Glycocalyx, cardiometabolic disease and inflammation
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Summary
The glycocalyx, meaning ‘sweet cell cover’ or ‘sweet husk’, was already introduced in 1963 by H.S. Bennet as a carbohydrate cover located over the surface of most eukaryotic cells. Electron microscopy also discovered a glycocalyx layer on endothelial cells covering the lumen of blood vessels but its functional relevance was unknown and the endothelial glycocalyx remained a mysterious structure for many years. In recent years, research on the glycocalyx has progressed substantially. Thus, it is increasingly becoming clear that the endothelial glycocalyx holds a vital position in many regulatory mechanisms operating at the endothelial surface layer. This feature makes the endothelial glycocalyx an interesting target for new drug therapies aiming to restore early endothelial damage and in this way prevent against atherosclerotic disease. Future research will therefore focus on unraveling glycocalyx structure and function and the question whether glycocalyx disruption truly is an important link in the development of atherosclerosis.

Presenting a modest step towards achieving the previously described goals, this thesis discusses the harming effect of cardiometabolic and inflammatory disease on the endothelial glycocalyx as well as a first attempt to measure glycocalyx dimension in vivo. Finally, we set out to provide proof-of-concept that damaging the glycocalyx has a direct impact on vascular homeostasis.

- **Part I** evaluates the consequences of glycocalyx disruption in different pathological settings and describes the development of techniques used to measure the glycocalyx.
- **Part II** describes strategies that were applied in order to restore glycocalyx damage in order to (hopefully) decrease disease consequences.

**Part I: Estimating glycocalyx dimensions and consequences of glycocalyx disruption.**

**Chapter 2** gives a survey of the literature and addresses what is known about normal structure and function of the endothelial glycocalyx. Also, current ideas about the physiopathology of disease processes in which the glycocalyx is thought to be involved are discussed. This chapter sets the stage for the overall hypothesis of this thesis by explaining how restoration of the glycocalyx layer may prevent cardiovascular disease.

The translation of an established experimental method for the estimation of intravital capillary glycocalyx dimension (SDF/OPS) to a measurement tool that can be used in large-scale clinical trials is described in **Chapter 3**. The reproducibility of sublingual capillary glycocalyx dimension assessed with orthogonal polarization spectral imaging was investigated in healthy individuals. Systemic glycocalyx volume estimated with the tracer dilution described in chapter 2 technique and cardiovascular risk profiles were evaluated in parallel. Microvascular glycocalyx thickness correlated with systemic glycocalyx volume and could be correlated with cardiovascular risk factors. Measuring glycocalyx dimension by OPS imaging is a non-invasive method which proved to be reproducible in humans.
However, although it is now readily applicable, the processing of the images is still a laborious job which makes this technique in its present form unsuitable for large patient trials. Also, the relation of glycocalyx dimension to the severity of vascular disease warrants further investigation prior to its wider implementation in clinical research as a surrogate marker for CV-risk.

The purpose of Chapter 4 was to further optimize the 'leukocyte passage' method for glycocalyx estimation (Chapter 3) using automated OPS/SDF imaging. In this study, we applied OPS/SDF to test whether in vivo assessment of temporal Red Blood Cell column width (RBC) variations and changes in RBC Perfused Diameter $D_{perf}$ in the human sublingual microcirculation is reproducible and whether these measurements were able to detect changes in the microvasculature and, derived from this, glycocalyx alterations in patients with DM2 with and without microalbuminuria. We show that median RBC column width is a reproducible measurement which can be performed in a fully automated fashion. The latter makes this technique attractive for use in large epidemiological studies. Median RBC column width is significantly increased in DM2 patients both with and without microalbuminuria compared to controls. Strikingly, DM2 patients with microalbuminuria also demonstrate an increase in $D_{perf}$, indicating damage of also the cell-impermeable part of the glycocalyx layer in patients with overt microvascular damage. Future studies are needed to unravel the predictive value of this measurement for early detection and/or therapeutic monitoring of treatment in patients at increased cardiometabolic risk.

In Chapter 5, we set out to provide proof-of-concept that glycocalyx perturbation actually bears consequences for 'vascular homeostasis' in vivo using an experimental setup. Thus, the effect of long-term enzymatic infusion on the endothelial surface layer and vascular barrier properties were studied in mice. The active or heat-inactivated enzyme hyaluronidase, an enzyme that degrades glycosaminoglycans like hyaluronan but also chondroitin and heparan sulphate, was infused for 4 weeks in apolipoprotein E-deficient $\text{apoE}^{-/-}$ mice on a Western-type diet. In this model, 'chronic' hyaluronidase infusion induced overt proteinuria and altered plaque composition without altering the course of atherogenesis. These results suggest an association between enzyme-mediated endothelial surface layer disruption and the induction of microalbuminuria. This could explain one of the mechanisms leading to deteriorating kidney function and increased plaque vulnerability in humans with cardiovascular risk factors like diabetes mellitus, as it has been shown before that hyaluronan is upregulated in diabetes $^2$.

The glycocalyx layer is an essential element for the maintenance of the vessel wall's permeability barrier. This study shows that the glycocalyx not only regulates systemic vascular permeability but is also very important for renal charge selectivity and permeability. In contrast to our expectation, enzymatic degradation of the glycocalyx only had a limited effect on atherogenesis in the present model. Several explanations could be responsible for this lack of atherosclerotic progression. First, we measured a pro-inflammatory reaction in all animals reflecting immune activation of both active as well as bovine derived heat-inactivated hyaluronidase. Since inflammation is a hallmark in atherosclerosis progression, this reactive inflammatory reaction may have masked a harmful effect
of active hyaluronidase in itself. Second, it should be taken into account that the duration of enzymatic exposure was too short to induce a significant difference in plaque size and composition. Therefore future studies, for instance aiming at genetic disruption of the glycocalyx in a pro-atherogenic environment are needed to find the answer to the question whether glycocalyx disruption truly influences atherosclerotic progression and if so, to explain the mechanism behind it.

Part II: Working towards glycocalyx restoration in Cardiometabolic diseases

In Chapter 6, we report the increased risk for coronary heart disease (CHD) and the beneficial effect of physical activity on this condition in middle-aged men and women people with MS enrolled in the EPIC-Norfolk prospective population study. The results of this study show a downward trend in CHD for increasing levels of physical activity in men and women and a lower risk for CHD in those who were physically active. Current guidelines recommend regular and moderate regimens of physical activity, but it is currently unknown whether individuals with MS who are physically active have less CHD consequences of MS and therefore a lower CHD risk. Chapter 6 provides evidence confirming the existing theory that being active decreases risk for cardiovascular disease and underlines the importance of interventions aiming at increasing physical activity and targeting the specific components of the metabolic syndrome. Despite the improved treatment options and prevention programs, cardiovascular disease and its complications still is a huge threat for public health. As the number of overweight people is still increasing, atherosclerosis and cardiovascular risk is rising too and it will be worthwhile to extend our options for primary prevention. In this perspective it seems useful to look beyond the traditional targets like a disbalanced lipid profile and elevated CRP and investigate other anti-inflammatory interventions together with remedies that strengthen the vessel walls own protective mechanism, including the glycocalyx.

The impact of a polyphenol rich extract on the chronic inflammatory state and the acute immune responses, triggered by LPS infusion, in men and women with clustered metabolic risk factors is investigated in Chapter 7. This study was designed to look at the effect on pro-inflammatory cytokines of 500 mg daily polyphenol rich extract per os (Frutologic, also know as VinitroxTM) or placebo for 4 weeks in a randomized, placebo-controlled, double-blind cross-over study in 34 subjects with 2 or more metabolic risk factors. At the end of the last treatment period, a subgroup of volunteers received an inflammatory challenge (LPS 1 ng/kg bolus). We observed a modest, but significant reduction in chronic circulating MCP-1 and MIF levels without affecting other inflammatory markers. The inflammatory response following the low-dose LPS challenge lowered MCP-1 production (AUC) over 6 hours in the polyphenol-rich extract treated individuals, whereas no differences could be detected for other inflammatory cytokines. For now, the clinical relevance of polyphenol ingestion on the development and progression of metabolic risk factors and CVD remains to be elucidated in larger trials.
Chapter 8 describes the unfavourable consequence of high glucose levels on endothelial barrier properties of human umbilical vein endothelial cells (HUVECs) in vitro and shows a modest attempt to restore increased permeability by addition of a mixture of 80% LMW heparin and 20% dermatan sulfate (Sulodexide®) but only in the absence of heparin. Hyperglycaemia for 24 hours increased albumin permeability through a monolayer of HUVECs which corresponds with previous studies performed in humans with diabetes mellitus showing increased urinary albumin loss and capillary leakage. Hyperglycemia had less effect on albumin permeability in HUVECs cultured in the presence of unfractionated heparin (UFH). The addition of Sulodexide to the media only slightly decreased albumin permeability without UFH in the medium. HUVECs were used in this study as a representation of endothelial cell behaviour in general and as a surrogate for conditions in vivo and is certainly not optimal. Efforts aiming to restore the endothelial surface layer by exogenous administration of GAG components could offer a new strategy to prevent complications in patients with DM. However, gathering evidence for this theory by studying endothelial cells under in vitro conditions reflect specific difficulties that have to be overcome before optimal results can be achieved.

Finally, Chapter 9 provides the first evidence in humans with type II diabetes mellitus that oral glycosaminoglycan administration (Sulodexide®) establishing enhanced precursor abundance for GAG-synthesis, improves endothelial glycocalyx dimension measured in two different vascular beds: the sublingual (by OPS/SDF) and retinal microvasculature (by FAG/ICG). This improvement coincides with a trend towards normalization of transcapillary albumin escape rate and a decrease in hyaluronan catabolism shown by a reduction of plasma hyaluronidase activity. These findings imply that restoration of endothelial glycocalyx in humans could be a promising target to attenuate vascular dysfunction in type 2 diabetes mellitus. Of course, this fascinating outcome has to be confirmed in other prospective large trials. However, when it holds true that endothelial glycocalyx improvement can reduce the development of complications from diabetes mellitus like albuminuria, diabetic retinopathy and cardiovascular disease, primary prevention in diabetes can be drastically improved. Supplementation of glycosaminoglycans as building stone abundance for GAG-synthesis could be one way to achieve this goal but future experiments testing other strategies that restore glycocalyx surface will have to investigate which treatment modality is best for this purpose in a clinical setting.
Future Perspectives

The studies and review shown in Part 1 of this thesis describe our attempts until now to develop a practical technique that gives a reliable estimate of glycocalyx dimensions in humans. For now, OPS/SDF imaging used to make an assessment of the width between the erythrocyte column and the vessel wall seems to be the most promising tool for this purpose. This technique, together with the establishment of biomarkers indicating glycocalyx shedding, will be improved and tested in larger groups of patients in the coming years and will hopefully prove to be a useful instrument for measurement of glycocalyx dimension in the microvasculature. In this perspective, we will set out to provide evidence for a technique that is both practical and relevant for the assessment of glycocalyx dimension in humans. However, for the development of treatment options and preventive strategies that can protect the glycocalyx layer, it is absolutely necessary to learn more about glycocalyx behaviour and structure.

This means that in the future our focus will be more on the composition of the glycocalyx layer rather than its dimension. We know now that the glycocalyx is constructed of several molecularly groups, mainly proteoglycans and glycosaminoglycans, with different enzymes regulating a perfect balance of constituents. It will be very interesting to clarify the specific role of single molecules in the glycocalyx structure and the effect of removal or malfunction of a molecule on the pathogenesis of atherosclerosis, diabetic retinopathy, renal disease and even cancer progression. Second, the glycocalyx is more than just a protective layer and a permeability border on top of the endothelial lining. Syndecan-1 heparan sulphate proteoglycans (HSPGs) present in the glycocalyx are for instance identified as remnant lipoproteins receptors. In diabetic mice, the gene heparin sulphate glycosamine-6-O-endosulfatase-2 (SULF2) was shown to be dysregulated, causing degradation of HSPGs which impaired catabolism of remnants and very low density lipoproteins (VLDL) resulting in hypertriglyceridemia. This effect could be completely undone by knockdown of SULF2. This study indicates that proteoglycans serve as receptors in important regulatory processes in the human. We also know that glycosaminoglycans, next to their function in lipid metabolism, are important in immune defence mechanisms against bacterial and viral infections. For instance, glycans play a role in the trafficking of lymphocytes and other immune cells, recognition of pathogens and regulation of the immune signalling response by protein-glycan binding to toll-like receptors. (J.D. Esko, Essentials in glycobiology) This initial binding of pathogen with glycosaminoglycans could also be valuable as a target for therapeutic intervention and prevention against infectious disease. And again, glycocalyx holds an important key.

The second part bundles four studies in which we successively showed that an active lifestyle improves health status in cardiometabolic disease and that ingestion of polyphenols can give a slight reduction in systemic inflammation in patients with cardiovascular risk factors. In addition, we describe differences in glycocalyx dimension in these patients with an increased cardiovascular risk profile compared to healthy individuals. Finally, we show a first attempt to restore glycocalyx
damage and reduce endothelial permeability caused by type II diabetes through supplementation of glycosaminoglycans. We hope that the perspective of glycovalyx restoration will be that new therapies can proof to be beneficial in protecting the vessel wall against shedding and disorganisation of the glycocayx resulting in endothelial dysfunction and may even be able to restore early damage. Following this proof of concept, showing that glycocalyx restoration is achievable and it does convey functional improvements (decreased vascular leakage), new efforts should target at correlating these short term functional improvements with decreasing end organ damage.

In atherosclerosis, understanding glycocalyx behaviour and composition means that we can develop intelligent methods to ‘treat’ or resupply this structure when it is damaged by factors like hyperglycemia, low grade inflammation or oxidative stress. One way could be by providing an abundance of certain precursor molecules for GAG synthesis, another could be by supplementation of drugs already known for reducing vascular permeability like ACE-inhibitors. It might even be possible to influence the production of organ specific proteoglycans and GAGs by gene modulation (like antisense) to target individual disease related complications. It is up to future research to teach us whether these hypothesis are feasible and so glycocalyx will remain a fascinating structure for many years to come.
Reference List

