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Introduction and outline

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The endothelial glycocalyx

The endothelial glycocalyx or endothelial surface layer (EG), is a negatively charged gel-like surface structure of proteoglycans with their covalently bound polysaccharide chains called glycosaminoglycans (GAGs), glycoproteins and glycolipids. Its main carbohydrate constituents are heparan sulfate (HS), chondroitin sulfate (CS) and hyaluronan (HA). The endothelial glycocalyx governs transcapillary fluid exchange and acts as biomechanical sensor to confer shear to the endothelium (EC) [1-3]. Glycosaminoglycans within the glycocalyx function as a molecular scaffold that facilitates protein binding in a very selective manner. In this way, circulating proteins, such as growth factors and chemokines are concentrated and spatially organized in gradients at the endothelial surface [4,5]. The proteins that bind to the glycocalyx include proteins involved in cell attachment, migration, differentiation, morphogenesis, blood coagulation, lipid metabolism, and inflammation, thus putting the endothelial surface layer at the very center of the pathophysiology of cardiovascular and renal disease (figure 1). However, despite its pivotal role in endothelial cell biology, glycocalyx function has proven to be hard to study due to its complex carbohydrate chemistry and the difficulties in interrogating its function in vivo and in vitro. Here we will describe the structure and main biological functions of the endothelial glycocalyx in the kidney and an give an outline of this thesis.
Figure 1: Schematic overview of the endothelial glycocalyx in a healthy and diseased condition. Left: In a physiological state, the endothelial glycocalyx protects against protein leakage, inflammation and coagulation. Heparan sulfates, bound to a heparan sulfate core protein, and hyaluronan, bound to e.g. CD44, are the main constituents of the endothelial glycocalyx (EG). Order and modification of disaccharide repeats within HS determine the binding site for specific proteins. Right: Upon endothelial activation, heparan sulfate disaccharide modification occurs, resulting in a change in protein binding sites. During a chronic disease condition, the EG gets damaged, mainly due to up regulation of degrading enzymes such as hyaluronidase, heparanase and proteinases. Both HS modification and EG degradation result in inflammation, coagulation and protein leakage.
**Biochemical structure of the endothelial glycocalyx**

The membrane bound part of the endothelial glycocalyx consists of proteoglycans, glycosaminoglycans, glycoproteins and glycolipids. Proteoglycans with their bound GAGs are the main contributors to the EG structure and function[6]. Of these GAGs, heparan sulfate and hyaluronan constitute up to 90% [7-9].

Heparan sulfate is a linear polysaccharide which consists of the repeating disaccharide β1–4-linked D-glucuronic acid (GlcA) and α1–4-linked N-acetyl-D-glucosamine (GlcNAc). This polymer is covalently attached to a limited number of core proteins at cell surface, called heparan sulfate proteoglycans (HSPGs). The proteoglycans found on the luminal endothelial side are syndecans 1 and 4, glypican 1, versican and thrombomodulin. HS chains are processed in the Golgi apparatus where they undergo a series of modifications in which subsets of glucosamine residues can become N-deacetylated and N-sulfated and where glucuronic acids may undergo epimerization to L-iduronic acid (IdoA). In particular, C2 of uronic acid and C6 (and rarely C3) of glucosamine residues may become sulfated [10]. After intracellular processing, further extracellular modification can occur. Heparanase (HPSE1) and endosulfatases (SULF1,2) can cleave the HS chain or further modify the sulfate groups within HS, respectively [11]. (figure 2) Although they do attribute to a wide variety of specific HS binding sites for protein interactions that determine endothelial cell biology, regulation of these modifications is still not well understood. While the core proteins can function independently of the HS chains they carry, the HS chains predominantly dictate ligand-binding capability and therefore the biological roles of HSPG. Some examples are discussed in the next section. HS is structurally related to heparin, a highly sulfated GAG [10-12].

Hyaluronan lacks the complex chemical editing of HS. It is a nonsulfated glycosaminoglycan composed of repeating polymeric disaccharides D-glucuronic acid and N-acetyl-D-glucosamine linked by a glucuronidic bond [2,3]. Under physiologic conditions, hyaluronan is synthesized by membrane bound synthases (HAS1, 2 and 3) as a macromolecule of 105–107 Da. Following its synthesis, hyaluronan is directed to the cell surface where it interacts with hyaluronan binding surface proteins (hyaladherins) such as CD44, or is assembled into the pericellular extracellular matrix [1]. The repeating nature of the polysaccharide and hence its protein binding sites, has been suggested to confer structural periodicity to the endothelial surface layer [13]. The main modifiers of hyaluronan are hyaluronidases, enzymes that cleave high molecular weight (HMW) hyaluronan chains into smaller low molecular weight hyaluronan fragments.
Figure 2: Schematic overview of production and modification of HA and HS. Top left: Hyaluronan (HA) is produced by HAS2 and transported over the cell membrane by ABC transporters, where it can bind to several HA binding proteins. Bottom left to upper right: Heparan sulfate (HS) containing proteoglycans are produced in the endoplasmic reticulum and Golgi apparatus. HS initiation starts with the addition of linker pentasaccharides to serine residues within the backbone protein (1). Two enzymes, exostosin 1 and 2 (EXT1/EXT2) form the initial heparan sulfate chain by adding alternating N-acetyl-glucosamine (GlcNAc) and glucuronic acid (GlcA) (2). 4 different isoenzymes of N-deacetylation/N-sulfation (NDST) enzymes modify the chain by removing N-acetyl groups from subsets of GlcNAc subunits and substituting this free group with sulfate (3). Glucuronic acid (GlcA) can be epimerized into iduronic acid (IdoA) by glucuronic acid epimerase (GLCE) (4). Isoforms of sulfotransferases transfer sulfate to 2-O, 3-O or 6-O residues (5). The HS proteoglycan is transported towards the endothelial membrane where it can be modified by the extracellular enzymes heparanase and sulfatase (6). Together all these enzymes are responsible for the structure and thus the ability to bind proteins such as fibroblast growth factor (FGF).
Introduction and outline

Function of the endothelial glycocalyx

Biomechanical properties
The endothelial glycocalyx is believed to be the primary molecular sieve for plasma proteins and, therefore, the origin of the oncotic forces that control transcapillary fluid exchange. This has led to a revised Starling principle in which the capillary filtration equilibrium is generated over the glycocalyx, rather than across the entire endothelial layer as had been widely assumed [14]. Within the glycocalyx, hyaluronan is a key determinant of many biomechanical and hydrodynamic properties such as its function in fluid exchange and shear sensing properties [15-19]. In particular, its physical properties in solution contribute to the function of hyaluronan [2,20,21]. Even small amounts of hyaluronan can bind large amounts of water molecules, forming a gel-like structure. Small molecules like water, electrolytes and nutrients can freely diffuse through the solvent. In contrast, large molecules, such as proteins, are excluded because of their hydrodynamic sizes in solution. In this way, hyaluronan contributes to the barrier function of the endothelial glycocalyx. The negative charge of the sulfated heparan groups in HS chains further adds to the barrier function of the glycocalyx, by repelling negatively charged circulating proteins like albumin.

An intact glycocalyx also serves as the primary sensor of shear stress on ECs. Hydrodynamic drag arising from flowing plasma through this layer transmits fluid shear stress through the thin sub-layer where proteoglycans, glycoproteins and glycolipids are directly linked to the endothelial cytoskeleton [22]. Both, the presence of hyaluronan, possibly by linking to the epithelial sodium channel (ENaC) in the endothelium, and HS appear to be critical for this function [23]. For example, enzymatic removal of hyaluronan or HS from the glycocalyx results in reduced nitric oxide (NO) release during shear [17]. Heparinase III, a bacterial enzyme that cleaves HS proteoglycans, has a dramatic effect on the ECs’ ability to produce NO to shear stress and transfer shear forces to the actin cytoskeleton [8,24]. Interestingly, the enzyme chondroitinase, employed to selectively degrade CS, did not affect shear-induced NO production, underpinning the importance of HS for the biomechanical properties of the endothelial glycocalyx [25].

Inflammation
The gel-like anti-adhesive properties of the endothelial glycocalyx preclude direct leukocyte interaction with adhesion molecules on the endothelial surface. However, during their passage through the capillaries the leukocytes will compress the endothelial glycocalyx thus allowing for engagement of the microvilli of the leukocyte with the GAGs in the glycocalyx. For example, HS on the endothelium acts as a direct ligand for L-selectin [26,27]. Interestingly, this interaction is modulated by very specific HS sulfation patterning. Endothelial deletion of the biosynthetic enzyme, N-acetylglicosamine N-deacetylase-N-sulfotransferase-1 (NDST1) which leads to decreased sulfation of the HS chains in the endothelium, reduces the binding of L-selectin, and leads to an increase in neutrophil rolling velocity [28]. Inflammatory stimuli, such as TNF-α, induce
NDST1 which results in expression of HS domains on glomerular endothelium that are associated with inflammation [29,30]. In agreement, we recently showed that endothelial deletion of NDST1 results in reduced leukocyte extravasation in experimental anti-GBM glomerulonephritis [30].

The migration of leukocytes across the endothelium occurs along chemoattractant gradients composed of chemokines that have bound to the endothelium [5]. Nearly all members of the chemokine family bind to HS by way of a positively charged C-terminal domain. Again, this binding may be modified by chemical editing of HS chains. For example, changing the domain structure of HS by inactivation of HS2ST (which causes a compensatory increase in glucosamine N-sulfation and 6-O-sulfation) results in enhanced binding of IL-8 to the endothelium and increases inflammation [31].

Endothelial HS has also been shown to be critical for the function of the complement system. The function of many of the components of the complement system, including C1, C1q, C1 inhibitor, C2, C4, C4b, FactorB, FactorD, FactorH, Properdin, and complement receptors CR3(CD11b/CD18) (24) and CR4 (CD11c/CD18) is dependent upon binding to specific glycosaminoglycan domains [32-34]. The clinical importance and the specificity of these interactions are illustrated by patients with mutations in the short consensus repeats 19 and 20 of the complement inhibitor, factor H. These mutations result in impaired binding of factor H to the glomerular endothelium, but not to other endothelial beds. Upon a trigger that activates the complement system this may result in glomerular endothelial injury and presents itself clinically as atypical hemolytic uremic syndrome [35].

In addition to HS, hyaluronan has also been demonstrated to modulate inflammatory processes. The HMW form of hyaluronan possesses anti-inflammatory, anti-angiogenic, and immunosuppressive properties and protects cells from injury, and leukocyte adhesion [36]. In part this is related to its gel-like physical properties. In addition, binding of tumor necrosis factor (TNF)-stimulated gene 6 (TSG-6) to hyaluronan inhibits chemokine-stimulated trans-endothelial migration of neutrophils via a direct interaction between TSG-6 and the glycosaminoglycan-binding site of CXCL8 [37]. However, during tissue injury and inflammatory processes hyaluronan can become depolymerized through oxidative stress and enzymatic cleavage by hyaluronidases. This results in the formation of low molecular weight (LMW) hyaluronan fragments [38]. Removal of LMW hyaluronan from sites of injury is dependent on their interaction with CD44, and the associated downstream signaling events have been implicated in cell proliferation, migration and activation. LMW hyaluronan also has been demonstrated to interact with Toll-like receptors enabling hyaluronan signaling in inflammatory cells [38,39].
Anticoagulation

Both the biomechanical properties as well as glycocalyx-protein interactions play a prominent role in preventing the blood from clotting. The gel-like properties of the glycocalyx prevent platelets from accessing the endothelium [40]. HS in the glycocalyx is required to activate antithrombin, a serine protease that inhibits thrombin and thus the conversion of fibrinogen to fibrin. By acting as a scaffold and simultaneously binding to both enzyme and substrate, HS greatly increases engagement of thrombin with antithrombin [41,42]. This interaction depends upon a specific pentasaccharide, an insight that led to the clinical development of fondaparinux. By binding the plasma protein Heparin cofactor II (HCII), heparan sulfate repeats in the glycocalyx further inhibit the pro-coagulant activity of thrombin. The latter pathway has been shown to counteract thrombus formation in the presence of endothelial injury [43].

A key regulator of the natural anticoagulant system is the endothelial glycocalyx protein thrombomodulin. Upon binding of thrombin it generates activated protein C (APC). APC regulates blood coagulation by cleaving and inhibiting two cofactors, activated factor V (FVα) and activated factor VIII (FVIIIa), which serve as phospholipid- membrane-bound cofactors to factor Xa (FXa) and factor IXa (FIXa), respectively. APC not only has anticoagulant properties, but also through PAR signaling induces a quiescent phenotype in the endothelium [44,45]. Activation of this pathway could prevent the development of diabetic nephropathy, underscoring the importance of APC formation in prevention of kidney disease [45]. Finally, thrombomodulin-thrombin binding activates thrombin-activatable fibrinolysis inhibitor (TAFI) thus suppressing fibrinolysis at the same time. This system is intensively modulated by interactions with glycosaminoglycans. First, thrombomodulin can bind chondroitin sulfate, greatly facilitating the interaction with thrombin and the formation of APC [46,47]. The activity of APC and thrombin in their turn are negatively regulated by protein C inhibitor which form together with different HS repeats ternary complexes [48]. While very complex and still only partially understood the intricate interaction of the coagulation system with the glycocalyx demonstrates the importance of the latter as a molecular scaffold.
**Risk factors for endothelial glycocalyx degradation**

The EG has a vulnerable location between the endothelium and flowing blood which might contain circulating risk factors such as glucose, lipids and inflammatory intermediates. In patients, acute hyperglycemia is associated with perturbation of EG and vascular permeability [49]. In diabetes mellitus type 1 (DM1) patients, increased levels of circulating hyaluronidase were observed [50]. In vitro, both an upregulation of the EG degrading enzyme heparanase [51] and a marked reduction in the biosynthesis of GAGs was observed upon high glucose stimuli in glomerular endothelial cells (GEncs) [52]. An association between plasma LDL cholesterol levels, reduced expression of endothelial GAGs and increased wall thickness at carotid lesion-prone sites was observed in previous studies, suggesting glycocalyx damage during hyperlipidemia [53,54]. These data concur with a study that demonstrates partial restoration of endothelial glycocalyx volume upon normalization of LDL levels [55]. Another type of risk factors affecting the endothelial glycocalyx are inflammatory mediators. For example, TNFa increases permeation of macromolecules into the ESL in hamsters and also LPS administration results in an induced dysfunction of the microcirculation accompanied by glycocalyx degradation [56]. Also, TNFa stimulation has been demonstrated to change expression of HS modifying enzymes and increase inflammatory epitopes within GEnc HS [29]. Endothelial damage upon stimulation with risk factors can occur both directly, such as by oxygen radicals produced during cellular stress that alter the composition of the EG [57-59], as well as indirectly through chronic stimulation resulting in changes in production and modification of GAGs by changing the expression of the involved synthesizing- and or degrading enzymes such as hyaluronidase and heparanase [50,58].
Current treatment options to stabilize the endothelial glycocalyx

The proposed role of the EG as target for cardiovascular risk factors implies that the EG could be an interesting target for therapies against the development of vasculature-born pathologies. First of all, as endothelial dysfunction seems to be closely associated with endothelial glycocalyx loss, current medication based on improving vascular or endothelial function will most likely also affect the endothelial glycocalyx. Stabilizing the endothelium, for example with angiopoietin 1 (Ang1), has been shown to enhance glycocalyx thickness in frog mesenteric cells and increase GAG production in human microvascular endothelium [60].

In contrast to these indirect effects, several options have been proposed to directly influence endothelial glycocalyx. This is done by GAG supplementation. One of the most well studied glycosaminoglycan supplements is sulodexide; a highly purified mixture of low molecular weight heparin (80%) and dermatan sulfate (20%). Although its function has been prescribed to different mechanisms over the past years, its anti-albuminuric effect has been demonstrated in several studies [61-63]. For example, in male participants with type 2 diabetes (T2D), sulodexide administration led to an increase in systemic glycocalyx thickness. It showed a reduction in plasma hyaluronidase, as well as a trend towards the normalization of systemic albumin clearance [64]. Although a recent study showed that sulodexide failed to demonstrate renoprotection in overt type 2 diabetic nephropathy patients [65], this can most likely be explained by the progression of type 2 diabetes. Restoring the EG has the most significant effects during the early stages of the disease, before irreversible morphological changes within the kidney occur. Indeed, animal studies showed that sulodexide ameliorates only early disease in models of radiation nephropathy and diabetic nephropathy in rats [66]. Nonetheless, the disadvantage of sulodexide is the heterogeneity of the composition, which might also explain the diversity in study results.

To solve this heterogeneity problem, a purified glycosaminoglycan should be produced. This targeting of specific heparan sulfate modification patterns seems to be a promising upcoming field. Several oligosaccharides and single chain antibodies directed against specific oligosaccharide compositions are being screened for their potential to block the HS moieties that initiate inflammation and angiogenesis on the endothelial surface [67-70]. The other way around, small peptides that resemble the HS binding region within a specific protein, might be developed to block the HS binding site. Furthermore, since increased amounts of heparanase and hyaluronidase are associated with a high variety of diseases, inhibiting such glycocalyx degrading enzymes might be interesting for future studies [71].
The endothelial glycocalyx in the glomerular filtration barrier

The kidney
In addition to the general composition and function of the endothelial glycocalyx in the vasculature, we studied the role of the endothelial glycocalyx in renal filtration. The human kidney contains approximately $0.8-1.5 \times 10^6$ nephrons, which are the functional units of the kidney. The normal glomerular filtration rate ranges from 130-180 liters per day [72]. Circulating blood enters the glomerulus via the afferent arterioles. In the glomerulus ultrafiltration takes place (figure 3). Here, water and small solutes, like salts, can pass through the glomerular filtration barrier, while larger molecular like proteins and cells are retained within the circulation.

Figure 3: Structure and function of the kidney and glomerulus. One of the main functions of the kidney is glomerular filtration. Blood enters the kidney (top left) and flows through the afferent artery into the glomerulus (bottom left). Here the blood is filtered: fluid containing small solutes passes the filtration barrier, while bigger components, such as proteins are maintained within the vasculature. The glomerular stalk (right) consists of mesangial cells, endothelial cells, glomerular basement membrane and podocytes. The combination of these cells is necessary to maintain the structure and function of the glomerulus. The filtration barrier, better shown in figure 4, is responsible for the filtration within the glomerulus. The bowman’s space, where the filtrate is collected, is lined by parietal epithelial cells. From here, the filtrate goes through the tubuli, where reabsorption of essential components such as glucose and sodium takes place.
Barrier function in the glomerulus

Although still under some debate [73], most evidence points towards the glomerular filtration barrier (GFB) as the main site of filtration. Farquhar demonstrated, using electron microscopy, that in intact glomeruli only about 0.06% of plasma albumin gets filtrated [74,75]. Although tubular reabsorption may play a role in fine-tuning the leakage of albumin, the GFB is assumed to play the key role in filtration. This filtration function of the GFB is maintained by the three layers of the filtration barrier: endothelium, glomerular basement membrane (GBM) and podocytes. Together with the mesangium, these components form the glomerulus: a unique non-clogging filter that can endure the filtration for over a lifetime. Although all layers and its specific roles within the GFB are often studied separately, it should always be taken into account that all these layers depend on each other’s structure and function to form this highly specialized glomerular filtration barrier. For example, disturbed VEGF signaling from the podocytes affects endothelial health [76], and perturbed PDGF signaling from the endothelium affects the mesangium [77].

Until recently, it was reasoned that the endothelial layer could not contribute to the filtration barrier because of its large fenestrae. These fenestrae lack a diaphragm and can therefore be considered as holes of about 60-80 nanometer [78]. These holes support the passage of high volumes of water, but are also large enough to allow proteins like albumin to pass the endothelial layer. Consequently, most studies focused on the GBM and podocytes as main components of the filtration barrier. However, recently Weil et al. showed that changes in the endothelial layer were associated with glomerular filtration rate and albumin-creatinine ratio in T2D patients [79,80].

A role for the endothelium in the filtration barrier is further supported by the little variation in reflection coefficient between fenestrated and continuous capillary beds [81,82]. Ryan et al. demonstrated that under physiological conditions hardly any albumin is observed in the GBM or underlying podocytes [83]. This can be explained by the observation that endothelial fenestrae are filled with endothelial glycocalyx [84,85]. Consequently, the endothelial glycocalyx within the fenestrae was proposed to be the first barrier within the GFB that excludes proteins [86]. Glycosaminoglycan-degrading enzymes such as chondroitinase and heparinase have been shown to alter the charge selectivity of the glomerular filter [87,88]. In addition, even local displacement of only the non-covalently bound components of the renal glycocalyx has been demonstrated to result in a 12-fold increase in fractional albumin clearance [89]. This indicates that besides GAGs, the loosely bound plasma proteins are also essential for the structure of the endothelial glycocalyx and for the barrier function of the glomerulus.

Although these data clearly indicate a role for the endothelial glycocalyx as filtration barrier, the exact mechanism is yet unknown. It has been postulated that glycocalyx polyanions such as heparan sulfate and sialated proteins on the surface of the endothelium and podocytes, as well as in the glomerular basement membrane would electrically repel
the negatively charged albumin [90,91]. Although this is supported by data with non-metabolizable negatively charged probes that would support that electric charge would modify filtration of albumin [92,93], this concept has been challenged by in vivo studies of the fractional clearance of negatively charged Ficoll as compared with Ficoll where negative charge selectivity did not exist [94,95].

Irrespective of the debate about charge exclusion, it is very plausible that the glycocalyx acts as a size barrier to albumin filtration. The glycocalyx basically functions as a hydrogel where glycosaminoglycans, i.e. heparan-, chondroitin sulfate and hyaluronan, constitute a fiber mesh with pores [20,96,97]. Plasma proteins bind multivalent to these glycosaminoglycans, thus creating steric hindrance to protein filtration. This barrier function will be further modified by the fluid drag through the glycocalyx which allows for dynamic equilibrium with glycocalyx-bound and free circulating proteins [98]. Such a size barrier function of the endothelial glycocalyx has in fact also been suggested from observations in the microcirculation in general, where the hydrostatic and osmotic forces that determine fluid filtration were shown to exist only across the glycocalyx and not across the endothelial cell towards the interstitium [99]. It is of interest that elongated molecules, such as bikunin and hyaluronan have >100-fold higher glomerular sieving coefficients than albumin, despite similar molecular weights and charges [100]. While such experiments may be challenged by confounding factors due to metabolism of albumin by the proximal tubulus [101], they can also be interpreted to demonstrate the importance of pore characteristics in relationship to the molecular conformation of the protein sieved. The latter is further supported by recent observations that large straight nanotubes (200-300 nm) may be filtered by the glomerulus similarly to small molecules [102].
Introduction and outline

Scope of this thesis

In the introduction we described a role for the EG in the onset of development in several vasculature-related pathologies like cardiovascular disease, diabetes, but also renal failure. In this thesis the structure and function of the EG and its components in the vasculature in general and in the glomerular capillaries have been studied. In addition, methods to determine changes in the EG and the relation with known vascular damage markers in healthy and diseased subjects have been newly developed.

In Chapter 2, we mainly focus on the challenges in visualization and quantification of the endothelial glycocalyx. Here, the different methods to study ESL thickness and composition, both in vitro and in vivo, are discussed.

Because it has been demonstrated that EG is absent in vitro, we hypothesized that mimicking the in vivo situation by subjecting the cells to prolonged shear stress would change the endothelial glycocalyx dimensions and composition. This will be further discussed in chapter 3.

The EG contributes to the permeability function of the endothelium throughout the whole vasculature. In the fenestrated endothelium of the kidney, the EG is also present within the fenestrae, suggesting a role for the EG in glomerular filtration. The main EG component that contributes to endothelial permeability has been proposed to be HA. In chapter 4 we studied the role of the EG, and specifically HA, in the filtration barrier. Therefore, we systemically removed HA with hyaluronidase and examined the effects on the function of the filtration barrier.

In chapter 5, we used a novel method to measure changes in the EG in end stage renal disease patients. Using SDF imaging of the sublingual microvasculature, we looked into variations in the width of the RBC column as a measure for changes in the EG. In addition, we looked into endothelial activation and glycocalyx shedding to determine the association between renal function, endothelial dysfunction and glycocalyx shedding. Finally we studied the ability of the EG to recover after successful kidney transplantation.

In chapter 6 we studied the EG in a cohort of healthy but obese participants. Here we demonstrated the relation between microvascular perfusion and EG thickness. Furthermore, these participants have a higher risk for the development of cardiovascular disease. Therefore this study functions as baseline measurement for future follow-up studies. Consequently the question whether early changes in EG predict cardiovascular events will be studied.

Finally, in chapter 7 this thesis is summarized and the relevance of these results in combination with the current knowledge in literature is discussed. Furthermore, some future perspectives are proposed.
References

Introduction and outline


