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Assessing the role of albumin in the formation of the endothelial glycocalyx layer using a microfluidic in vitro model

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Declaration of Authorship

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02.02.2019

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1 Abstract

Background: Cardiovascular diseases (CVD) are the leading causes of mortality, responsible for 31% of all deaths worldwide. Endothelial dysfunction plays an important role in CVD in terms of development, progression and clinical manifestation of atherosclerosis by predisposing to thrombosis, leukocyte adhesion and smooth muscle cell proliferation. Low serum albumin levels have been described in CVD like ischemic heart disease, stroke and venous thromboembolism. Albumin has an effect on the coronary flow, most likely due to shear stress-transmission via the endothelial glycocalyx. The glycocalyx is a complex, carbohydrate-rich layer on the luminal side of vascular endothelial cells and in direct contact with the blood, forming a key element for the homeostasis in blood vessels. It is composed of membrane-bound proteoglycans with glycosaminoglycan side-chains like heparan sulfate and glycoproteins with carbohydrate side-chains. The consequences of glycocalyx shedding are a pro-coagulant and pro-inflammatory phenotype of the endothelium, increased capillary permeability, adhesion of immune cells, vascular inflammation and thrombosis. In the present study an in vitro microfluidic perfusion system was used to assess the role of albumin in the formation and preservation of the glycocalyx of wild type porcine aortic endothelial cells (PAEC) and to check whether additional albumin in the perfusion medium has an effect on their shape and alignment in flow direction.

Methods: PAEC were cultured under static conditions in micro channels of 550 μm diameter until confluency. A peristaltic perfusion with a flow of 860 μl/min, corresponding to a shear stress of 15 dyn/cm^2, was applied for 48 h. Perfusion medium (DMEM + 10 % FBS + 1 % Penicillin/Streptomycin + 4 % Dextran) was used, with or without additional bovine serum albumin (BSA). After perfusion the cells were fixed if needed and incubated under static condition with fluorescence-labeled WGA-lectin or anti-HS, anti-BSA, anti-F Actin or anti-CD31 antibody. The fluorescence imaging was performed by confocal microscopy.

Results: Perfusion with additional 0.5 % BSA significantly increased the expression of heparan sulfate on PAEC as shown by anti-HS staining. Perfusion with additional 1 % BSA increased the expression of Neu5Ac and GlcNac residues on PAEC as assessed by WGA-lectin. Furthermore, PAEC perfused with additional 1 % BSA showed fewer gaps in the confluent cell monolayer and a significantly increased elongation and alignment in flow direction compared to PAEC perfused without additional BSA.

Conclusion: Our data suggest that albumin is important for the formation of the endothelial glycocalyx as well as to maintain the functionality and health of endothelial cells. Addition of 1 % BSA to the perfusion medium improves the microfluidic in vitro model, making it more physiological by supporting a more in vivo-like structure of the endothelial cells. The serum albumin concentration may therefore also play an important role in cardiovascular disease.

2   Introduction

2.1   Cardiovascular disease

Cardiovascular disease (CVD) is a group of diseases comprising coronary heart disease (CHD), cerebrovascular disease, peripheral arterial disease, rheumatoid and congenital heart disease and venous thromboembolism. CVD is the leading cause of mortality, responsible for 31 % of all deaths worldwide. The prevalence of risk factors for CVD is raising in low risk countries and the rate of CVD is therefore predicted to increase worldwide. However the World Health Organization (WHO) estimates that more than 75 % of premature CVD-related deaths could be prevented [1] [2].
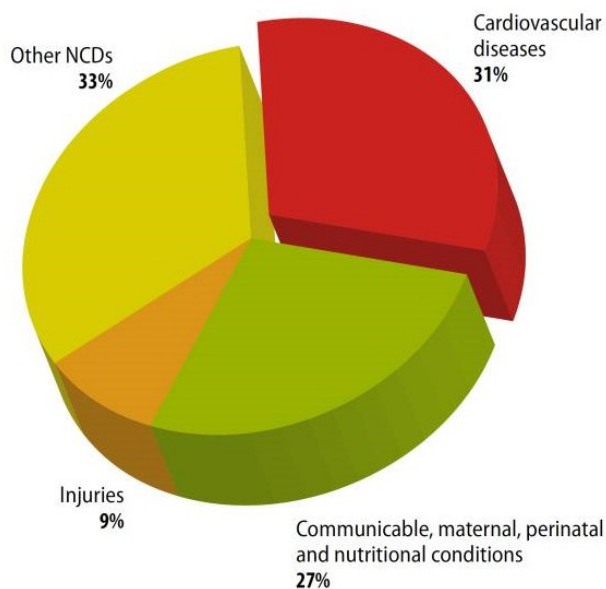
Figure 1: Distribution of major causes of death worldwide in 2008 including cardiovascular diseases (CVD), other non-communicable diseases (NCD), injuries and communicable, maternal, perinatal and nutritional conditions. Figure from WHO report [1].

Peripheral arterial disease (PAD) for example is a frequent atherosclerotic syndrome which is associated with a high morbidity and mortality [3]. Endothelial dysfunction plays an important role in development, progression and clinical manifestation of atherosclerosis by predisposing to thrombosis, leukocyte adhesion and smooth muscle cell proliferation [4].

Low serum albumin levels are closely related to ischemic heart disease, heart failure, atrial fibrillation, stroke and thromboembolism. Looking at the anti-inflammatory, antioxidant, anticoagulant and antiplatelet-aggregational physiological properties of serum albumin, hypoalbuminemia could be a modifiable risk factor for cardiovascular disease [5].

2.2   Endothelium / Endothelial cells

Blood vessel walls form a selective barrier between blood and tissues. The endothelial cell layer is the closest cell layer to the bloodstream and serves as a large surface area for the exchange of materials between blood and tissue. The endothelium is a continuous cell-monolayer with an approximately 350 m^2 large surface in human. The endothelial cells are linked to each other by different types of adhesive structures and cell junctions forming a tight interface [6]. The surface of a healthy endothelial cell monolayer is antithrombotic, expressing anticoagulant and anti-inflammatory proteins and is secreting different molecules like prostacyclin (PGI2) and nitric oxide (NO) which are important for the regulation of blood coagulation and platelet function [7]. Therefore, the complement- and coagulation systems are strongly linked to endothelial cell activation. Vessel damage, exposure to certain cytokines for example interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α) or other proinflammatory stimuli can shift the balance towards a procoagulant and prothrombotic phenotype of the endothelial cells [8] [6].

Endothelial cell dysfunction is an important promoter of atherosclerosis and can lead to several non-adaptive alterations of the functional phenotype. Alterations of the healthy endothelial cell phenotype can have an important impact on the regulation of hemostasis and thrombosis, local vascular tone and the regulation of acute and chronic inflammatory reactions within the vessel wall [9].

Fluid mechanical forces generated by arterial blood flow play a major role in maintenance of the morphology and functional phenotype of endothelial cells. It has been shown that application of an adequate laminar shear stress to vascular bovine aortic endothelial cells in vitro on coverslips for 72 hours leads to a change in shape from polygonal to ellipsoidal and the cells become uniformly oriented with the flow [10]. An application of higher shear stress (15 dyn/cm^2) to bovine aortic endothelial cells (BAEC) in vitro led to a fusiform shape and to an orientation in flow direction of the cells already after 24 h [11]. In addition to the alignment and the morphology change of the endothelial cells under laminar shear stress, changes in surface glycocalyx and cytoskeletal organization which mimics the morphology of aortic endothelium in vivo could be observed [12] [10] [13].

In a 3D in vitro culture system with porcine aortic endothelial cells (PAEC) it was also demonstrated that application of a peristaltic flow (10 dyn/cm^2) has an impact on the expression pattern of endothelial cell markers such as CD31 and VE-cadherin. PAEC cultured under pulsatile flow were more aligned with the direction of the flow than PAEC cultured under static conditions. These findings suggest that the expression of these endothelial cell markers as well as the shape of the cells is affected by shear stress dependent mechanotransduction [14] [15].

For a homeostatic function of the endothelium, an intact endothelial glycocalyx is very important to provide an effective vascular barrier [16].

2.3 The endothelial glycocalyx

2.3.1 Overview / Function

The endothelial glycocalyx is a complex, carbohydrate rich layer on the luminal side of vascular endothelial cells. The glycocalyx is in direct contact with the blood and therefore a key element for the homeostasis in blood vessels. It is composed of membrane-bound proteoglycans with glycosaminoglycan side-chains (GAG-chains) like heparan sulfate (HS) and glycoproteins with carbohydrate side-chains. The endothelial glycocalyx is known to play important roles in vascular physiology and pathology, in the mechanotransduction as a shear stress sensor, in hemostasis, signaling and blood cell-vessel wall interactions. Glycocalyx dysfunction arising by its degradation, damage or shedding is closely linked to diabetes, atherosclerosis, ischemia/reperfusion injury and xenotransplantation settings [17] [18]. The consequences of glycocalyx shedding are a pro-coagulant and pro-inflammatory phenotype of the endothelium, increased capillary permeability, adhesion of immune cells, vascular inflammation and thrombosis. The vasculoprotective role in micro-and macro circulation is therefore a very important function of the glycocalyx [18] [19].

The endothelial glycocalyx is a dynamic, flow dependent structure. Shear stress exposure results in a dynamic reorganization of the glycocalyx, linked to membrane rafts and actin cytoskeleton which may underlie alterations in endothelial mechanotransduction mechanisms. The adaptive remodeling of the glycocalyx due to shear stress needs a balance between the synthesis of its components such as GAG and core proteins, and their degradation which is modulated by enzymes like heparinase and metalloproteases [11].

Shear stress is known to stimulate porcine endothelial cells to synthesize HS and chondroitin/dermatan sulfate. The secretion of HS is manly on the cell surface and into the extracellular matrix while chondroitin/dermatan sulfate is secreted into the medium [12].

A shear stress in the thoracic aorta up to 30 dyn/cm^2 was estimated to be typical. In smaller vessels such as arterioles with a diameter of around 20-40 μm, the shear stress is estimated to be about 5-25 dyn/cm^2 [20].

The GAG synthesis can be increased when PAEC are exposed to fluid laminar shear stress in vitro. A stimulation of the GAG synthesis such as HS and chondroitin/dermatan sulfate in PAEC requires more than 24 h of shear stress application [12].

The thickness of the endothelial glycocalyx increases with the vascular diameter of arteries reaching from 2 μm to 3 μm in smaller arteries up to 4.5 μm ± 1 μm in larger arteries like the

carotid artery of mice [21] [22] [17]. The thickness and organization of the glycocalyx is shown to be shear stress dependent [12] [11].
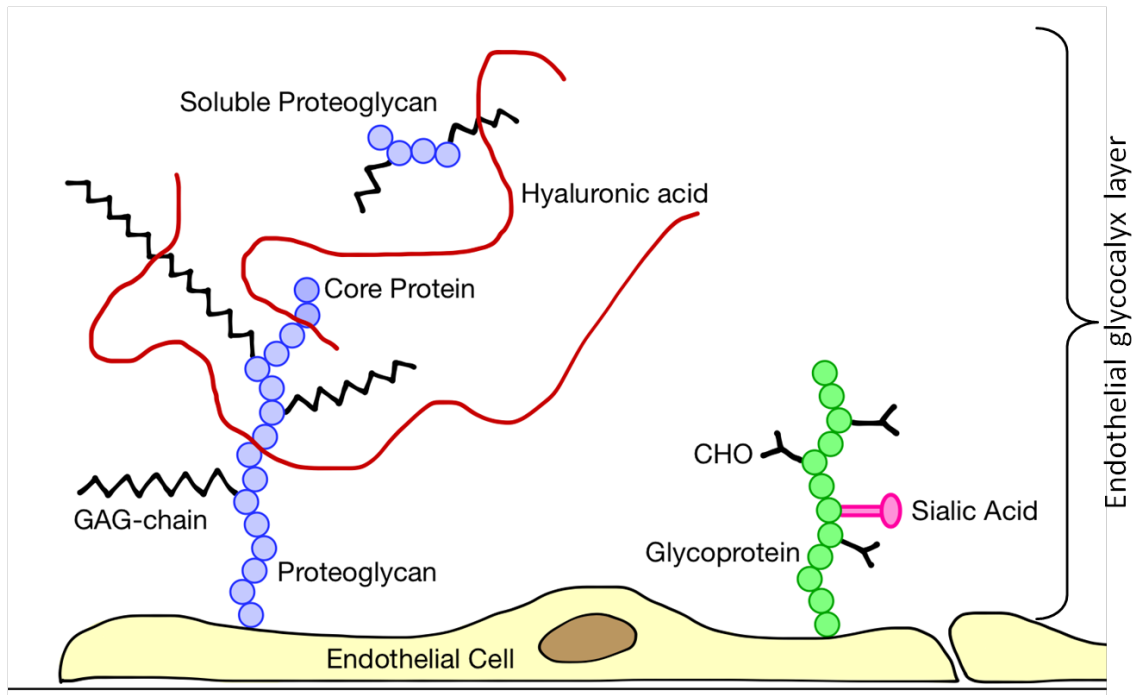
Figure 2: Schematic representation of the main components of the endothelial glycocalyx layer such as proteoglycans and glycoproteins. Figure based on ref. [17].

2.3.2 Composition of the endothelial glycocalyx

2.3.2.1 Proteoglycans

Proteoglycans are the most important molecules of the endothelial glycocalyx and therefore also known as "backbone" molecules of the glycocalyx. They consist of a core protein to which one or several negatively charged glycosaminoglycan (GAG) chains are attached. Proteoglycans vary depending on their size, number of attached GAG chains and whether they are bond to the cell membrane or not [17] [16].

The two most common core proteins of proteoglycans are syndecans and glypicans. The core protein has a strong connection to the endothelial cell membrane via a membrane-spanning domain, namely syndecans or a glycosylphosphatidylinositol anchor, called glypicans. Other proteoglycans like mimecan, perlecan and biglycan are secreted after their assembly and they can either stay in the glycocalyx or diffuse into the blood stream [17]. One proteoglycan core protein can contain several types of GAG chains. There are five different types of GAG chains identified on endothelial cells: Heparan Sulfate (HS), chondroitin sulfate, dermatan sulfate, keratan sulfate and hyaluronan/hyaluronic acid. HS represent around 50 % - 90 % of all GAG chains. [17]

GAG chains are linear polymers of disaccharides with different lengths and are modifiable by sulfation and/or (de)acetylation to different variations. Each disaccharide is built with an uronic acid and a hexosamine. The GAG classification depends on the pattern of sulfation and on which uronic acid or hexosamine is incorporated [17].

2.3.2.2 Glycoproteins

Glycoproteins are contributing to the connection of the glycocalyx with the endothelial cell membrane. Therefore certain glycoproteins are also regarded as "backbone" molecules of the glycocalyx. The endothelial glycoproteins manifest relatively small and branched carbohydrate (CHO) side chains. The level of glycoprotein expression on the surface of the endothelial cell membrane varies with the cell activation or stimulation [17]. Glycoproteins act as adhesion molecules and are therefore closely linked to the coagulation, fibrinolytic and haemodynamic systems [16].

N-Acetylneuraminic acid (Neu5Ac) residues also called sialic acid residues, are terminal CHO side chains on top of glycoproteins which protect the endothelium from proteolysis [23]. Neu5Ac contributes to the negative charge of cell surfaces which is important to form a protective electrostatic shield. This electrostatic shield is important for the antiadhesive state between circulating cells and the vessel wall [24] [25].

The endothelial cell adhesion molecules (ECAM) are glycoproteins which play an important role in blood cell recruitment and cell signaling. There are three different families of cell adhesion molecules existing in the endothelial glycocalyx: The selectin family, the integrin family and the immunoglobulin superfamily [17].

ECAMs of the **selectin family** which are present on the vascular endothelium are E-selectin and P-selectins. Both are important for leukocyte-endothelial cell interactions. In resting vascular endothelial cells, P-selectin is basically produced and stored in Weibel-Palade bodies [17] [26]. Stimuli like activated platelets, thrombin and histamine induce Weibel-Palade body secretion leading to a release of P-selectin at the cell surface [27] [28].

In endothelial cells E-selectin is neither stored in granules nor expressed under basal conditions but it requires de novo mRNA and protein synthesis to be expressed on the cell surface. E-selectin expression is up regulated through stimulation of endothelial cells by cytokines like interleukin-1 (IL-1), tumor necrosis factor α (TNF-α) and lipopolysaccharides [17] [29].

ECAMs of the **integrin family** are important mediators and regulators of angiogenesis and vascular homeostasis. Integrins are necessary for the physical interaction with the extracellular matrix such as cell adhesion, migration and positioning, and are the main receptors for extracellular matrix proteins such as fibronectin, laminin and collagen [30].

The most common members of the **immunoglobulin superfamily** of glycoproteins are: The intercellular adhesion molecule 1 and 2 (ICAM-1 and 2), the vascular cell adhesion molecule 1 (VCAM-1) and the platelet/ endothelial cell adhesion molecule 1 (PECAM-1). All these molecules act as ligands for integrins for leukocytes and platelets and are essential for the leukocyte attachment to, and diapedesis through the endothelium [31].

2.4 Albumin

2.4.1 General function

Human albumin represents around 50 % of the total protein content in human plasma with a healthy concentration of 3.5 g/dl - 5 g/dl. Human albumin is a small and highly soluble protein with an elliptical shape and a molecular weight of 66.5 kDa. It is a very stable protein consisting of a 585 amino acid long single chain [32].

One of the most important properties of serum albumin is the capability to bind a wide range of endogenous and exogenous ligands, such as fatty acids, metal ions, steroids, amino acids and a lot of drugs [33].

The high concentration in the plasma together with its strong negative charge makes albumin to the main responsible protein for plasma oncotic pressure and therefore crucial for the distribution of fluid between compartments [32].

Albumin is synthesized in hepatocytes at a rate of 9 g- 12 g per day under physiological conditions. Since there is no storage of albumin in the liver, it cannot be just released on demand. However the synthesis can be adjusted in a large span and is stimulated by hormones such as insulin, cortisol and thyroxine. Under normal conditions just 20 % - 30 % of hepatocytes produce albumin, therefore the synthesis can be increased on demand by 200 % - 300 % [34]. At the other hand albumins mRNA levels can be decreased by pro-inflammatory substances like interleukin-6 and tumor necrosis factor-α [35] [36].

Albumin is known to play an important role in the stabilization of the endothelial layer leading to normal capillary permeability and maintenance of the liquid balance [32].

Albumin has the main circulating antioxidant function of the body [37]. Albumin has the capacity to bind metal ions like copper, cobalt, nickel, zinc and iron which otherwise would catalyze chemical reactions generating free radicals [38] [32].

Reducing oxidative damage and modulating inflammation, albumin demonstrates an antithrombotic effect which also seems to be related to the capacity of binding nitric oxide (NO). The binding of NO to albumin thereby prevents the rapid inactivation of NO and prolongs its anti-aggregant effect on platelets [39].

2.4.2 Albumin in cardiovascular disease

The development of atherosclerosis and many cardiovascular diseases are related to endothelial dysfunction, inflammation and oxidative stress, via not yet fully understood processes [5].

It is well known that hypoalbuminemia is a powerful prognostic marker in many pathological settings mainly in malnutrition, inflammation, cancer and mortality in general [40] [41] [42].

After all, there are indications that low serum albumin levels play a role in development of several cardiovascular diseases, such as ischemic heart disease, heart failure, atrial fibrillation, stroke and venous thromboembolism. Hypoalbuminemia is also shown to be a powerful prognostic parameter in many cardiovascular diseases independent of traditional prognostic markers. Regarding the properties of serum albumin, such as colloid osmotic effect, anti-inflammatory, antioxidant, antiplatelet aggregation and anticoagulant activity, S. Arques [5] proposes that hypoalbuminemia could represent a modifiable risk factor in some cardiovascular diseases. However a benefit of the correction of low serum albumin levels in patients with cardiovascular disease could not be proven so far [5].

Even though serum albumin is known as the most important anti oxidant in the whole blood, its anti-inflammatory activity is not well understood [37] [5].

It was shown that albumin leads to a significant increase in coronary flow in an isolated perfused organ model with a guinea pig heart. This manifestation is most likely connected to the shear stress-transmission via the endothelial glycocalyx. This phenomenon leads to the generation of NO in the guinea pig heart model. Stripping away the main part of the endothelial glycocalyx or inhibiting the NO synthase, result in a decrease in the coronary flow. Albumin causes a way larger effect than infusion of artificial colloids, leading to the assumption that there is a specific interaction of natural colloid, such as albumin, with the endothelial glycocalyx [43].

2.4.3 Albumin in an in vitro cell culture system

Albumin interacts with several ligands or bioactive factors such as hormones, growth factors, lipids, amino acids, metal ions and reactive oxygen species (ROS). With this interaction, albumin in the culture medium has a potential influence on the metabolic and biosynthetic activity, proliferation, survival and growth of cells in culture [33].

In serum-free cell culture, bovine serum albumin (BSA) was often used as an essential component in the culture media. Albumin is the major protein in fetal bovine serum (FBS) and was therefore seen as a non-omissible factor in serum-free cell culture [44] [33].

Albumin interacts with several substances present in cell culture medium. The antioxidant properties of albumin in mammals represent a key interaction with cells. Therefore the role of albumin as a transporter and antioxidant might be also relevant in cell culture, especially in microfluidic culture. Albumin is the second largest carrier of Cu in the blood responsible for its safe transport and preventing from its pro-oxidant activity. In his review, G. L. Francis [33] assumes that it possibly provides the same function in cell culture medium, thereby prevent harmful actions of free Cu and facilitate its transport into the cells.

Albumin is used to study the endothelial glycocalyx in vitro. It is shown to be important to prevent the endothelial glycocalyx from collapsing [45]. Therefore in previous in vitro studies, BSA was used either to prevent the glycocalyx from collapsing and to support it in his function and growth, or to provoke a degradation of the glycocalyx by starving the endothelial cells for BSA and FBS which also contains BSA. It could be shown that BSA is absorbed and well integrated in the structure of the endothelial glycocalyx of BAEC [46].

2.5 In vitro model

2.5.1 Cell culture

Culturing cells in vitro is an important technique in today's science. Cell culture-based assays are very common in drug discovery since decades. These days two-dimensional (2D) cell culture models are efficiently used for predicting in vivo drug responses and are still very useful in drug discovery. Nevertheless 2D cell culture based assays have some disadvantages like loss of tissue-specific architecture, mechanical and biomechanical limitations and lack of cell-to-cell / cell-to-matrix interactions. In the past decade, the development of three-dimensional (3D) cell culture systems strongly increased. A 3D cell culture has some important advantages compared to a 2D cell culture: Greater predictability of efficacy and toxicity in drug testing, co-culture setups are possible, and a more in vivo–like context is generated. 3D cell culture using human cells allows to replace certain animal experiments, eliminating the species differences of animal models. Therefore, 3D cell culture has a great potential and should be further developed [47].

2.5.2 Microfluidic in vitro model

Microfluidic cell culture is becoming more common within experimental cell biology and is determined by its capacity to permit new insights into cellular function, which would otherwise be difficult or impossible to obtain with macroscopic (conventional) cell culture [48].

Microfluidics is characterized by the science and technology of systems that handle small amounts of fluids (10^{-9} to 10^{-18} liters) within channels with a diameter of tens to hundreds of micrometers. Microfluidic technologies have a lot of helpful properties such as the ability to use small quantities of samples and reagents, they can perform high resolution and high sensitivity separations and detections, they are time saving and can provide low cost analysis [49]. Further advantages of the microfluidic cell culture are: A high flexibility of the design of the devices, a low cell number is required, single cell handling is possible, real time and on chip analysis can be achieved, controlled co-culture can be obtained and it has the ability to perform perfusion culture [48].

An example of microfluidic cell culture is the 3D microfluidic in vitro cell culture setup with PAEC which has been developed to study endothelial cells and their glycocalyx under peristaltic flow conditions and in xenotransplantation settings [14]. This particular setup is very promising and opens a door to many new scientific experiments. This model is very flexible, can be adjusted and there is still a potential for improvement that has to be worked out.

2.6 Aim of the project

The aim of the present study was to assess the role of albumin in the formation and preservation of the endothelial glycocalyx layer of PAEC and to assess whether additional albumin in the perfusion medium has an effect on the shape and alignment in flow direction of the PAEC using a microfluidic in vitro model.

A second aim of the present study was to improve our microfluidic in vitro model making it more physiological by adding albumin to the perfusion medium.

2.6.1 First hypothesis:

We hypothesized that perfusion of PAEC with additional albumin in the perfusion medium has a positive effect on the formation, support and preservation of the endothelial glycocalyx.

2.6.2 Second hypothesis:

We hypothesized that a better pronounced endothelial glycocalyx by the presence of albumin will result in a more efficient mechanotransduction of the shear stress to the endothelial cells, leading to an increased elongation and alignment of the endothelial cells with the flow direction.

3   Materials and methods

3.1   Cell culture

Wild type (WT) porcine aortic endothelial cells (PAEC) passage two to five which were stored at -150 °C were thawed in a 37 °C water bath and removed when still a small amount of ice was left. The cell suspension was re-suspended until this small amount of ice melted. 1 ml of the cell suspension was then seeded in a T-75 flask (TPP, Trasadingen, Switzerland) and cultured with cell culture medium (DMEM(1x) + GlutaMAX^{TM}-1 (Gibco, Thermo Fisher Scientific, Rockford, IL, USA) supplemented with 10 % fetal bovine serum (FBS) (Biochrom AG, Berlin, Germany) and 1 % penicillin/streptomycin (Gibco, Thermo Fisher Scientific, Rockford, IL, USA). The cells were incubated at 37 °C and 5 % CO_2 and the medium was changed the first time after 5 h- 24 h to remove all traces of DMSO remained from the freezing medium. Later the medium was changed every 48 h until the flask was confluent.

3.2   PDMS microchip fabrication

The microchips were produced by mixing polydimethylsiloxane (PDMS) and curing agent (Sylgard 184, Dow Corning, Wiesbaden, Germany) in a ratio of 10:1. The mixture was poured into a petri dish and bubbles were reduced with a vacuum pump. The mold needles (cut syringe needles) with a thicknes of 550 µm (B.Braun, Melsungen, Hessen, Germany) were washed with isopropanol and dried on a tissue paper. Two support needles with a diameter of 120 µm and a length of 3 cm (Seirin, Hamburg, Germany) per chip were placed horizontally in the liquid PDMS mixture on the bottom of the petri dish and four mold needles were placed on top of the support needles, perpendicularly to the latter, with the biggest distance possible to each other. The petri dish with the liquid PDMS mixture and the needles was cured in an oven at 60 °C over night. After curing, the chips were cut out of the petri dish and cut into single chips. The mold needles were then removed with tweezers. The support needles were cut away from both sides of each chip. To make an access from the top of the chip to the microchannels, an inlet and an outlet was punched with a 2 mm biopsy puncher (kai Europe GmbH, Solingen, Germany, REF; BPP-20F) at a distance of 1 cm. The two parts of each channel where the mold needles were pulled out, were sealed again with PDMS and cured overnight in the oven at 60 °C. Afterwards the chips were cleaned and covered with scotch tape to store.
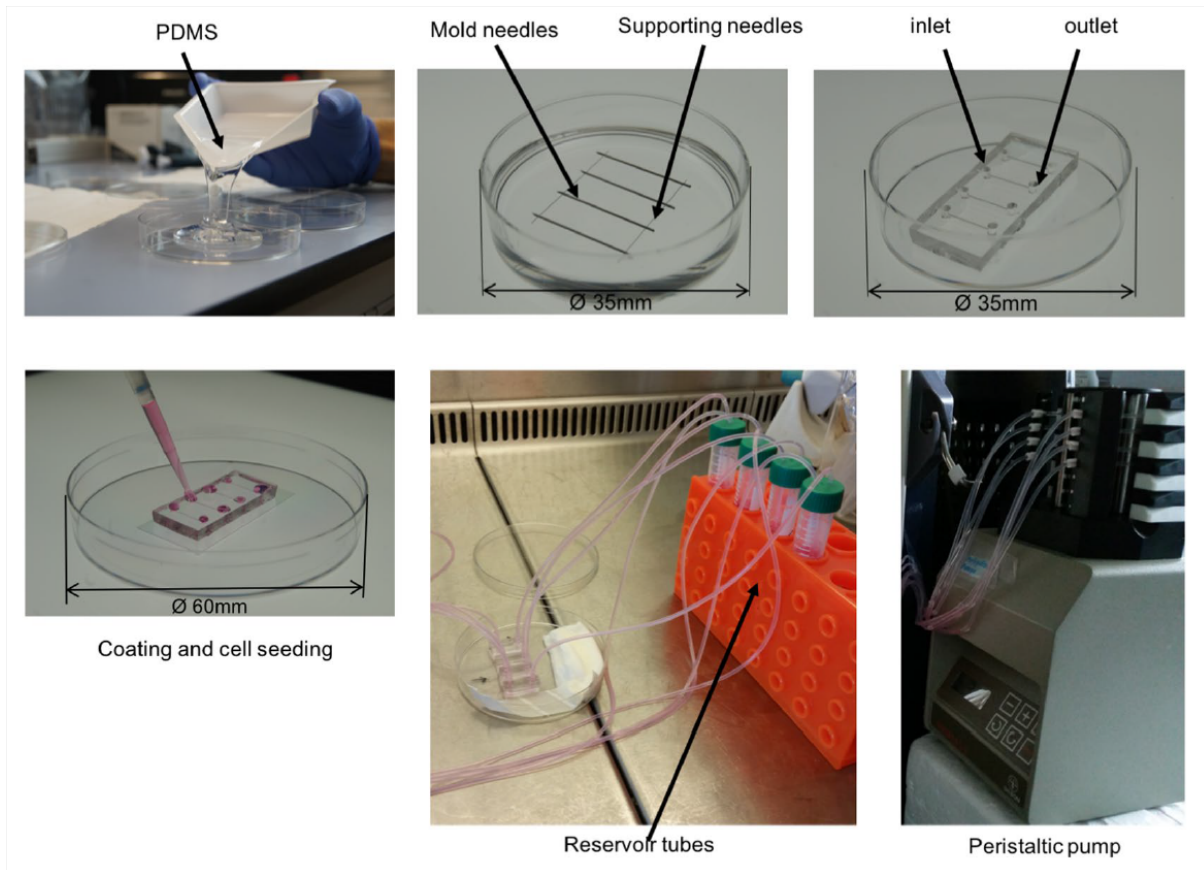
Figure 3: Stepwise representation of the microchip fabrication and pump connection. PDMS is poured into an empty petri dish. The supporting needles were placed horizontally and mold needles perpendicularly on top as shown in the picture. After the incubation at 60 °C over night, the chips were cut and the needles removed. An inlet and an outlet to the channels were created with a 2 mm biopsy puncher and the lateral wholes were sealed with liquid PDMS. The chip was bond on a glass slide after plasma oxygen treatment and coated with fibronectin and collagen I. Cells are seeded in the channels and incubated for one day. Then reservoir tubes and a peristaltic pump were connected to apply a shear stress of 15 dyn/cm^2. Figure from [14].

3.3 PDMS surface modification

The chip was cut with a scalpel into single channel blocks which were then placed on a scotch tape. A cover glass slide (24 mm x 60 mm #1) was first cleaned with ethanol, soap water and distilled water and then dried with a nitrogen gun. The surface of the PDMS chips and of the cleaned glass slide were activated in an oxygen plasma cleaner (Harrick Plasma, NY, USA) at 650 mTorr for 3 min. In this process, the surface chemistry is altered by adding silanol (SiOH) groups. Directly afterwards the chip and the glass slide were bonded together. Immediately after the bonding, the surface of the channels was treated with 5 % (3-Aminopropyl)triethoxysilane (APTES) (Sigma-Aldrich, St.Louis, MO, USA) for 20 min at room temperature to regain a hydrophilic state of the PDMS surface in the channels. The channels were washed with ultrapure water and treated with 0.1 % glutaraldehyde (Sigma-

Aldrich, St.Louis, MO, USA) for 30 min at room temperature. After washing again with ultrapure water the channels were incubated with 50 µg/ml human fibronectin (Chemicon, Temecula, CA, USA) in phosphate-buffered saline (PBS) at 37 °C for 1 h. The channels were then directly treated with 100 µg/ml bovine collagen I (Gibco, Thermo Fisher Scientific, Rockford, IL, USA) in 0.02 M acetic acid (Gibco, Thermo Fisher Scientific, Rockford, IL, USA) for 1.5 h at room temperature. The channels were then washed and incubated with cell culture medium at 37 °C for at least 30 min before seeding the cells in the channels.
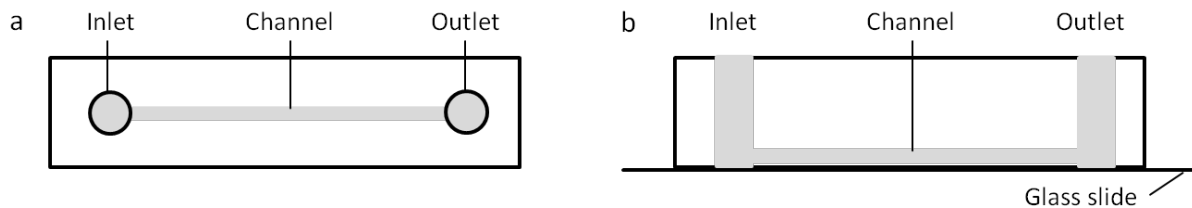
Figure 4: a) Top view of a PDMS channel block. b) Side view of a PDMS channel block bond to a glass slide. The connections to the 550 µm thick channel are labeled as inlet and outlet and have a diameter of 2 mm. For better imaging with the confocal microscope, the channel is placed right at the lower border of the PDMS. The binding of the PDMS channel block to a glass slide closes the lower whole of the inlet and the outlet.

3.4 Cell seeding and pump connection

For the current study WT PAEC passage two to six were used. Confluent PAEC in the flask were washed with PBS and incubated with 0.05 % Trypsin-EDTA (Gibco, Thermo Fisher Scientific, Rockford, IL, USA) at 37 °C until the cells were detached. Cell culture medium was added to dilute and inactivate the Trypsin. The cell suspension was centrifuged at 1200 rpm (0.2 rcf) for 8 min and the supernatant was removed. The pellet was then resuspended in 1 ml perfusion medium (cell culture medium with 4 % Dextran from Leuconostoc spp. (MW ~ 70'000, Sigma-Aldrich, St.Louis, MO, USA, REF; 31390)). The cells were counted and diluted with perfusion medium to a cell suspension containing 10^6 cells/ml. The chip was fixed with scotch tape on the petri dish and the cell suspension was seeded in the microchannels with a 200 µl pipette. The chip was then incubated upside down for 15 min at 37 °C. Afterwards new cell suspension was seeded in the microchannels and the chip was placed upright in the incubator at 37 °C for 15 min. After the seeding the channels were washed and filled with perfusion medium and put in the incubator at 37 °C. The medium was changed 3-4 times every 3-12 h and left overnight. After 24 h the cells in the channels were confluent and then connected to the pump as described below.

A peristaltic pump Minipuls 3 with 8 channels (Gilson, Villiers le bel, France) was used for the perfusion. Extension silicon tubings (Maagtechnic, Dübendorf, Switzerland) were autoclaved.

The specific PVC pumphead tubings (Gilson, Villiers le bel, France, REF; F117938) were rinsed with isopropanol, ultrapure water, ethanol and again with ultrapure water. After rinsing, the pvc pumphead tubings were treated with ozone gas in a CoolCLAVE Laboratory Bench Top Sterilizer (Genlantis, San Diego, CA, USA). All tubes were connected together and finally to the pump. The tubes were flushed with autoclaved ultrapure water, with PBS and then with perfusion medium. One 15 ml Falcon tube filled with 10 ml perfusate was used as a reservoir for each channel. Then the chip and the reservoir were connected to the tubes. The tubing end coming from the outlet of the channel was set at the 8 ml mark and the tubing end going to the inlet of the channel was set at 2 ml mark of the falcon tube.

3.5 Perfusion

Perfusion medium or perfusion medium with additional bovine serum albumin (BSA) (Sigma-Aldrich, St.Louis, MO, USA) in different concentrations depending on the experiment, was filtered with 0.22 μm sterile Rotilabo-syringe filters (Carl Roth GmbH+Co., Karlsruhe, Germany, REF: P668.1), filled in the reservoirs and used for the perfusion. After the connection of the pump to the chip, the reservoir falcon tubes and the chip were placed in the incubator at 37 °C at 5 % CO_2. The pump remained outside of the incubator and was set to 10 rpm corresponding to 100 beats per minute (bpm) and to a flow of 860 μl/min = 51 ml/h leading to a shear stress for the cells of around 15 dyn/cm^2. The channels were perfused for 48 h using 10 ml perfusate per channel, which was changed every 24 h.

3.6 Immunofluorescent staining

In case of a live cell staining with WGA-Lectin, the channels were washed with pure cell culture medium (DMEM(1x) + GlutaMAX^{TM}-1 (Gibco, Thermo Fisher Scientific, Rockford, IL, USA). Then fluorescently labeled WGA-Lectin in pure cell culture medium was applied on the channels and the chip was placed in the incubator at 37 °C for 15 min. The channels from the live cell staining and the other channels for the death cell staining were then washed with PBS and fixed with 4 % paraformaldehyde (PFA) at room temperature for 10 min. The channels were washed well with PBS. In case of an intracellular staining of F-actin the cells were permeabilized with 0.5 % Triton X (Sigma-Aldrich, St.Louis, MO, USA) in PBS for 10 min at room temperature on a shaker. In all channels (except in the channels stained for BSA) PBS-3 % BSA was used for blocking for 30 min at room temperature. The channels stained for albumin were blocked with PBS-3 % milk powder (Rapilait, Migros, Zürich, Switzerland). After the blocking step, the channels were incubated with the primary

antibodies, diluted in PBS-1 % BSA (and in PBS-1 % BSA-0.05 % Tween 20 if cells were permeabilized) or in PBS – 0.05 % Tween 20 if the channels were stained for BSA, for 1 h at room temperature on a shaker. Then the channels were washed with PBS and incubated with the secondary and the directly labeled antibodies, diluted in PBS-1 % BSA (and in PBS-1 % BSA-0.05 % Tween 20 if cells were permeabilized) or in PBS – 0.05 % Tween if the channels were stained for albumin, for 1 h at room temperature on a shaker in the dark. The channels were washed, filled with PBS and stored in the dark at 4 °C.

The different antibodies used in the present study are listed in the table below:

Primary antibodies	Dilution	Company / Ref. Nb.
Mouse anti-human HS	1:100	Amsbio; 370255-1
Rat anti-porcine CD31	1:100	R&D Systems; MAB 338-71
Rabbit anti-BSA IgG	1:100	Thermo Fisher; Cat # A11133

Secondary antibodies	Dilution	Company / Ref. Nb.
Goat anti-mouse IgM FITC	1:500	Jackson Immuno Research; 115-097-020
Goat anti-rat IgG Cy3	1:500	Jackson Immuno Research; 112-166-003
Goat anti–rabbit IgG FITC	1:500	SouthernBiotech; Catno. 4050-02

Directly labeled antibodies	Dilution	Company / Ref. Nb.
F-Actin Alexa Fluor 555	1:200	Cytoskeleton; Cat. # PHDH1
WGA-Lectin FITC	1:100	Sigma; L4895
DAPI	1:1000	Sigma; 32670-25MG-F

3.7 Image analysis

A confocal laser-scanning microscope (LSM 710, Zeiss, Feldbach, Switzerland) with 10 x and 20 x objectives was used to acquire images of the channels. For the image analysis of the raw integrated density of the staining, ImageJ (National Institutes of Health, Bethesda, MD, USA) was used.

The analysis of the elongation and alignment of the cells was performed with our own "Cell-Metrology" software. The program was created using LabVIEW professional development system (National Instruments, TX, USA). The software can load the confocal laser-scanning microscope images. The images are red, green, blue color coded depending on the selected fluorescence channels. Using the program, single color channels can be selected or color-combinations can be adjusted to enhance the visibility of certain features. Additionally contrast and brightness can be optimized. The adjusted images are loaded in the

cell evaluation module for cell evaluation. The outlines of individual cells can be manually marked with the polygon line tool. For all marked cells the following characteristics are determined: Length and width of the cells, the aspect ratio, the area and the orientation with respect to the vertical channel direction. The orientation of the polygon is determined as the direction of the axis with the lowest moment of inertia (cell axis) [50] [51] [52]. The determined orientation is also the base for calculating the length and the width of the polygon. The length is determined as the longest distance between two points in the direction of the cell axis. The width is the longest distance between two points perpendicular to the cell axis. For all evaluated cells the mean parameter values and their standard deviations are calculated. Furthermore a ratio weighted angle is determined. It is the absolute value of the angular deviation with respect to the vertical direction weighted with the aspect ratio. This means that long cells with a clear orientation have a higher weight than round cells with weak orientation. Other features of the software are the exportation of evaluated images with the cell markups, zooming and pixel scaling. If the pixel size is provided the calculated length, width and area are given in the corresponding unit.

3.8 Statistical analysis

All data are given as mean ± standard deviation (SD). The statistical analysis were performed with GraphPad Prism 7 software (GraphPad, San Diego, CA, USA) using unpaired T-test or using one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test to compare the means of all groups. p values < 0.05 were considered as statistically significant.

4 Results

4.1 Heparan sulfate (HS) expression increased with additional BSA

To examine whether the addition of BSA in the perfusion medium has a positive impact on the endothelial glycocalyx expression, PAEC were perfused with and without addition of 0.5 % BSA. Heparan sulfate (HS) was stained after the cells were exposed to peristaltic flow for 48h.

A significantly increased expression of HS was observed on PAEC perfused with additional 0.5 % BSA (Figure 5).
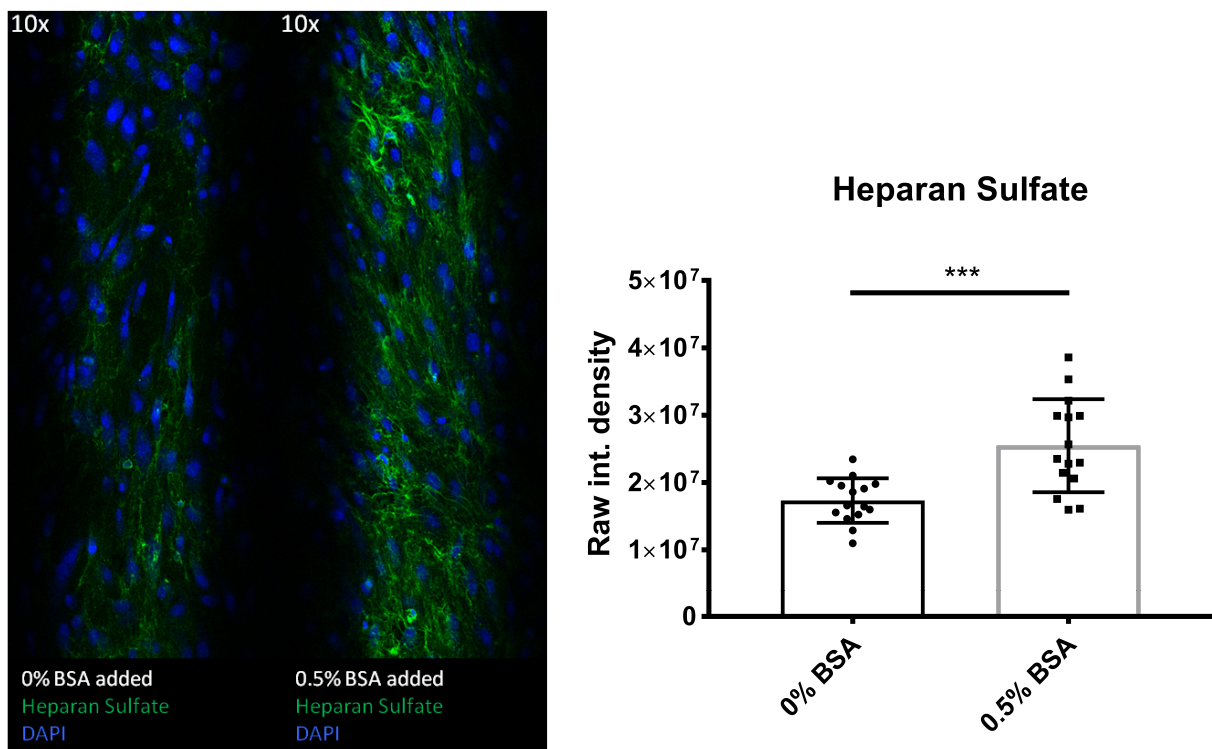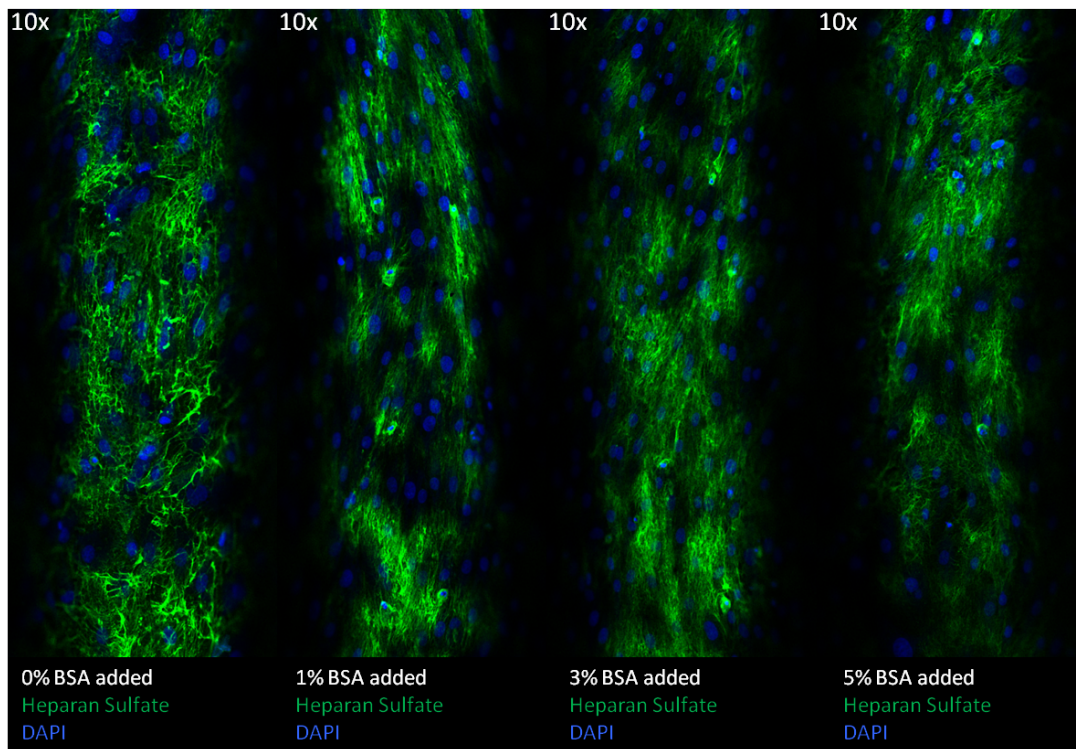

Figure 5: Left: Confocal imaging of HS on PAEC. Peristaltic perfusion for 48 h with medium without and with additional 0.5 % BSA. **Right: Quantification of the HS raw integrated density.** Three channels perfused without additional BSA and three channels perfused with additional 0.5 % BSA. Evaluation of five pictures per channel captured from different spots of each channel. Statistic: Unpaired t test, p-value = 0,0003.

## 4.2 HS pattern on PAEC vary with different BSA concentrations

To determine whether different BSA concentrations in the perfusion medium have an effect on the expression and the pattern of HS, PAEC were perfused with and without additional 1 %, 3 %, and 5 % BSA. After the exposure to peristaltic flow for 48 h, the channels were stained for HS.

Visual inspection of the confocal images show a difference in the HS pattern on PAEC perfused with additional BSA compared to PAEC perfused without additional BSA.

The analysis of the HS intensity showed no significant difference between the channels perfused with different BSA concentrations (Figure 6 and 7).

Figure 6: Confocal imaging of HS on PAEC. Peristaltic perfusion for 48 h with and without additional 1 %, 3 %, and 5 % BSA.
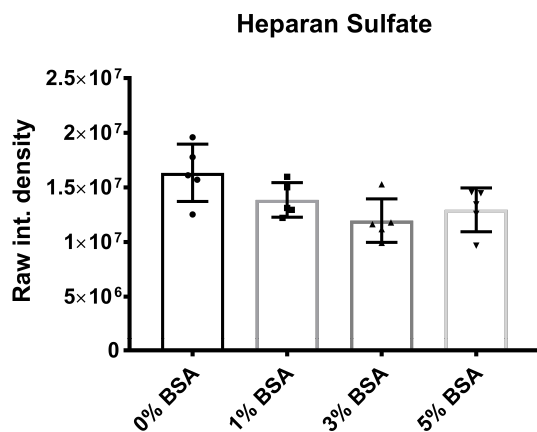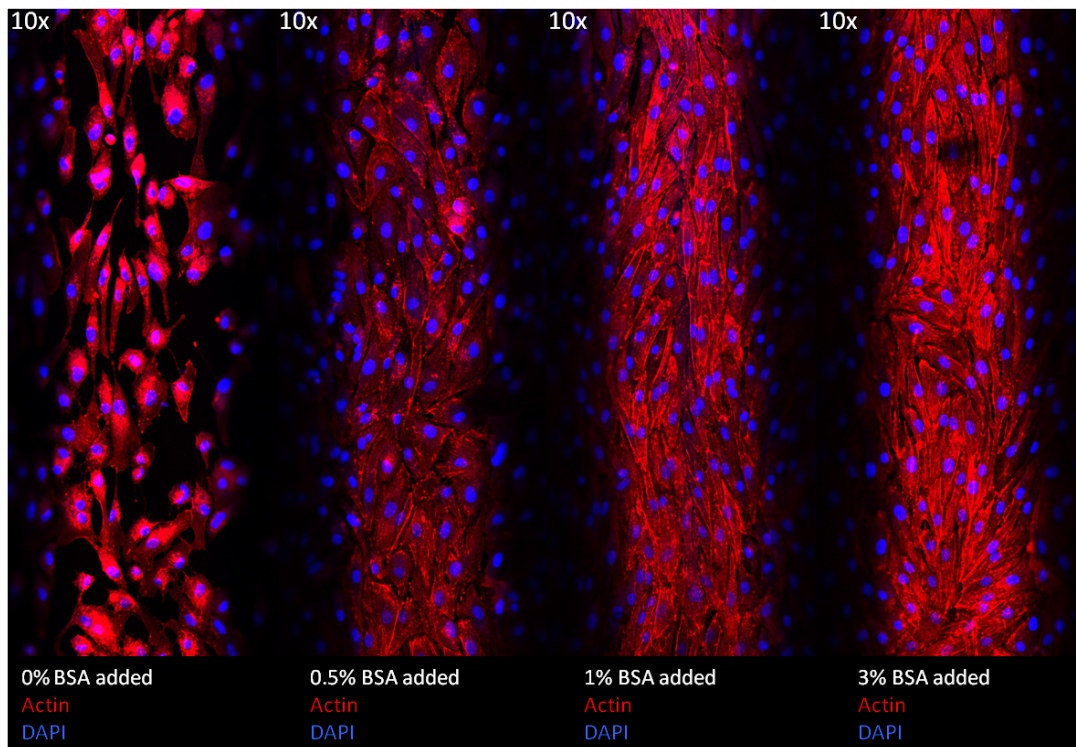

Figure 7: Quantification of the HS raw integrated density. Perfusion with and with without additional 1 %, 3 %, and 5 % BSA. One channel for each concentration was used. Evaluation of five pictures per channel captured from different spots of each channel.

4.3 Cell-elongation and alignment in flow direction increased with additional BSA

To check whether additional BSA in the perfusion medium has a positive impact on the elongation and alignment in flow direction of the endothelial cells, PAEC were perfused with and without additional 0.5 %, 1 %, and 3 % BSA. After the peristaltic perfusion for 48 h, F-actin was stained to visualize the shape of the PAEC.

The results show that PAEC perfused with additional BSA show fewer gaps in the confluent cell monolayer and a significantly increased elongation and alignment in flow direction compared to PAEC perfused without additional BSA. The results further indicate that additional 1 % or 3 % BSA is sufficient to reach an elongated and in flow direction orientated confluent cell monolayer (Figure 8, 9 and 10).

Figure 8: Confocal imaging of F-actin in PAEC. Elongation and alignment in flow direction of PAEC under peristaltic perfusion for 48 h with and without additional 0.5 %, 1 %, and 3 % BSA.
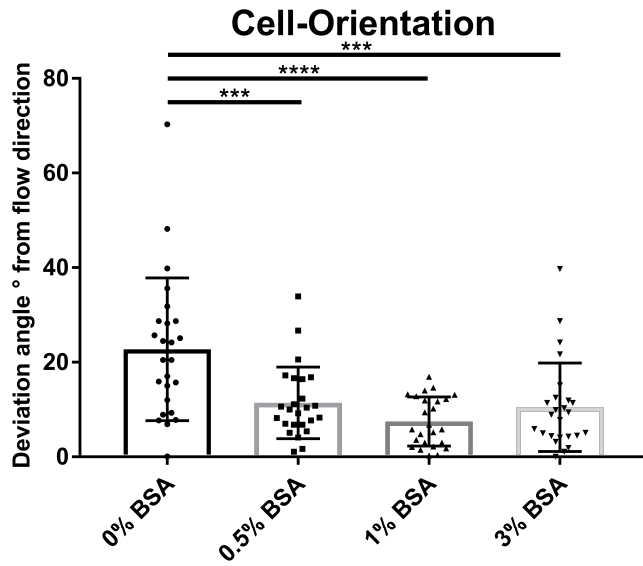
Cell-Orientation

Figure 9: Cell-orientation by quantification of the deviation angle [°] from flow direction of PAEC. Perfusion with and without additional 0.5 %, 1 %, and 3 % BSA. One channel for each concentration was used. Evaluation of 25 different cells per channel. Statistic: Ordinary one-way ANOVA, Bonferroni's multiple comparison test. p-values: ***p < 0.001 and ****p < 0.0001.
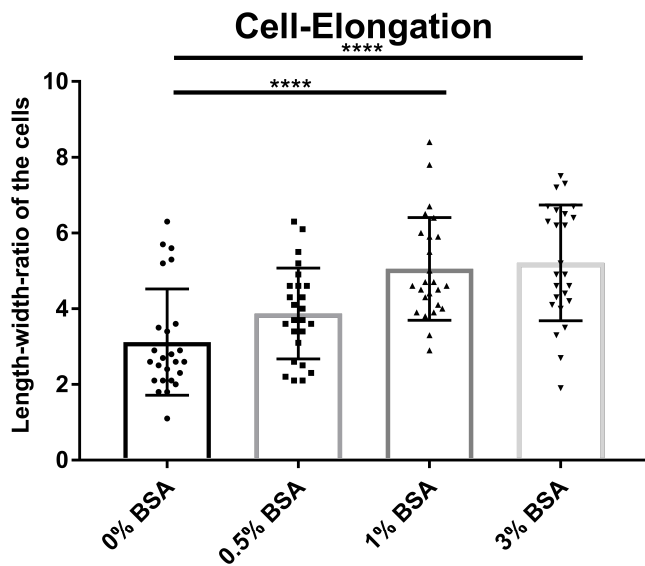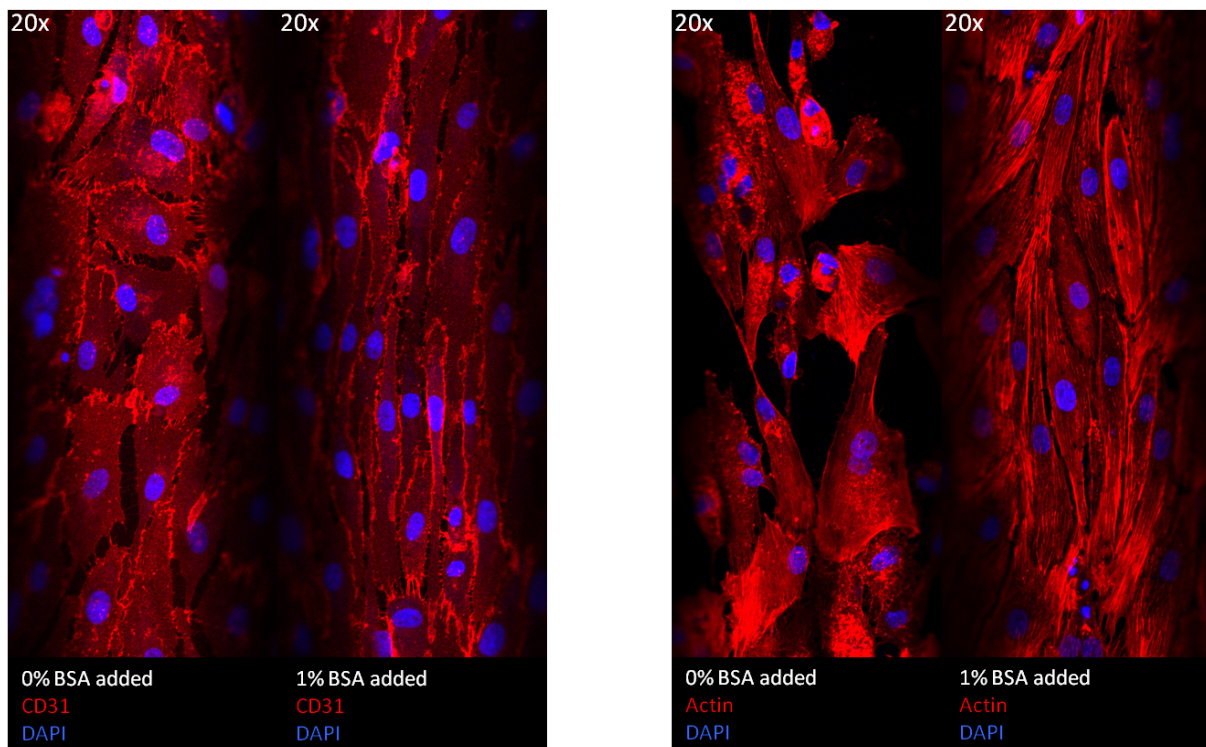
Cell-Elongation

Figure 10: Cell-elongation by quantification of the length-width-ratio of PAEC. Perfusion with and without additional 0.5 %, 1 %, and 3 % BSA. One channel for each concentration was used. Evaluation of 25 cells per channel. Statistic: Ordinary one-way ANOVA, Bonferroni's multiple comparison test. p-values: ****p < 0.0001

4.4 Cell-elongation and alignment in flow direction increased with additional 1 % BSA

To confirm that additional 1 % BSA in the perfusion medium has a positive effect on the elongation and alignment in flow direction of endothelial cells, PAEC were perfused with and without additional 1 % BSA. After the peristaltic perfusion for 48 h either CD31 which is an endothelial cell marker or F-actin was stained to characterize and visualize the shape of the PAEC. For the confocal microscopy a 20 x objective was used in this experiment instead of the usually used 10 x objective.

The results confirm that PAEC perfused with additional 1 % BSA show a significantly increased elongation and alignment in flow direction compared to PAEC perfused without additional BSA. (Figures 11, 12 and 13)

Figure 11: Left: Confocal imaging of CD31 on PAEC. Right: Confocal imaging of F-actin in PAEC. Elongation and alignment in flow direction of the PAEC under peristaltic perfusion for 48 h with and without additional 1 % BSA.
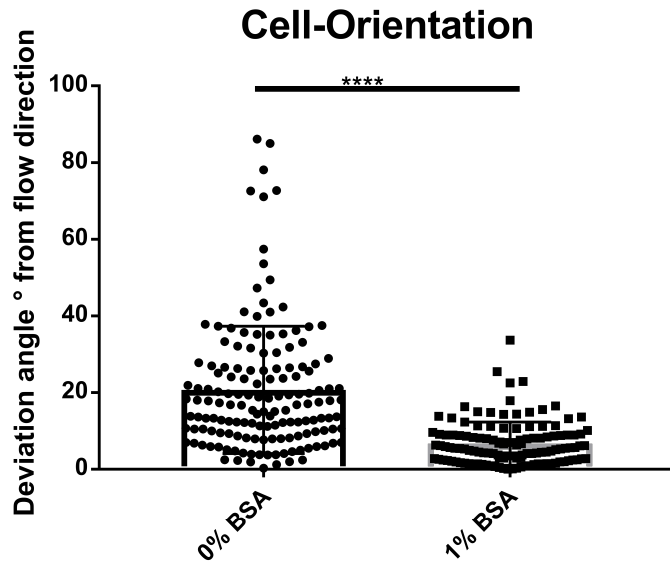
Cell-Orientation

Figure 12: Cell-orientation by quantification of the deviation angle [°] from flow direction of PAEC. Six channels were perfused without additional BSA (four channels stained with CD31 and two channels stained with F-actin). Five channels were perfused with additional 1 % BSA (three channels stained with CD31 and two channels stained with F-actin). Evaluation of 25 different cells per channel. Statistic: Unpaired t test, p-value: ****p < 0.0001
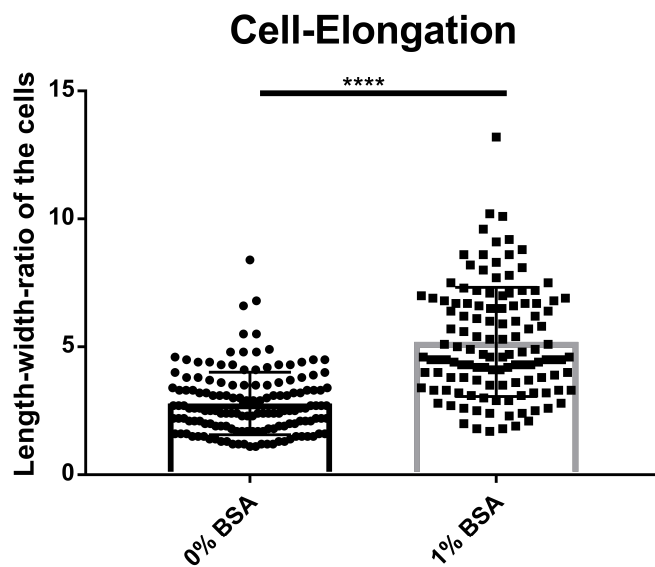
Cell-Elongation

Figure 13: Cell-elongation by quantification of the length-width-ratio of PAEC. Six channels were perfused without additional BSA (four channels stained with CD31 and two channels stained with F-actin). Five channels were perfused with additional 1 % BSA (three channels stained with CD31 and two channels stained with F-actin). Evaluation of 25 different cells per channel. Statistic: Unpaired t test, p-value: ****p < 0.0001

4.5 Neu5Ac and GlcNac residue expression increased with additional 1 % BSA

To examine whether 1 % additional BSA in the perfusion medium has an impact on the expression of N-Acetylneuraminic acid (Neu5Ac) and N-acetyl-D-glucosaminyl (GlcNac) residues present in the glycocalyx, PAEC were perfused with and without additional 1 % BSA. After the exposure to peristaltic flow, PAEC were stained with fluorescently labeled WGA-lectin which binds to Neu5Ac and GlcNac residues present in the glycocalyx.

The results show that PAEC perfused with additional 1 % BSA show a significantly increased expression of GlcNac and Neu5Ac residues compared to PAEC perfused without additional BSA (Figure 14).
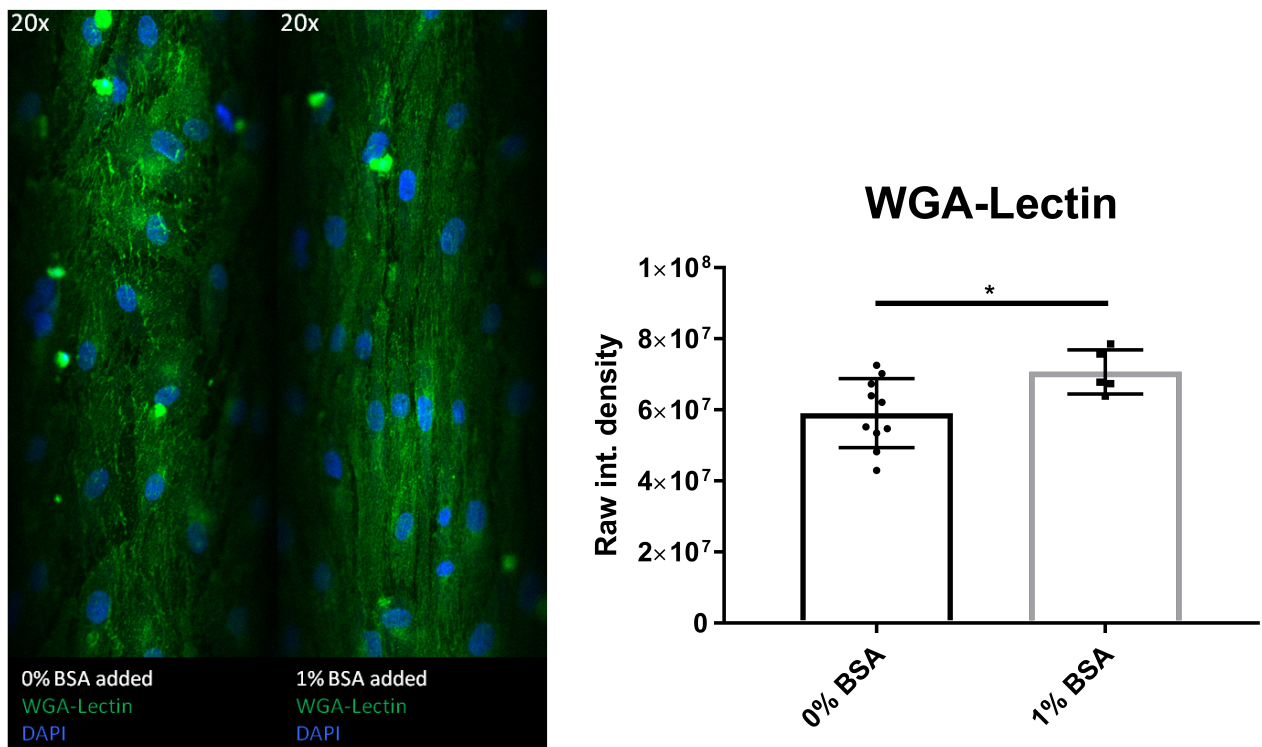
**Figure 14: Left: Confocal imaging of WGA-lectin on PAEC.** Peristaltic perfusion for 48 h with and without additional 1 % BSA. **Right: Quantification of the WGA-lectin raw integrated density.** Two channels perfused without and one channel perfused with additional 1 % BSA. Evaluation of five pictures per channel captured from different spots of each channel. Statistic: Unpaired t test, p-value: *p < 0.05

4.6   Albumin staining of PAEC perfused with additional 1 % BSA

To examine whether BSA binds to the endothelial glycocalyx under peristaltic flow conditions, PAEC were perfused with and without additional 1 % BSA in two channels each. BSA was stained after the cells were exposed to peristaltic flow for 48h.

Visual inspection shows a similar BSA staining in all the channels. The staining is also present at cell-less spots of the channels perfused without additional BSA. Closer visual inspection shows that the staining is more uniform in the channels perfused with additional 1 % BSA. Furthermore a less dense cytoplasmic staining in the cells perfused without additional BSA is visible (Figure 15).
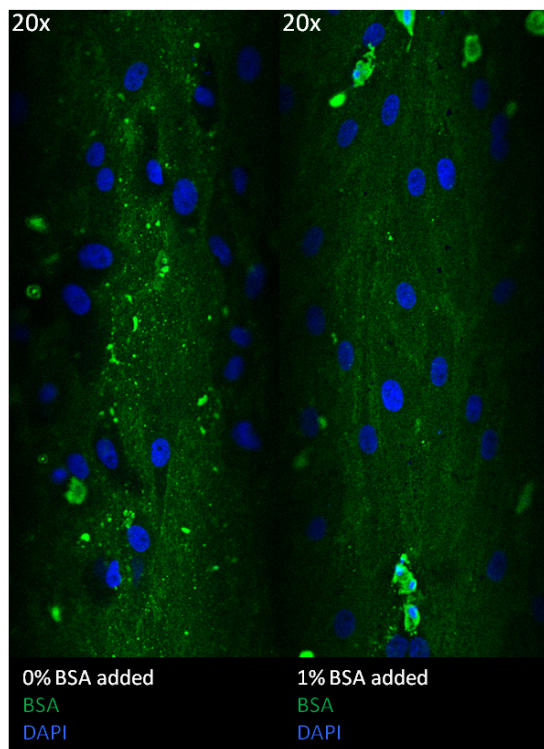
Figure 15: Confocal imaging of albumin on PAEC. Peristaltic perfusion for 48 h with and without additional 1 % BSA.

5   Discussion

Our study shows that additional albumin in the perfusion medium has a positive effect on the formation, support and preservation of the endothelial glycocalyx of PAEC.
In the first experiment we used a concentration of additional 0.5 % BSA, to check whether there are any indications that albumin supports the endothelial glycocalyx structure. This is more than threefold of the amount of BSA which was contained in the 10 % FBS, used in our usual microfluidic protocol. Our findings that additional 0.5 % BSA increased the expression of HS on PAEC were very astonishing and point at the potential of albumin to support the endothelial glycocalyx in formation and preservation.

We decided to try different BSA concentrations and to study the pattern of the HS staining on the PAEC. Our results indicate that the HS pattern on PAEC vary with different BSA concentrations. Nevertheless the analysis of the intensity of the HS staining showed no significant difference in between the different BSA concentrations. The channel without additional BSA shows even a slightly higher intensity of the HS staining than the channels with higher BSA concentrations. One reason for this could be that some cells were detached from the channel perfused without additional BSA what led to a more intensive HS staining on cell less spots. This could be because HS from the basal lamina of the PAEC stays attached to the channel even when the rest of the cell is detached, and is therefore better accessible for the staining antibodies. The findings that HS is also secreted into the extracellular matrix [12] would confirm this explanation. In our case it could be that the PAEC secrete HS into the fibronectin and collagen I layer from the coating of the channels.

To examine the endothelial glycocalyx in more detail we decided to visualize GlcNac and Neu5Ac residues on top of PAEC, using a WGA-lectin staining. Our results show that perfusion with additional 1% BSA increases the expression of GlcNac and Neu5Ac residues on PAEC, leading to an increased formation and preservation of the endothelial glycocalyx. Nevertheless this experiment should be repeated to collect more data and to confirm these first results.

To check whether albumin interacts with the endothelial glycocalyx we stained BSA on PAEC perfused with and without additional 1 % BSA. Looking at the confocal images of this experiment one can notice a similar BSA staining in all the channels. The staining is also present at cell-less spots of the channels perfused without additional BSA. A possible explanation for the staining of the cell-less spots could be that the staining antibodies against albumin were reacting with remains of the detached cells. Another explanation could be that parts of the milk powder of the blocking solution or BSA from the FBS attached to the channel at cell-less spots and were then targeted by the staining antibodies. Closer

inspection shows that the staining is more uniform in the channels perfused with additional 1 % BSA. Looking carefully at the surrounding of the nuclei of the PAEC, a less dense staining of the cytoplasm or the cell surface itself is visible in the channels perfused without additional BSA. Comparing this finding with the more uniform staining of the channels perfused with additional 1 % BSA it could be that BSA is indeed present on the surface or PAEC perfused with additional BSA. However one should repeat this experiment trying to get a clearer staining and better data.

Taking the findings of increased HS as well as increased GlcNac and Neu5Ac residue expression, all reflecting important components of the endothelial glycocalyx, together, we can assume that albumin has indeed a supporting and preserving effect on parts of the endothelial glycocalyx and therefore **we could verify our first hypothesis (2.6.1).**

Furthermore, our results show that PAEC perfused with additional 1 % BSA showed fewer gaps in the confluent cell monolayer and a significantly increased elongation and alignment in flow direction compared to PAEC perfused without additional BSA.

In previous experiments we could observe a difference in the cell orientation and elongation between the different BSA concentrations by light microscopy. Therefore we decided to check whether additional BSA in the perfusion medium has an impact on the orientation and the elongation of endothelial cells. Our results show a significant increase of the cell elongation and alignment in flow direction of PAEC perfused with additional BSA in different concentrations compared to PAEC perfused without additional BSA. We could further show that perfusion with additional 1 % BSA was already enough to reach an elongation and an alignment in flow direction of PAEC in our microfluidic in vitro model. Additional 3 % BSA had no beneficial effect compared to additional 1 % BSA. We decided to confirm these findings with further experiments.

Therefore we chose 1 % additional BSA in the next experiment, because it is easier and cheaper to use smaller BSA concentrations regarding the aim of the establishment of an addition of BSA in our microfluidic protocol. We used a higher number of channels and also a bigger magnification of the confocal microscope (20 x objective) to be able to select the outlines of the cells more precisely.

Our results confirm that perfusion with additional 1 % BSA significantly increased the elongation and the alignment in flow direction of PAEC. A more elongated shape combined with a more in flow direction aligned orientation corresponds to a more in vivo like situation of endothelial cells under high shear stress [10]. With those results **we could verify our second hypothesis (2.6.2)**

In addition we successfully established an improvement of our microfluidic in vitro model making it more physiological by adding 1 % BSA to the perfusion medium.

The BSA concentration used in the perfusion medium of our usual microfluidic protocol was around 0.23 % (in our study declared as 0 % additional BSA) because it contains 10 % FBS, with a concentration of around 2.3 % BSA [53]. The physiological concentration of albumin in human plasma is around 3.5-5 g/dl [32], what corresponds to around 3.5 % to 5 %. The addition of 1 % BSA to the perfusion medium leads to a total BSA concentration in the perfusion medium of around 1.23 %. This value is still lower than the physiological albumin concentration in human blood, but our results show that this concentration is already high enough to change the shape and the alignment of PAEC to a more physiological appearance in our microfluidic in vitro model.

Our results from the HS and the GlcNac and Neu5Ac residue experiments combined with the results from the elongation and alignment experiments, are in line with the findings from M. Jacob *et al.* [43], which indicate that albumin plays an important role in the shear stress transmission via the endothelial glycocalyx and suggest that there could be a specific interaction of albumin with the endothelial glycocalyx. Our results are further supported with the findings of other studies which show that shear stress exposure of endothelial cells results in a dynamic reorganization of the endothelial glycocalyx, linked to membrane rafts and actin cytoskeleton which may underlie alterations in endothelial mechanotransduction mechanisms [11].

This leads us to the assumption that a 'healthier' endothelial glycocalyx by the presence of albumin will result in a more efficient mechanotransduction of the shear stress to the endothelial cells and therefore lead to an increased change of their shape and alignment in flow direction.

A first explanation for a better pronounced endothelial glycocalyx due to an increased albumin concentration could be that indeed albumin supports the endothelial glycocalyx structure physically by binding to it. This assumption is in line with M. Jacob *et al.* [43], which assume that there is a specific interaction of natural colloid, such as albumin, with the endothelial glycocalyx. Furthermore, other in vitro studies which show that BSA is absorbed and well integrated in the structure of the endothelial glycocalyx of bovine aortic endothelial cells (BAEC) cultured in static conditions [46], support our findings.

To verify this explanation, by checking whether albumin binds to the endothelial glycocalyx, we suggest repeating our microfluidic experiment with the albumin staining. One should try to

get a confluent cell layer in channels perfused without additional BSA to be able to compare the results with the confluent cell layer in the channels perfused with additional 1 %BSA.

A second explanation for a better pronounced endothelial glycocalyx supported by albumin could be that an application of shear stress leads to an increased production and release of NO from the endothelial cells. In the circulating system of our microfluidic in vitro model, the additional albumin in the perfusion medium will bind the NO and can thereby prevent its rapid inactivation and prolong the vasculoprotective effect. This would lead to reduced activation and better conditions of the endothelial cells, resulting in less shedding and in an increased formation of the endothelial glycocalyx. The more pronounced endothelial glycocalyx can then detect the shear stress more efficiently leading again to an increased production of NO. This explanation is supported by the findings of previous studies which show that the binding of NO to albumin prevents the rapid inactivation of NO and prolongs its anti-aggregant effect on platelets [39]. It goes also along with other studies which show that albumin leads to a significant increase in coronary flow in an isolated perfused organ model what is most likely connected to the shear stress-transmission via the endothelial glycocalyx leading to an increased generation of NO. Stripping away the main part of the endothelial glycocalyx or inhibiting the NO synthase, result in a decrease of the coronary flow [43].

A third explanation for a better pronounced endothelial glycocalyx due to the support of albumin could also be that adding albumin to the perfusion medium would increase its viscosity and therefore increase the shear stress (according to Newton's Law), leading to a stronger stimulus of the endothelial cells which then respond by building a better pronounced glycocalyx. This assumption can be explained with the findings from previous studies which show that shear stress exposure of endothelial cells leads to dynamic reorganization of the endothelial glycocalyx [11] and that shear stress exposure to endothelial cells leads to an increased thickness of the proteoglycan layer on the endothelial surface [12].
To prove this explanation we suggest measuring the viscosity of the perfusion medium with additional 1 % BSA, then to make an experiment with perfusion medium without additional BSA but with additional dextran instead reaching the same viscosity, and compare the results.

In conclusion, the current study demonstrates that albumin is an important supporter of the endothelial glycocalyx structure to maintain the functionality and health of endothelial cells in our microfluidic in vitro model. With this in mind and with regard to the anti-inflammatory, antioxidant and antiplatelet aggregational properties of albumin, and based on the fact that hypoalbuminemia is a powerful prognostic parameter in some cardiovascular disease, our

results support the suggestion that albumin plays an important role in cardiovascular diseases as a modifiable risk factor [5].

In addition, we could successfully establish an improvement of the protocol of our microfluidic in vitro model making it more physiological by supporting a more in vivo-like structure of the endothelial cells.

6   Outlook

Regarding the low amount of data from the GlcNac and Neu5Ac residue experiment we suggest to repeat the WGA–lectin staining to prove the findings that perfusion with additional 1 % BSA leads to an increased expression of GlcNac and Neu5Ac residues on PAEC. In addition, we suggest to perform 3D imaging of the channels with the confocal microscope to be able to see the exact localization of the GlcNac and Neu5Ac residues.

To examine the exact localization of the HS one should also perform 3D imaging of the channels. Especially with regard to the findings that HS remained attached to the channel even when the rest of the cells were detached. Thereby we could investigate if the PAEC secretes HS into the extracellular matrix.

The evaluation of the confocal images with our own cell metrology software concerning the deviation angle from flow direction of PAEC, compared the cell axis with the axis of the channels. With regard to the U – shape of our channels, formed by the inlet and the outlet, it might be that the flow is not perfectly linear along the channel and that a turbulent or a spiral-like flow could occur. Like the flow, the cells could also be aligned in spiral form along the channel. Therefore, we suggest to analyze the cell alignment comparing the axis of the cells among each other and not with the axis of the channel.

With regard to the BSA staining we should repeat the experiment with and without additional 1 % BSA in the perfusion medium but this time starve the cells for FBS during the perfusion in order to have a control with no albumin at all. One could also try to get a confluent cell monolayer in all the channels and to use a more specific antibody for BSA which does not react with other substances.

Since albumin binds NO and thereby prevent its rapid inactivation and prolong its vasculoprotective effect [39], we suggest to measure NO in the remaining perfusate of our circulating microfluidic perfusion system and see whether it is increased with additional BSA in the perfusion medium. In addition we suggest to measure inflammatory cytokines such as

IFNγ, IL-6 or TNF-α in the remaining perfusate, and to stain PAEC for endothelial cell activation markers for instance E-selectin and VCAM to check whether there is less activation and inflammation when the cells are perfused with additional BSA.

In our experiments we used endothelial cells from pigs and serum and albumin from cows. Since this is quite a xenogeneic setting it could be that the endothelial cells do not behave in the most physiological way possible. It is conceivable that the endothelial cells are already activated just by using serum and albumin from another species. Therefore, we suggest to make also experiments in allogenic settings, meaning to use endothelial cells, serum and albumin from the same species. One could use BAEC perfused with medium supplemented with FBS and BSA. In addition we suggest making experiments with human aortic endothelial cells (HAEC), and human albumin, to see whether the same outcome can be observed.

7   Acknowledgements

8 References

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