Usage of a Microfluidic System to Investigate the Endothelial Glycocalyx in vitro

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Written by

Fabian Luther

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Thesis Supervisors
Prof. Dr. R. Rieben, Department of Biomedical Research
Riccardo Sfriso, PhD Student

1 Abstract

The endothelial glycocalyx is a key player in inflammatory regulation, regulation of blood flow and blood coagulation. It acts as a protective layer to hide important adhesion molecules within its structure so that they are only accessible during inflammation, where the endothelial glycocalyx is no longer fully intact. The endothelial glycocalyx is able to bind different anticoagulant factors, i.e. antithrombin III (AT-III), and binding to the endothelial glycocalyx even enhances its anticoagulant capabilities. It is obvious, that an intact endothelial glycocalyx is necessary to keep the vascular endothelium in a healthy condition.
In this study, porcine aortic endothelial cells were cultured in a microchannel and exposed to physiological pulsatile flow. Different antibodies were tested for their ability to stain the endothelial glycocalyx to find the most suitable one. Furthermore, microchannels were then stained with the previously tested antibody to assess the time it takes to fully build up a covering layer of the endothelial glycocalyx on top of the cells under the same physiological pulsatile flow conditions. Additionally, microchannels with pig aortic endothelial cells were perfused with human plasma, human heat-inactivated plasma and pig plasma to assess the binding of AT-III to the endothelial glycocalyx in a xenotransplantation setting and to compare the binding of the AT-III to the allogenic (pig plasma perfused) situation.
The WGA-Lectin staining resulted in the best visible staining for the endothelial glycocalyx. The ability to stain the microchip in vivo enhanced the result of the staining. Fixation process in other staining trials affected the endothelial glycocalyx layer on the endothelial cells and had a negative impact on the staining result too. Assessing of the time for building up the glycocalyx resulted in at least 5 days under perfusion to see a full coverage of the cells by the endothelial glycocalyx. Repetitive experiments need to be conducted to further prove these results. First trials of human plasma / heat-inactivated human plasma / pig plasma perfusion showed mainly staining of the basal membrane. Further improvement to the experimental setting as well as a repetition of the experiment need to be performed to get better visual results of the AT-III binding.
To conclude, the WGA-Lectin staining turned out to be the most appealing staining due to different factors, such as in vivo staining as well as the target of the antibody (the sialic acid residues) being on the glycocalyx. Furthermore, a full covering layer of the endothelial glycocalyx on top of the cells is observable after 5 days of pulsatile physiological flow conditions.

2 Xenotransplantation

Xenotransplantation is defined as a procedure that involves the transplantation, implantation or infusion into a human recipient of live cells, tissues and organs from a non-human animal source or human body fluids, cells, tissues or organs that had ex vivo contact with live non-human animal cells, tissues or organs [1].
Organ transplantation is a well-known therapy for end-stage organ diseases and the only effective therapy for it [1,2]. Over 114'000 organ transplants are carried out every year in the world, which is less than 10% of global needs [3]. The supply-demand discrepancy could be bridged by the usage of xenografts. A reasonable group of animals for xenotransplantation would be non-human primates (NHP) as they are closely related to humans. However, NHP are not the best source for xenotransplantation because of the high risk of cross-species transmission of infections, ethical issues and the organ size disparities compared to adult human organs. Pigs, on the other hand, have a lot of similarities in organ size and physiology with humans. Breeding of pigs is very rapid and makes them more appealing for trials with genetic modifications. Over 600 million pigs are used every year in China to produce meat as well as heparin and more than 100 million pigs are slaughtered just in the US as a source of meat, so from an ethical perspective, there should not be an issue with the usage of pig organs as an alternative source for humans [3]. With the shift of possible donors from NHP to pigs, new immunological hurdles became apparent. Xenorejection, defined as hyper acute rejection (HAR); acute vascular rejection (AVR) and delayed xenograft rejection (DXR), is triggered by humoral and cellular human immune response against endothelial cells of vascularized pig xenografts. The different types of rejection will be discussed later.

A major concern for the usage of pigs as organ donors is the risk of transmitting a disease or infection from pigs (in general all vertebrate animals) to humans, also called zoonosis. Porcine endogenous retroviruses are the most important worth mentioning. The risk is nowadays small, mainly because the pigs will be held under specific pathogen-free and biosecure conditions and are under regular monitoring[4]. If these pigs are compared with the average human donor, one can almost certainly say that they are superior in terms of carrying any infectious agents.

There are other approaches which could solve the issue with the gap of donor organs. Artificial organs and mechanical devices could help for specific types of organ failure but the likelihood of them displacing transplantation as a long-term solution is low. Stem-cell derived organs are also worth mentioning as this is a thriving field which could potentially be the solution for the lack of organs. However, it will still take quite a lot of time until this field moves on towards clinical organ replacement [2].

3 Microfluidic system and chip design

For many years scientists have adapted and perfected protocols for macroscopic cell cultures and there are numerous studies on all the different materials that have been used for this type of cell culture. Culturing cells in a microfluidic system is an approach which is used more and more nowadays. Microfluidics describes the science and technology of systems that use a small amount of fluids (10^{-9} to 10^{-18} litres) and work with channels in a range of tens to hundreds of micrometres [5]. With the new approach to culturing and analysing cells, there are also new hurdles to overcome. Advantages and challenges of this recent system will be discussed in more detail in this chapter.

3.1 Advantages of microfluidic systems

Microfluidic systems can be adapted greatly to the needs of the specific cell type which is going to be used for a project. It is even possible to coculture different types of cells on the same chip. Microfluidics allows to create a microenvironment that is closer to the natural environment cells are used to have, i.e. by continuously perfusing a culture and/or by adding a chemical gradient to the system. Obviously, this system does not require the same number of reagents compared to a macroscopic cell culture and the risk of contamination is lowered drastically. PDMS (Polydimethylsiloxane) has a high flexibility and is convenient to work with and allows the design of complex microfluidic systems. In vitro culture systems struggle to represent the physiological architecture of human organs or vessels. The microfluidic systems allow reproducing designs that are similar to the complex structures of human organs and vessels [6]. It is possible to design microfluidic systems as perfusion systems. Medium will continuously flow through the system and this setup allows the removal of waste products and supplies the cells with fresh medium.

3.2 Challenges of microfluidic systems

Moving cells from a macroscopic culture environment to a microscopic culture is a big change. Most of the culturing protocols are designed for macroscopic system and need therefore revision or extensive trials on the microscale culture system.
On Macroscale cell culture level, oxygen and CO_2 diffuses from the air inside of the incubator into the culture medium and provide enough supply of oxygen for cell growth and proliferation, as well as CO_2 for medium buffering [6]. It is crucial to control the levels of oxygen and CO_2 in microscale cell culturing because even minor changes have a huge impact on the condition of the cells because of the lower cell-to-media ratio. Aerobic respiration and buffering of the medium pH is necessary and can be affected by low levels of CO_2 and O_2.
Macroscopic cell cultures usually have medium unmoved and in excess to ensure a high amount of nutrients for the culture to feed over several days. Microscale systems rely on a regular exchange of medium to provide enough nutrients for the number of cells because, like mentioned above, the cell surface-to-volume ratio is lower.

3.3 Polydimethylsiloxane and surface modification

Silicone is referred to as a synthetic polymer with a repeating chain of Si-O molecules with various organic groups in the backbone attached to the silicone [6]. PDMS, which belongs to the group of the silicones, has two methyl groups attached to the silicon. A curing agent is required for the silicone to allow the polymers to crosslink. This silicone is simple, cost-effective and is appealing because of its gas permeability, optical transparency (crucial for microscopy), biocompatibility and non-toxicity. Like mentioned before, PDMS is easy to handle – by simply mixing PDMS with the curing agent and heating it to accelerate the curing process the final polymerized product is produced. Permanent bonding to glass or plastic is achieved by oxygen plasma bonding. Mammalian cell adhesion on the PDMS surface is a key point for microfluidic systems and crucial

for cell viability, proliferation and differentiation. The surface of PDMS is naturally hydrophobic, which does not favour the formerly mentioned points[7].
Oxygen plasma activates the synthetic surface of PDMS reducing the hydrophobicity and switching it to a hydrophilicity. Addition of APTES (3-Aminopropyl)triethoxysilane) strengthens the

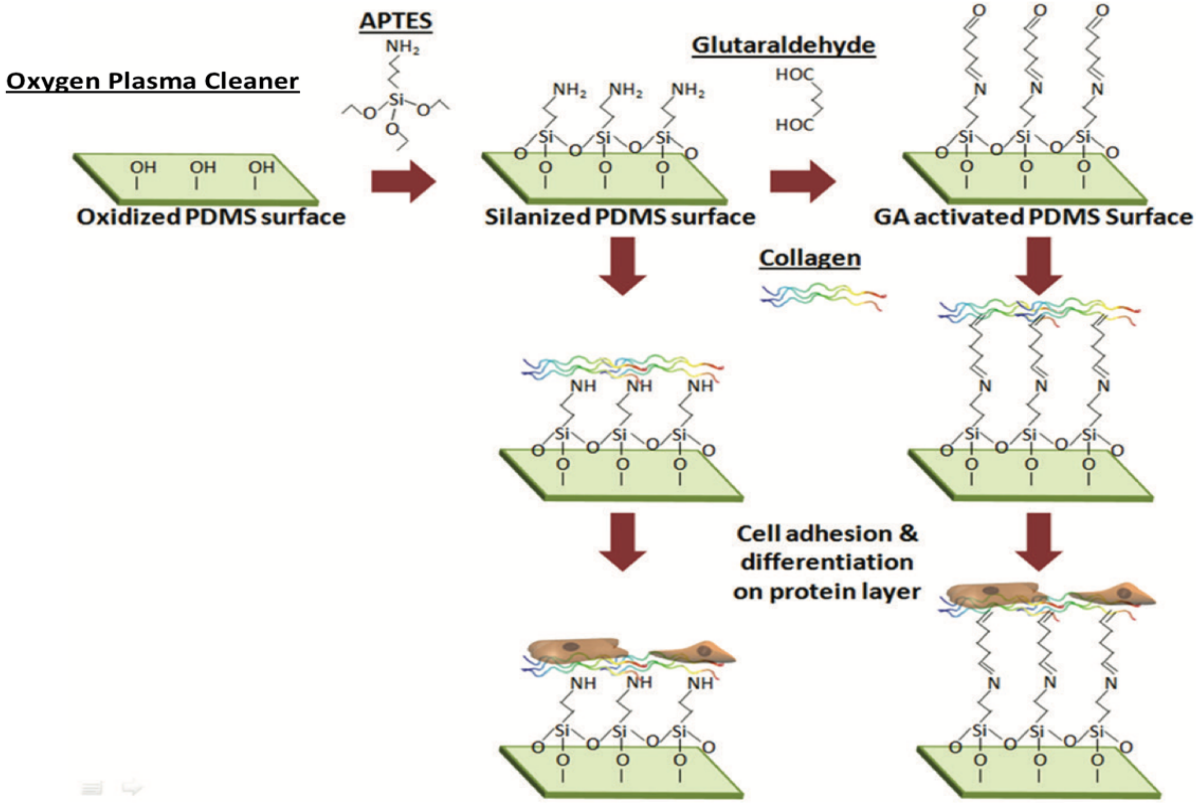

Figure 1: Schematic illustration of PDMS surface modification by APTES ± GA + Protein crosslinking and subsequent cell culture (modified from Chuah et al. 2015)

hydrophilicity of the surface and by adding glutaraldehyde (GA) matrix proteins can be attached, such as collagen and fibronectin, to the cross-linkage of GA and APTES (visualized in figure 1). The combination of APTES and GA results in stable covalent attachment of matrix proteins on the active surface functional groups of the PDMS [7,8]. The integration of extracellular matrix proteins allows cell seeding onto the PDMS. Experiments were done to check if the combination of fibronectin and collagen type 1 resulted in a better support for endothelial cells or if one of the two extracellular matrix proteins provides enough help. It has been shown that the combination of collagen 1 and fibronectin resulted in the best cell adherence [9].

4 Rejection

4.1 Hyperacute rejection (HAR)

HAR is induced by preformed, natural antibodies that are directed against the terminal carbohydrate epitope Galα1-3Galβ1-4GlcNAc (short α1,3-Gal). This omnipresent carbohydrate epitope is existing on the porcine vascular endothelium and is a crucial factor to consider in xenotransplantation.
α1,3 Gal is produced by the enzyme α1,3-galactosyltransferase, which is functional in most species. Humans and their most recent ancestors, the Old-World monkeys, are a special case because the enzyme is not functional in them. Because of the non-functional enzyme, humans and Old-World monkeys express α1,3Gal-specific natural antibodies in their serum. One important reason for the occurrence of these antibodies is the presence of microorganisms expressing α1,3 Gal in the intestinal flora.

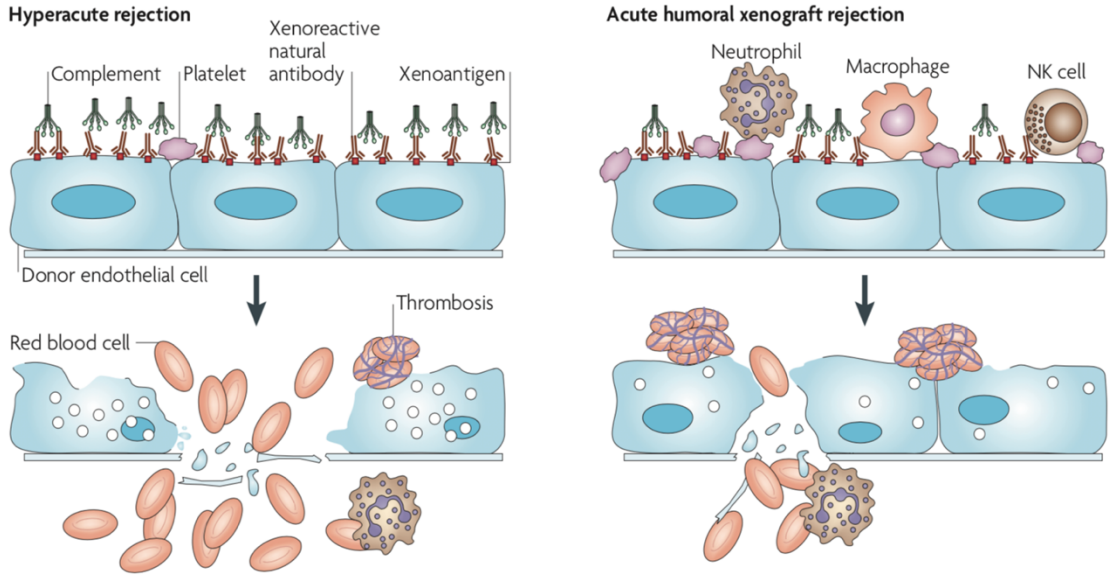

Figure 2: Overview of the hyper acute rejection and the acute humoral xenograft rejection (Yang and Sykes, 2007)

After binding of α1,3 Gal-specific natural antibodies to the endothelium of the vascularized xenograft, a cascade of reactions begins (shown in figure 2). The binding of the antibody activates the complement system, which further triggers the coagulation cascade. The final result of these complex biological processes is the rapid (minutes to hours) graft rejection process. Typical characterizations of HAR are haemorrhage, oedema and thrombosis of small vessels [2,10]

4.2 Acute humoral xenograft rejection (AHXR)

AHXR, also known as acute vascular rejection (AVR) can be induced by a very low level of α1,3Gal-specific natural antibodies and develop over days or weeks after the transplantation. AHXR even develops in primates after getting an organ from an α1,3 GalT-deficient pig and in concordant xenotransplantation settings (α1,3 Gal antibodies are absent) which implies that xenoreactive antibodies which are specific for non-α1,3Gal antigens can trigger the AXHR [2]. Endothelial cell activation and injury, which are important features of AXHR, can cause thrombotic microangiopathy and disseminated intravascular coagulopathy (short DIC) by interfering with the anti-coagulant properties of the endothelium [10].

Molecular incompatibilities between the recipient and donor can enhance the activation of coagulation pathways and intravascular thrombosis. Regulators of coagulation on the endothelium of the xenograft are unable to bind their soluble targets in the recipients' circulation due to these incompatibilities, i.e. the inability of the porcine inhibitor of the tissue-factor pathway that is not capable of neutralizing the human coagulation factor Xa [10].

4.3 Innate-immune cell-mediated xenograft rejection

PAMPs (pathogen-associated molecular patterns) can trigger cells of the innate immune system, i.e natural killer cells (NK cells), macrophages and neutrophils. These cells of the innate immune system can be downregulated by recognizing their own molecules, also called as self-molecules. Species differences in terms of the cells occur in the scenario of a xenotransplantation. The graft has different glycosylation patterns. The immune system of the host detects these differences as PAMPs and activates the cells mentioned above.
NK-cells require regular self-MHC-mediated (self-major-histocompatibility-complex-mediated) inhibitory signals to keep them inactivated. They receive these inhibitory signals from autogeneic MHC molecules. After xenotransplantation the ratio between autogeneic and xenogeneic MHC molecules is different. The likelihood to receive self-MHC-mediated inhibitory signals is less due to higher numbers of xenogeneic MHC molecules. NK cells can get activated by the interplay between their activating receptors and the ligands on xenogeneic cells[2].
Antibody-dependent cell-mediated cytotoxicity (ADCC) is a mechanism by which NK cells are able to recognize IgG molecules bound to the surface of target cells and to mediate cytolysis by secreting cytotoxic factors such as perforin and granzymes. This cytotoxic mechanism implies that natural and induced IgG antibodies may be a crucial factor in NK-cell-mediated rejection. Macrophages and endothelial cells (EC) can be activated through cytokines, i.e. IFNγ, and tumour-necrosis factor (TNF) and induce inflammation. Once activated NK cells can produce and secrete these pro-inflammatory factors contributing to endothelial cell activation[11].

5 Transgenic pig modification

Porcine xenografts could be a valuable resource to lower the growing number of patients with terminal organ failure. The physiology, anatomy and genetics of pigs have a lot in common with those of humans, which further enhances their importance for possible organ donors [12]. Pigs and primates evolved independently over time, this resulted in numerous differences in terms of biological processes and is a striking issue for xenotransplantation. These discrepancies affect the regulation of complicated molecular cascades, i.e. coagulation and complement and can shorten the survivability of a xenograft drastically.
CRISPR (clustered regularly-interspaced short palindromic repeats)/Cas (CRISPR-associated) ZFNs (zinc finger proteins) and TALENs (transcription activator-like effector nucleases) are discoveries that pushed the modification of complex mammalian genomes to new levels and precise as well as fast modification of porcine genome was suddenly possible due to improvements in genomic mapping of humans and pigs [12].

There are different genetic approaches to protect the xenograft from dysregulation of coagulation. By removing xenoantigens, i.e. αGal (*GTKO*) and/or Neu5Gc (*CMAH KO*) one can create a xenograft that is less easily detectable by the recipients' immune system[13].
Expressing molecules like human CRP ameliorate the xenografts capability of regulating the complement activation and increase the survivability of the xenograft. Expression of human TBM (thrombomodulin), TFPI (tissue-factor pathway inhibitor) and EPCR (endothelial protein-C receptor) eliminate the issue of molecular incompatibilities between the recipient and donor and furthermore strengthen the anticoagulant defence of the xenograft [13].

The hyper acute rejection (HAR) can be prevented by the use of α1,3-galactosyltransferase gene knockout xenografts from pigs. Yamada and colleagues were able to extend the survival of pig-to-baboon xenotransplantation with a kidney of a pig (GTKO) up to 83 days and even after the autopsy of the organ they could only see a mild thrombotic microangiopathy[14].

6 Endothelial Glycocalyx

The carbohydrate-rich layer coating the vascular endothelium is called endothelial glycocalyx (EG). It is in contact with the endothelium through backbone molecules, mainly proteoglycans and glycoproteins (will be discussed later in detail). The EG interacts with plasma proteins and lipids. The EG has a large surface area where a lot of exchange of materials between blood and tissue takes place. It is safe to say, that this layer plays an important role in many crucial biological processes, i.e. regulation of blood flow, inflammatory response and blood coagulation[15]. The glycocalyx changes from inactive to the physiologically active endothelial surface layer once plasma constituents are bound to it [16].

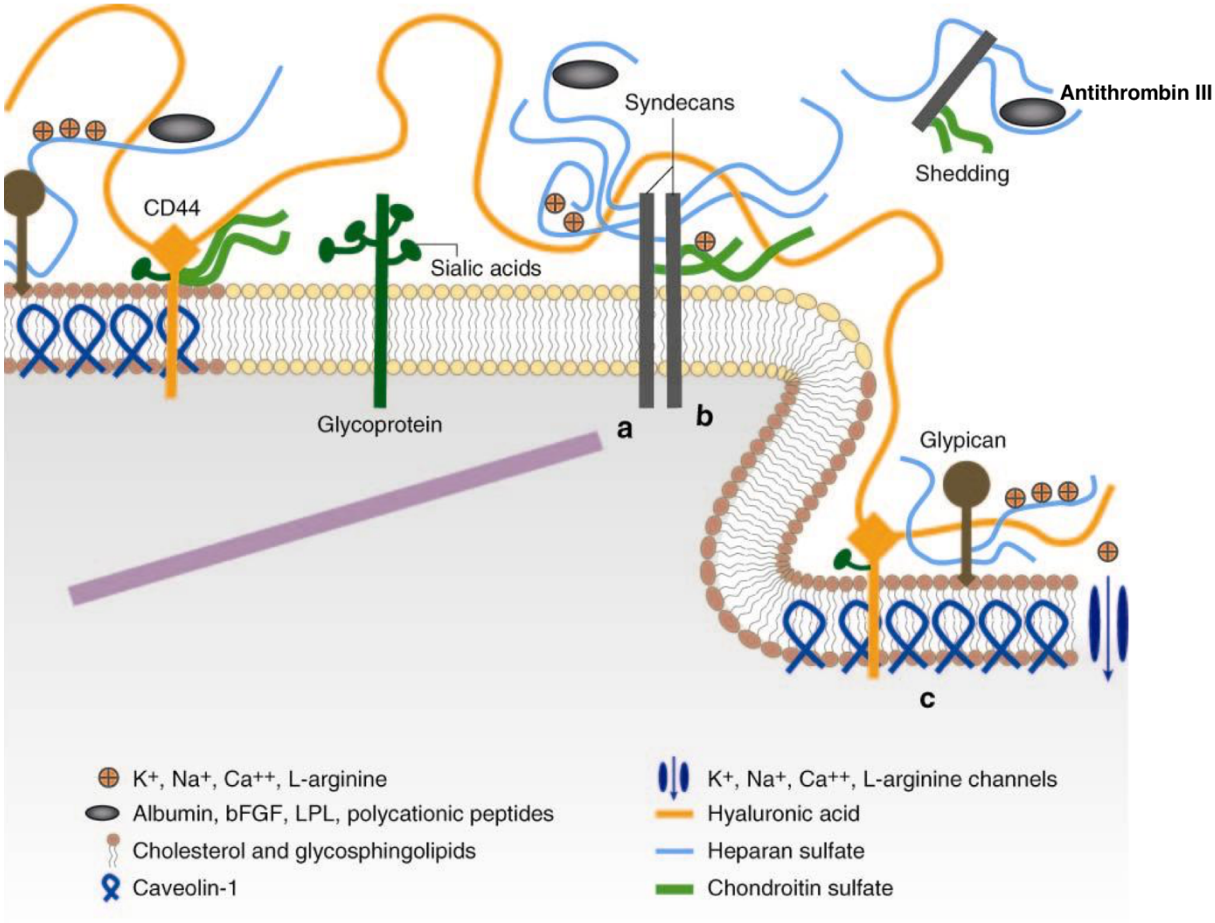

Figure 3: Representation of proteoglycans and glycoproteins on the endothelial glycocalyx (modified from Weinbaum et al. 2007)

The composition and thickness of the endothelial glycocalyx is affected by the flowing blood and there is continuous replacement of the sheared material [16,17].
The associated plasma proteins and soluble glycosaminoglycans and the composition of the membrane-bound proteoglycans, glycoproteins and glycosaminoglycans (GAGs) is always under change and cannot be seen as a static situation [17]. The observed thickness varies between 0.1 and 4.5 μm [15-17] and changes throughout the body depending on which type of vessel you look at. The EG is overall negatively charged which has an impact on the interaction with plasma constituents. The charge is dependent on the sulfation levels of the GAG side chains and changes in the sulfation level influence the vascular permeability and the protein binding [16]. The net negative

charge pushes negatively charged molecules, white blood cells, red blood cells and platelets away [16,18].

6.1 Composition of the endothelial glycocalyx

6.1.1 Proteoglycans

Proteoglycans consist of a core protein with one or more glycosaminoglycan chains linked to it. GAGs are linear heteropolysaccharides that are characterized by specific disaccharide unit repeats[18]. There is a huge variety among the proteoglycan core proteins regarding to their size, number of attached glycosaminoglycan chains and being bound to the cell membrane or not. Syndecans and glypicans, two groups of core proteins, have a tight connection to the cell membrane through a membrane-spanning domain (syndecans) or a glycosylphosphatidylinositol anchor (glypicans) (visible on figure 3). Some proteoglycans, like perlecan, mimecan and biglycan are secreted after their assembly and glycosaminoglycan chain modification, which leads to soluble proteoglycans than can either stay in the glycocalyx or diffuse into the blood stream [16,17].
There are in total five types of glycosaminoglycan chains: heparan sulphate, chondroitin sulphate, dermatan sulphate, keratan sulphate and hyaluronic acid [16-18]. Heparan sulphate makes up 50-90% of the total amount of proteoglycans in the glycocalyx [16,17,19]. Hyaluronic acid is the only proteoglycan that is synthesised on the cell surface and is not covalently attached to a core protein [16,18].

6.1.2 Glycoproteins

Glycoproteins belong to the backbone molecules of the glycocalyx. These glycoproteins have typically small and branched carbohydrate side chains (2-15 sugar residues). The main focus will be held on the endothelial cell adhesion molecules that belong to the group of glycoproteins. The three families of cell adhesion molecules in the endothelial glycocalyx are selectins, integrins and immunoglobulins [17].
Selectins that are mainly present on the vascular endothelium are E- and P-Selectin which are key components for the interaction with leukocytes and endothelial cells.
Integrins are able to bind to collagen, fibronectin and laminin in the subendothelial matrix and play an important role in the interaction of platelets with endothelial cells.
Intercellular adhesion molecules 1 and 2 (ICAM-1 and ICAM-2), vascular cell adhesion molecule 1 (VCAM-1) and platelet/endothelial cell adhesion molecule 1 (PECAM-1) belong to the group of immunoglobulins. These cell adhesion molecules are ligands for integrins on leucocytes and platelets and are crucial for the adhesion to the endothelium and diapedesis [16].
In addition to the cell adhesion molecules, the EG accommodates glycoproteins that influence the coagulation, fibrinolysis and haemostasis.

6.2 Functional importance of the endothelial glycocalyx

The unique position of the endothelial glycocalyx, namely being between the blood stream and the endothelium, highlights the importance of this layer[20]. The EG is a determinant of the vascular permeability. It has a tendency to be more permeable for smaller molecules and therefore the ability to restrict the access of certain molecules to the endothelial cell membrane[17]. Electrostatic charges of the glycocalyx and its permeating substance has a big impact on the permeability. With the glycocalyx being negatively charged due to the highly sulphated glycosaminoglycans it acts as an additional barrier for negatively charged permeating substances. Adhesion molecules, i.e. PECAM, VCAM and ICAM, are covered within the structure of the endothelial glycocalyx[17]. They get more exposed during inflammation which then makes cell rolling and adhesion during diapedesis easier. Integrins and selectins which boost the adhesion of monocytes and polynuclear neutrophils get more accessible as well during the inflammation[16]. By binding of cytokines and / or attenuating the binding of cytokines to the cell surface receptors the endothelial glycocalyx is able to regulate

inflammatory responses. Removing of heparan sulphate through shedding from the glycocalyx boosts the endothelial cell sensitivity to the activation by cytokines[17].

As mentioned before, the glycocalyx is able to push away red blood cells. There is an observable exclusion zone of red blood cells which runs parallel to the endothelium and shrinks in size when the glycocalyx breaks down [16].

Endothelial cells get protection of blood flow by the endothelial surface layer. Endothelial shear stress increases the production of nitric oxide (NO) which then expands vessels and reduces the stress. HUVEC (Human umbilical vein endothelial cells) that were under shear stress doubled their amount of hyaluronic acid in the glycocalyx, which could be a second mechanism to control shear stress [16,17]. Damage to the EG compromises these mechanisms and the response of the endothelium to shear stress.

Different important anticoagulant factors can bind to the glycocalyx. Antithrombin III (see figure 3) inhibits thrombin and activated factors IX and X[15]. The binding to heparan sulphate (found on the glycocalyx) enhances the anti-coagulant activity of Antithrombin III. Dermatan sulphate (also a part of the endothelial glycocalyx) activates the Heparin cofactor II which is a thrombin-specific protease inhibitor. The protein C anticoagulant pathway gets activated by the interaction of thrombomodulin and thrombin. This converts thrombin from a procoagulant enzyme to anticoagulant. Factor VIIa and Xa get inhibited by the binding of tissue factor pathway inhibitor (TFPI) to heparan sulphate[16]. The presence of all these anticoagulant molecules in the glycocalyx supports the thromboresistant nature of a healthy endothelium. To conclude, the anti-coagulant and anti-inflammatory mechanisms are tightly regulated by the glycocalyx through increasing of the activity of TFPI and antithrombin III [21].

The glycocalyx is also capable of binding enzyme that scavenge oxygen radicals, i.e. superoxide dismutase. Superoxide dismutase helps to reduce the oxidative stress and maintain NO bioavailability to prevent endothelial dysfunction [16,18].

7 Complement system

7.1 Origin of complement

The origin of the complement system is back in the 1890s. It was named due to its ability to complement the antibacterial properties of antibody in the heat-stabile part of the serum [22,23]. The complement system is a highly complex compound which involves plasma and membrane-associated serum proteins. These proteins are capable of launching a highly efficient inflammatory immune response against infectious organisms such as viruses, bacteria and parasites as well as damaged tissue (physically and chemically) and surfaces that are not recognized as “self” [22].
Over 30 proteins are a part of the complement system, either soluble proteins in the blood or as membrane-associated proteins on cell surfaces [22-24]. Apoptosis and chemoattraction are just a few of the potent physiological responses that result from the activation of the complement system and the formation of the anaphylatoxins C3a and C5a. The activation process of the complement system and its three pathways an intricate cascade-like action which starts with the identification of potential pathogenic surfaces and will be discussed later in detail. At first, complement was associated only with the innate immunity, where a rapid response is mediated against invading pathogens. Nowadays it is indisputable, that the complement system is a key player in the adaptive immunity, which involves the B and T cells that support the removal of pathogens [23] and in sustaining the immunological memory to prevent pathogenic reinvasion.

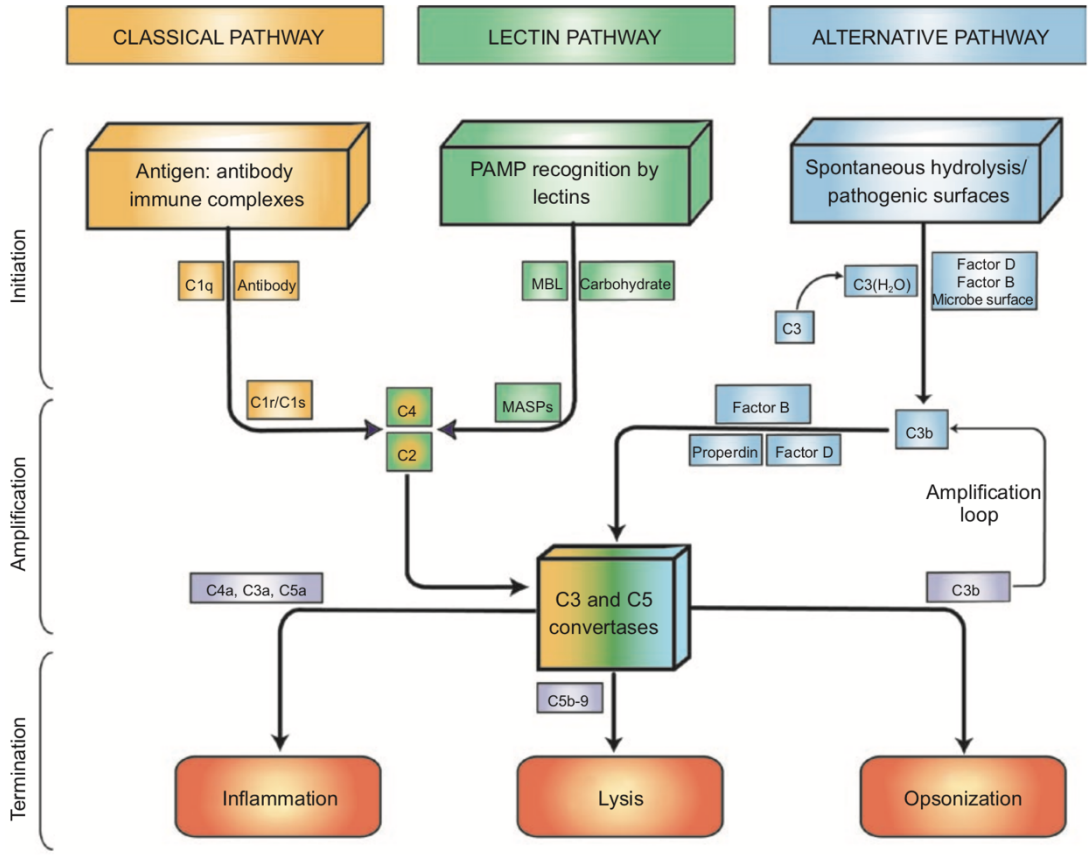

Figure 4: The complement pathway - Overview of the three different pathways, classical pathway, lectin pathway and alternative pathway (from Dunkelberger et al. 2010)

7.2 Classical pathway

IgM or IgG bind to pathogens or foreign and non-self-antigens and build immune complexes that trigger the classical pathway (CP). C1, a multimeric complex which inheres C1q, C1r and C1s molecules, adheres to the Fc (fragment crystallizable region) portion of the IgG and IgM that are attached to pathogenic surfaces. The C1q adherence to the Fc region of the IgG or IgM activates C1s and C1r. C1s cleaves C4 and C2, which results in the cleaving of C4 and C2 into C4a, C4b, C2a and C2b [22,23]. The larger fragments (C2a and C4b) build the C4bC2a complex, also called CP C3 convertase. The C3 convertase of the CP and LP and the C3bBb of the AP cleave C3 to release C3a and C3b. This is the point, where all the different complement activation come together. C3b acts as an opsonin, which results in enhanced phagocytosis and further amplification of the complement activation. C3b can also adhere to the C3 convertase to produce the C5 convertase C3bBbC3B and C4bC2aC3b[23]. Cleaving of C5 to C5a and C5b by the C5 convertase results in the formation of the membrane attack complex (MAC, C5b-9) [25]. C6 and C7 bind to C5b and C8 and several C9 molecules bind to the C5bC6C7 complex to launch the MAC. Cell lysis is achieved due to the insertion of MAC into the cell membrane [22,23].

7.3 Lectin pathway

The lectin pathway (LP) operates immunoglobulin-independent. It detects pattern-recognition receptors (PRRs), i.e. mannose-binding lectin (MBL) and ficolins, to execute nonself recognition [22]. The antigen-recognition receptors of antibodies or T-cell receptors of the adaptive immune system are able to recognize pretty much every possible antigen due to their somatic diversity. PRRs are specifically scanning for highly conserved structures that are mainly present in large groups of microorganisms. These highly conserved structures are also known as pathogen-associated molecular patterns, short PAMPs. MBL is able to adhere to the carbohydrate PAMPs on Gram-negative and Gram-positive bacteria, as well as on viruses, parasites and yeast. MBL and Ficolin are circulating in the serum as complexes with so called MBL-associated proteins, short MASPs [23]. So far, there are four structurally related MASPs, MASP1, MASP2, MASP3 and a cropped version of MASP2 called MAP19 [22,23]. Cleaving of C4 to C4a and C4b happens after binding of pathogens that lead to a conformational change and activate MASP2 [23]. C4b is able to stick to the surface of pathogens and gather C2 to bind to it. The C2 is cleaved by MASP2 and produces C2b and C2a. C4b and C2a converge and build the LP C3 convertase also known as C4bC2a [22].

7.4 Alternative pathway

The alternative pathway (AP) is activated by lipids, carbohydrates and proteins detected on foreign and non-self surfaces [23]. C3 is continually hydrolysed to build C3b, which is capable of adhering to bacteria[26]. C3b binds to the Factor B which then allows the cleaving of Factor B to Bb and Ba by Factor D. This mesh together forms the initial alternative pathway C3 convertase C3bBb [23]. This complex is strengthened by the presence of plasma properdin, which is released by activated neutrophils. Properdin binds C3b and prevents the cleavage of it by Factors H and I [23]. The C3bBb builds the foundation of the amplification loop which converts C3 into C3b and C3a in a similar way to the C4bC2a (C3 convertase) of the classical and lectin pathway [22]. The C3b that was produced by this loop binds to the surfaces in the contiguity, link with Factor B, which then can be triggered by Factor D to build the C3bBb, the most abundant alternative pathway C3 convertase [22].

7.5 Regulation of complement

New discoveries led to the conclusion that MBL is able to trigger C3 independent of C2 which leads to the activation of the lectin pathway[26]. Thrombin is able to activate C5 directly, not relying on C3. This is a new bridge between coagulation and complement and the formation of MAC without being regulated by CD46 or CD55 [26].

CD46, CD55 and CD59 are some of the membrane-bound regulators of the complement system. CD55 quickens the degeneration of the C3 and C5 convertase; CD46 is a cofactor for the cleavage of the C3b by the Factor I and CD59 blocks the formation of the membrane attack complex (MAC) [26].

The complement activation has calamitous possibilities due to its amplification loop of the AP and it is therefore inevitable to regulate the complement system. The activity has to be limited to specific pathogenic surfaces and the production of powerful effectors has to be controlled rigorously to protect the host tissue from collateral damage. The control of the complement activation is regulated on many different levels. This results in a strict balance between the competent detection and neutralization of pathogens and the diminution of unnecessary tissue damage.
Regulation of the complement system happens at two specific situations in the cascade: the assembly and enzymatic activity of the convertase and the construction of the membrane attack complex (MAC) [22,23]. C4b and C3b fragments that are covalently linked to cellular surfaces are regulated by two ways. One of the two is the prevention of building active convertases. This is achieved by catabolism of C3b and C4b through the active serine protease Factor I, which leads to inactive fragments of C3b and C4b, like iC3b, C3c and C3dg [22,23]. To obstruct nonspecific C3b degeneration Factor I rely on cofactors for its proteolytic activity [22]. There are membrane cofactor proteins (MCP, CD46) complement receptor 1 (CR1) and Factor H[22,23]. These cofactors are either intrinsic membrane proteins on host cells or have specific mechanisms to assure the proper cofactor activity on the host surfaces. This specific cofactor activity ensures to prevent unnecessary tissue damage [22]. The second way to regulate deposited C3b/C4b fragments is by building active convertases that have their biological imperative. In the case of uncontrollable C3b deposition, which leads to high levels of C3 convertase formation, there are several complement inhibitors that have either inhibitory or decay-accelerating activities for the C3 convertase. One example of these complement inhibitors is CD55, also known as decay-accelerating factor. CD55 prevents the assembly of new C3 convertases and also shortens the half-life of the preformed convertases [22,23]. This limits the ability to activate the complement system even further. Other complement inhibitors with decay-accelerating activity that should be mentioned are CR1, Factor H, C4-binding protein (C4BP).
Host-specific expression patterns of the complement regulators ensure that there is host-specific protection. Factor H, a fluid-phase inhibitor, is able to provide host-specific protection through the binding of sialic acids and heparin (also called polyanions) that are main components of the eukaryotic (but not prokaryotic) cell surfaces [22]. This leads to the consequence, that Factor H binds to host surfaces and uses cofactors and decay-accelerating activities to inhibit C3 convertase formation and ultimately leads to the catabolic degradation of C3b [22,23].
The inhibition of the assembly of MAC is usually achieved by membrane-bound (CD59) or fluid phase (S protein) inhibitors, that act in situation where the complement activation is very rapid and extensive[22].

8 Coagulation

Under normal circumstances, the coagulation process is under control by numerous inhibitors which prevent the clot formation and furthermore avoid the thrombus propagation. This complex equilibrium is disturbed when the procoagulant activity of the coagulation factors is enhanced or the inhibitory capabilities of the inhibitors is lowered.

8.1 Primary Haemostasis

Formation of an initial platelet plug due to complex interactions between platelets, the vessel wall and adhesive proteins is called primary haemostasis. The subendothelial layer is highly thrombogenic and contains several procoagulant factors like collagen, von Willebrand factor (vWf) and proteins that play an important role in platelet adhesion [27].

8.1.1 Platelet structure

Platelets are cell fragments, that are disc shaped and anucleate. They contain three different types of granules, which are able to release their content after being activated. The alpha granule is the most abundant type of granule. It contains proteins that provide a surface for platelet adhesion, such as vWf and fibrinogen [28]. Alpha granules are also capable of secreting factors that mediate blood vessel formation and inflammation. They contain both type of factors, the ones that promote new blood vessel formation (angiogenic factors) and the ones that stabilize the established vessels (angiogenesis inhibitors).

8.1.2 Platelet adhesion

Once the vessel wall is injured platelets are able to interact with it in different ways depending on the shear rate. In high shear stress situations, collagen that is exposed binds to the A3 domain of the vWf [28]. After the binding, the A1 domain of vWF is exposed and as a consequence the platelet is able to bind via their platelet surface receptor GpIb-V-IX[28]. The platelet glycoprotein complex I (GP-Ib) is the main receptor for vWf [27]. Binding of the GP-Ib-V-IX receptor to the collagen-bound vWf links the platelets at the site of the injury [28]. Therefore, under shear stress the interaction between GpIb-V-IX and vWf leads to platelet agglutination at the site of injury.

8.1.3 Platelet secretion

The adhesion process of platelets leads to the degranulation of the platelet. This degranulation process releases different factors. One important is calcium. The calcium is able to bind to the phospholipids that appear after the platelet activation. This binding acts as a surface for the assembly of coagulation factors [27].

8.1.4 Platelet aggregation

Further platelet aggregation is stimulated by Thromboxane A2 which is produced by activated platelets. Adenosine-5-diphosphate (ADP) together with TxA2 enlarge the platelet aggregate ending in a formation of a platelet plug which has the ability to seal off a vascular injury temporarily. ADP binding has an impact on the GpIIb/IIIa receptor on the surface of the platelet and changes its conformation. This conformational change allows the deposition of fibrinogen [27]. The conversion of fibrinogen to fibrin is initiated by the thrombin generation. Fibrin adds more stability to the platelet plug and this stabilization is known as secondary haemostasis [27,28].

8.2 Coagulation proteins

Von Willebrand factor is present in the blood plasma and is a glycoprotein. It is produced in the endothelium and the subendothelial connective tissues. Its job is to mediate platelet adhesion to

the subendothelial surface, as mentioned above. It is a carrier protein for the coagulant activity of Factor VIII.
Prothrombin is an unstable protein, which gets cleaved by the activated factor X into two proteins. One of these small proteins is thrombin, which has pro inflammatory effects. The cleaving process is enhanced, when the activated factor X complexes with its the activated co-factor V. Together they are called prothrombinase-complex (further explained later in the common pathway) and are in a procoagulant state [29].
The precursor of fibrin is fibrinogen, a protein which gets synthesised in the liver. It has an important role as a coagulation protein because it defines the strength of the clot.
Tissue factor, also known as Factor III is a membrane bound glycoprotein with procoagulant abilities. It is usually present in the subendothelial tissue and also in fibroblasts. There are a lot of possibilities to activate TF such as inflammation, direct vascular injury, physical injury or hypoxia [27,30].

8.3 Natural anticoagulants in the blood

Antithrombin (AT) is a key regulator of the coagulation system by acting as a main inhibitor for thrombin. AT is a serine protease inhibitor which is capable of binding and inactivating thrombin, factor IXa, Xa, Xia, and XIIa[27,31]. AT is capable of binding to the heparan sulphate binding sites that are present on the endothelial cell surface layer. If the endothelial surface layer is disrupted, heparan sulphate gets removed and also the AT that is bound to it, which sets this location into a procoagulant state (also visible in figure 3).
Thrombomodulin, an endothelial membrane protein, is a crucial anticoagulant and anti-inflammatory player[32]. It is capable of changing thrombin's activity by altering the favour substrate range. This switch prevents the dissociation of fibrinogen and activation of the protease activated receptor 1 (PAR-1). It also enhances the activation of protein C and the thrombin-activated fibrinolysis inhibitor (TAFI) [32,33].

8.3.1 Protein C pathway

Another serine protease which is important to mention is the protein C. Protein C has important anticoagulant and anti-inflammatory properties and is profibrinolytic [27,28]. Thrombin activates this serine protease which is called activated protein C (APC) in its activated status and APC inhibits Va and VIIIa. The endothelial protein C receptor is a transmembrane receptor that has the ability to activate the protein C as well.
Protein S is a glycoprotein synthesised by endothelial cells and has a free form, which is in the plasma and a bound form, which is bound to the C4b-binding protein [27]. The free form is capable of acting as an anticoagulant while the bound form is an inhibitor for the complement system.

8.4 Coagulation cascade

8.4.1 Extrinsic pathway

The TF activates the first step in the plasma mediated haemostasis. Tissue factor is located in the subendothelial tissue. The chances of TF to get into contact with plasma procoagulants is minimized under healthy physiological conditions of the vascular endothelium. As soon as the vascular endothelium is damaged, the TF is able to bind to factor VIIa and calcium. This binding results in enhanced conversion of factor X to Xa [27,28].

8.4.2 Intrinsic pathway

The intrinsic pathway is parallel to the extrinsic pathway and end ultimately in the activation of factor X. High-molecular-weight kininogen together with factor XII, XI and prekallekerin (serine protease) result in the activation of factor XI[27]. The activation of factor XI leads to the activation of factor IX.

With the help of a cofactor (factor VIII) the IXa forms tenase complexes on the phospholipid surface to activate factor X (overview on figure 4).

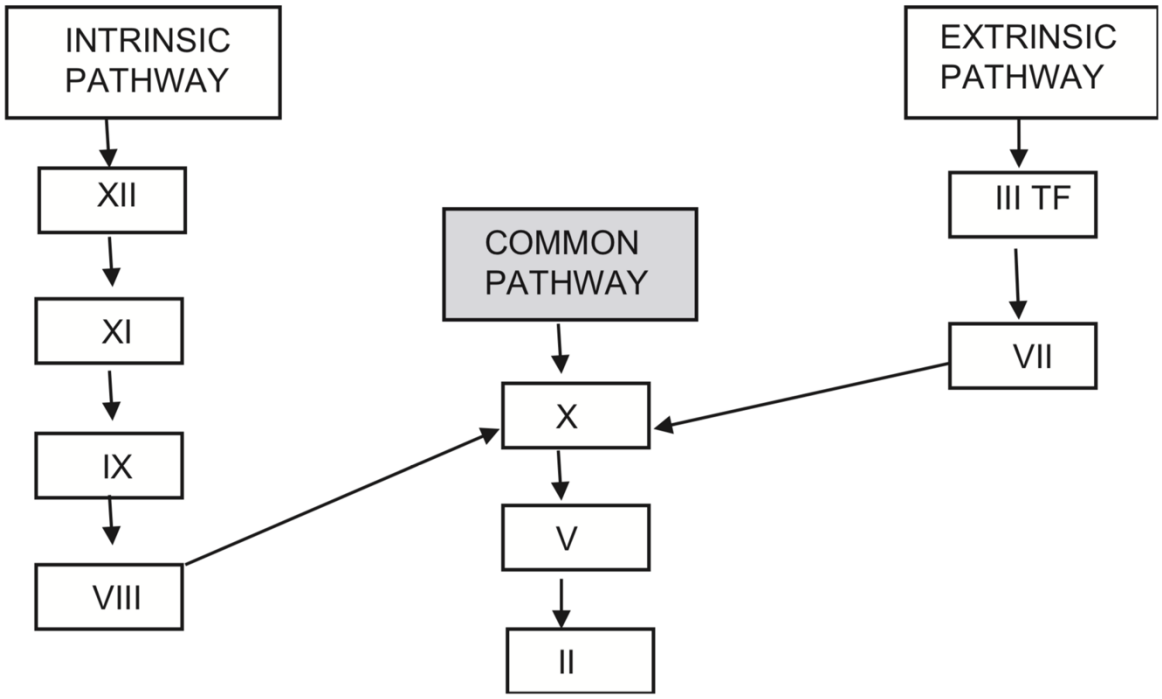

Figure 5: Overview of the pathways of coagulation (from Palta et al. 2014)

8.4.3 Common pathway

Factor X can be activated by the intrinsic and extrinsic pathway and plays a key role in the common pathway. Tissue phospholipids, platelet phospholipids, calcium, cofactor factor V and the activated factor X build the prothrombinase complex. This complex is able to turn prothrombin into thrombin, which later cleaves circulating fibrinogen to the insoluble fibrin and activates the factor XIII [27]. The activated factor XIII covalently crosslinks all the fibrin polymers that are meshed together in the platelet plug. This crosslinking builds a connection between the fibrins and stabilizes the clot further.

9 Aim of the study

A good visualization of the endothelial glycocalyx is needed to assess the integrity or the damage of the endothelial glycocalyx in transplantation settings to perform other experiments or to get accurate measurements of this layer. This led to the first aim of the experiment, being the visualization of the endothelial glycocalyx by immunofluorescence.
One of the previously mentioned further experiments is to assess the time it takes to build a full cover of the cells by the endothelial glycocalyx. Determining this time is the second aim of the study, which cannot be performed if we do not have a suitable antibody to visualize the endothelial glycocalyx.

10 Materials and Methods

10.1 Cultivation of pig aortic endothelial cells (PAEC)

Pig aortic endothelial cells (PAEC) between passage two and five, that were previously isolated and stored at -150°C, were cultivated in a T75 flask. DMEM Glutamax 1g/l D-Glucose (Gibco, 21883-025) supplemented with 10% Fetal Bovine Serum (FBS) (Biochrom AG, Cat. No. S0615), 1% penicillin/streptomycin (Life Technologies; Cat. No. 15140-114), 0,4% Endothelial Cell SupplementMix (Promocell, Cat. No. C-39216) and 25 µl Heparin (Liquemin 25'000 U/5ml heparinium natricum, Drossapharm AG) was used for the cultivation of the PAEC. We had some issues with the general health of the cells and later decided to exclude Heparin and Endothelial Cell SupplementMix from the medium. Cells were grown until confluency at 37°C in a 5% CO_2 incubator and the medium was exchanged every other day.

10.2 Production of microfluidic channels

The microchannels were produced out of polydimethylsiloxane (PDMS, Dow corning) mixed together with curing agent (Dow corning) in a ratio of 10:1. The mixture was poured into a petri dish and a vacuum pump was used to get rid of the air bubbles. Two support needles (Ø120µm, Seirin) were placed vertically and four molding needles (Ø 550µm, Braun) were placed horizontally on top of the support needles. The PDMS was cured overnight in a 60°C oven. After curing, the chips were cut out of the petri dish and the molding needles were removed horizontally to prevent damage to the channel. A 2mm biopsy puncher (kai medical) was used to create the inlet and outlet of the channels in a distance of one cm. The small parts of the channel before and after the inlet / outlet were sealed with PDMS and cured overnight.

10.3 Surface modification of PDMS chips

The surface of the channels was modified to allow the binding of extracellular matrix proteins. PDMS chips and glass slides were cleaned with isopropanol, soap water and distilled water and dried with a nitrogen gun. Glass slides and the chip were put into an oxygen plasma cleaner to activate the surface at 650 mTorr for 3 minutes. After the oxygen plasma treatment, the chip was mounted onto the glass slides. Immediately after the oxygen plasma treatment the hydrophobic surface of the PDMS chip was permanently switched to hydrophilic by filling up the channels with 5% 3-Triethoxysilylpropylamine (APTES, Sigma Aldrich A3648-100m) and leaving them like this for twenty minutes. The chip was washed with ultrapure water and then incubated for thirty minutes with 0,1% glutaraldehyde (Sigma G-6257). Glutaraldehyde was used to provide a crosslinker for the immobilization of extracellular matrix proteins [34]. Channels were washed with ultrapure water and incubated with 50µg/ml human fibronectin (Merck, FC010) in phosphate-buffered saline (PBS) overnight in the laminar flow at RT under UV light. We later switched to incubating the channels with human fibronectin for 60 minutes at 37°C in a 5% CO_2 incubator. After the fibronectin incubation the channels were directly filled with 100µg/ml bovine collagen I (Gibco A10644-01) in 0.02M Acetic acid (Gibco, Thermo Fisher Scientific). We switched to diluting the collagen I in sterile distilled water and switched back in the end to 0.02M acetic acid. We included an additional washing step with PBS after the incubation with collagen I. After washing, the channels were incubated with cell culture medium for at least 30 minutes in the incubator at 37°C at 5% CO_2 to prevent unspecific protein binding with the collagen I and as an additional washing step.

10.4 Cell seeding and connection of the pump

Confluent PAEC were washed with PBS, trypsinized using 0,05% Trypsin-EDTA (Gibco Life technologies, 25300-054) and diluted in cell culture medium to inactivate the Trypsin. The suspension was centrifuged at 1200 rpm for 8 minutes and the pellet was resuspended in 1 ml of cell culture medium with 4% Dextran from Leuconstoc spp. (Sigma, 31390-100G). Cells were counted and diluted to a cell suspension with 10^6 cells/ml. Cells were injected at the inlet of the microchannels with a 200µl pipette and the chip was incubated for 15 minutes inside the incubator at 37°C at 5% CO_2 upside down. The chip was fixed with some scotch tape to prevent it from falling down. The unattached cells were aspirated and some more cells were loaded into the channels. The chip was incubated for 60 more minutes in the normal orientation in the incubator. After the cell loading, the chip was washed with cell culture medium containing 4% Dextran to remove all unattached cells. The medium was changed every 2 hours under the static conditions.

Cells in the channels had to be confluent before the pump was connected to the chip. The peristaltic pump Minipuls 3 with 8 channels (Gilson) was used to create a pulsatile flow. The tubings were sterile silicon tubings (Gilson) with specific pump head adaptors and longer extension silicone tubings (Gobatec). All the tubes were autoclaved and connected to the pump. The tubes were first flushed with sterile distilled water, PBS and cell culture medium with 4% dextran. A 15 ml falcon tube per channel was used as a medium reservoir and filled with 10 ml of cell culture medium with 4% dextran. The outlet of the channel was set at 8 ml, the inlet at 2ml height of the falcon tube. We later switched this around to ensure, that any waste products or unattached cells would stay at the bottom of the reservoir tube, therefore preventing recirculation of unattached cells in the system. We started to put some more medium inside of the reservoir tubes and started the pump without connecting the outlets of the channels. This was done to ensure, that all the cell debris and unattached cells were flushed through the channel at first to get rid of them. We changed this due to a high amount of cell detachment in the experiments. The reservoir tubes and the chip were put into the incubator at 37°C at 5% CO_2 and the pump remained on the outside of the incubator. The pump was initially set to 6 RPM directly. We adapted the flow rate during the experiment extensively. We had trials with 1 RPM overnight and then increasing the flow at 1RPM/h the next day, which resulted in being too low flow for the cells and led to cell detachment. We tried 2 RPM over the weekend, which also ended up with empty channels. We finally found a satisfying way by putting the cells directly at 10 RPM, which resulted in good survival. The desired flow rate was kept for several days, depending on the experiment. Medium was changed every other day.

10.5 Perfusion of PAEC with human plasma and pig plasma

PAEC were under 10 RPM flow for 24 hours before the perfusion. Human plasma and pig plasma were diluted 1:10 in cell culture medium with 4% dextran. The reservoir tubes were changed from cell culture medium with 4% dextran to the cell culture medium with 4% dextran and 10% human or pig plasma. The channels were perfused for 2 hours at 10 RPM. After perfusion, all the tubings were removed, the channels were washed with serum free medium and used for immunofluorescence staining.

10.6 Immunofluorescence staining

Immunofluorescence staining was done to characterize the PAEC, to visualize the endothelial glycocalyx with different antibodies, to check for AT-III binding, to check for endothelial cell activation and to perform live-cell staining. If live cell staining was performed channels were washed with serum free medium and the WGA-Lectin was added on the channels and sucked through with a vacuum boy. Channels were incubated for 15 minutes at 37°C and 5% CO_2. All channels were washed with PBS^{++} and fixed with 4% paraformaldehyde for 10 minutes at room temperature. Channels were washed again with PBS, then permeabilized (if needed) with 0.5% Triton X in PBS

for 10 minutes on a shaker. PBS-3% BSA was used for the blocking step at room temperature for 30 minutes. Directly after the blocking, the channels were incubated with the primary antibody diluted in PBS-1%BSA (and 0.05% Tween 20 if permeabilized) overnight at 4°C or at room temperature for 1 hour on a shaker. After the incubation with the primary antibody, the channels were washed with PBS and then incubated with the secondary antibody and DAPI, diluted in the same solutions as the primary antibody. Incubation time for the secondary antibody with DAPI was 1 hour at room temperature on a shaker. The channels were washed again with PBS after the final incubation and stored at 4°C in the fridge covered with aluminium foil and parafilm to prevent any bleaching and evaporation.
Here is a list of the different antibodies used, grouped in primary, secondary and directly labelled antibodies:

Primary Antibodies	Dilution	Company
AT-III C(-18) Goat polyclonal IgG	1:50	Santa Cruz
CD 31 Rat Anti Porcine	1:100	R&D Systems
CD68 Mouse Anti Human	1:100	Dako
HS Mouse Anti human	1:100	Amsbio
Mouse anti porcine E-Selectin	1:100	Sigma
Mouse anti porcine V-Cam 1	1:100	Homemade
Perlecan Rat monoclonal	1:100	Abcam
Rabbit anti vWf IgG	1:100	Dako
Syndecan Goat polyclonal IgG Anti Human	1:50	Santa Cruz Biotechnology
Ve-cadherin mouse monoclonal IgG	1:100	Santa Cruz

Secondary Antibodies	Dilution	Company
Donkey Anti-Goat IgG Alexa Fluor 633	1:500	Thermo Fisher Scientific
Goat Anti Mouse 546 Alexa Fluor	1:500	Invitrogen
Goat Anti-Mouse IgG Alexa Fluor 488	1:500	Life technologies
Goat Anti-Mouse IgM FITC	1:500	Jackson Immuno Research
Goat Anti-Rabbit FITC	1:500	Southern Biotech
Goat Anti-Rat IgG Cy3	1:500	Jackson Immuno Research

Directly labelled Antibodies	Dilution	Company
4',6-diamidino-2-phenylindole (DAPI)	1:1000	Boehringer Mannheim GmbH
F-Actin FITC	1:100	Sigma
Fibrinogen FITC	1:500	Dako
WGA-Lectin FITC	1:100	Sigma

A confocal laser-scanning microscope (LSM 710, Zeiss) was used at 10x, 40x and 63x magnification. ImageJ (National Institutes of Health [35]) was used for image analysis. The overall thickness of the microfluidic chip is 0.5 cm and the distance between the bottom of the microchannel and the bottom of the device is 120μm, which allows a good image acquisition [34].

11 Results

11.1 Pig aortic endothelial cell characterization

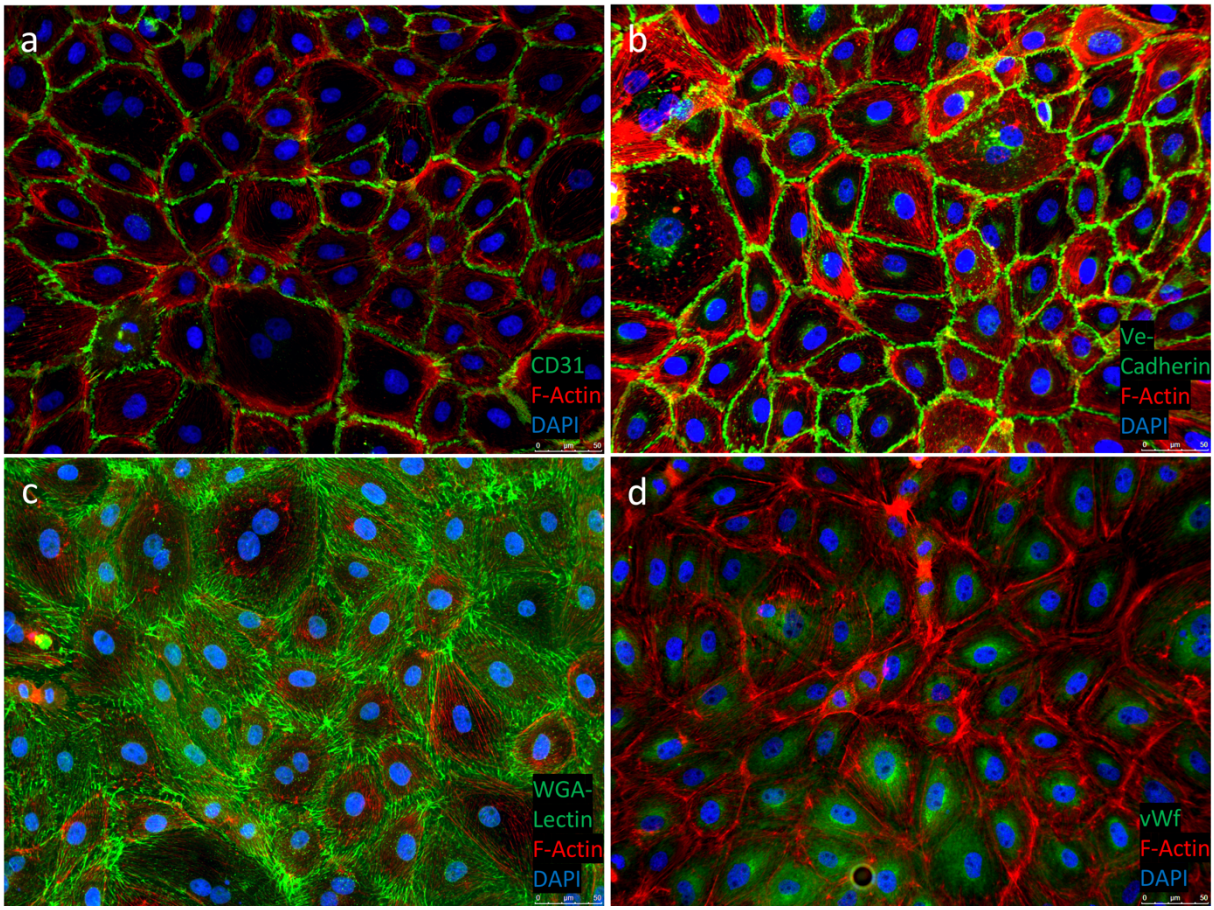

Figure 6: Characterization of PAEC WT P6 (pig 7, isolated 15.4.15) – (**a**) CD31 (**b**) Ve-Cadherin (**c**) WGA-Lectin (**d**) vWf – all cells were stained with F-Actin and DAPI as well. Cells were kept under static condition in a chamber slide until confluency and then fixed and stained. Images were taken by a fluorescence microscope (Leika DMI 4000b).

Pig aortic endothelial cells (Passage 6, pig 7, isolated on the 15.4.15) were characterized by their expression of typical endothelial cell markers such as CD31, VE-Cadherin, vWf and WGA-Lectin. The cells were cultivated in a fibronectin coated glass chamber slide until they reached confluency. They were fixed and then stained for the previously named markers. The cells expressed all of the markers that we stained for and we were able to clearly define these cells as endothelial cells.

11.2 Perlecan and syndecan are both colocalized with heparan sulfate

We were experimenting with different stainings to find the best solution for the staining of the endothelial glycocalyx. All three (heparan sulfate, syndecan and perlecan) stainings worked out fine. Syndecans, as mentioned before, are core proteins that have a tight connection to the cell membrane through a membrane spanning domain and perlecan is a proteoglycan. Syndecan II is mainly present at the basal membrane. This is visible in the ortho view of the staining in *figure 7e*. There is a red staining on both the y-z and x-z axis that is fully covering the bottom layer of the cells. This bottom layer is the connection point from the EC-layer and the chip, which is the basal membrane. Due to colocalization of heparan sulphate and syndecan we know that heparan sulphate is present on the basal membrane as well. The perlecan is colocalized with the heparan sulphate (see *figure 8e* and the comparison of *figure 8c* and *figure 8d*). So heparan sulphate,

perlecan and syndecan are all present on the basal membrane. It was not possible for us to determine a best fit candidate in terms of the staining for the endothelial glycocalyx.

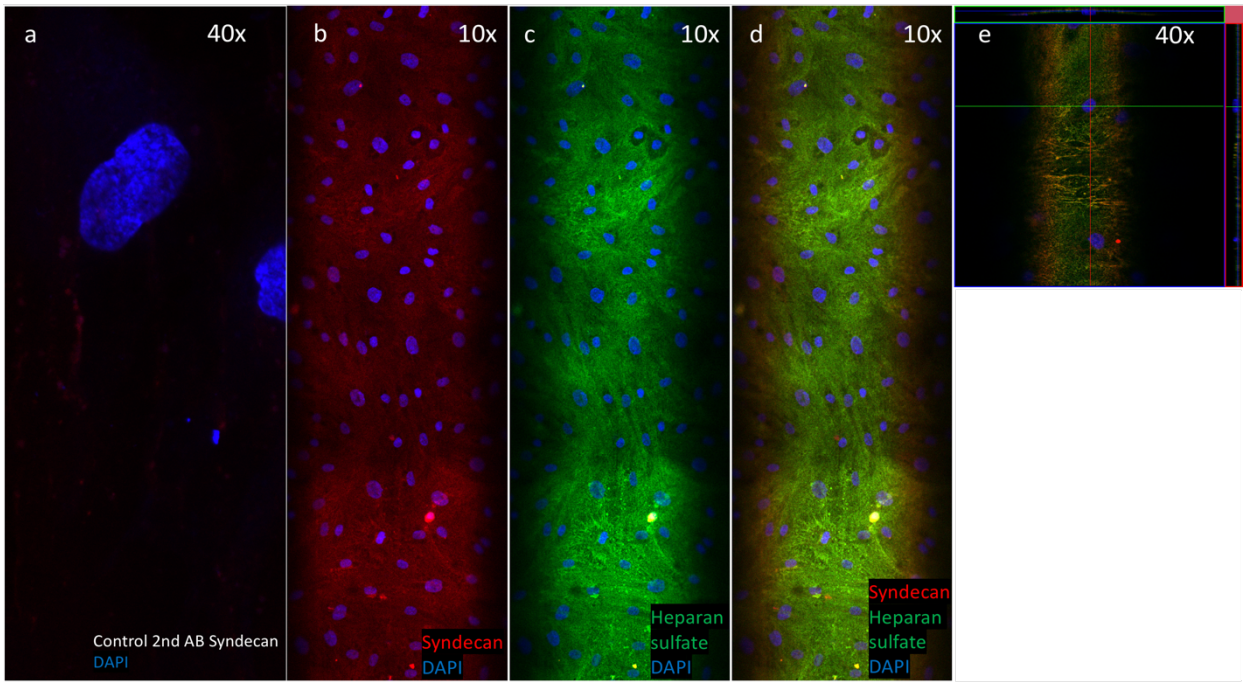

Figure 7: Staining of PAEC WT P6 that were cultured on a microchip under static conditions for 3 days. The medium was changed every 2 hours. **(a)** control of the secondary antibody for the syndecan staining **(b)** syndecan II staining **(c)** heparan sulphate staining **(d)** combined picture of the syndecan and heparan sulphate staining **(e)** ortho view of the combined staining. All channels were stained for DAPI to detect the cell nuclei.

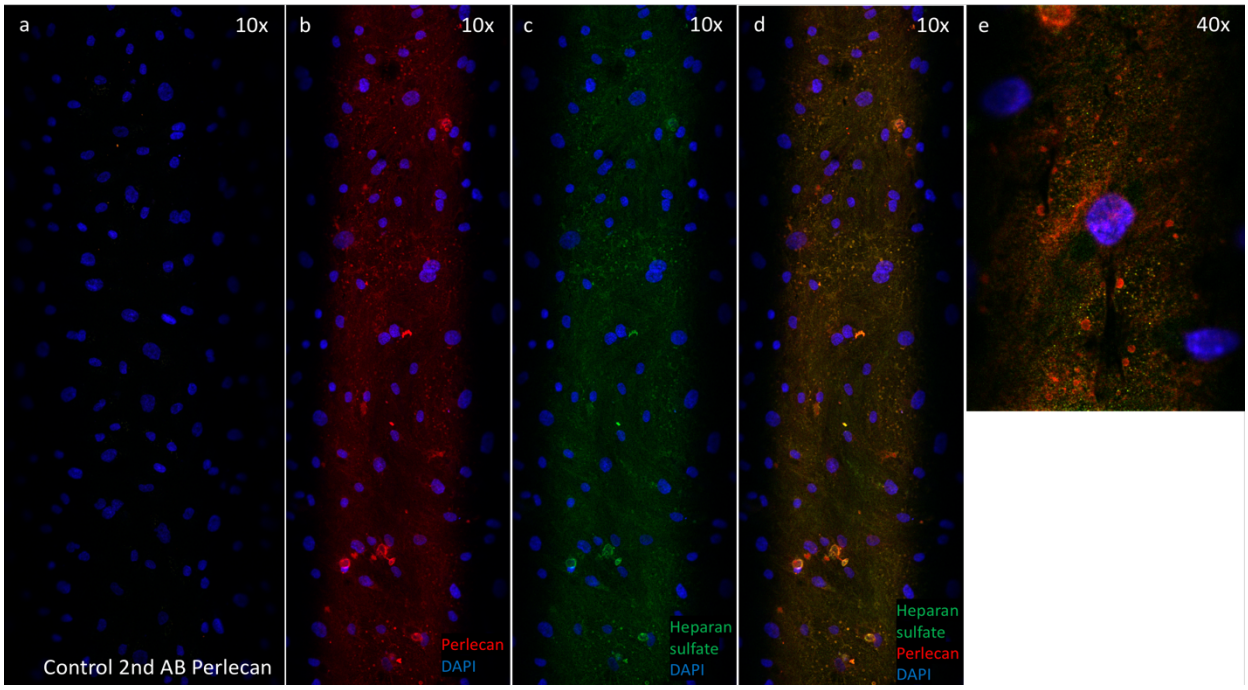

Figure 8: Staining of PAEC WT P6 that were cultured on a microchip under static conditions for 3 days. The medium was changed every 2 hours. **(a)** control of the secondary antibody for the perlecan staining **(b)** perlecan staining **(c)** heparan sulphate staining **(d)** combined picture of the perlecan and heparan sulphate staining **(e)** 40x view of the combined staining. All channels were stained for DAPI to detect the cell nuclei.

11.3 *In vivo* WGA-Lectin staining of P2 PAEC WT 1 day under flow

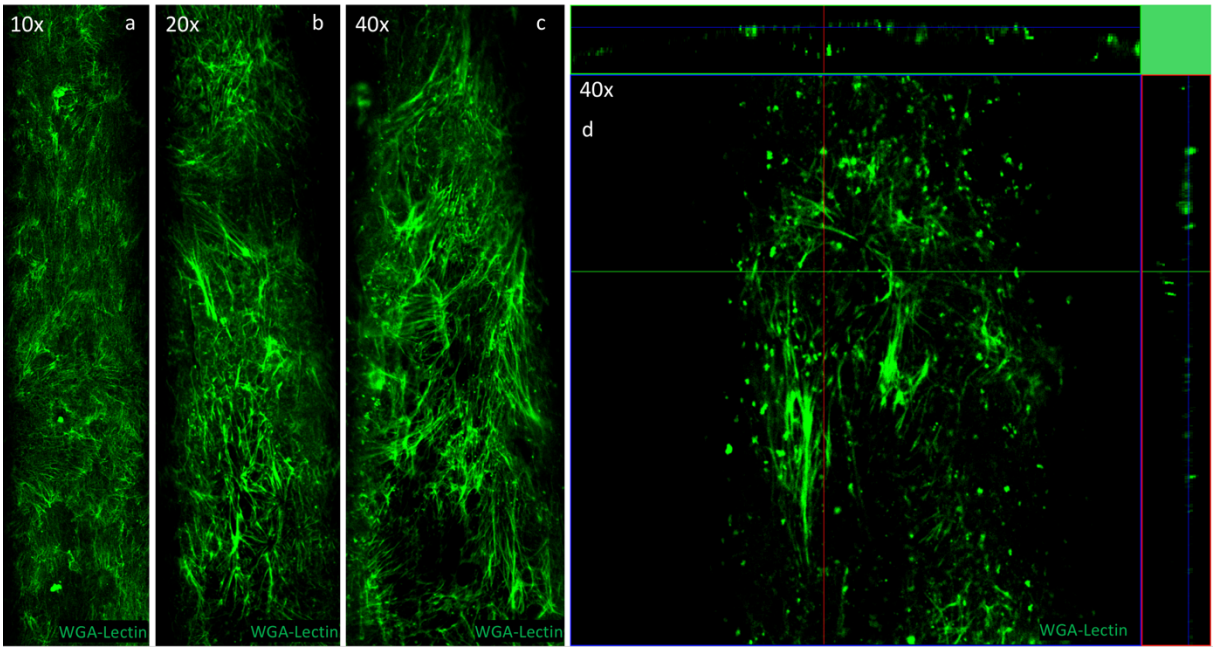

Figure 9: In vivo WGA staining of Sialic Acid Residues (Neu5Ac, N-Acetylneuraminic Acid) and N-acetyl-D-glucosaminyl Residues(GlcNAc) of P2 PAEC that were 1 day under 6 RPM flow. **(a)** 10x magnification of the WGA-Lectin staining **(b)** 20x magnification of the WGA-Lectin staining **(c)** 40x magnification of the WGA-Lectin staining **(d)** ortho view of the WGA-Lectin staining

The sialic acids are components of the endothelial glycocalyx *(*see *figure 3)* and are on the connective layer between blood and the endothelium. The staining was done on living cells, meaning that the cells were stained before they were fixed. The staining resulted in a filamentous looking structure on the surface of the cells. There is no nuclei staining here, because we don't have a nuclei staining antibody for *in vivo* staining. A nucleus covering shape is visible on the x-z and the y-z axis of the ortho view (*figure 9d)*, even though there is no full coverage yet after only one day under flow. Still, the staining is strong, and the antibody seems to be a suitable choice for the staining of the endothelial glycocalyx. We performed more stainings with WGA-Lectin on chamber slides and microchips that will be presented later.

11.4 *In vivo* WGA-Lectin staining of P2 PAEC WT one day under flow

We managed to keep cells under flow for 5 days and stained these with the WGA-Lectin. The amount of fluorescence detected is different in the two time points. The main difference that is visible is seen in *figure 11c* on the x-axis between 100 and 120μm. A nucleus (stained in blue, looks purple on the picture) is fully covered with the green staining (WGA-Lectin). If we compare this staining to the WGA-Lectin staining 1 day under flow, even though there is no nucleus staining, a clear difference is visible. In the day 1 under flow (*figure 9c*) a patchy pattern is observable that "covers" the nucleus of a cell. It is not yet really a full layer, but it is already observable where the nucleus is, even though they are not stained.
The coverage of the WGA-Lectin staining in *figure 10a&b* is hard to compare. PAEC under flow for 5 days (*Figure 10b*) appeared aligned and elongated to the flow (flow direction from top to bottom). The green staining is clearly on the surface of the cells, but it is hard to tell how heavily covered they are from this 2D picture. Gaps between the cells are visible in the picture as well. The F-Actin staining is weak on the 10x picture in *figure 10b* and is more visible on the 40x picture on *figure 10c*. The cells in the 5 days under flow look healthy, even though half of the channel was already detached. The WGA-Lectin staining in *figure 10a* looks like as if it is stronger compared to *figure*

10b which is probably due to the fact that the cells are not yet under the same shear stress duration and are not as aligned and elongated as the ones that were 5 days under flow. The picture in *figure 11a* was acquired after visualizing the WGA-Lectin staining previously without the DAPI staining. The sample was stained again later for DAPI and the WGA-Lectin staining was already bleached a bit. The important thing to see in this picture compared to *figure 11b* is, that a clear difference between the coverage of the nuclei with the green staining is observable. 1 day under flow has barely any green on the nuclei, 5 days in comparison has green spots on top of the nuclei.

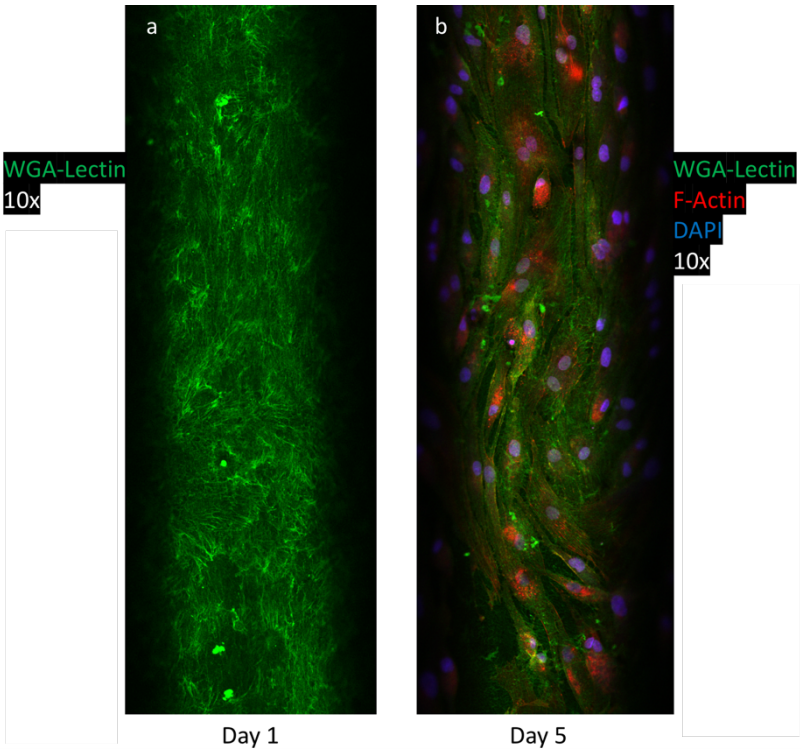

Figure 10: Comparison of WGA-Lectin in vivo staining after 1 day or 5 days under flow of PAEC P2 WT. (**a**) WGA-Lectin staining (**b**) WGA-Lectin staining, F-Actin staining and nuclei are stained with DAPI.

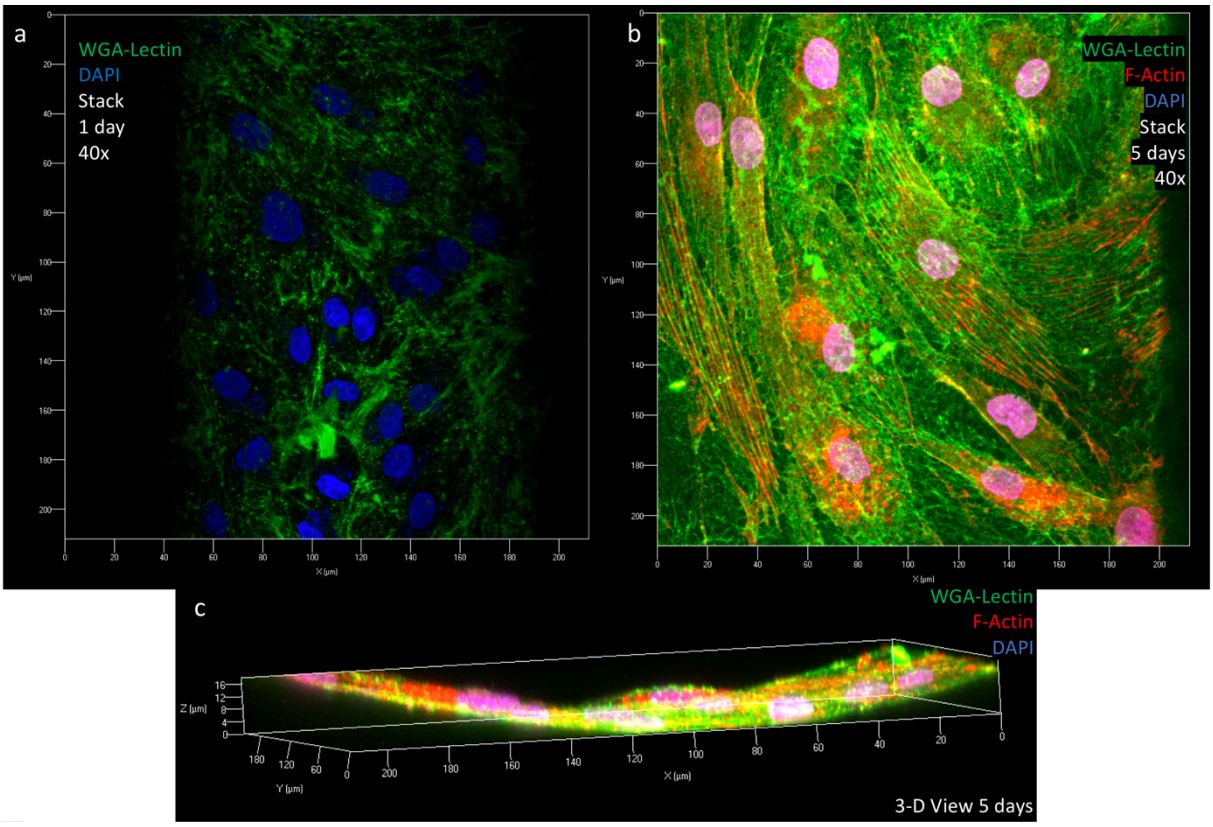

Figure 11: Comparison of WGA-Lectin staining in vivo after 1 day or 5 days under flow of PAEC P2 WT. (**a**) stack of WGA-Lectin staining and nuclei stained with DAPI (staining was done after fixation for DAPI) (**b**) stack of WGA-Lectin, F-Actin and DAPI staining (**c**) 3D view of WGA-Lectin, F-Actin and DAPI staining

11.5 Heparan sulphate and WGA-Lectin staining in chamber slides of PAEC P6 WT

After we had issues with detachment of cells from the channels, due to reasons we don't know up to today, we decided to cultivate PAEC WT under static conditions in chamber slides to see if we have the same problems of detachment. We stained for HS and WGA-Lectin to compare these two stainings, as both of them seemed to be more or less suitable for endothelial glycocalyx visualization. The settings for the picture acquirement were the same for each staining, so that we would be able to compare the results. We managed to culture cells up to 7 days (see *figure 12e*) and stained them for WGA-Lectin. We had some cell loss during the staining and the overall health of the cells was not so pleasing in the bottom line of the chamber slide. This is especially visible in *figure 12c* – the nuclei got stained green even though there was no permeabilization before the WGA-Lectin staining. If we compare *figure 12c*, *figure 12d* and *figure 12e* it is visible that there is more green staining in the day 7 staining of WGA-Lectin compared to day 2 and day 3. The F-Actin staining is similar in all three timepoints, and only in the day 7 chamber the nuclei did not get stained.

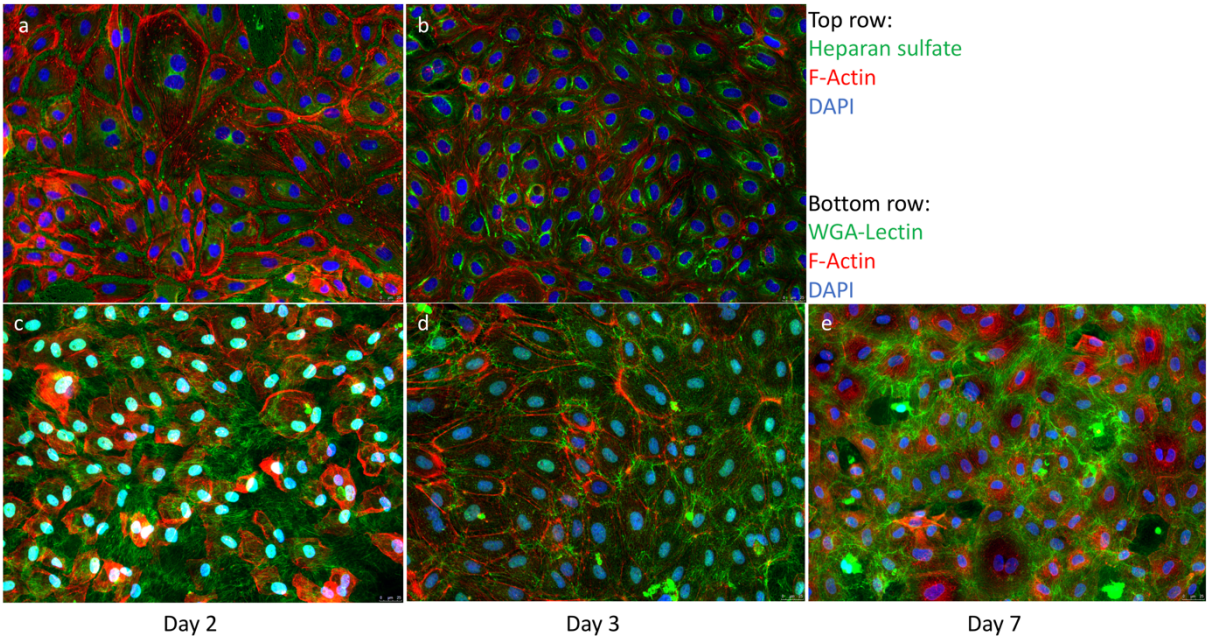

Figure 12: PAEC P6 WT (pig 7, isolated 15.4.15) were cultured in fibronectin coated chamber slides for multiple days under static conditions and stained for HS and WGA-Lectin, as well as F-Actin and DAPI. (**a**) HS, F-Actin and DAPI staining after 2 days (**b**) HS, F-Actin and DAPI staining after 3 days (**c**) WGA-Lectin, F-Actin and DAPI staining after 2 days (**d**) WGA-Lectin, F-Actin and DAPI staining after 3 days (**e**) WGA-Lectin, F-Actin and DAPI staining after 7 days.

The heparan sulfate staining worked fine. At both timepoints, the cells were perfectly healthy, but we still had some loss during the staining. The structure of the green staining is definitely different between the two timepoints in *figure 12a* and *figure 12b*. In *figure 12a* it seems to be mainly outside of the cell and looks like a sketch drawing and we were able to see clear connections between the cells, whereas in *figure 12b* the green staining is blurrier and the "sketch drawing"-like structure is no longer observable. One specific difference that is visible by eye is the fact that the green staining is way more concentrated around the nuclei in *figure 12b* compared to *figure 12a*.

11.6 Staining for the activation of endothelial cells with E-Selectin and VCAM-1

Due to issues with detachment of cells and the general health of the cells we decided to check the cells of a microchip that showed apoptotic behavior for endothelial cell activation by staining them for E-Selectin and VCAM.

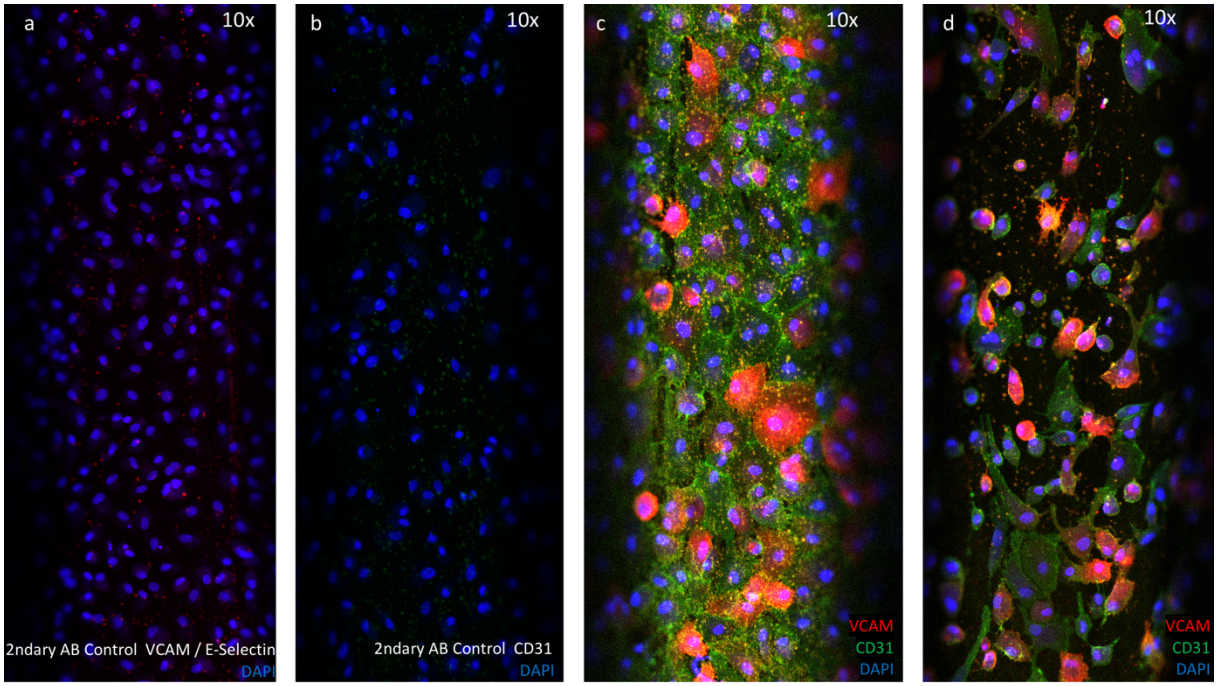

Figure 14: Staining of P6 WT PAEC (Pig 7, isolated 15.4.15) for VCAM, CD31 and DAPI after roughly two days of 2 RPM flow. (**a**) Secondary AB for VCAM / E-Selectin and DAPI staining (**b**) Secondary AB for CD31 and DAPI staining(**c**) VCAM, CD31 and DAPI staining of channel one(**d**) VCAM, CD31 and DAPI staining of channel three.

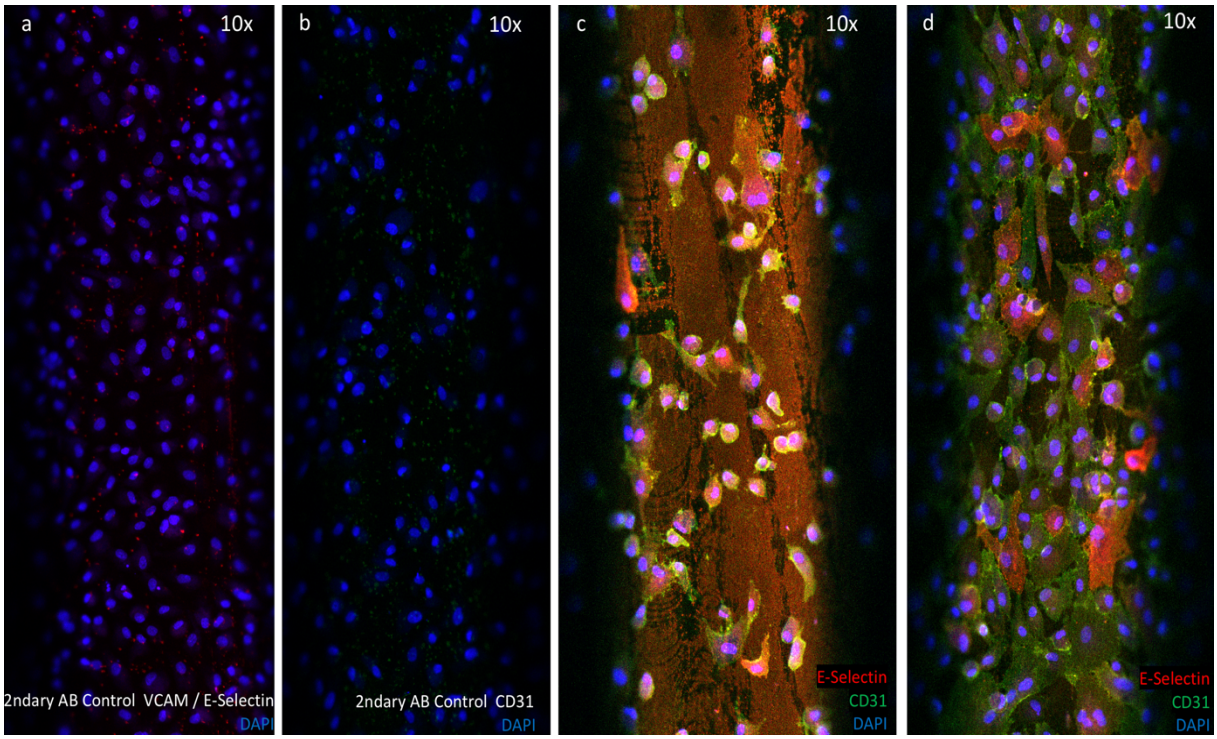

Figure 13: Staining of P6 WT PAEC (Pig 7, isolated 15.4.15) for E-Selectin, CD31 and DAPI after roughly two days of 2 RPM flow. (**a**) Secondary AB for VCAM / E-Selectin and DAPI staining(**b**) Secondary AB for CD31 and DAPI staining (**c**) E-Selectin, CD31 and DAPI staining of channel two (**d**) E-Selectin, CD31 and DAPI staining of channel 4

In *figure 13c* and *figure 13d* it is visible, that the cells are not healthy. The cells are roundish and the connections between the cells are no longer intact. The CD31 staining is usually like a monolayer around the cells, but in *figure 13c* and *figure 13d* the staining is punctual around the cells and no clear monolayer is observable. The VCAM staining is visible and expressed in many cells and the cell nuclei are shrunken.

The same picture is shown in *figure 14c* and *figure 14d*. The cell nuclei started to shrink and there are numerous round cell, which are clearly apoptotic. We see the same situation with the CD31 staining being mainly randomly scattered across the surface, but no clear layer is visible. The E-selectin staining is expressed in a lot of cells, even though there is a lot of background fluorescence in *figure 13c*.

11.7 Perfusion of PAEC WT with human and pig plasma

The perfusion with plasma was done to assess the binding of AT-III to the endothelial glycocalyx in a xenotransplantation setting. Cells were kept under flow for 3 days at 10 RPM with daily medium exchange. Heat inactivation of the human plasma was done at 56°C for 30 minutes.

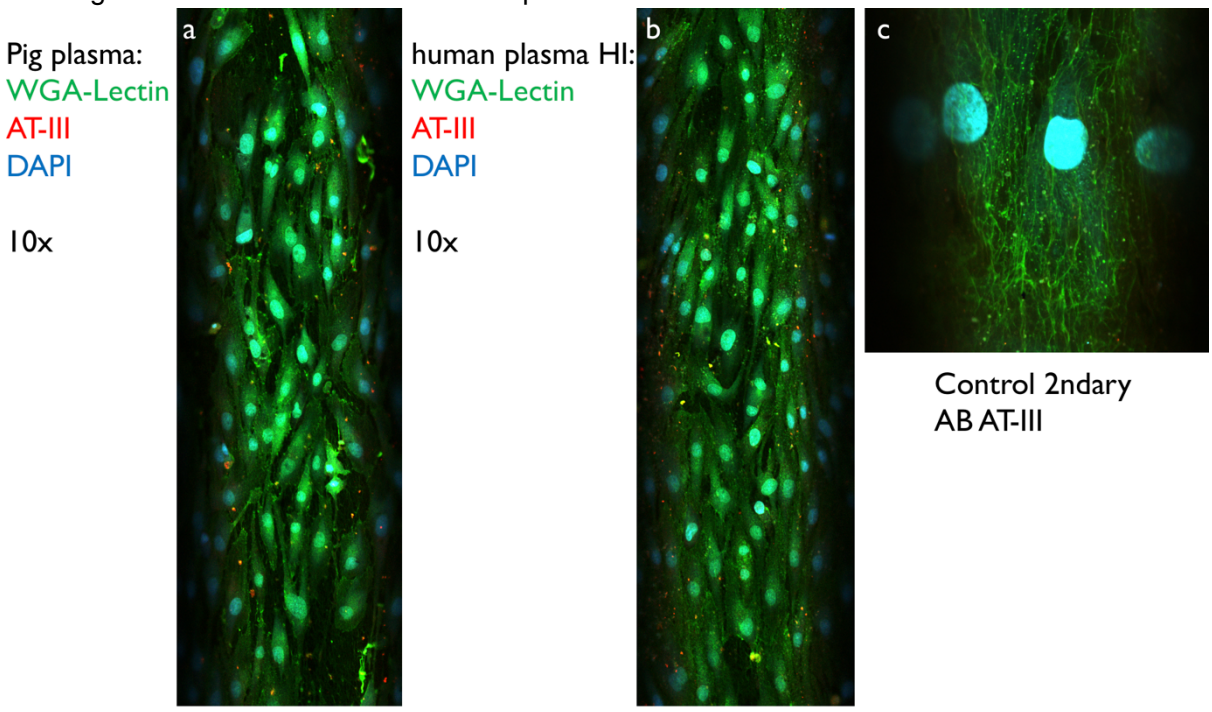

*Figure 15: Staining of PAEC WT P4 for WGA-Lectin, AT-III and DAPI after 3 days of 10 RPM flow and 45 minutes of perfusion with 10% human plasma heat inactivated or 10% pig plasma. (**a**) Staining of WGA-Lectin, AT-III and DAPI after pig plasma perfusion (**b**) Staining of WGA-Lectin, AT-III and DAPI after heat inactivated human plasma perfusion (**c**) Control of the secondary antibody used for AT-III staining and DAPI staining.*

The secondary antibody control in *figure 15c* showed no red staining. Staining that is colocalized with the WGA-Lectin staining is visible at the borders of the picture and on the green filaments that connect the cells to each other in *figure 15a*. There is also barely any cell loss visible after the perfusion with the allogenic plasma. The xenotransplantation setting showed severe cell loss in the channel, that was perfused with non-heat-inactivated human plasma. These pictures were excluded, because they were not really usable. In the heat-inactivated human plasma perfusion (*figure 15b*) a similar pattern of AT-III staining is visible. There is a tendency of more peripheral staining compared to the pig plasma perfused channel.

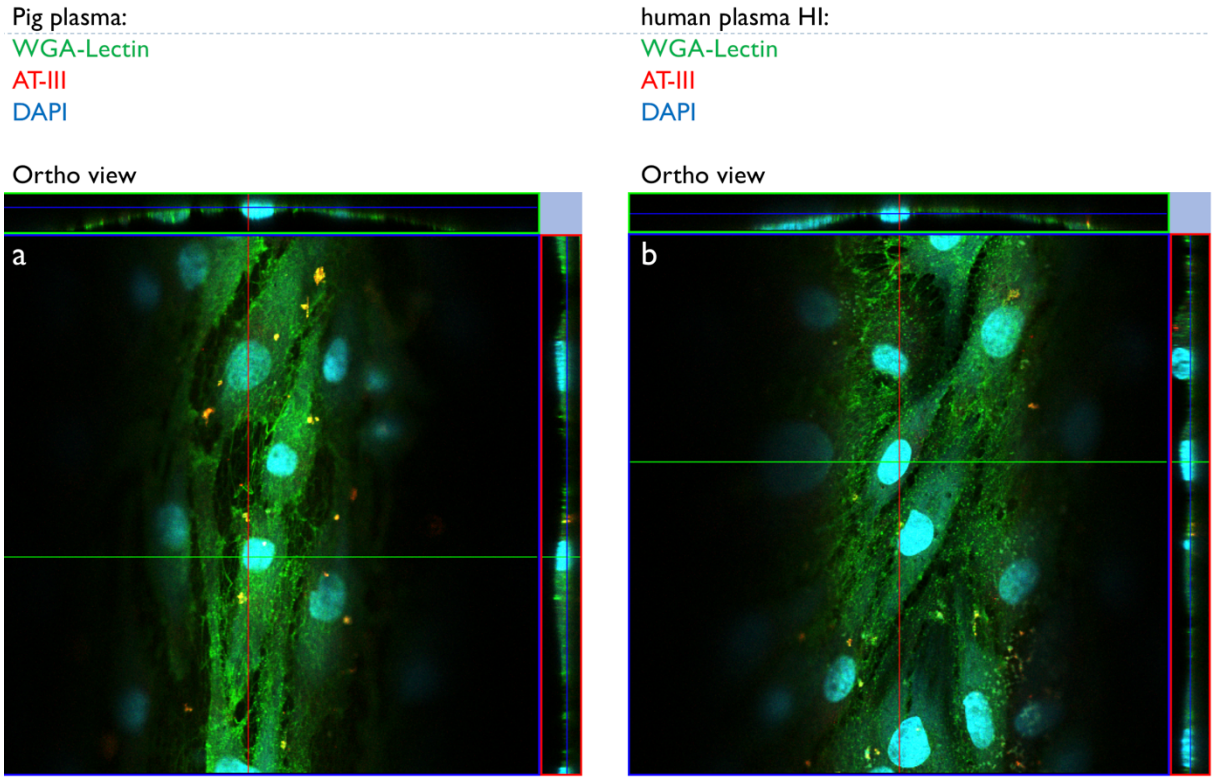

*Figure 16: Ortho view of the WGA-Lectin, AT-III and DAPI staining of both perfused channels. (**a**) Staining of WGA-Lectin, AT-III and DAPI after perfusion with Pig Plasma (**b**) Staining of WGA-Lectin, AT-III and DAPI after perfusion with heat inactivated human plasma.*

There is not yet a fully covered glycocalyx visible on both the y-z and x-z of *figure 16a* and *figure 16b*. The red AT-III staining is mainly present on the basal membrane for both perfused channels. A small red spot on the y-z axis of *figure 16b* shows a staining on top of the cell layer, but it is hard to judge the quality of the staining due to the not yet fully built up glycocalyx.

12 Discussion

Characterization of our cells that were used for the microfluidic system was important to ascertain that the cells were actually endothelial cells. We were certain, that we were working with endothelial cells after visualizing the typical endothelial cell markers (vWF, CD31, Ve-cadherin and WGA-Lectin) and analysing the pictures. Different antibodies were tested by using them as a staining for microfluidic chips that were under static conditions for at least 3 days. We wanted to see if one of the antibody is more suitable as an endothelial glycocalyx staining candidate than the heparan sulphate proteoglycan staining antibody. The results from the perlecan and syndecan staining showed us, that the staining mainly occurred on the basal membrane of the cell layer and that there was a lot of colocalization with the heparan sulphate staining. The colocalization can be explained by the fact that heparan sulphate is a side chain and perlecan and syndecan are both core proteins. So, it is obvious that the heparan sulphate and syndecan / perlecan have a colocalization if for example a heparan sulphate chain is linked to the syndecan or perlecan. At this point of the experiment we were thinking, that the syndecan and perlecan stainings are not suitable. We were not able to see a staining on top of the cell layer and this was, what we were looking for. Looking back to this staining experiment, one could argue, that 3 days under static conditions are simply not long enough for the endothelial glycocalyx to build up. This assumption would also explain, why we were not able to see any cell surface staining – simply because there was not enough glycocalyx built up to be visualized properly. Further experiments with microfluidic chips under static conditions showed us, that it takes more time to build up a fully covering glycocalyx under static condition compared to the “under flow situation”. This strengthens the argument, that the endothelial glycocalyx was not enough built up for the staining trials under static conditions.
WGA-Lectin might be used for live-cell staining for Sialic Acid Residues (Neu5Ac, N-Acetylneuraminic Acid) and N-acetyl-D-glucosaminyl residues (GlcNAc). Live-cell stainings are appealing because the cells are not fixed before the staining. Fixation might disrupt the endothelial glycocalyx and an live-cell staining can offer more accurate results. The sialic acid residues are short side chains of the glycoproteins that are mainly present on the surface of the endothelial glycocalyx. These facts are all promising for a better visualization of the endothelial glycocalyx. The results of the WGA-Lectin staining were very pleasing. The comparison of 1 day to 5 days under flow showed already, that after 5 days a full coverage of the cells by the endothelial glycocalyx was visible by immunofluorescence staining and looking at the ortho-view of the confocal microscopy pictures. The cells were also more aligned and elongated after 5 days under flow compared to the cells that were one day under flow. After these staining experiments, we were able to conclude, that it takes at least 5 days under flow (6 RPM) for a full coverage of the cells by the endothelial glycocalyx and that this staining is, in our view, the best staining for the endothelial glycocalyx. Further experiments with microfluidic chips that were at least 5 days under flow need to be done to replicate the results that we got from our experiments and to really determine the time that is needed for an endothelial glycocalyx to be built up under our experimental conditions. Replicates of the experiments would be a way to determine the thickness of the glycocalyx by analysis with ImageJ.
We had problems with cells detaching from the microfluidic chip during the experiments. The cells looked healthy and had a good morphology and up to today we are not really sure what the reasoning for the cell detachment was. We started cultivating cells in chamber-slides under static conditions to see if we have the same cell detachment. We managed to keep cells for 7 days under static conditions before we stopped the experiment. The cells were no longer looking healthy, so we decided to fix them and stain them. We stained cells in the microfluidic chip that were roundish and started to detach for endothelial cell activation markers, namely E-Selectin and VCAM. The staining showed us clear endothelial cell activation. We hypothesized, that the activation could have resulted because of the cells being under “too low” flow (only 2 RPM). The 2 RPM were used

to ensure medium exchange, which is a crucial factor in microfluidic systems due to the low cell-to-media ratio. Due to the fact that we are using aortic endothelial cells, low flow could result in cell detachment because aortic cells are used to higher flow situations.
Nevertheless, we are currently doing trial experiments with PAEC that were under flow (10 RPM) and perfuse them with human plasma, human heat-inactivated plasma and pig plasma to assess the binding of AT-III to the endothelial glycocalyx in a xenotransplantation setting. We have no more issues with cell detachment and the morphology of the cells looks perfectly fine. The first staining trials with the AT-III antibody show already staining after 3 days but further improvement needs to be done. 3 days under 10 RPM flow is quite a shear stress for the cells and they need some more time to build up the glycocalyx (around 5 days at least). The AT-III binds to the heparan sulphate side chains in the endothelial glycocalyx and a proper binding can only be achieved once the endothelial glycocalyx is fully built up. Staining occurred mainly on the basal membrane, which could also be an indicator the fact, that the endothelial glycocalyx was not built up fully yet. Once it is fully built, the antibody should no longer be able to reach the basal membrane – firstly because we do not permeabilize in the staining procedure and secondly due to the endothelial glycocalyx being a protective layer for the endothelial cells (the basal membrane is no longer exposed). The heat-inactivation of the human plasma is a big improvement for the experimental setting. First stainings without heat inactivation resulted in a huge cell loss inside of the microfluidic chips. The additional step ensures, that the complement proteins are no longer functional in the plasma and prevents lysis of the cells due to complement activation in the xenotransplantation setting.
Overall there was no big difference between the allogenic situation (perfusion with pig plasma) and the xenotransplantation setting. It is safe to say, that we have to improve the experimental setting to ensure a fully built up glycocalyx. Once we achieve this, we can start to compare the different perfusion set ups.
To conclude, we were able to determine the time it takes to build up an endothelial glycocalyx in a microfluidic chip under flow and managed to find a suitable staining for the visualization of the endothelial glycocalyx by immunofluorescence staining.

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14 Declaration of consent

Erklärung

gemäss Art. 28 Abs. 2 RSL 05

Name/Vorname:	Luther Fabian
Matrikelnummer:	14-204-184
Studiengang:	Bachelor in Biology, Specialisation in Cell Biology
	Bachelor ☑ Master ☐ Dissertation ☐
Titel der Arbeit:	Usage of the Microfluidic system to investigate the Endothelial Glycocalyx of PAEC in vitro
LeiterIn der Arbeit:	Prof. Dr. R. Rieben, Departement of Biomedical Research Riccardo Sfriso, PhD Student

Ich erkläre hiermit, dass ich diese Arbeit selbständig verfasst und keine anderen als die angegebenen Quellen benutzt habe. Alle Stellen, die wörtlich oder sinngemäss aus Quellen entnommen wurden, habe ich als solche gekennzeichnet. Mir ist bekannt, dass andernfalls der Senat gemäss Artikel 36 Absatz 1 Buchstabe r des Gesetzes vom 5. September 1996 über die Universität zum Entzug des auf Grund dieser Arbeit verliehenen Titels berechtigt ist. Ich gewähre hiermit Einsicht in diese Arbeit.

Ort/Datum

Unterschrift

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