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General introduction
The process of atherosclerosis

Cardiovascular disease (CVD) is one of the major causes of death worldwide, leading to 40,129 deaths in 2008 in the Netherlands (CBS). The majority of CVD is caused by atherosclerosis. Atherosclerosis is a chronic disease evolving around the accumulation of low density lipoprotein cholesterol (LDL-C) in the subendothelial space of the arterial wall in middle and large sized arteries. This process is already present in adolescence. The development of atherosclerosis and cardiovascular disease depends on the combination of genetic predisposition, lifestyle and environmental factors. Important risk factors that predispose to cardiovascular disease are; age, inactivity, diabetes, abdominal obesity, hypertension, smoking, dyslipidemia but also chronic systemic inflammation.

Early atherosclerosis begins with activation of the endothelial cells lining the vessel wall. Excess amounts of circulating lipoproteins trigger an inflammatory process that accelerates the formation of fatty streaks and plaques. Leukocytes and macrophages are drawn to the site of endothelial ‘injury’ in order to clean the area from the cholesterol intruder. Macrophages and monocytes scavenge (oxidized) LDL containing fragments, which leads to the formation of foam cells and enhances the accumulation of subendothelial cholesterol that forms the lipid core of the plaque. This process gradually enlarges the atherosclerotic plaque and a fibrous cap is formed over the lesion to separate the thrombogenic material of the core from the bloodstream (Figure 1). The attracted macrophages in the lesion produce proinflammatory cytokines and chemokines which amplify the signalling cascade and attract even more inflammatory cells 1. The active inflammation, together with the ongoing expenditure of the lesion, cause thinning of the fibrous cap through decreased collage synthesis and increased enzymatic breakdown, which leads to destabilization of the plaque and makes it more susceptible to rupture 2. Eventually, when rupture occurs, the lipid rich content comes in contact with the bloodstream which activates the coagulation system, inducing the formation of a dangerous thrombus with the potential to cause ischemia and/or infarction in distal tissues.

Over the last decades, the amount of people with excess weight has been rapidly increasing to the point that it now has become a serious problem worldwide which is increasingly aggravating healthcare costs. The presence of obesity is accompanied by a wide array of health risks, including an increased risk for cardiovascular disease. Thus, these patients invariably develop multiple risk factors for cardiovascular disease like elevated blood pressure, dyslipidemia and insulin resistance. Obesity is often accompanied by chronic low grade inflammatory activation and it has come forward that this inflammation probably contributes to the clustering of cardiovascular risk factors known as the metabolic syndrome 3.
Obesity, atherogenic dyslipidemia, hypertension and hyperglycaemia or insulin resistance are the key features of the metabolic syndrome and predispose to increased cardiovascular risk. Patients with the metabolic syndrome or type 2 diabetes are characterised by elevated levels of circulating triglyceride-rich lipoproteins and the accumulation of abdominal fat, which leads to enlargement of abdominal adipocytes. The excess amount of fat triggers the production of, among others, tumour necrosis factor $\alpha$ (TNF-$\alpha$) through the NF-kb pathway $^{4,5}$ followed by downstream secretion of various inflammatory mediators like interleukin 6 (IL-6), macrophage migration inhibitory factor (MIF)-1 and monocyte chemoattractant protein (MCP)-1. Inflammatory cytokine release by abdominal adipocytes is thought to modulate the paracrine function of the adipocytes, which eventually contributes to systemic inflammation together with insulin resistance $^6$ and the development of diabetes (Figure 2). As the source of inflammation has been recognised in excess abdominal fat and enlargement of abdominal adipocytes, it readily follows that exercise and weight reduction can dramatically alter a patients health status. Hence, lifestyle modification is regarded as the primary treatment option in order to prevent future cardiovascular events in this group of high-risk patients, before starting lipid-lowering therapy.

**The endothelial glycocalyx as a protective barrier**

The vessel wall is fundamental in the pathogenesis of atherosclerosis. The endothelial cells of all blood vessels are covered by a tight matrix of glycosaminoglycans anchored in the cell membrane by a backbone of glycoproteins $^7$. This endothelial coating has many functions and facilitates a smooth journey of circulating blood cells along the vessel wall and is called the glycocalyx (Figure 3). Located between the endothelial cell lining and flowing blood, the glycocalyx forms a protective
barrier that shields the endothelial cells from circulating atherogenic and inflammatory factors. The anti-atherogenic effects of the glycocalyx comprise fending off transendothelial lipid migration but also through modulation of platelet and leukocyte activation. The glycocalyx is at least 0.5µm thick, exceeding the length of adhesion molecules on the endothelial surface. This aspect of the glycocalyx prevents leukocytes and platelets from reaching molecules like VCAM, ICAM and von Willebrand factor and are thereby kept from adhesion and activation. Last but not least, the tight structure of the matrix, for which hyaluronan is crucial, and the negative charge of glycosaminoglycans like heparan sulphate and chondroitin sulphate, seals the lumen and makes it inaccessible for larger molecules. This quality makes the glycocalyx instrumental for the regulation of vascular permeability.

**Figure 2.** Obesity-induced inflammatory changes in adipose tissue, adapted from KE Wellen, J Clin Invest 2003.

The endothelial glycocalyx is present in the macro- as well as the microvasculature and its thickness progresses with increasing vascular diameter. More than 95% of the endothelium is located within capillaries and therefore most of the glycocalyx mass is present in the microvasculature. Damage to the glycocalyx may contribute to an imbalance in vascular permeability and is associated with many unfavourable changes on the endothelial surface which, in theory, makes perturbation of the glycocalyx layer an early step in the development of atherosclerosis. For this reason, the glycocalyx could be an interesting target for future therapies that prevent atherosclerosis, as restoration of this layer may strengthen the natural defence mechanisms of the vessel wall.
**Figure 3.** Healthy and damaged endothelial glycocalyx, adapted from M Nieuwdorp, Curr Opin Lipidol 2005. NO = nitric oxide, EC-SOD = extracellular superoxide dismutase, vWF = von Willebrand factor, TFPI = tissue factor pathway inhibitor, VCAM = vascular cell adhesion molecule, ICAM = intercellular adhesion molecule.
Measuring the Glycocalyx

In recent years, various attempts have been undertaken to measure and understand the glycocalyx. Estimating the dimensions of the glycocalyx is not an easy job, as it is a very delicate structure of 0.5 and 3 µm that does not maintain well outside the body. The first images of the glycocalyx, made with electron microscopy, revealed a small irregular shaped layer of approximately 50-100 nm. Subsequent approaches used systemically injected fluorescein isothiocyanate (FITC) labelled dextrans in animals implementing the selective barrier properties of the glycocalyx in the measurement of glycocalyx dimensions. By these methods it was observed that there is a wide variation in thickness of the endothelial surface layer according to the diameter of the micro-and macrovasculature and that the dimension of the glycocalyx also depends on where it is located, as the layer is substantially thinner near arterial bifurcations.

In humans, the method most frequently used to estimate glycocalyx dimension is by subtracting the intravascular volume of a patient’s own glycocalyx impermeable erythrocytes labelled with fluorescein from the intravascular volume of the glycocalyx permeable tracer Dextran 40. Although feasible, this method is certainly not infallible. Up till now, studies have only been performed in small patients groups. This underscores the need for other strategies in order to allow glycocalyx measurements in a larger clinical setting. For this purpose, efforts have already been made to estimate local glycocalyx with sidestream darkfield (SDF) imaging (or orthogonal polarization spectroscopy (OPS)), which uses a little camera that visualises the sublingual microcirculation and can be handled by patients themselves. This thesis describes the glycocalyx measurement strategies that are currently under development as well as the first steps in the search for therapeutic options that restore the damaged glycocalyx and which could therefore protect against cardiovascular disease.

Outline of this thesis

This thesis consists of two parts. Part one focuses on the development of methods by which we can measure the endothelial glycocalyx in humans and animals, which is a prerequisite in order to evaluate potential future clinical applications. Part two describes the influence of cardiovascular risk factors on the glycocalyx, endothelial function and coronary heart disease and discusses potential therapeutic strategies to restore the glycocalyx.


Chapter 2 describes the components of the glycocalyx and its functions in protecting the vessel wall against ‘detrimental’ stimuli like hyperglycaemia and dyslipidemia. It also provides an overview of the methods that have been used to estimate glycocalyx dimensions. In chapter 3 and 4 we use two imaging modalities to estimate local glycocalyx thickness: Sidestream DarkField imaging (SDF)
of the sublingual microcirculation and Fluoresce AngioGram/IndoCyanine Green (FAG/ICG) imaging of the retinal vasculature. We validated both techniques for the measurement of glycocalyx thickness in humans and compared healthy individuals to patients with various metabolic and inflammatory diseases. Finally, chapter 5 shows that an infusion with hyaluronidase, an enzyme that degrades the glycocalyx component hyaluronan, results in albuminuria in hypercholesterolemic mice but does not significantly alter atherosclerotic plaque progression.

Part II: Working towards glycocalyx restoration in Cardiometabolic diseases

Chapter 6 focuses on risk factors and the role of inactivity on cardiovascular risk in men and women with the metabolic syndrome (MS) selected from the EPIC-Norfolk cohort and reports that increasing levels of physical activity in middle aged men and women with MS reduce risk for coronary heart disease. Chapter 7 describes the effect of a polyphenol rich abstract on chronic systemic inflammation and on an acute inflammatory response in men and women with multiple risk factors for cardiovascular disease showing a modest reduction in the pro-inflammatory cytokines MIF and MCP-1 after treatment. Subsequently, chapter 8 evaluates the effect of Sulodexide on the glycocalyx of human umbilical vein endothelial cells (HUVECs) under hyperglycaemic conditions. High glucose levels caused increased trans-endothelial albumin transport which could in part be restored by the addition of GAGs demonstrating that supplementation of GAGs alters the endothelial surface. In chapter 9, we provide an addition to the latter chapter as it assesses the SUGAR study, designed to evaluate glycocalyx degradation and restoration by Sulodexide® [a mixture of glycosaminoglycans (GAGs)] in patients with type 2 DM. A summary of these studies is provided in chapter 10, as well as a perspective on the future of glycocalyx estimation in a clinical setting and the possible preventive treatment options for patients at risk for CVD.
Reference List

