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The glycocalyx: a diagnostic and therapeutic target in cardiometabolic diseases

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CHAPTER 7

Summary and General Discussion

Summary

The endothelial glycocalyx covers the endothelium throughout the whole vasculature. After years of research, the important functional role of the glycocalyx in vascular homeostasis and several pathophysiological processes is widely accepted. In diabetes, perturbation of the glycocalyx is related to the pathogenic diabetic milieu with elevated glucose, increased lipids and circulating inflammatory stimulants. Sustained exposure to this diabetic milieu facilitates activation of endothelial cells, with influx of macrophages in response to MCP-1 and heparan sulphate breakdown by HPSE-1, inducing alterations in the endothelial glycocalyx [1, 2]. Consequently, alterations within the endothelial glycocalyx is proposed as an early hallmark for vascular dysfunction. Detecting and monitoring these changes can be used to identify early risk for the development of vascular complications or monitor the effects of therapeutic interventions. As some ethnic groups are more prone to develop vascular complications [3, 4], unravelling the pathogenesis of these cardiovascular health disparities and investigating interventions aimed at reducing cardiovascular risk could lead to improved cardiovascular risk management.

This thesis studied several aims: (1) can detection of endothelial glycocalyx changes be used as a diagnostic marker, (2) explore the relation between the two biomarkers, HPSE-1 and MCP-1, and microvascular glycocalyx changes in type 2 diabetes mellitus (T2DM) and (3) whether dietary interventions result into measurable microvascular restoration in diabetes.

Study findings

One of the first signs of cardiovascular disease are changes in the microcirculation. In **chapter 2** we showed that obese individuals at high risk for developing cardiovascular disease, defined by the Framingham risk score, were characterized by changes in the microvasculature. A new software system allowed the measurements of capillary red blood cell velocity and analyzed the capillaries according to diameters class (each 1 µm). These measurements were incorporated into new microvascular parameters. We explored if correcting the PBR for red blood cell velocity minimized the variability in the estimated PBR. However, we still found high variability and overlap in the PBR between the Framingham risk groups. It would be expected that the PBR was increased in individuals in the high-risk group, reflecting degradation of the endothelial glycocalyx. The high variability within- and between the risk groups may be to individual variability in the PBR or compensatory increased glycocalyx synthesis. Although the risk groups had overlap in microvascular parameters, the highest risk group was characterized by loss of the smallest capillaries. This was accompanied by an increase in red blood cell velocity indicating

impaired capillary recruitment in the high risk group. Within this study, we show that detecting early microvascular changes using SDF-imaging, can be a useful tool to monitor individuals before the development of cardiovascular disease.

MCP-1 and HPSE-1 are upregulated in patients with diabetic nephropathy [5-7]. Through increasing influx of monocytes to areas of inflammation and modifying the HS glycosaminoglycans, these factors can perturb the glomerular filtration barrier, inducing urinary protein leakage. Besides possible increased plasma levels, urinary levels of MCP-1 and HPSE-1 activity could reflect the degree of inflammation and glomerular damage in the diabetic kidney. As there are striking ethnic differences in the development and progression of diabetic nephropathy, we studied such ethnic differences in a multi-ethnic cohort of individuals with T2DM measuring urinary MCP-1 and HPSE-1 activity levels in **chapter 3.** Of the 6 ethnicities (Dutch, South Asian- and African Surinamese, Ghanaian, Moroccan and Turkish), Moroccan and South-Asian Surinamese participants had higher urinary MCP-1 levels compared to the Dutch, independently of confounding factors. In addition, urinary MCP-1 levels were associated with albumin-creatinine levels in all ethnic minorities, confirming the link between renal inflammation and albuminuria in diabetic nephropathy. These associations were the strongest in diabetic individuals from South-Asian and African Surinamese descent. In contrast, low urinary HPSE-1 activity levels were found in all participants. Compared to Dutch, African Surinamese and Ghanaian had lower urinary HPSE-1 activity levels. Only in Dutch participants, HPSE-1 activity levels correlated with the degree of albuminuria.

In **chapter 4,** we investigated two dietary interventions, a supplement to strengthen the endothelial glycocalyx or a fasting mimicking diet (FMD) to strengthen cellular response, in South-Asian Surinamese patients with T2DM. At baseline, we observed that South-Asian Surinamese T2DM patients have a reduced functional capillary network compared to the pre-clinical cohort in chapter 2, reflecting poor microvascular health in South Asian individuals. After 3 months of supplementation with glycocalyx mimetics, the RBC-velocity independent $PBR_{dynamic}$ and overall microvascular health score improved in the South Asian Surinamese T2DM patients. No changes were seen in clinical markers such as blood pressure and albuminuria, or in glycocalyx degrading markers such as MCP-1 and HPSE-1 activity. This showed that supplementation with glycocalyx mimetics is able to improve the endothelial glycocalyx beyond the HPSE-1 inhibiting effects that has been shown *in vitro*.

In contrast, after 3 months of a monthly 5-day FMD regime, BMI and Hba1c levels were significantly lower compared to baseline. However, endothelial markers worsened after the diet intervention, revealed by an increase in $PBR_{dynamic}$ and a decrease in capillary blood volume and microvascular health score. No differences were seen in albumin-creatinine

ratio, MCP-1 or HPSE-1 levels after the fasting mimicking diet. As one patient experienced an temporary decline of the kidney function after the FMD cycle, the FMD should be used with caution in patients with decreased kidney function.

Parallel to this clinical intervention study we also tested both dietary interventions in a diabetic mouse model, with specific emphasis on the possible effects on the diabetic kidney. In **chapter 5 and 6,** we investigated the effect of the supplement and fasting mimicking diet on the glomerular endothelial glycocalyx in a streptozotocin-induced diabetic nephropathy mouse model in ApoE-KO mice on a standard diet enriched with cholesterol. After 10 weeks of supplementation with glycocalyx mimetics, the glomerular endothelial surface coverage with heparan sulfate and hyaluronan was preserved and capillary loop rarefaction was prevented compared to control diabetic mice (**chapter 5**). These effects were accompanied with a reduction of CD11b positive renal tissue macrophages and dendritic cells, while the major myeloid cell population macrophage-like dendritic cells were not affected. During this 10-week period in both diabetic control mice and diabetic mice receiving the glycocalyx mimetics, increased levels of albuminuria and HPSE-1 activity returned to normal levels, without significance between the two groups. In **chapter 6** we showed that the diabetic ApoE-KO mice switched to lipid metabolism during the fasting mimicking cycles, which was accompanied with reduced glucose levels and loss of lean mass. After 5 FMD cycles, glomerular endothelial glycocalyx breakdown and the loss of capillaries was partially prevented (not for hyaluronan presence). However, metabolite analysis revealed increased oxidative stress and reduced presences of hyaluronan precursors, suggesting negative effects on kidney metabolism after the FMD cycles. In contrast to our previous studies with this mouse model, we monitored and maintained blood glucose levels to preferred set glucose levels (15-20 mmol/L) throughout the study in chapter 5 and 6. These set glucose levels were used since pilot experiments revealed that STZ-induced diabetes in the apoE-KO mice was accompanied by blood glucose levels above the detection limit and a total body metabolic switch already to predominant lipid oxidation. While our strategy prevented the preliminary shift to lipid usage for energy metabolism, it resulted in a much milder nephropathic disease phenotype. Along with reduced morphologic changes, maintaining set glucose levels also could have played a role in reducing proteinuria during the experimental period.

Endothelial glycocalyx as a diagnostic marker for microvascular health

In this thesis, we show that changes in the sublingual endothelial glycocalyx can be detected before the onset of clinical signs of cardiovascular disease. In recent years, changes in the sublingual microcirculation has been studied in specific patient groups and correlated with disease severity. In sepsis patients, microvascular changes are more prominent and sublingual microvascular changes have a high correlation with disease severity [8]. In patients with diabetes, microvascular changes were associated with an increased coronary

artery calcium score [9] and with the degree of albuminuria [10]. We revealed that several microvascular changes, such as loss of the smallest sublingual capillaries, can be detected in a pre-clinical cohort with individuals at risk for developing cardiovascular disease. This loss of capillaries is a phenomenon that is called capillary rarefaction, i.e. a decrease in functional capillary density. Functional rarefaction is a reversible state with arteriolar vasoconstriction leading to less perfusion of capillaries whereas structural rarefaction is the irreversible anatomic loss of capillaries. It is considered to be a sign of endothelial dysfunction and has been linked to cardiovascular disease [11]. A lower functional and total perfused capillary density in several microvasculature beds has been found in patients with hypertension, chronic heart failure, diabetes and chronic kidney disease [12-14]. It would be interesting to investigate whether the microvascular parameters that we found can be used for risk stratification for developing cardiovascular disease in the future. One study with a 6 year follow up in a cohort without established cardiovascular disease was the first study that showed that the PBR_{static} was associated with the risk of future cardiovascular events even after adjustment for conventional atherosclerotic risk factors [15]. However, more studies need to be conducted in the future to corroborate these findings.

In the current study, the new software included measurements of the longitudinal RBC velocity (V_{RBC}). This resulted in several new parameters such as: $PBR_{dynamic}$ (PBR corrected for variation in local V_{RBC}), V_{RBC} in capillaries and in feed vessels (> 10µm), perfused capillary density, capillary blood volume. While current calculations are still dependent on per-group slope calculations between V_{RBC} and PBR_{static} to get $PBR_{dynamic}$, new recording and analysis strategies are under investigation to allow these parameters calculated from intra-person measurements. This will result in a more person specific estimate of endothelial glycocalyx health.

In addition, for use in clinical practice, specific cut off points for interpretation of the microvascular parameters have yet to be determined. This will make it more accessible to use the technique in daily practice.

Concluding, there is a role for the endothelial glycocalyx to be used as a diagnostic marker for microvascular health. Ultimately, it may even have a role in the cardiovascular risk management (CVRM) in current practice, identifying and monitoring individuals at risk for developing cardiovascular disease.

MCP-1 and HPSE-1 in type 2 diabetes mellitus

Monocyte chemoattractant protein 1 (MCP-1) and heparanase-1 (HPSE-1) are upregulated in diabetes and result in endothelial activation and glycocalyx perturbation. Several studies found that MCP-1 and HPSE-1 are released in renal tissue and urine of patients with T2DM, correlating with the degree of albuminuria and kidney function [5-7, 16-19].

The ROADMAP study even showed that serum and urinary MCP-1 levels were a strong independent predictor for the development of albuminuria over time, suggesting that elevated MCP-1 levels may be an early indicator of diabetic nephropathy [20]. In the HELIUS study in chapter 3, we corroborated this association with urinary MCP-1 levels and degree of albuminuria in type 2 diabetic individuals from different ethnic origins. These findings reveal that urinary MCP-1 activity can also be used as a biomarker for diabetic nephropathy in these ethnic groups and indicate that these patients may also benefit from therapeutic interventions aimed at inhibiting MCP-1 activity, such as Emapticap [21, 22].

In our studies, low urinary HPSE-1 activity levels were found in diabetic patients from different ethnic origins (chapter 3) and our cohort of South Asian Surinamese patients (chapter 4). Increased glomerular and tubular HPSE-1 expression has been found in T2DM patients, but the histological findings have not been correlated with the urinary HPSE-1 activity levels [5, 23]. It therefore needs to be established whether urinary HPSE-1 activity levels actually reflect the local HPSE-1 expression in kidney and how much of this activity is still detectable in urine. Given previous results in literature, we expected higher HPSE-1 urinary and plasma levels in patients with T2DM than we observed in the clinical studies in chapter 3 and 4. We used a TAKARA HPSE ELSA kit, a sandwich ELSA that measures HPSE activity by detecting cleaved HS fragments in supernatant. A probable disadvantage of this method is that HPSE-1 could already be bound to shedded HS, from other downstream renal tubular or bladder cells, as every cell in the urinary tract expresses heparan sulphates. In the presence of high HS levels in urine samples the current ELSA possibly will give an underestimation of the amount of active HPSE-1. Therefore, a standard test to detect active HPSE-1 and inactive pro-HPSE-1 directly in urine is needed in future research [1, 24]. One such approach could be the use of activity-based probes (ABPs). Activity-based probes are designed to covalently bind to the active cleavage site of the target molecule, allowing detection, visualization and even inactivation of the enzymatic activity. A β-glucuronidase specific ABP has been developed for rapid and quantitative visualization of HPSE-1 in biological samples [25]. This ABP has the ability to label active HPSE-1 but also the inactive pro-HPSE-1, even in the presence of shed HS. Labelling and quantifying both active and non-active HPSE-1 is of importance as it gives a better understanding on real HPSE-1 release in urine or tissue. The current method to detect and analyse urinary HPSE-1 may not be sufficient and is not yet suitable as a biomarker in type 2 diabetes.

Dietary interventions to restore the endothelial glycocalyx

In the introduction, we elucidated on several therapeutic interventions aimed at restoring the endothelial glycocalyx, by inhibiting heparanase activity or supplementing glycocalyx substitutes. Several of those interventions, mainly heparanase inhibitors, are not yet approved for clinical trials. Only sulodexide, a heparan sulphate mimetic, has been studied in clinical trials [26, 27].

We investigated if two dietary interventions were able to restore the endothelial glycocalyx or reduce glycocalyx breakdown in a clinical trial with South-Asian Surinamese with type 2 diabetes and in a mouse model of diabetes. The first intervention was a food supplement, consisting of glycocalyx components such as hyaluronic acid and fucoidan, a heparan sulphate mimetic. In the experimental study, we showed that supplementation with glycocalyx mimetics preserved the glomerular endothelial surface coverage of heparan sulfate and hyaluronan and capillary loop rarefaction was prevented. In the clinical study, the PBR, an indirect marker of the endothelial glycocalyx, improved after supplementation with glycocalyx mimetics. These result suggest that the supplement is able to partially restore the endothelial glycocalyx, or prevent further damage to the glycocalyx induced by the diabetic environment. We previously showed that fucoidan was able to restore the endothelial glycocalyx and permeability barrier in human pulmonary microvascular endothelial cells *in vitro* [28]. The *in vitro* study in chapter 4 showed that fucoidan was able to dose dependently inhibit HPSE-1 activity in glomerular endothelial cells. Despite this, in both the experimental and clinical study, no significant improvement in HPSE-1 or albumin-creatinine levels were detected. Interestingly, in 2 patients with macro-albuminuria at baseline, albuminuria levels dropped to normo-albuminuria after 3 months of daily supplementation with glycocalyx mimetics. Larger studies with more patients are needed to corroborate these findings, investigating if fucoidan supplementation can restore the glomerular barrier function and reduce albuminuria in individuals with diabetes. Long-term follow-up studies are needed to investigate if the supplement is able to slow down the progression of cardiovascular disease. An advantage of supplementation with glycocalyx mimetics is that the supplement consists of natural ingredient as opposed to other therapeutic interventions, with no major side effects in the clinical setting. If proven effective in larger trials, it might be used as an add-on therapeutic intervention in the battle against development of cardiovascular disease in patients with diabetes.

Fasting interventions have become increasingly popular over the years for their potential to improve longevity and cardiometabolic risk factors [29-31]. We investigated a periodic 5 day fasting regime that previously revealed to improve cardiovascular and diabetes risk factors in a healthy population [32]. Besides this, it is believed that cellular pathways that regulate autophagy and reduce inflammation are upregulated after periods of fasting [31].

In the clinical study, 3 FMD cycles showed beneficial effects on BMI and Hba1c levels. In contrary to the earlier published clinical study with the FMD in healthy persons, systolic blood pressure, inflammatory markers such as hs-CRP or HPSE-1 and albuminuria were not affected. In one patient in our study, kidney function deteriorated on day 5 of one FMD cycle. Kidney function recovered after intravenous fluid resuscitation, pointing at dehydration as the probable cause of this decline in kidney function. Interestingly, in the mouse model, water and muscle loss was the main source of weight loss during the FMD

cycles. The loss of water during fasting may be due to glycogen depletion in the liver and muscles, which is mainly bound to water. More than half of the weight loss that can occur after fasting periods are a result of the loss of this excess water, which has been found by several studies [33, 34]. In addition, no positive effects were seen on the microvasculature, as the PBR worsened after the FMD cycles. In the mouse model, in dept metabolite analysis revealed negative effects on kidney metabolism with increased oxidative stress and reduced UDP-GlcA levels, a precursor of hyaluronan. Reduced levels of UDP-GlcA could prevent optimal hyaluronan synthesis, impairing endothelial glycocalyx integrity.

An advantage of this FMD is that it is easier to adhere compared to continues diet regimes, while still having positive effects on weight loss and glucose levels. However, we observed that these effects disappeared when the diet regime was discontinued. This is a common phenomenon for diet intervention studies when individuals are not monitored as intensively as in a clinical trial, which underlies challenges for the use of lifestyle interventions in daily practice [35, 36]. Overall, FMD is not suitable as an intervention to restore the endothelial glycocalyx and may even have adverse effects on the microvasculature and glycocalyx integrity. Furthermore, we advise that the FMD should not be used or used with caution in patients with CKD due to the possibility of dehydration.

Other interventions aimed at reducing HPSE-1 activity may be more promising to target the endothelial glycocalyx. A recent study showed that the use of HPSE-2 protein and peptides prevented streptozotocin-induced kidney injury, showing possible HPSE-1 inhibiting effects in this diabetic nephropathy mouse model [37]. The above mentioned activity-based probes also could be a possible therapeutic intervention, as the probes can covalently and irreversible bind to the active HPSE-1 enzyme and inhibit its activity. Recently, the first promising proof of principle experimental study with a selective covalently binding HPSE-1 inhibitor has been published in the field of cancer research [38]. It remains to be seen if these therapeutic interventions emerge as a possible therapy in type 2 diabetes.

Challenges for South-Asian Surinamese individuals

In the current thesis, we investigated individuals with type 2 diabetes from South-Asian Surinamese descent. As mentioned in the introduction, South-Asians are more prone to develop diabetes and cardiovascular complications, despite standard therapeutic management. The onset and progression of cardiovascular complications remains higher compared to individuals from European descent. Type 2 diabetes affects South-Asians at an earlier age and at a lower BMI, they have a higher rate of myocardial infarction, a higher prevalence of proteinuria and a faster decline in eGFR compared to individuals of European descent [39, 40]. The cardiovascular health burden of South-Asians has been

well described over the years, however, representation in clinical trials, adequate risk calculators and therapeutic strategies are lacking.

South-Asians are a difficult group to recruit for randomized controlled trials, especially for lifestyle intervention studies. Conducting lifestyle intervention studies in the South-Asian population has been proven to be extremely difficult due to low response rates, high drop-out rates and conflicting effects. An intensive 1 and 2 year targeted lifestyle intervention in general practice revealed no significant weight loss or improvement in metabolic profiles in South-Asian Surinamese participants in The Hague [41, 42]. In our study, we also experienced a low response rate but did achieve significant improvement of BMI and Hba1c after 3 months. A meta-analysis with randomized controlled trials also showed that lifestyle interventions can be successful to reduce the risk of diabetes in South-Asian individuals [43]. A large part of the current studies conducted with South-Asians are directed towards preventive lifestyle interventions [44, 45]. Other studies are directed towards pathophysiological explanations for the disparities in cardiovascular or diabetes risk. Altered high-density lipoprotein composition [46] or different inflammatory pathways [47] are examples of recent discovered differences between South-Asian and European individuals. Therefore, unravelling the differences in pathophysiology of cardiovascular complication development may lead to more tailored and successful therapeutic interventions for South-Asians in the future.

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