Endothelial glycocalyx hyaluronan biosynthesis and function in microcirculation

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CHAPTER 1

General Introduction & Outline of the thesis

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Endothelial glycocalyx hyaluronan: regulation and role in prevention of diabetic complications

The endothelium has emerged as the key regulator of vascular homeostasis and integrity. As a barrier between blood flow and organs, It is optimally placed and is able to respond to physical and chemical signals by production of a wide range of factors that regulate vascular tone, cellular adhesion, thromboresistance, smooth muscle cell proliferation and vessel wall inflammation [1].

The endothelial cell is surrounded by a negative-charged gel like layer, the glycocalyx, which serves as a barrier between blood and vessel wall. This membrane bound part of the endothelial glycocalyx consists of proteoglycans, glycosaminoglycans (GAGs), glycoproteins and glycolipids. GAGs are the main contributors to the endothelial glycocalyx structure and function, of which, heparan sulfate (HS) and hyaluronan (HA) constitute up to 90% [2-4]. Plasma proteins such as albumin, orosomucoid, antithrombin III, extracellular superoxide dismutase, lipases, growth factors and chemokines associate with the glycocalyx [4], thus constituting a very bio- active surface layer. Its thickness ranges from 0.2-0.5 μm to 2-3 μm in small arteries and 4.5 μm in carotid arteries [4]. Previous studies, especially when specific approaches were applied to stabilize anionic carbohydrate structures to prevent loss and or collapse of these structures, gave evidence for a thick endothelial surface layer throughout the whole vascular tree [5]. (figure 1) Injection of hyaluronidase, heparinase and chondroitinase into rat mesenteric postcapillary venules reduced glycocalyx thickness by 26.1%, 43.3% and 34.1% respectively, and 89.7% with a mixture of all three enzymes analyzed by intravital microscopy [6].

The endothelial glycocalyx is critically involved in vascular integrity and homeostasis, where it regulates endothelial cell mechanotransduction, vascular permeability, coagulation and inflammation [4, 7]. The binding capacity of glycocalyx, and in particular its HS component, to various growth factors and chemokines regulates endothelial activation state and cross communication with neighboring cells [4, 8]. In a physiological state, glycocalyx synthesis and degradation are dynamically regulated to maintain and adapt endothelial function. During chronic disease conditions, such as diabetes, atherosclerosis, hypertension and sepsis, the endothelial glycocalyx may lose its structure or become compositionally modified. Both modification and degradation of the endothelial glycocalyx can further result in endothelial dysfunction, vascular inflammation, coagulation and transendothelial protein leakage and thus contribute to the development of (micro)vascular disease [4, 9, 10].

Endothelial cell surface hyaluronan (HA) appears to be key in many of the glycocalyx functions. In the current thesis we will specifically discuss how this particular endothelial glycocalyx component is regulated and how it plays a role in regulation of vessel function and in disease conditions such as diabetes and tumor angiogenesis.

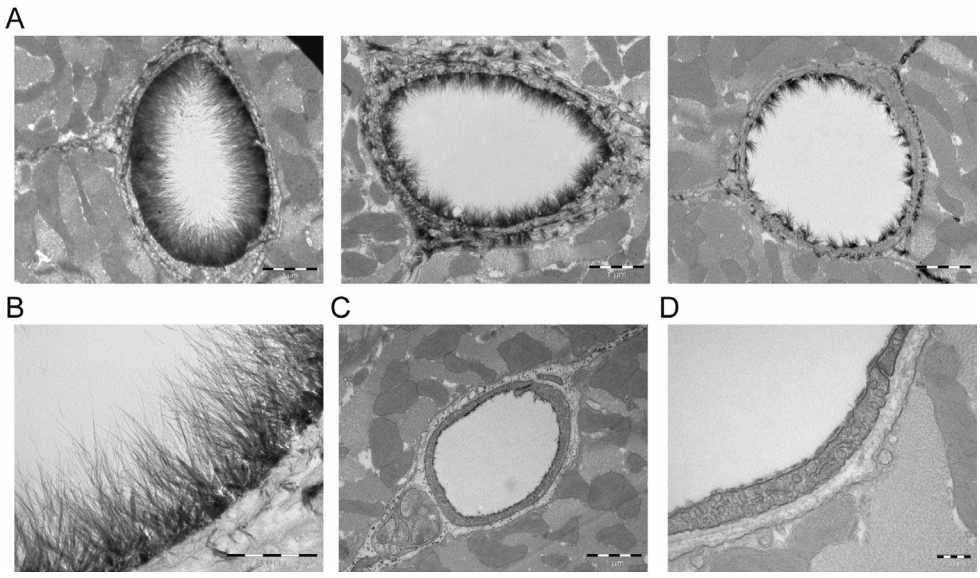

Figure 1. Removing endothelial glycocalyx hyaluronan by hyaluronidase treatment leads to loss of the endothelial glycocalyx structure. A. Upon perfusion staining of rat left ventricular vasculature with Alcian blue 8GX, capillary endothelial cell surfaces are stained with a thick layer varying in dimension as shown in the transmission electron microscopic overviews (bar = 1 μm). B, Detail of normal capillary glycocalyx (bar = 0.5 μm). C. Degrading hyaluronan from the endothelial surface reveal a residual staining with loss of the hair-like structures resulting in a different appearance and smaller dimension (bar = 1 μm). D, Detail of remaining capillary glycocalyx after hyaluronidase treatment (bar = 20 nm). The experiments setting and staining protocol were published previously by BM van den Berg, et al. [50]

Hyaluronan biosynthesis

HA is a linear polysaccharide that is composed of repeating units of glucuronic acid (GlcA) and *N*-acetyl-glucosamine (GlcNAc) linked together through glycosidic bonds [11] (Figure 2). In contrast to synthesis of the other GAGs, which takes place in the Golgi apparatus, HA is synthesized at the inner plasma membrane and subsequently secreted into the extracellular space by membrane protruding hyaluronan synthases (HAS1, HAS2 and HAS3). Following its synthesis, HA interacts with specific surface proteins (hyaladherins) such as CD44, or is assembled into the pericellular extracellular matrix [9, 12]. In mammalian cells, the three

HAS isoenzymes differ in distribution, functional properties and respond to different stimuli [11, 13, 14]. Among the three HASs, HAS1 expression is the lowest in healthy cells [15], it requires higher substrate concentrations [16] and production of HA is slowest [16]. Upon stimulation, HAS3 can produce large amounts of HA but of a lower molecular weight [11]. HAS2 is the most widespread isoform which is also correlated to HA distribution [13]. HAS2 knockout mice die early in gestation due to major defects in cardiovascular development, suggesting that HA may function as molecular platform for vascular signaling [17]. All three HAS' have been shown to produce extracellular HA, while HAS1 also produces intracellular HA [15].

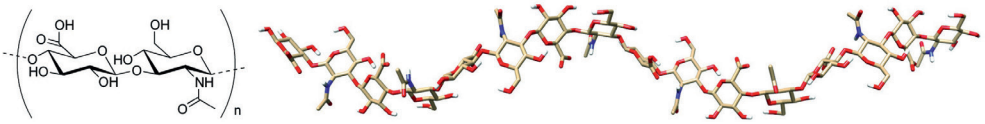

Figure 2. Structure model of hyaluronan. Chemical and 3D ribbon structure of the disaccharide repeats of glucuronic acid (GlcA) and *N*-acetyl-glucosamine (GlcNAc). In the 3D ribbon structure the carbon backbone (brown), oxygen (red), hydrogen (white) and nitrogen (blue) are depicted.

The HA biosynthesis rate in vitro is 10-30 monosaccharides/s for recombinant streptococcal HAS [18]. In optimal conditions (pH 6.5-10.5), purified streptococcal HAS could polymerize HA in at a rate of ~240 monosaccharides/s [19]. This very high synthesis rate raises the point that the cytosolic availability of uridine diphospho-glucuronic acid (UDP-GlcA) and uridine diphosphate *N*-acetylglucosamine (UDP-GlcNAc), the two substrates for HA synthesis, could be critical for HA synthesis [20-23]. Due to the high affinity of transporters located on the Golgi membrane for sugar nucleotides, UDP-sugars are quickly pumped into the Golgi to keep levels at saturation in the Golgi but at the same time may result in low cytosolic availability of UDP-GlcA and UDP-GlcNAc levels for HA biosynthesis. Furthermore, cytosolic UDP-GlcA concentration is also lower than UDP-GlcNAc [16], which makes it a potential rate-limiting factor for HA production by HAS. In agreement, overexpression of UGDH induces HA production without changing the synthesis of the other GAGs [21], further underscoring the dependency of glycocalyx HA synthesis upon the substrate UDP-GlcA. UDP-GlcNAc, the other component of the polysaccharide HA is more abundant in the cytosol. Besides acting as a substrate for HA synthesis, it also directly regulates HAS2 protein stability and mRNA expression through O-GlcNAcylation [24, 25].

The synthesis rate of HA also depends upon the activity of the HAS2 enzyme. HAS2 protein has a very rapid turnover with a half-life of 17 min in the absence of O-GlcNAcylated serine 221 while O-

GlcNAcylation of serine 221 on HAS2, regulated by UDP-GlcNAc, greatly increases its stability at the membrane [24]. This allows for a substantial increase in HA production. Other factors are also involved in the modulation of *HAS2* gene expression, HAS2 activity and thus HA synthesis (Figure 3). HAS2 forms dimers or oligomers to form the pores in the membrane, necessary for protruding the newly synthesized HA. This pore formation is regulated by ubiquitination [26]. For example, mutation of the ubiquitin site on lysine 190 of HAS2 leads to inactivation of its enzymatic activity [26]. HAS2 is the sole GAG producing enzyme that can be regulated by the main energy sensor, AMP activated protein kinase (AMPK), where phosphorylation of threonine 110 of HAS2 by AMPK inhibits HAS2 enzymatic activity [27]. The AMPK mediated inhibition of HA production probably serves cellular energy homeostasis as HA production is a high energy consuming process given the very high synthesis rate: the biosynthesis of UDP-GlcA and UDP-GlcNAc require 1 ATP and 2 ATP respectively [20]. The subsequent translocation of one HA disaccharide unit across the membrane costs energy equivalent to 1 ATP [28].

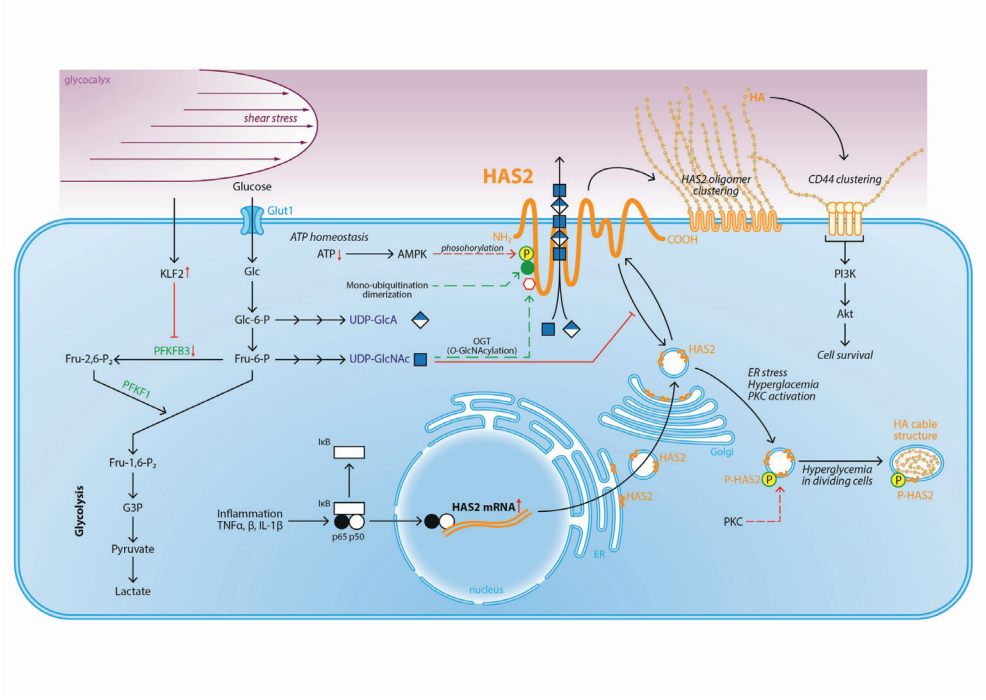

Figure 3. Schematic overview of hyaluronan biosynthesis regulation. Hyaluronan synthase 2 (Has2) is the main enzyme involved in endothelial homeostasis and HA production. The availability of UDP-GlcA and UDP-GlcNAc determine HA synthesis by HAS2. UDP-GlcNAc formation also leads to O-GlcNAcylation of

serine 221 on HAS2 protein, which greatly increases HAS2 stability on the membrane and reduces its endocytosis. Ubiquitination of HAS2 protein activates its enzymatic activity through forming HAS2 dimers or oligomers. As an ATP homeostasis sensor, AMP activated protein kinase (AMPK) inhibits HAS2 activity by regulating the phosphorylation of threonine 110 of HAS2. Upon endoplasmic reticulum (ER) stress triggered by hyperglycemia induced PKC pathway in dividing cells or proinflammatory cytokines, HAS2 can be activated on the ER membrane to produce cable-like HA structure embedded in aggresomes intracellularly. Proinflammatory cytokines such as TNFα, TNFβ and IL1β can induce HAS2 expression through NFκB pathway activation. Normal HA increases CD44 clustering and promotes cell survival.

Hyaluronan degradation

In the vasculature, HA is mainly incorporated into the glycocalyx and extracellular matrix, while the plasma level of HA is low in healthy people, due to the rapid removal of HA from the circulation by liver and kidney [29]. Degradation of HA is very efficient, with a half-life of 2-6 min and a total normal turnover of 10-100 mg/day in the blood of adult human [30]. This process is greatly dependent on the activity of hyaluronidases, which are a family of enzymes that can degrade HA into HA fragments by hydrolyzing the disaccharides at hexosaminidic β (1–4) linkages. Six hyaluronidases have been identified in man: HYAL1, HYAL2, HYAL3, HYAL4, SPAM1 (PH-20) and HYAL6P [31], of which HYAL1 and HYAL2 are the predominant isoforms in most tissues. HYAL2 is at the cell surface and anchored to the plasma membrane by a glycosylphosphatidylinositol (GPI) link. It can cleave high molecular weight HA (HMW-HA) to a product of approximately 20 kDa (≈ 50 disaccharide units) [7, 32]. These fragments functionally enhance inflammatory and angiogenic signaling [33, 34]. HA fragments, bound to CD44, e.g. promote endothelial cell proliferation and migration [33], and stimulate monocyte activation in a TLR4 dependent manner [34]. The HYAL2 generated HA fragments are hypothesized to subsequently become internalized and delivered to endosomes and lysosomes, where intracellular HYAL1 degrades the 20-kDa fragments to small HA oligosaccharides [32]. Interestingly, it is has been proposed that HYAL1 can also be taken up from the circulation and subsequently become activated in the endosome, as it requires a low pH (3-4) to be functional [7]. In support for this model of HA degradation, HYAL2 deficient mice show a thicker endothelial glycocalyx than control mice [35]. While no human HYAL2 deficiency has been reported, knockdown of HYAL2 in mice showed extremely high HA plasma levels, underpinning the role of HYAL2 in HA homeostasis [35, 36]. HYAL1-deficient mice likewise display a thicker glycocalyx and are also protected from endothelial dysfunction in early diabetes mellitus [37], underscoring the relevance of this enzyme system in

regulating glycocalyx function. Injection of active bovine testes HYAL, which is active at a higher pH, in the circulation of rats also greatly reduces the endothelial HA surface presence and damage the endothelial glycocalyx structure (Figure 1). Finally, a rare case of genetic HYAL1 deficiency in turn also showed a dramatic elevation of plasma HA [38].

Cell migration inducing hyaluronidase 1 (CEMIP) and cell migration inducing hyaluronidase 2 (CEMIP2) are the most recent identified HA binding proteins exhibiting HA degrading activity [39, 40]. CEMIP, which is present in various organs [41], is a secreted protein that contains a N-terminal signal sequence [42],and requires participation of the clathrin-coated pit pathway to degrade HA [39]. CEMIP2, also known as transmembrane protein 2 (TMEM2), is expressed on the cell surface as a transmembrane structure. It can specifically degrade HMW-HA into ~5 kDa fragments at pH 6-7 in a contact-dependent manner. CEMIP2 is thought to be involved in the initial step of HA catabolism before internalization and degradation in the lysosome. [40, 42]

Except for enzymatic degradation by hyaluronidase, HA can also be directly chemically degraded. Especially reactive oxygen species (ROS), derived from superoxide anion radicals (O_2^-) and nitrogen monoxide (•NO), including hydrogen peroxide, peroxynitrite, and hypochlorous acid have been shown to cause direct depolymerization of HA [43,44,45,46].

Hyaluronan function in the endothelial glycocalyx

As a main component of the endothelial glycocalyx, HA contributes to its structure and gel-like properties. The nonsulfated HA is not covalently linked to any core protein, unlike e.g. HS, but instead it deeply penetrates the glycocalyx, attached to endothelial membrane bound proteins, such as CD44 [4]. Various techniques have been used to visualize the polysaccharide composition of the endothelial glycocalyx [3, 47]. Recently, using super-resolution optical microscopy, HA was shown as long molecules that form a hexagonal network covering the endothelial luminal surface, while HS is a shorter molecule

perpendicular to the cell surface [48]. This HA network is critical to the integrity and function of the endothelial glycocalyx. Permeation of the luminal capillary glycocalyx is by and large determined by its HA component. Treatment of the endothelium with hyaluronidase leads to loss of the capillary filtration barrier to plasma proteins and results in edema formation and proteinuria [49,6,50]. Furthermore, the presence of HA at the endothelial surface is required for endothelial mechanosensing and maintenance of endothelial quiescence [51, 52]. The endothelial glycocalyx has long been recognized as a molecular

scaffold critical for growth factor binding, generation of chemokine gradients and surface-receptor organization [8]. While this research has been mostly focused on the HS component, where the negatively charged sulfate groups and iduronic acid (IdoA) residues in the HS chain are critical for presentation and concentration of protein ligands [53], more data is becoming available that shows a role for HA as well in modulation of growth factor and chemokine gradients at the endothelial surface. For example, binding of tumor necrosis factor (TNF) α induced protein 6 (TSG-6) to HA inhibits chemokine-stimulated trans-endothelial migration of neutrophils via a direct interaction between TSG-6 and the HA-binding site of CXCL8 [54]. HA and HA fragments can also interact with the CD44 and the receptor for HA-mediated motility (RHAMM) to co-regulate endothelial activation. CD44 is a classical transmembrane receptor that is present on various cell types and is regulated by inflammation [55]. For example, T lymphocytes use CD44 to bind to HA and start engaging with the endothelium [55]. The endothelium itself can also express CD44 which then stimulates angiogenic signaling events in the endothelium [56, 57]. RHAMM functions on the one hand as an intracellular receptor where it associates with microtubules in the cell and is involved in endothelial cell migration [58]. It can, however, also be exported to the extracellular surface during cytokine exposure where it binds both HA and CD44, and further induces endothelial cell activation and angiogenesis [59]. Finally, glycocalyx components, including HA, have also been shown to regulate plasma membrane shape, increasing membrane extensions that can further serve communication between cells and the extracellular matrix [60].

Endothelial hyaluronan in diabetes

Endothelial cells play an essential role in glucose and insulin delivery from blood to organs. Impaired insulin signaling in the endothelial cells, with reduction of insulin receptor substrate 2 (Irs2) expression and insulin-induced eNOS phosphorylation, reduces insulin-induced glucose uptake by the skeletal muscle via decreased capillary recruitment and decreased interstitial insulin concentrations in the skeletal muscle [61]. Insulin rapidly increases glycocalyx accessibility for circulating blood in muscle, and this is associated with an increased blood volume in individual capillaries [62]. Hyaluronidase treatment of the glycocalyx abolishes the effects of insulin on capillary blood volume and impairs insulin-mediated glucose disposal [62,63]. These studies suggest that endothelial HA in the glycocalyx is related to insulin homeostasis.

Endothelial dysfunction is not only associated with insulin resistance but also one of the major causes of diabetic vascular complications including macro- and microangiopathies. However, the role of endothelial HA in the development of diabetic vascular complications is still not clear.

In type 1 diabetic patients, using glycocalyx permeable and impermeable tracers and orthogonal polarization spectral microscopy, systemic glycocalyx volume was reported to be decreased while microvascular sublingual glycocalyx properties were affected allowing more red blood cells to penetrate this layer [64]. Furthermore, plasma HA and HYAL1 levels were also elevated [64, 65]. Similarly, these changes have also been reported for type 2 diabetic patients, in which also retinal glycocalyx dimensions were reduced and the transcapillary escape rate of albumin was increased [66].

Regulation of endothelial glycocalyx hyaluronan in diabetes.

As diabetes is associated with altered cellular glucobiosynthesis and redox state [67], it may perhaps not come as a surprise that endothelial HA homeostasis, which is so closely interconnected with these processes becomes dysregulated as well. Impaired biosynthesis secondary to reprioritization of glucose metabolism away from the HA glucobiosynthetic pathways appears, however, a less likely explanation for the loss of endothelial glycocalyx HA. During hyperglycemia, excess glucose uptake activates the metabolic hexosamine pathway resulting in increased production of UDP-GlcNAc [68]. Increased UDP-GlcNAc concentrations enable, as discussed above, the increase of both HAS2 gene expression and enzyme stability through O-GlcNAcylation, which should result in elevated HA production. It is on the other hand still not clear how the UDP-GlcA pool, which is regarded the rate-limiting factor for HA biosynthesis, changes in diabetes. Interestingly, UDP-GlcA also functions as a substrate for the glucuronidation reaction to decrease the intracellular lipid and fatty acid toxicity [69, 70], which could limit its availability for HA synthesis in diabetic conditions.

ROS production has been well established as a cause of endothelial dysfunction in diabetes [71]. Under diabetic conditions, endothelial cells produces large amounts of ROS both in cytosol and mitochondria, mainly caused by activation of the PKC, polyol and hexosamine pathways as well as reduction of the pentose phosphate pathway (PPP) flux [72]. Besides changing cellular metabolic pathways, ROS induced excess formation of advanced glycation end products (AGEs), which cause photo-induced HA degradation [73]. Proinflammatory cytokines, such as TNFα, TNFβ and IL1β, in diabetes also can induce HAS2 expression through NFκB pathway activation [74]. Furthermore, diabetes-induced endothelial activation, via induced PKC activation, has been shown to induce the formation of intracellular HA-cable like structures that are secreted as HA aggregates and have been shown to trigger monocyte adhesion [75, 76].

Upon endothelial cell activation, platelet derived HYAL2 degrades HA from the surface of endothelial cells into fragments, capable of further inducing immune responses by monocytes [77]. HYAL2 has been shown

to induce glycocalyx impairment in cultured endothelial cells under low shear stress [78]. Both ROS and pro-inflammatory cytokines have been reported to induce HYAL2 expression in epithelial cells [79, 80]. How endothelial HYAL2 activity changes in response to diabetes has not been investigated thus far. HYAL1 is found to be elevated in the blood of type 1 diabetes, both in human and animal models, as well as in type 2 diabetic patients [37, 64, 66, 81], which is correlated with the plasma HA content [65]. Pro-inflammatory cytokines, such as TNFα and IL1β, have been shown to increase HYAL1 expression and activity in multiple cell types via activation of NFκB pathway [79, 82, 83]. Moreover, early growth response 1 (EGR1), a master transcription factor that coordinates endothelial activation and which has been reported to be upregulated in diabetic endothelium, binds to the promoter in HYAL-1-expressing cells [83], including endothelial cells [84, 85]. Is therefore likely that the altered redox state and ensuing microinflammation are directly related to the induction of hyaluronidases and loss of the glycocalyx. As discussed before, circulating plasma HYAL1 can possibly be endocytosed and activated by endothelial cells [7], which may contribute to the generalized glycocalyx dysfunction induced by diabetes. In addition, in concert with HYAL2, CEMIP2 could also play a role in endothelial surface HA loss although its regulation in diabetes is still unknown [42].

Endothelial hyaluronan as a pharmacological target

Preserving and restoring the endothelial glycocalyx HA could be a potent drug target to prevent diabetic organ complications. Druggable targets may include the biosynthesis of HA as well its degradation through hyaluronidases.

Increased vascular endothelial growth factor (VEGF) A levels have been demonstrated in the development of diabetic retinopathy and early diabetic nephropathy. By counteracting the VEGFA effect, VEGFC treatment reduces the development of early diabetic nephropathy and protects against albuminuria in an endothelial glycocalyx dependent manner by increasing glomerular endothelial HA synthesis [86, 87]. Interestingly as a hypoglycemic drug, metformin improves the endothelial glycocalyx in db/db mice [88]. It is also shown to protect endothelial function in diet-induced obese mice by inhibition of ER stress through activating AMPK [89]. The metformin regulated ER stress inhibition and AMPK activation could be postulated to also have a beneficial effect on endothelial HA biosynthesis.

Activation of hyaluronidases is one of the main causes of HA shedding in diabetes. Inhibition of HYAL1 activity has also been considered as another strategy to prevent the endothelial glycocalyx damage in diabetes. HYAL1 deficiency dramatically increases HA incorporation into the endothelial glycocalyx and

results in a thicker glycocalyx layer in 4 week STZ-induced diabetic mice [37]. It also prevents diabetes induced endothelial dysfunction and glomerular barrier dysfunction [37]. HYAL1 deficiency also potentially decrease the local accumulation HA fragments in lesion area of diabetes [37]. In this aspect, HYAL1 inhibitors could be developed and used as a therapeutic approach to prevent the early stage of vascular complication in diabetes. However, complete HYAL1 deficiency causes pathologic storage of mucopolysaccharide in lysosomes of histiocytes and fibroblasts, named as mucopolysaccharidosis IX, in both human and mouse [38, 90], although a gene dose dependency of this severe phenotype has been described as well [38]. HYAL2 deficient mice show increased markers of endothelial damage and microvascular fibrin deposition, which induces thrombotic microangiopathy with hemolytic anemia [35]. These phenotypes point to the complexity of hyaluronidase inhibition, particularly with respect to the therapeutic window. Sulodexide, a mix of HS and dermatan sulfate supplied as GAG precursor, increases the endothelial glycocalyx and also reduces the plasma HYAL1 activity in type 2 diabetic patients [66]. However, it failed to demonstrate renoprotection in overt type 2 diabetic nephropathy patients [91].

The use of antioxidants has been shown to effectively increase the endothelial glycocalyx and preserve endothelial function upon stimulation in human and animal models [92-94]. Infusion of the antioxidant *N*-acetylcysteine could prevent the acute hyperglycemia induced reduction of endothelial HA and increase the endothelial glycocalyx volume in healthy people [92].

Summary and open questions

Endothelial HA plays a critical role in glycocalyx integrity and endothelial homeostasis. In physiological circumstances, the balance between HA biosynthesis and catabolism is in a dynamic equilibrium where glycocalyx functions are maintained. The presence of HA in the glycocalyx is a prerequisite for (micro)vascular stability both through its mechanosensitive properties as well as through its role as a matrix for vascular stability factors. In diabetes, the metabolic changes in endothelial cells lead to increased HA degradation, potentially increased intracellular HA-cable structures and loss of glycocalyx function (Figure 4).

Despite these results, how exactly endothelial cells regulate their surface HA expression, with processes involved, still needs to be further explored. In addition, what role can endothelial HA play in relation to interaction with growth factors, stabilizing factors, necessary for endothelial integrity and vascular function.

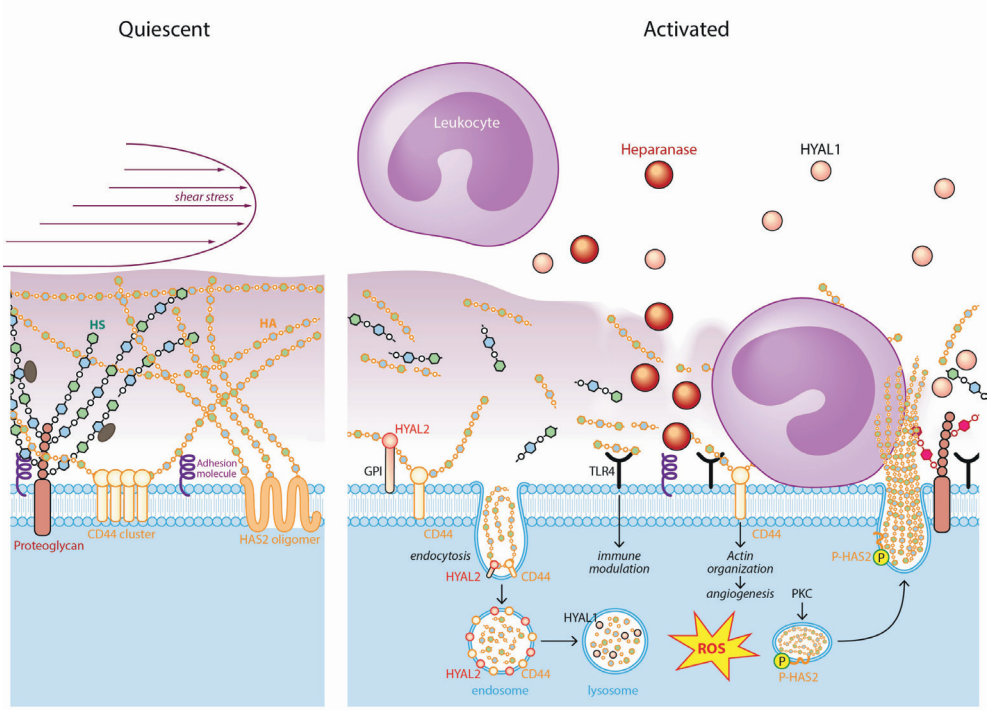

Figure 4. Schematic overview of hyaluronan metabolism disorder results in the endothelial glycocalyx dysfunction in diabetes. In a physiological state (left panel), the dynamic metabolism of HA regulated by laminar shear stress keeps the endothelial glycocalyx integrity and protects against endothelial dysfunction. Long polymeric hyaluronan is interwoven with proteoglycan-bound polysaccharides such as heparan sulfate. Direct access to the cell membrane for circulating factors and cells is shielded, and specific binding domains in the polysaccharides create gradients of growth factors and chemokines that together with the shear-sensing properties of this layer determine endothelial cell behavior. In diabetes (right panel), the glycocalyx layer is degraded by heparanase and hyaluronidases. HA biosynthesis in activated endothelial cells but now leads to increased intracellularly HA-cabled structures. Together with increased HYAL2 activity this could lead to excess HA degradation and accumulation of longer HA fragments extracellularly. These can also be further degraded into small HA oligosaccharides by HYAL1 in lysosomes. These fragments can increase inflammation and angiogenesis through association with TLR4 and CD44 respectively. In addition, the released HA aggresomes on the membrane attract monocytes adhesion. This generalized HA metabolism dysregulation leads to the endothelial glycocalyx damage and vascular destabilization, which can contribute to the develop of vascular complications in diabetes.

Outline of this thesis

In this thesis, we address the role of endothelial HA/glycocalyx in vascular function in health and disease. In **Chapter 2**, we focus on the roles of endothelial HA in vascular integrity in different organs, especially kidney. This chapter presents new insights how the endothelial HA is critical in preservation of vascular and glomerular stability using a new inducible endothelium specific *HAS2* deletion mouse model. We also investigate the link with vessel destabilization such as can be observed in diabetes.

Chapter 3 continues with a same endothelial HA deletion mouse model further studying the role of endothelial HA in microvascular perfusion and adaptation to ischemic insults. We use vascular ischemia after a single ligation of the common femoral artery, and we put forward a possible mechanistic explanation of failed angiogenic therapy in diabetic patients with critical limb ischemia.

Loss of endothelial HA and very high expression of HA (formation of HA cable structures and LMW-HA fragments) are both detrimental for endothelial integrity (see above) . It is therefore key that HA synthesis is regulated in a very tight way. In **Chapter 4**, we study the regulation of HA synthesis under physiological adaptation to shear. We show that endothelial HA biosynthesis is closely linked to glucose metabolism, and in particular the glycolytic flux This new physiological concept is not only relevant to our fundamental understanding of vascular biology in general, but is also important for the field of cardiovascular diseases (diabetic nephropathy, retinopathy, atherosclerosis) and cancer biology where disturbances in shear regulation of endothelial cells have been demonstrated.

In **Chapter 5**, we finally explored the metabolic regulation of endothelial HA biosynthesis in a known hyperglycolytic melanoma liver metastasis model, and its effects on endothelial integrity. In this chapter, we show a direct link between glucose flux, glycocalyx synthesis and vessel stability in tumor endothelium, and further suggest that restoration of endothelial glycocalyx function by manipulating the endothelial glycolysis rate could be a target to achieve vessel normalization.

Chapter 6 provides a summary and discussion of the observations in this thesis, including future perspectives.

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