incubated in 1% periodic acid for 10 minutes at 60°C, and then stained in a silver solution at 60°C until the glomerular basement membranes became black. The sections were then incubated in 0.2% gold chloride until the sections became light grey. Finally, the sections were incubated for 2 minutes in 5% sodium thiosulfate, 10 minutes in tap water, and 30 seconds in eosin.

Paraffin-embedded sections were stained with Sirius Red by incubating sections with 0.2% phosphomolybdic acid for 5 minutes, 0.1% Sirius Red for 90 minutes, and saturated picric acid for 1 minute.

Primer sequences

Hprt1: 5'-GGCTATAAGTTCTTTGCTGACCTG-3' and 5'-AACTTTTATGTCCCCCGTTGA-3'; human *APOC1*: 5'-AGCCGCATCAAACAGAGTGA-3' and 5'-TCCTGGGATGTCACCCTTCA-3'; mouse *ApoC1*: 5'- GTCCGGAACATTGGAGAGCA-3' and 5'-CCTGAAAGGTCCTAACCGGG-3'; mouse *Tnf-alpha*: 5'-GGTGCCTATGTCTCAGCCTC-3' and 5'-GCTCCTCCACTTGGTGGTTT-3'; mouse *Tgf-beta*: 5'-AGCTGCGCTTGCAGAGATTA-3' and 5'-AGCCCTGTATTCCGTCTCCT-3'; mouse *Pdgf-beta*: 5'-TGTGAGACAGTAGTGACCCCT-3' and 5'-AAACTTTCGGTGCTTGCCTTT-3'; mouse *Il1-beta*: 5'-GCTCTCCACCTCAATGGACA-3' and 5'-GTGGGTGTGCCGTCTTTCAT-3'.



Chapter 3

The VEGF-A inhibitor sFLT-1 improves renal function by reducing endothelial activation and inflammation in a mouse model of type 1 diabetes

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Abstract

Aims/hypothesis Animal models of diabetic nephropathy show increased levels of glomerular vascular endothelial growth factor (VEGF)-A, and several studies have shown that inhibiting VEGF-A in animal models of diabetes can prevent albuminuria and glomerular hypertrophy. However, in those studies, treatment was initiated before the onset of kidney damage. Therefore, the aim of this study was to investigate whether transfecting mice with the VEGF-A inhibitor *sFlt-1* (encoding soluble fms-like tyrosine kinase 1) can reverse pre-existing kidney damage in a mouse model of type 1 diabetes. In addition, we investigated whether transfection with *sFlt-1* can reduce endothelial activation and inflammation in these mice.

Methods Subgroups of untreated 8-week-old female C57BL/6J control (n=5) and diabetic mice (n=7) were euthanised 5 weeks after the start of the experiment in order to determine the degree of kidney damage prior to treatment with sFLT-1. Diabetes was induced by three i.p. injections of streptozotocin (75 mg/kg) administered at 2 day intervals. Diabetic nephropathy was then investigated in diabetic mice transfected with *sFlt-1* (n=6); non-diabetic, non-transfected control mice (n=5); non-diabetic control mice transfected with *sFlt-1* (n=10), and non-transfected diabetic mice (n=6). These mice were euthanised at the end of week 15. Transfection with *sFlt-1* was performed in week 6.

Results We found that transfection with *sFlt-1* significantly reduced kidney damage by normalising albuminuria, glomerular hypertrophy and mesangial matrix content (i.e. glomerular collagen type IV protein levels) ($p<0.001$). We also found that transfection with *sFlt-1* reduced endothelial activation ($p<0.001$), glomerular macrophage infiltration and glomerular TNF-α protein levels ($p<0.001$). Finally, sFLT-1 decreased VEGF-A-induced endothelial activation *in vitro* ($p<0.001$).

Conclusions/interpretation These results suggest that sFLT-1 might be beneficial in treating diabetic nephropathy by inhibiting VEGF-A, thereby reducing endothelial activation and glomerular inflammation, and ultimately reversing kidney damage.

Introduction

Diabetic nephropathy is characterised by damage and dysfunction of the microvasculature[1]. A critical factor in maintaining the microvasculature is vascular endothelial growth factor (VEGF)-A, which regulates many aspects of vascular physiology, including vascular permeability and the migration, proliferation and survival of endothelial cells (for review, see Bartlett *et al.*)[2]. Several studies in both human and animal models have indicated that proper glomerular function requires tight regulation of VEGF-A levels, as both upregulation and downregulation of VEGF-A can lead to kidney disease[3].

Animal models of diabetic nephropathy develop increased levels of glomerular VEGF-A[4,5], possibly due to the effect of high glucose on VEGF-A production in podocytes[6]. Therefore, inhibiting VEGF-A may be beneficial in treating renal complications. Consistent with this notion, antibodies directed against VEGF-A have been shown to prevent albuminuria[7,8] and glomerular hypertrophy[9] in animal models of diabetes. However, in these studies, the inhibition of VEGF-A was initiated prior to the onset of diabetic kidney disease (i.e. prior to the development of albuminuria, glomerular hypertrophy, and mesangial expansion/matrix production); thus, whether this strategy is feasible for treating diabetic people with existing kidney damage is currently unknown.

In addition to its role in maintaining vascular homeostasis, VEGF-A also facilitates the migration of monocytes and macrophages. Several studies found that macrophages play a role in diabetic nephropathy[10-12]. VEGF-A–induced migration of monocytes and macrophages is mediated by the binding of VEGF-A to VEGF receptor (VEGFR)-1 (also known as fms-related tyrosine kinase (FLT)-1) expressed on these cells[13-15]. In addition, both VEGF-A[16] and high glucose levels[17] can activate endothelial cells, leading to increased levels of vascular cell adhesion molecule (VCAM)-1 and intercellular adhesion molecule (ICAM)-1, thereby promoting monocyte infiltration.

Here, we investigated whether the VEGF-A inhibitor soluble FLT-1 (sFLT-1; also known as soluble VEGFR-1) can reduce renal complications, including albuminuria and mesangial matrix expansion, in a mouse model of type 1 diabetes and pre-existing kidney damage. In addition, because diabetic nephropathy is accompanied by endothelial

activation[1] and macrophage infiltration[11,12,18], both of which are mediated by VEGF-A, we also investigated the effect of inhibiting VEGF-A on these variables. Last, we investigated whether transfection with *sFlt-1* reduces glomerular TNF-α protein levels (a measure of inflammation) in diabetic mice.

Methods

sFlt-1 transfection

pcDNA3.1 vectors (Invitrogen, Breda, the Netherlands) containing either mouse *sFlt-1-VSV* or the luciferase gene, both of which are driven by the cytomegalovirus promoter, were constructed as described previously[19]. The plasmids were amplified in *Escherichia coli* DH5α (Invitrogen), purified using the QIAfilter Plasmid Maxi-prep kit (Qiagen, Venlo, the Netherlands), and dissolved in EndoFree Tris–EDTA buffer (Qiagen). The mice were co-transfected with the *sFlt-1-VSV* and luciferase constructs in both calf muscles (20 μg each) using electroporation, as described previously[19]. To monitor transfection efficiency, the mice were injected with i.p. luciferin at 2-week intervals. Five minutes after the luciferin injection, luciferase activity was visualized using a NightOWL bioluminescence camera (Xenogen Ivis Spectrum, Alameda, CA, USA), as described previously[19].

Tube formation assay

To confirm functional expression of the *sFlt-1* construct, we performed a tube formation assay as described previously[20]. In brief, human umbilical vein endothelial cells (HUVECs) ($1.5x10^3$ cells per well; Promocell, Heidelberg, Germany) were plated on Matrigel-coated 96-well plates (Corning, Amsterdam, the Netherlands). The HUVECs were incubated for 6 h with culture medium obtained from human embryonic kidney 293 (HEK293) cells (ATCC, Manassas, VA, USA) transfected with an *sFlt-1* construct (2 μg), or a luciferase construct (2 μg). The HEK293 cells were transfected using 6 μl X-tremeGENE (Roche, Basel, Switzerland); 2 days after transfection, the culture medium was collected and applied to the HUVECs in the presence or absence of VEGF-A (10 ng/ml; R&D Systems, Minneapolis, MN, USA). The number

of tube branch points was counted in five 400X fields. Images were taken using a Moticam camera (Motic, Xiamen, China). This experiment was performed three times.

Animals

This study used 8-week-old female C57BL/6J mice (specific pathogen free; Harlan Laboratories, Indianapolis, IN, USA), weighing 17.8 ± 1.1 g (mean ± SD). All experiments were conducted in accordance with national guidelines for the care and use of experimental animals (DEC license 13163). Mice were housed in individually ventilated cages in groups of five mice, with food and water *ad libitum*. C57BL/6J mice were chosen because this study was a follow-up of a previous study that investigated podocyte-specific VEGF-A knock-down on a C57BL/6 background[21]. Moreover, C57BL/6J mice respond well to the streptozotocin (STZ) regimen in terms of blood glucose levels[22]. Diabetes was induced by three i.p. injections of STZ (75 mg/kg body weight; Sigma-Aldrich, St. Louis, MO, USA) administered at 2 day intervals. Blood glucose levels were measured (Accu-Chek, Roche) at the end of weeks 1, 5 and 15 after diabetes induction. Mice with a blood glucose level of 15 mmol/L or higher were considered diabetic. Mice were randomly divided into groups. Subgroups of untreated control mice (n=5) and diabetic mice (n=10) were killed 5 weeks after the start of the experiment in order to determine the degree of kidney damage prior to treatment with sFLT-1. In week 6, the mice were transfected with a plasmid containing *sFlt-1*. Diabetic nephropathy was then investigated in diabetic mice transfected with *sFlt-1* (n=10), non-diabetic, non-transfected control mice (n=5), non-diabetic control mice transfected with *sFlt-1* (n=10), and non-transfected diabetic mice (n=10). These mice were killed at the end of week 15. Three diabetic mice 5 weeks after the induction of diabetes, four diabetic mice transfected with *sFlt-1* and four diabetic mice 15 weeks after the induction of diabetes were excluded from the study as they did not met the inclusion criteria of a blood glucose level of 15 mmol/L or higher.

Measurement of the urine albumin excretion ratio

To measure the urine albumin excretion ratio, spot urine was collected in weeks 5 and 15. Urine albumin levels were measured using

rocket immunoelectrophoresis with rabbit anti-mouse albumin; purified mouse serum albumin (Sigma-Aldrich) was used as a standard. Urine creatinine was measured using a creatinine assay, with picric acid, sodium hydroxide and creatinine standards (Sigma-Aldrich); the albumin:creatinine ratio was then calculated.

Immunohistochemistry

Paraffin-embedded kidney tissues (4 µm thickness) were cut using a Leica microtome (Wetzlar, Germany) and stained with periodic acid-Schiff's reagent using a standard protocol. Rabbit anti-mouse platelet/endothelial cell adhesion molecule 1 (PECAM-1; 1:400; Santa Cruz Biotechnology, Dallas, TX, USA), rabbit anti-human Wilms tumour (WT)1 (1:500; Santa Cruz Biotechnology) and rabbit anti-mouse collagen type IV (1:200; Abcam, Cambridge, UK) primary antibodies were used for immunostaining, followed by the anti-rabbit-Envision HRP-conjugated secondary antibody (undiluted; Dako, Glostrup, Denmark), with diaminobenzidine (DAB+; Dako) as the chromogen. The rabbit anti-human WT1 antibody cross-reacts with mouse WT1 (data not shown). As a negative control, non-specific isotype matched antibodies were used.

Frozen kidney tissues (4 µm thickness) were cut using a Leica cryostat. Rabbit anti-mouse fibronectin (1:2400; Sigma-Aldrich), rat anti-mouse CD68 (1:15; Abcam), rat anti-mouse VCAM-1 (1:1400; BD Pharmingen, San Diego, CA, USA), rat anti-mouse ICAM-1 (1:200; ATCC), rabbit anti-mouse TNF-α (1:100; Abcam), and rabbit anti-vesicular stomatitis virus (VSV; 1:2500; Sigma-Aldrich) primary antibodies were used for immunostaining, followed by the appropriate Envision (undiluted; Dako) or Impress (undiluted; Vector Laboratories, Burlingame, CA) HRP-conjugated secondary antibody, with DAB+ as the chromogen. As a negative control, non-specific isotype matched antibodies were used. Antibodies were tested for specificity with western blot analysis (PECAM-1, WT1, fibronectin, CD68, ICAM-1, TNF-α, VSV), immunoprecipitation (VCAM-1) or immunogen affinity purified (collagen type IV).

Digital image analysis

Sections were digitized using the Philips Ultra-Fast Scanner 1.6 RA (Amsterdam, the Netherlands). The surface area of the glomerular tuft (in μm^2) was measured in periodic acid-Schiff's reagent-stained slides with 25 glomeruli per section using Philips Ultra-Fast Scanner 1.6 RA software (Philips). ImageJ software (https://imagej.nih.gov/ij/) was used to measure the levels of fibronectin, collagen type IV, PECAM-1, VCAM-1, ICAM-1, and TNF-α. The positive area per glomerulus was determined by measuring the respective positively stained area, corrected for the total area of the glomerulus (ten and 25 glomeruli per frozen and paraffin-embedded section, respectively) at x400 magnification. The number of podocytes in each sample was determined by counting the number of WT1-positive nuclei per glomerulus in 25 glomeruli. The number of macrophages was determined by counting the number of CD68-positive cells in 10 glomeruli. The glomeruli used for these measurements were selected at random. Experimenters were blind to group assignment and outcome assessment.

Endothelial activation assay

HUVECs that were confluent for 2 days were incubated with VEGF-A (20 ng/ml; R&D Systems) for 2, 4, 6 and 8 h. To determine the effect of sFLT-1 on VEGF-A-induced endothelial activation, HUVECs were incubated for 4 h with sFLT-1 (0, 10, 100 or 1000 ng/ml; R&D Systems) in the presence of 20 ng/ml VEGF-A. These experiments were performed three times. Cell lines were negative for mycoplasma contamination.

To quantify changes in gene expression, total RNA was extracted from HUVECs using TRIzol extraction buffer (ThermoFisher Scientific, Waltman, MA, USA) and converted to cDNA with AMV reverse transcriptase (Roche) using random hexamer primers. Quantitative real-time PCR was performed using IQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) on a Bio-Rad CFX real-time system. Cycle threshold values were normalized to the housekeeping gene *HPRT1*. The following primers were used in this study: *HPRT1*: 5'-AGATGGTCAAGGTCGCAAGC-3' and 5'-TCAAGGGCATATCCTACAACAAAC-3'; *ICAM-1*: 5'-CAGAGGTTGAACCCCACAGT-3' and 5'-CCTCTGGCTTCGTCAGAATC-3'; *SELE*: 5'-AGCCCAGAGCCTTCAGTGTA-3' and 5'-AACTGGGATTTGCTGT-

GTCC-3'. Primers for amplifying *VCAM-1* were obtained from Sino Biological (North Wales, PA, USA).

Statistical analyses

Data are expressed as means ± SD. Data were analyzed using the two-tailed Student's *t* test or one-way ANOVA. We also used a one-way ANOVA to analyse the effect of sFLT-1 treatment in diabetic mice (week 15), corrected for the effect of time. Differences were considered significant at $p<0.05$.

Results

Transfection with *sFlt-1* reduced endothelial tube formation *in vitro*

To confirm functional expression of the *sFlt-1* construct, we performed a tube formation assay. First, HUVECs were cultured in medium obtained from luciferase-transfected HEK293 cells. The addition of VEGF-A (10 ng/ml) to the culture medium led to increased tube formation (Figure 1A, B), reflected by an increased number of branch points (Figure 1E). VEGF-A–induced tube formation was significantly inhibited by medium obtained from *sFlt-1*–transfected HEK293 cells (Figure 1D), confirming that expression of the *sFlt-1* construct inhibits VEGF-A–induced tube formation. As a control, culturing HUVECs with medium obtained from *sFlt-1*–tranfected HEK293 cells had no effect on tube formation in the absence of VEGF-A (Figure 1C).

Expression of sFLT-1 in mice by co-transfection with the *sFlt-1-VSV* and luciferase constructs

Diabetes was induced in mice by i.p. injections of STZ (see Methods). In week 6, mice were transfected with the *sFlt-1-VSV* and luciferase constructs by bilateral injection in the calf muscle. Transfection was confirmed by injecting mice with luciferin (see electronic supplementary material [ESM] Figure 1). Staining for VSV was used to confirm the presence of exogenous sFLT-1 in the renal vasculature (data not shown).

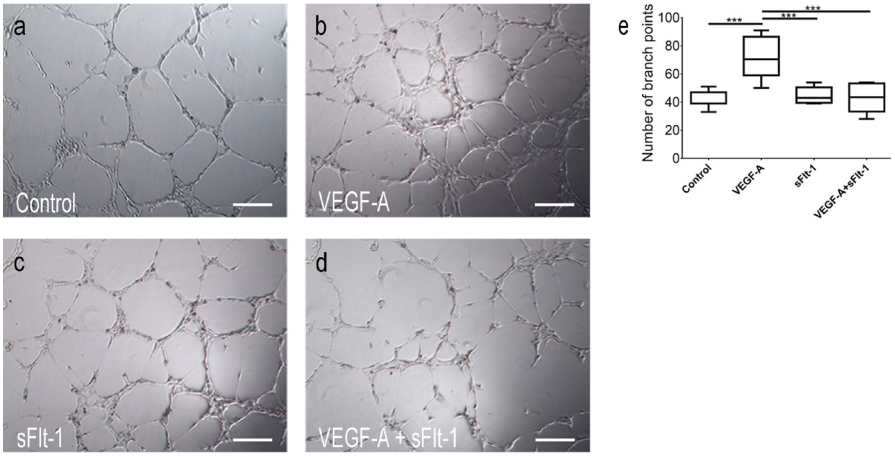

Figure 1: sFLT-1 inhibited VEGF-A-induced tube formation *in vitro*. (a-d) HUVECs were cultured in the presence or absence of VEGF-A (10 ng/ml) and/or sFLT-1, and the number of branch points was measured. E. Summary of the total number of branch points measured in five fields under each condition. Boxes represent 1st and 3rd quartiles; whiskers represent minimum and maximum number of branch points; horizontal line represents median number of branch points. ***$p<0.001$, one-way ANOVA. Scale bars, 100 μm.

Transfection with *sFlt-1* reduced kidney damage in diabetic mice

We first determined the development of kidney damage in diabetic mice 5 weeks after diabetes was induced. Inducing diabetes led to albuminuria, reflected by an albumin:creatinine ratio of 8.53 ± 2.59 mg/mmol, which was significantly higher than in control mice (3.06 ± 0.98 mg/mmol; $p<0.001$) (Figure 2A). In addition, compared with control mice, diabetic mice developed glomerular hypertrophy ($p<0.001$) (Figure 2B). Podocyte numbers did not differ between diabetic and control mice (Figure 2C). The protein levels of both collagen type IV and fibronectin—two markers of mesangial matrix expansion—were higher in the diabetic mice compared with control mice ($p<0.001$) (Figure 2D-F and Figure 2G-I, respectively).

Having confirmed that kidney damage develops in these mice within 5 weeks, we next examined the effect of *sFlt-1* transfection; transfection with *sFlt-1* was performed in week 6 and the mice were analyzed 9 weeks after transfection (i.e. 15 weeks after diabetes was induced). Our analysis revealed that sFLT-1 significantly reduced all

markers of kidney damage in the diabetic mice, including albuminuria, glomerular hypertrophy, and mesangial matrix expansion (p<0.001) (Figure 2A, B, D, G). Compared with control-transfected diabetic mice, *sFlt-1*–transfected diabetic mice had significantly fewer podocytes (p<0.01) (Figure 2C). Transfecting control (i.e. non-diabetic) mice with

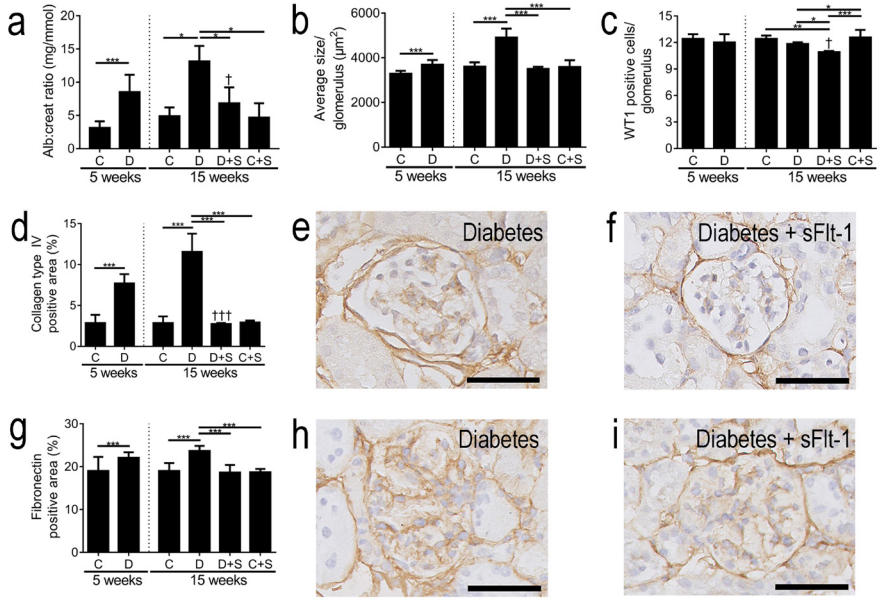

Figure 2: sFLT-1 reversed kidney damage in diabetic mice. Mice were injected with STZ to induce diabetes. In week 6, diabetic ("D") and control ("C") mice were transfected with a construct expressing *sFlt-1* ("S"). At 5 and 15 weeks, albuminuria (a; albumin:creatinine ratio [ACR]), glomerular hypertrophy (b), glomerular podocytes (c), collagen type IV positivity (d), and fibronectin positivity (g) were measured. e, f. Representative images of collagen type IV immunostaining in an untreated diabetic mouse at week 15 (e) and a diabetic mouse transfected with *sFlt-1* (f). h, i. Representative images of fibronectin immunostaining in an untreated diabetic mouse at week 15 (h) and a diabetic mouse transfected with *sFlt-1* (i). ***p<0.001, Student's t test between groups at week 5. *p<0.05, **p<0.01, and ***p<0.001, one-way ANOVA between groups at week 15. †p<0.05 and †††p<0.001 vs the corresponding diabetic mice at 5 weeks, one-way ANOVA after correcting for the time effect. Bars represent means ± SD. Number of animals: non-diabetic, non-transfected control mice at 5 and 15 weeks (n=5 mice each); non-transfected diabetic mice at 5 and 15 weeks (n=7 and n=6, respectively); non-diabetic control mice transfected with *sFlt-1* (n=10); and diabetic mice transfected with *sFlt-1* (n=6). Scale bar: 50 μm.

sFlt-1 had no effect on any of the markers investigated (Figure 2). Finally, compared with diabetic mice at week 5, *sFlt-1*–transfected diabetic mice at week 15 had significantly lower levels of albuminuria and collagen type IV ($p<0.05$ and $p<0.001$, respectively), indicating that transfection with *sFlt-1* can reverse pre-existing kidney damage (Figure 2A, D).

Transfection with *sFlt-1* reduced endothelial activation and inflammation in diabetic mice

Next, we measured endothelial activation in diabetic and control mice at the 5-week time point. Compared with control mice, the diabetic mice had increased glomerular endothelial activation, reflected by increased levels of VCAM-1, ICAM-1 and PECAM-1 ($p<0.001$) (Figure 3A-C). The diabetic mice also had increased levels of glomerular TNF-α ($p<0.001$) (Figure 3D) and increased numbers of glomerular macrophages ($p<0.001$) compared with control mice (Figure 3E, F). At week 15, all three markers of glomerular endothelial cell activation remained increased in the diabetic mice compared with control (non-diabetic) mice ($p<0.001$) (Figure 3A-C). At week 15, the diabetic mice also had more infiltration of glomerular macrophages and increased levels of glomerular TNF-α compared with control mice ($p<0.001$). Strikingly, transfection with *sFlt-1* significantly reduced all of these markers of glomerular endothelial activation and inflammation in the diabetic mice ($p<0.01$); in most cases, the marker was reduced to control levels (Figure 3A-E). Transfecting control (non-diabetic) mice with *sFlt-1* had no effect on any of the markers investigated (Figure 3A-E). Compared with diabetic mice at week 5, *sFlt-1*–transfected diabetic mice at week 15 had significantly lower levels of ICAM-1 and PECAM-1 ($p<0.01$) (Figure 3B, C).

sFLT-1 reduced VEGF-A-induced endothelial activation in a dose-dependent manner

Our data suggest that *sFlt-1* transfection in diabetic mice reduces kidney damage by reducing the glomerular infiltration of macrophages and by lowering the production of pro-inflammatory molecules such as TNF-α. Activation of endothelial cells is a key factor in this

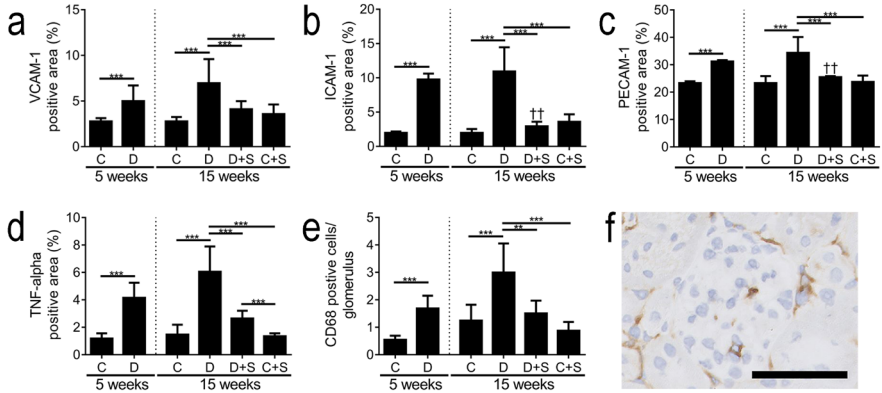

Figure 3: **sFLT-1 reduced glomerular endothelial activation, the number of glomerular macrophages and glomerular inflammation in diabetic mice**. Mice were injected with STZ to induce diabetes. In week 6, diabetic ("D") and control ("C") mice were transfected with a construct expressing *sFlt-1* ("S"). At 5 and 15 weeks, VCAM-1 (a), ICAM-1 (b), PECAM-1 (c), TNF-α (d) and the number of glomerular macrophages (e) were measured. ***p<0.001, Student's t test between groups at week 5. **p<0.01 and ***p<0.001, one-way ANOVA between groups at week 15. ††p<0.01 vs the corresponding diabetic mice at 5 weeks, one-way ANOVA after correcting for the time effect. Bars represent means ± SD. Number of animals: non-diabetic, non-transfected control mice at 5 and 15 weeks (n=5 mice each); non-transfected diabetic mice at 5 and 15 weeks (n=7 and n=6, respectively); non-diabetic control mice transfected with *sFlt-1* (n=10); and diabetic mice transfected with *sFlt-1* (n=6). f. Representative image of macrophages present in a glomerulus of a diabetic mouse at week 15 after staining for CD68. Scale bar: 50 μm.

process, as it mediates the vascular adhesion of monocytes and their migration from the bloodstream into the tissue. Therefore, we investigated the *in vitro* effect of sFLT-1 on VEGF-A–induced endothelial activation. First, we measured the time course of VEGF-A–induced endothelial activation. Incubating HUVECs with 20 ng/ml VEGF-A induced endothelial activation, reflected by significant increases in expression of the genes encoding E-selectin (*SELE*) and VCAM-1 (*VCAM-1*) compared with unstimulated HUVECs; the mRNA levels of *SELE* and *VCAM-1* peaked 6 and 4 h, respectively, after stimulation (Figure 4A, B). In contrast, the expression of *ICAM-1* was not significantly affected by VEGF-A stimulation (data not shown).

Next, we investigated the effect of applying various concentrations of sFLT-1 on endothelial activation in HUVECs 4 h after stimulation with VEGF-A (Figure 4C, D). We found that sFLT-1 significantly decreased the VEGF-A–induced upregulation of *VCAM-1* ($p<0.001$) in a dose-dependent manner. sFLT-1 did not significantly affect the VEGF-A-induced upregulation of *SELE*. sFLT-1 had no effect on the mRNA levels of *SELE* or *VCAM-1* in unstimulated cells.

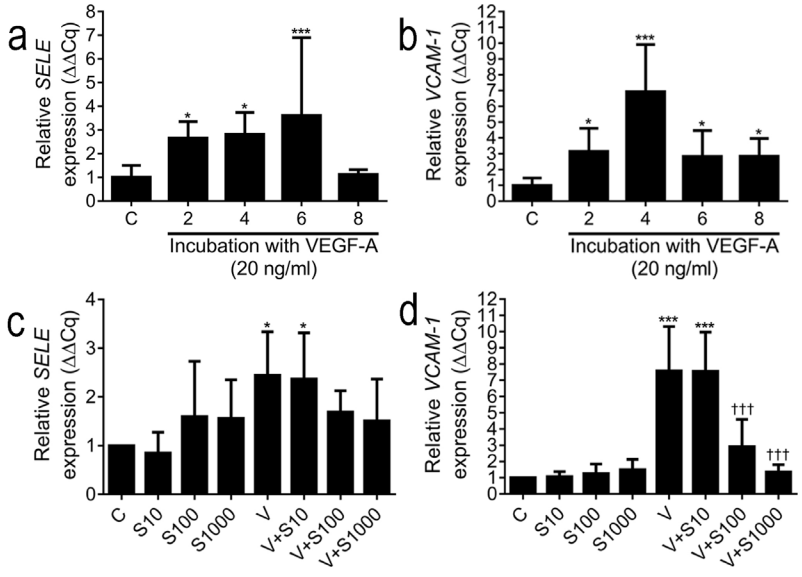

Figure 4: ***In vitro*** **treatment with sFLT-1 reduced VEGF-A–induced endothelial activation in a dose-dependent manner.** (a) *SELE* and (b) *VCAM-1* mRNA levels were measured in HUVECs incubated with 20 ng/ml VEGF-A for 2, 4, 6 or 8 h; each mRNA level is plotted relative to the respective level in untreated HUVECs. (c) *SELE* and (d) *VCAM-1* mRNA levels were measured in HUVECs incubated with 20 ng/ml VEGF-A for 4 h in the presence of 10, 100 or 1000 ng/ml sFLT-1 (S10, S100 or S1000, respectively). C, cells that were not treated with either VEGF-A or sFlt-1; V, cells stimulated with VEGF-A but were not treated with sFLT-1. Each mRNA level is plotted relative to the respective level in untreated cells. Bars represent average ± SD. *$p<0.05$ and ***$p<0.001$ vs the respective untreated control group, one-way ANOVA. †††$p<0.001$ vs the respective VEGF-A–stimulated group.

Discussion

Here, we show that transfection with the VEGF-A inhibitor gene *sFlt-1* in mice with diabetic nephropathy reverses pre-existing kidney damage by normalising albumin:creatinine levels and mesangial matrix content. Furthermore, transfection with *sFlt-1* in diabetic mice also reduced endothelial activation (measured as VCAM-1, ICAM-1, and PECAM-1 protein levels), glomerular infiltration of macrophages, and glomerular TNF-α protein levels. Finally, treating HUVECs with sFLT-1 decreased VEGF-A-induced endothelial activation in a dose-dependent manner. Taken together, these data suggest that treatment with sFLT-1 may be beneficial in individuals with diabetic nephropathy.

Animal models of diabetic nephropathy develop increased levels of glomerular VEGF-A[4,5], and inhibiting VEGF-A in diabetic animal models can prevent the development of albuminuria, glomerular hypertrophy and podocyte loss[7-9,23]. Consistent with these findings, podocyte-specific overexpression of *sFlt-1* has been reported to reduce mesangial expansion and glomerular basement membrane thickening in diabetic mice[24]. However, this study did not investigate the effect of systemic sFLT-1 treatment, which will likely be required to treat individuals with diabetes. In contrast, other studies have found that anti-VEGF-A treatment has no effect on early renal pathology[25], and that podocyte-specific deletion of *Vegfa* in diabetic mice causes increased proteinuria and kidney damage[21]. Moreover, although another study reported that treating diabetic mice with sFLT-1 decreased albuminuria, it did not reduce glomerular matrix deposition and led to an increase in tubular damage[26]. These conflicting results could be due to a variety of factors, including the time at which treatment is initiated, and the dose and/or type of anti-VEGF-A treatment used (e.g. an anti-VEGF-A antibody, a VEGFR2 inhibitor or sFLT-1). For example, using a construct in which domain 2 of FLT-1 is linked to human IgG1Fc may lead to increased inflammation due to binding to Fc receptors on macrophages (for review, see Guilliams *et al.*[27]), leading to increased tubular damage[26] independent of sFLT-1. In contrast, we used a full-length sFLT-1 construct without an Fc tag. In addition, VEGF-A inhibitors such as native sFLT-1 may have beneficial functions in addition to binding VEGF-A. For example, sFLT-1 has been reported to bind to lipid mi-

crodomains in podocytes, thereby affecting the actin cytoskeleton and the function of the glomerular barrier[28]. Podocyte-specific deletion of *Flt-1* expression causes reorganization of the cytoskeleton, leading to proteinuria and kidney damage; these effects are rescued by expressing a kinase-deficient mutant of *Flt-1*, suggesting that physiological levels of sFLT-1 are necessary for the proper structure and function of podocytes[28]. Therefore, with respect to kidney damage, treating individuals with sFLT-1 may provide improved outcomes compared with anti-VEGF-A antibodies and VEGFR2 inhibitors.

Importantly, the studies discussed above investigated the prevention—rather than the treatment—of diabetes-induced kidney damage, as therapy was initiated before the onset of kidney damage. Therefore, it is difficult to estimate the effects of such treatments in diabetic individuals who have already developed kidney damage. Fioretto *et al.* reported that kidney lesions in diabetic individuals were reversed by normalizing glycaemia levels as a result of pancreatic transplantation[29]. Therefore, we tested the effect of treating diabetic mice with the VEGF-A inhibitor sFLT-1 after the onset of kidney damage, including albuminuria and mesangial matrix accumulation. We found that even though transfection with *sFlt-1* did not normalize blood glucose levels in diabetic mice (ESM Figure 2), kidney damage was reversed, as both albuminuria and mesangial matrix accumulation were reduced.

Several studies have reported that macrophages play a role in the development of diabetic nephropathy[10-12]. Moreover, VEGF-A plays a role in the migration of monocytes and macrophages[13] by binding the FLT-1 receptor on these cells[14,30]. In addition, incubating endothelial cells with either glucose[17] or VEGF-A[16] results in endothelial activation, a key event in the adhesion and migration of monocytes from the circulation into the tissue. Consistent with this finding, both animals and people with diabetes have increased levels of endothelial activation[31-33]. Furthermore, we found that incubating HUVECs with VEGF-A increased endothelial activation, and that this effect was reversed by treating the cells with sFLT-1. We also found that transfection with *sFlt-1* normalized both the number of glomerular macrophages and the level of TNF-α in diabetic mice. Taken together, these findings suggest that the VEGF-A inhibitor sFLT-1 reduces endothelial activation and subsequent macrophage infiltration. Treatment with sFLT-1

has reported benefits in treating other diseases, including arthritis[34,35], vascular disease[36,37], sepsis[38], and psoriasis[39]; these clinical benefits are attributed primarily to reduced numbers of infiltrating macrophages and reduced inflammation. The current results indicate that sFLT-1 may be a valuable treatment for diabetic nephropathy, as well as other diseases in which inflammation plays an important role. Macrophages produce cytokines such as TNF-α and TGF-β, which increase the production of matrix proteins by mesangial cells[40]. Thus, reducing the number of glomerular macrophages using sFLT-1 might also reduce mesangial matrix expansion in diabetic nephropathy.

As reviewed by Deeds *et al.*[41], techniques using STZ (such as dosage and administration) and consistency with respect to the resulting diabetes mellitus in small animal models have not been standardised. In our study, we used a moderate dosing regimen of three doses of 75 mg/kg STZ, for two reasons: (1) this regimen is less nephrotoxic than a single high dose; and (2) this regimen induces more diabetes-related histological damage compared with several low doses, which result in a relatively mild phenotype. In rodents, STZ can cause nephrotoxicity; however, Kraynak *et al.* have reported that STZ-induced cellular and molecular damage resolves within 3 weeks[42]. This suggests that the albuminuria seen in our diabetic mice at 5 weeks was probably related to diabetes rather than STZ. This is supported by the histological characteristics typical of diabetic nephropathy seen in these diabetic mice (i.e. mesangial matrix expansion and glomerular hypertrophy). Although some groups reported albuminuria and histological lesions at this time point[43-45], other groups did not find albuminuria at this time point[31,46]; this discrepancy may be due to differences in the dose and/or route of administration of STZ. It is important to note that although present, the albuminuria in our STZ-injected diabetic mice is not exceedingly high, and we suggest that the importance of albuminuria in C57BL/6 mice must be considered in combination with the presence (or absence) of histological findings.

Importantly, we found a small, but significant, decrease in podocyte numbers in *sFlt-1*–transfected diabetic mice; decreased numbers of podocytes have also been reported in pre-eclampsia, which is characterized by high circulating levels of sFLT-1[47]. Despite this decrease in podocyte numbers, albuminuria was significantly reduced in *sFlt-1*–

transfected diabetic mice. It is possible that the decrease in podocyte numbers in these *sFlt-1*–transfected mice was too small to functionally affect the filtration barrier. This notion is supported by previous reports that a substantial decrease in podocyte numbers is required for increased albuminuria[48,49]. Nevertheless, we cannot exclude the possibility that longer treatment and/or higher levels of sFLT-1 expression could affect the glomerular filtration barrier. Thus, we hypothesise that sFLT-1 will likely have a beneficial effect in people with diabetes until the production of VEGF-A by podocytes drops below a certain threshold, given that decreased VEGF-A levels also result in kidney damage[21]. In this respect, it is important to note that both VEGF-A and sFLT-1 levels should be adjusted with care, as both increased and decreased levels of VEGF-A can lead to renal pathology[3,50].

In conclusion, we report that normalising VEGF-A levels with sFLT-1 may represent a viable approach for treating patients with existing diabetic nephropathy by reducing endothelial activation, glomerular macrophage infiltration and glomerular inflammation, thereby reversing kidney damage.

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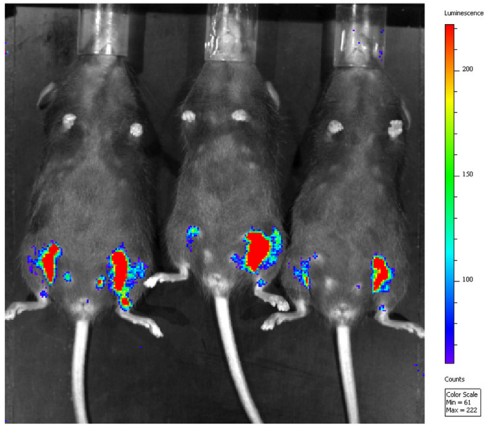
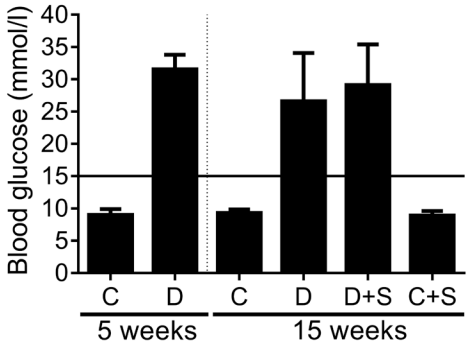
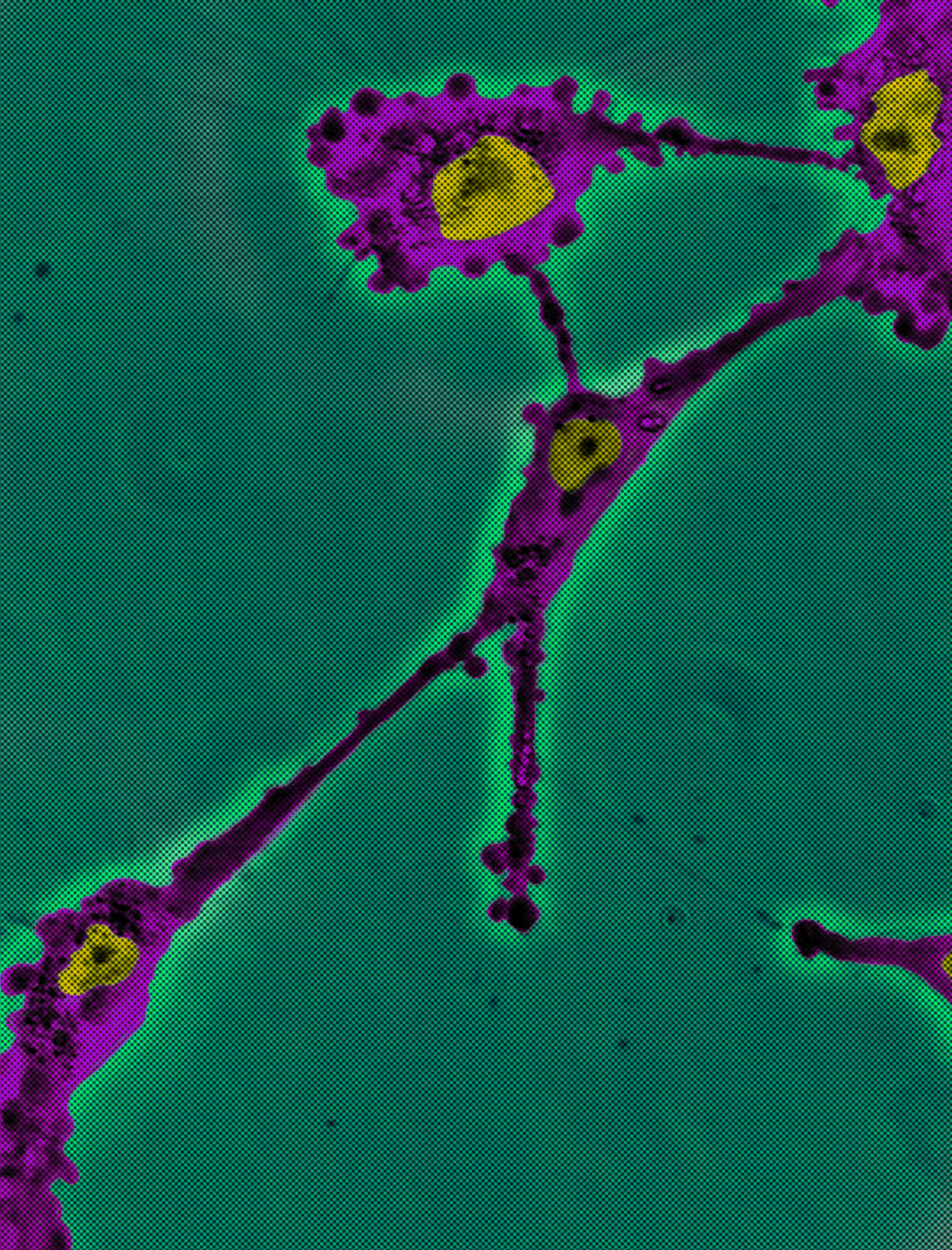
Electronic supplementary material



ESM Figure 1: **Transfection efficiency**. The *sFlt-1* and luciferase constructs were co-transfected bilaterally into both calf muscles. Following an i.p. injection of luciferin, luminescence was measured and is shown on an arbitrary pseudocolor scale; red color indicates high luminescence.



ESM Figure 2: Blood glucose levels were measured in control ("C") and diabetic mice ("D") at 5 and 15 weeks. Where indicated, mice were transfected with *sFlt-1* ("S"). The horizontal line at 15 mmol/L indicates the threshold used in this study to define diabetic mice. Transfection with *sFlt-1* had no significant effect on blood glucose levels measured at week 15 in diabetic mice. Bars represent average ± SD. Number of animals: non-diabetic, non-transfected control mice at 5 and 15 weeks (n=5 mice each); non-transfected diabetic mice at 5 and 15 weeks (n=7 and n=6, respectively); non-diabetic control mice transfected with sFlt-1 (n=10); and diabetic mice transfected with sFlt-1 (n=6).



Chapter 4

Endoglin mediates VEGF-A-induced endothelial cell activation by regulating Akt signaling

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Submitted

Abstract

In diabetic nephropathy, differential expression of growth factors leads to vascular changes, including endothelial cell activation, monocyte infiltration, and inflammation. Endoglin plays an important role in endothelial function and is also associated with inflammation. In the kidney, vascular endoglin expression is increased in animal models of renal injury, where it contributes to disease severity – possibly by promoting endothelial cell activation and inflammation. Here, we investigated whether endoglin expression is associated with diabetic nephropathy. In addition, we examined whether reducing endothelial endoglin expression *in vitro* affects endothelial cell activation and monocyte adhesion, and if so, which intracellular pathways are involved. Finally, we analyzed whether glomerular endoglin expression is correlated with endothelial cell activation in patients with diabetic nephropathy. Endoglin levels were significantly increased in mice with type 1 diabetes compared to control mice. Reducing endoglin expression in cultured endothelial cells significantly impaired the VEGF-A–induced upregulation of activation markers and monocyte adhesion. This was mediated by increased phosphorylation of Akt, thereby inhibiting ATF-2 phosphorylation which regulates *VCAM1* gene transcription in these cells. Lastly, endoglin co-localized with VCAM-1 in the glomeruli of diabetic patients, glomerular VCAM-1 expression was significantly increased in these patients, and this increase in VCAM-1 expression was correlated with increased glomerular endoglin expression. Thus, targeting endoglin function may have therapeutic value in patients at risk for diabetic nephropathy.

Introduction

Diabetic nephropathy develops in 20-40% of diabetic patients and is the leading cause of end-stage renal disease worldwide[1]. Diabetic nephropathy is characterized by microvascular injury resulting from hyperglycemia, high blood pressure, and dyslipidemia, and is associated with increased production of reactive oxygen species, an accumulation of advanced glycation end-products, and the activation of intracellular signaling molecules such as protein kinase C[2]. As a consequence of these processes, the expression of several growth factors changes – including vascular endothelial growth factor A (VEGF-A) – causing an angiogenic imbalance, ultimately leading to, but not limited to, endothelial dysfunction and activation[3], monocyte infiltration[4], and inflammation[5].

Endothelial cell activation is characterized by *i)* the increased surface expression of adhesion molecules such as vascular cell adhesion molecule (VCAM)-1, intercellular adhesion molecule (ICAM)-1, and E-selectin[6], *ii)* increased endothelial permeability, and a *iii)* prothrombotic and proinflammatory phenotype[7]. VEGF-A induces endothelial activation by binding to vascular endothelial growth factor receptor 2 (VEGFR2/KDR), leading to activation and internalization of this receptor and subsequent phosphorylation and activation of extracellular signal–regulated kinases 1 and 2 (ERK1/2). Phosphorylated ERK1/2 translocates to the nucleus and promotes activating transcription factor 2 (ATF-2) phosphorylation, which regulates *VCAM1* gene transcription[8]. Increased expression of VCAM-1 on the endothelial cell surface in turn aids in leukocyte extravasation[9], ultimately leading to inflammation. A link between VEGF-A-induced endothelial changes and endoglin have been suggested; however, it is currently unknown by which mechanism.

Endoglin is an important molecule involved in physiological and pathological vascular changes. Studies using endoglin-deficient mice showed that endoglin plays an essential role in angiogenesis and vascular remodeling[10,11]. Under physiological conditions, endoglin is expressed in endothelial cells at relatively low levels; however, endoglin expression increases during angiogenesis[12] and in response to vascular damage[13]. Furthermore, the expression of endoglin in endo-

thelial cells increases during inflammation and is associated with the infiltration of inflammatory cells such as macrophages[14].

Recently it has been demonstrated that endoglin co-localizes with VEGFR2 in early endosomes upon stimulation with VEGF-A and that endothelial cells with decreased endoglin expression display increased phosphorylation of Akt[15]. Activation of the Akt pathway has been shown to inhibit adhesion molecule expression after VEGF-A stimulation[3], possibly by reducing the phosphorylation of ATF-2[16]. Therefore, decreasing the expression of endoglin might reduce VEGF-A-induced endothelial activation and subsequent leukocyte extravasation by increasing the phosphorylation of Akt, thereby downregulating ATF-2-induced transcription of *VCAM1*.

Here, we investigated whether glomerular endoglin expression is associated with diabetic nephropathy using both mice and patient samples. Moreover, we examined whether reducing endothelial endoglin expression affects endothelial activation and/or monocyte adhesion upon stimulation with VEGF-A, and if so, which intracellular signaling pathways are involved. Finally, we studied whether glomerular endoglin expression is correlated with endothelial activation in patients with diabetic nephropathy.

Materials and methods

Animal studies

The diabetic mouse model used in this study has been described previously[17]. All experiments were conducted in accordance with national guidelines for the care and use of experimental animals (DEC license 13163). In brief, diabetes was induced in 8-week-old female C57BL/6J mice (obtained from Harlan Laboratories, Indianapolis, IN,) by three intraperitoneal injections of streptozotocin (75 mg/kg body weight; Sigma-Aldrich, Saint Louis, MO) administered at 2-day intervals. Mice that developed a blood glucose level ≥15 mmol/L were considered diabetic. Diabetic mice (n=10 per time point) and age-matched non-diabetic control mice (n=5 per time point) were sacrificed at 5 and 15 weeks after the induction of diabetes, and renal tissues were collected, fixed, and embedded in paraffin for immunohistochemical analysis.

Cell culture

Immortalized human endothelial cells (ECRF)[18] that were transduced with a lentiviral vector expressing either an shRNA against endoglin ($ECRF^{+/-}$) or a non-targeting control shRNA ($ECRF^{+/+}$) were generously provided by Dr. Luuk Hawinkels (Department of Gastroenterology, Leiden University Medical Center, the Netherlands). Compared to $ECRF^{+/+}$, endoglin mRNA and proteins levels in $ECRF^{+/-}$ were reduced by approximately 30% and 40%, respectively (Figure S1A,C). ECRF were cultured at 37˚C in 5% CO_2 in Medium 199 (Gibco Laboratories, Gaithersburg, MD) supplemented with 10% fetal bovine serum (Sigma-Aldrich), 0.4% penicillin-streptomycin (Gibco Laboratories), 0.2% unfractionated heparin (LEO Pharma, Ballerup, Denmark), and 25 mg bovine pituitary extract (Gibco Laboratories). THP-1 cells (American Type Culture Collection, Manassas, VA) were cultured at 37˚C in 5% CO_2 in RPMI 1640 Medium (Gibco Laboratories) supplemented with 10% fetal bovine serum (Sigma-Aldrich) and 1% penicillin-streptomycin (Gibco Laboratories). Human umbilical vascular endothelial cells (HUVECs) were cultured in EGM-2 media (Lonza, Basel, Switzerland) at 37˚C in 5% CO_2.

Endothelial cell activation assay

$ECRF^{+/+}$ and $ECRF^{+/-}$ that were confluent for two days were incubated with 20 ng/ml vascular endothelial growth factor-A_{165} (VEGF-A; R&D Systems, Minneapolis, MN) for 2, 4, 6, or 8 hours[3]. As control for VEGF-A, cells were incubated with 0.1% (w/v) bovine serum albumin (BSA). To quantify changes in gene expression, total RNA was extracted using TRIzol (Ambion, Foster City, CA) in accordance with the manufacturer's instructions and transcribed into cDNA. Quantitative real-time PCR (qPCR) was performed using the IQTM SYBR Green Supermix (Bio-Rad, Hercules, CA) with a CFX real-time system (Bio-Rad). Ct (cycle threshold) values were normalized to the housekeeping gene *HPRT1*. The following primers were used in this study: endoglin (*ENG*) forward: 5'-CACTAGCCAGGTCTCGAAGG-3' and reverse: 5'-CTGAGGACCAGAAGCACCTC-3'; *Eng* forward: 5'-CTTCCAAGGACAGCCAAGAG-3' and reverse: 5'- GTGGTTGCCATTCAAGTGTG-3'; *Eng* (short isoform) forward: 5'-TAGCACCTTGTCCCAGGAAG-3' and reverse: 5'-GTGGAG

GCTTGGGATACTCA-3'; *Eng* (long isoform) forward: 5'-TAGCACCTTGTC-CCAGGAAG-3' and reverse: 5'-GGCCACGTGTGTGAGAATAG-3; *HPRT-1* forward: 5'-AGATGGTCAAGGTCGCAAGC-3' and reverse: 5'-TCAAGGG-CATATCCTACAACAAAC-3'; *ICAM1* forward: 5'-CAGAGGTTGAACCCCA-CAGT-3' and reverse: 5'-CCTCTGGCTTCGTCAGAATC-3'; E-selectin (*SELE*) forward: 5'-AGCCCAGAGCCTTCAGTGTA-3' and reverse: 5'-AACT-GGGATTTGCTGTGTCC-3'; *VCAM1* (Sino Biological, Beijing, China); plasminogen activator inhibitor-1 (*PAI-1*) forward: 5'-ACTGGAAAGGCAACAT-GACC-3' and reverse: 5'-TGACAGCTGTGGATGAGGAG-3'; and connective tissue growth factor (*CTGF*) forward: 5'-CCTGGTCCAGACCACAGAGT-3' and reverse: 5'-TTGAGATTTTGGGAGTACGG-3. These experiments were performed in triplicate.

Immunoblotting

$ECRF^{+/+}$ and $ECRF^{+/-}$ that were confluent for two days were serum-starved for 5 hours. Cells were then incubated with 20 ng/ml VEGF-A (R&D Systems) for 5, 10, 15, 30, and 60 minutes. Cells were washed with ice-cold PBS and lysed in buffer containing 1% (w/v) SDS, Tris-buffered saline, 10mM EDTA, and protease and phosphatase inhibitor cocktails (Roche, Basel, Switzerland). Protein concentration was determined by using a detergent compatible protein assay (Bio-Rad). A two-fold serial dilution of a HUVEC lysate (from 20 µg to 0.16 µg) was used to generate a calibration curve in order to calculate relative endoglin protein levels in unstimulated and VEGF-A–stimulated $ECRF^{+/-}$ and $ECRF^{+/+}$. Protein lysates were subjected to SDS–PAGE and subsequent western blotting. Membranes were blocked for 1 hour in 5% BSA/TBST (Tris-buffered saline with Tween 20) and then incubated over night with primary antibodies against phosphorylated Akt (Ser473), Akt, phosphorylated ERK1/2 (Thr202/Tyr204), ERK1/2, phosphorylated ATF-2 (Thr71) and ATF-2 (all from Cell Signaling Technology, Danvers, MA); GAPDH (Cell Signaling Technology) was used as loading control. Secondary antibodies were horseradish peroxidase-conjugated. Signals were visualized by chemiluminescent detection according to the manufacturer's protocol (SuperSignal West Pico, Thermo Fisher Scientific, MA, USA). Band intensity was quantified using Image Lab software (Bio-Rad). These experiments were performed in triplicate. Membranes

were also incubated in goat anti-human endoglin (R&D Systems) as the primary antibody, followed by an IRDye Infrared Fluorescent (LI-COR Biosciences, Lincoln, NE) secondary antibody. Signals were visualized using the LI-COR Odyssey Infrared Imaging System (LI-COR Biosciences). Band intensity was quantified using Odyssey V3.0 software (LI-COR Biosciences). HUVEC protein concentrations and band intensities were used to calculate a calibration curve for measuring endoglin protein levels; this calibration curve was then used to quantify endoglin protein levels in $ECRF^{+/+}$ and $ECRF^{+/-}$.

To inhibit Akt phosphorylation, $ECRF^{+/+}$ and $ECRF^{+/-}$ that were confluent for two days were incubated for 1 hour with wortmannin (3 nM, Selleckchem, Houston, TX), followed by 15 minutes stimulation with 20 ng/ml VEGF-A (R&D Systems). The cells were then washed with ice-cold PBS and lysed in buffer containing 1% (w/v) SDS, Tris-buffered saline, 10 mM EDTA, and protease and phosphatase inhibitor cocktails (Roche). Protein concentration was determined using a detergent-compatible protein assay (Bio-Rad). Protein lysates were subjected to SDS-PAGE and subsequent western blot analysis. Membranes were blocked for 1 hour in 5% BSA/TBST, and then incubated overnight with primary antibodies against phosphorylated Akt (Ser473), Akt, phosphorylated ATF-2 (Thr71), and ATF-2 (all from Cell Signaling Technology); GAPDH (Cell Signaling Technology) was used as a loading control. IRDye Infrared Fluorescent antibodies (LI-COR Biosciences) were used as the secondary antibody, and the signals were visualized using the LI-COR Odyssey Infrared Imaging System (LI-COR Biosciences). Band intensity was quantified using Odyssey V3.0 software (LI-COR Biosciences). The ratio of phosphorylated to non-phosphorylated proteins was used to calculate whether the differences between $ECRF^{+/+}$ and $ECRF^{+/-}$ were significant.

Monocyte adhesion assay

$ECRF^{+/+}$ and $ECRF^{+/-}$ were plated in 96-well plates and grown to confluence. The cells were then stimulated with 20 ng/ml VEGF-A (or 0.1% BSA) for 2, 4, 6, or 24 hours, after which 150,000 Hoechst-labeled THP-1 cells were added to each well, and the cells were incubated for 30 minutes at 37˚C under static conditions. Subsequently, ECRF were

washed with phosphate-buffered saline (PBS) to remove the unbound THP-1 cells. The cells were then fixed in 100 µl 4% paraformaldehyde in PBS (Merck Millipore, Billerica, MA) for 10 minutes, followed by two washes with PBS. The number of attached THP-1 cells was measured with an ArrayScan XTI High Content Platform (Thermo Fisher Scientific) using a 5X objective; nine fields of view were measured, and these experiments were performed in triplicate.

Human kidney samples

Renal biopsy samples from eleven patients with histologically confirmed diabetic nephropathy were obtained from the pathology archives of the Leiden University Medical Center. Of these eleven patients, three (27%) had class II diabetic nephropathy, seven (64%) had class III diabetic nephropathy, and one (9%) had class IV diabetic nephropathy. As a control group, we used tumor-free renal resection material obtained from seven patients with impaired renal function who had a tumor elsewhere in the kidney; the tissue sections used for stainings were morphologically normal. Tissue samples in both groups were not matched for age and gender, but were included based on tissue morphology. Clinical data are provided in Table S1. All human samples were collected and handled in accordance with Dutch national ethics guidelines (Code of Conduct for Proper Secondary Use of Human Tissue).

Immunohistochemistry

Sections (4-µm thick) of paraffin-embedded kidney tissues were cut using a Leica microtome. For staining endoglin, sections were subjected to heat-induced antigen retrieval using Tris-EDTA (pH 9), and then stained with goat anti-human endoglin (1:800; R&D Systems); this antibody also cross-reacts with mouse endoglin. For staining VCAM-1, sections were subjected to heat-induced antigen retrieval with citrate buffer (pH 6), and then stained with mouse anti-human VCAM-1/CD106 (1:50; LSBio, Seattle, WA). The sections were then stained with either rabbit anti-goat HRP (1:150; Dako, Glostrup, Denmark) or rabbit anti-mouse Envision HRP (Dako) secondary antibodies; diaminobenzidine (DAB+; Dako) was used as the chromogen.

Immunofluorescence

Double-labeled immunofluorescence was performed on paraffin-embedded tissue sections (4-µm thickness). Antigen retrieval was performed as described in Table S2, and the sections were co-stained with goat anti-human endoglin (1:800; R&D Systems) together with mouse anti-human CD31 (1:200; Dako), mouse anti-human CD68 (1:2000; Dako), or rabbit anti-mouse platelet endothelial cell adhesion molecule 1 (PECAM1; 1:400; Santa Cruz Biotechnology, Dallas, TX). Additional sections were also co-stained with goat anti-human endoglin (1:200; R&D Systems) together with mouse anti-human VCAM-1/CD106 (1:50; LSBio) or rabbit anti-human nephrin (1:1000; LSBio). The stained sections were then incubated with the appropriate secondary IgG Alexa antibody (see Table S2) and mounted with ProLong Gold antifade reagent containing DAPI (Life Technologies, Carlsbad, CA).

Digital image analysis

Immunohistochemically stained sections were imaged using a Philips Ultra-Fast Scanner 1.6 RA. ImageJ 1.8.0 was used to measure the glomerular levels of endoglin (mouse and human) and the glomerular levels of VCAM-1 (human). Mouse kidney sections were imaged at 400X magnification, and the positive area per glomerulus was measured in 25 glomeruli per section and corrected for the total area of each glomerulus. Human kidney sections were imaged at 200X magnification, and the positive area per glomerulus was measured in 10 glomeruli per section (resection material) or in all, non-global sclerotized glomeruli (biopsy material), and corrected for the total area of each glomerulus. The examined glomeruli were selected at random, and the same glomeruli were used to measure VCAM-1 and endoglin in the human samples.

Statistical analyses

Data of the independent experimental repeats are expressed as the mean ± the standard deviation. Differences between groups were analyzed using the two-tailed unpaired Student's *t* test or by using one-way ANOVA where appropriate. To analyze the relationship between VCAM-1 and endoglin levels, a linear regression was performed.

To this end, the glomerular endoglin and glomerular VCAM-1–positive areas in patients with diabetic nephropathy were log-transformed in order to obtain a linear relationship. A linear mixed model was used to account for the correlation of repeated measurements within patients. Differences were considered significant at $p<0.05$.

Results

Diabetic mice have increased glomerular endoglin expression

First, we measured the expression of endoglin in the glomeruli of mice 5 and 15 weeks after the induction of diabetes. At both 5 and 15 weeks, diabetic mice had significantly higher levels of endoglin compared to age-matched non-diabetic mice ($p<0.001$; Figure 1A-C). Further analysis revealed that endoglin is expressed primarily in endothelial cells (Figure 1D-F). Two isoforms of endoglin exist, the long and short isoform, which have been described to have opposite effects on angiogenesis[19]. Therefore, a shift in the expression of these isoforms might be involved in the development of endothelial cell activation and subsequent renal disease. We found no significant difference in either the short or long endoglin isoform ($p=0.11$ and $p=0.35$, respectively; Figure S2) between diabetic mice and age-matched non-diabetic controls; we also found no significant difference in total endoglin mRNA levels ($p=0.78$; Figure S2). This suggests that the increased endoglin protein levels in diabetic mice is likely caused by changes at the translational and/or posttranslational level, and not by an increase in endoglin transcription or mRNA stability.

Endoglin mediates the activation of endothelial cells

Because endoglin expression was upregulated in the glomerular endothelial cells of diabetic mice, we hypothesized that endoglin might play a role in mediating the activation of these cells, as well as subsequent inflammation. To test this hypothesis, we used two immortalized human endothelial cell lines that express either normal ($ECRF^{+/+}$) or 30% reduced ($ECRF^{+/-}$) levels of endoglin (Figure S1). These cells were stimulated with 20 ng/ml VEGF-A for 2-8 hours, after which the mRNA levels of *VCAM1*, *SELE*, and *ICAM1* (which encode vascular cell

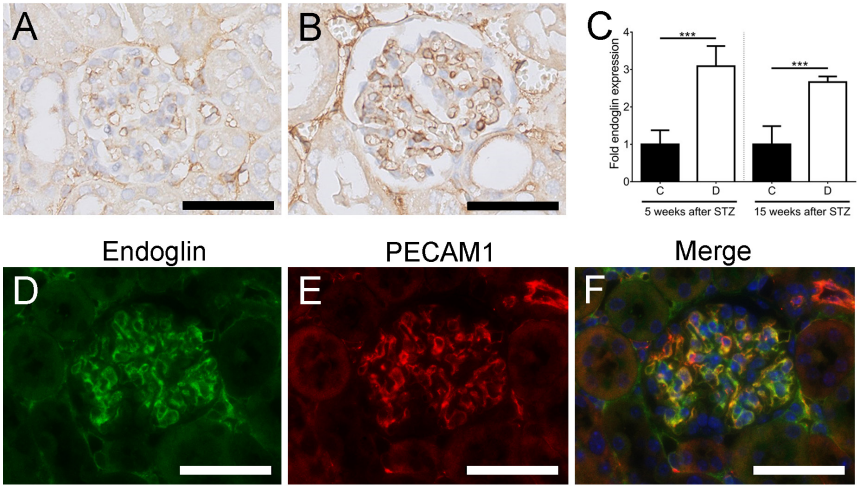

Figure 1: **Glomerular endoglin expression is upregulated in mice with experimentally induced diabetic nephropathy**. A-B. Representative images of endoglin immunostaining in the glomeruli of an age-matched, non-diabetic control mouse (A) and a diabetic mouse 5 weeks after streptozotocin-induced diabetes (B). C. Quantitative analysis of endoglin protein levels in the glomeruli of diabetic mice 5 and 15 weeks after streptozotocin (STZ)-induced diabetes ("D"), relative to the protein levels measured in age-matched non-diabetic mice ("C"). D-F. Representative images of endoglin (green; D) and PECAM1 (red; E) immunofluorescence in a kidney section obtained from a diabetic mouse. The nuclei were counterstained with DAPI (blue). ***p<0.001 vs. the respective non-diabetic group, Student's t test. Scale bars: 50 μm.

adhesion molecule 1, E-selectin, and intercellular adhesion molecule 1, respectively) were measured using qPCR. VEGF-A induced the rapid and robust activation of ECRF$^{+/+}$, as illustrated by increased expression of both *VCAM1* and *SELE* (Figure 2). In contrast, VEGF-A had little effect on *VCAM1* or *SELE* expression in ECRF$^{+/-}$, indicating that these cells have impaired VEGF-A-induced activation. *ICAM1* expression was not affected by VEGF-A stimulation in either ECRF$^{+/+}$ or ECRF$^{+/-}$ (Figure 2). To investigate whether endoglin affects endothelial cell activation by modifying TGF-β signaling, we measured the mRNA levels of *PAI-1* and *CTGF*, both of which are downstream target genes of TGF-β signaling[20,21]. Stimulating either ECRF$^{+/+}$ or ECRF$^{+/-}$ with VEGF-A had no effect on the mRNA levels of *PAI-1* or *CTGF* (Figure 2). This finding suggests that the effect of endoglin on VEGF-A–induced endothelial cell activation is independent of TGF-β signaling.

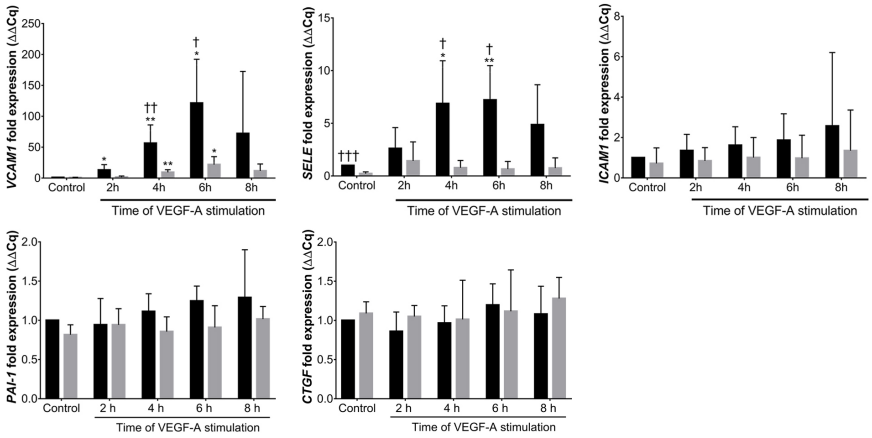

Figure 2: Endoglin mediates VEGF-A–induced endothelial cell activation independent of TGF-β signaling. *VCAM1*, *SELE*, *ICAM1*, *PAI-1*, and *CTGF* mRNA levels were measured in ECRF$^{+/+}$ (black bars) and ECRF$^{+/-}$ (gray bars). Where indicated, the cells were incubated for the indicated times with 20 ng/ml VEGF-A. All expression levels are expressed relative to the corresponding unstimulated (control) ECRF$^{+/+}$ group. *p<0.05 and **p<0.01 vs. the respective control, Student's t test; †p<0.05, ††p<0.01, and †††p<0.001 vs. the respective ECRF$^{+/-}$ group at the same time point, Student's t test.

Reduced endoglin levels diminish endothelial cell activation by increasing Akt phosphorylation

Because reduced endoglin expression resulted in reduced endothelial cell activation upon VEGF-A stimulation, we also investigated whether VEGF-A stimulation affects endoglin protein levels. As shown in Figure S1B-C, VEGF-A stimulation reduced endoglin protein levels in ECRF$^{+/+}$; in contrast, endoglin protein levels were unaffected by VEGF-A stimulation in ECRF$^{+/-}$. Next, we investigated whether changes in endoglin expression altered VEGFR2 downstream signaling. Stimulation of ECRF$^{+/+}$ with VEGF-A led to a rapid increase in ERK1/2 phosphorylation (Figures 3 and S3). Basal Akt phosphorylation was strong in unstimulated ECRF$^{+/+}$, which might be due to serum starvation[22]. Akt phosphorylation initially decreased after VEGF-A stimulation, but then increased over time and peaked at 15-30 min. In addition, VEGF-A induced ATF-2 phosphorylation thereby confirming other studies in endothelial cells[8]. In ECRF$^{+/-}$, VEGF-A induced ERK1/2 phosphorylation

to a similar degree as in ECRF$^{+/+}$ (Figures 3 and S3). Yet, phosphorylation of Akt was stronger in ECRF$^{+/-}$ than in ECRF$^{+/+}$, both after VEGF-A stimulation and under basal conditions. In contrast to ECRF$^{+/+}$, phosphorylation of ATF-2 was significantly reduced in ECRF$^{+/-}$ upon stimulation with VEGF-A. Wortmannin inhibited Akt phosphorylation in both ECRF$^{+/+}$ and ECRF$^{+/-}$ and increased the pATF-2/ATF-2 ratio upon VEGF-A stimulation (Figures 4 and S4). Taken together, these results suggest that endoglin affects VEGF-A-mediated activation of endothelial cells by reducing Akt phosphorylation and thereby promoting ATF-2 induced transcription of *VCAM1*.

Endoglin mediates the adhesion of monocytes to activated endothelial cells

Next, we investigated the consequences of impaired endothelial cell activation via reduced endoglin expression by measuring the adhesion of monocytes to endothelial cells. Both ECRF$^{+/+}$ and ECRF$^{+/-}$ were stimulated with 20 ng/ml VEGF-A for 2, 4, 6, or 24 hours, after which THP-1 cells (a human leukemia monocytic cell line) were applied for 30 minutes. We then counted the number of THP-1 cells that adhered to the ECRF$^{+/+}$ and ECRF$^{+/-}$. In ECRF$^{+/+}$, VEGF-A significantly increased the adhesion of monocytes; in contrast, VEGF-A stimulation had no effect on the adhesion of monocytes to ECRF$^{+/-}$ (Figure 5). These results indicate that endoglin expression plays a role in the adhesion of monocytes to endothelial cells activated by VEGF-A.

Endoglin is expressed in human glomerular endothelial cells

Next, we examined the clinical relevance of endoglin expression in the glomeruli of human kidneys by immunostaining kidney sections obtained from non-diabetic controls and from patients with diabetic nephropathy. Sections were co-immunostained for endoglin and the endothelial marker CD31 (Figure 6), showing that endoglin is expressed in glomerular endothelial cells. Interestingly, the co-localization of endoglin and CD31 was less robust in the patients with diabetic nephropathy, which is likely due to reduced CD31 expression in these patients[23]. A close examination of the endoglin-positive cells in our immunohistochemical and immunofluorescence experiments suggests

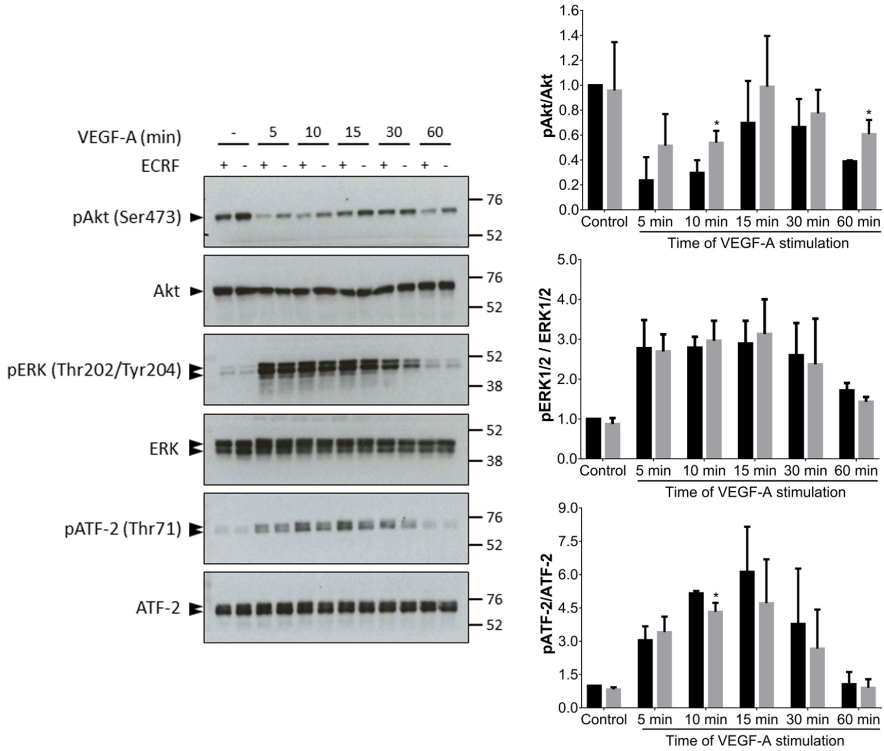

Figure 3: **Akt phosphorylation is increased in endothelial cells with reduced endoglin expression.** $ECRF^{+/+}$ (+; black bars) and $ECRF^{+/-}$ (-; gray bars) were incubated with VEGF-A (20 ng/ml) for the indicated times. VEGF-A induced Akt phosphorylation was higher in $ECRF^{+/-}$ compared to $ECRF^{+/+}$, whereas ATF-2 phosphorylation was lower in $ECRF^{+/-}$. The VEGF-A-mediated effects were significantly different between the two cell lines after 10 minutes of stimulation; $*p<0.05$. VEGF-A also increased ERK1/2 phosphorylation; however; this increase was similar between $ECRF^{+/+}$ and $ECRF^{+/-}$. The summary data are presented as the mean ± the standard deviation of three independent experiments.

that mesangial cells may also express endoglin, a finding supported by a previous study[24]. Furthermore, co-immunostaining for endoglin together with either nephrin or CD68 revealed that endoglin is not expressed in either podocytes or glomerular macrophages (Figure S5).

In contrast to our findings in diabetic mice, glomerular endoglin levels were similar between patients with diabetic nephropathy and non-diabetic controls (p=0.874) (Figure 7A). However, the level of

endoglin expression varied widely between glomeruli within some patients, as shown in Figures 7B and 7C. This suggests that activation of glomerular endothelial cells also varies within patients.

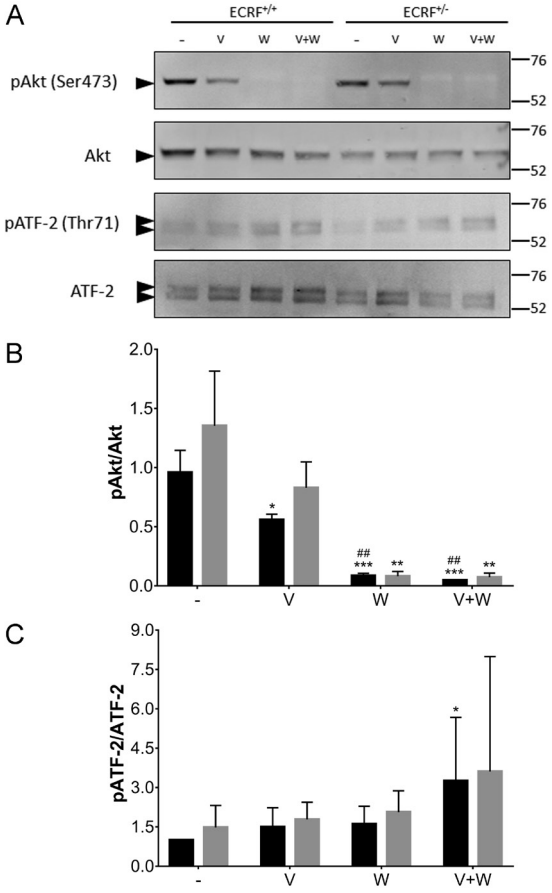

Figure 4: **Inhibiting Akt phosphorylation increases VEGF-A–induced phosphorylation of ATF-2**. ECRF$^{+/+}$ and ECRF$^{+/-}$ were incubated with wortmannin (W; 3 nM) for 1 hour, then stimulated with VEGF-A (V; 20 ng/ml) for 15 minutes. A. Representative western blot. B-C. Quantification of the western blot data shows that treating cells with wortmannin and VEGF-A reduces Akt phosphorylation (B) and increases ATF-2 phosphorylation (C). The summary data are presented as the mean ± the standard deviation of four experiments and are plotted relative to unstimulated ECRF$^{+/+}$. *p<0.05, **p<0.01, and ***p<0.001 versus the respective unstimulated control; $^{\#\#}p$<0.01 versus VEGF-A stimulated ECRF$^{+/+}$, one-way ANOVA.

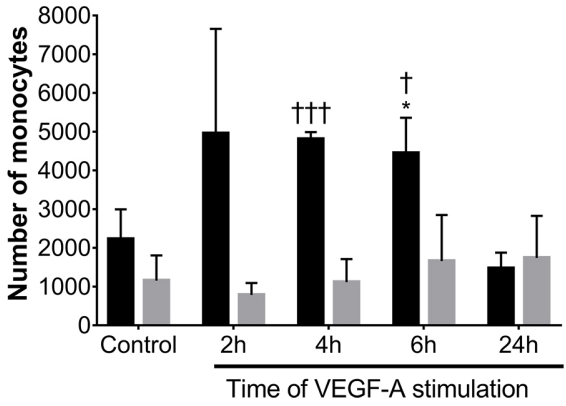

Figure 5: **Endoglin mediates the adhesion of monocytes to activated endothelial cells.** ECRF$^{+/+}$ (black bars) and ECRF$^{+/-}$ (gray bars) were incubated with VEGF-A (20 ng/ml) for the indicated times, and the number of adherent Hoechst-stained THP-1 cells was counted. *p<0.05 vs. the respective unstimulated control group; $^{\dagger}p$<0.05 and $^{\dagger\dagger\dagger}p$<0.001 vs. the respective ECRF$^{+/-}$ group at the same time point, Student's t test.

VCAM-1 expression is increased in the glomerular capillaries of patients with diabetic nephropathy

Finally, we investigated whether endothelial cell activation is increased in patients with diabetic nephropathy, and we examined whether high glomerular endoglin expression is correlated with endothelial cell activation. We therefore co-immunostained kidney sections for endoglin and the endothelial cell activation marker VCAM-1 (Figure 7G-I). We found that VCAM-1 co-localized with endoglin in the glomeruli of patients with diabetic nephropathy. In addition, immunohistochemistry showed that the relative glomerular VCAM-1-positive area was significantly higher in patients with diabetic nephropathy compared to non-diabetic controls (p<0.001; Figure 7D). Similar to our observation with respect to endoglin-stained sections, we observed high variability within patients with respect to glomerular VCAM-1 levels (Figures 7E and 7F). Importantly, when we plotted the glomerular VCAM-1–positive area against the glomerular endoglin-positive area, we found that the difference in -2 log-likelihood (β=1.09) between the models was highly significant (p<0.001). This indicates that glomerular endoglin levels are significantly correlated with

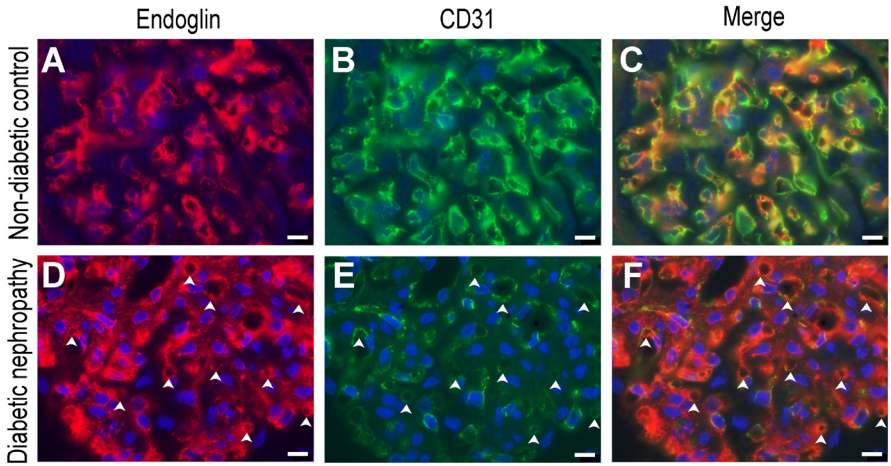

Figure 6: **Endoglin is expressed in glomerular endothelial cells in patients with diabetic nephropathy**. Representative images of endoglin (red; A and D) and CD31 (green; B and E) immunofluorescence in kidney sections obtained from a non-diabetic control (A-C) and a patient with diabetic nephropathy (D-F). Note the reduced CD31 immunostaining in the patient with diabetic nephropathy (E). The arrowheads in D-F indicate examples of co-localization between endoglin and CD31. The nuclei were counterstained with DAPI (blue). Scale bars: 10 μm.

glomerular VCAM-1 levels in patients with diabetic nephropathy (Figure 7J); meaning that glomeruli with increased levels of endoglin also have increased levels of VCAM-1. This finding supports our *in vitro* data and suggests that endoglin likely plays a role in mediating endothelial cell activation in patients with diabetic nephropathy.

Discussion

Here, we show that the expression of glomerular endoglin is increased in a mouse model of type 1 diabetes, and that endoglin plays an important role in VEGF-A-induced endothelial cell activation and in monocyte adhesion to VEGF-A-activated endothelial cells by regulating VEGFR2 signaling. Furthermore, we show that glomerular endoglin expression is positively correlated with glomerular VCAM-1 levels in patients with diabetic nephropathy, providing clinical evidence that endoglin mediates endothelial cell activation in diabetic nephropathy.

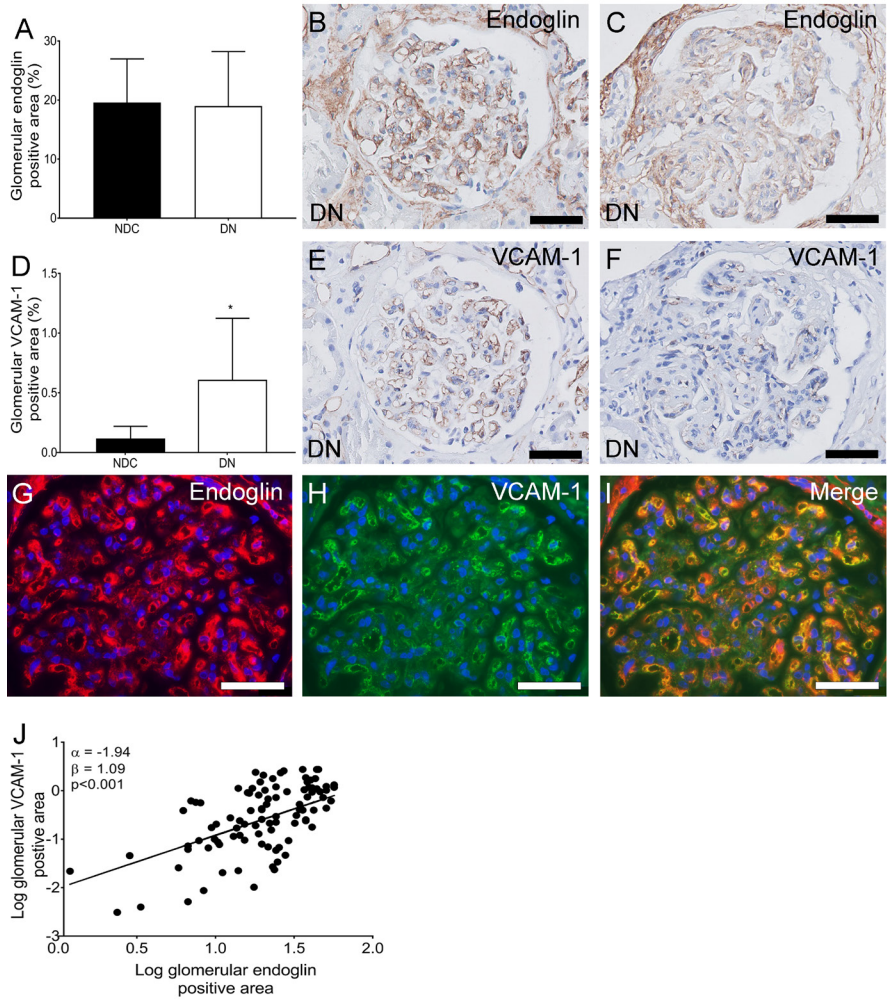

Figure 7: Glomerular endoglin protein levels are correlated with endothelial cell activation in patients with diabetic nephropathy. A-F. Endoglin (A-C) and VCAM-1 (D-F) immunohistochemistry and quantification. Endoglin-positive glomerular area (A) and VCAM-1–positive glomerular area (D) were measured in patients with diabetic nephropathy (DN, white bars) and non-diabetic controls (NDC, black bars). *p<0.05, Student's t test. The images show glomerular endoglin (B and C) and glomerular VCAM-1 (E and F) immunohistochemistry in adjacent sections from a single patient with diabetic nephropathy; note the high variability in both endoglin and VCAM-1 staining. Note that the glomerulus with high endoglin staining (B) also has high VCAM-1 staining (E), and the glomerulus with low endoglin staining (C) also has low VCAM-1 staining (F).

Endothelial cell activation is characterized by the increased surface expression of adhesion molecules such as VCAM-1, ICAM-1, and E-selectin[6], increased endothelial permeability, and a prothrombotic and proinflammatory phenotype[7]. In the context of diabetes, a variety of factors can activate endothelial cells, including hyperglycemia[25], advanced glycation end-products[26], oxidative stress[27], hyperlipidemia[28], hyperinsulinemia[29], proinflammatory cytokines[30], and growth factors such as VEGF-A[3]. The increase in adhesion molecules on endothelial cells facilitates the binding and infiltration of monocytes[9], which then produce cytokines that induce the typical glomerular changes associated with diabetic nephropathy, including mesangial matrix expansion[31].

Using animal models of diabetic nephropathy, we and other groups previously showed that the expression of both VCAM-1 and ICAM-1 is increased in glomerular capillaries[17,32,33], suggesting that endothelial cell activation may play a role in the pathogenesis of diabetic nephropathy. Accordingly, deleting ICAM-1 expression in diabetic mice leads to decreased glomerular and interstitial macrophages, reduced albuminuria, and reduced renal fibrosis[34,35]. Here, we report that the TGF-β co-receptor endoglin may play a role in endothelial activation in diabetic nephropathy.

Several studies using animal models showed that endoglin is upregulated following renal injury and contributes to the severity of renal damage[36-38], possibly by boosting endothelial cell activation and the subsequent infiltration of macrophages[37]. In our study, we found that glomerular endothelial endoglin levels were increased in an animal model of type 1 diabetes both 5 and 15 weeks after diabetes was induced. Using an *in vitro* system, we found that stimulation with VEGF-A caused endothelial cell activation, as evidenced by increased

G-I. Example images of endoglin (red; G) and VCAM-1 (green; H) immunofluorescence in a kidney section obtained from a patient with diabetic nephropathy. The nuclei were counterstained with DAPI (blue). Scale bars: 50 μm. J. Linear mixed model regression analysis of the correlation between log-transformed endoglin-positive area (on the *x*-axis) and VCAM-1-positive area (on the *y*-axis) in the glomeruli of patients with diabetic nephropathy. Each symbol represents an individual glomerulus, and the solid line shows the linear regression. The difference in -2 log-likelihood (β=1.09) between the models is highly significant ($p<0.001$), indicating that glomerular endoglin levels are significantly correlated with glomerular VCAM-1 levels.

levels of *VCAM1* and *SELE* expression. However, in contrast with other studies[3,39], we found no change in the expression of *ICAM1*. This apparent discrepancy may be due—at least in part—to the fact that we measured mRNA levels rather than protein levels. Interestingly, the activation of endothelial cells with downregulated endoglin expression ($ECRF^{+/-}$) was reduced following stimulation with VEGF-A, with virtually no upregulation in *VCAM1* or *SELE* expression. This finding may be due to endoglin's effect on the kinetics and downstream signaling of the VEGFR2[15]. Because expression of the TGF-β target genes *PAI-1* and *CTGF* was unaffected by VEGF-A stimulation in both $ECRF^{+/+}$ and $ECRF^{+/-}$, it is unlikely that TGF-β signaling plays a significant role in mediating endoglin's effects in endothelial cells.

Analysis of VEGFR2 downstream signalling demonstrated that, in contrast to $ECRF^{+/+}$, $ECRF^{+/-}$ had a stronger phosphorylation of Akt, as reported earlier[15]. Furthermore, $ECRF^{+/-}$ had a reduced phosphorylation of ATF-2 compared to $ECRF^{+/+}$. ATF-2 is essential for VEGF-A induced endothelial cell activation[8] and ATF-2 phosphorylation is decreased by the Akt-pathway[16]. Therefore, endothelial cell activation may be diminished in $ECRF^{+/-}$ by increased Akt phosphorylation which in turn downregulates the activation of ATF-2. This notion is supported by the finding that inhibiting Akt phosphorylation results in an increase in the pATF-2/ATF-2 ratio upon stimulation with VEGF-A. Figure 8 schematically illustrates our hypothesis on VEGF-A-induced endothelial cell activation in cells with different endoglin levels. Briefly, upon VEGF-A stimulation of endoglin proficient endothelial cells, endoglin co-localizes with VEGFR2 in early endosomes[15] and is associated with ERK1/2 phosphorylation[8]. In contrast, when endoglin expression is reduced, phosphorylation of Akt is increased, thereby reducing ATF-2 phosphorylation and subsequent *VCAM1* expression[15,16]. The Akt-pathway can be activated by plasma membrane bound VEGFR2 – whereas the ERK1/2-pathway cannot[40] – suggesting that endoglin regulates the internalization and intracellular signaling processes of the VEGFR2. Pan *et al.* reported that upon stimulation with VEGF-A, both VEGFR2 and endoglin are internalized in endosomes and may be subsequently degraded, resulting in decreased endoglin protein levels[41]. Our results support these findings, showing that endoglin protein levels are reduced in VEGF-A–stimulated $ECRF^{+/+}$ (Figure S1B-C). In contrast, in

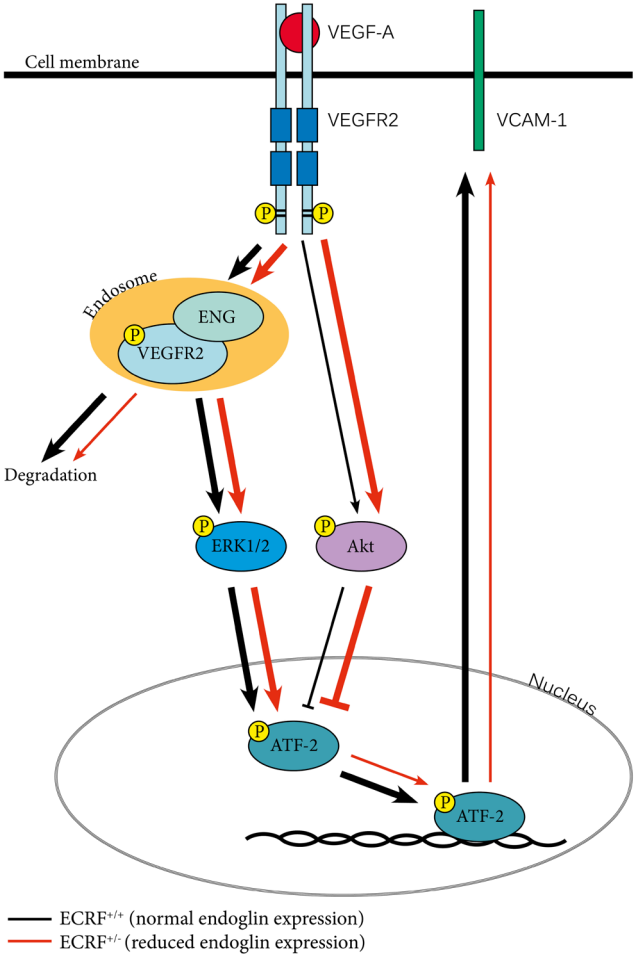

Figure 8: Schematic model of endoglin-dependent endothelial cell activation upon VEGF-A stimulation. In endoglin proficient cells (ECRF$^{+/+}$), VEGF-A drives endothelial cell activation via phosphorylation of VEGFR2, which is then internalized in endosomes and in turn phosphorylates ERK1/2. Activated pERK1/2 then translocates to the nucleus, where it phosphorylates ATF-2, thereby upregulating *VCAM1* expression, increasing VCAM-1 levels at the cell surface (signaling depicted by the black lines). In cells with reduced endoglin expression (ECRF$^{+/-}$), VEGF-A stimulation causes increased Akt phosphorylation, which in turn inhibits ATF-2 activation, thereby resulting in reduced endothelial cell activation (signaling depicted by the red lines). The level of activation is indicated by the thickness of the line; a thicker line indicates stronger activation. Note: the residual endoglin levels in ECRF$^{+/-}$ cells allows ERK1/2 to still be activated.

cells with reduced endoglin expression, VEGFR2 recycling is increased upon stimulation with VEGF-A[15]. Because endoglin co-localizes with VEGFR2 in endosomes, this may also result in increased endoglin recycling. This would also explain why endoglin protein levels are unchanged in ECRF$^{+/-}$ upon stimulation with VEGF-A (Figure S1C).

We also found that in addition to reduced VEGF-A-induced endothelial cell activation, ECRF$^{+/-}$ also have reduced monocyte adhesion to VEGF-A activated endothelial cells. During leukocyte extravasation, E-selectin is involved primarily in the rolling of leukocytes on the vessel's endothelial cell lining, whereas ICAM-1 and VCAM-1 arrest leukocytes at the endothelium[9]. This suggest that endoglin facilitates monocyte adhesion by promoting endothelial cell activation. This hypothesis is supported by a recent study showing that the inflammation-induced transendothelial migration of leukocytes in the peritoneum and lungs is reduced in heterozygous endoglin-knockout mice (which have an approximately 50% reduction in endoglin levels) compared to wild-type mice[42]. However, in their study, the authors found that endoglin binds to the integrin α5β1 on leukocytes via its RGD motif. Taken together, these results suggest that endoglin increases the adhesion and trans-endothelial migration of inflammatory cells by at least two distinct mechanisms: *i*) by a direct interaction with leukocytes, and *ii*) by activating endothelial cells.

Patients with diabetic nephropathy are reported to have increased plasma levels of soluble VCAM-1 and soluble ICAM-1[43,44], and these changes are correlated with albuminuria and vascular disease[44-46]. In addition, E-selectin, P-selectin, and ICAM-1 levels are increased in the glomerular capillaries of patients with diabetic nephropathy[47], whereas VCAM-1 is not expressed[48]. In contrast, and consistent with previous studies using diabetic animal models[17,33], we found increased levels of VCAM-1 protein in the glomerular capillaries of patients with diabetic nephropathy. This difference between previous studies and our results with respect to VCAM-1 expression in patients with diabetic nephropathy may be attributed to recent improvements in antibody specificity and/or signal amplification techniques. Moreover, in our patients with diabetic nephropathy, VCAM-1 was co-localized with endoglin in glomerular endothelial cells. Interestingly, although the levels of glomerular endoglin were similar between patients with diabetic nephro-

pathy and non-diabetic controls, within-sample endoglin expression varied widely among glomeruli. Finally, a mixed model regression analysis revealed that glomeruli with high VCAM-1 expression also have high levels of endoglin. Taken together, these results suggest that *i*) endoglin may promote endothelial cell activation in patients, and *ii*) endothelial cell activation is not always uniform within the kidney of a given patient.

In conclusion, our findings suggest that reducing endoglin function in patients at risk for diabetic nephropathy may be a viable strategy for inhibiting endothelial cell activation and monocyte adhesion, thereby preventing and/or reducing inflammation and the subsequent development of nephropathy.

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Supplementary Materials

Table S1: Clinical characteristics of non-diabetic controls and patients with DN

	Non-diabetic controls (n=7)	Patients with diabetic nephropathy (n=11)	*p* value
Age, years[1]	50 (42-59)	62 (32-70)	0.042
Sex, male[2]	3 (43)	10 (91)	0.047
DM type 1[2]	N.A.	2 (18)	N.A.
DN Class[2]			
Class I	N.A.	0 (0)	N.A.
Class II	N.A.	3 (21)	N.A.
Class III	N.A.	7 (64)	N.A.
Class IV	N.A.	1 (9)	N.A.
HbA1c, % units[1]	N.A.	6.6 (5.4-8.2)	N.A.
Serum creatinine, µmol/l[1]	99 (88-247)	108.5 (154-230)	0.928
eGFR, ml/min per 1.73m2[1]	61 (25-66)	61 (25-90)	0.660
Proteinuria, g/24 hours[1]	0.24 (0.04-0.45)	5.4 (0.06-11.00)	0.068

DM: diabetes mellitus; DN: diabetic nephropathy; HbA1c: glycated hemoglobin; eGFR: estimated glomerular filtration rate. N.A.: not applicable.

[1]Data are presented as the median (range).

[2]Data are presented as n (%).

Table S2: Antibodies used for double-labeled immunofluorescence staining

Tissue	Co-staining	Antigen retrieval	Primary	Dilution/Company	Secondary	Dilution/Company
Human	Endoglin + CD31	TRIS/EDTA pH 9	Goat anti-human endoglin	1:800; R&D Systems, Minneapolis, MN	Donkey anti-goat IgG Alexa 546	1:200, Thermo Fisher Scientific, Waltham, MA
			Mouse anti-human CD31	1:200; Dako, Glostrup, Denmark	Donkey anti-mouse IgG Alexa 488	1:200, Thermo Fisher Scientific
	Endoglin + CD68	TRIS/EDTA pH 9	Goat anti-human endoglin	1:800; R&D Systems	Donkey anti-goat IgG Alexa 546	1:200, Thermo Fisher Scientific
			Mouse anti-human CD68	1:2000; Dako	Donkey anti-mouse IgG Alexa 488	1:200, Thermo Fisher Scientific
	Endoglin + nephrin	Citrate pH 6	Goat anti-human endoglin	1:200; R&D Systems	Donkey anti-goat IgG Alexa 488	1:200, Thermo Fisher Scientific
			Rabbit anti-human nephrin	1:1000; LSBio	Donkey anti-rabbit IgG Alexa 546	1:200, Thermo Fisher Scientific
	Endoglin + VCAM-1	Citrate pH 6	Goat anti-human endoglin	1:200; R&D Systems	Donkey anti-goat IgG Alexa 546	1:200, Thermo Fisher Scientific
			Mouse anti-human CD106 (VCAM-1)	1:50; LSBio, Seattle, WA	Donkey anti-mouse IgG Alexa 488	1:200, Thermo Fisher Scientific
Mouse	Endoglin + PECAM1	TRIS/EDTA pH 9	Goat anti-human endoglin	1:800; R&D Systems	Donkey anti-goat IgG Alexa 488	1:200, Thermo Fisher Scientific
			Rabbit anti-mouse PECAM1	1:400; Santa Cruz Biotechnology, Dallas, TX	Donkey anti-rabbit IgG Alexa 546	1:200, Thermo Fisher Scientific

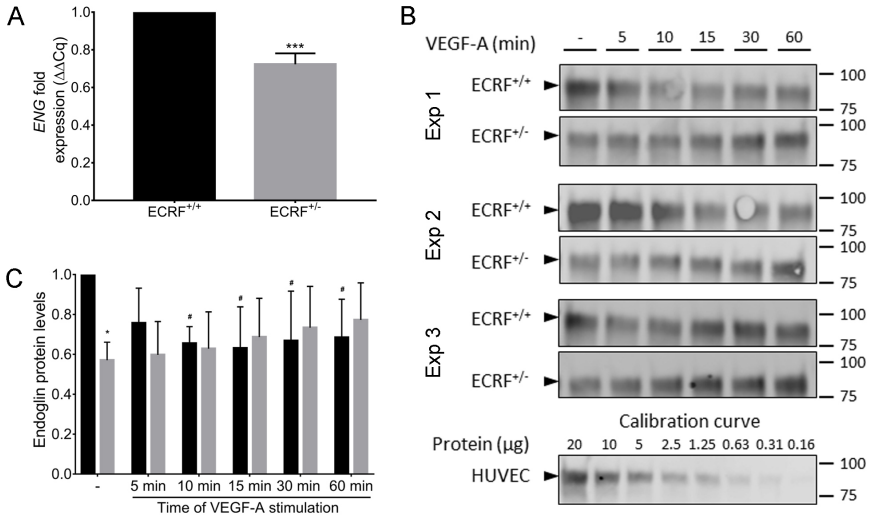


Figure S1: Relative endoglin mRNA and protein levels in ECRF$^{+/+}$ and ECRF$^{+/-}$. A. Relative endoglin mRNA levels (*ENG*) in ECRF$^{+/+}$ and ECRF$^{+/-}$. B. Western blot analysis of endoglin protein levels in unstimulated ECRF$^{+/+}$ and ECRF$^{+/-}$ and following stimulation with VEGF-A. The results of three independent experiments are shown. Endoglin levels in HUVECs were used to generate a calibration curve (bottom panel). C. Relative levels of endoglin protein in unstimulated ECRF$^{+/+}$ (black bars) and ECRF$^{+/-}$ (gray bars) and after stimulation with VEGF-A for the indicated times. The summary data are presented as the mean mRNA or protein level (± standard deviation) from three experiments. Endoglin levels are displayed relative to unstimulated ECRF$^{+/+}$. *p<0.05, ***p<0.001, and $^{\#}p$<0.05 versus unstimulated ECRF$^{+/+}$, Student's t test.

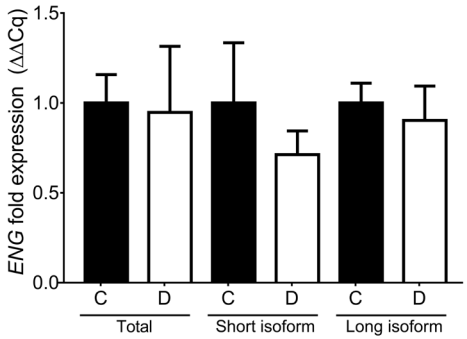


Figure S2: *ENG* mRNA levels are similar between diabetic mice and non-diabetic control mice. Total *ENG* mRNA levels, as well as the short and long isoforms, were measured in total kidney lysates obtained from diabetic mice ("D") 15 weeks after the induction of diabetes (with STZ) and in age-matched non-diabetic control mice ("C").

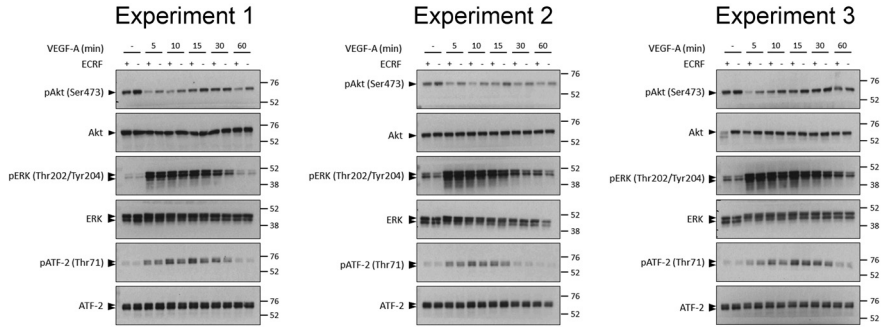


Figure S3: **Cells with reduced endoglin expression have increased Akt phosphorylation and decreased ATF-2 phosphorylation**. $ECRF^{+/+}$ (+) and $ECRF^{+/-}$ (-) were incubated with VEGF-A (20 ng/ml) for the indicated times. Compared to $ECRF^{+/+}$, $ECRF^{+/-}$ had increased levels of phosphorylated Akt and decreased levels of phosphorylated ATF-2. In contrast, ERK1/2 phosphorylation was similar between $ECRF^{+/+}$ and $ECRF^{+/-}$ following stimulation with VEGF-A.

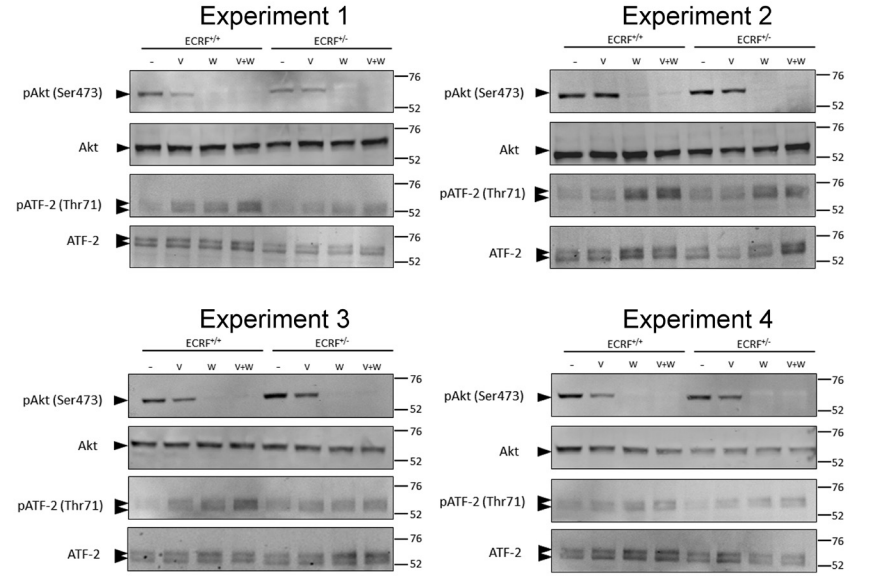


Figure S4: **Inhibiting Akt phosphorylation increases ATF-2 phosphorylation in VEGF-A–stimulated cells**. $ECRF^{+/+}$ and $ECRF^{+/-}$ were incubated with wortmannin (W; 3 nM) for 1 hour, and then stimulated with VEGF-A (V; 20 ng/ml) for 15 minutes. Cells treated with wortmannin had reduced Akt phosphorylation and increased ATF-2 phosphorylation.

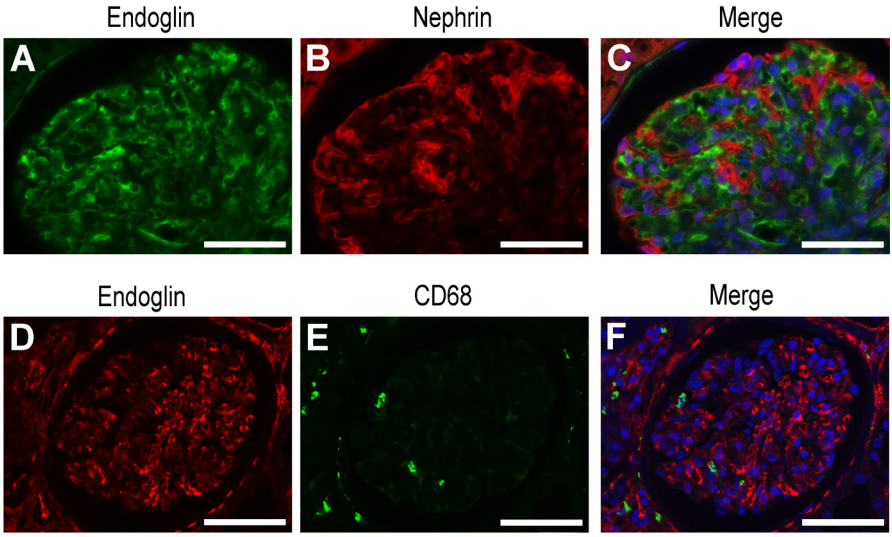
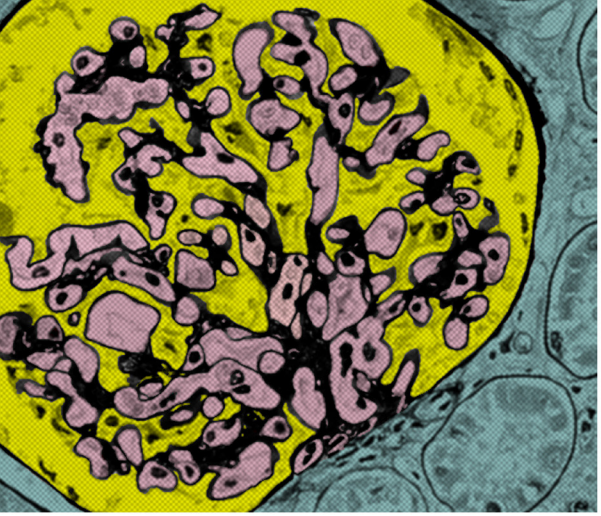
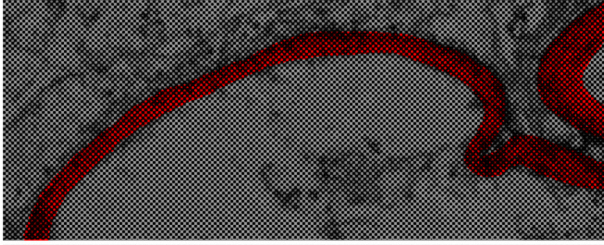
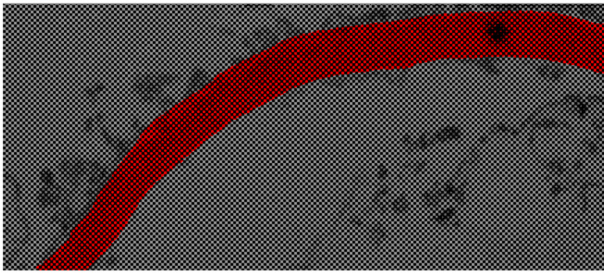
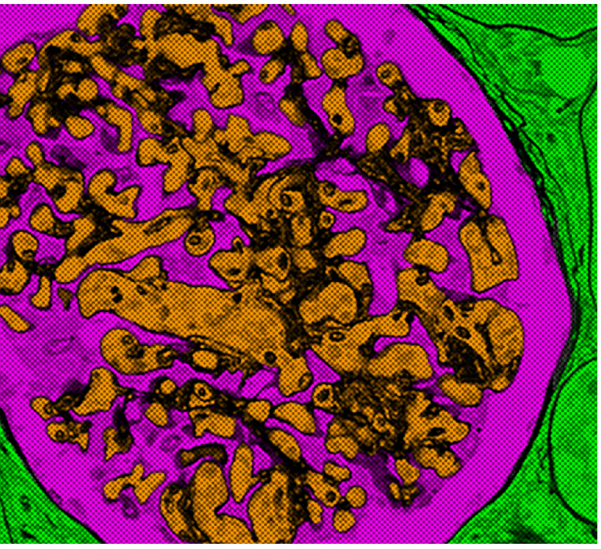
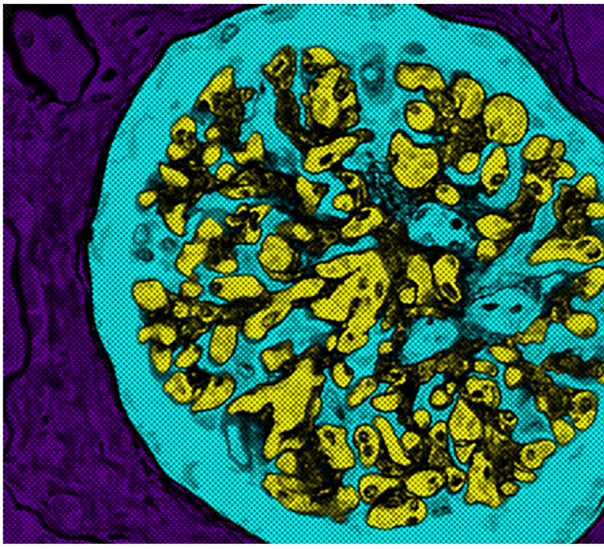
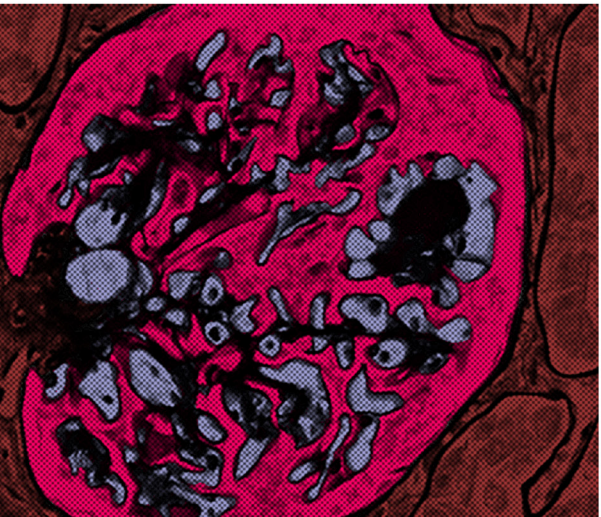
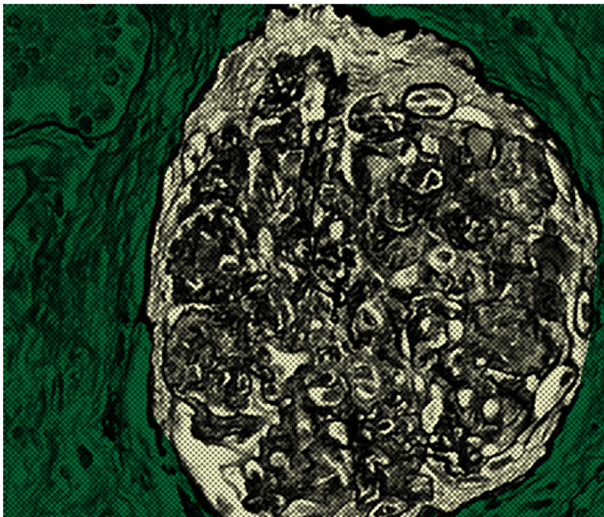


Figure S5: Endoglin is not expressed in podocytes or glomerular macrophages. Example images of kidney sections obtained from a patient with diabetic nephropathy and co-stained with endoglin (A and D) together with nephrin (B) or CD68 (E). The nuclei were counterstained with DAPI (blue). Note the absence of co-localization between endoglin and nephrin immunoreactivity (C) and between endoglin and CD68 immunoreactivity (F). Scale bars: 25 µm.

Chapter 5

Complement activation in patients with diabetic nephropathy

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Abstract

Introduction Complement activation plays a role in various organs in patients with diabetes. However, in diabetic nephropathy (DN), the role of complement activation is poorly understood. We examined the prevalence and clinical significance of complement deposits in the renal tissue of cases with type 1 and type 2 diabetes with and without DN.

Methods We measured the prevalence of glomerular C4d, C1q, mannose-binding lectin (MBL), and C5b-9 deposits in 101 autopsied diabetic cases with DN, 59 autopsied diabetic cases without DN, and 41 autopsied cases without diabetes or kidney disease. The presence of complement deposits was scored by researchers who were blinded with respect to the clinical and histological data.

Results C4d deposits were more prevalent in cases with DN than in cases without DN in both the glomeruli (46% vs. 26%) and the arterioles (28% vs 12%). C1q deposits were also increased in the glomerular hili (77% vs. 55%) and arterioles (33 vs. 14%) and were correlated with DN ($p<0.01$). MBL deposits were only rarely observed. C5b-9 deposits were more prevalent in the cases with diabetes mellitus (DM) than in the cases without DM (69% vs. 32%; $p<0.001$). Finally, glomerular C4d and C5b-9 deposits were correlated with the severity of DN (rho = 0.341 and 0.259, respectively; $p<0.001$).

Discussion Complement activation is correlated with both the presence and severity of DN, suggesting that the complement system is involved in the development of renal pathology in patients with diabetes and is a promising target for inhibiting and/or preventing DN in these patients.

Introduction

Diabetic nephropathy (DN), the leading cause of end-stage renal disease worldwide, can occur in patients with either type 1 or type 2 diabetes mellitus (DM)[1-3]. DN is characterized by a gradual increase in proteinuria and blood pressure, and a gradual decrease in glomerular filtration rate that may result in the need for renal replacement therapy. Prolonged hyperglycemia can lead to the development of DN via a number of pathways with complex interactions; however, the precise cellular and molecular mechanisms that underlie this process are poorly understood[4-6].

The first evidence for a possible role of the complement system in the development of DN was provided by the finding that serum, urine, and renal samples obtained from patients with diabetes often contained activated complement proteins, and that these proteins are associated with DN[7-14]. Serum levels of mannose-binding lectin (MBL) are correlated with the severity of DN, which suggest a role for the lectin complement pathway[15]. In addition, advanced glycation end-products can directly bind C1q and activate the complement system[16], and DN has been associated with increased renal expression of complement factors C3, C4, and C9 (at the protein level), as well as increased expression of C1q, C1s, and C1r (at the mRNA level), which also suggest a role for the classical complement pathway[8,17]. Furthermore, hyperglycemia can cause glycation-induced dysfunction and/or inactivation of complement regulatory proteins, including CD59, which inhibits C5b-9 under physiological conditions[11,18]; this glycation-induced complement dysregulation leads to increased C5b-9 levels in patients with DN[11]. In a rat model of type 2 diabetes, treating diabetic rats with a C3a receptor antagonist improved renal function and reduced both albuminuria and the deposition of extracellular matrix proteins[19].

Recently, 2 groups reviewed the role of complement activation in DM[7,14]. They concluded that the relative role of complement in the development of DM-related complications, including DN, are unknown. Moreover, it remains unclear whether these mechanisms are similar between type 1 DM and type 2 DM[7]. To address these questions, we examined whether complement activation occurred in renal autopsy samples obtained from a large cohort of cases with diabetes with

and without DN. Specifically, we measured the prevalence, localization, and staining patterns of renal C4d and C5b-9 deposits. Furthermore, because both the lectin and classical complement pathways can lead to C4d deposits, and because both pathways might be involved in the development of DN, we also determined which complement pathway was associated with the deposition of C4d in cases with DN. We validated our findings in renal biopsy samples. Finally, we correlated complement deposition with histopathology, and examined differences in complement deposition between cases with type 1 and 2 DM.

Methods

This study group includes a selection of a previously described cohort[20]. In brief, we retrospectively searched the database of our pathology department for native kidneys from adult cases with either type 1 or type 2 DM who were autopsied from 1984 through 2004. We initially included 184 autopsied kidneys that were prepared for light microscopy, electron microscopy, and immunohistochemistry. We subsequently excluded 25 cases due to poor tissue quality or missing tissue for immunostaining. Thus, we included a total of 159 samples from cases with diabetes for whom we confirmed the histopathological presence or absence of DN according to the classification for DN[21]. In addition, we included a control group consisting of autopsy samples obtained from 41 cases without diabetes without renal pathology. We validated our findings with autopsy tissue by examining 12 kidney biopsies from patients with DN and 10 biopsies obtained from healthy living transplantation donors.

Clinical data

The clinical information was obtained retrospectively via the medical records and autopsy reports available at Leiden University Medical Center, and the general practitioners of the patients. The following laboratory parameters were collected from a period starting 1 year before the patient died: serum creatinine, estimated glomerular filtration rate (eGFR) (calculated using the Modification of Diet in Renal Disease formula), microalbuminuria (defined as 30-300 mg/l), protein-

uria (defined as >300 mg/l) measured via a 24-hour urine or dipstick test, systolic and diastolic blood pressures, serum hemoglobin, serum cholesterol, and serum glycosylated hemoglobin (HbA1c)[20]. The clinical data were analyzed to reflect a stable representation of the serum and/or urine levels, thereby excluding data that were clearly affected by an unstable clinical condition (for example, patients who were clinically unstable in an intensive care unit before death). Cause of death was categorized into the following 5 general categories: cancer, cardiovascular, infection and/or sepsis, multiple pathologies, and other (e.g., high-impact trauma).

Histopathology and transmission electron microscopy

Renal tissue was fixed in 10% buffered formalin and embedded in paraffin. Sections were cut and then stained with hematoxylin and eosin, periodic-acid Schiff, and silver using standard protocols. Glomerular lesions, interstitial lesions, and vascular lesions were scored according to the histopathological classification for DN[21]. Discrimination between class 0 (i.e., no DN) and class I DN was determined using transmission electron microscopy, as described previously[20].

Immunohistochemistry and immunofluorescence

To measure renal complement activation, immunohistochemistry was performed on adjacent kidney sections using primary antibodies against the following proteins: C4d (1:150; Biomedica Gruppe, Vienna, Austria), which is a cleavage product of C4 that binds covalently to the target tissue and can arise from both the classical and MBL pathways; C1q (1:1200; DakoCytomation, Glostrup, Denmark), which reflects activation of the classical complement pathway; MBL (1:300; Sigma-Aldrich Biotechnology, Saint Louis, MO), which reflects activation of the MBL pathway; and SC5b-9 (1:500; Quidel, San Diego, CA), which is formed by activation of any of the aforementioned pathways. To investigate the presence of natural antibodies, immunofluorescence was performed on sections using a fluorescein isothiocyanate-labeled anti-IgM antibody (1:20; DakoCytomation).

The immunostained tissue samples were scored semi-quantitatively as follows: staining of glomeruli was scored as absent (representing

either an absence of staining or trace levels of staining in <5% of glomeruli) or present (representing staining in ≥5% of glomeruli). If present, the distribution of glomerular staining was scored as focal (5%-50% of the glomeruli) or diffuse (>50% of the glomeruli), and the staining pattern was scored as segmental (<50% of the glomerular tuft) or global (>50% of the glomerular tuft). In addition, if present, glomerular staining was scored as present in the glomerular capillary walls, mesangial cells, or both. Immunohistochemical staining in the glomerular hilus, arterioles, and arterial branches, was scored as absent or present (i.e., the presence of staining in ≥1 glomerular hilus, arteriole, and/or arterial branch was scored as positive). Renal tissue specimens containing ≥100 glomeruli were scored by two investigators who were blinded with respect to the clinical data of the cases.

Statistical analysis

The SPSS statistical software package (version 20.0; IBM, Armonk, NY) was used for all statistical analyses. Categorical variables were compared using the chi-squared test or the Fisher exact test, where appropriate. Continuous variables were compared using the Student's *t* test or the Mann-Whitney *U* test, where appropriate. Spearman's rank correlation coefficient was used to analyze the correlation between diabetes class and the presence of glomerular complement deposits. Differences with a p value <0.05 were considered statistically significant.

Results

Clinical and histological characteristics

We included 159 diabetic cases; histologically confirmed DN was present in 101 cases (64%) and absent in 58 cases (36%). We also included an age- and sex-matched control cohort of 41 renal samples from autopsied nondiabetic cases without renal pathology. The clinical characteristics of the cases are summarized in Table 1. The duration of diabetes was significantly higher in the cases with DN than in the cases without DN (p=0.017); however, we found no difference between these 2 groups with respect to age, sex, diabetes type, presence of

hypertension, serum creatinine, eGFR, or HbA1c levels.

The histological features of the cases with diabetes are summarized in Table 2. Among the 101 cases with DN, DN was distributed as follows: 20% with class I, 20% with class IIA, 10% with class IIB, 45% with class III, and 5% with class IV. Compared with the cases without DN, the cases with DN had a significantly higher prevalence of glomerular hyalinosis, glomerular capsular drop, arteriosclerosis, and arteriolar hyalinosis ($p<0.05$). In addition, the cases with DN had more interstitial fibrosis and tubular atrophy (IFTA) compared with the cases without DN ($p=0.028$).

C4d deposition is associated with DN

C4d deposits in the glomeruli, glomerular hili, and arterioles were significantly more prevalent in cases with diabetes than in the control cases without diabetes ($p<0.05$) (Table 3), in contrast to C4d deposits in arterial branches ($p=0.171$) (Figure 1). In the diabetic cohort, C4d deposits were present in the glomeruli, glomerular hili, and arterioles of 38%, 48%, and 22% of the cases, respectively. In the nondiabetic control cohort, C4d deposits were rarely observed in any vascular structure.

In the cohort of cases with diabetes, cases with DN had a significantly higher prevalence of C4d deposits in the glomeruli and arterioles than cases without DN (Figure 1) ($p=0.019$ and $p=0.022$, respectively). The cases with DN had a higher prevalence of C4d in the glomerular capillary walls (45% vs. 26% of cases without DN; $p=0.019$) and in the mesangial cells (26% vs. 12%, respectively; $p=0.041$) than cases without DN. The distribution of glomerular C4d did not differ significantly between the 2 groups. However, the global staining pattern of C4d was significantly more prevalent in cases with DN than patients without DN (15% vs 5%, $p=0.044$).

Within our cohort of cases with DN, eGFR was significantly lower in the cases with glomerular C4d than in the cases without glomerular C4d (39.8 ± 28.6 ml/min per 1.73m^2 vs. 60.3 ± 33.5 ml/min per 1.73m^2; respectively; $p=0.004$). In contrast, we found no other correlation between C4d deposits and clinical data (Table 4).

Table 1: Clinical characteristics of the control cases and diabetic cases.

Clinical characteristics	Non-diabetic controls (n=41)	Diabetic patients without DN (n=58)	Diabetic patients with DN (n=101)	All diabetic patients (n=159)	*p* value
Age, years	63.8 ± 16.8	69.1 ± 12.6	69.0 ± 12.8	69.1 ± 12,7	0.100[a]
Female, n (%)	16 (39)	24 (41)	47 (47)	71 (45)	0.754[a]
T1DM, n (%)	NA	5/48 (10)	12/89 (14)	17/137 (12)	0.603[b]
Duration of diabetes, years	NA	8.4 ± 7.3	15.3 ± 13.1	13.3 ± 10.0	0.003[b]
T1DM (median, IQR)	NA	17.5 (2)	28.0 (33)	18.0 (28)	0.215[b]
T2DM (median, IQR)	NA	5.0 (5)	10.0 (14)	8.0 (15)	0.004[b]
Creatinine serum, mmol/L	NA	155 ± 169	167 ± 112	163 ± 135	0.625[b]
eGFR, ml/min/1.73m^2	NA	59 ± 36	50 ± 33	54 ± 34	0.184[b]
HbA1c, % unit	NA	7.5 ± 1.8	8.5 ± 2.4	8.1 ± 2.3	0.117[b]
Hypertension, n (%)	NA	27/48 (56)	44/85 (52)	71/133 (53)	0.618[b]
Systolic pressure, mmHg	NA	134 ± 29	155 ± 29	135 ± 29	0.780[b]
Diastolic pressure, mmHg	NA	75 ± 14	75 ± 12	75 ± 13	0.961[b]
Cause of death, n (%)					0.199[a]
Cancer	4 (10)	9 (16)	5 (5)	14 (9)	
Cardiovascular	16 (39)	20 (34)	49 (48)	69 (43)	
Infection/Sepsis	4 (10)	7 (12)	12 (12)	19 (12)	
Multiple pathologies	13 (31)	14 (24)	17 (17)	31 (20)	
Other	4 (10)	8 (14)	18 (18)	26 (16)	

DN, diabetic nephropathy; eGFR, estimated glomerular filtration rate; HbA1c, glycosylated hemoglobin; IQR: interquartile range; T1DM, type 1 diabetes mellitus; T2DM, type 2 diabetes mellitus. Data are presented as the mean ± standard deviation, unless stated otherwise.

[a] Between nondiabetic controls without renal disease, diabetic cases without DN and diabetic cases with DN.

[b] Between diabetic cases without DN and diabetic cases with DN.

Evidence for classical complement activation in diabetic patients

Next, to investigate which complement pathway(s) led to the deposition of C4d in cases with DN, we stained renal tissue for MBL (to measure the lectin pathway) and C1q (to measure the classical pathway).

MBL was only observed in 6% of the kidneys from cases with diabetes and was not observed in the control cases without diabetes. When present, the staining pattern of MBL in the glomeruli was predominantly focal and segmental; MBL was not observed in the glomerular hili, arterioles, or arterial branches. We found no significant differences between MBL deposition in the cases with diabetes and control cases without diabetes or between the cases with diabetes with DN and the cases with diabetes without DN (Figure 2A and Table 3).

Table 2: Histological characteristics of the diabetic cases

Histological characteristics	Diabetic patients without DN (n=58)	Diabetic patients with DN (n=101)	All diabetic patients (n=159)	*p* value[a]
DN class				
0 (no DN)	58 (100)	0 (0)	58 (37)	NA
I	0 (0)	20 (20)	20 (13)	
IIA	0 (0)	21 (20)	21 (13)	
IIB	0 (0)	10 (10)	10 (6)	
III	0 (0)	45 (45)	45 (28)	
IV	0 (0)	5 (5)	5 (3)	
Glomerular hyalinosis	19 (33)	85 (84)	104 (65)	<0.001
Glomerular capsular drop	2 (3)	15 (15)	17 (11)	0.025
FSGS	2 (3)	12 (12)	14 (9)	0.071
IFTA				
absent	22 (38)	17 (17)	39 (25)	0.028
10-25%	25 (43)	56 (55)	81 (51)	
25-50%	5 (9)	15 (15)	20 (13)	
>50%	6 (10)	13 (13)	19 (12)	
Arteriosclerosis	46 (79)	92 (91)	138 (87)	0.035
Arteriolar hyalinosis	46 (79)	92 (91)	138 (87)	0.035
Cholesterol emboli	3 (5)	3 (3)	6 (4)	0.483

DN, diabetic nephropathy; FSGS, focal segmental glomerulosclerosis; IFTA, interstitial fibrosis and tubular atrophy.

[a] Between diabetic cases without DN and diabetic cases with DN.

Data are presented as the number of cases (%).

Glomerular C1q was present in 36% of the cases with diabetes, and the staining pattern was predominantly focal and global. The prevalence of glomerular C1q was not significantly different between the cases with diabetes and control cases without diabetes (37% vs. 49%, respectively; p=0.150) (Figure 2B and Table 3). In contrast, the prevalence of C1q in the glomerular hili, arterioles, and arterial branches was significantly higher in the cases with diabetes than in the control cases without diabetes ($p \leq 0.001$). Furthermore, among the cases with diabetes, the cases with DN had a significantly higher prevalence of C1q in the glomerular hili and arterioles compared with the cases without DN ($p<0.05$), whereas the prevalence of C1q in the arterial branches did not differ significantly between these 2 groups (p=0.106). Finally, the presence of C1q deposits was correlated with the presence of C4d deposits in the glomeruli (p=0.006), glomerular hili (p=0.027), and arterioles ($p<0.001$).

Table 3: Summary of complement deposition in nondiabetic controls, diabetic cases without nephropathy, and diabetic cases with nephropathy

	Presence of C1q				Presence of MBL				Presence of C4d				Presence of C5b-9			
	NDC	DM+ DN-	DM+ DN+	*p* value	NDC	DM+ DN-	DM+ DN+	*p* value	NDC	DM+ DN-	DM+ DN+	*p* value	NDC	DM+ DN-	DM+ DN+	*p* value
Glomerular presence	20 (49)	17 (29)	41 (41)	0.132	0 (0)	1 (2)	8 (8)	0.057	3 (7)	15 (26)	45 (46)	<0.001	13 (32)	35 (60)	74 (73)	<0.001
Glomerular location				<0.001				0.184				<0.001				<0.001
Mesangium only	5 (12)	0 (0)	0 (0)		0 (0)	0 (0)	0 (0)		2 (5)	0 (0)	0 (0)		1 (2)	2 (3)	2 (2)	
Glomerular capillary wall only	0 (0)	2 (3)	18 (18)		0 (0)	1 (2)	4 (4)		0 (0)	8 (14)	19 (19)		0 (0)	0 (0)	0 (0)	
Both mesangium and capillary wall	15 (37)	15 (26)	23 (23)		0 (0)	0 (0)	4 (4)		1 (2)	7 (12)	26 (26)		12 (29)	34 (59)	72 (71)	
Glomerular distribution				0.023				0.215				<0.001				<0.001
Focal	7 (17)	13 (22)	25 (25)		0 (0)	1 (2)	7 (7)		3 (7)	10 (17)	27 (27)		3 (7)	8 (14)	14 (14)	
Diffuse	13 (32)	4 (7)	16 (16)		0 (0)	0 (0)	1 (1)		0 (0)	5 (9)	18 (18)		10 (24)	27 (47)	60 (59)	
Glomerular staining pattern				0.027				0.197				<0.001				<0.001
Segmental	2 (5)	8 (14)	14 (14)		0 (0)	1 (2)	5 (5)		3 (7)	12 (21)	30 (30)		2 (5)	11 (19)	26 (26)	
Global	18 (44)	9 (16)	27 (27)		0 (0)	0 (0)	3 (3)		0 (0)	3 (5)	15 (15)		11 (27)	24 (41)	48 (48)	
Glomerular hili presence	4 (10)	32 (55)	78 (77)	<0.001	0 (0)	0 (0)	2 (2)	0.372	0 (0)	22 (38)	54 (54)	<0.001	32 (78)	55 (95)	101 (100)	<0.001
Arteriolar presence	1 (2)	8 (14)	33 (33)	<0.001	0 (0)	0 (0)	0 (0)	NA	3 (7)	7 (12)	28 (28)	0.005	31 (82)	58 (100)	101 (100)	<0.001
Arterial branches presence	0 (0)	10 (17)	29 (29)	<0.001	0 (0)	0 (0)	0 (0)	NA	0 (0)	1 (2)	6 (6)	0.149	39 (98)	58 (100)	101 (100)	0.136

DM, diabetic cases without diabetic nephropathy; DN, diabetic cases with diabetic nephropathy; NDC, non-diabetic controls. All data are presented as the number of non-diabetic controls or patients (%).

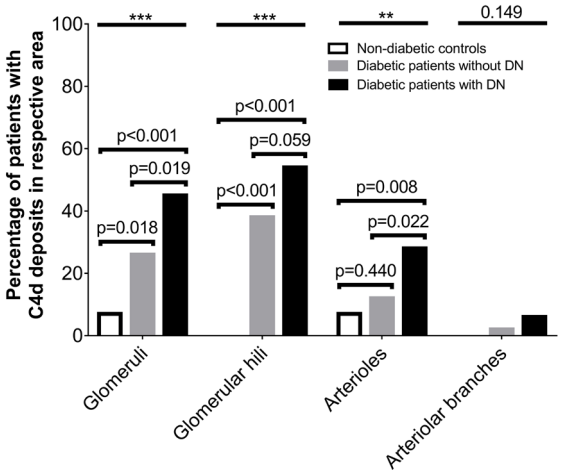

Figure 1: **Prevalence of C4d deposits in cases and controls**. The percentage of cases and controls with complement factor C4d is shown for the indicated renal structures. Asterisks represent the overall differences between the nondiabetic controls, the diabetic cases without diabetic nephropathy (DN), and the diabetic cases with DN; The *p* values shown between the 2 groups represent *post hoc* analyses. ***p*<0.01 and ****p*<0.001.

C5b-9 deposits are associated with diabetes, but not with DN

In the cases with diabetes, glomerular C5b-9 staining was predominantly diffuse and global. Although the prevalence of C5b-9 deposits was significantly higher in the glomeruli, glomerular hili, and arterioles of the cases with diabetes compared with the control cases without diabetes ($p<0.001$) (Figure 2C and Table 3), the prevalence of C5b-9 was still relatively high in the control cases without diabetes. Only the prevalence of C5b-9 deposits in the glomerular hili were significantly higher in the patients with DN than in patients without DN ($p=0.047$; Figure 2C). The presence of glomerular C5b-9 deposits was correlated with the presence of glomerular C4d deposits ($p=0.002$).

The prevalence, location, distribution, and staining patterns of complement proteins in the glomeruli, glomerular hili, arterioles, and arterial branches of the cases with diabetes are listed in Table 3. Representative images of C1q, C4d, MBL, and C5b-9 staining in these structures are shown in Figure 3.

	Glom C4d -	Glom C4d +	*p* value	Glom hilus C4d -	Glom hilus C4d +	*p* value	Art C4d -	Art C4d +	*p* value	Art branch C4d -	Art branch C4d +	*p* value
Age, years	67.6 ± 12.7	70.8 ± 12.7	0.202	68.9 ± 12.4	69.2 ± 13.1	0.896	68.3 ± 13.4	71.0 ± 10.9	0.339	68.9 ± 12.9	70.8 ± 10.6	0.723
eGFR, ml/min/1.73m^2	60.3 ± 33.5	39.8 ± 28.6	0.004	58.3 ± 33.8	44.1 ± 30.8	0.053	53.6 ± 31.2	43.8 ± 35.4	0.211	51.3 ± 32.4	39.8 ± 37.6	0.414
HbA1c, % unit	8.0 ± 2.4	9.0 ± 2.4	0.182	8.1 ± 3.0	8.7 ± 2.1	0.536	8.4 ± 2.7	8.7 ± 2.1	0.725	8.4 ± 2.4	10.3 ± 4.2	0.285
SBP, mmHg	129.6 ± 27.8	141.6 ± 29.3	0.092	139.3 ± 36.0	132.4 ± 23.0	0.381	133.3 ± 28.9	139.4 ± 29.3	0.425	133.9 ± 28.0	156.3 ± 40.3	0.135
DBP, mmHg	75.4 ± 12.9	74.5 ± 12.1	0.769	74.7 ± 12.9	75.2 ± 12.3	0.889	75.4 ± 12.6	74.1 ± 12.3	0.706	74.9 ± 12.6	76.3 ± 12.5	0.838
Duration of DM, years	14.1 ± 12.0	16.6 ± 14.3	0.469	15.8 ± 17.0	14.9 ± 9.8	0.808	13.0 ± 10.2	20.9 ± 17.5	0.085	15.5 ± 13.2	12.0 ± 12.5	0.660

C4d and C5b-9 deposits are correlated with histological lesions and DN class

Among the cases with diabetes, the presence of glomerular C4d was correlated with glomerular hyalinosis (p=0.020), IFTA (p<0.001), arteriosclerosis (p=0.017), and arteriolar hyalinosis (p=0.017) (Supplemental Table S1A and S1B). Moreover, the presence of C4d in the glomerular hili was correlated with glomerular hyalinosis (p=0.002), and the presence of C4d in the arterioles was correlated with IFTA (p<0.001), arteriosclerosis (p=0.041), and arteriolar hyalinosis (p=0.041). Although glomerular C1q was not correlated with histological lesions, the presence of C1q in the arterioles was correlated with the presence of glomerular capsular drop (p=0.034), arteriolar hyalinosis (p=0.048), and IFTA (p=0.027). The presence of MBL in the glomeruli, arterioles, and arterial branches was not correlated with any renal lesion. In addition, the presence of glomerular C5b-9 was correlated with IFTA (p=0.008). Finally, with respect to DN class, the prevalence of glomerular C4d

Table 4: **Correlation between clinical data and C4d deposition in autopsied cases with diabetic nephropathy**

Art, arterioles; Art branch, arteriolar branches; DBP, diastolic blood pressure; Glom, glomerular; HbA1c, glycosylated hemoglobin; SBP, systolic blood pressure. All data are presented as the mean ± SD.

and C5b-9 was correlated with more severe classes of DN ($p \leq 0.001$) (Figure 4), particularly with class III and class IV DN.

Differences in complement deposition between type 1 DM and type 2 DM

Data on diabetes type was available for 137 patients (86% of cases with diabetes); 17 cases (12%) had type 1 DM and 120 cases (88%) had type 2 DM. Compared to cases with type 2 DM, cases with type 1 DM had a significantly higher prevalence of C4d in glomeruli (64% vs 33%; $p=0.012$), C4d in arterioles (47% vs 18%; $p=0.007$), and C5b-9 in glomeruli (94% vs 64%; $p=0.013$). Within the subgroup of cases with DN, cases with type 1 DM also had a significantly higher prevalence of C4d in glomeruli (75% vs 35%; $p<0.01$), C4d in arterioles (50% vs 22%; $p=0.040$), and C5b-9 in glomeruli (100% vs 68%; $p<0.05$).

Evidence for the activation of the classical complement pathway in biopsies

To support our findings with autopsy tissue, we found that the prevalence of glomerular C4d was significantly higher in biopsies from cases with DN compared with a control group of biopsies obtained from healthy living transplantation donors (75% vs. 10%, respectively; $p=0.002$); similar results were obtained with respect to glomerular C5b-9 deposits (75% vs. 0%, respectively; $p=0.001$; Figure 5). In contrast, the prevalence of glomerular C1q deposits did not differ significantly between these 2 groups (58% vs. 40%, respectively; $p=0.392$), and glomerular MBL deposits were not observed in either group. The prevalence of glomerular IgM deposits was significantly higher in the cases with DN compared with control cases (82% vs. 30%, respectively; $p=0.017$), and the prevalence of glomerular IgM deposits were significantly correlated with the prevalence of glomerular C1q ($p=0.003$) and C4d ($p=0.001$) deposits. Finally, glomerular IgM deposits co-localized with glomerular C1q and C4d deposits (Figure 6), which suggested that the classical complement pathway was activated in cases with DN.

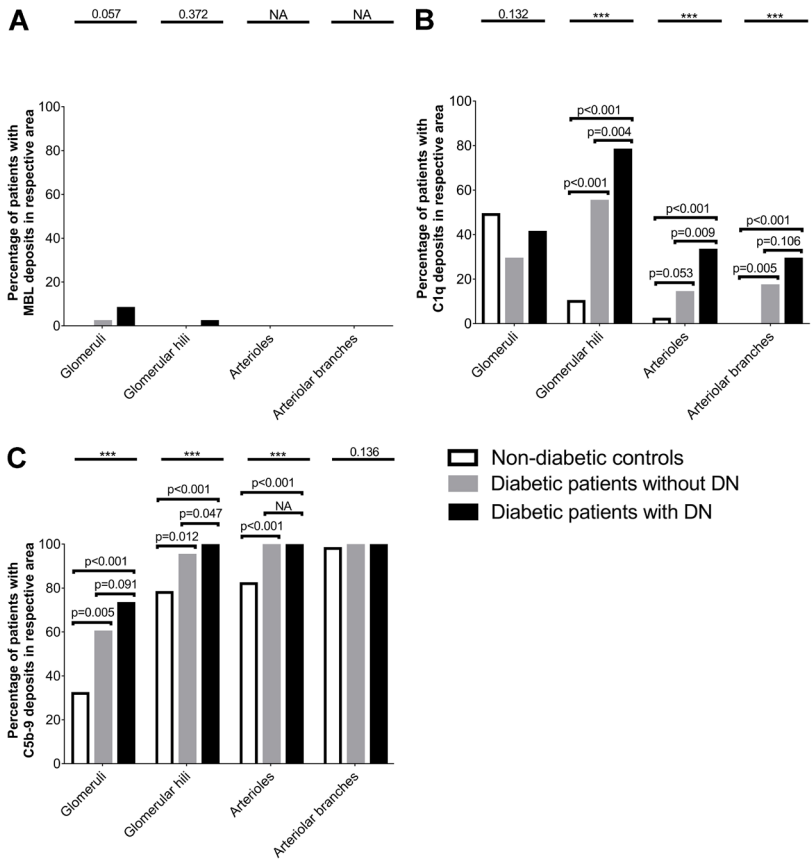

Figure 2: **Prevalence of mannose-binding lectin (MBL), C1q, and C5b-9 deposits in cases and controls**. The percentage of cases and controls with complement factor MBL (a), C1q (b), and/or C5b-9 (c) is shown for the indicated renal structures. The *p* values shown between the 2 groups represent post hoc analyses. ****p*<0.001, chi-squared test between nondiabetic controls, diabetic cases without diabetic nephropathy (DN), and diabetic cases with DN.

Discussion

DN is a microvascular complication that affects 20% to 40% of patients with diabetes, making it the leading cause of end-stage renal disease[1-3]. An increasing body of evidence has suggested that com-

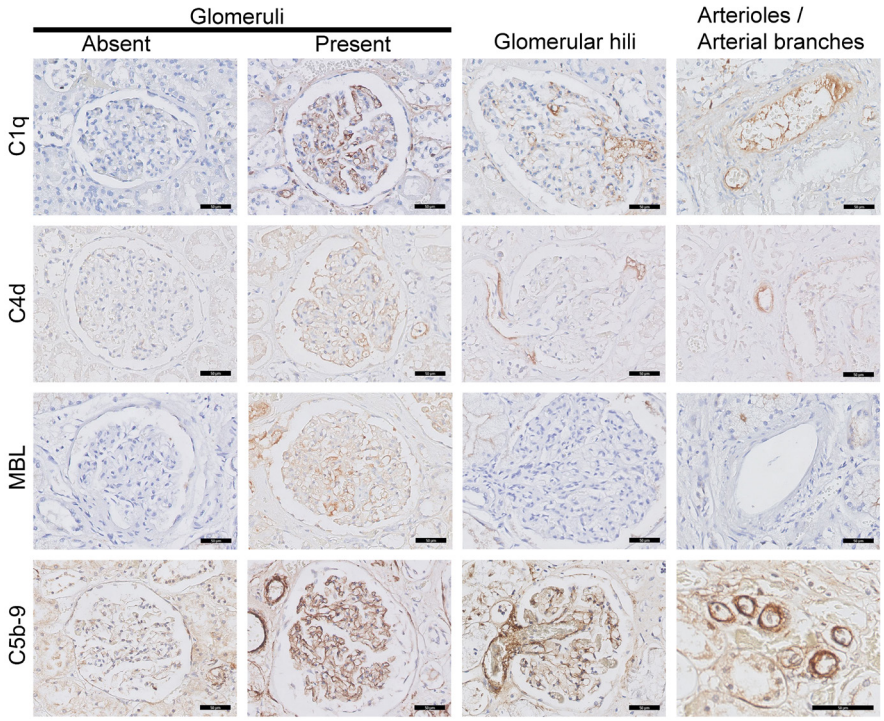

Figure 3: **Representative images of complement staining in patients**. Kidney sections were immunostained for the indicated proteins, and representative images containing the glomeruli, glomerular hili, arterioles, and arterial branches are shown. Mannose-binding lectin (MBL) staining was negative in the glomerular hilus, arterioles, and arterial branches. Scale bars: 50 μm

plement activation via the lectin and/or classical complement pathway plays a role in the development of DN[4-7,14]. Here, we investigated the deposition of complement proteins in a relatively large cohort of cases with diabetes with DN and without DN. We found that the complement activation marker C4d was correlated with DN, as well as with the severity of DN, microvascular and interstitial lesions, and lower eGFR in cases with DN, suggesting that complement activation might play a role in the development of DN.

Because C4d binds covalently to its target cells, C4d can be observed long after the factors that activated the pathway have disso-

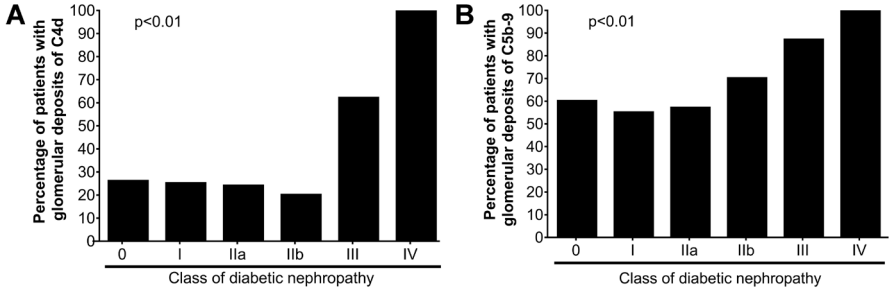

Figure 4: **Percentage of patients with glomerular C4d deposits and glomerular C5b-9 deposits plotted against diabetic nephropathy class.** The presence of glomerular C4d (a) and C5b-9 (b) was correlated with diabetic nephropathy class. For C4d and C5b-9, the Spearman rank correlation coefficient (rho) was 0.344 and 0.228, respectively (both $p<0.01$). Cases without diabetic nephropathy were classified as class 0.

ciated, making it a commonly used biomarker for complement activation[22]. The prevalence of C4d deposits in the glomeruli and arterioles was significantly higher in cases with DN than cases without DN and in cases without diabetes without renal pathology, both in the autopsy cohort and in the biopsy cohort. Moreover, glomerular C4d and arteriolar C4d were more prevalent in the cases with vascular and chronic renal lesions, and glomerular C4d was correlated with the severity of DN. These data suggested that complement activation, together with the renal microvasculature, might be involved in the development of DN. This notion is supported by the general absence of C4d deposits among the control cases without diabetes. To investigate which complement pathway could underlie the deposition of C4d, we studied the prevalence and localization of C1q (to measure the classical pathway) and MBL (to measure the lectin pathway) deposits. The presence of C1q, IgM, and C4d deposits were associated with each other, and co-localized in the same renal vascular structures, whereas MBL was detected rarely in our cohort. These findings suggest that the presence of C4d reflected activation of the classical complement pathway. However, because MBL is not covalently bound and because other proteins such as ficolins can initiate the lectin pathway, we could not exclude the possibility that activation of the lectin pathway led to C4d deposition.

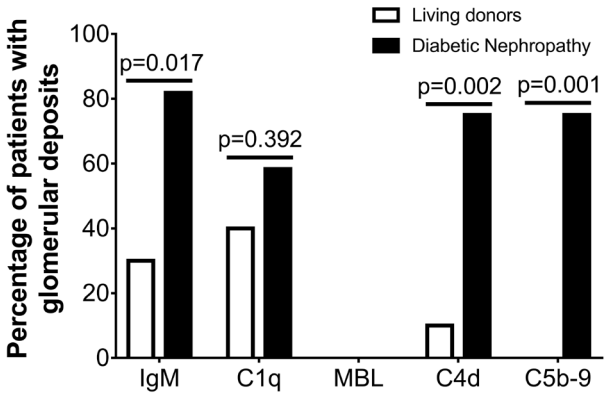

Figure 5: **Percentage of renal sections containing IgM, C1q, mannose-binding lectin (MBL), C4d, or C5b-9 deposits**. Biopsy samples were obtained from living patients with diabetic nephropathy (n=12) and healthy living renal transplantation donors (n=10) and stained for IgM, C1q, MBL, C4d, and C5b-9. *P* values were calculated using the chi-square test.

C4d deposits were significantly more prevalent in kidneys from cases with type 1 DM than in kidneys from cases with type 2 DM, both in the total cohort of cases with diabetes and in the subgroup of cases with DN. The presence of DN was not a confounder in this association because DN was not more prevalent in type 1 DM compared with type 2 DM. Rowe *et al.* showed similar differences in pancreatic C4d deposition among cases with type 1 DM, type 2 DM, and control cases without diabetes[23]. The higher C4d prevalence between cases with type 1 DM and type 2 DM could possibly reflect a different pathogenesis. Nevertheless, we could not exclude the possibility that the difference observed in complement deposition might be attributed, at least in part, to the duration of diabetes rather than the type of diabetes.

Glomerular C4d was a common finding in our cases with class III DN. Paueksakon *et al.* suggested that Kimmelstiel-Wilson lesions, which are a hallmark lesion of DN and a diagnostic requirement for class III DN, might be a form of thrombotic microangiopathy. Specifically, they found that a subset of cases with DN had fragmented red blood cells exclusively in Kimmelstiel-Wilson lesions[24]. Recently, we reported that complement factor C4d was a common denomina-

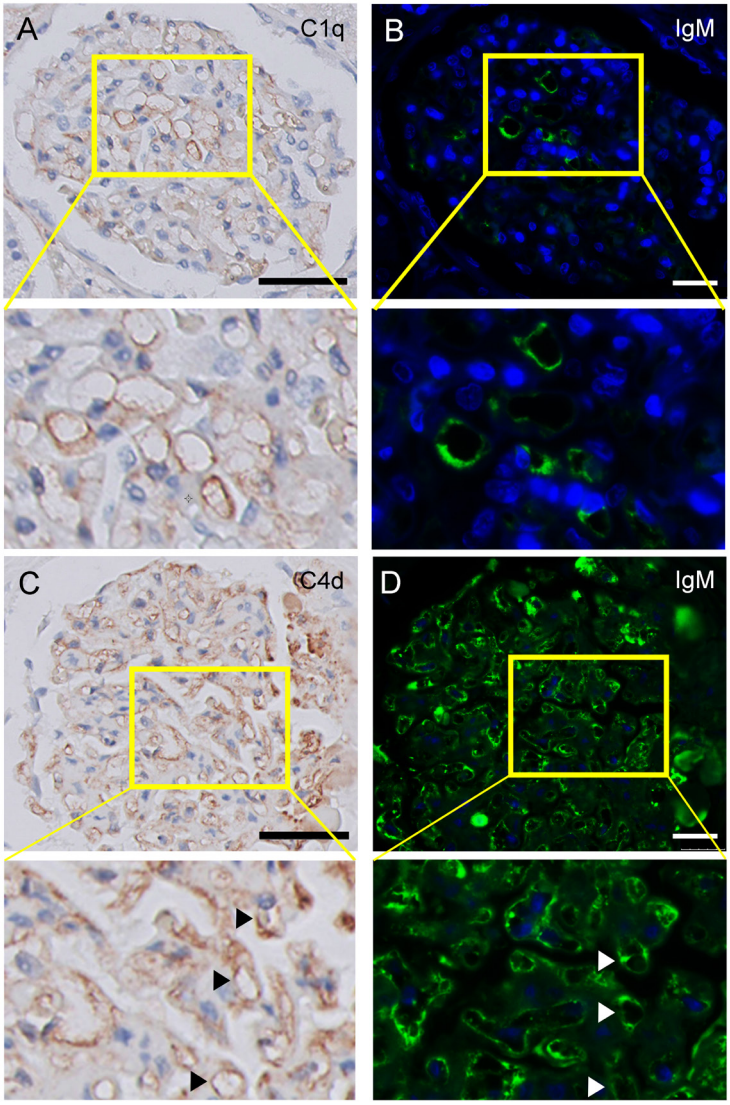

Figure 6: **Glomerular IgM deposits co-localize with glomerular C1q and C4d**. Adjacent sections of an autopsied kidney from a case with diabetic nephropathy were stained for C1q (a) or IgM (b). Adjacent sections of an autopsied kidney from a case with diabetic nephropathy were stained for C4d (c) or IgM (d); the arrowheads indicate co-localization between C4d and IgM. The scale bars in (a) and (c) represent 50 μm; the scale bars in (b) and (d) represent 25 μm.

tor in several thrombotic microangiopathies[25]. Thus, our data further supported the hypothesis that Kimmelstiel-Wilson lesions might be a form of thrombotic microangiopathy that arises from complement activation. The underlying cause of C4d deposits in the arterioles, arteries, and glomerular hilus (the junction between afferent and efferent arterioles) is currently unknown. Because both renal afferent arterioles and renal efferent arterioles play a role in regulating renal blood flow, these vessels can be exposed to extremely turbulent blood flow and high shear stress conditions[26-29], which may increase vulnerability to vascular injury, complement deposition, and/or the development of renal pathology.

We found that C5b-9 deposits were more prevalent in our cases with diabetes than in control cases without diabetes; however, the clinical relevance of these deposits in our cohort is difficult to interpret, because C5b-9 was relatively frequently prevalent in control cases without diabetes (albeit to a lesser extent than in the cases with diabetes). This finding might be related in part to our use of autopsy samples because we did not observe these deposits in renal biopsies obtained from living donors, which was consistent with findings reported by Qin *et al.*[11] Nevertheless, in our cohort of cases with diabetes with DN, the presence of glomerular C5b-9 was correlated with the severity of DN, which suggested that C5b-9 might play a role in the progression of renal damage. Our data suggested that the process that leads to C4d deposition also leads to C5b-9 deposition. However, C5b-9 could have also been activated due to a direct effect of hyperglycemia on regulatory proteins in the complement system. Hyperglycemia can lead to the glycation of CD59, which inhibits C5b-9 under physiological conditions[11,18]. This glycation-induced inactivation of CD59 could lead to the formation of C5b-9.

In the setting of DN, several factors could have led to the deposition of C4d. For example, autoantibodies can activate the complement system[30]. This notion is supported by the report of linear IgG staining along the glomerular basement membrane in 60% of type 2 DM cases with DN[31]. In addition, high glucose levels in cases with DM can lead to increased levels of glycated proteins, including advanced glycation end-products and oxidized proteins[32,33], which can activate the classical and/or lectin pathways either directly or by reacting with

autoantibodies[16,34-36]. The complement system can also be activated by the binding of natural (i.e., IgM) antibodies to either hypoxic or apoptotic cells in the setting of DN[37,40,41]. Natural antibodies play an important role in clearing damaged cells via intracellular antigens that are externalized during apoptosis and/or hypoxic conditions[37-41]. We found a significantly higher prevalence of glomerular IgM deposits in biopsies from cases with DN compared with healthy living transplantation donors. Moreover, glomerular IgM deposits co-localized with—and were significantly correlated with—both C1q and C4d, which supported the hypothesis that IgM antibodies activate the classical complement pathway in cases with DN.

It is currently unknown whether complement activation is a cause and/or consequence of microvascular damage in DN. Our data suggested that complement activation was involved in the progression of renal damage in cases with diabetes mellitus because complement activation was correlated with more severe renal damage, including higher DN class and increased IFTA levels. These findings were consistent with other studies with regard to complement activation in the development of DN and other diabetes-associated microvascular and/or macrovascular complications[7,14]. Furthermore, type 2 diabetic rats treated with a complement inhibitor had improved renal function and morphology compared with untreated rats[19], which supported the notion that complement activation plays a role in the progression of diabetes-associated kidney disease. In the context of DM, hyperglycemia can both directly and indirectly lead to complement deposition, which can then lead to the increased production of reactive oxygen species, activation of protein kinase C, and upregulation of nuclear factor-kB, thereby inducing the release of proinflammatory, prothrombotic cytokines, and growth factors[7]. Both complement-dependent and complement-independent mechanisms can then lead to inflammation, proliferation, and thrombosis, which together characterize the diabetes-associated complications in target organs. In contrast, complement activation may also be a consequence of renal vascular damage, possibly following activation via natural antibodies. This hypothesis is consistent with our findings regarding the presence of C4d and IgM antibodies in other renal microangiopathies[25,42]. Moreover, we previously reported that C4d deposits were associated with remo-

deling of the glomerular basement membrane[43], which was consistent with cases with DN that presented with a remodeled glomerular basement membrane. Nevertheless, despite our relatively large cohort and our ability to examine >100 glomeruli per case, the autopsy-based nature of this study precluded our ability to measure causality. Therefore, future studies should be designed to determine whether complement activation is a cause, consequence, or mediator of DN.

Our study has several limitations. First, the use of autopsy samples precluded our ability to investigate whether the prevalence of complement deposition in the glomeruli, glomerular hili, and/or vessels was associated with patient survival and/or renal survival. Second, our study might have had a selection bias because not all cases with diabetes had an autopsy; generally speaking, most cases that are autopsied were in the hospital at the time of death. Third, because autopsy samples were used, we could not exclude the possibility that post-mortem changes and/or cause of death might have affected the tissue in terms of protein expression and/or tissue morphology. However, to address this possibility, we examined control samples obtained from autopsied cases without diabetes or renal pathology. Furthermore, because cause of death was not significantly correlated with the prevalence of complement deposits, and because our findings were supported by examining renal biopsy samples, we concluded that our data were not likely affected by autopsy-related artifacts. In contrast, the strength of our study was the relatively high number of cases combined with our ability to examine >100 glomeruli per case.

In conclusion, complement activation is associated with DN, and both glomerular C4d and glomerular C5b-9 deposits are correlated with the class of DN. Furthermore, complement deposits in several renal vascular structures are correlated with more severe renal damage. Our data suggest that complement activation is involved with the development of renal damage in cases with diabetes, and that inhibition or modulation of complement activity could be a promising therapeutic strategy for patients with DN.

Ethics statement

All tissue samples were coded and then handled and analyzed anonymously in accordance with the Declaration of Helsinki. Approval for the study was obtained from the medical ethics committee of Leiden University Medical Center.

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Supplementary Tables

Supplemental Table 1: C1q, MBL, C4d, and C5b-9 deposits and histologic lesions in all diabetic cases.

Table S1A	Glom C1q -	Glom C1q +	*p* value	Glom hilus C1q -	Glom hilus C1q +	*p* value	Art C1q -	Art C1q +	*p* value	Art branch C1q -	Art branch C1q +	*p* value
Glomerular capsular drop	10 (9.9)	7 (12.1)	0.670	6 (12.2)	11 (10.0)	0.672	9 (7.6)	8 (19.5)	0.034	13 (10.8)	4 (10.3)	0.919
Glomerular hyalinosis	66 (65.3)	38 (65.5)	0.983	30 (61.2)	74 (67.3)	0.459	72 (61.0)	32 (78.0)	0.048	76 (63.3)	28 (71.8)	0.334
FSGS	9 (8.9)	5 (8.6)	0.950	2 (4.1)	12 (10.9)	0.161	10 (8.5)	4 (9.8)	0.803	11 (9.2)	3 (7.7)	0.778
IFTA			0.712			0.109			0.027			0.053
0	26 (25.7)	13 (22.4)		18 (36.7)	21 (19.1)		35 (29.7)	4 (9.8)		33 (27.5)	6 (15.4)	
1	53 (52.5)	28 (48.3)		20 (40.8)	61 (55.5)		59 (50.0)	22 (53.7)		63 (52.5)	18 (46.2)	
2	12 (11.9)	8 (13.8)		5 (10.2)	15 (13.6)		11 (9.3)	9 (22.0)		14 (11.7)	6 (15.4)	
3	10 (9.9)	9 (15.5)		6 (12.2)	13 (11.8)		13 (11.0)	6 (14.6)		10 (8.3)	9 (23.1)	
Arteriosclerosis	88 (87.1)	50 (86.2)	0.869	43 (87.8)	95 (86.4)	0.811	101 (85.6)	37 (90.2)	0.449	101 (84.2)	37 (94.9)	0.086
Arteriolar hyalinosis	88 (87.1)	50 (86.2)	0.869	43 (87.8)	95 (86.4)	0.811	101 (85.6)	37 (90.2)	0.449	101 (84.2)	37 (94.9)	0.086
Cholesterol emboli	4 (4.0)	2 (3.4)	0.870	3 (6.1)	3 (2.7)	0.300	4 (3.4)	2 (4.9)	0.667	3 (2.5)	3 (7.7)	0.139

Table S1B	Glom MBL -	Glom MBL +	*p* value	Glom hilus MBL -	Glom hilus MBL +	*p* value	Art MBL -	Art MBL +	*p* value	Art branch MBL -	Art branch MBL +	*p* value
Glomerular capsular drop	17 (11.3)	0 (0)	0.285	17 (10.8)	0 (0)	0.622	NA	NA	NA	NA	NA	NA
Glomerular hyalinosis	98 (65.3)	6 (66.7)	0.935	102 (65.0)	2 (100)	0.301	NA	NA	NA	NA	NA	NA
FSGS	12 (8.0)	2 (22.2)	0.144	14 (8.9)	0 (0)	0.658	NA	NA	NA	NA	NA	NA
IFTA			0.361			0.583			NA			NA
0	38 (25.3)	1 (11.1)		39 (24.8)	0 (0)							
1	74 (49.3)	7 (77.8)		79 (50.3)	2 (100)							
2	19 (12.7)	1 (11.1)		20 (12.7)	0 (0)							
3	19 (12.7)	0 (0)		19 (12.1)	0 (0)							
Arteriosclerosis	130 (86.7)	8 (88.9)	0.848	136 (86.6)	2 (100)	0.579	NA	NA	NA	NA	NA	NA
Arteriolar hyalinosis	130 (86.7)	8 (88.9)	0.848	136 (86.6)	2 (100)	0.579	NA	NA	NA	NA	NA	NA
Cholesterol emboli	6 (4.0)	0 (0)	0.541	6 (3.8)	0 (0)	0.778	NA	NA	NA	NA	NA	NA

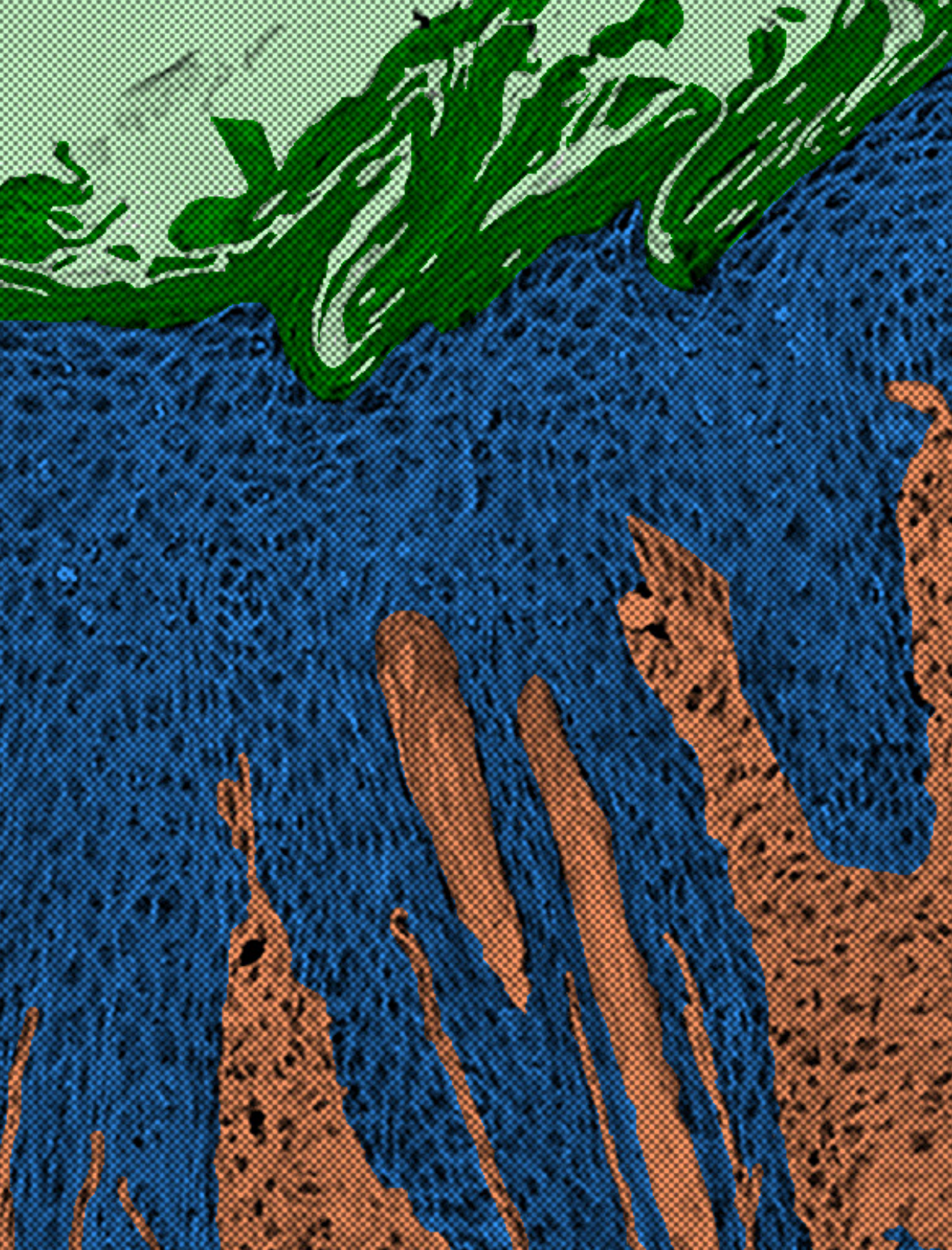
Glom: glomeruli; glom hilus: glomerular hilus; Art: arterioles; Art Branch: arterial branches; FSGS: focal segmental glomerulosclerosis; IFTA: interstitial fibrosis and tubular atrophy. All data are presented as the number of patients (%).

Supplemental Table 1: C1q, MBL, C4d, and C5b-9 deposits and histologic lesions in all diabetic cases.

Table S1C	Glom C4d -	Glom C4d +	*p* value	Glom hilus C4d -	Glom hilus C4d +	*p* value	Art C4d -	Art C4d +	*p* value	Art branch C4d -	Art branch C4d +	*p* value
Glomerular capsular drop	10 (10.1)	7 (11.7)	0.757	6 (7.2)	11 (14.5)	0.140	12 (9.7)	5 (14.3)	0.436	17 (11.2)	0 (0)	0.349
Glomerular hyalinosis	58 (58.6)	46 (76.7)	0.020	45 (54.2)	59 (77.6)	0.002	74 (59.7)	30 (85.7)	0.004	97 (63.8)	7 (100)	0.049
FSGS	8 (8.1)	6 (10.0)	0.679	6 (7.2)	8 (10.5)	0.464	11 (8.9)	3 (8.6)	0.956	12 (7.9)	2 (28.6)	0.059
IFTA			<0.001			0.202			<0.001			0.167
0	34 (34.3)	5 (8.3)		26 (31.3)	13 (17.1)		37 (29.8)	2 (5.7)		39 (25.7)	0 (0)	
1	49 (49.5)	32 (53.3)		39 (47.0)	42 (55.3)		65 (52.4)	16 (45.7)		78 (51.3)	3 (42.9)	
2	9 (9.1)	11 (18.3)		10 (12.0)	10 (13.2)		9 (7.3)	11 (31.4)		18 (11.8)	2 (28.6)	
3	7 (7.1)	12 (20.0)		8 (9.6)	11 (14.5)		13 (10.5)	6 (17.1)		17 (11.2)	2 (28.6)	
Arteriosclerosis	81 (81.8)	57 (95.0)	0.017	70 (84.3)	68 (89.5)	0.339	104 (83.9)	34 (97.1)	0.041	131 (86.2)	7 (100)	0.291
Arteriolar hyalinosis	81 (81.8)	57 (95.0)	0.017	70 (84.3)	68 (89.5)	0.339	104 (83.9)	34 (97.1)	0.041	131 (86.2)	7 (100)	0.291
Cholesterol emboli	3 (3.0)	3 (5.0)	0.528	4 (4.8)	2 (2.6)	0.470	4 (3.2)	2 (5.7)	0.495	5 (3.3)	1 (14.3)	0.135

Table S1D	Glom C5b-9 -	Glom C5b-9 +	*p* value	Glom hilus C5b-9 -	Glom hilus C5b-9 +	*p* value	Art C5b-9 -	Art C5b-9 +	*p* value	Art branch C5b-9 -	Art branch C5b-9 +	*p* value
Glomerular capsular drop	4 (8.0)	13 (11.9)	0.457	1 (33.3)	16 (10.3)	0.200	NA	NA	NA	NA	NA	NA
Glomerular hyalinosis	30 (60.0)	74 (67.9)	0.331	1 (33.3)	103 (66.0)	0.238	NA	NA	NA	NA	NA	NA
FSGS	2 (4.0)	12 (11.0)	0.148	0 (0)	14 (9.0)	0.587	NA	NA	NA	NA	NA	NA
IFTA			0.008			0.802			NA			NA
0	20 (40.0)	19 (17.4)		1 (33.3)	38 (24.4)							
1	24 (48.0)	57 (52.3)		2 (66.7)	79 (50.6)							
2	3 (6.0)	17 (15.6)		0 (0)	20 (12.8)							
3	2 (6.0)	16 (14.7)		0 (0)	19 (12.2)							
Arteriosclerosis	40 (80.0)	98 (89.9)	0.087	2 (66.7)	136 (87.2)	0.299	NA	NA	NA	NA	NA	NA
Arteriolar hyalinosis	40 (80.0)	98 (89.9)	0.087	2 (66.7)	136 (87.2)	0.299	NA	NA	NA	NA	NA	NA
Cholesterol emboli	3 (6.0)	3 (2.8)	0.318	0 (0)	6 (3.8)	0.729	NA	NA	NA	NA	NA	NA

Glom: glomeruli; glom hilus: glomerular hilus; Art: arterioles; Art Branch: arterial branches; FSGS: focal segmental glomerulosclerosis; IFTA: interstitial fibrosis and tubular atrophy. All data are presented as the number of patients (%).



Chapter 6

Soluble fms-like tyrosine kinase-1 reduces inflammation in *APOC1* transgenic mice

Pascal Bus, Malu Zandbergen, Leendert A. van Es, Jan A. Bruijn, and Hans J. Baelde

Abstract

A polymorphism in the *APOC1* gene, which encodes apolipoprotein C-I (apoCI), has been associated with the development of diabetic nephropathy. In addition, patients with diabetes have increased plasma levels of apoCI compared to non-diabetic control subjects. *APOC1* transgenic (*APOC1*-tg) mice develop nodular glomerulosclerosis by the age of 15 months; however, the slow disease progression in this model makes it poorly suited for studying therapeutic and preventive interventions. In an attempt to accelerate the progression of glomerulosclerosis in *APOC1*-tg mice, we introduced a second genetic 'hit' by transfecting these mice with the *sFlt-1* gene, which encodes soluble fms-like tyrosine kinase-1 (sFLT-1), an inhibitor of vascular endothelial growth factor A (VEGF-A). We then investigated whether inducing a systemic increase in sFLT-1 levels for 15 weeks in 8-week-old *APOC1*-tg mice accelerates the development of glomerulosclerosis. Our results demonstrate that a systemic increase in sFLT-1 levels for 15 weeks does not induce the development of glomerulosclerosis in *APOC1*-tg mice. Instead, *APOC1*-tg mice transfected with *sFlt-1* had significantly fewer glomerular macrophages compared to non-transfected *APOC1*-tg mice ($p<0.01$). Surprisingly, the atopic dermatitis that normally develops in *APOC1*-tg mice was resolved following transfection with *sFlt-1*, presumably by reducing the infiltration of inflammatory cells into the skin. Moreover, we found no significant change in glomerular endothelial cell activation or glomerular *VEGF-A* mRNA levels in *sFlt-1*–transfected *APOC1*-tg mice compared to non-transfected *APOC1*-tg mice. Taken together, these data suggest that sFLT-1 has immune-modulating properties independent of its inhibitory effects on VEGF-A.

Introduction

Diabetic nephropathy remains the leading cause of end-stage renal disease worldwide[1]. Although the risks of acute myocardial infarction, stroke, and amputation among diabetic patients have decreased considerably over the past two decades, the risk of developing end-stage renal disease has remained high in these patients[2]. Thus, new preventive and therapeutic strategies, as well as a clearer understanding of the mechanisms that underlie the development and repair process in diabetic nephropathy, are urgently needed.

The risk factors associated with the development of diabetic nephropathy include hyperglycaemia, hyperlipidaemia, hypertension, and genetic factors. With respect to genetics, a polymorphism in the *APOC1* gene, which encodes apolipoprotein C-I (apoCI), has been associated with an increased risk of developing diabetic nephropathy[3]. Furthermore, compared to non-diabetic control subjects, patients with diabetes have increased plasma levels of apoCI[4]. Recently, we reported that patients with diabetic nephropathy have higher levels of glomerular apoCI protein compared to both diabetic patients without nephropathy and non-diabetic controls[5]. We also reported that transgenic mice carrying the *APOC1* transgene (*APOC1*-tg mice), but not wild-type mice, develop albuminuria and nodular glomerulosclerosis, features that are highly reminiscent of diabetic nephropathy. In addition, apoCI increases the inflammatory response of activated macrophages *in vitro*; specifically, these cells have increased expression of tumour necrosis factor alpha (TNF-alpha)[5,6]. Moreover, TNF-alpha production by glomerular macrophages has been suggested to promote the development of diabetic nephropathy[7]. Together, these data support the hypothesis that apoCI increases the inflammatory response of glomerular macrophages in *APOC1*-tg mice, resulting in the development of nodular glomerulosclerosis. Unfortunately, however, *APOC1*-tg mice do not develop glomerulosclerosis until 15 months of age[5]; thus, the relatively slow disease progression in this model makes it unsuitable for studying therapeutic and preventive interventions.

Vascular endothelial growth factor A (VEGF-A) is another important mediator of kidney disease, and studies have shown that VEGF-A plays an important role in the maintenance and survival of endothe-

lial cells[8]. A decrease in glomerular VEGF-A levels below physiological levels results in the development of renal disease. Specifically, mice that lack VEGF-A expression in the glomeruli are not viable and have a wide range of endothelial cell defects; moreover, mice that are heterozygous for VEGF-A selectively in podocytes (i.e. mice with podocyte-specific VEGF-A haploinsufficiency) develop proteinuria, endotheliosis, and progressive loss of endothelial cells[9]. Similarly, treating rats with soluble fms-like tyrosine kinase-1 (sFLT-1) – an inhibitor of VEGF-A – causes endothelial cell damage and loss, hypertension, and proteinuria[10]. Together, these findings indicate that too little VEGF-A and too much sFLT-1 can both lead to glomerular endothelial dysfunction.

In an attempt to accelerate the progression of glomerulosclerosis in *APOC1*-tg mice, we introduced a second genetic 'hit' by transfecting 8-week-old mice with *sFlt-1*. We then examined whether systemically increasing sFLT-1 levels leads to an accelerated development of glomerulosclerosis by monitoring the mice for 15 weeks post-transfection.

Methods

sFlt-1 transfection

Two pcDNA3.1 vectors (Invitrogen, Breda, the Netherlands) containing either *sFlt-1-VSV* or the luciferase gene were generated as described previously[11,12]. The resulting plasmids were amplified in DH5α *E. coli* (Invitrogen), purified using the QIAfilter Plasmid Maxi-prep kit (Qiagen, Venlo, the Netherlands), and dissolved in Endo-Free Tris–EDTA buffer (Qiagen). Mice were transfected by electroporation of the *sFlt-1-VSV* and luciferase constructs into both gastrocnemius muscles (20 μg each) as described previously[11]. To monitor transfection efficiency, the mice were injected intraperitoneally with luciferin at 2-week intervals. Five minutes after each luciferin injection, luciferase activity was visualised at the transfection sites using a Night-OWL bioluminescence camera (Xenogen Ivis Spectrum, Alameda, CA) as described previously[11].

Animals

For this study, we used 8-week-old female C57BL/6J mice (Harlan Laboratories, Indianapolis, IN) and *APOC1*-tg mice. All experiments were conducted in accordance with national guidelines for the care and use of experimental animals (DEC licence 13163). Mice were housed in individually ventilated cages in groups of up to five mice/cage, with food and water provided *ad libitum*. For the experiments, the mice were randomly assigned to groups. At 8 weeks of age, the mice were transfected with *sFlt-1*. The development of glomerulosclerosis was then monitored in *sFlt-1*–transfected and non-transfected *APOC1*-tg mice (n=6 mice/group), as well as in *sFlt-1*–transfected and non-transfected C57BL/6J (wild-type) mice (n=6 mice/group). Fifteen weeks after transfection, the mice were sacrificed; this time point was chosen because we previously found that treatment with sFLT-1 for 15 weeks results in significant changes in kidney function and morphology[12]. One mouse in the non-transfected *APOC1*-tg group was removed from the study due to the development of severe atopic dermatitis complicated by open wounds.

Measurement of the urine albumin:creatinine ratio

To measure the urine albumin:creatinine ratio, spot urine was collected at 5 and 15 weeks after the start of the experiment. Urine albumin levels were measured using rocket immunoelectrophoresis with a rabbit anti-mouse albumin; purified mouse serum albumin (Sigma-Aldrich, Saint Louis, MO) was used as a standard. Urine creatinine was measured using a creatinine assay, with picric acid, sodium hydroxide, and creatinine standards (Sigma-Aldrich); the albumin:creatinine ratio was then calculated.

Immunohistochemistry

Paraffin-embedded kidney tissues were cut at 4-µm thickness using a Leica microtome and stained with periodic acid-Schiff (PAS) using a standard protocol. A rabbit anti-human Wilms tumour 1 (WT1; 1:500; Santa Cruz Biotechnology, Dallas, TX) primary antibody was used for immunostaining, followed by an anti-rabbit-Envision HRP-conjugated

secondary antibody (Dako, Glostrup, Denmark), with diaminobenzidine (DAB+; Dako) as the chromogen. This rabbit anti-human WT1 antibody cross-reacts with mouse WT1 (data not shown). A non-specific isotype-matched antibody was used as a negative control.

Paraffin-embedded skin tissues were cut at 4-µm thickness using a Leica microtome and stained with hematoxylin and eosin (H&E) using a standard protocol. The rat anti-mouse F4/80 (1:100; kindly provided by the Department of Human Genetics, LUMC) primary antibody was used for immunostaining, followed by the anti-rat-Impress (Vector Laboratories, Burlingame, CA) HRP-conjugated secondary antibody, with DAB+ as the chromogen.

Frozen kidney tissues were cut at 4-µm thickness using a Reichert cryostat and immunostained using the following primary antibodies: rat anti-mouse CD68 (1:15; Abcam, Cambridge, MA), rat anti-mouse vascular cell adhesion molecule-1 (VCAM-1; 1:1400; BD Pharmingen, San Diego, CA), rat anti-mouse intercellular adhesion molecule-1 (ICAM-1; 1:200; ATCC, Manassas, VA), and rabbit anti-vesicular stomatitis virus (VSV; 1:2500; Sigma-Aldrich), followed by the appropriate Envision (Dako) or Impress (Vector Laboratories) HRP-conjugated secondary antibody, with DAB+ as the chromogen. For each immunostaining experiment, the respective non-specific isotype-matched antibody was used as a negative control.

Frozen skin tissues were cut at 4-µm thickness using a Reichert cryostat and immunostained with rat anti-mouse CD3 (1:20; Abcam), followed by the appropriate Impress (Vector Laboratories) HRP-conjugated secondary antibody, with DAB+ as the chromogen.

Digital image analysis

Images of tissue sections were digitised using a Philips Ultra-Fast Scanner 1.6 RA. The surface area of the glomerular tuft was measured in PAS-stained slides containing 25 glomeruli per section using Philips Ultra-Fast Scanner 1.6 RA software. ImageJ (NIH, Bethesda, MD) was used to quantify the levels of VCAM-1 and ICAM-1. The positive area per glomerulus was determined by measuring the positively stained area (corrected for the total area of the glomerulus) at 400X magnification with 10 glomeruli per section. The number of podocytes in each sample was determined by counting the number of WT1-positive

nuclei per glomerulus, measured in 25 glomeruli. The number of macrophages was determined by counting the number of CD68-positive cells in 10 glomeruli per section. The glomeruli that were used for these measurements were selected at random. The average thickness of the epidermal layer was determined by averaging 10 measurements per field in 5 fields at 20X magnification using Philips Ultra-Fast Scanner 1.6 RA software.

RNA isolation and quantitative real-time PCR

Frozen kidney tissues were chopped and then dissolved in 1 ml TRIzol (Ambion, Foster City, CA) to obtain total RNA. Quantitative real-time PCR (qRT-PCR) was then performed using SYBR Green I master mix (Bio-Rad, Hercules, CA) in accordance with the manufacturer's instructions; the reactions were run on a Bio-Rad CFX real-time system. Ct (cycle threshold) values were normalised to the housekeeping gene *Hprt1*. The following primers were used: *Hprt1*: 5'-GGCTATAAGTTCTTTGCTGACCTG-3' and 5'-AACTTTTATGTCCCCCGTTGA-3'; *Vegf-a*: 5'-CTGGACCCTGGCTTTACTGC-3' and 5'-GCTTCGCTGGTAGACATCCA-3'.

Statistics

Data were analysed using a one-way ANOVA. Differences were considered significant at $p<0.05$.

Results

Transfection efficiency

First, we tested the efficacy and efficiency of our transfection protocol. Transfected and non-transfected mice were injected intraperitoneally with luciferin, and luciferase activity was visualised using a Night-OWL bioluminescence camera. As shown in Figure 1, non-transfected mice had no luminescence in the gastrocnemius muscles following injection with luciferin (Figure 1, mice 1 and 2); in contrast, transfected mice had robust luminescence at the site of transfection (Figure 1, mice 3-5). Staining for VSV (to measure the VSV-tagged sFLT-1 protein) confirmed the presence of exogenous sFLT-1 in the renal vasculature (data not shown).

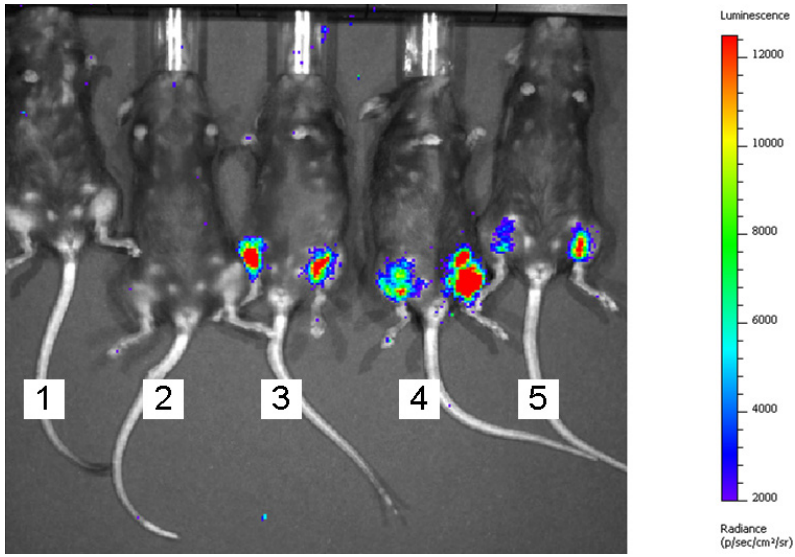


Figure 1: Transfection efficiency. The *sFlt-1* and luciferase constructs were co-transfected bilaterally by injection into the gastrocnemius muscles. Luciferase activity was visualised using a Night-OWL bioluminescence camera and is depicted using an arbitrary pseudocolour scale with red and blue corresponding to high and low luminescence, respectively. 1 and 2: two representative non-transfected *APOC1*-tg mice following an i.p. injection of luciferin; 3-5: three representative *sFlt-1*–transfected *APOC1*-tg mice following an i.p. injection of luciferin.

Albuminuria and glomerulosclerosis

APOC1-tg mice and age-matched wild-type mice have a similar albumin:creatinine ratio (Figure 2A). Moreover, *APOC1*-tg mice that were transfected with *sFlt-1* had a similar albumin:creatinine ratio as non-transfected *APOC1*-tg mice. Furthermore, the glomerular surface area was similar between *APOC1*-tg mice and wild-type mice (Figure 2B), and transfection with *sFlt-1* did not significantly affect glomerular surface area. Non-transfected *APOC1*-tg mice had significantly fewer podocytes compared to wild-type mice, and transfecting *APOC1*-tg mice with *sFlt-1* had no further effect on the number of podocytes (Figure 2C). No other changes in glomerular histology were observed (data not shown). These data indicate that transfecting *APOC1*-tg mice with *sFlt-1* does not accelerate the development of glomerulosclerosis.

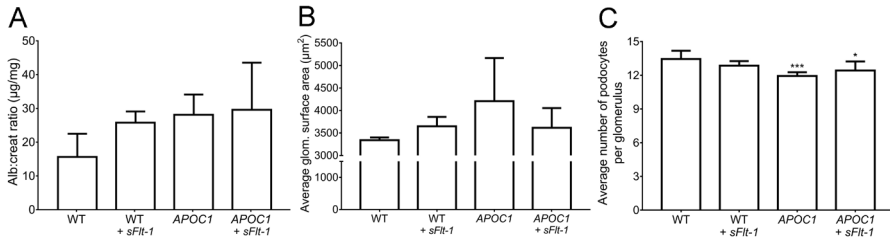


Figure 2: Overexpressing *sFlt-1* in *APOC1*-tg mice for 15 weeks does not cause albuminuria or glomerulosclerosis. At 8 weeks of age, wild-type (WT) and *APOC1*-tg mice were transfected with *sFlt-1*; 15 weeks later, the albumin:creatinine ratio (A), glomerular surface area (B), and number of podocytes per glomerulus (C) were measured. In this and subsequent figures, summary data are presented as the mean ± the standard deviation. *p<0.05 and ***p<0.001 versus the respective wild-type group, one-way ANOVA.

Endothelial cell activation

Glomerular VCAM-1 protein levels were similar between *APOC1*-tg mice and wild-type mice (Figure 3A). In contrast, glomerular ICAM-1 protein levels were significantly higher in the *APOC1*-tg mice compared to wild-type mice (Figure 3B); however, transfection with *sFlt-1* had no effect on either glomerular VCAM-1 or glomerular ICAM-1 protein levels compared to the respective non-transfected groups. Lastly, glomerular *Vegf-a* mRNA levels were similar between all four groups (Figure 3C).

sFlt-1–transfected *APOC1*-tg mice have fewer glomerular macrophages compared to non-transfected *APOC1*-tg mice

Consistent with our previous findings[5], we found that *APOC1*-tg mice have significantly more glomerular macrophages compared to wild-type mice (Figure 4). Interestingly, however, we found that *APOC1*-tg mice transfected with *sFlt-1* have significantly fewer glomerular macrophages compared to non-transfected *APOC1*-tg mice; 15 weeks after transfection, the number of glomerular macrophages was reduced to wild-type levels (Figure 4).

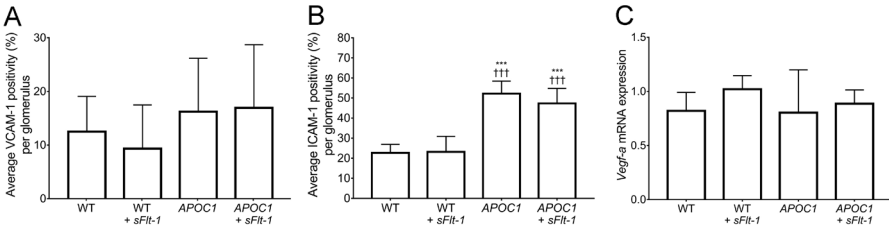


Figure 3: ***APOC1*-tg mice have increased glomerular endothelial cell activation**. *APOC1*-tg and wild-type mice were treated as in Figure 2, and glomerular VCAM-1 (A), ICAM-1 (B), and *Vegf-a* mRNA (C) were measured 15 weeks after transfection. ***$p<0.001$ versus wild-type mice and $^{\dagger\dagger\dagger}p<0.001$ versus *sFlt-1*–transfected wild-type mice, one-way ANOVA.

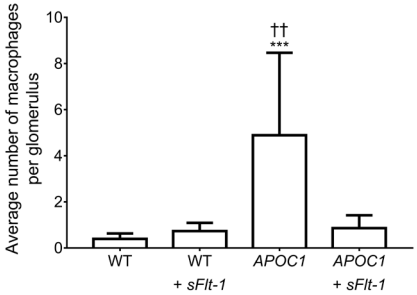


Figure 4: **Expressing *sFlt-1* in *APOC1*-tg mice reduces the glomerular infiltration of macrophages to wild-type levels**. *APOC1*-tg and wild-type mice were treated as in Figure 2, and the number of glomerular macrophages was counted 15 weeks after transfection. ***$p<0.001$ versus non-transfected wild-type mice and $^{\dagger\dagger}p<0.01$ versus both *sFlt-1*-transfected wild-type mice and *sFlt-1*-transfected *APOC1*-tg mice, one-way ANOVA.

Atopic dermatitis is resolved in *APOC1*-tg mice following transfection with *sFlt-1*

Consistent with a previous report[13], *APOC1*-tg mice developed atopic dermatitis, with increased thickness of the epidermal layer compared to wild-type mice (Figure 5). Interestingly, these lesions were not present in the *APOC1*-tg mice that were transfected with *sFlt-1* (Figure 5C, F). The quantification of the thickness of the epidermal layer is provided in Figure 5G. In addition, we found that inflammatory cells were present in the skin of *APOC1*-tg mice (Figure 5H), including macrophages (Figure 5I) and few T cells (Figure 5J). In contrast,

inflammatory cells were not present in the skin of *sFlt-1*–transfected *APOC1*-tg mice (Figure 5F).

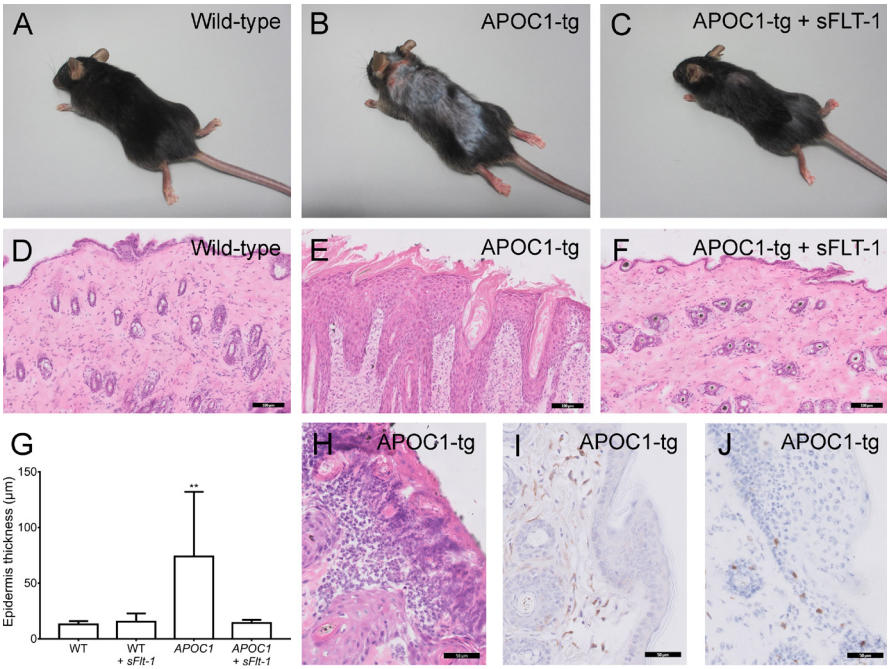


Figure 5: Transfecting *APOC1*-tg mice with *sFlt-1* prevents atopic dermatitis. Representative images of the skin of a wild-type mouse (A, D), an *APOC1*-tg mouse (B, E, H-J), and an *sFlt-1*–transfected *APOC1*-tg mouse (C, F). (G) Summary of epidermal thickness measured in the indicated groups. The sections shown in D-F and H were stained with H&E. Example images of F4/80 (I) and CD3 (J) immunohistochemical staining in skin sections from an *APOC1*-tg mouse. The scale bars represent 100 µm (D-F) and 50 µm (H-J). $^{**}p<0.01$ versus all three other groups, one-way ANOVA.

Discussion

APOC1-tg mice develop nodular glomerulosclerosis by the age of 15 months[5], making them poorly suited for use in therapeutic or preventive intervention studies. Therefore, we attempted to accelerate the development of glomerulosclerosis by transfecting these mice with *sFlt-1* at 8 weeks of age; we then looked for signs of glome-

rulosclerosis 15 weeks after transfection. To our surprise, *APOC1*-tg mice transfected with *sFlt-1* did not develop glomerulosclerosis within the investigated time frame; instead, these mice had fewer glomerular macrophages compared to non-transfected *APOC1*-tg mice, and they showed no signs of atopic dermatitis.

VEGF-A plays a key role in the health and function of endothelial cells, controlling their migration, proliferation, and survival[8]. Decreased levels of glomerular VEGF-A result in damage to the glomerular endothelium; specifically, homozygous glomerular VEGF-A knockout mice are not viable, present with a wide range of glomerular endothelial defects, and fail to develop a glomerular filtration barrier[9]. Moreover, diabetic mice that are heterozygous for VEGF-A selectively in podocytes have increased proteinuria, mesangial matrix expansion, and increased glomerular cell death compared to wild-type diabetic mice[14]. Furthermore, deleting VEGF-A in the kidneys in an inducible VEGF-A knockout mouse induces vascular damage by promoting thrombotic microangiopathy[15]. Similarly, increasing the serum levels of sFLT-1, a decoy receptor for VEGF-A, causes hypertension, glomerular endotheliosis, and proteinuria in rats[10]. In patients with kidney cancer, breast cancer, or colorectal cancer, treatment with an anti-VEGF-A antibody in order to inhibit tumour angiogenesis leads to proteinuria in a dose-dependent manner[16]. Finally, treating wild-type mice with either anti-VEGF-A antibodies or sFLT-1 causes a loss of glomerular endothelial cells, glomerular hypertrophy, and proteinuria[17]. Taken together, these findings indicate that decreasing glomerular VEGF-A levels with sFLT-1 results in glomerular endothelial damage and subsequent glomerular disease. Therefore, we hypothesised that transfecting mice with *sFlt-1* would lead to a systemic increase in sFLT-1, thereby affecting the health and function of glomerular endothelial cells, consequently accelerating the progression of glomerulosclerosis in *APOC1*-tg mice. However, we found that transfecting 8-week-old *APOC1*-tg mice with *sFlt-1* did not lead to the development of glomerulosclerosis, even after 15 weeks. Furthermore, similar to a previous report[13], *APOC1*-tg mice develop atopic dermatitis, a skin disease characterised by scaling, lichenification, excoriations, and pruritus, with thickening of the epidermis and influx of inflammatory cells. The majority of these inflammatory cells were macrophages, although a

few T cells were also present (Figure 5I-J). Interestingly, however, atopic dermatitis was resolved in *sFlt-1*-transfected *APOC1*-tg mice, resulting in normal epidermal thickness and a lack of inflammatory cells in the skin (Figure 5). We previously reported that type 1 diabetic mice transfected with *sFlt-1* have fewer glomerular macrophages, normal glomerular morphology, and improved renal function compared to non-transfected type 1 diabetic mice[12].

One possible explanation for the lack of accelerated glomerulosclerosis in *sFlt-1*–transfected *APOC1*-tg mice may be that the systemic levels of sFLT-1 were insufficient to induce glomerular endothelial damage within the investigated time frame; in other words, glomerular VEGF-A levels may have remained within physiological levels, thereby maintaining the health and function of glomerular endothelial cells. On the other hand, the higher levels of sFLT-1 may have affected other VEGF-A targets aside from its effect on endothelial cells, for example by affecting VEGF-A–induced migration of monocytes and/or macrophages[18,19], which is mediated by the binding of VEGF-A to the FLT-1 receptor on these cells[19]. This possible reduction in cell migration may be the result of VEGF-A sequestering by sFLT-1. In addition, VEGF-A can induce the activation of endothelial cells[12,20]; however, the observed increase in glomerular endothelial cell activation (measured as an increase in glomerular ICAM-1 levels) in *APOC1*-tg mice was not reduced by transfecting *APOC1*-tg mice with *sFlt-1*. Finally, glomerular *Vegf-a* mRNA levels were similar between *APOC1*-tg mice and wild-type mice. Taken together, these results suggest that VEGF-A does not appear to play a role in the increase in glomerular macrophages in *APOC1*-tg mice.

Interestingly, Jin *et al.* recently reported that sFLT-1 has other functions in addition to sequestering VEGF-A[21]. Specifically, sFLT-1 plays an essential role in podocyte cell morphology and glomerular barrier function – independent of VEGF-A – by binding directly to the glycosphingolipid monosialodihexosylganglioside (GM3) in lipid rafts on the surface of podocytes. Monocytes also express GM3, and this expression is increased upon differentiation of monocytes into macrophages and during inflammatory responses[22,23]. Therefore, sFLT-1 may bind to monocytes/macrophages in a similar fashion as sFLT-1 binds to podocytes (i.e. via GM3), subsequently altering cellular function.

This notion is supported by the finding that sFLT-1 downregulates the FLT-1 receptor in leukocytes by decreasing the activity of the *FLT-1* promoter, thereby preventing subsequent migration of these cells upon stimulation with VEGF-A[24]; thus, the reduction in monocyte/macrophage migration with sFLT-1 may be independent of VEGF-A sequestering. Preliminary *in vitro* data by our group suggest that sFLT-1 does indeed affect monocyte/macrophage function. For example, monocytes that differentiate into macrophages in the presence of sFLT-1 develop a different morphology than monocytes that differentiate in the absence of sFLT-1; specifically, the former are more circular in shape and have fewer – or no – filopodia. In addition, the cytokine expression pattern in macrophages that are activated with lipopolysaccharide (LPS) differs depending on whether the macrophages were differentiated in the presence or absence of sFLT-1; specifically, LPS-activated macrophages that were differentiated in the presence of sFLT-1 have reduced expression of several inflammation-related proteins, including interleukin-6 and TNF-alpha (our unpublished data). Taken together, these data suggest that transfecting *APOC1*-tg mice with *sFlt-1* reduces the migration of monocytes by downregulating *FLT-1* promoter activity in these cells, potentially affecting the cells' differentiation into macrophages, thereby reducing both atopic dermatitis and the number of glomerular macrophages.

Similar results have been reported by groups that investigated the use of sFLT-1 to treat a variety of other conditions, including vascular disease[25,26], arthritis[27,28], sepsis[29], and psoriasis[30]; specifically, these studies showed that sFLT-1 was beneficial in terms of reducing both systemic and local inflammation, as well as reducing the number of macrophages at the site of pathology.

In summary, we report that transfecting *APOC1*-tg mice with *sFlt-1* to increase systemic sFLT-1 levels does not accelerate the development of glomerulosclerosis in the investigated time frame; rather, this treatment significantly reduces the number of glomerular macrophages and completely resolves the atopic dermatitis that normally develops in *APOC1*-tg mice. Future studies are needed in order to determine whether these effects of sFLT-1 treatment are dependent upon VEGF-A or whether sFLT-1 has other immunosuppressing properties that are independent of its effect on VEGF-A.

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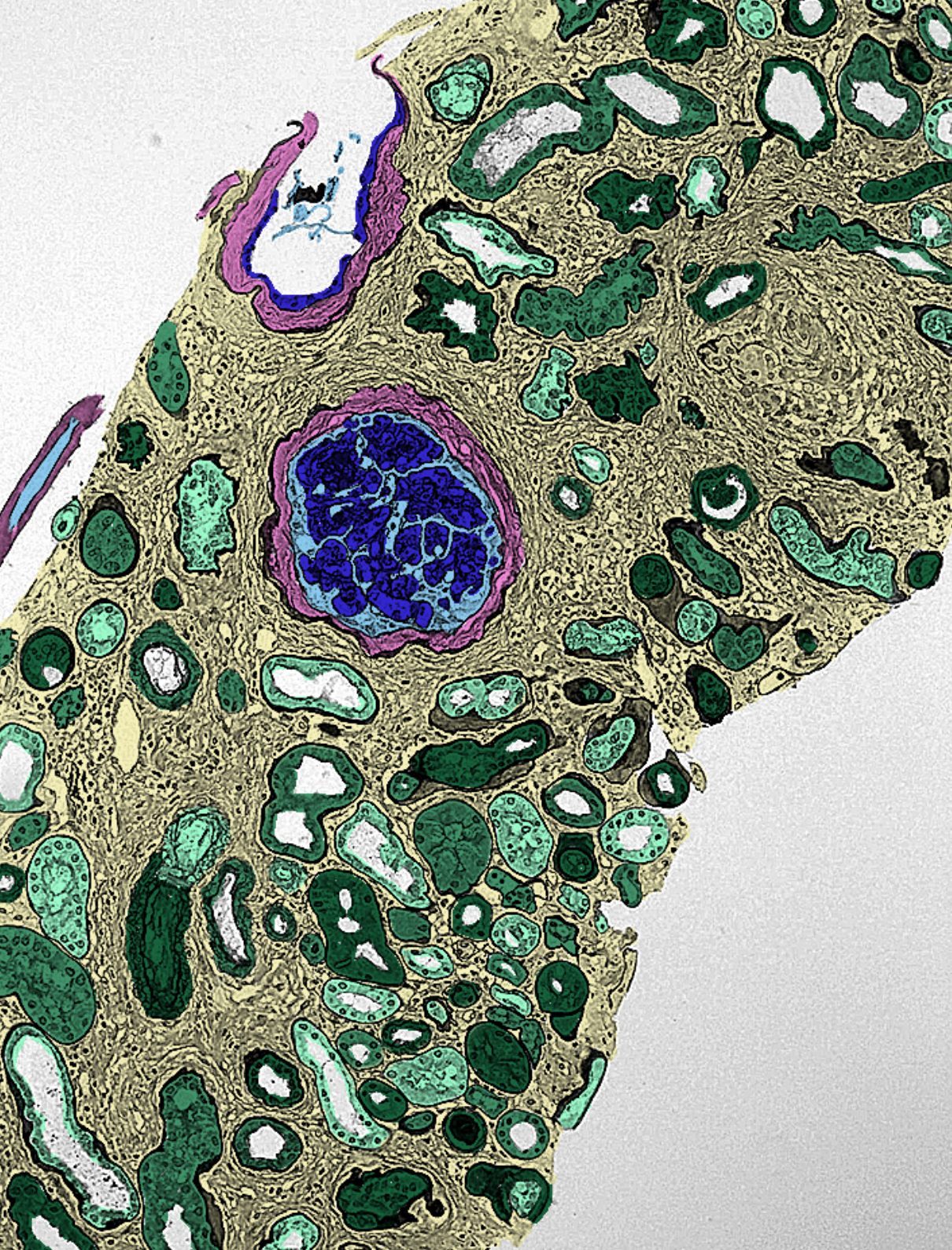

Chapter 7

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.

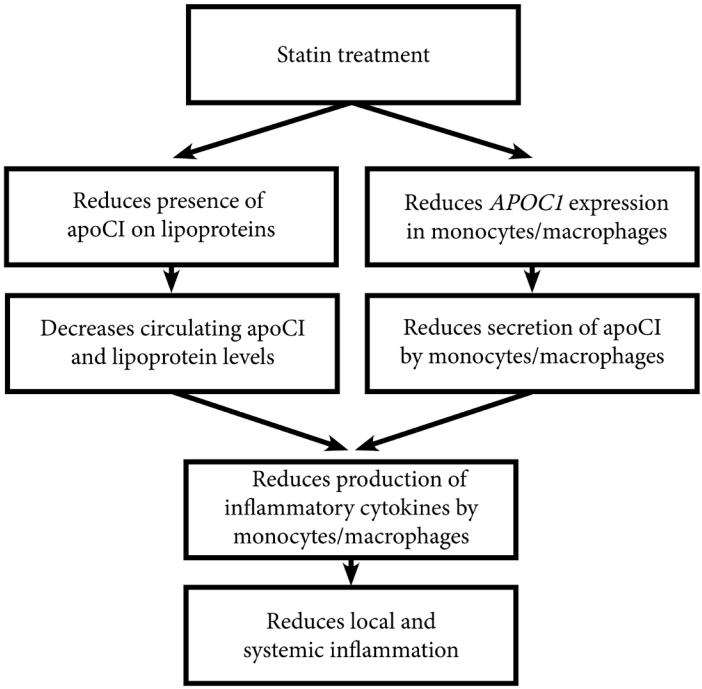

Figure 1: Hypothetical scheme by which statins may confer clinically beneficial effects. Statins reduce expression of the *APOC1* gene, reducing apoCI in lipoproteins, thereby reducing the inhibitory effect of apoCI on lipid uptake and lowering serum lipoprotein levels. Reduced *APOC1* expression also 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.

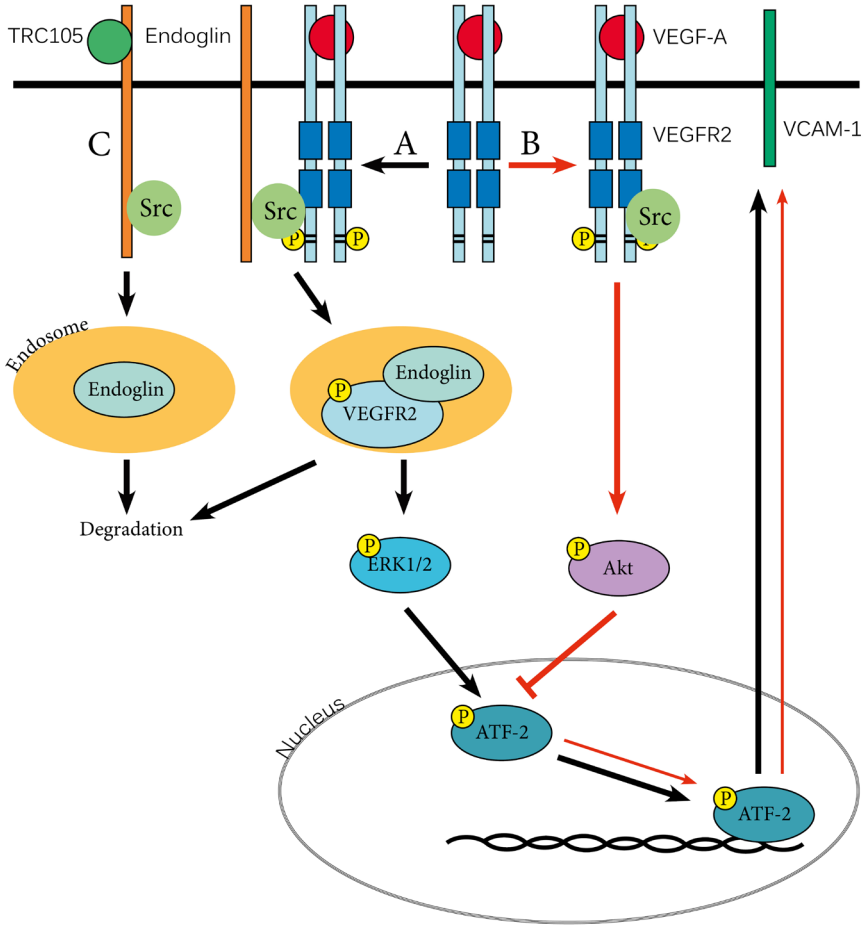

Figure 2: Schematic representation of VEGF-A–induced endothelial cell activation. A. VEGF-A binds to VEGFR2, which in turn becomes phosphorylated (P) and associates with both Src and endoglin. Phosphorylated VEGFR2 is then internalised via endosomes and subsequently activates ERK1/2. Phosphorylated ERK1/2 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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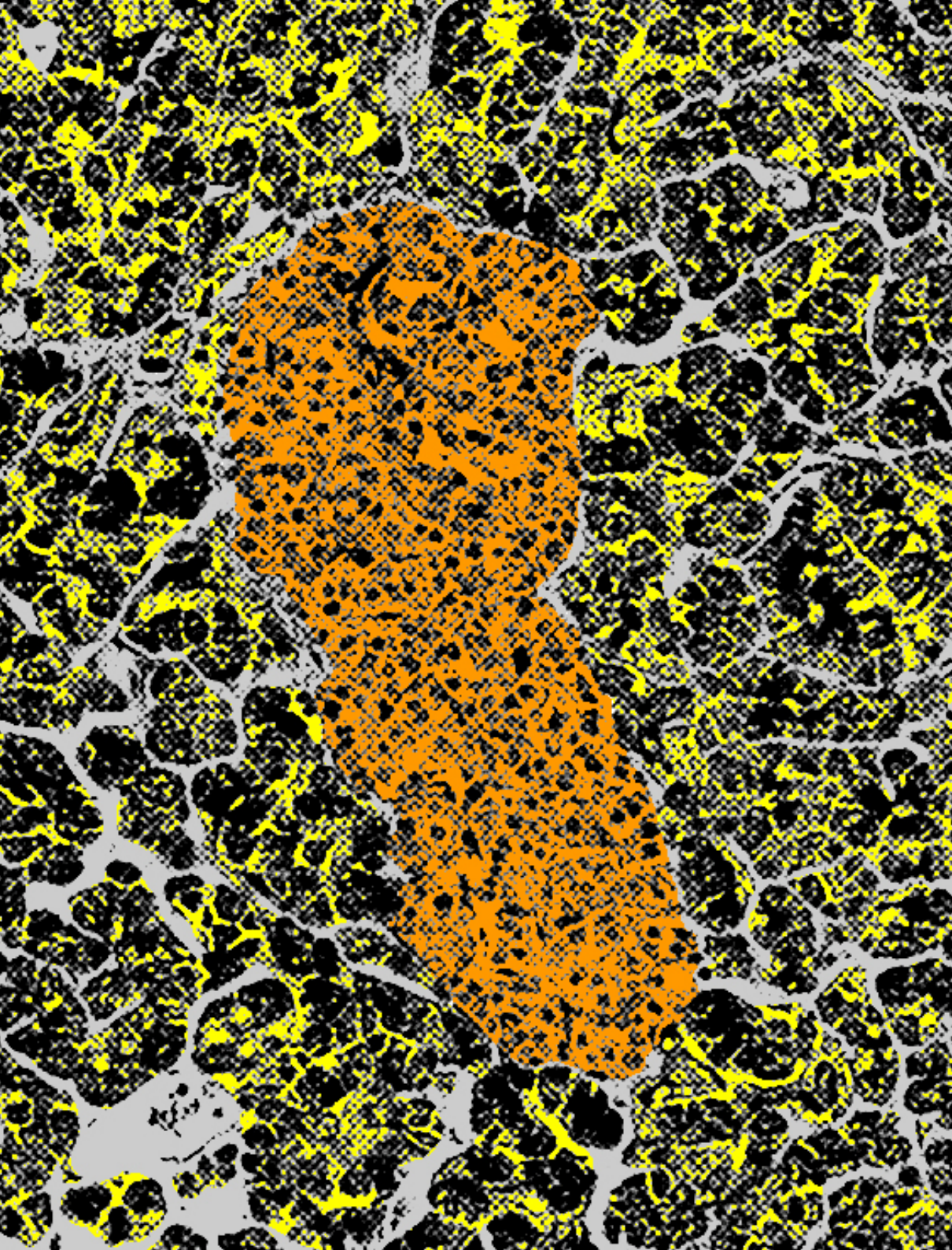


Chapter 8

Nederlandse samenvatting

Diabetes mellitus, ook wel suikerziekte genoemd, ontwikkelt zich wanneer specifieke cellen van de alvleesklier – de β-cellen – minder of geen insuline meer maken, of als bepaalde cellen ongevoelig worden voor insuline. Door een verlaagde insulineproductie, een verlaagde reactiviteit op insuline, of een combinatie hiervan, raakt de glucosehuishouding verstoord. Door deze verstoorde glucosehuishouding ontwikkelen patiënten met diabetes mellitus verschillende soorten complicaties, waaronder diabetische nierschade – ook wel diabetische nefropathie genoemd. In Nederland neemt het aantal patiënten met diabetes mellitus snel toe: van 2,8 procent van de bevolking in 2001 naar 4,5 procent in 2013. Diabetes mellitus beïnvloedt de levens van ongeveer 750.000 Nederlanders. Ongeveer twintig tot veertig procent van de mensen met diabetes mellitus ontwikkelen diabetische nefropathie.

Diabetische nefropathie wordt gekenmerkt door vasculaire schade en is de grootste oorzaak voor eindstadium nierfalen. Dit houdt in dat de nieren niet goed genoeg meer functioneren, waardoor patiënten dialyse-afhankelijk worden of zelfs een niertransplantatie moeten ondergaan. Vanzelfsprekend heeft dit een grote invloed op de levenskwaliteit van deze mensen.

Traditionele behandelmethoden van diabetisch mellitus, zoals het verlagen van de bloedsuikerspiegel, helpen de ontwikkeling van bepaalde complicaties te voorkomen of te remmen. Desondanks is het percentage patiënten met diabetes mellitus dat eindstadium nierfalen ontwikkelt de afgelopen decennia nauwelijks gedaald. Dit geeft aan dat er naast – of in combinatie met – een verstoorde glucosehuishouding ook andere factoren moeten zijn die bijdragen aan de ontwikkeling van diabetische nefropathie.

Diabetes mellitus is historisch gezien altijd beschouwd als een metabolische ziekte. Steeds meer publicaties beschrijven echter de betrokkenheid van het immuunsysteem in deze ziekte en de ontwikkeling van bijbehorende complicaties. Het centrale doel van de studies beschreven in dit proefschrift was om de bijdrage van het immuunsysteem en vasculaire veranderingen te onderzoeken in de ontwikkeling van diabetische nefropathie.

Genetische studies hebben aangetoond dat een afwijking in het apolipoproteïne C1 gen (*APOC1*) leidt tot een verhoogd risico voor

patiënten met diabetes mellitus om diabetische nefropathie te ontwikkelen. Recent is ook gevonden dat patiënten met diabetes mellitus verhoogde concentraties van apolipoproteïne C-I (apoCI) in het bloed hebben ten opzichte van gezonde mensen. ApoCI speelt een belangrijke rol in het lipiden metabolisme, maar is ook betrokken bij ontstekingsreacties. ApoCI versterkt namelijk de ontstekingsreactie van geactiveerde macrofagen. Het is eerder aangetoond dat de ontstekingsreactie van macrofagen bijdraagt aan de ontwikkeling van diabetische nefropathie. In **hoofdstuk 2** van dit proefschrift laten we zien dat muizen die transgeen zijn voor het humane *APOC1* gen (*APOC1*-tg muizen) albuminurie, een verminderde nierfunctie en glomerulosclerose ontwikkelen. Daarnaast hebben deze *APOC1*-tg muizen een verhoogd aantal macrofagen in glomeruli ten opzichte van het aantal in wildtype muizen. Met celkweekexperimenten hebben we gevonden dat activatie van macrofagen afkomstig uit *APOC1*-tg muizen leidt tot een sterkere ontstekingsrespons dan activatie van macrofagen afkomstig uit wildtype muizen. Ten slotte hebben we in deze studie laten zien dat er meer apoCI deposities aanwezig zijn in glomeruli van patiënten met diabetische nefropathie dan in diabetespatiënten zonder nierschade en in gezonde mensen. Samengevat suggereren deze data dat toename van apoCI de ontwikkeling van diabetische nefropathie bevordert. Dit komt mogelijk door het versterken van de ontstekingsreactie van macrofagen aanwezig in glomeruli van deze patiënten.

Vasculair endotheel groei factor A (VEGF-A) is belangrijk voor verschillende functies van endotheelcellen, de cellaag aan de binnenzijde van bloedvaten. Het is cruciaal dat VEGF-A levels binnen bepaalde fysiologische waarden blijven, omdat zowel een verhoging als een verlaging van VEGF-A niveaus leidt tot de ontwikkeling van nierschade. In diabetische dieren zijn VEGF-A niveaus hoger dan in niet-diabetische dieren. Ook is laten zien dat een verhoging van VEGF-A niveaus specifieke cellen van de glomerulus de ontwikkeling van diabetische nefropathie verergert.

VEGF-A is op twee manieren betrokken bij de ontwikkeling van glomerulaire ontsteking. Ten eerste zorgt VEGF-A ervoor dat monocyten (ontstekingscellen) naar de glomerulus migreren. Ten tweede draagt VEGF-A bij aan de extravasatie van monocyten door het activeren van endotheelcellen. Een belangrijk onderdeel van glomerulaire ont-

steking is activatie van het glomerulaire endotheel. Dit houdt in dat bepaalde adhesiemoleculen worden opgereguleerd op het endotheel. Monocyten kunnen vervolgens aan deze adhesiemoleculen binden, die op deze manier bijdragen aan de extravasatie van monocyten. VEGF-A zorgt voor de opregulatie van deze markers op het endotheel. Zodra monocyten extravaseren, differentiëren deze cellen naar macrofagen. Zoals eerder genoemd dragen macrofagen bij aan de ontwikkeling van diabetische nefropathie.

In **hoofdstuk 3** van dit proefschrift beschrijven we dat het remmen van VEGF-A in diabetische muizen ertoe leidt dat deze muizen minder albuminurie en minder glomerulaire schade ontwikkelen dan diabetische muizen die niet behandeld zijn met VEGF-A-remmers. Daarnaast zorgt remming van VEGF-A in diabetische muizen voor minder glomerulaire ontsteking ten opzichte van onbehandelde diabetische muizen. Deze data suggereren dat het remmen van VEGF-A in patiënten mogelijk de ernst van de diabetische nefropathie zou kunnen verminderen.

Endoglin is een type 1 membraan glycoproteïne aanwezig op het oppervlak van endotheelcellen. Endoglin is belangrijk voor het onderhoud van endotheelcellen en is daarnaast geassocieerd met endotheelactivatie. Er zijn aanwijzingen dat er een interactie is tussen VEGF-A-signalering en endoglin. Dit mechanisme is echter nog onduidelijk. In **hoofdstuk 4** van dit proefschrift laten we zien dat er meer endoglin in endotheelcellen in glomeruli van diabetische muizen aanwezig is dan in niet-diabetische muizen. Vervolgens hebben we met celkweekexperimenten de betrokkenheid van endoglin in VEGF-A geïnduceerde endotheelactivatie onderzocht. We stelden vast dat een verlaging van de aanwezigheid van endoglin leidt tot verminderde endotheelactivatie na stimulatie met VEGF-A ten opzichte van de endotheelactivatie in endotheelcellen met een normale aanwezigheid van endoglin. Dit had ook tot gevolg dat minder monocyten bonden aan deze endotheelcellen met een verminderde endoglin aanwezigheid. De verlaging van endoglin had tot gevolg dat de intracellulaire signalering van VEGF-A, die normaal gesproken leidt tot endotheelactivatie, was veranderd. In nierweefsel van patiënten vonden we dat de hoeveelheid endoglin in de glomerulus correleert met endotheelactivatie. Dit wil zeggen: hoe meer endoglin, hoe meer endotheelactivatie, en vice

versa. Deze data suggereren dat het verlagen van endoglin levels in diabetespatiënten – en daarmee glomerulaire ontsteking – mogelijk een nieuwe behandelmethode zou kunnen zijn om de ontwikkeling van diabetische nefropathie te voorkomen, dan wel te remmen.

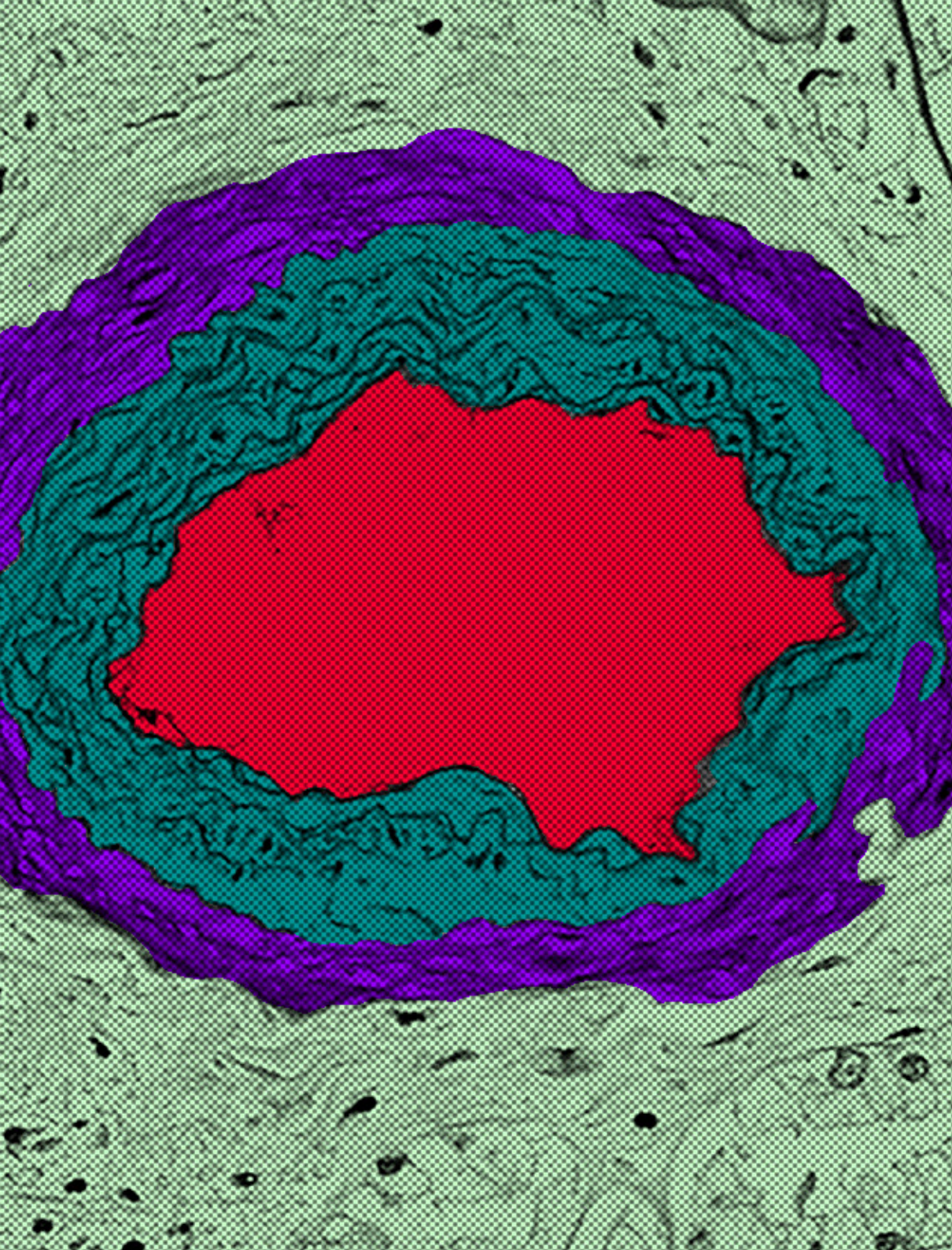
Het complementsysteem is onderdeel van het aangeboren immuunsysteem. Een belangrijke taak van het complementsysteem is beschermen tegen infectie. Het systeem bestaat uit drie verschillende routes, elk geactiveerd door specifieke stimuli. Er is zowel klinisch als experimenteel bewijs dat het complementsysteem betrokken is bij het ontstaan van diabetische complicaties zoals diabetische oogschade. Er is echter minder bekend over de betrokkenheid van het complementsysteem bij de ontwikkeling van diabetische nefropathie.

In **hoofdstuk 5** van dit proefschrift laten we zien dat complementactivatiemarkers worden opgereguleerd in patiënten met diabetische nefropathie ten opzichte van diabetes patiënten zonder diabetische nefropathie en ten opzichte van niet-diabetische controles. Daarnaast vonden we een sterke positieve correlatie tussen de aanwezigheid van het complementsysteem in vasculaire structuren van de nier, onder andere in glomeruli, en de ernst van de diabetische nefropathie. Ook vonden we een sterke negatieve correlatie tussen de aanwezigheid van het complementsysteem en de nierfunctie van patiënten met diabetische nefropathie. Deze bevindingen suggereren dat het complementsysteem betrokken is bij de ontwikkeling van diabetische nefropathie. De remming van de activatie van het complementsysteem zou daarom mogelijk diabetespatiënten kunnen beschermen tegen het ontwikkelen van diabetische nefropathie, of de ontwikkeling van diabetische nefropathie kunnen vertragen.

Zoals in hoofdstuk 2 van dit proefschrift beschreven, ontwikkelen *APOC1*-tg muizen nodulaire glomerulosclerose. Deze laesies zijn echter in deze muizen pas aanwezig vanaf de leeftijd van 15 maanden. De relatief trage ontwikkeling van deze ziekte maakt dit model daarom minder geschikt om therapeutische en preventieve interventiestudies mee uit te voeren. In **hoofdstuk 6** van dit proefschrift hebben wij geprobeerd om de progressie van de ziekte in dit model te versnellen door VEGF-A te remmen. Een langdurige verlaging van VEGF-A leidt namelijk tot de ontwikkeling van nierschade, voornamelijk door de invloed van VEGF-A op glomerulaire endotheelcellen. Onze hypothese

was dat de combinatie van *APOC1*-overexpressie en VEGF-A remming zou leiden tot een versnelde progressie van de ziekte in dit model. Onze bevindingen lieten echter zien dat dit niet het geval was. *APOC1*-tg muizen met verlaagde VEGF-A levels hadden juist minder ontstekingscellen in glomeruli dan onbehandelde *APOC1*-tg muizen. Dit suggereert dat het remmen van VEGF-A een anti-inflammatoire werking zou kunnen hebben.

De centrale conclusie van het werk in dit proefschrift is dat het immuunsysteem een belangrijke rol speelt in de ontwikkeling en progressie van diabetische nefropathie. Daarom zou de focus van toekomstige studies, betreffende de behandeling en preventie van diabetische nefropathie, moeten zijn om het immuunsysteem en renale ontsteking te remmen.



Chapter 9

Curriculum vitae en Dankwoord

CURRICULUM VITAE

Pascal Bus is geboren op 25 januari 1990 in Groningen. In 2008 behaalde hij zijn VWO-diploma aan het Kamerlingh Onnes college in Groningen. Datzelfde jaar begon hij met de studie Biomedische Wetenschappen aan de Rijksuniversiteit Groningen. Tijdens zijn master Biomedische Wetenschappen heeft hij negen maanden aan de Universiteit van New South Wales in Sydney, Australië, een wetenschapsstage gelopen in het laboratorium van prof. dr. John Whitelock. Naast zijn master volgde hij het Master's Honoursprogramma van de Rijksuniversiteit Groningen. In 2013 begon hij in het Leids Universitair Medisch Centrum (LUMC) met zijn promotieonderzoek naar diabetische nefropathie onder begeleiding van dr. J.J. Baelde en prof. dr. J.A. Bruijn. De resultaten van dit onderzoek zijn beschreven in dit proefschrift. Tijdens zijn onderzoek presenteerde Pascal op verschillende congressen zijn onderzoek, onder andere op de American Society of Nephrology Kidney Week in Philadelphia (2014), San Diego (2015), Chicago (2016) en New Orleans (2017). Tijdens zijn onderzoek heeft Pascal meerdere congres- en onderzoeksbeurzen ontvangen van Stichting Preventie Diabetes. In 2018 zal Pascal zijn carrière vervolgen als postdoc onderzoeker in het laboratorium van dr. Sumant Chugh in Chicago.

DANKWOORD

Zoals een van mijn stellingen aangeeft ('No man is an Island'), heeft dit proefschrift niet tot stand kunnen komen zonder de hulp van velen. Een aantal mensen zou ik in het bijzonder willen noemen.

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