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Endothelial glycocalyx restoration by growth factors in diabetic nephropathy

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Abstract

The endothelial glycocalyx (eGlx) constitutes the first barrier to protein in all blood vessels. This is particularly noteworthy in the renal glomerulus, an ultrafiltration barrier. Leakage of protein, such as albumin, across glomerular capillaries results in albumin in the urine (albuminuria). This is a hall mark of kidney disease and can reflect loss of blood vessel integrity in microvascular beds elsewhere. We discuss evidence demonstrating that targeted damage to the glomerular eGlx results in increased glomerular albumin permeability. EGlx is lost in diabetes and experimental models demonstrate loss from glomerular endothelial cells. Vascular endothelial growth factor (VEGF)A is upregulated in early diabetes, which is associated with albuminuria. Treatment with paracrine growth factors such as VEGFC, $VEGF_{165}b$ and angiopoietin-1 can modify VEGFA signalling, rescue albumin permeability and restore glomerular eGlx in models of diabetes. Manipulation of VEGF receptor 2 signalling, or a common eGlx biosynthesis pathway by these growth factors, may protect and restore the eGlx layer. This would help to direct future therapeutics in diabetic nephropathy.

Contents

1. List of abbreviations

Ang-1	Angiopoietin-1
A disintegrin and metalloproteinase domain 17	ADAM17
Chondroitin sulphate	CS
eGLX	Endothelial glycocalyx
Endoplasmic reticulum	ER
Endothelial surface layer	ESL
GAG	Glycosaminoglycans
Glomerular endothelial cells	(GEnC)
Glomerular basement membrane	GBM
Glomerular filtration barrier	GFB
Glomerular filtration rate	GFR
Hyaluronidase	HYAL
Hyaluronic acid	HA
Hyaluronic acid synthase	HAS
Heparanase	HPSE
Heparan sulphate	HS
Hydroxyethylstarch	HES
Matrix metalloproteinase	MMP
Munich Wistar Frompter	MWF
Urine albumin creatinine ratios	uACR
Vascular endothelial growth factor	VEGF
Vascular endothelial growth factor receptor	VEGFR
Wheat germ agglutinin	WGA

2. Review overview

The focus of this review is to examine how components of the endothelial glycocalyx (eGlx) influence vascular permeability, particularly in the diabetic glomerulus, and the role they may have as a potential therapeutic target in diabetic nephropathy. First an overview of the eGlx will be given, followed by evidence demonstrating its importance in vascular permeability in health, diabetes and diabetic nephropathy. Finally, this review will present approaches that restore glomerular eGlx through common pathways, thereby highlighting therapeutic potential.

3. Endothelial glycocalyx (eGlx)

The eGlx is a complex carbohydrate meshwork, present on the luminal surface of blood vessels. It is primarily composed of glycoproteins (not a focus of this review) and proteoglycans, which have a protein core, embedded into the endothelial cell surface. Glycoprotein cores are attached to acidic oligosaccharides and terminal sialic acids, while proteoglycan cores are attached to multiple, long, unbranched glycosaminoglycan (GAG) chains [1]. The eGlx is often referred to as an extended endothelial surface layer (ESL) to incorporate the structural contribution of absorbed plasma proteins. It is a dynamic structure [2], with a high turnover rate, that is greatly influenced by surrounding factors.

Proteoglycan synthesis begins when ribosomes, bound to the endoplasmic reticulum (ER), synthesise a protein core backbone and feed it into the ER lumen. From here it is transported to the lumen of the Golgi apparatus where a serine residue of the core protein is attached to a tetrasaccharide via an O-glycosidic bond [3]. GAG chains are then extended from the non-reducing end by glycosyltransferase enzymes that alternately attach an amino and an acidic sugar [4-6]. The main core proteins found in the eGlx are transmembrane syndecans and membrane-bound glypicans [7], while the three main GAG are heparan sulphate (HS) chondroitin sulphate (CS) and hyaluronic acid (hyaluronan, HA). During polymerisation, GAG can be modified by sulfation (the addition of a sulfate group to an individual monosaccharide),

which is what gives the eGlx an overall negative charge. GAG can also be modified by de-acetylation (removal of an acetyl group from a monosaccharide) and/or epimerisation (the alteration of a monosaccharide at one stereochemical position only e.g. in the transformation of glucuronic acid to iduronic acid) [8]. GAG distribution on core proteins is highly variable. For example, endothelial syndecans-1, -2 and -4 exhibit three GAG attachment sites, which predominantly bind HS. However, syndecan-1 has two additional sites specifically for chondroitin sulphate [9]. HA is a unique GAG as it is not sulphated, is unbranched, and is not linked to a core protein. It is the largest GAG with repeating disaccharide units reaching in excess of 10^6 Da [5]. These lengths are achievable as it is not constrained by the Golgi space during chain extension. It is synthesised at the inner surface of the plasma membrane by hyaluronic acid synthases (HAS1, HAS2 and HAS 3) [10].

4. Contribution of eGlx to microvascular permeability

Vascular permeability is the movement of water and molecules across the capillary wall and was originally modelled on Starling principles [11];

$(Jv/A) = Lp[(P_c - P_i) - \sigma (\Pi_p - \Pi_i)]$

Where: Jv/A is the volume filtered per unit area, Lp is the hydraulic conductance, P_c is the capillary hydrostatic pressure, P_i is the interstitial hydrostatic pressure, σ is the osmotic reflection co-efficient, Π_p is the oncotic pressure on plasma-side of endothelial surface layer and Π_i is the oncotic pressure in the interstium. However, recently these principles have been revised. It is now recognised that there is no steady state fluid absorption in capillaries [12]; Π_p cannot draw fluid back into capillaries (although it can reduce flow from capillaries) and Π_i has no effect as a filtration force [13]. This is because the eGlx exerts an oncotic force (Π_g), caused by the plasma proteins in the ESL, that regulates filtration of water at the EnC surface [65]. There is also a protein free zone below the GAG chains of the eGlx and above the

endothelial cells, the sub eGlx space. This acts as a buffer zone to oncotic forces. Thus, Π_i is replaced by Π_g in the revised Starling's principles as follows;

$(Jv/A) = Lp[(P_c - P_i) - \sigma (\Pi_p - \Pi_g)]$ [12] Experimental models demonstrate that eGlx damage leads to vascular complications [14]. For example, in isolated coronary arterioles the movement of fluorescently labelled albumin into the surrounding bath was used to demonstrate that pronase (which removes the eGlx) increased albumin permeability 2.3-fold [15]. Pronase was also used to show that, when perfused into individually cannulated frog mesenteric vessels, eGlx depth was reduced and hydraulic conductivity (water and small solute permeability) was increased without impacting tight junctions [16]. Notably, restoration of eGlx depth in these vessels recovered hydraulic conductivity. Similarly, we demonstrated that neuraminidase removed eGlx in perfused, single cannulated, frog mesenteric vessels, resulting in increased albumin permeability [17]. Of note, in isolated perfused guinea pig hearts, eGlx degradation by heparinase I led to increased extravasation of albumin and hydroxyethylstarch (HES), yet not small solutes. The authors confirmed that under "normal" conditions there was low filtration from venules; under hypoalbuminuric conditions (i.e. no colloid perfusion) the increased (colloid free) extravasate occurred independently of eGlx; but when eGlx was damaged in the presence of colloid, more colloid rich extravasation occurred. Interestingly, extravasation was less with albumin vs HES, despite the fact that the albumin had a lower colloidal pressure than HES. It is thought that albumin seals the damaged eGlx [18], thereby restoring the ESL. Accordingly, these authors have further modified Starling's principles as follows;

$(Jv/A) = Lp[(P_c - P_i) - \sigma (\Pi_e - \Pi_g)]$

Where Π_e=colloid osmotic pressure within the ESL and Π_g= colloid osmotic pressure directly below it. [18]

Vascular leakage and oedema are important concerns post-surgery with those experiencing perioperative ischemia (e.g. due to aortic clamping or cardiopulmonary by-pass). The importance of eGlx in vascular leakage in these patients was highlighted for the first time by Rehm et al. [19]. SDC1 and HS were measured in arterial blood of patients before, during and after vascular surgery. Both SDC1 and HS shedding were increased after different operative procedures in association with conditions of reperfusion (but not due to surgery itself) [19]. Using a complementary animal model of ischemia reperfusion, the authors confirmed that increased SDC1 and HS shedding were seen in association with reduced coronary microvascular eGlx depth, by electron microscopy, in guinea pig hearts.

5. Diabetes and eGlx

The eGlx has been shown to be altered in a number of pathological situations including ischemia-reperfusion injury and inflammation [20-22], hyperglycaemia [23], in chronic kidney disease [24]. and during the progression of diabetes [25, 26]. Diabetes is a major health concern worldwide. In the UK between 2015-2016, diabetes-related drugs made up to 10.6% of prescribed drugs used in primary care and 6.4% of individuals going to their GPs aged 17yr and over were diagnosed with diabetes [27]. In general, diabetic patients suffer ill health, and there is strong correlation between diabetes and cardiovascular diseases [28, 29]. More specifically, many of those with diabetes will suffer from complications associated with microvascular damage, which include retinopathy, neuropathy and nephropathy (collectively known as microangiopathy).

5.2 Diabetes and albuminuria

Diabetic nephropathy is the main cause of end stage renal failure in the western world [30]. It is considered a relatively late complication of diabetes, progressively occurring in susceptible patients 15-25yr after the initial onset of diabetes [31, 32]. Albuminuria is correlated with the progression of diabetic nephropathy [33] and can be categorised as normoalbuminuric (less

than 30mg/day), microalbuminuric (30-300mg/day) or macroalbuminuria (300+mg/day). It is also associated with endothelial dysfunction, decline in glomerular filtration rate (GFR) and increased risk of cardiovascular disease [34, 35]. Of note, increased GFR also predisposes those with diabetes to the development of diabetic nephropathy [36]. Interestingly, even normoalbuminuria at the top end of the physiological range is an independent risk factor for cardiovascular disease in an otherwise health population [37-39].

4.2 Diabetes and eGlx dysfunction

Damage to the eGlx has been shown to occur in both Type-I and Type-II diabetes. Nieuwdorp *et.al* analysed the depth of the eGlx in patients with Type-I diabetes by imaging the erythrocyte-endothelium gap in sublingual microcirculation using side-stream darkfield imaging, before and after leukocyte passage [40]. Using this method, they demonstrated that; eGlx volume was reduced in Type-I diabetics; was exacerbated in those diabetics with microalbuminuria; and was correlated with increased blood plasma levels of HA and hyaluronidase (which cleaves HA). Indeed, in hyaluronidase knock out mice, the development of Type I diabetes-associated albuminuria was prevented [41]. EGlx volume has also been shown to be reduced in patients with Type-II diabetes in both the sublingual and retinal vascular beds. This was analysed using side-stream darkfield imaging and measuring the intravascular distribution of two fluorescent tracers of different sizes [26].

In addition to hyaluronidase, other enzymes have been implicated in GAG shedding in diabetes. For example, circulating matrix metalloproteinases (MMP) 2 and 9, known to cleave components of the eGlx [42], were significantly increased in Type I diabetic patients [43]. We suggest that TNF-α, an inflammatory mediator in the diabetic milieu, induces MMP-9 activation, leading to HS cleavage from Sydecan-4 in glomerular endothelial cells [44]. Also, vascular endothelial growth factor (VEGF)A which is upregulated in the diabetic milieu (discussed in more detail below), can induce glomerular MMP9 expression [45]. Further to

this, we have shown that, in an experimental model of albuminuria with glomerular eGlx dysfunction, MMP 2/9 inhibition restored glomerular eGlx and prevented albuminuria [46]. Another enzyme involved in GAG shedding in diabetes is heparanase (HPSE). Active HPSE degrades HS and is upregulated during diabetes in humans and in animal models [47-49]. Increased HPSE levels have also been demonstrated in the urine and plasma from Type II diabetic patients, and were found to positively correlate with increased glucose levels [48]. An increase in vascular permeability is also seen in mice that overexpress heparanase [50] and this enzyme has been further linked to the pathophysiology of childhood steroid-sensitive nephrotic syndrome [51].

Hyperglycaemia, an important mediator of the diabetic milieu in Type I and II diabetes, is thought to impact eGlx health. Nieuwdorp *et.al* showed that acute induction of hyperglycaemia in healthy individuals reduced eGlx volume within 6 hours [52]. In culture, hyperglycaemia inhibits synthesis of sulphated and non-sulphated GAG, with no demonstrable change in proteoglycan expression [53]. Together, these studies demonstrate that eGlx damage is strongly associated with the development of albuminuria in diabetes, indeed, the eGlx has been proposed as the site of initial damage leading to microalbuminuria [54]. Therefore, the eGlx may be a promising target to reduce albuminuria and protect against other microvascular complications.

6. Glomerular eGlx

Understanding how the glomerulus regulates filtration and maintains selective (restricted) protein passage is key to understanding the development and progression of renal pathologies in which this restriction is lost, such as in diabetic nephropathy. Blood is filtered across the glomerular capillary through a highly specialised filtration barrier. This multi-layered structure prevents large proteins, such as albumin, from leaving the blood whilst allowing minimally restricted movement of water and small solutes. Traditionally the glomerular filtration barrier

(GFB) is presented as a three-layer structure consisting of podocytes (epithelial cells), the glomerular basement membrane (GBM) and fenestrated glomerular endothelial cells (GEnC) [55]. However, the glomerular eGlx, highlighted in **figure 1**, has also been shown to significantly contribute towards the GFB [56]. Tissue culture studies show that GEnC synthesise and express HS [57], CS and HA [58]. CS and HA can be seen within human glomeruli in **figure 2**. In the glomerulus, HS has been identified not only in the eGlx but also in the GBM where it is one of the 4 main components along with laminin, collagen IV and nidogen. Here it is primarily found as part of the proteoglycan, agrin [59, 60]. Whether GBM HS plays a role in protein restriction is under debate [59, 61-63], although a general note for these and all Glx studies is that there is a need to discriminate between GAG of eGlx and elsewhere (i.e GBM, podocytes and mesangial cells).

6.1 EGlx and glomerular permeability

The glomerular eGlx has been suggested to restrict both solutes and fluid (not just large, charged proteins) according to a mathematical simulation of the GFB [64]. Its contribution to water permeability was confirmed experimentally when a bolus of hyaluronidase or heparinase, given *in vivo* for up to an hour, led to an increase in GFR [65]. EGlx damage in experimental models of diabetes was associated with increased glomerular hydraulic conductivity (a measure of single nephron GFR). Further, treatment that restored eGlx depth also rescued hydraulic conductivity [66]. In cultured human GEnC, targeted eGlx removal led to an increase in trans-endothelial electrical resistance, indicating increased water and solute flux [57, 58].

Various studies have highlighted the importance of eGlx in glomerular large solute (i.e. albumin) permeability. For example, glomerular eGlx removal, by hypertonic saline in rats, caused an increase in fractional albumin clearance by 12-fold [67]. Additionally, both hyaluronidase and chondroitinase individually increased the fractional clearance of albumin in isolated, perfused, mouse kidneys *ex vivo* (although not *in vivo*) [65, 68]. This was associated

with reduced glomerular eGlx depth, although measurements were indirect. However, it is worth noting that chondroitinase and hyaluronidase can digest HA and CS respectively, albeit at a slower rate [69, 70]. Hyaluronidase given over 4wk in mice reduced glomerular eGlx coverage and caused an increase in albumin passage across the GFB (but not urinary albumin creatinine ratio (uACR)) [71]. This was determined by anti-albumin immunogold staining in the GFB. We have recently shown that a low dose combination of hyaluronidase and chondroitinase, given acutely (30min) or chronically (over 2wk), reduced eGlx coverage without off-target effects [72, 73]. Under the same conditions we directly measured changes in glomerular albumin permeability, which was significantly increased following enzyme treatment. By measuring glomerular albumin permeability directly, we could bypass tubular reabsorption of albumin which impacts on the sensitivity of uACR measurements [72]. *In vitro,* low dose, targeted enzymatic removal of CS, HS, and HA on cultured GEnC (quantified by immunofluorescence) demonstrated that removal of CS and HS, but not HA, increased albumin passage across GEnC monolayers [57, 58]. These treatments demonstrate that specific removal of eGlx leads directly to a rapid and sustained increase in glomerular albumin permeability.

Other non-enzyme-based studies have also demonstrated how the eGlx contributes towards GFB function. Aged Munich-Wistar Frömter (MWF) rats with spontaneous proteinuric kidney disease have decreased eGlx and increased glomerular albumin permeability [74]. Additionally, intravenous administration and subsequent absorption of wheat germ agglutinin (WGA) lectin decreased glomerular albumin permeability in aged rats, indicating that modifications to the eGlx can improve glomerular function [74]. Genetic deletion of the linker between HS chains and perlecan (a core protein) caused mice to become proteinuric [75]. We have recently shown that aldosterone, in combination with high salt, damages glomerular eGlx *in vivo*, leading to an increased glomerular sieving coefficient of albumin [46]. This follows from interesting work by Oberleithner *et al* on salt overload and eGlx stiffness. Atomic force

microscopy studies demonstrated that salt overload increased eGlx stiffness and reduced eGlx depth in cultured endothelial cells in an aldosterone dependent manner [76], a potential mechanism for increased sodium accumulation in the tissues. These experiments highlight the importance of the eGlx in preventing excess protein passage.

7. EGlx restoration and diabetic nephropathy

The key question is; how can we restore eGlx? Significant progress has been made in directly targeting the eGlx in diabetic patients. Sulodexide, a mixture of low-molecular weight GAG (heparan (80%) and dermatan sulfate (20%)), has been used to treat microvascular complications in diabetic patients [77]. Initially, several small scale clinical studies demonstrated successful, therapeutic use of Sulodexide, showing an increase in sublingual eGlx depth (using side-stream darkfield imaging), a decrease in albuminuria (most effective in those with microalbuminuria), and slowed progression of diabetic nephropathy [26, 78]. Unfortunately, two larger randomized double-blinded placebo control studies later demonstrated that treatment with Sulodexide did not reduce albuminuria, therefore the need for a new and effective treatment remains [77, 79]. However, it should be noted that many consider the effects of Sulodexide to have been undervalued in these later studies [80-83]. Overall, these data suggest that the eGlx may be amenable to therapeutic intervention in diabetes and that targeting this structure could prove the key for treating this disease. Experimentally, restoration of the eGlx has been demonstrated to be achievable and effective in blood vessels outside of the kidney in the coronary [84], pulmonary [85] and mesenteric microcirculation [16]. We have also demonstrated that the eGlx can be restored in glomeruli [46, 66, 86] which will be key in targeting albuminuria.

8.1 Glomerular vascular endothelial growth factors (VEGF)

Podocyte-GEnC crosstalk is critical to maintain normal glomerular function. Growth factors form a major part of this communication, but during disease states, these can become

imbalanced. One example of these is the VEGF family of proteins. In humans there are five VEGF variants, VEGF-A, -B, -C, -D and placenta growth factor [87]. These proteins each have distinct biological roles and are important in blood and lymph vessel formation and vascular homeostasis. Alternative splicing of the 8 exon, 7 intron VEGF-A gene gives rise to a number of mature isoforms with different biological activities. Of these, $VEGF_{165}$ is the most commonly found variant throughout the body [88, 89]. $VEGF_{165}$ is important for GEnC fenestration formation and health via its main signalling receptor, VEGF receptor 2 (VEGFR2)[90-92]. However, transgenic overexpression of $VEGF_{165}$ can cause increased GFR in the short term, followed by loss of GFR in the long term [93]. In addition, podocyte-specific overexpression of $VEGF_{165}$ was shown to induce albuminuria, increase glomerular VEGFR2 phosphorylation and increase glomerular MMP-9 expression [45]. Additionally, $VEGF_{165}$ has also been shown to be upregulated in early diabetic nephropathy in human disease [94, 95] and in experimental models [96-99]. VEGFR2 was also shown to be upregulated in early experimental diabetic nephropathy and was associated with increased GEnC $VEGF_{165}$-VEGFR2 signalling [96]. This has been shown to cause loss of permselectivity and albuminuria [100]. Of note, $VEGF_{165}$ may also induce eGlx loss. As well as promoting glomerular MMP9 expression [45], it also promoted ADAM17 (another member of the MMP family of proteins) activation in cultured human GEnC [58]. Furthermore, $VEGF_{165}$ has been shown to increase shedding of sulphated GAG in cultured human GEnC [58]. In the following section, paracrine growth factors will be described that may counterbalance the detrimental effects of glomerular $VEGF_{165}$ to protect from albuminuria in diabetes. We suggest they may act by antagonising $VEGF_{165}$/VEGFR2 signalling to restore the glomerular eGlx. Of course, $VEGF_{165}$ signalling is just one mediator of diabetic nephropathy. As well as counterbalancing the effects of the VEGF axis, we believe the end point of these paracrine growth factors, restoring eGlx, would go some way to counteract the generic effects of the diabetic milieu.

8.2 Podocyte paracrine growth factors

i. VEGF$_{165}$b: In 2002, Bates and colleagues described a novel family of VEGFA isoforms, where the last 6 amino acids differ due to alternative splicing of exon 8, such that the terminal end of exon 8 is transcribed instead of the proximal end. These are depicted as VEGF$_{xxx}$b. For example, VEGF$_{165}$b has been shown to bind VEGFR2 with the same affinity as VEGF$_{165}$ [101]. Podocyte-specific VEGF$_{165}$b expression in mice has been shown to block the increase in glomerular hydraulic conductivity induced by VEGF$_{165}$ [102]. Reduced glomerular hydraulic conductivity has also been demonstrated in glomeruli isolated from diabetic mice, rats and humans treated *ex vivo* with VEGF$_{165}$b [66]. Further, in a mouse model of Type I diabetes, podocyte-specific expression of VEGF$_{165}$b significantly reduced albuminuria [66]. This was also achieved when a dual insult of VEGF$_{165}$ overexpression was induced alongside the induction of diabetes, supporting the hypothesis that VEGF$_{165}$b reduces albuminuria through antagonising VEGF$_{165}$. Recombinant human VEGF$_{165}$b protected against albuminuria in a Type II model of diabetes, but only when administered before the development of albuminuria. Thus, VEGF$_{165}$b appears to have an impact on water/small solute and large solute permeability in the GFB. The protective effects of VEGF$_{165}$b on glomerular hydraulic conductivity in diabetic rats was shown to be VEGFR2 dependent [66]. The activation of VEGFR2 by VEGF$_{165}$b appears to be endothelial cell-type dependent. In human umbilical vein (large vessel) endothelial cells, VEGF$_{165}$b could bind, but not phosphorylate, VEGFR2 yet in human microvascular endothelial cells it resulted in the phosphorylation of Akt [101]. Of note, overexpression of VEGF$_{165}$b in glomeruli caused increased VEGFR2 expression and phosphorylation in both healthy and diabetic mouse glomeruli [66]. This suggests that VEGF$_{165}$b could have differential VEGFR2 signalling effects in different vascular beds. Critically, VEGF$_{165}$b has also been shown to modify the eGlx. This was demonstrated in an early model of Type I diabetes in mice where human recombinant VEGF$_{165}$b administration

restored glomerular eGlx depth. This may be via delayed downstream signalling by $VEGF_{165}b$-induced activation of VEGFR2, potentially involving a role for VEGFR2/VEGFR1 heterodimer formation [103].

ii. Ang1: Angiopoietins are a family of endothelial cell growth factors, with essential roles in regulating vascular growth, development and maturation. The complex interaction between the angiopoietins and vascular endothelial growth factors determine endothelial cell behaviour, therefore they are key players in microvascular permeability, both in health and diabetic nephropathy [104-107]. The two major members of the angiopoietin family are angiopoietin-1 (Ang1) and angiopoietin-2 (Ang2) [108]. Tie2 is the main signalling receptor and binds both Ang1 and Ang2, although in most circumstances only Ang1 induces receptor phosphorylation [109-111]. Ang2 predominantly antagonises Ang1-induced phosphorylation of Tie2 [110]. Tie2 together with VEGFR2 activation is required for endothelial cell survival and vessel stabilization [92, 112, 113]. In healthy mice, Ang1 reduced the vascular leakage of 70kDa macromolecules from newly formed mouse blood vessels [114] and from mature vessels (mouse and human) following treatment with permeabilising reagents [115, 116]. Moreover, Ang1 reduced hydraulic conductivity in rat glomeruli and in continuous frog mesenteric vessels [16]. The reflection coefficient (a measure of macromolecular restriction) in continuous vessels was also increased. Critically, Ang1 increased eGlx depth in continuous vessels. All these data indicate that Ang1 decreased vascular water and solute permeability, potentially via eGlx. Therefore, we hypothesised that Ang1 could modify glomerular eGlx to restrict albuminuria. Of note, Ang-2 reduced eGlx depth in human umbilical vein endothelial cells in a heparanase dependent manner, which was blocked by Ang-1 [117]. Further, in an *in vivo* model, Ang-2 was shown to increase vascular leak which was blocked by heparanase inhibition [117]. In diabetes the balance between Ang1 and Ang-2 is lost, which also impacts on $VEGF_{165}$. Ang2 mRNA was upregulated in human diabetic glomeruli and Ang1 was significantly decreased in

a Type 1 diabetic mouse model [104]. In these mice, when Ang1 was overexpressed by podocytes, albuminuria was reduced and Tie2 phosphorylation was increased, highlighting the protective effects of Ang1 in diabetes. Of note, in the diabetic mice, glomerular VEGFA expression was increased and glomerular VEGFR2 expression was decreased, but VEGFR2 phosphorylation was increased. Notably, podocyte-specific overexpression of Ang1 significantly reduced the increased VEGFR2 phosphorylation [104]. Also, kidney cortex soluble VEGFR1 expression was increased, which can sequester VEGFA. Therefore, the effects of Ang1 may be two-pronged; Tie2 activation and $VEGF_{165}$ signalling suppression.

Our group investigated if the protective capabilities of Ang1 in diabetes may be mediated by the eGlx. First, we confirmed that Ang1 (given *ex vivo)* significantly reduced albumin permeability in glomeruli isolated from Type 1 diabetic rats [72]. We then demonstrated that recombinant Ang1, given i.v. 30min before sacrifice, significantly restored glomerular eGlx in diabetic animals, shown by quantitative electron microscopy. This suggests that Ang1 effects glomerular function through glomerular eGlx [72].

iii. VEGFC: VEGFC is most commonly associated with developmental lymphangiogenesis, where it is required for normal development of the endothelial lymphatic system, signalling through VEGFR3 [118]. It also signals through VEGFR3 to maintain the differentiated lymphatic endothelium in adults [119]. Like VEGFA, VEGFC can signal to vascular endothelial cells, increasing migration and proliferation by stimulating VEGFR2 [120]. Binding to VEGFR2 by VEGFC occurs at the same binding site as $VEGF_{165}$, as indicated by the ability of each growth factor to displace the other [120]. In the glomerulus, VEGFC is expressed by podocytes and acts on GEnC [121]. Unusually, we could not demonstrate phosphorylation of VEGFR3 by VEGFC in cultured human GEnCs, but could show phosphorylation of VEGFR2, albeit over a longer time course than VEGFA (30min v.s. 2min) [121]. We examined phosphorylation of VEGFR2 tyrosine residues by VEGFC, however we

did not find significant phosphorylation at Tyr 951, 1175 or 1214; sites involved in $VEGF_{165}$ activation and downstream signalling (**Figure 3**). This suggests that VEGFR2 phosphorylation is delayed and perhaps total phosphorylation is reduced in GEnC by VEGFC. In these cells VEGFC reduced macromolecular protein passage [121]. We suggest that this is due to the synthesis of unsulfated and sulphated GAG by VEGFC (discussed in more detail below) [122]. Using our glomerular albumin permeability assay, we demonstrated that *ex vivo* treatment of isolated mouse glomeruli with VEGFC blocked the increased permeability induced by $VEGF_{165}$. In addition, VEGFC could rescue raised glomerular albumin permeability in glomeruli isolated from Type II diabetic (and albuminuric) mice [73]. We have also demonstrated that the effects induced by VEGFC, like the other protective growth factors discussed, may be mediated via the eGlx. Glomerular albumin permeability was increased in mice administered GAG enzymes, both acutely (30min) and chronically (2wk), but VEGFC blocked this effect whilst restoring eGlx depth and/or coverage [73].

We went on to investigate the effect of VEGFC on albuminuria and eGlx *in vivo* using podocyte-specific overexpression of VEGFC. Of note, VEGFC reduced glomerular VEGFA mRNA expression and had no long term detrimental effects on glomerular function [73], in contrast to that described for podocyte-specific overexpression of VEGFA [45]. Glomerular VEGFR2/VEGFR3 heterodimer formation was significantly increased in podocyte-specific VEGFC overexpressing mice [73]. This suggests that VEGFC may antagonise $VEGF_{165}$ signalling by competitively binding VEGFR2, but may induce differential signalling through modification by VEGFR3. We confirmed that podocyte-specific overexpression of VEGFC could rescue albuminuria in a Type I diabetic mouse model, if it was expressed before albuminuria developed [73]. Increased glomerular VEGFR2 expression in diabetic mice was significantly reduced by VEGFC, supporting our hypothesis that VEGFC antagonises

VEGF$_{165}$/VEGFR2 signalling. Together, this data suggests that VEGFC protects from albuminuria through modification of VEGFR2 signalling and eGlx restoration.

8. A common pathway for therapeutic targeting of eGlx?

VEGF$_{165}$b, Ang1 and VEGFC all protect from increased glomerular VEGF$_{165}$ signalling and from albuminuria in diabetes, preferentially when administered during early development. Critically, these growth factors counterbalance VEGFA signalling, either through expression/sequestering of VEGF$_{165}$ and/or potential modification of VEGFR2 signalling. Blockade of the glomerular VEGF$_{165}$ system in disease needs to be moderate to avoid additional glomerular dysfunction [92, 123-125]. Perhaps counterbalancing by these important endogenous mediators provides the key. In addition, restoration of the glomerular eGlx by these growth factors was associated with reduced glomerular small solute (VEGF$_{165}$b and Ang1) and albumin permeability (VEGF$_{165}$b, Ang1, VEGFC). We know that VEGF$_{165}$b restores glomerular eGlx and that this leads to increased WGA lectin binding [66]. WGA binds N-acetyl glucosamine, a component of HS and HA, and N-acetyl neuraminic acid, which is a major sialic acid. This gives some clues as to the components of the VEGF$_{165}$b-restored eGlx. More extensive investigations have been carried out for Ang1 and VEGFC. Ang1 can restore/enhance eGlx in frog mesentery vessels and glomerular capillaries *in vivo* within 30min. Whether this is due to *de novo* synthesis or not has yet to be proven, but inhibition of vesicle translocation by Brefeldin A blocked the effect, indicating that restoration may come from pre-existing pools of GAG [16]. In human microvascular endothelial cells *in vitro*, Ang1 increased sulphated GAG content of cell supernatant [16]. GAG were also shown to be effected by Ang-1 in cultured human GEnC, where treatment induced *de novo* synthesis of both sulphated and un-sulphated GAG, as determined by ^{3}H glucosamine and liquid chromatography, within 1hr (Desideri et al, unpublished). These experiments suggest that rapid *de novo* synthesis *in vivo* is a plausible explanation. Using the same technique, *de novo* GAG synthesis was also seen in

GEnC in response to VEGFC after 24hr [58]. 76% of sulphated GAG synthesised by VEGFC were highly sulphated, compared to only 32% under control conditions. Additionally, using immunofluorescence, VEGFC was shown to induce HA and CS synthesis. Furthermore, VEGFC significantly increased NDST2 mRNA, a sulfotransferase, which supports observations of increased eGlx charge. As mentioned previously, these effects by VEGFC are in contrast to $VEGF_{165}$, which may promote GAG shedding [45, 58]. This leads to a complex interaction between these families of growth factors, $VEGF_{165}$/VEGFR2 signalling and eGlx modifications as summarised in **figure 4**.

In conclusion, there are similarities arsing in the mechanism of protection of these paracrine growth factors. The next step is to identify the common pathways influenced by these growth factors that promote restoration of glomerular eGlx, and those that protect from degradation. This will prove to be a powerful, multi-targeted approach to albuminuric diabetic nephropathy. In addition, eGlx restoration by the same means has the potential to treat other microvascular disorders where capillary leak to small solutes and large proteins is a key step in the progression of the disease.

9. References

1.	Haraldsson B, Nyström J, Deen WM. Properties of the glomerular barrier and mechanisms of proteinuria. Physiol Rev. 2008;88(2):451-87.
2.	Pries AR, Secomb TW, Gaehtgens P. The endothelial surface layer. Pflugers Arch. 2000;440(5):653-66.
3.	Sugahara K, Kitagawa H. Heparin and heparan sulfate biosynthesis. IUBMB life. 2002;54(4):163-75.
4.	Alberts B, Johnson A, Lewis J, Raff M, Roberts K, Walter P. Molecular Biology of the Cell. 5th ed: Garland Science; 2007. p. 1181.
5.	Laurent TC, Fraser JR. Hyaluronan. The FASEB Journal. 1992;6(7):2397-404.
6.	Harvey R, Ferrier D. Lippincott's Illustrated Reviews: Biochemistry. Wilkins LW, editor2011.
7.	Rosenberg RD, Shworak NW, Liu J, Schwartz JJ, Zhang L. Heparan sulfate proteoglycans of the cardiovascular system. Specific structures emerge but how is synthesis regulated? J Clin Invest. 1997;100(11 Suppl):S67-75.
8.	Harvey SJ. Proteoglycans Methods and Protocols Humana Press; 2012. Available from: http://download.springer.com/static/pdf/396/bok%253A978-1-61779-498-8.pdf?auth66=1406739568_80ba5ddccc001efa9117b14e737a254b&ext=.pdf.
9.	Kokenyesi R, Bernfield M. Core protein structure and sequence determine the site and presence of heparan sulfate and chondroitin sulfate on syndecan-1. J Biol Chem. 1994;269(16):12304-9.
10.	Spicer AP, Seldin MF, Olsen AS, Brown N, Wells DE, Doggett NA, et al. Chromosomal localization of the human and mouse hyaluronan synthase genes. Genomics. 1997;41(3):493-7.
11.	Starling EH. On the Absorption of Fluids from the Connective Tissue Spaces. J Physiol. 1896;19(4):312-26.
12.	Levick JR, Michel CC. Microvascular fluid exchange and the revised Starling principle. Cardiovascular research. 2010;87(2):198-210.
13.	Hu X, Adamson RH, Liu B, Curry FE, Weinbaum S. Starling forces that oppose filtration after tissue oncotic pressure is increased. Am J Physiol Heart Circ Physiol. 2000;279(4):H1724-36.
14.	Nieuwdorp M, Meuwese MC, Vink H, Hoekstra JB, Kastelein JJ, Stroes ES. The endothelial glycocalyx: a potential barrier between health and vascular disease. Curr Opin Lipidol. 2005;16(5):507-11.
15.	Huxley VH, Williams DA. Role of a glycocalyx on coronary arteriole permeability to proteins: evidence from enzyme treatments. Am J Physiol Heart Circ Physiol. 2000;278(4):H1177-85.
16.	Salmon AH, Neal CR, Sage LM, Glass CA, Harper SJ, Bates DO. Angiopoietin-1 alters microvascular permeability coefficients in vivo via modification of endothelial glycocalyx. Cardiovascular research. 2009;83(1):24-33.
17.	Betteridge KB, Arkill KP, Neal CR, Harper SJ, Foster RR, Satchell SC, et al. Sialic acids regulate microvessel permeability, revealed by novel in vivo studies of endothelial glycocalyx structure and function. J Physiol. 2017;595(15):5015-35.
18.	Jacob M, Bruegger D, Rehm M, Stoeckelhuber M, Welsch U, Conzen P, et al. The endothelial glycocalyx affords compatibility of Starling's principle and high cardiac interstitial albumin levels. Cardiovascular research. 2007;73(3):575-86.
19.	Rehm M, Bruegger D, Christ F, Conzen P, Thiel M, Jacob M, et al. Shedding of the endothelial glycocalyx in patients undergoing major vascular surgery with global and regional ischemia. Circulation. 2007;116(17):1896-906.

20.   Mulivor AW, Lipowsky HH. Inflammation- and ischemia-induced shedding of venular glycocalyx. Am J Physiol Heart Circ Physiol. 2004;286(5):H1672-80.

21.   Henry CB, Duling BR. TNF-alpha increases entry of macromolecules into luminal endothelial cell glycocalyx. Am J Physiol Heart Circ Physiol. 2000;279(6):H2815-23.

22.   Mulivor AW, Lipowsky HH. Role of glycocalyx in leukocyte-endothelial cell adhesion. Am J Physiol Heart Circ Physiol. 2002;283(4):H1282-91.

23.   Zuurbier CJ, Demirci C, Koeman A, Vink H, Ince C. Short-term hyperglycemia increases endothelial glycocalyx permeability and acutely decreases lineal density of capillaries with flowing red blood cells. J Appl Physiol. 2005;99(4):1471-6.

24.   Padberg JS, Wiesinger A, di Marco GS, Reuter S, Grabner A, Kentrup D, et al. Damage of the endothelial glycocalyx in chronic kidney disease. Atherosclerosis. 2014;234(2):335-43.

25.   Nussbaum C, Cavalcanti Fernandes Heringa A, Mormanova Z, Puchwein-Schwepcke AF, Bechtold-Dalla Pozza S, Genzel-Boroviczeny O. Early microvascular changes with loss of the glycocalyx in children with type 1 diabetes. The Journal of pediatrics. 2014;164(3):584-9.e1.

26.   Broekhuizen LN, Lemkes BA, Mooij HL, Meuwese MC, Verberne H, Holleman F, et al. Effect of sulodexide on endothelial glycocalyx and vascular permeability in patients with type 2 diabetes mellitus. Diabetologia. 2010;53(12):2646-55.

27.   Kmietowicz Z. Diabetes drugs in England cost nearly pound1bn last year. Bmj. 2016;354:i4324.

28.   Pan WH, Cedres LB, Liu K, Dyer A, Schoenberger JA, Shekelle RB, et al. Relationship of clinical diabetes and asymptomatic hyperglycemia to risk of coronary heart disease mortality in men and women. Am J Epidemiol. 1986;123(3):504-16.

29.   Kannel WB, McGee DL. Diabetes and cardiovascular risk factors: the Framingham study. Circulation. 1979;59(1):8-13.

30.   Rossing P. The changing epidemiology of diabetic microangiopathy in type 1 diabetes. Diabetologia. 2005;48(8):1439-44.

31.   Mogensen CE. The effect of blood pressure intervention on renal function in insulin-dependent diabetes. Diabete Metab. 1989;15(5 Pt 2):343-51.

32.   Mogensen CE. Microalbuminuria and hypertension with focus on type 1 and type 2 diabetes. J Intern Med. 2003;254(1):45-66.

33.   Wylie EC, Satchell SC. Diabetic nephropathy. Clinical medicine (London, England). 2012;12(5):480-2; quiz 3-5.

34.   Young BA, Maynard C, Boyko EJ. Racial differences in diabetic nephropathy, cardiovascular disease, and mortality in a national population of veterans. Diabetes Care. 2003;26(8):2392-9.

35.   Tarnow L, Rossing P, Nielsen FS, Fagerudd JA, Poirier O, Parving HH. Cardiovascular morbidity and early mortality cluster in parents of type 1 diabetic patients with diabetic nephropathy. Diabetes Care. 2000;23(1):30-3.

36.   Mogensen CE, Christensen CK. Predicting diabetic nephropathy in insulin-dependent patients. N Engl J Med. 1984;311(2):89-93.

37.   Diercks GF, van Boven AJ, Hillege JL, de Jong PE, Rouleau JL, van Gilst WH. The importance of microalbuminuria as a cardiovascular risk indicator: A review. The Canadian journal of cardiology. 2002;18(5):525-35.

38.   Matsushita K, van der Velde M, Astor BC, Woodward M, Levey AS, de Jong PE, et al. Association of estimated glomerular filtration rate and albuminuria with all-cause and cardiovascular mortality in general population cohorts: a collaborative meta-analysis. Lancet. 2010;375(9731):2073-81.

39.   Smink PA, Lambers Heerspink HJ, Gansevoort RT, de Jong PE, Hillege HL, Bakker SJ, et al. Albuminuria, estimated GFR, traditional risk factors, and incident cardiovascular disease: the PREVEND (Prevention of Renal and Vascular Endstage Disease) study. Am J Kidney Dis. 2012;60(5):804-11.

40.   Nieuwdorp M, Mooij HL, Kroon J, Atasever B, Spaan JAE, Ince C, et al. Endothelial glycocalyx damage coincides with microalbuminuria in type 1 diabetes. Diabetes. 2006;55(4):1127-32.

41.   Dogne S, Rath G, Jouret F, Caron N, Dessy C, Flamion B. Hyaluronidase 1 Deficiency Preserves Endothelial Function and Glycocalyx Integrity in Early Streptozotocin-Induced Diabetes. Diabetes. 2016;65(9):2742-53.

42.   Lipowsky HH. Protease Activity and the Role of the Endothelial Glycocalyx in Inflammation. Drug discovery today Disease models. 2011;8(1):57-62.

43.   Gharagozlian S, Svennevig K, Bangstad HJ, Winberg JO, Kolset SO. Matrix metalloproteinases in subjects with type 1 diabetes. BMC clinical pathology. 2009;9:7.

44.   Ramnath R, Foster RR, Qiu Y, Cope G, Butler MJ, Salmon AH, et al. Matrix metalloproteinase 9-mediated shedding of syndecan 4 in response to tumor necrosis factor alpha: a contributor to endothelial cell glycocalyx dysfunction. FASEB J. 2014;28(11):4686-99.

45.   Veron D, Reidy KJ, Bertuccio C, Teichman J, Villegas G, Jimenez J, et al. Overexpression of VEGF-A in podocytes of adult mice causes glomerular disease. Kidney Int. 2010;77(11):989-99.

46.   Butler M, Ramnath R, Kadoya H, Onions K, Ferguson J, Ogier A, et al. Aldosterone induces albuminuria via matrix metalloproteinase dependent glomerular endothelial glycocalyx damage. Kidney International. 2018;In press.

47.   van den Hoven MJ, Rops AL, Bakker MA, Aten J, Rutjes N, Roestenberg P, et al. Increased expression of heparanase in overt diabetic nephropathy. Kidney Int. 2006;70(12):2100-8.

48.   Shafat I, Ilan N, Zoabi S, Vlodavsky I, Nakhoul F. Heparanase levels are elevated in the urine and plasma of type 2 diabetes patients and associate with blood glucose levels. PLoS One. 2011;6(2):e17312.

49.   Wang F, Wan A, Rodrigues B. The function of heparanase in diabetes and its complications. Canadian journal of diabetes. 2013;37(5):332-8.

50.   Zcharia E, Metzger S, Chajek-shaul T, Aingoran H, Elkin M, Friedmann Y, et al. Transgenic expression of mammalian heparanase uncovers physiological functions of heparan sulfate in tissue morphogenesis, vascularization, and feeding behavior. The FASEB Journal. 2004;18(2):252-63.

51.   Holt RCL, Webb NJA, Ralph S, Davies J, Short CD, Brenchley PEC. Heparanase activity is dysregulated in children with steroid-sensitive nephrotic syndrome. Kidney Int. 2005;67(1):122-9.

52.   Nieuwdorp M, van Haeften TW, Gouverneur MC, Mooij HL, van Lieshout MH, Levi M, et al. Loss of endothelial glycocalyx during acute hyperglycemia coincides with endothelial dysfunction and coagulation activation in vivo. Diabetes. 2006;55(2):480-6.

53.   Singh A, Friden V, Dasgupta I, Foster RR, Welsh GI, Tooke JE, et al. High glucose causes dysfunction of the human glomerular endothelial glycocalyx. Am J Physiol Renal Physiol. 2011;300(1):F40-8.

54.   Salmon AH, Satchell SC. Endothelial glycocalyx dysfunction in disease: albuminuria and increased microvascular permeability. J Pathol. 2012;226(4):562-74.

55.   Rhodin J. Electron microscopy of the glomerular capillary wall. Exp Cell Res. 1955;8(3):572-4.

56. Salmon AH, Neal CR, Harper SJ. New aspects of glomerular filtration barrier structure and function: five layers (at least) not three. Curr Opin Nephrol Hypertens. 2009;18(3):197-205.

57. Singh A, Satchell SC, Neal CR, McKenzie EA, Tooke JE, Mathieson PW. Glomerular endothelial glycocalyx constitutes a barrier to protein permeability. J Am Soc Nephrol. 2007;18(11):2885-93.

58. Foster RR, Armstrong L, Baker S, Wong DW, Wylie EC, Ramnath R, et al. Glycosaminoglycan regulation by VEGFA and VEGFC of the glomerular microvascular endothelial cell glycocalyx in vitro. Am J Pathol. 2013;183(2):604-16.

59. Harvey SJ, Jarad G, Cunningham J, Rops AL, van der Vlag J, Berden JH, et al. Disruption of glomerular basement membrane charge through podocyte-specific mutation of agrin does not alter glomerular permselectivity. The American journal of pathology. 2007;171(1):139-52.

60. Genovese F, Manresa AA, Leeming DJ, Karsdal MA, Boor P. The extracellular matrix in the kidney: a source of novel non-invasive biomarkers of kidney fibrosis? Fibrogenesis & Tissue Repair. 2014;7:4-.

61. Chen S, Wassenhove-McCarthy DJ, Yamaguchi Y, Holzman LB, van Kuppevelt TH, Jenniskens GJ, et al. Loss of heparan sulfate glycosaminoglycan assembly in podocytes does not lead to proteinuria. Kidney Int. 2008;74(3):289-99.

62. Aoki S, Saito-Hakoda A, Yoshikawa T, Shimizu K, Kisu K, Suzuki S, et al. The reduction of heparan sulphate in the glomerular basement membrane does not augment urinary albumin excretion. Nephrol Dial Transplant. 2018;33(1):26-33.

63. Borza DB. Glomerular basement membrane heparan sulfate in health and disease: A regulator of local complement activation. Matrix biology : journal of the International Society for Matrix Biology. 2017;57-58:299-310.

64. Punyaratabandhu N, Kongoup P, Dechadilok P, Katavetin P, Triampo W. Transport of Spherical Particles Through Fibrous Media and a Row of Parallel Cylinders: Applications to Glomerular Filtration. Journal of biomechanical engineering. 2017;139(12).

65. Jeansson M, Haraldsson B. Glomerular size and charge selectivity in the mouse after exposure to glucosaminoglycan-degrading enzymes. J Am Soc Nephrol. 2003;14(7):1756-65.

66. Oltean S, Qiu Y, Ferguson JK, Stevens M, Neal C, Russell A, et al. Vascular Endothelial Growth Factor-A165b Is Protective and Restores Endothelial Glycocalyx in Diabetic Nephropathy. J Am Soc Nephrol. 2015;26(8):1889-904.

67. Friden V, Oveland E, Tenstad O, Ebefors K, Nystrom J, Nilsson UA, et al. The glomerular endothelial cell coat is essential for glomerular filtration. Kidney Int. 2011;79(12):1322-30.

68. Jeansson M, Haraldsson B. Morphological and functional evidence for an important role of the endothelial cell glycocalyx in the glomerular barrier. Am J Physiol Renal Physiol. 2006;290(1):F111-6.

69. Mio K, Carrette O, Maibach HI, Stern R. Evidence that the serum inhibitor of hyaluronidase may be a member of the inter-alpha-inhibitor family. J Biol Chem. 2000;275(42):32413-21.

70. Hamai A, Hashimoto N, Mochizuki H, Kato F, Makiguchi Y, Horie K, et al. Two distinct chondroitin sulfate ABC lyases. An endoeliminase yielding tetrasaccharides and an exoeliminase preferentially acting on oligosaccharides. J Biol Chem. 1997;272(14):9123-30.

71. Dane MJ, van den Berg BM, Avramut MC, Faas FG, van der Vlag J, Rops AL, et al. Glomerular endothelial surface layer acts as a barrier against albumin filtration. Am J Pathol. 2013;182(5):1532-40.

72.   Desideri S, Onions KL, Qiu Y, Ramnath RD, Butler MJ, Neal CR, et al. A novel assay provides sensitive measurement of physiologically relevant changes in albumin permeability in isolated human and rodent glomeruli. Kidney Int. 2018;93(5):1086-97.

73.   Onions KL, Gamez M, Buckner NR, Baker SL, Betteridge KB, Desideri S, et al. VEGFC Reduces Glomerular Albumin Permeability and Protects Against Alterations in VEGF Receptor Expression in Diabetic Nephropathy. Diabetes. 2019;68(1):172-87.

74.   Salmon AH, Ferguson JK, Burford JL, Gevorgyan H, Nakano D, Harper SJ, et al. Loss of the endothelial glycocalyx links albuminuria and vascular dysfunction. J Am Soc Nephrol. 2012;23(8):1339-50.

75.   Morita H, Yoshimura A, Inui K, Ideura T, Watanabe H, Wang L, et al. Heparan sulfate of perlecan is involved in glomerular filtration. J Am Soc Nephrol. 2005;16(6):1703-10.

76.   Oberleithner H, Peters W, Kusche-Vihrog K, Korte S, Schillers H, Kliche K, et al. Salt overload damages the glycocalyx sodium barrier of vascular endothelium. Pflugers Arch. 2011;462(4):519-28.

77.   Lewis EJ, Lewis JB, Greene T, Hunsicker LG, Berl T, Pohl MA, et al. Sulodexide for kidney protection in type 2 diabetes patients with microalbuminuria: a randomized controlled trial. Am J Kidney Dis. 2011;58(5):729-36.

78.   Abaterusso C, Gambaro G. The role of glycosaminoglycans and sulodexide in the treatment of diabetic nephropathy. Treatments in endocrinology. 2006;5(4):211-22.

79.   Packham DK, Wolfe R, Reutens AT, Berl T, Heerspink HL, Rohde R, et al. Sulodexide fails to demonstrate renoprotection in overt type 2 diabetic nephropathy. J Am Soc Nephrol. 2012;23(1):123-30.

80.   Dedov I, Shestakova M, Vorontzov A, Palazzini E. A randomized, controlled study of sulodexide therapy for the treatment of diabetic nephropathy. Nephrol Dial Transplant. 1997;12(11):2295-300.

81.   Rossini M, Naito T, Yang H, Freeman M, Donnert E, Ma LJ, et al. Sulodexide ameliorates early but not late kidney disease in models of radiation nephropathy and diabetic nephropathy. Nephrol Dial Transplant. 2010;25(6):1803-10.

82.   Coccheri S. Game not over for sulodexide. Am J Kidney Dis. 2012;59(3):467.

83.   Gambaro G. Discounting the efficacy of sulodexide in diabetic nephropathy is premature. Am J Kidney Dis. 2012;60(1):169-70.

84.   Chappell D, Jacob M, Hofmann-Kiefer K, Bruegger D, Rehm M, Conzen P, et al. Hydrocortisone preserves the vascular barrier by protecting the endothelial glycocalyx. Anesthesiology. 2007;107(5):776-84.

85.   Giantsos-Adams K, Lopez-Quintero V, Kopeckova P, Kopecek J, Tarbell JM, Dull R. Study of the therapeutic benefit of cationic copolymer administration to vascular endothelium under mechanical stress. Biomaterials. 2011;32(1):288-94.

86.   Desideri S, Onions KL, Qiu Y, Ramnath RD, Butler MJ, Neal CR, et al. A novel assay provides sensitive measurement of physiologically relevant changes in albumin permeability in isolated human and rodent glomeruli. Kidney Int. 2018.

87.   Olsson AK, Dimberg A, Kreuger J, Claesson-Welsh L. VEGF receptor signalling - in control of vascular function. Nature reviews Molecular cell biology. 2006;7(5):359-71.

88.   Kretzler M, Schroppel B, Merkle M, Huber S, Mundel P, Horster M, et al. Detection of multiple vascular endothelial growth factor splice isoforms in single glomerular podocytes. Kidney Int Suppl. 1998;67:S159-61.

89.   Ferrara N, Gerber HP, LeCouter J. The biology of VEGF and its receptors. Nature medicine. 2003;9(6):669-76.

90.   Ostendorf T, Kunter U, Eitner F, Loos A, Regele H, Kerjaschki D, et al. VEGF(165) mediates glomerular endothelial repair. J Clin Invest. 1999;104(7):913-23.

91.   Satchell SC, Tasman CH, Singh A, Ni L, Geelen J, von Ruhland CJ, et al. Conditionally immortalized human glomerular endothelial cells expressing fenestrations in response to VEGF. Kidney Int. 2006;69(9):1633-40.

92.   Sison K, Eremina V, Baelde H, Min W, Hirashima M, Fantus IG, et al. Glomerular structure and function require paracrine, not autocrine, VEGF-VEGFR-2 signaling. J Am Soc Nephrol. 2010;21(10):1691-701.

93.   Liu E, Morimoto M, Kitajima S, Koike T, Yu Y, Shiiki H, et al. Increased expression of vascular endothelial growth factor in kidney leads to progressive impairment of glomerular functions. J Am Soc Nephrol. 2007;18(7):2094-104.

94.   Bailey E, Bottomley MJ, Westwell S, Pringle JH, Furness PN, Feehally J, et al. Vascular endothelial growth factor mRNA expression in minimal change, membranous, and diabetic nephropathy demonstrated by non-isotopic in situ hybridisation. J Clin Pathol. 1999;52(10):735-8.

95.   Hovind P, Tarnow L, Oestergaard PB, Parving HH. Elevated vascular endothelial growth factor in type 1 diabetic patients with diabetic nephropathy. Kidney Int Suppl. 2000;75:S56-61.

96.   Cooper ME, Vranes D, Youssef S, Stacker SA, Cox AJ, Rizkalla B, et al. Increased renal expression of vascular endothelial growth factor (VEGF) and its receptor VEGFR-2 in experimental diabetes. Diabetes. 1999;48(11):2229-39.

97.   Sung SH, Ziyadeh FN, Wang A, Pyagay PE, Kanwar YS, Chen S. Blockade of vascular endothelial growth factor signaling ameliorates diabetic albuminuria in mice. J Am Soc Nephrol. 2006;17(11):3093-104.

98.   Chou E, Suzuma I, Way KJ, Opland D, Clermont AC, Naruse K, et al. Decreased cardiac expression of vascular endothelial growth factor and its receptors in insulin-resistant and diabetic States: a possible explanation for impaired collateral formation in cardiac tissue. Circulation. 2002;105(3):373-9.

99.   Tsuchida K, Makita Z, Yamagishi S, Atsumi T, Miyoshi H, Obara S, et al. Suppression of transforming growth factor beta and vascular endothelial growth factor in diabetic nephropathy in rats by a novel advanced glycation end product inhibitor, OPB-9195. Diabetologia. 1999;42(5):579-88.

100.   de Vriese AS, Tilton RG, Elger M, Stephan CC, Kriz W, Lameire NH. Antibodies against vascular endothelial growth factor improve early renal dysfunction in experimental diabetes. J Am Soc Nephrol. 2001;12(5):993-1000.

101.   Woolard J, Wang WY, Bevan HS, Qiu Y, Morbidelli L, Pritchard-Jones RO, et al. VEGF165b, an inhibitory vascular endothelial growth factor splice variant: mechanism of action, in vivo effect on angiogenesis and endogenous protein expression. Cancer research. 2004;64(21):7822-35.

102.   Oltean S, Neal CR, Mavrou A, Patel P, Ahad T, Alsop C, et al. VEGF165b overexpression restores normal glomerular water permeability in VEGF164-overexpressing adult mice. Am J Physiol Renal Physiol. 2012;303(7):F1026-36.

103.   Cebe Suarez S, Pieren M, Cariolato L, Arn S, Hoffmann U, Bogucki A, et al. A VEGF-A splice variant defective for heparan sulfate and neuropilin-1 binding shows attenuated signaling through VEGFR-2. Cell Mol Life Sci. 2006;63(17):2067-77.

104.   Dessapt-Baradez C, Woolf AS, White KE, Pan J, Huang JL, Hayward AA, et al. Targeted glomerular angiopoietin-1 therapy for early diabetic kidney disease. J Am Soc Nephrol. 2014;25(1):33-42.

105.   Satchell SC, Mathieson PW. Angiopoietins: microvascular modulators with potential roles in glomerular pathophysiology. J Nephrol. 2003;16(2):168-78.

106.   Satchell SC, Anderson KL, Mathieson PW. Angiopoietin 1 and vascular endothelial growth factor modulate human glomerular endothelial cell barrier properties. J Am Soc Nephrol. 2004;15(3):566-74.

107.   Woolf AS, Gnudi L, Long DA. Roles of angiopoietins in kidney development and disease. J Am Soc Nephrol. 2009;20(2):239-44.

108.   Koh GY, Kim I, Kwak HJ, Yun MJ, Leem JC. Biomedical significance of endothelial cell specific growth factor, angiopoietin. Experimental & molecular medicine. 2002;34(1):1-11.

109.   Davis S, Aldrich TH, Jones PF, Acheson A, Compton DL, Jain V, et al. Isolation of angiopoietin-1, a ligand for the TIE2 receptor, by secretion-trap expression cloning. Cell. 1996;87(7):1161-9.

110.   Maisonpierre PC, Suri C, Jones PF, Bartunkova S, Wiegand SJ, Radziejewski C, et al. Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis. Science. 1997;277(5322):55-60.

111.   Tsigkos S, Koutsilieris M, Papapetropoulos A. Angiopoietins in angiogenesis and beyond. Expert Opin Investig Drugs. 2003;12(6):933-41.

112.   Satchell SC, Harper SJ, Tooke JE, Kerjaschki D, Saleem MA, Mathieson PW. Human podocytes express angiopoietin 1, a potential regulator of glomerular vascular endothelial growth factor. J Am Soc Nephrol. 2002;13(2):544-50.

113.   Potente M, Gerhardt H, Carmeliet P. Basic and therapeutic aspects of angiogenesis. Cell. 2011;146(6):873-87.

114.   Thurston G, Suri C, Smith K, McClain J, Sato TN, Yancopoulos GD, et al. Leakage-resistant blood vessels in mice transgenically overexpressing angiopoietin-1. Science. 1999;286(5449):2511-4.

115.   Thurston G, Rudge JS, Ioffe E, Zhou H, Ross L, Croll SD, et al. Angiopoietin-1 protects the adult vasculature against plasma leakage. Nature medicine. 2000;6(4):460-3.

116.   Svedas E, Islam KB, Nisell H, Kublickiene KR. Vascular endothelial growth factor induced functional and morphologic signs of endothelial dysfunction in isolated arteries from normal pregnant women. Am J Obstet Gynecol. 2003;188(1):168-76.

117.   Lukasz A, Hillgruber C, Oberleithner H, Kusche-Vihrog K, Pavenstadt H, Rovas A, et al. Endothelial glycocalyx breakdown is mediated by angiopoietin-2. Cardiovascular research. 2017;113(6):671-80.

118.   Karkkainen MJ, Haiko P, Sainio K, Partanen J, Taipale J, Petrova TV, et al. Vascular endothelial growth factor C is required for sprouting of the first lymphatic vessels from embryonic veins. Nat Immunol. 2004;5(1):74-80.

119.   Joukov V, Pajusola K, Kaipainen A, Chilov D, Lahtinen I, Kukk E, et al. A novel vascular endothelial growth factor, VEGF-C, is a ligand for the Flt4 (VEGFR-3) and KDR (VEGFR-2) receptor tyrosine kinases. Embo j. 1996;15(2):290-98.

120.   Joukov V, Sorsa T, Kumar V, Jeltsch M, Claesson-Welsh L, Cao Y, et al. Proteolytic processing regulates receptor specificity and activity of VEGF-C. Embo J. 1997;16(13):3898-911.

121.   Foster RR, Slater SC, Seckley J, Kerjaschki D, Bates DO, Mathieson PW, et al. Vascular endothelial growth factor-C, a potential paracrine regulator of glomerular permeability, increases glomerular endothelial cell monolayer integrity and intracellular calcium. Am J Pathol. 2008;173(4):938-48.

122.   Foster RR, Armstrong L, Baker S, Wong DWL, Wylie EC, Ramnath R, et al. Glycosaminoglycan Regulation by VEGFA and VEGFC of the Glomerular Microvascular Endothelial Cell Glycocalyx in Vitro. The American Journal of Pathology. 2013;183(2):604-16.

123.   Zhu X, Wu S, Dahut WL, Parikh CR. Risks of proteinuria and hypertension with bevacizumab, an antibody against vascular endothelial growth factor: systematic review and meta-analysis. Am J Kidney Dis. 2007;49(2):186-93.

124.   Eremina V, Sood M, Haigh J, Nagy A, Lajoie G, Ferrara N, et al. Glomerular-specific alterations of VEGF-A expression lead to distinct congenital and acquired renal diseases. J Clin Invest. 2003;111(5):707-16.

125.   Eremina V, Jefferson JA, Kowalewska J, Hochster H, Haas M, Weisstuch J, et al. VEGF inhibition and renal thrombotic microangiopathy. N Engl J Med. 2008;358(11):1129-36.

10. Figure legends

Figure 1. Transmission electron micrograph of the glomerular filtration barrier showing endothelial glycocalyx. Representative image from an FVB strain mouse, anaesthetised and perfusion fixed with Glutaraldehyde containing the cation, Alcian blue. This enabled visualisation of the electron dense, negatively charged glycocalyx on GEnCs. Of note, podocytes are also coated in glycocalyx.

Figure 2. Hyaluronic acid and chondriotin sulphate expression in human glomeruli. Fresh frozen sectioned human glomeruli were treated with 250mg/ml HYAL (**B**), 100mU/ml chondroitinase (**D**), to show specificity, or left untreated (**A** and **C**). They were then fixed and immunostained with biotinylated hyaluronic acid binding protein (HABP) in conjunction with strepavidin-AF488(green) and anti-PECAM-1(red) (**A** and **B**), or anti-CS in conjunction with anti-mouse IgM AF488 (green) anti-PECAM-1(red) (**C** and **D**), and counterstained with DAPI (blue). Confocal Z stacks are shown. Architecture is not optimal in fresh frozen tissue and capillary loops are not apparent. Arrows indicate co-localisation of GAG with GEnCs, suggesting eGlx.

Figure 3. VEGFC does not induce VEGFR2 phosphorlyation at tyrosine (ty) sites 1175, 1214 or 951 in cultured glomerular endothelial cells (GEnC). GEnC were stimulated with 1nM VEGFA (VA), 10nM VEGFC (VC) or vehicle (Veh) for 2 min (**A**) or 30min (**B**). Cells were lysed and samples subjected to Western blotting for specific VEGFR2 tyrosine residues ty1175, ty1214 or ty951 and total VEGFR2. Representative blots are shown. Densitometry summary data is shown in **C**. One-way ANOVA, Bonferroni post hoc tests indicated. *=p<0.05, ***=p<0.001, n=3-6.

Figure 4. The effect of protective paracrine growth factor signalling on restoration of eGlx in the glomerular filtration barrier. $VEGF_{165}$ podocyte overexpression in early diabetic nephropathy leads to enhanced glomerular endothelial VEGFR2 activation, a damaged eGlx

29

and increased albuminuria (A). The overexpression of paracrine growth factors $VEGF_{165}b$, Ang1 and VEGFC can impact the detrimental signalling of $VEGF_{165}$ in a number of ways; podocyte $VEGF_{165}$ expression or availability may be reduced; VEGFR2 signalling may be reduced/altered; enzymes associated with the synthesis, modification or shedding of GAG may be rebalanced such that the eGlx layer is restored and albuminuria is reduced.
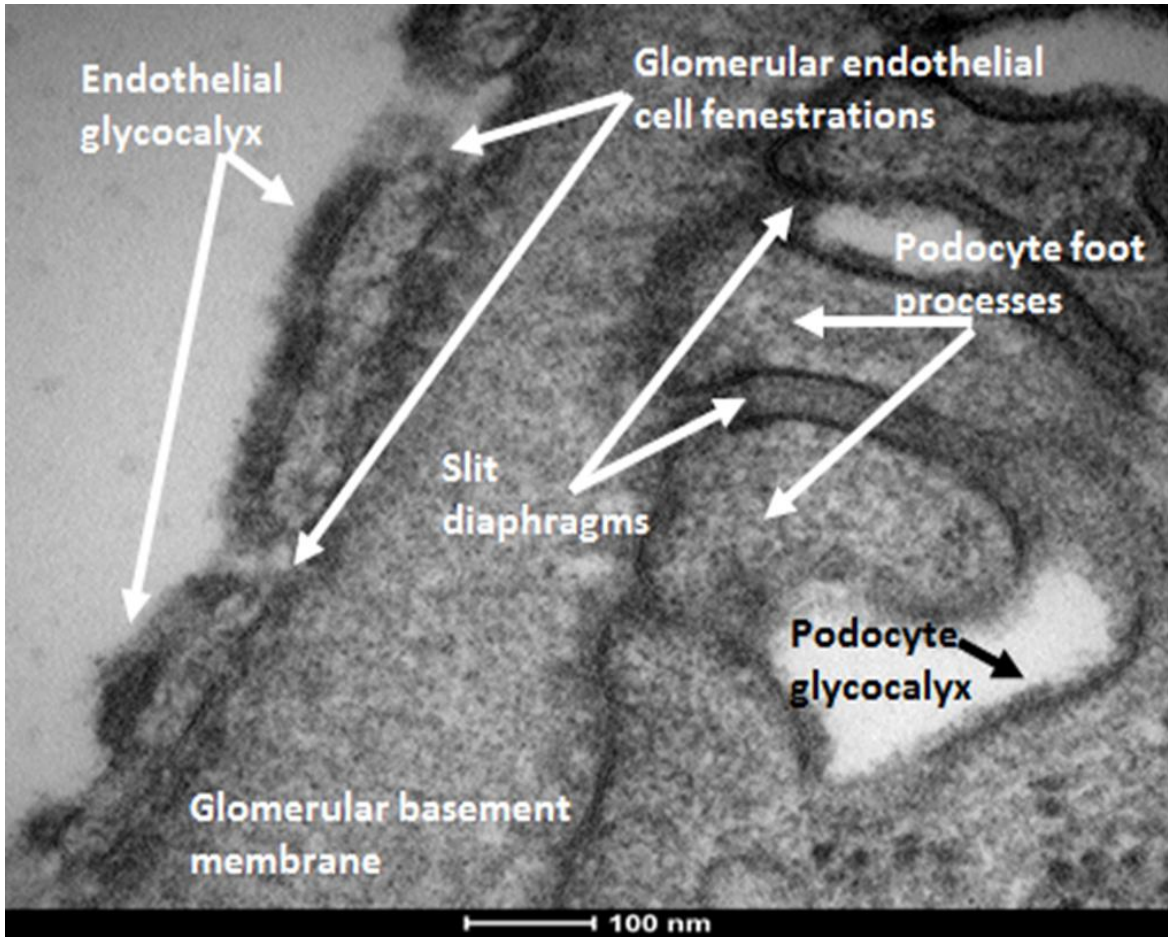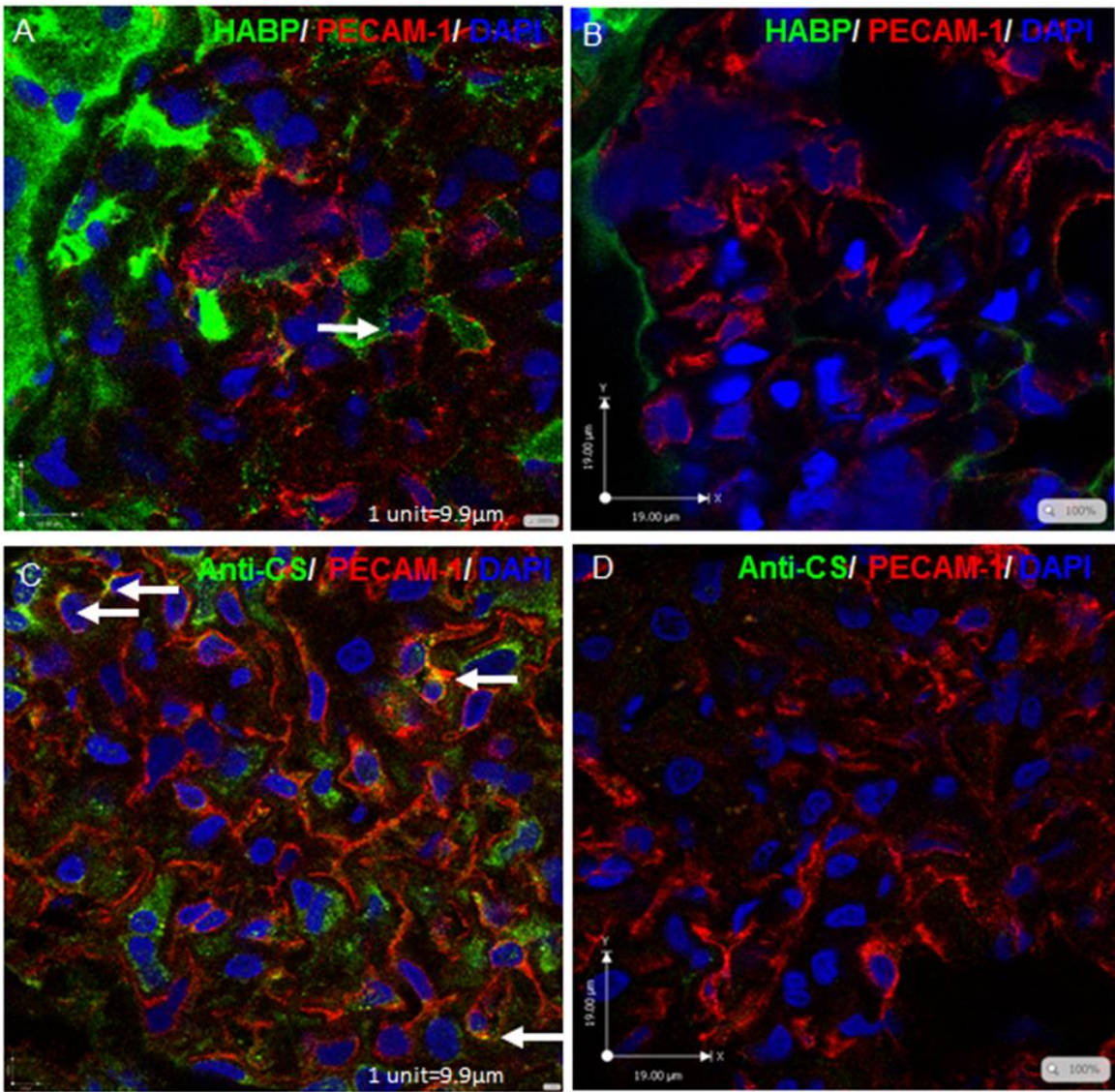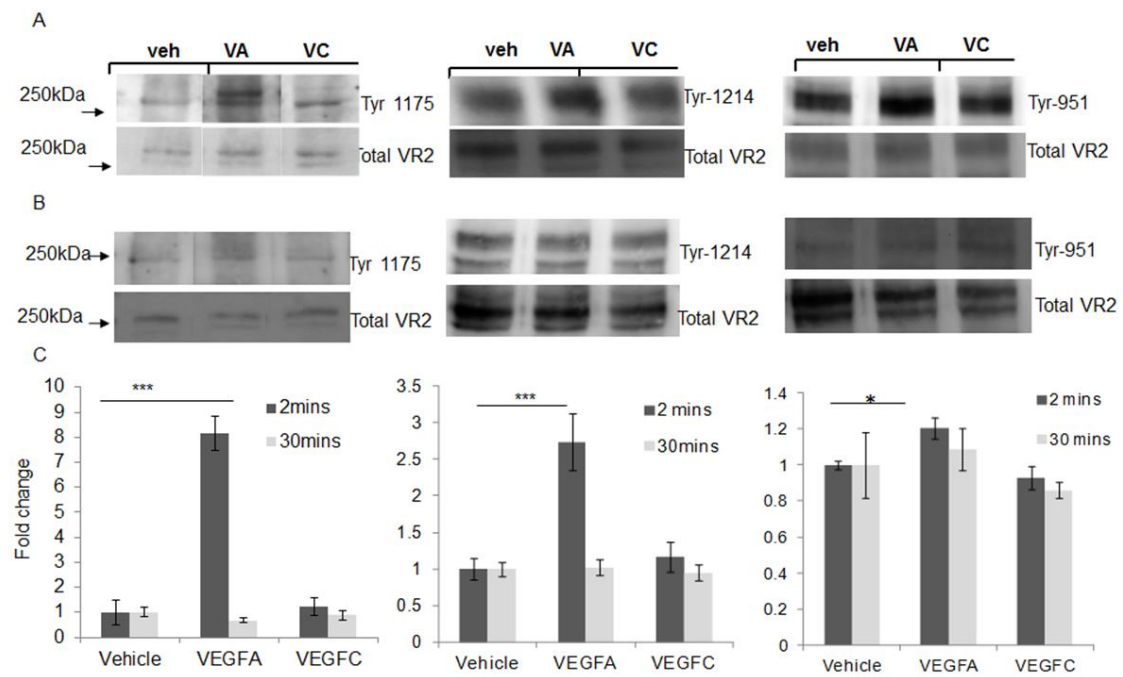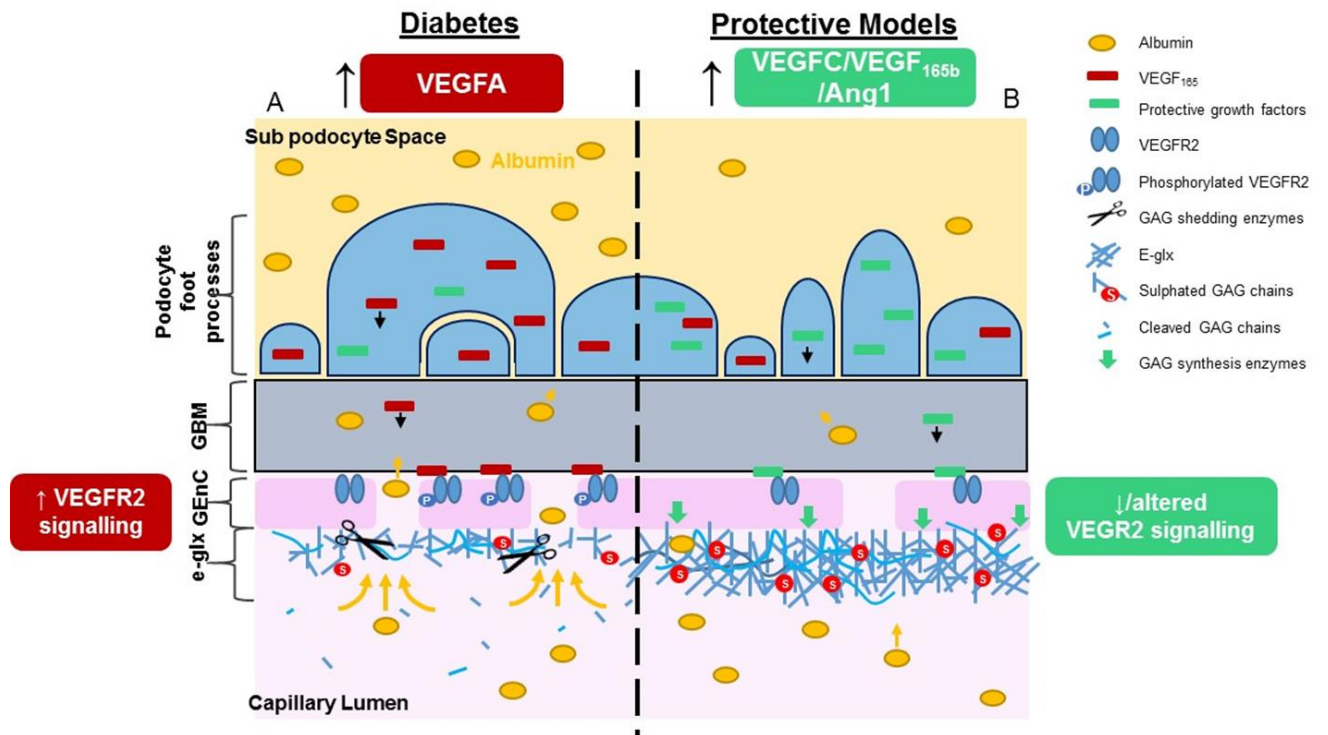
Figure 1.

Figure 2.

Figure 3.

Figure 4.