The glycocalyx: a diagnostic and therapeutic target in cardiometabolic diseases

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CHAPTER 1

General Introduction

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Diabetes mellitus is a chronic metabolic disorder with a complex pathogenesis. Patients with diabetes are characterized by hyperglycemia, obesity, hypertension, hypercholesterolemia and endothelial dysfunction. In the long term, type 2 diabetes mellitus (T2DM) can lead to various microvascular and macrovascular complications that are responsible for the high morbidity and mortality associated with this disease. Intensive management of glucose and lipid concentrations as well as blood pressure control are used to minimize the risk of developing vascular complications. However, current therapies are not sufficient enough to stop vascular disease progression.

Micro- and macrovascular complications in diabetes have been pathophysiologically linked with endothelial dysfunction [1] and one of the first signs of vascular damage results from endothelial dysfunction. The metabolic milieu of T2DM, including hyperglycemia, hypercholesterolemia and low grade inflammation affects endothelial cells and their glycocalyx. This glycocalyx is a negatively charged dynamic mesh of glycoproteins, glycolipids, glycosaminoglycans (GAG) and proteoglycans surrounding the cells and is the first barrier between the vascular wall and flowing blood. The endothelial glycocalyx exerts various important functions to maintain vascular homeostasis; It acts as a mechanosensor for shear stress induced release of nitric oxide [2], plays an important role in regulation of the inflammatory- and thrombotic response of the endothelium and acts as a permeability barrier for circulating blood components. The main glycosaminoglycans (GAGs) represented in the endothelial glycocalyx are heparan sulphate (HS) and hyaluronan, accounting for up to 90% of the total GAGs. Other less abundant GAGs are chondroitin sulphate, keratan sulphate and dermatan sulphate. The glycocalyx is highly interactive with circulating cytokines, proteins, growth hormones and other blood components. Under normal conditions, there is a dynamic balance between biosynthesis and shedding of glycocalyx constituents. However, the endothelial glycocalyx is very susceptible for stressors in pathophysiological conditions such as diabetes [3, 4].

Metabolic alterations in the diabetic environment, with production of toxic metabolites (advanced glycation end products, reactive oxygen species, free fatty acids and inflammatory cytokines), together with hemodynamic changes, provides a milieu for sustained activation of the endothelium. Such activation represents the switch from a quiescent phenotype towards a pro-inflammatory pro-thrombotic phenotype, which together with expression of chemokines and adhesion molecules, results in increased interactions with leukocytes and platelets [5]. Furthermore, upregulation of glycocalyx degrading enzymes such as heparanase-1 (HPSE-1), hyaluronidase-1 (HYAL-1), monocyte chemoattractant protein-1 (MCP-1) and matrix metalloproteinases (MMP) impairs the endothelial glycocalyx layer. These glycocalyx degrading enzymes play an important role in the pathophysiol-

ogy of diabetic vascular complications, as genetic deletion of these enzymes in diabetic mouse models prevented the development of endothelial dysfunction and albuminuria [6, 7]. Hyaluronidase-1 (HYAL-1) cleaves hyaluronan, a major glycosaminoglycan of the glycocalyx and increased plasma hyaluronan levels and HYAL-1 activity have been found in patients with diabetes [3, 4]. MCP-1 is a potent chemokine that regulates the renal influx of macrophages and activation of tissue resident macrophages. HPSE-1 is a beta-glucuronidase capable of cleaving heparan sulphate in the endothelial glycocalyx. It is secreted as the inactive precursor pro-heparanase and activation requires cleavage of its pro enzyme by proteases such as cathepsin-L [8, 9]. The heparan sulphate fractions cleaved by HPSE-1 can in turn serve as toll-like receptor ligands inducing inflammatory cytokines secretion by macrophages [10]. In patients with diabetes, HPSE-1 and MCP-1 have been found in renal tissue and urine, correlating with the degree of albuminuria and kidney function [11-17]. Prolonged exposure to the diabetic environment results in chronic endothelial activation, endothelial dysfunction and eventually structural vascular damage affecting the brain, retina, peripheral nerves and kidneys.

The renal glomerulus contains a specialized microvascular bed, as it excretes waste products from the plasma toward the urinary tract collection system via the glomerular filtration barrier (GFB). The glomerular filtration barrier is composed of the fenestrated endothelium which are covered with a dense glycocalyx layer [18], the glomerular basement membrane and podocyte foot processes with their slit diaphragms. The glomerular glycocalyx contributes significantly to the perm-selective filtration properties of the GFB [19, 20]. Altering the composition of the glomerular glycocalyx via enzymatic removal of the glycocalyx or indirect alterations results in albuminuria in several experimental models [18]. Albuminuria has therefore been implicated as indicator of glomerular glycocalyx damage in the context of direct glomerular injury or glomerular involvement in generalized vascular dysfunction [21, 22]. In diabetic nephropathy, albuminuria is considered the first hallmark of diabetic kidney disease. However, the glycocalyx is present throughout all capillary beds, preventing protein filtration across the endothelium. Loss of the glycocalyx not only drives the development of albuminuria but also contributes to cardiovascular diseases. In addition, albuminuria may not only be a sign of kidney disease, but is an expression of systemic dysfunction of the vascular endothelium. This is seen in various studies were there is a strong association between albuminuria and vascular disease [22-24]. The mechanism was first proposed as the Steno hypothesis in 1989, which states that excessive urinary albumin loss is the result of widespread peripheral vascular damage or endothelial dysfunction [25]. This resulted in the acceptance of albuminuria and endothelial function as therapeutic target in the prevention of cardiovascular disease [26, 27].

Ethnic disparities in vascular complications in patients with T2DM

The incidence and prevalence of T2DM is higher among ethnic minorities. Compared with Caucasians, individuals from Black African, African Caribbean and South-Asian ethnic origin suffer disproportionately from type 2 diabetes and its long-term vascular complications. Diabetic nephropathy is one of the major complications of T2DM, as it is the main cause of end-stage renal disease (ESRD) worldwide. Among the different ethnic groups and across various regions of the world there is a striking difference in prevalence and progression of diabetic nephropathy [28-30].

Patients with T2DM from South-Asian or African descent seem to be more prone to develop albuminuria [31-34], show a faster decline of kidney function [32, 35] and have a 40 times higher risk at developing ESRD compared to patients with diabetes from European origin [36, 37]. The cause for these ethnic differences in disease phenotype have not been fully elucidated.

Proposed underlying mechanisms for the high rate of vascular complications in South-Asian individuals with diabetes are increased visceral adipose tissue, systemic inflammation and endothelial dysfunction, in addition to the classic diabetic risk factors [38, 39]. Interestingly, signs of endothelial activation have already been found in South-Asian neonates, compared to European neonates [40]. Other studies have shown that even healthy South-Asians are characterized by endothelial dysfunction compared to Europeans [41, 42]. As we know, signs of endothelial dysfunction can be evident before the occurrence of clinically detectable vascular complications [43-45]. Sustained endothelial dysfunction in individuals of South-Asian descent may explain their predisposition to develop vascular complications. As this ethnic group suffers from an excessive rate of vascular complications, there is a need for therapeutic interventions aimed at slowing down vascular disease progression. Therefore, South-Asian patients with T2DM may benefit from therapeutic interventions aimed at improving endothelial function.

In the Netherlands, the biggest South-Asian group are the immigrated South-Asians from Suriname. In this thesis, this group is mostly studied and referred to as South-Asian Surinamese. These South-Asian immigrants originally descent from the Indian Subcontinent (India, Pakistan, Bangladesh). They migrated to Suriname due to the economic situation in North India and worked mostly on the plantation upon arrival to Suriname. Around 1975 and 1980, two political migration waves caused the South-Asian population to migrate to the Netherlands where they mainly settled in The Hague, Rotterdam and Amsterdam.

The glycocalyx as a therapeutic target in diabetes

Increased understanding of the role that the endothelial glycocalyx plays in the development of vascular complications has led to novel interventions and therapeutic treatments

aimed at the improvement of the endothelial glycocalyx. Inhibiting glycocalyx degrading enzymes and supplementing glycocalyx mimetics are strategies used to preserve and restore the endothelial glycocalyx. Heparan sulphate mimetics aimed at inhibiting HPSE-1 activity are mainly developed and investigated in the field of cancer research but may also be of interest in the diabetic field. So far, HPSE-1 inhibitors which also have been studied in experimental diabetes are PI-88 [46], PG545 [47] and SST0001 [48]. PI-88 is a mixture of highly sulfated oligosaccharides derived from yeast. PI-88 has been investigated in autoimmune type 1 diabetes, where it preserved HS content in the pancreatic islet [49]. PG545 is a sulfated tetra-saccharide with the addition of a lipophilic moiety which has been studied in experimental diabetic retinopathy and ischemic reperfusion during acute kidney injury (I/R AKI), were it was able to inhibit the inflammatory response and upregulation of HPSE-1 [50, 51]. SST0001, a non-anticoagulant *N*-acetylated glycol split heparin, showed to reduce renal damage and albuminuria in experimental diabetes [7]. However, none of these HPSE-1 inhibitors have been approved for clinical application.

One of the most extensively studied heparan sulphate mimetic in the diabetes field is sulodexide, a purified mixture of low-molecular-weight heparin and dermatan sulfate. In vitro, sulodexide showed to be a potent inhibitor of HPSE-1 and MMP [52, 53]. Broekhuizen et al. showed that in type 2 diabetes patients both sublingual as retinal glycocalyx dimensions increased after 2 months of sulodexide administration, whereas plasma hyaluronidase decreased [4]. In a larger trail with both type 1 and type 2 diabetic patients, sulodexide improved albuminuria in a dose dependent manner, indicating the effect of this GAG supplementation on the glomerular glycocalyx layer and filtration barrier [54]. However, phase II studies with sulodexide in overt diabetic nephropathy failed to demonstrate beneficial effects and were therefore discontinued [55]. An explanation for the disagreement in clinical trials may be due to different sources of the drug components [56]. It may also be that stabilization and preservation of the glycocalyx is only effective in early diabetes, before the occurrence of irreversible morphological changes in the kidney. Currently, sulfated polysaccharides derived from marine algae are under investigation, as they resemble biological properties of especially heparan sulfate glycosaminoglycans.

Assessment of the endothelial glycocalyx in vivo

Despite the important role of the endothelial glycocalyx in vascular homeostasis, it has been challenging to study the endothelial glycocalyx *in vivo*. The microvasculature covers more than 95% of the total vascular surface area, therefore most glycocalyx volume resides in the microvasculature. The development of non-invasive methods that are able to assess the endothelial glycocalyx *in vivo* has become of major importance and new techniques have been developed over the years.

Sidestream dark field (SDF) imaging is a non-invasive intravital microscopy imaging technique that allows visualization of the sublingual microcirculation in a clinical setting at bedside. The SDF imaging camera can be used in various vascular beds, however, not every microvascular bed can be easily visualized at bedside. The sublingual microcirculation is one of the most easily accessible surface in human and has proved to be a clinically relevant location, as alterations in the microvascular bed have been associated with several clinical parameters and outcomes in different patient groups [57-61]. The SDF camera uses green light-emitting stroboscopic diodes to detect the hemoglobin of passing red blood cells (RBCs). Over the years, several visual scoring systems and automated analysis software have been developed to standardize and improve acquisition and analysis of the microcirculation. The Glycocheck^{TM} software automatically detects, records and analysis the microvessels with a diameter between 4 and 25 µm. Several videos per individual are recorded and subjected to predefined quality criteria. Around 3000 valid vascular segments are collected and analyzed in one measurement. The software automatically generates several microvascular parameters based on these valid vascular segments, such as the vascular density, red blood cell velocity, blood volume and the perfused boundary region (PBR, an inverse estimation of the endothelial glycocalyx).

Damage to the glycocalyx allows RBCs to penetrate deeper towards the endothelial surface, which is expressed by an increased perfused boundary region (PBR). In previous studies, the PBR was found to be increased in dialysis patients [57], sepsis patients [62], patients with SARS-CoV-2 [63] and patients with type 2 diabetes [64]. In addition, the PBR has proven to be a valuable additive predictor for adverse cardiovascular events [65]. However, the variability of the PBR has been an issue of concern. Due to its dynamic nature, substantial differences in measured PBR have been reported, especially in healthy individuals. Average PBR in healthy volunteers ranged from 3.3 to 1.8 µm in two different studies [57, 64]. Only in patients with severe disease states, such as sepsis patients, the PBR revealed to have an acceptable intra- and interobserver variability, with comparable PBR values across different studies [66, 67]. To improve reliability of the PBR, the average of more than one measurement per individual used to be estimated [66, 68]. Recently, it was hypothesized that PBR dimensions are dependent on the velocity of passing RBCs in the vessels. Therefore, to minimize this flow dependent variability in PBR estimation, the slope of measured PBR values (called PBR_{static}) with corresponding red blood cell velocity (V_{RBC}) is used to estimate the PBR in the absence of RBC velocity (V_{RBC} =0 µm/s). This V_{RBC} independent PBR is called the $PBR_{dynamic}$ and is expected to have a much lower variation [59]. In the current thesis, we use SDF-imaging with the new Glycocheck software to assess the microvascular health in patients at risk for developing vascular complications and to monitor the effect of dietary interventions on the microvascular health.

Thesis objectives and outline

The objectives of this thesis are first to investigate if microvascular changes can be detected and used as a diagnostic marker in individuals at risk of developing cardiovascular disease. Secondly, we investigated if urinary HPSE-1 and MCP-1 can serve as biomarkers in individuals with type 2 diabetes. Lastly, we explored two dietary interventions aimed at stabilizing and preserving the endothelial glycocalyx in diabetes. The effect on the glomerular glycocalyx will be investigated in an experimental mouse model next to the effect on the sublingual microvasculature in South-Asian Surinamese patients with type 2 diabetes.

We first investigated in **chapter 2** whether changes in the microcirculation already could be detected in individuals with increased cardiovascular risk with the newly developed Glycocheck^{TM} software. Therefore, we assessed SDF-imaging parameters in individuals of the Netherlands Epidemiology of Obesity (NEO) Study stratified by risk groups according to the Framingham risk score, which is used to assess the risk of developing cardiovascular disease within 10 years.

Because individuals of South-Asian descent have a predisposition to develop diabetes and vascular complications, we wanted to investigate inflammatory markers involved in degradation of the endothelial glycocalyx in a multi-ethnic cohort of patients with type 2 diabetes in **chapter 3.** We determined ethnic differences in MCP-1 and HPSE-1 activity in participants of the HELIUS study. In addition, we investigated associations between these markers and the degree of albuminuria per ethnic group.

Next, we focus on two dietary interventions aimed at stabilizing or preservation of the endothelial glycocalyx and investigate the effect of these interventions on the diabetic kidney.

In **chapter 4,** we conducted a randomized controlled trial to investigate the effect of supplementation of glycocalyx mimetics or a repeated fasting mimicking diet on microvascular health in South-Asian Surinamese patients with type 2 diabetes. In addition, the effect on metabolic markers, inflammatory markers and albuminuria are investigated.

If supplementation with glycocalyx mimetics could preserve the glomerular endothelial glycocalyx in an experimental diabetic nephropathy mouse model was studied in **chapter 5**.

In **chapter 6**, the fasting mimicking diet intervention was studied in an experimental diabetic nephropathy mouse model. The effect on weight loss, inflammatory markers, albuminuria and the glomerular glycocalyx was investigated.

Finally, this thesis is summarized in **chapter 7** where the relevance of the results are discussed and future perspectives are proposed.

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