Primary cilia on endothelial cells : component of the shear stress sensor localized to athero-prone flow areas

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Chapter 1

General Introduction

1.1 Hemodynamics in development and pathology

Mechanobiology is receiving enormous interest in the field of cardiovascular research, because of emerging evidence that it plays a major role in cardiovascular development and pathology. Biomechanical forces that act upon the vessel wall include shear stress and (cyclic) stretch. They modulate endothelial structure and function through mechanosensing/transducing mechanisms, ultimately resulting in regulation of endothelial gene expression (reviewed by Lehoux *et al.*[1]). Shear stress is the parallel frictional drag imposed on endothelial cells by the viscous blood. Stretch, which is related to blood pressure, acts perpendicular to the endothelium. Stretch primarily affects the vascular smooth muscle cells, whereas endothelial cells are most responsive to shear stress. The focus of this thesis is on shear stress and the endothelial biosensor for shear stress. Background information is provided for shear stress and its role in cardiovascular development and pathology.

1.1.1 Shear stress

Shear stress is the force per unit area imposed by a fluid in motion on a solid boundary in parallel to the direction of the fluid in motion. In case of the cardiovascular system blood induces shear stress on the endothelium. Shear stress is measured in dyne/cm^2 or N/m^2 (= Pascal (Pa), 1Pa = 10 dyne/cm^2). When fluid flows through a straight tube flow velocity is highest at the center and drops towards the wall. Shear stress is proportional to the velocity gradient (shear rate) near the wall. This velocity gradient is due to frictional forces between adjacent layers of the fluid and between the fluid and the wall. These forces arise from the viscous properties of the fluid. Viscosity is dependent on the composition of the fluid. Blood is a mixture of erythrocytes, leukocytes, thrombocytes, and plasma. Plasma behaves as a Newtonian fluid, i.e., it has a constant viscosity at all shear rates. Whole blood, on the other hand, is non-Newtonian, i.e., blood viscosity depends on shear rate. Because analyzing the behavior of a non-Newtonian fluid is far more complex than analyzing a Newtonian fluid, whole blood is considered a Newtonian fluid in many computational studies. In vessels with a diameter smaller than 300 μm, the fluid layer over the endothelium is indeed Newtonian, as a cell-free layer of plasma with reduced viscosity is present near the wall. This is known as the Fåhraeus-Lindqvist effect[2]. Steady, laminar flow of a Newtonian fluid in a cylindrical tube with rigid walls develops into a parabolic velocity profile, with the highest velocity at the center of the tube. This type of flow is termed Poiseuille flow. Shear stress can subsequently be calculated by the Hagen-Poiseuille formula: $\tau = 4\mu Q/\pi R^3$, where τ is the shear stress, μ is the blood viscosity, Q is the volumetric flow rate, and R is the lumen radius. However, considering blood flow as Poiseuille flow requires simplifications that are not physiologically relevant, as blood vessels, and in particular the heart, are not straight and rigid tubes. Nevertheless, these simplifications are acceptable to obtain approximations of wall shear stress values[3].

With respect to the cardiovascular system the viscosity and pulsatility of blood flow and the curvature of blood vessels should be considered. The Reynolds number (Re), which is dependent on the viscosity, density, and velocity of the blood, and on the lumen radius of the blood vessel, represents the ratio of inertial forces to viscous forces. It is a measure of the stability of flow, i.e., whether it is laminar or turbulent. Inertia is the property of an object to remain at constant velocity unless acted upon by an outside force. When Re is very low (< 1) viscous forces dominate over inertial forces, the flow is laminar and follows the vessel geometry. This is the case in the embryonic system. In the adult system, on the

other hand, inertial forces dominate over viscous forces (Re >> 1). Between a Re of 500 and 1500 flow is still laminar but turbulence can occur, dependent on the vessel geometry (bends and bifurcations) and flow pulsatility[4]. Turbulent flow is characterized by regions of separation, recirculation, and temporal as well as spatial gradients of shear stress and is termed oscillatory (multi- or bidirectional) shear stress. Laminar, non-turbulent flow, is unidirectional and termed steady (veins) or pulsatile (arteries) shear stress. The effect of pulsations on the flow profile is described by the Womersley number (α). In cases in which α is greater than unity, the flow profile will flatten compared to the parabolic poiseuille flow profile. In the embryo, α does not exceed unity. Furthermore, as blood vessels and the heart are not straight tubes the Dean number (Dn) should be taken into account. Dn describes the effect of the curvature of a vessel on the flow profile. With large values of Dn in the adult situation the maximum flow velocity shifts toward the outer wall of the curvature. However, the combination of the strong curvature of the embryonic heart and the low Re results in a small Dn and a shift of the maximum velocity toward the inner curvature[5,6].

Shear stress drives gene expression in endothelial cells *ex vivo*[7,8] and *in vivo*[9,10]. As a consequence, shear stress plays a crucial role in remodeling of the heart and vasculature. A key component in the endothelial flow response is the shear-responsive gene *Krüppel-like factor-2* (*KLF2* or *LKLF*), a zinc finger transcription factor. *KLF2* expression is confined to areas of high shear stress in the embryonic[9] and adult cardiovasculature[10]. *In vitro, KLF2* expression is upregulated by high steady or pulsatile shear stress[8,10] but is downregulated by prolonged oscillatory shear stress[11]. KLF2 acts as a switch between the quiescent and activated state of the endothelium through regulation of multiple genes[12]. Furthermore, it plays a role in vascular tone regulation by upregulating expression of *endothelial nitric oxide synthase* (*eNOS*), which produces the vasodilator nitric oxide (NO), and downregulating expression of *endothelin-1* (*ET1*), a vasoconstrictor[10]. Alterations in shear stress, therefore, result in adjustment of the vessel diameter and subsequent maintenance of shear stress level.

1.1.2 Shear stress and development

Shear stress-related gene expression patterns of *KLF2*, *ET1*, and *eNOS* are already present in early cardiovascular development[9]. Consequently, shear stress is believed to play a substantial role in cardiovascular development. The early embryonic heart is a nearly straight tube that develops into a four-chambered pump through extensive growth and remodeling. This generates changes in luminal diameter and accordingly shear stress. The animal model we use to study shear stress and heart development is the chicken embryo. This is a suitable model as shear stress levels are in the same range as in the human adult arterial system (up to 70 dyne/cm^2)[13]. The velocity gradients near the wall and concomitant shear stress can be measured in the beating chicken embryonic heart, *in ovo,* with a high speed optical technique called micro particle image velocimetry (μPIV). A maximum wall shear stress of 50 dyne/cm^2, was measured in the outflow tract (OFT) of chicken embryos[14].

The chicken hatches after 21 days, which corresponds to Hamburger and Hamilton stage (HH)[15] 46. At HH10 the heart begins to contract[16], which is just before looping starts (see below). A relatively stable laminar blood flow pattern is present from HH12 on[17] but oscillations in flow still occur due to movement of the cardiac wall. Below, heart development is described correlated to the stages of chicken development, as reviewed by Martinsen[18].

Heart development starts at HH7 with the formation of the bilateral endocardial heart tubes. These tubes fuse and form the early embryonic heart tube[19] at HH8. This tube consists of an inner endothelial layer and an outer myocardial layer, which are separated by cardiac jelly, an extracellular matrix (ECM)[20]. The first stage of looping (C-shape) (reviewed by Männer[21], Gittenberger-de Groot *et al.*[22], Taber[23]) occurs from HH10 to HH12/13 and is regulated by several genes essential in left-right signaling in embryos[24] but independent from blood flow[23,25]. At the beginning of the second phase of looping (S-shape), which occurs between HH12/13 to HH18, blood flow is initiated and shear stress becomes a crucial factor. The heart lumen is wide and shear stress is expected to be moderate. Endocardial cushion development (HH12-36) and cardiac septation (HH17-46) separate the heart into four chambers, resulting in local changes in shear stress. The endothelial/endocardial cells overlying the cushions undergo the process of epithelial-to-mesenchymal transformation (EMT) and invade the cushion ECM[26]. The endocardial cushions, subsequently, develop into the semilunar valves and the AV valves (mitral and tricuspid valves), which are fully developed at HH34 and HH36, respectively. The endocardial cushions cover the narrowest passages of the heart and are, therefore, exposed to high levels of shear stress, which is exemplified by the patterning of *KLF2* expression[9]. Cushion development also contributes to septation. Formation of the interatrial, interventricular, and OFT (aorticopulmonary) septa alters lumen diameter and, consequently, shear stress. All three septa are completed at approximately HH34. However, the atrial septum undergoes another conformational change after birth when the foramen ovale closes. During atrial septum development shunting of blood from the right atrium to the left atrium is always possible due to multiple fenestrations, rendering intricate flow patterns. The ventricular trabeculations start to form at HH16 and become more pronounced over time, affecting shear stress patterning as well.

The pharyngeal arch artery (PAA) system, the connection between the OFT of the heart and the aorta, remodels from a symmetrical to an asymmetrical system. This asymmetric remodeling is dependent on genetic factors and shear stress, as was demonstrated in mouse embryos. The genetic programme induces a morphological change in the OFT, which affects shear stress in the PAAs. The alterations in shear stress regulate the persistence or regression of the PAAs[27]. Eventually, the PAA system of the chicken embryo includes the brachiocephalic arteries (left and right 3rd), an aortic arch (right 4th), and the ducti arteriosi (left and right 6th)[28,29].

Several animal models show that disturbing hemodynamics during development results in congenital heart malformations[30-33]. The model we use to disturb hemodynamics during chicken embryonic development is the chicken venous clip model[30] in which blood flow through the embryo is experimentally-altered through ligation of the right lateral vitelline vein, which is one of the veins that drains blood from the yolk sac vessels into the embryo. Vitelline vessel development is related to intracardiac flow patterns. Blood from specific yolk sac regions follows a specific stage-dependent intracardiac route[17]. Venous clip reduces preload[34] and shifts blood flow towards the inner curvature of the heart[35]. A decrease in mean and peak blood flow up till 5 hours after clip is reported for the dorsal aorta[34]. This is followed by a rapid shift, within 3 hrs, in *KLF2* expression, which is suggestive for an increase in cardiac shear stress and a decrease in aortic shear stress[36]. Permanent ligation results in a spectrum of congenital cardiovascular anomalies[30,35] and leads to structural adaptations of the heart that becomes less compliant[37-39]. In conclusion,

venous clip induces shear stress alterations that eventually result in cardiac malformations, demonstrating the importance of hemodynamics on cardiovascular development.

1.1.3 Shear stress and disease: atherosclerosis

Besides the major impact of hemodynamics on development it plays an equally important role in adult life. The main cause of cardiovascular death is a disease of the arterial wall, called atherosclerosis. Atherosclerosis is an inflammatory disease characterized by, e.g., the accumulation of lipids within the arterial wall. Atherosclerotic lesions can lead to ischemia (infarction) of the heart, brain, or extremities. Risk factors for atherosclerosis include elevated and modified low-density lipoprotein (LDL); free radicals caused by smoking, hypertension, and diabetes; genetic susceptibility; elevated plasma homocysteine concentrations; and infectious micro-organisms. Atherosclerosis originates at specific sites in the circulation (see below), where the endothelium is activated or primed for atherosclerosis. At these sites, endothelial cells express adhesion molecules that are responsible for the adherence, migration, and accumulation of monocytes and lymphocytes (reviewed by Ross[40]). In the presence of cardiovascular risk factors, activated endothelium can become dysfunctional, which is the start of lesion formation. Endothelial dysfunction is followed by an inflammatory response, which in turn stimulates migration and proliferation of smooth muscle cells into the lesion. The resulting thickening of the arterial wall is compensated by gradual dilation to maintain lumen diameter, a process termed outward remodeling. Continued inflammation results in invasion of monocytes and lymphocytes from the blood into the lesion, where they proliferate and differentiate, leading to further enlargement of the lesion. The lesion then restructures and becomes covered by a fibrous cap overlying a lipid core and necrotic tissue. At some point further remodeling is not possible and the lesion protrudes into the lumen, resulting in alterations in blood flow[40,41].

Although the etiology of atherosclerosis is systemic, lesions develop locally, i.e., exclusively at sites of low and disturbed blood flow and concomitant low and multi- or bidirectional (oscillatory) shear stress. Therefore, the role of shear stress in atherosclerosis has gained enormous interest in the last decade. Low and disturbed flow is invoked by the geometry of the vasculature, i.e., by arterial bifurcations, branch points, and the curvature of arteries. The endothelium plays an essential role in atherogenesis as it translates the mechanical signal into a biological response. The endothelium is differentially affected by high or low shear stress and by unidirectional or bidirectional shear stress, causing the flow-determined occurrence of lesions. Low and bidirectional shear stress correlates with endothelial activation (athero-prone), whereas high and unidirectional shear stress is associated to a quiescent phenotype of endothelial cells (athero-protective)[42,43]. Athero-prone flow profiles prime the endothelium for atherosclerosis, resulting in atherosclerosis when cardiovascular risk factors are present. Athero-prone shear stress induces formation of reactive oxygen species through uncoupling of the eNOS enzyme[44] and it increases the permeability of the endothelial layer through disruption of the glycocalyx[45]. It induces inflammatory gene expression profiles[4], downregulates expression of *KLF2*[11] rendering the endothelium in an activated state[12], and induces expression of adhesion molecules[40].

The animal model we use to study shear stress and atherosclerosis is the apolipoprotein-E-deficient (*Apoe*$^{-/-}$) mouse. This is a suitable model as *Apoe*$^{-/-}$ mice are hyperlipidemic, develop atherosclerosis spontaneously, i.e., not diet-induced, and have lesions similar to those in man[46]. Lesion formation can be accelerated by feeding *Apoe*$^{-/-}$ mice a high-fat diet[47] or by experimental induction of low and/or disturbed shear stress[48].

To gain insight into the participation of specific proteins in atherosclerosis, genetically modified mice are interbred with *Apoe*$^{-/-}$ mice. In this respect, we used Connexin (Cx) deficient (*Gja4*$^{-/-}$) *Apoe*$^{-/-}$ mice (Chapter 9). Connexins are the structural components of gap junctions that mediate intercellular communication. Endothelial cells possess 3 types of connexins, i.e., Cx 37, 40, and 43[49], which can form homo- or heteromeric channels. Cx37 was believed to play a role in atherosclerosis after the observation of genetic polymorphisms of the human gene coding for Cx37 in patients with atherosclerosis[50]. Lesion formation is accelerated in *Gja4*$^{-/-}$ *Apoe*$^{-/-}$ mice when compared with *Gja4*$^{+/+}$ *Apoe*$^{-/-}$ mice[51].

1.2 The primary cilium

The differential response of endothelial cells to various velocity patterns is directed by the mechanosensing/transducing machinery. The shear stress sensing apparatus could therefore be involved in pathological alterations of endothelial cells. Recently, primary cilia were shown to function as shear stress sensors of several flow exposed cell types. We hypothesized that primary cilia function as shear stress sensors of endothelial cells. Background information is provided for cilia structure and function.

1.2.1 Ciliogenesis

Cilia are membrane-covered, rod-like cellular protrusions. They contain microtubules, which are a major constituent of the cytoskeleton. Microtubules consist of two subunits, i.e., α- and β-tubulin that form protofilaments by alternating addition of subunits at the plus-ends of the protofilaments. One microtubule contains 13 protofilaments organized in a tubular structure. The template on which microtubules nucleate is γ-tubulin. γ-tubulin is found in the centrosome, which functions as the microtubule organizing center (MTOC) of the cell. The centrosome consists of two interconnected[52] centrioles, i.e., the mother (oldest) and daughter centriole, and pericentriolar material (PCM) located around the centrioles. γ-tubulin is present in the PCM and in subdistal appendages attached to the mother centriole[53]. The cytoskeletal microtubules nucleate from the γ-tubulin in both these structures[54,55]. The microtubules of a cilium, the ciliary axoneme, nucleate on the distal site of a centriole, which is located just beneath the plasma membrane. When a centriole nucleates a cilium it is called a basal body. The axoneme of the basal body consists of 9 triplets of microtubules, whereas the ciliary axoneme has 9 microtubule doublets. Two types of cilia exist, i.e., motile and immotile cilia. If the 9 doublets surround a central pair of singlet microtubules and have axonemal dynein arms, which are responsible for ciliary movement, attached the cilia are motile (9+2). If they lack central microtubules and axonemal dynein arms they are immotile, termed primary cilia (9+0). Nucleation of the central pair of singlet microtubules is dependent on the presence of γ-tubulin within the distal central part of the basal body[56]. The basal body of a motile cilium forms in a centrosome-dependent or independent pathway and migrates towards the cell surface[57]. The basal body of a primary cilium is always the mother centriole of the centrosome[58], which does not contain γ-tubulin in its distal central part and can, therefore, not nucleate a central pair of microtubules[56]. As the mother centriole functions as the basal body of a primary cilium and as a nucleation site for cytoskeletal microtubules the primary cilium and the cytoskeletal microtubules are physically connected. Due to this connection to the centrosome the occurrence of primary cilia is cell cycle-dependent. Cell surface protruding primary cilia are present during the G0 and G1 phase of the cell cycle, whereas primary

cilia immersed in the cytoplasm can be observed during the S and G2 phase[58]. This is due to ciliary resorption for disassembly in the cytoplasm upon entry into the cell cycle[59]. In contrast, motile cilia are cell cycle independent as they form on terminally differentiated cells[60]. Cells can present multiple motile cilia but only one primary cilium. The immotile primary cilium has sensory functions[61](see below) and are found on nearly all mammalian cell types[62]. Motile cilia beat in a wave-like manner. The axonemal dynein arms on one microtubule doublet generate force against the adjacent doublet, causing them to slide past one another. The central pair of microtubules regulate dynein movement[63]. Motile cilia can propel a cell (single-celled organisms) or transport fluid over the surface of the cell, e.g., on epithelial cells lining the airways and reproductive tracts and epithelial cells of the ependyma and choroid plexus in the brain[64]. Two additional types of motile cilia exist, i.e., motile 9+0 cilia and flagella. Motile 9+0 cilia are located on the mammalian embryonic node (homologous to Hensen's node in the chicken and the dorsal lip of the amphibian blastopore), the organizing center of the early embryo, and function in determining left-right asymmetry[65-67]. In contrast to primary cilia they do contain axonemal dynein arms but as they lack the central pair of microtubules, movement is not regulated, resulting in a twirling motion. The axoneme of flagella is identical to that of motile cilia (9+2) but flagella are longer and cells have only 1 or 2 flagella (spermatozoa and protists)[60].

Ciliary assembly and maintenance requires microtubule motor-based transport of axoneme subunits from the body of the cell to the ciliary tip as cilia themselves lack the machinery for protein synthesis. This process is bidirectional and is called intraflagellar transport (IFT), which was discovered in the green alga *Chlamydomonas*. Besides the continuous supply of microtubule subunits, which is necessary because of the constant turnover of microtubules at their distal plus-ends, IFT is required for the transport of specialized proteins to the ciliary membrane and the transport of various signals from the cilium to the cell body and vice versa. Microtubule motor proteins move in a single direction between the ciliary membrane and the outer doublet microtubule. Kinesins are responsible for anterograde movement of IFT particles. Dyneins direct retrograde movement of IFT particles and motors, which are thus recycled. Between the cell cytoplasm and the ciliary cytoplasm is a boundary demarcated by transition fibers that controls movement of particles between both compartments. Proteins destined for the cilium contain a specific amino-acid sequence through which they are targeted to the cilium and carried through the transitional zone (reviewed by Rosenbaum and Witman[68]).

Mutations in any of the IFT components lead to defective ciliogenesis. Defects in ciliogenesis or ciliary function are associated to several pathologies. A defect in motile cilia, rendering them immotile, is called primary ciliary dyskinesia (for example Kartagener syndrome). This disease can affect all tissues that possess motile cilia. It encompasses respiratory tract defects, infertility, laterality disturbances, ectopic pregnancies, and hydrocephalus[69]. Defects in primary cilia were associated to human disorders such as Senior-Loken, Alström, Orofaciodigital, Jeune, Usher, Meckel(-Gruber), and Bardet-Biedl syndrome. These syndromes can include anosmia, laterality defects, sensorineural deafness, vestibular impairment, retinitis pigmentosa, and polycystic kidney diseases[60,68-70]. Ciliopathy phenotypes include obesity, diabetes, hypertension, and cardiac abnormalities[71].

1.2.2 Primary cilia function

Although the existence of primary cilia is known for more than a century, investigations into their functionality arose in the last decade after they were associated to several human

syndromes. They were demonstrated to function in olfaction, photoreception, chemosensation, and mechanosensation. The mechanosensory function makes primary cilia attractive for our study on blood flow-induced shear stress sensing by endothelial cells. Primary cilia are very sensitive mechanosensors[72], responding to shear stresses as low as 0.007 dyne/cm^2. They function as flow sensors of kidney epithelial cells[73], nodal cells[65,66], osteocytes and osteoblasts occupying the fluid filled cavities (lacunae) in bone matrix[74,75], and bile duct epithelial cells[76]. The various ciliary functions, as reviewed by Satir and Christensen[64] and by Singla and Reiter[70], are described below.

Olfaction
Primary cilia of olfactory sensory neurons function in olfaction. An odorant binds to a G-protein-coupled receptor (GPCR) localized in the membrane of the primary cilium, producing the second messenger cyclic adenosine monophosphate (cAMP). Elevated levels of cAMP then depolarize the cell by opening a cyclic nucleotide-gated channel, located in the ciliary membrane.

Photoreception
Light photons activate opsin GPCRs located in the ciliary membrane of rod and cone cells of the retina by increasing hydrolysis of cyclic guanosine monophosphate (cGMP), thereby closing cGMP-gated channels.

Chemosensation
The chemosensing function of primary cilia is gaining much interest after the discovery that they play a role in the Hedgehog and Wnt signaling pathways, key regulators of development that have been implicated in stem cell function and carcinogenesis[77]. The Hedgehog receptor Patched 1[78], its downstream mediators Smoothened[79] and Gli transcription factors[80], and the Wnt mediator Inversin[81] are localised in the primary cilium and are dependent on IFT. In neuronal cells, SST_3 somatostatin[82] and 5-HT_6 serotonin[83] receptors were observed in the ciliary membrane. Primary cilia of connective tissue cells, such as chondrocytes and fibroblast, are embedded in the ECM. Chondrocyte primary cilia sense mechanical (see below), physiochemical, and osmotic stimuli through ciliary receptors and ion channels. Platelet-derived growth factor receptor α (PDGFRα) localizes to fibroblast primary cilia and is involved in growth control[84]. In the absence of PDGF the primary cilium functions as a mechanosensor (see below) but in the presence of PDGF it acts as a chemoreceptor.

Whether primary cilia function as chemosensors on the embryonic organizing center is a matter of debate. The node contains two populations of cilia, i.e., motile 9+0 cilia in the center of the node and immotile 9+0 cilia at the periphery of the node. The motile cilia generate a left-ward (nodal) flow[67]. Two hypotheses about the function of cilia in determining left-right asymmetry exist. The flow establishes a gradient of signaling proteins, such as fibroblast growth factor, present in the nodal fluid that (I) induce signaling through activation of receptors present on the nodal cilia[85] and/or (II) the immotile cilia at the periphery of the node sense the left-ward flow, resulting in asymmetric Ca^{2+} signaling, triggering left-sided gene expression[65,66] (see below).

Mechanosensation
Besides functioning in chemosensation chondrocyte and fibroblast primary cilia function in mechanosensation. They make contact with the ECM through specific receptors on the

ciliary membrane and can therefore transmit mechanical forces (reviewed by Satir and Christensen[64]). In the ciliary membrane of the fruit fly *Drosophila Melanogaster* and the nematode *Caenorhabditis elegans* transient receptor potential (TRP) ion channels are located that respond to mechanical stimuli. In the cilia of auditory sensory neurons of *D. Melanogaster* they sense vibrations and in cilia of sensory neurons of *C. elegans* they sense touch[70]. In *C. elegans* a mechano-responsive complex of PKD-2 (vertebrate homolog polycystin-2 (PC2)) and LOV-1 (vertebrate homolog polycystin-1 (PC1)) localizes to the cilia of male-specific sensory neurons[86]. PC1 is an integral membrane protein with a large extracellular part, a transmembrane domain, and a C-terminal cytoplasmic tail, whereas PC2 is a Ca^{2+}-permeable, non-selective cation channel that is a member of the TRP channel family[87]. The link between polycystins and primary cilia is conserved from nematodes to mammals. Polycystins play a role in cilia-mediated sensing of another mechanical force: flow-induced shear stress. PC2 is present in the two populations of nodal cilia in mice[65], strengthening the mechanosensory hypothesis, and both PC1 and PC2 localize to the primary cilium of murine kidney epithelial cells, where they form a mechano-responsive complex[88]. Mutations in one of the polycystin genes in humans cause polycystic kidney disease (PKD). PKD is characterized by clonal expansion of kidney epithelial cells, resulting in cyst formation that causes kidney failure. In addition, both polycystins are present in ciliated osteocytes and osteoblasts[89] and in ciliated cholangiocytes[76]. In cholangiocytes flow induces ciliary- and polycystin-dependent rises in intracellular Ca^{2+}. Bending of bone primary cilia by flow results in altered gene expression, suggesting they function as mechanosensors, but cilium-mediated mechanosensing in bone is different, as the Ca^{2+} transient upon flow is independent of primary cilia or PC2[74].

A comparable role for primary cilia in endothelial mechanosensing is likely as (I) primary cilia are also present on endothelial cells[90-93], (II) a similar flow-induced rise in intracellular Ca^{2+} is seen[94], and (III) both polycystins were detected in these cells[95].

1.3 Endothelial mechanotransduction

Several cell components, other than primary cilia, have been put forward as potential endothelial shear stress sensors. They are described as sensors because blockage leads to abrogation of the shear stress response. However, they are more likely to be downstream of the shear stress sensor and are, therefore, termed mechanotransducers in this thesis. In this section background information on these mechanotransducing components, as reviewed by Lehoux *et al.*[1], Resnick *et al.*[7], and Li *et al.*[96], and the potential pathway from shear stress sensing to gene expression is provided.

1.3.1 Mechanotransduction

Cell-extracellular matrix and cell-cell junction molecules

Shear stress induces a conformational change of integrins, which are αβ heterodimers involved in cell-ECM interactions. The extracellular domain binds specific ECM components, such as fibronectin, vitronectin, collagen, and laminin. The cytoplasmic domain of integrins is functionally linked to cytoskeletal proteins and to focal adhesion sites. Interference of integrin-ECM interactions abrogates the shear stress response. Another mechano-responsive complex is located at the endothelial cell-cell junction. Platelet endothelial cell adhesion molecule-1 (PECAM-1; also known as CD31), vascular endothelial (VE)-cadherin, and vascular endothelial growth factor receptor 2 (VEGFR 2;

also known as FLK-1 or KDR) form a complex at adherens junctions. This complex is connected to the cytoskeleton via anchoring molecules such as catenins. Activation by shear stress results in activation of the NF-κB pathway[97].

Membrane structures
Several shear stress responsive components reside in the endothelial cell membrane, such as ion channels, including Na^+, K^+ and Ca^{2+} channels, receptors, and caveolae. Tyrosine kinase receptors, such as VEGFR2, are phosphorylated upon exposure to shear stress. Activation is independent of their ligands, suggesting a double role in chemo- and mechanosensation. In addition, nicotinamide adenine dinucleotide phosphate (NADPH) oxidases and G-proteins are activated upon the onset of shear stress. G-proteins are also located in caveolae, which are membrane domains that contain signaling molecules. Caveolae are rich in cholesterol and consequently more rigid than other parts of the plasma membrane. Upon shear stress caveolae are directed to the cell surface where they mediate the shear stress response. Another shear stress transducer connected to the plasma membrane is a hydrated polysaccharide coat covering the apical membrane, termed the glycocalyx. The glycocalyx might mediate mechanotransduction as degradation of the glycocalyx results in a reduced shear stress response[98,99].

Cytoskeleton
A structure that can tie all the shear stress responsive cell components together is the cytoskeleton. The cytoskeleton rearranges in response to flow and cells elongate along the flow direction[100]. The cytoskeleton comprises microtubules, actin microfilaments, and intermediate filaments that are interconnected[101], transduce force to various cell compartments, and translocate signaling molecules. Davies[102] proposed the decentralization model of endothelial mechanotransduction, i.e., shear stress is transduced by the cytoskeleton to multiple sites that are directly or indirectly connected to the cytoskeleton[100], which each generate a shear stress response.

1.3.2 Intracellular signaling pathways and gene expression

Several intracellular signaling pathways are activated in response to shear stress, which modulate expression of genes, including those involved in proliferation or growth arrest, inflammation or anti-inflammation. Shear stress modulates the activity of small GTPases (including Ras, RhoA, Rac1, Cdc42, Arf, Rab and Ran), which mediate both cytoskeletal reorganization and transcriptional activation of target genes[103], through induction of the NF-κB or MAP kinase cascades. The latter comprises activation of MEKK, MEK, and MAPK, involving ERK1/2, JNK or p38[1]. KLF2, the key regulator in shear-induced gene expression, is induced by high shear stress through the MEK5-ERK5-MEF2 and PI3K-nucleolin signaling pathways[104,105]. Activation of transcription factors such as NF-κB and KLF2 depends on the pattern of shear stress, i.e., steady, pulsatile, or oscillatory, resulting in a pro- or anti-inflammatory gene profile. NF-κB regulates expression of VCAM-1, ICAM-1, and E-selectin, these products are involved in vascular inflammation and cell viability. KLF2, on the other hand, is a potential mediator of the anti-inflammatory effects of shear stress, as it modulates pro-inflammatory signaling pathways, and can suppress NF-κB activation[106].

In conclusion, the exact mechanism of endothelial shear stress sensing remains to be elucidated but the cytoskeleton may play a central role. The cytoskeleton can transduce the

force throughout the cell to the connected shear-responsive sites. A consequence of that would be that blood flow-induced shear stress is sensed by the endothelium from the inside-out. A way to amplify cytoskeletal deformation upon shear stress could be the protrusion of a primary cilium into the lumen. In this thesis the precise relation between endothelial primary cilia and shear stress is described.

1.4 Chapter outline

Chapter 2 describes the distribution of endothelial primary cilia in chicken cardiovascular development. We show that the occurrence of primary cilia on endothelial cells is shear stress-related.
In *Chapter 3* the effect of venous clip on shear stress and the distribution of endothelial primary cilia is investigated. We demonstrate that venous clip appears to alter shear stress levels but not primary cilia distribution.
Chapter 4 describes the role of shear stress in looping of the embryonic chicken heart.
In *Chapter 5* the correlation between primary cilia occurrence and different flow profiles is studied. We show that endothelial primary cilia are induced by flow reversals.
Chapter 6 demonstrates that the endothelial shear stress response is dependent on an intact microtubular cytoskeleton and that primary cilia sensitize the endothelium for shear stress.
Chapter 7 describes a double mechanistic role for endothelial primary cilia in shear stress sensing.
Chapter 8 presents the distribution of endothelial primary cilia in the aortic arch and common carotid artery of wild-type and *Apoe*$^{-/-}$ adult mice. The effect of experimentally-altered shear stress on primary cilia distribution was analyzed by placement of a restrictive cast around an artery. A correlation between the presence of endothelial primary cilia and disturbed flow is demonstrated.
In *Chapter 9* the effect of Cx37 deficiency on primary cilia occurrence in the aortic arch of *Apoe*$^{-/-}$ adult mice is investigated. The absence of Cx37 does not affect the prevalence of primary cilia on endothelial cells.
The obtained results are discussed in *Chapter 10*.

1.5 References

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